SANDRA REGINA LEPRI

AVALIAÇÃO *IN VITRO* DO EFEITO DAS ISOFLAVONAS GENISTEÍNA E DAIDZEÍNA NO METABOLISMO DE XENOBIÓTICOS, CICLO CELULAR E ANTIMUTAGENICIDADE/MUTAGENICIDADE

Londrina
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Tese apresentada ao Programa de Pós–Graduação em Patologia Experimental, da Universidade Estadual de Londrina, como requisito para a obtenção do título de Doutor.

Orientador: Prof. Dr. Mario Sergio Mantovani.

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***À* minha família natural,**
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Meu esposo Rafael Fuentes e meu filho Rafael Lepri, incentivadores, amigos e aliados de todos os momentos;

Sem vocês a vida não teria o menor sentido;

Mantenhamos a nossa aliança.

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RESUMO

Genisteína e daidzeína, as principais isoflavonas de soja, são moléculas de baixo peso molecular com amplo espectro de atividades biológicas. Muitos estudos sugerem que estas isoflavonas tem ação protetora contra várias doenças crônicas incluindo câncer. Estas moléculas podem exercer seus efeitos através de múltiplos mecanismos que entre outros destacam-se: atividade antioxidante, inibição de crescimento de células neoplásicas, regulação do ciclo celular e apoptose, modulação de enzimas do metabolismo de xenobióticos (fase I e II) e modulação de várias vias de sinalização celular. No presente trabalho avaliamos os efeitos das isoflavonas genisteína e daidzeína sobre três linhagens de células tumorais: hepatoma de rato (HTC), hepatoma humano (HepG2) e carcinoma de cólon humano (HT29). Os parâmetros avaliados tiveram foco sobre atividade antiproliferativa (HTC, HepG2 e HT29), efeitos genotóxicos e antigenotóxicos (HTC), atividade sobre modulação de enzimas do metabolismo de xenobióticos (HTC, HepG2) e efeito sobre a via de sinalização celular Wnt/β-catenina (HT29). Os principais resultados obtidos no presente trabalho foram: (i) Em HTC, o ensaio do micronúcleo mostrou que nenhuma das isoflavonas utilizadas teve efeito genotóxico. Genisteína a 10 μM apresentou efeito antimutagênico quando associada aos agentes de danos ao DNA de ação direta (DXR) e indireta (2AA). Os níveis de *GST* mRNA não foram diferencialmente modulados por genisteína ou daidzeína. (ii) Em HepG2, genisteína e daidzeína (10 a 100 μM) inibiram crescimento celular de forma dose e tempo dependente. Resultados através de PCR em tempo real mostraram que a suplementação com 50 μM de genisteína causou uma redução na expressão de *CYP1A1* enquanto a daidzeína promoveu aumento de expressão nas duas concentrações testadas (10 e 50 μM). Os níveis de *GST* mRNA não foram diferencialmente modulados por genisteína ou daidzeína. (iii) Em HT29, genisteína inibiu proliferação celular em concentrações de 25 a 100 μM. Daidzeína somente inibiu proliferação na concentração mais alta (100 μM). Genisteína (50 μM) reduziu significativamente a expressão do gene *CTNNBIP1*(β-catenin) e não interferiu na expressão de APC (*Adenomatous polyposis coli)* ou *BIRC5* (survivina). Daidzeina não interferiu na expressão dos genes *CTNNBIP1*(β-catenin), APC (*Adenomatous polyposis coli)* ou *BIRC5* (survivina) em nenhuma concentração testada. Estes estudos indicam que especialmente a genisteína apresenta grande potencial a ser explorado para tratamento e prevenção de câncer.

Palavras-chave: Genisteína. Daidzeína. HTC. HepG2. HT29.

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ABSTRACT

Genistein and daidzein, the soy isoflavones, are low molecular weight molecules with a wide spectrum of biological activities. Studies suggest that these isoflavones have protective action against chronic diseases including cancer. These molecules may exert effects through multiple mechanisms as antioxidant activity, inhibition of growth of neoplastic cells, cell cycle regulation and apoptosis, the modulation of xenobiotic metabolizing enzymes (phase I and II) and modulation signaling pathways. In this study we evaluated the effects of isoflavones genistein and daidzein on three tumor cell lines: rat hepatoma (HTC), human hepatoma (HepG2) and human colon carcinoma (HT29). The parameters studied had focused on antiproliferative activity (HTC, HepG2 and HT29), genotoxic and antigenotoxic effects (HTC), modulation of xenobiotic metabolism enzymes (HTC, HepG2) and effect on the signaling pathway Wnt / β - catenin (HT29). The main results obtained in this study were: (i) in HTC, the micronucleus assay showed antimutagenic effect of genistein at 10 μM when associated with DNA damage direct (DXR) and indirect (2AA) agents. In the regulation of enzymes of phase II metabolism, the study indicated an increase in total GST activity in response to genistein and daidzein at 10 μM. *GST* mRNA levels were not differentially modulated by genistein or daidzein. (ii) in HepG2, genistein and daidzein (10 to 100 μM) inhibited cell growth in a dose and time dependent. Real-time PCR showed that 50 μM of genistein caused a reduction in the expression of *CYP1A1* while daidzein increased expression at 10 and 50 μM. GST mRNA levels were not differentially modulated by genistein or daidzein. (iii) in HT29, genistein inhibited cell proliferation at concentrations 25 to 100 μM. Daidzein inhibited proliferation only at the highest concentration (100 μM). Genistein (50 μM) significantly reduced gene expression *CTNNBIP1* (β-catenin) and did not affect the expression of APC (*Adenomatous polyposis coli*) or *BIRC5* (survivin). Daidzein did not affect the expression of genes CTNNBIP1 (β-catenin), APC (Adenomatous polyposis coli) or *BIRC5* (survivin) at any concentration tested. These studies indicate that genistein has especially great potential to be exploited for cancer treatment and prevention.

Keywords: Genistein. Daidzein. HTC. HepG2. HT29.

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LISTA DE SIGLAS E ABREVIATURAS

APC	polipose adenomatosa do cólon
CYP	citocromo
DMBA	7,12-dimetilbenzilantraceno
DNA	Ácido desoxirribonucléico
IQ	2-amino-3-metillimidazo[4,5-*f*]quinoline
MTT	3-4,5-Brometo de dimetiltiazol-2-il -2,5-difenil tetrazolium
NQO1	NADPH: quinona oxidoredutase 1
RT-PCR	Reação em cadeia da polimerase em tempo real
HTC	células de hepatoma de rato
HepG2	células de hepatoma humano
HT29	células de carcinoma de cólon
ODMA	O-desmetilangolensina
GST	glutaiona-S-transferase
B[a]P	benzo[a]pireno
TCDD	2,3,7,8-tetraclorodibenzo-p-dioxina
PHA	hidrocarbonetos poliaromáticos
AhR	receptores de aril hidrocarboneto
DMSO	Dimetilsulfóxido
MTT	3-(4-5-dimetiltiazol-2-il)-2,5-difenil brometo de tetrazólio
2AA	2-aminoantraceno
DXR	doxorrubicina
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
PBS	soro fetal bovino
MN	micronúcleo
RNA	ácido ribonucleico
CDNB	1-cloro-2,4-dinitrobenzeno

SUMÁRIO

Avaliação *in vitro* do efeito das isoflavonas genisteína e daidzeína no metabolismo de xenobióticos, ciclo celular e antimutagenicidade/mutagenicidade

1. INTRODUÇÃO

A extensa pesquisa sobre isoflavonas da soja, ao longo das últimas décadas concentrou-se em dois conjuntos de observações: (i) investigações sobre os fatores responsáveis pela menor incidência de doenças crônicas em países Asiáticos e (ii) investigações em busca de agentes farmacológicos com potencial quimiopreventivo e quimioterapêutico. Em vários modelos experimentais, as isoflavonas exibem propriedades que sugerem a prevenção de doenças crônicas tais como osteoporose, doenças do coração, cânceres e diabetes (KLEIN *et al*., 2007). A baixa incidência de câncer de mama nas mulheres asiáticas comparadas com mulheres de países ocidentais foi atribuída ao consumo de produtos a base de soja (PARK & SUHR, 2004). Outros tipos de cânceres como próstata, endométrio e cólon também apresentam relação inversa entre a ingestão de soja e incidência de tumor (BIRT *et al*., 2001; GOODMAN *et al*., 1997). Estudos com animais corroboram tais evidências, mostrando que ratos mantidos com dieta a base de soja foram protegidos contra tumor mamário induzido por dimetilbenzeno[a]antraceno (DMBA) e N-metil-N-nitrosourea (NMU) (SIMMEN *et al*., 2005).

O conceito de quimioprevenção pelo qual compostos naturais ou sintéticos podem prevenir, retardar ou reverter o desenvolvimento de cânceres tem grande relevância clínica. É estimado pela Sociedade Americana de Câncer que um terço de todos os cânceres podem ser prevenidos simplesmente pela modificação da dieta, manutenção de peso corporal e atividade física regular (American Cancer Society, 2009).

Os agentes candidatos a quimioprotetores podem ser classificados em dois grupos principais: (i) agentes bloqueadores e (ii) agentes supressores (WATTENBERG, 1985; MORSE & STONER, 1993). Os agentes bloqueadores são inibidores da fase inicial da carcinogênese e incluem entre outros,

mecanismos como alterações transcricionais de enzimas metabolizadoras da fase I e fase II, atividade antioxidante, inibição de replicação celular e indução de reparo do DNA. Os agentes supressores são descritos especialmente como inibidores de promoção e progressão de tumor e incluem mecanismos de supressão de crescimento celular e apoptose, entre outros (WATTENBERG, 1985; MORSE & STONER, 1993; DE FLORA & FERGUSON, 2005).

A soja aparece neste contexto com elevado interesse dietético, apresentando propriedades biológicas suficientes para atrair a atenção de muitos pesquisadores para seus efeitos benéficos, dentre os quais se destacam a prevenção de doenças crônicas tais como osteoporose, doenças do coração, câncer e diabetes (ESTEVES & MONTEIRO, 2001). Em adição, ela é a única entre os alimentos mais comuns que contém de 1 – 3 mg/g de isoflavonas (COWARD *et al*., 1993; citado por BOERSMA *et al*., 2001), não tem efeitos colaterais e apresenta baixa toxicidade fazendo-as excelentes candidatas a agentes quimiopreventivos. Os efeitos antimutagênicos e anticarcinogênicos destes compostos têm sido atribuídos a vários mecanismos incluindo atividade estrogênica e antiestrogênica, inibição de crescimento celular, regulação do ciclo celular e apoptose, atividade antioxidante, inibição de angiogênese, modulação de vias de sinalização celular e modulação de atividade enzimática que resulta em decréscimo dos efeitos tóxicos de vários xenobióticos (BIRT *et al*., 2001; SARKAR & LI, 2004; MOON *et al*., 2006). Os principais mecanismos de ação das isoflavonas de soja são sumarizados na Figura 1.

Figura 1. Principais mecanismos de ação das isoflavonas de soja em sistemas biológicos.

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1.1. Considerações gerais sobre as isoflavonas de soja

1.1.1. Isoflavonas – caracterização e biodisponibilidade

As isoflavonas são compostos químicos fenólicos pertencentes à família dos flavonóides e estão amplamente distribuídos no reino vegetal, sendo que suas concentrações são relativamente maiores nas leguminosas e em particular, na soja (*Glycine max*) (ESTEVES & MONTEIRO, 2001) O grão de soja contém especialmente 3 tipos de isoflavonas que se apresentam normalmente em duas diferentes formas: na forma conjugada (IFG), os glicosídeos daidzina, genistina e glicitina e na forma estrutural não conjugada (IFA), as agliconas daidzeína, genisteína e gliciteína (FIGURA 1) (ESTEVES & MONTEIRO, 2001). As primeiras são as formas predominantes em produtos não fermentados de soja (DAJANTA *et al*., 2009). Apresentam entretanto taxa de absorção intestinal muito baixa quando comparada às agliconas (IZUMI *et al*., 2000).

Isoflavonas	R1	R2	R3
Genisteína	OH	H	OH
Daidzeína	OH	H	H
Gliciteína	OH	OCH_3	H
Genistina	$C_6O_5H_{11}$	H	OH
Daidzina	$C_6O_5H_{11}$	H	H
Glicitina	$C_6O_5H_{11}$	OCH_3	H

Figura 2. Estrutura química das principais isoflavonas encontradas em grãos de soja. AGUIAR (2002) modificado.

Após a ingestão, as formas glicosídicas são hidrolisadas por enzimas intestinais ou pela microflora colônica liberando as principais agliconas, daidzeína, gliciteína e genisteína. Estas podem ser absorvidas e/ou metabolizadas por bactérias intestinais e produzir metabólitos específicos, tais como equol e/ou O-desmetilangolensina (ODMA) a partir da daidzeína e p-etinilfenol a partir da genisteína (FIGURA 2). Extensivas modificações ocorrem ainda durante o curso de absorção destes flavonóides por conjugação nas

células intestinais e mais tarde no fígado por metilação, sulfatação e/ou glucuronidação (SCALBERT & WILLIAMSON, 2000). Como conseqüência, as principais formas destas isoflavonas encontradas no sangue e nos tecidos são diferentes daquelas presentes na dieta e podem apresentar considerável variação individual, uma vez que as reações de metabolismo são catalisadas por enzimas que apresentam polimorfismos genéticos e são também induzíveis pela dieta (SCALBERT & WILLIANSON, 2000; FRANKENFELD, 2004).

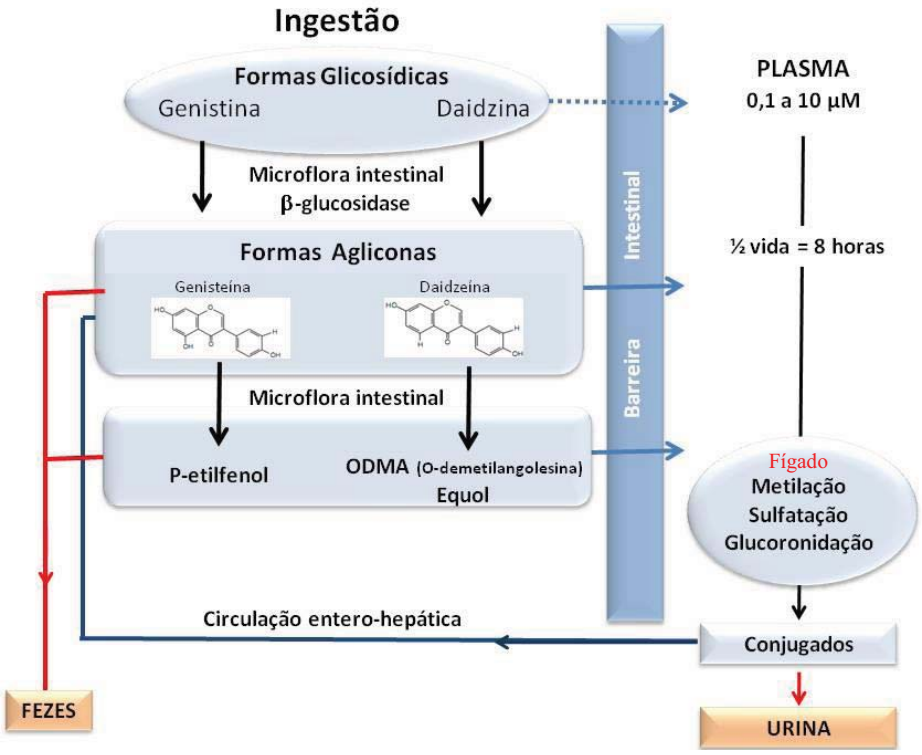

Figura 3. Representação esquemática da absorção, metabolismo e excreção das principais isoflavonas de soja genisteína e daidzeína. FERNANDES (2006) modificado.

As concentrações dos flavonóides no plasma humano encontram-se na faixa de 0,1 a 10 µM, sendo geralmente inferior a 1 µM (PENG & KUO, 2003), embora concentrações acima de 20 µM de genisteína e daidzeína tenham sido encontradas após ingestão de altas doses de isoflavonas agliconas (IZUMI *et al.*, 2000). As concentrações plasmáticas, entretanto podem ter menor relevância do que níveis teciduais. Recentemente, alguns estudos com humanos e animais mostraram que as concentrações de flavonóides incluindo genisteína, daidzeína e equol em macerados de tecidos de mama, próstata,

ovários, entre outros, podem exceder em várias vezes as concentrações encontradas no plasma (MAUBACH *et al*., 2003; BREINHOLT *et al*.; 2004, GARDNER *et al*., 2009). Estes estudos indicam que concentrações plasmáticas de polifenóis podem não estar diretamente correlacionadas com as concentrações nos tecidos alvo.

1.1.2. Principais propriedades biológicas das isoflavonas

As isoflavonas são moléculas de baixo peso molecular com amplo espectro de atividades biológicas. São compostos bioativos e apresentam estrutura química similar ao estradiol, o que justifica sua habilidade em comportar-se como estrógenos em sistemas biológicos (FERRARI & DEMIATE, 2001). Contudo, genisteína e daidzeína apresentam efeitos agonista e antagonista estrogênicos dependendo de suas concentrações. Em baixas concentrações (3,7 μM) e na ausência de estrógenos, genisteína promove proliferação celular agindo como um agonista em células intestinais positivas para receptor de est´rgenos (ER+), enquanto que em concentrações mais altas (26 – 111 μM) inibe a proliferação celular (CHEN & DONOVAN, 2004). Resultados semelhantes foram também reportados para células de câncer de mama (SARKAR & LI, 2002; 2004). Similarmente, a daidzeína apresenta efeito bifásico sobre a proliferação celular em células de câncer de cólon (LoVo) (GUO *et al*., 2004). Concentrações menores que 1 μM aumentam a proliferação celular enquanto concentrações mais altas entre 10 a 100 μM inibem o crescimento celular de maneira dose-dependente.

No entanto, a ação das isoflavonas sobre a proliferação de células tumorais vai além de seus efeitos hormonais. Estudos experimentais mostram que genisteína e daidzeina exercem efeitos inibitórios em uma variedade de células neoplásicas incluindo células hormônio-independentes de carcinoma gástrico e mama (LI *et al*., 1999; ZHOU *et al*., 2004; CUI *et al*., 2005; GUO *et al*., 2004).

Os efeitos da genisteína e daidzeína sobre crescimento e proliferação celular podem se explicados em parte pela habilidade destas moléculas em induzir apoptose e/ou interromper o ciclo celular (CHANG *et al*., 2004; CHEN &

DONOVAN, 2004; SHENOUDA *et al*., 2004; ZHOU *et al*., 2004; CUI *et al*., 2005; LOUIS JEUNE *et al*., 2005; CHODON *et al*., 2007; CHOI & KIM, 2008; JIN *et al*., 2010). Outros estudos mostram que o efeito inibitório das isoflavonas sobre a carcinogênese e progressão do câncer pode ser mediado por regulação de várias vias de sinalização celular incluindo a via Wnt (SARKAR *et al*., 2009)

1.1.3. Via de sinalização celular Wnt/β-catenina (genes *CTNNBIP1* (β-catenin), *APC* (adenomatous polyposis coli))

A via Wnt/β-catenina, também denominada de via de sinalização Wnt canônica, regula aspectos fundamentais para manutenção da homeostase dos tecidos. Com importante papel no controle do crescimento e diferenciação celular, sua característica principal é a estabilização da β-catenina citosólica (SARKAR, 2004). Os mecanismos de sinalização celular da via Wnt/β-catenina são sumarizados na Figura 2.

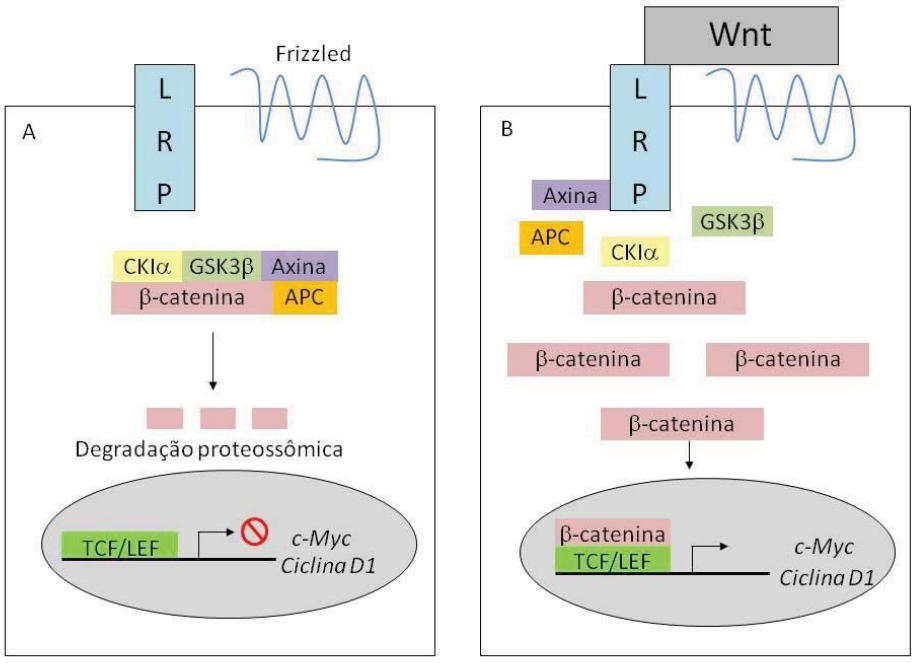

Figura 4. Via de sinalização celular Wnt/β-catenina. BIENZ & CLEVERS (2000) modificado.

Na ausência de ligantes (A) para via canônica Wnt, os níveis citoplasmáticos de β-catenina são mantidos através da degradação mediada por proteossomos, que é controlada pelo complexo GSK-3 (glicogênio sintase quinase-3β)/APC/axina (YANG *et al*., 2005). Ao contrário, a ativação desta via (B) promove dissociação do complexo GSK-3/APC/axina, impossibilitando a fosforilação da β-catenina que consequentemente acumula-se no citoplasma. Beta-catenina é então translocada para o núcleo resultando em ativação transcricional de genes específicos envolvidos no desenvolvimento do câncer como *c-myc* e *ciclina D1* (YANG *et al*., 2005).

Bem caracterizadas para câncer de cólon, alterações na proteína APC são consideradas um "gatilho" para o surgimento de distúrbios proliferativos na mucosa colônica (PINHO, 2006). Mutações desta proteína estão presentes em 80% dos adenomas em fase inicial, sendo esta mutação considerada hoje como a alteração mais precoce no processo de carcinogênese da mucosa colônica, motivo pelo qual é a ela atribuída a função de guardiã ("gatekeeper") (PINHO, 2006).

Vários fotoquímicos dietéticos têm mostrado inibir a sinalização via β-catenina como parte de seu mecanismo de quimioproteção. Em células de câncer de prostata, recente estudo mostrou que isoflavonas (uma mistura de 83.3% genisteína, 14.6% daidzeína, and 0.26% gliciteína – contendo de 25 a 50 μM de genisteína) aumentaram a expressão de GSK-3β (LI *et al*., 2008). O aumento de GSK-3β promove um aumento da fosforilação de β-catenina levando-a a degradação e consequentemente inativando a via Wnt (LI *et al*., 2008). Um estudo de microarray em tecido de tumores de rato mostrou que a genisteína inibiu a via Wnt e reduziu na expressão de genes alvo da via Wnt como ciclina D1 (SU *et al*., 2007). Outros flavonoides como curcumina e licopeno também tem mostrado regular a expressão ou atividade da via Wnt/β-catenina (SARKAR *et al*., 2010).

1.1.4. *BIRC5* (survivina)

Um outro possível mecanismo pelo qual isoflavonoides exercem sua atividade antiproliferativa podem ser mediados pela inibição da survivina. Os mecanismos reguladores da expressão de survivina em células malignas ainda não estão suficientemente esclarecidos, porém estudos têm demonstrado que a inibição desta proteína através do silenciamento gênico por RNAs de interferência, resulta em significativa inibição de crescimento tumoral em células humanas de carcinoma gastrico e pâncreas (MIAO *et al*., 2007; SHEN *et al*., 2010). Assim como a beta-catenina, a expressão celular de survivina é também inibida pela presença da proteína APC (PINHO, 2006). Survivina é um membro da família das proteínas inibidoras da apoptose (IAP), e regula dois processos essenciais na célula: inibe apoptose e promove proliferação celular (RYAN *et al*., 2009). Abundantemente expressa em tecidos tumorais e raramente expressa em tecidos saudáveis adultos, survivina tem despertado grande interesse como um biomarcador e uma nova forma de tratamento para muitos cânceres (RYAN *et al*., 2009). Estudos recentes mostram inibição da expressão da survivina pela genisteína em células de câncer de mama (MAI *et al*., 2007), pulmão (ZOU *et al*., 2008), próstata (SUZUKI *et al*., 2002) e pâncreas (EL-RAYES *et al*., 2006).

1.1.5. Enzimas metabolizadoras de fase I e II – CYP1A1 e GST

No organismo humano a biotransformação constitui uma etapa fundamental no processo de eliminação e diminuição de substâncias xenobióticas. No entanto, nem sempre estas substâncias são inativadas, pelo contrario, alguns metabólitos apresentam atividade aumentada e muitas vezes tóxica. O metabólito ativo intermediário neste caso, representa a ponte entre a biotransformação e a toxicidade e é capaz de causar vários danos no DNA podendo levar à perda de informação e finalmente ao câncer (OSHIMA-FRANCO & FRANCO, 2003).

As enzimas envolvidas na metabolização de xenobióticos estão distribuídas especialmente em dois principais complexos enzimáticos: 1)

enzimas da fase I (Citocromo P450), que convertem compostos hidrofóbicos em compostos hidrofílicos reativos, e 2) enzimas da fase II como glutationa-S-transferases (GSTs), UDP-glucuronil transferase (UGT) e NAD(P)H quinina oxidoredutase (QR) que catalisam reações de conjugação (POOL-ZOBEL *et al*., 2005). Asssim, enzimas da fase I, frequentemente resultam em bioativação, comparado com a inativação que resultam da fase II. De acordo com Manson *et al*. (1997), o resultado da exposição a uma toxina ambiental em termos de toxicidade aguda ou crônica, largamente depende do balanço entre estes dois processos.

As enzimas da família CYP1 do citocromo P450, consiste nos membros 1A1, 1A2 e 1B1 e são conhecidos por participar da ativação de vários pro-carcinógenos hidrocarbonetos aromáticos policíclicos (PHAs) como benzo[a]pireno (B[a]P) e dimetilbenzoantraceno (DMBA). Alta expressão e atividade de CYP1A1 está normalmente associado com risco de câncer de pulmão e cólon (MOON *et al*., 2006) enquanto CYP1B1 é expresso em alta freqüência em uma variedade de cânceres incluindo mama, cólon, pulmão, esôfago, pele, cérebro e testículo (ARROO *et al*., 2008).

O possível mecanismo de regulação de enzimas da família CYP1 pelos flavonóides parece estar relacionado com a reatividade de PHAs com receptores aril de hidrocarboneto (AHR), ativando a transcrição de genes que codificam proteínas envolvidas na metabolização de xenobióticos (NGUYEN & BRADFIELD, 2008).

A habilidade dos flavonóides em modular enzimas da fase I (P450) foi recentemente revisada por Moon *et al*. (2006) com ênfase na inibição dos efeitos causados por vários agentes químicos como DMBA, TCDD e B[a]P. Muitos flavonóides tais como dimetoxiflavona (WEN *et al*., 2005) e campferol (PUPPALA *et al*., 2007) foram capazes de inibir a expressão de CYP1A1 por PAHs em HepG2 (carcinoma de fígado) e BEAS-2B (células epiteliais de pulmão) respectivamente. Biochanina A (um derivado metilado da genisteína) inibiu transcrição e atividade de CYP1A1 e CYP1B1 em células epiteliais brônquicas (BERGE *et al*., 2004) e MCF-7 (carcinoma mamário) (CHAN *et al*., 2003). Outros flavonóides também tem mostrado decréscimo na atividade de CYPs em numerosos tipos celulares (MOON *et al*., 2006).

Contudo, flavonóides também podem aumentar atividade e/ou expressão de CYP. Galangina, curcumina, quercetina e baicaleina aumentaram a expressão de CYP1A1 ao mesmo tempo em que são relatados como protetores de câncer (CIOLINO *et al*., 1988; CIOLINO & Yeh, 1999a; CIOLINO *et al*., 1999b). Os autores sugerem que em adição à inibição da ativação de pro-carcinógenos através da inibição da expressão e/ou atividade de CYPs, os flavonoides podem exercer seus papeis quimiterapêuticos quando são submetidos a metabolismo oxidativo. Baseado nos dados obtidos na literatura, Arroo *et al*. (2008) sugerem que uma variedade de compostos naturais (notavelmente os fitoestrogenos) agem como pro-drogas. Estes compostos são substratos para enzimas expressas em células tumorais (CYP1A1 e CYP1B1), e os produtos de conversão inibem seletivamente o crescimento celular. Desta forma, CYP1B1 e CYP1A1 podem ser usados como alvos para desenhar pro-drogas específicas que somente são transformadas intratumoralmente em agentes citotóxicos ou citostáticos (ANDROUTSOPOULOS *et al*., 2010).

Um contraste com esse consenso, de que CYP1A1 induzível é prejudicial para o organismo humano, foi recentemente demonstrado *in vivo* com geração de camundongos knockout CYP1A1 (-/-). Camundongos com ausência do gene CYP1A1 morreram dentro de 30 dias de tratamento com 125 mg/kg/dia de B[a]P, enquanto ratos CYP1A1 (+/+) sobreviveram sem sinais evidentes de toxicidade (UNO *et al*., 2004). Estes dados mostraram pela primeira vez, que essa enzima é mais importante para a detoxificação de B[a]P no fígado e no intestino, do que para a ativação metabólica em produtos cânceríge nos.

A indução de enzimas da fase II, por outro lado, catalisam a conjugação de metabólitos oriundos da fase I a várias moléculas hidrossolúveis e aceleram sua excreção metabólica. Passíveis de indução e inibição, a modulação destas enzimas tem sido considerada um adicional mecanismo de quimioprevenção (MOON *et al*., 2006; POOL-ZOBEL *et al*., 2005). Uma correlação inversa entre a atividade de GST e a incidência de tumor ao longo do trato gastrointestinal foi demonstrada por Peters *et al*. (1993).

Indução da atividade de NAD(P)H quinina oxidoredutase (QR) pela genisteína foi observada em células neoplásicas de cólon (Colo205) e mama

((MCF-7 e MDA-MB-231) (WANG *et al*., 1998; BIANCO *et al*., 2005). Steiner *et al*. (2007) reportaram que a genisteína promoveu um aumento da expressão e atividade de GSTP1 em células de mama (MCF-10A) em concentrações de 1 – 30 μM. Adicionalmente este estudo mostrou uma redução significativa dos danos causados por 4-hidroxinonenal e B[a]P. Estudos *in vivo* mostraram que genisteina aumentou significativamente os níveis de expressão de GSTA2 enquanto os níveis de GSTM2 e GSTP1 foram significativamente diminuidos (WIEGAND *et al*., 2009). Dieta rica em genisteína e daidzeina também resultou em elevação das atividades de GST no rim e QR no cólon de ratos fêmeas *in vivo* (APPELT & REICKS, 1999).

1.1.6. Genotoxicidade e antigenotoxicidade

O potencial antimutagênico de uma substância pode ser avaliado em sistemas biológicos diversos, os mesmos empregados para o estudo e identificação dos agentes mutagênicos. Sistemas de células de mamíferos utilizados para a avaliação da mutagenicidade e/ou antimutagenicidade, abrangem os testes *in vitro* e *in vivo*. Nos sistemas *in vitro*, testes como o ensaio do micronúcleo ou ensaio do Cometa são frequentemente utilizados. O ensaio do micronúcleo é mais amplamente utilizado para a detecção de agentes clastogênicos (que quebram cromossomos) e aneugênicos (que induzem aneuploidia ou segregação cromossômica anormal), sendo internacionalmente aceito como parte da bateria de testes recomendada para a avaliação do potencial mutagênico e para o registro de novos produtos químicos que entram anualmente no mercado mundial. Os micronúcleos são núcleos pequenos, separados e adicionais ao núcleo principal das células, produzidos durante a telófase da mitose por perda de fragmentos cromossômicos ou cromossomos inteiros.

Os mecanismos de ação dos antimutagênicos dietéticos parecem ocorrer por numerosas vias incluindo, mecanismos extracelulares como inibição da captação do agente mutagênico, inibição da formação endógena do mutagênico, limpeza de espécies reativas, antioxidantes, proteção dos sítios nucleofílicos do DNA, indução dos caminhos de detoxificação, modulação do

metabolismo do DNA e processos de reparo, controle do ciclo celular e aumento de apoptose e modulação de enzimas que metabolizam xenobióticos (FERGUSON *et al*., 2004).

Os relatos com abordagem mutagênica e/ou antimutagênica das isoflavonas ainda são escassos e apresentam resultados negativos e positivos. Entre as isoflavonas, as formas agliconas tem sido as mais estudadas. Efeitos genotóxicos das isoflavonas genisteína, daidzeína e equol, um metabólito da daidzeína, foram avaliados por Di Virgilio *et al*. (2004) em cultura de linhagem de células pulmonares de hamster V79. A genisteína causou indução de micronúcleo em concentrações de 5 – 25 μM, declinado em doses mais altas. A daidzeína induziu um discreto aumento no número de micronúcleos em concentrações que variaram entre 25 – 100μM e equol causou um aumento de micronúcleo a partir de 25 μM mantendo-se estável neste patamar em concentrações de até 50 μM. Testes utilizando ensaio cometa demonstraram que a genisteína induziu danos somente em concentrações muito altas. Em células de linfoma de rato a genisteína induziu aumento de pequenas colônias indicando uma ação clastogênica a este composto (McCLAIN *et al*., 2006). Efeitos clastogênicos para genisteína em sistemas *in vitro* também foram reportados por Stopper *et al*. (2005) e Kulling *et al*. (2002).

Por outro lado McClain *et al*. (2006) mostraram que a genisteína não foi mutagênica em testes *in vitro* pelo ensaio AMES em células de linfoma de rato e *in vivo* através do ensaio do micronúcleo em ratos e camundongos. Efeitos antimutagênicos da genisteína foram também reportados células de linfoma de rato protegendo as células contra agentes indutores de danos ao DNA diretos e indiretos (POLIVKOVA *et al*., 2006).

2. JUSTIFICATIVA

Quimioprevenção ganhou considerável atenção nas últimas décadas como meio de de prevenir, retardar ou reverter o processo de carcinogênese uma vez que não é mais apenas um estratégia teórica, mas uma abordagem com resultados experimentais e clínicos. É estimado pela Sociedade Americana de Cancer que um terço de todos os canceres podem ser prevenidos simplismente pela modificação da dieta, manutenção de peso corporal e atividade física regular (American Cancer Society, 2009).

Neste contexto, há considerável interesse na soja e em seus componentes, em especial às isoflavonas, como potencial quimiopreventivo. Vários estudos epidemiológicos sugerem redução das taxas de cânceres associado com o consumo de alimentos a base de soja. Estudos *in vivo* e *in vitro* corroboram com estas evidências. No entanto os mecanismos de ação específicos pelos quais estes compostos exercem suas ações protetoras contra o câncer ainda não são claros podendo ser variados, complementares e/ou sobrepostos.

Desta forma, esforços direcionados na identificação do potencial antimutagênico e anticarcinogênico das isoflavonas de soja na proteção contra doenças ambientais, são de fundamental importância e podem reforçar a idéia de que a dieta é um fator chave na determinação da estabilidade genômica e prevenção de doenças crônicas. Em adição podem levar ao desenvolvimento de novas e mais efetivas drogas quimioterápicas.

Os resultados deste trabalho são apresentados na forma de 3 artigos:

- Chemoprotective activity of isoflavones genistein and daidzein on mutagenicity induced by direct and indirect mutagens in cultured HTC cells
- Analysis of expressed gluthatione-S-transferase (GST) and cytochrome P450 (CYP1A1) genes in human hepatoma cells (HepG2) exposed to genistein and daidzein
- The effects of genistein and daidzein on cell proliferation kinetics in HT29 colon cancer cells: the expression of *CTNNBIP1* (β-catenin), *APC* (adenomatous polyposis coli) and *BIRC5* (survivin)

3. OBJETIVOS

3.1. Objetivo geral

O presente estudo teve por objetivo avaliar os efeitos das isoflavonas genisteína e daidzeína em três linhagens de células tumorais: hepatoma de rato (HTC), hepatoma humano (HepG2) e carcinoma de cólon humano (HT29). Os parâmetros avaliados tiveram foco sobre: efeitos genotóxicos e antigenotóxicos, atividade sobre modulação de enzimas do metabolismo de xenobióticos, atividade antiproliferativa e efeito sobre a via de sinalização celular Wnt/β-catenina.

3.2. Objetivos específicos

- Investigar os potenciais genotóxicos e antigenotóxicos da genisteína e daidzeína em células de hepatoma de *Rattus novergicus* (HTC), através do ensaio do micronúcleo;
- Avaliar os efeitos da genisteína e daidzeína sobre a proliferação celular em células de hepatoma de *Rattus novergicus* (HTC), e células de carcinoma de cólon (HT29) através do ensaio de cinética de proliferação celular;
- Avaliar os efeitos da genisteína e daidzeína sobre enzimas do metabolismo celular CYP1A1 e GST em células de hepatoma de *Rattus novergicus (*HTC) e células de hepatoma humano (HepG2) através de PCR em tempo real;
- Avaliar os efeitos da genisteína e daidzeína sobre os genes *CTNNBIP1* (β-catenin), *APC* (adenomatous polyposis coli) and *BIRC5* (survivina) e células de carcinoma de cólon (HT29) através de PCR em tempo real;

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5. ARTIGO 1

Chemoprotective activity of isoflavones, genistein and daidzein on mutagenicity induced by direct and indirect mutagens in cultured HTC cells

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Chemoprotective activity of isoflavones genistein and daidzein on mutagenicity induced by direct and indirect mutagens in cultured HTC cells

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Abstract

Isoflavones are phenolic compounds widely distributed in plants and found in a high percentage in soybeans. They have important biological properties and are regarded as a potential chemopreventive agent. The aim of this study was to verify the preventive effect of two soy isoflavones (genistein and daidzein) by a micronucleus assay, analysis of GST activity, and real-time RT-PCR analysis of GSTa2 gene expression. Mutagens of direct (doxorubicin) and indirect (2-aminoanthracene) DNA damage were used. Hepatoma cells (HTC) were treated with genistein or daidzein for 26h at non-cytotoxic concentrations; 10 μM when alone, and 0.1, 1.0 and 10 μM when combined with genotoxic agents. The micronucleus test demonstrated that both isoflavones had no genotoxic effect. Genistein showed antimutagenic effects at 10 μM with both direct and indirect DNA damage agents. On phase II enzyme regulation, the current study indicated an increase in total cytoplasmic GST activity in response to genistein and daidzein at 10 μM supplementation. However, the mRNA levels of GSTa2 isozymes were not differentially modulated by genistein or daidzein. The results point to an in vitro antimutagenic activity of genistein against direct and indirect DNA damage-induced mutagenicity.

Keywords: Hepatoma cell line, antimutagenicity, genistein, daidzein, phase II enzyme.

Introduction

The intake of soy isoflavones (genistein and daidzein) has been associated with a reduced risk of cancer particularly estrogen-related cancers, such as breast cancer), heart disease, osteoporosis, and menopause symptoms (Linseisen et al., 2004; Klein and King, 2007). Genistein and daidzein have received considerable attention for their chemopreventive

properties against cancer initiation and development. Both isoflavones belong to the chemical group of flavonoids, which have little to no toxicity and have a long history of human consumption, making them excellent candidates for chemopreventive agents (Moon et al., 2006)

Cancer chemoprevention refers to the use of natural or synthetic compounds that are able to inhibit, delay, or reverse the multi-step process of carcinogenesis. The protective action of these agents is explained as a combination of various proposed mechanisms involving molecular association with carcinogens, the modulation of enzymes involved in biotransformation reactions (such as cytochrome p450 and glutathione S-transferase), anti-oxidant, anti-inflammatory, anti-angiogenesis, and immunomodulatory actions, apoptosis induction, cell-cycle arrest, cell-differentiation induction, antimicrobial effects, tyrosine kinase inhibition, and others (Akiyama et al. 1987; Chen et al., 2003; Foti et al., 2005; Moon et al., 2006; Ruffer and Kulling, 2006; Chodon et al., 2007; Choi and Kim, 2008; Naithani et al., 2008).

Previous studies on the genotoxic or antigenotoxic effects of genistein are controversial. Genistein was able to induce micronuclei and DNA strand breaks in Chinese hamster lung fibroblasts (V79 cells) (Kulling and Metzler, 1997), chromosomal structural aberrations in human lymphocytes (Kulling et al., 1999), and micronuclei in mouse splenocytes and V79 cells (Record et al, 1995; Di Virgilio et al., 2004). There is little evidence for genotoxic activity associated with daidzein, as there was only a slight increase in micronucleus formation in V79 cells treated with 75 μM daidzein (Di Virgilio et al., 2004). Genistein and daidzein have also been reported as antigenotoxic compounds (Polivokova et al., 2002; Pugalendhi et al., 2009).

The glutathione S-transferases (GSTs) represent a major group of detoxification enzymes that work by conjugating xenobiotics to reduced glutathione to facilitate dissolution in the aqueous cellular and extracellular media. Some GSTs are free in the cytoplasm (alpha, mu, pi, sigma, theta, zheta, and omega), and some are bound to cellular membranes (microssomal GST, and leukotriene C4 synthetase). Xenobiotics are able to increase the expression of certain GST genes (alpha, mu, and pi isoenzymes) by interaction with the following: a) the antioxidant-responsive element (ARE); b) the xenobiotic-responsive element (XRE); GST P enhancer I (GPE); or c) the

glucocorticoid-responsive element (GRE) (Hayes and Pulford, 1995; Singh and Michael, 2009).

The present study evaluated the antiproliferative, mutagenic, and antimutagenic potential of genistein and daidzein on hepatoma cells from *Rattus norvegicus* (HTC) in vitro. For the induction of DNA lesions, we used one direct agent (doxorubicin, DXR) and one indirect agent dependent on metabolic activation via cytochrome P450s (2-aminoanthracene, 2AA). The DNA lesions were assessed by the frequency of micronucleus formation, and the participation of GST in the chemopreventive effects was evaluated by total cytoplasmic GST activity and GSTalpha2 (GSTa2) gene expression.

Material and Methods:

Chemical Agents

For this study, doxorubicin (CAS nº 25316-40-9) (DXR, Pharmacia) was used as a direct genotoxic agent and 2-aminoanthracene (CAS nº613-13-8) (2-AA, Acros Organics) as an indirect genotoxic agent. DXR was generously supplied by the University Hospital of North Parana, Londrina – Paraná – Brazil. DXR was dissolved in PBS, and 2-AA was dissolved in dimethyl sulfoxide (DMSO, Mallinckrodt Chemicals). Genistein (CAS nº 446-72-0) and Daidzein (CAS nº 486-66-8) were obtained from Acros Organics and dissolved in dimethyl sulfoxide (DMSO) to prepare the appropriate concentrations. The final DMSO concentration in the medium culture did not exceed 1%.

HTC cell culture conditions

HTCs from a *Rattus novergicus* hepatoma were obtained from the Cell Bank of Rio de Janeiro (RJCB – Brazil). The cells were grown as monolayer cultures in 25 cm^2 culture flasks in DMEM/F12 (Gibco) with the addition of 10% fetal bovine serum (FBS, Gibco). The cultures were incubated in a humidified incubator at 37°C and 5% CO_2.

MTT cytotoxicity assay

The cytotoxic effect of the isoflavones, genistein and daidzein on the rat HTC cell line was determined by the 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay based on the protocol described by Mossmann (1983). Briefly, cells were plated into 96-well tissue culture dishes at a density of 2.5 x 10^4 cells/well in 100 µL of medium. The cells were cultured for 24h at 37°C and 5% CO_2 to adhere to the well. The supernatant solution was removed and replaced with fresh medium without FBS, containing genistein or daidzein at concentrations of 0.1, 1, 10, 50, and 100 µM. After 4h, 150 µL of culture medium plus MTT (0.5 mg/mL) was added to each well. The MTT solution was carefully decanted off and formazan was extracted from the cells with 200 µL of DMSO in each well. Color was measured with a 96-well ELISA plate reader at 550 nm. Data were plotted as relative cell survival rate (%) = (Absorbance Test/Absorbance control) ×100. MTT assays were performed in triplicate.

Cell proliferation kinetics

HTCs (5 x 10^4 cells/flask) were seeded in 10 cm^2 tissue flasks and cultured for 24, 48, 72 and 96h supplemented with genistein or daidzein at 10 µM. The number of cells were counted every 24h. Cells were trypsinized and counted using a Neubauer hemocytometer. Cell viability was evaluated by trypan blue staining. The population doubling time was calculated using the algorithm provided by http://www.doubling-time.com. Experiments were carried out in triplicate.

The micronucleus assay with a cytokinesis block (MNCtB)

Exponentially growing HTCs were seeded at a density of 10^6 cells/flask in 25 cm^2 tissue flasks and incubated for 24h before treatment allowing cell adhesion. After this period, the cells were washed with PBS and treated for 26h with fresh medium containing cytochalasin B at a final concentration of 3.0 µg/mL and containing the chemicals in the respective treatment protocols. For the genotoxicity evaluation protocol, the cells were treated with genistein or daidzein at 10 µM. For antigenotoxicity, cells were treated with genistein or daidzein at 0.1, 1 or 10 µM along with either DXR at 0.2 µM or 2-AA at 13 µM.

One thousand binucleated cells with well-preserved cytoplasm were scored on coded slides in three experimental repetitions using a microscope at 400× magnification, which resulted in the analysis of 1000 cells per treatment. The criteria for the identification of binucleated cells and micronuclei were described by Fenech (2000). The capacity of genistein or daidzein to reduce the DNA damage induced by DXR or 2-AA was calculated according to Waters *et al*. (1990) using the following formula:

Percent Reduction (DR%) = (nº MN cells in A - nº MN cells in B) / (nº MN cells in A - nº MN cells in C) x 100

Where, A = DNA damage-inducing agent, B = associated treatment and C = negative control

For cell cycle analysis, 500 cells per treatment group were scored for the presence of one, two, or more than two nuclei and the nuclear division index (NDI) was calculated as described by Fenech (2000). Experiments were performed in triplicate.

Real-time quantitative RT-PCR

The study of GSTa2 mRNA was chosen based on the study by Wiegand *et al*. (2009), which showed a significant increase in the expression levels of this enzyme in Wistar rat liver tissue. The HTCs (10^6/flask) were treated with genistein or daidzein at 10 µM for 12h. Total RNA was prepared from genistein or daidzein-treated HTCs using Trizol-LS® reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. RNA samples were incubated with DNase (1U) (CAS 18068-015, Invitrogen Life Technologies). The quantity of RNA was determined by spectrophotometer analysis (BioPhotometer - Eppendorf), and the integrity by eletrophoresis in a 1% agarose gel. The cDNA synthesis was carried out in reactions containing 1 µg total RNA (20 µl), 10 pmol/µL oligo dT primer (1µL), 10 mM dNTPs (2µL), RNAseOUT (0.1µL) and reverse transcriptase M-MLV (1µL) (Invitrogen Life Technologies). The real-time RT-PCR was performed using Platinum® SYBR Green qPCR SuperMix-UDG (Invitrogen Life Technologies). In each reaction, 0.4 µM of primer (forward and reverse) and 2 µL of template cDNA was added to make a final volume of 20 µL. The reactions were performed in PTC 200 DNA Engine Cycler using a Chromo4 Detection System (MJ Research). The oligonucleotides utilized in

these experiments are *Rattus novergicus* glutathione S-transferase *(GST)* and β-actin as follows:

The primer pairs used in this study for PCR amplification.

	Sequence	Size (bp)	NCBI	
B-actin	Forward - 5′ TTG CTG ACA GGA TGC AGA AGG AGA 3′ Reverse - 5′ ACT CCT GCT TGC TGA TCC ACA TCT 3′	159	BC063166	Present study
GSTa2	Forward - 5′ AGC CAT GGC CAA GAC TAC CTT GTA 3′ Reverse - 5′ AGA GGT CAG AAG GCT GGC ATC AAA 3′	108	FJ179397	Present study

The reaction conditions were as follows: 95ºC for 3 min, 40 cycles (95ºC for 30s, 60ºC for 30s, 72ºC for 20 s), 95ºC for 10s and 40ºC for 1 min., followed by melting curve analysis temperature between 50 to 90ºC (at 0.5^0C every 5 s). The data were normalized by using the *β-actin* internal standard gene. The relative differences between HTC cell control (DMSO) and treatment groups (genistein or daidzein) were calculated according to the method describe by Pfaffl (2001).

Each experimental protocol was performed in triplicate in two independent experiments. Data are expressed as mean values ± standard derivation (SD).

GST activity

Total cytoplasmic glutathione S-transferase (GST) activity was measured according to the protocol described by Houghton *et al*. (2007). This method is based on the GST-catalyzed reaction between GSH and 1-Chloro-2,4-dinitrobenzene (CDNB, Sigma), which acts as an electrophilic substrate for GSTs. HTCs (10^6) were seeded in 25 cm^2 culture flasks with 5 ml of complete culture medium. After this period, cells were treated with genistein or daidzein at 10 μM concentration for 12 or 24 hours. After incubation, the cells were trypsinized and kept in an ice bath. The cells were disrupted using 3 cycles of freezing at -80ºC followed by vortexing. To guarantee 100% cell disruption, the cells were evaluated with trypan blue staining. The cell lysates were centrifuged at 90 x g for 5 min at 4ºC, and the cytosolic solutions were obtained from the supernatant. For measurement of the enzymatic activity, the cytosolic solution

was pre-incubated for 5 min at 30ºC (Houghton et al., 2007). For each measurement, 100 µl of the reaction mixture (GSH + CDNB at final concentrations of 8.3 mM GSH and 3.3 mM CDNB) was added. A kinetic analysis was performed in which the absorbance of the mixture at 340 nm was measured every minute for 5 min using Libra S32 spectrophotometer (Biochrom) coupled to a temperature regulator TE-2005 (Tecnal). To calculate the GST activity, the following formula was used: **GST activity = Slope (ΔAbs/ml)/9.6mM^{-1}cm^{-1} x protein concentration (mg/ml).** The activity was expressed as nmol/min/mg of protein, where 1 enzyme unit is the amount of enzyme required to conjugate 1000 nmol/min/mg at 30°C. The protein concentration was measured by the Bradford method (Bradford, 1976). Each enzymatic measurement was performed in duplicate, and the average of the results obtained was used in the calculation of GST activity. The experiments were performed in triplicate.

Statistical analyses

The MTT assay, the micronucleus assay, and the GST activity data were analyzed by analysis of variance (ANOVA) followed by Tukey's test. The cell proliferation kinetic was estimated by exponential regression as described by Weisstein <http://mathworld.wolfram.com/LeastSquaresFittingExponential.html>. Point to point analysis was performed by analysis of variance (ANOVA) followed by Dunnett's test.

For RT-PCR, REST (Relative Expression Software Tool – 384, REST-384 © - version 2) (Pfaffl et al., 2002) software was used. For the mRNA induction or repression studies, only expression levels above 2-fold with statistically significant differences were seen as relevant.

RESULTS

MTT cytotoxicity assay

Figure 1 shows the effect of various concentrations of genistein or daidzein on the relative cell survival percentage of HTCs after 24 h. Survival of vehicle-treated control groups, not exposed to isoflavones or DNA damage inductors,

was defined as 100% survival. Concentrations ranging from 0.1 to 10 μM showed poor inhibition of HTC growth. However, at the higher concentrations of 50 and 100 μM, a marked inhibition occurred for genistein and daidzein in comparison to the control.

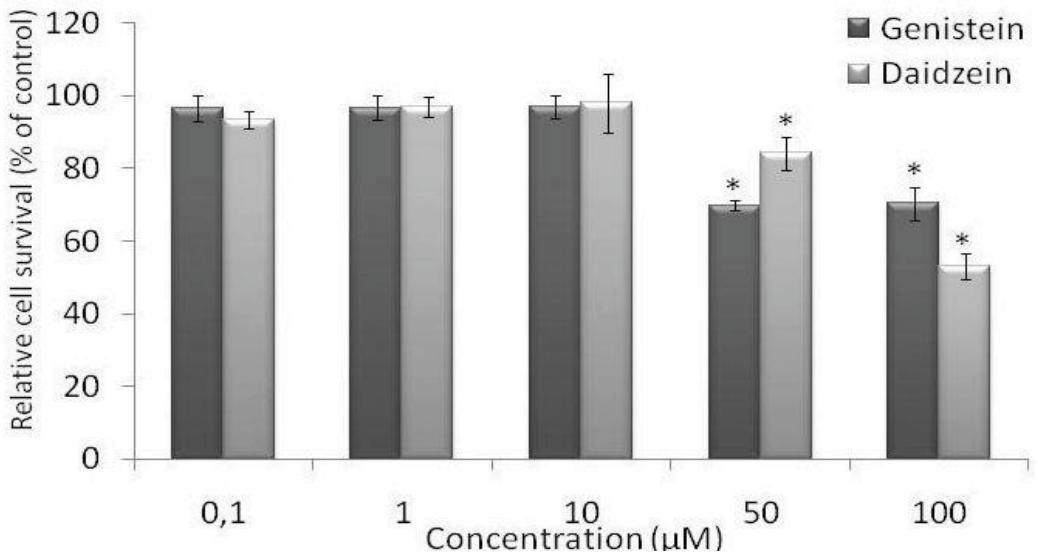

Figure 1. Relative HTC cell survival determined by the cytotoxicity assay. Cells were treated with different concentrations of genistein or daidzein (0.1 – 100 μM), and the cytotoxicity was determined using the MTT assay for 24 h. Data are represented as the mean ± S.D. of the triplicate experiments. The *asterisk* (*) indicates a significant difference (p<0.01) between the group treated and the group control. The statistic analysis was performed using the absorbance data.

Cell proliferation kinetics

The effect of genistein and daidzein on the growth kinetics of HTCs is shown in Figure 2. Cells were incubated with genistein or daidzein at 10 μM. Cell counting was used to measure cell proliferation. Both isoflavones displayed similar growth rates compared with control treatment (Figure 2). The R^2 of >0.97 in these models suggest a successful regression on proliferation data. The doubling time for control, genistein and daidzein was estimated to be 23.17, 23.5 and 23.2 h, respectively

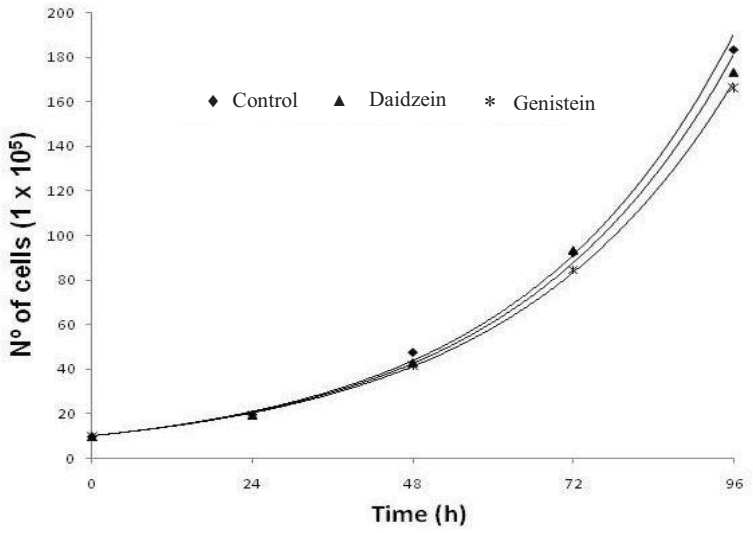

Figure 2. Cell proliferation kinetics for genistein and daidzein (10μM) on HTC cell..

The Micronucleus assay

In Tables 1 and 2, the total micronuclei formation, means, standard deviation and damage reduction percentage in HTCs are presented. No significant difference in the induction of micronuclei was observed between the group treated with genistein or daidzein and the negative control ($P > 0.05$). These findings indicate the absence of a genotoxic effect of genistein or daidzein at the concentrations tested. Direct or indirect DNA damage agents, used as positive controls, demonstrated the sensitivity of the micronucleus assay and yielded a clear positive response at the concentrations used. The results also show that genistein ($P > 0.001$) at a 10 μM concentration in combination with DXR or 2AA significantly reduced the frequency of micronuclei (Tables 1 and 2). This reduction was 38.59 and 71.43% in the micronucleus assay for DXR and 2AA, respectively. However, no significant difference in the frequency of micronuclei was observed for cultures treated with low genistein or daidzein concentrations (0.1 and 1 μM) that were associated with DNA damage inductors, and daidzein 10 μM showed no protective effect. The nuclear division indices (NDI) observed demonstrate that genistein and daidzein did not influence the cell cycle kinetics between treatments.

Table 1. Evalution of mutagenicity and anti mutagenicity against doxorubicin for genistein or daidzein on HTC cells, determined by Micronucleus assay.

Treatments	Means ± S.D.	DR%	NDI
Control	8.33 ± 0.57[b]		1.86
DXR	65.66 ± 0.57[a]***		1.84
Genistein 10 µM	8.33 ± 0,57[a]		1.87
Daidzein 10 µM	9.33 ± 0.57[a]		1.9
Genistein 0.1 µM + DXR	61.66 ± 3.21[b]	6.43	1.89
Genistein 1 µM + DXR	56.00 ± 10.44[b]	16.37	1.85
Genistein10 µM + DXR	43.33 ± 2.88[b]***	38.59	1.83
Daidzein 0.1 µM + DXR	62.00 ± 2.64[b]	-0.01	1.88
Daidzein 1 µM + DXR	59.66 ± 4.72[b]	-1.76	1.85
Daidzein 10 µM + DXR	65.00 ± 5.00[b]	0.58	1.85

***Statistically significant difference. DR%: percent reduction; NDI: nuclear division index; DXR: doxorubicin (0.2 µM). [a]Compared statiscally to the control. [b]Compared statiscally to DXR. Values are expressed as the mean of micronucleus in 1000 analysed cells ± S.D.

Table 2. Evalution of mutagenicity and antimutagenicity against 2-aminoanthracene for genistein or daidzein on HTC cells, determined by Micronucleus assay.

Treatments	Means ± S.D.	DR%	NDI
Control	9.00 ± 1.00[b]		1.78
2AA	30.00 ± 3.00[a]***		1.82
Genistein 10 µM	10.33 ± 1.53[a]		1.76
Daidzein 10 µM	11.33 ± 2.08[a]		1.78
Genistein 0.1 µM + 2AA	30.00 ± 1.00[b]	0.00	1.80
Genistein 1 µM + 2AA	29.00 ± 1.00[b]	4.76	1.78
Genistein10 µM + 2AA	15.00 ± 3.60[b]***	71.43	1.75
Daidzein 0.1 µM + 2AA	30.66 ± 0.57[b]	-3.17	1.80
Daidzein 1 µM + 2AA	30.00 ± 1.00[b]	0.00	1.80
Daidzein 10 µM + 2AA	27.33 ± 2.08[b]	12.70	1.78

***Statistically significant difference. DR%: percent reduction; NDI: nuclear division index; 2AA, 2-amino-anthracene (13 µM).[a]Compared statiscally to the control. [b]Compared statiscally to 2AA. Values are expressed as the mean of micronucleus in 1000 analysed cells ± S.D.

GST activity

Figure 3 shows activities of GST, a phase II xenobiotic-metabolizing enzyme, in the HTC cell lineage. The activities of phase II enzymes were significantly increased in cells treated with genistein and daidzein for 12 h compared to control. This effect was not observed with cells treated for 24 h (Figure 3).

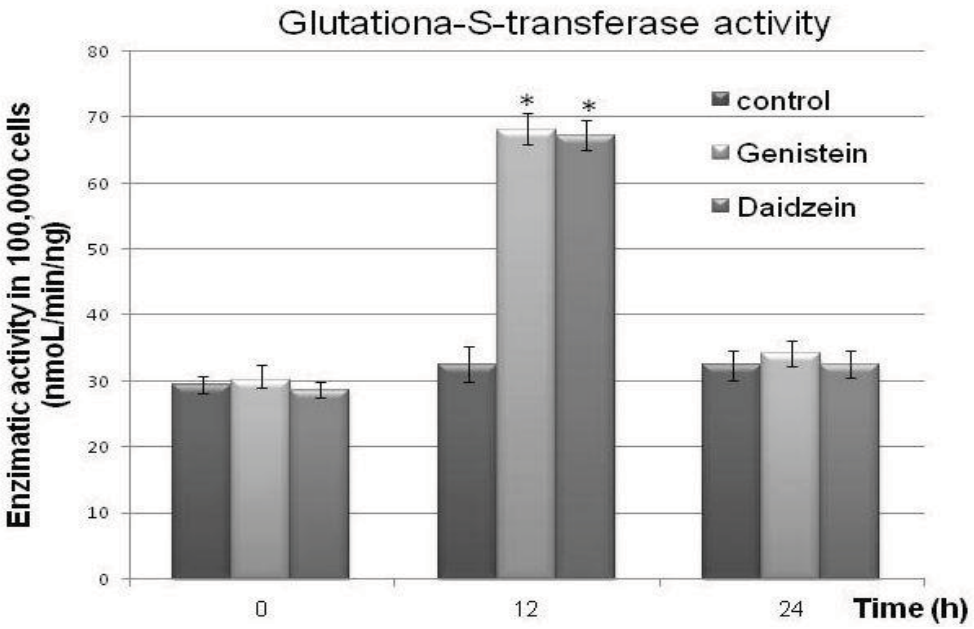

Figure 3 - Measurement of GST in HTC cells submitted to treatment with 10μM genistein and daidzein, in the days 12 and 24 hours of treatment. The asterisks (**) represent significant differences compared to the enzyme dosage control of the respective time period ($p < 0.001$).

GST gene expression

As observed in Figure 4, the exposure of HTCs to 10 μM of genistein and daidzein for 12h did not affect GSTa2 expression, as shown by real time PCR. Data are expressed as fold change between treatment and control, which is the difference between the normalized RNA expression level (ΔC_t, where C_t is the threshold cycle) of the gene in the control group and that of the gene in the treatment group.

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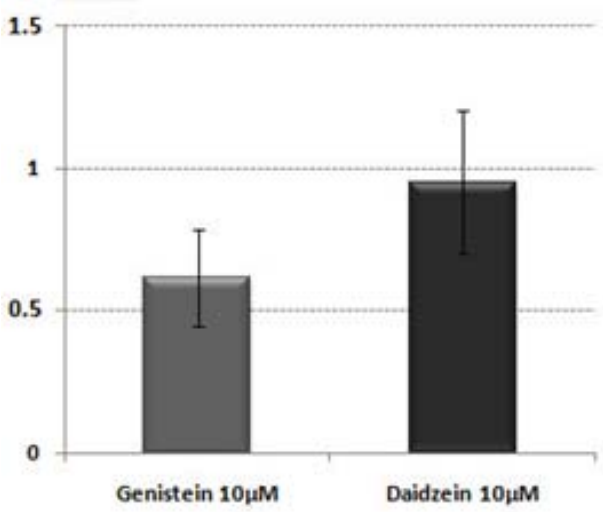

Figure 4. Fold change of GSTa2 mRNA after 12 hours exposure to genistein or daidzein in HTC cells. Data represents the means ± SD of two experiments

DISCUSSION

HTC cell lineage is a drug-metabolizing cell line that has been used for the direct and indirect identification of antigenotoxic agents (Bellini et al., 2006; Oliveira *et al*., 2006; Macarini *et al*., 2010). We investigated the protective effects of genistein and daidzein on DNA damage induced by doxorubicin (a direct-acting mutagen) and 2-aminoanthracene (an indirect-acting mutagen) on HTCs by using the micronucleus test. We first examined the cytotoxic effect of genistein and daidzein at concentrations ranging from 0.1 to 100 μM for 24 h to avoid false positives in the genotoxicity test. The highest noncytotoxic doses (10 μM) of genistein and daidzein were further evaluated at 96 h to explore the cellular kinetic proliferation. Our results of the MTT assay show that genistein and daidzein have similar cytotoxic effects on HTCs. Only the highest concentrations of both (50 and 100 μM) showed cytotoxic effect. Both genistein and daidzein at 10 μM did not alter the proliferation rate of HTCs at 96 h. Control, genistein, and daidzein showed similar doubling times (22.7, 23.4 and 23.2 h, respectively), excluding the possibility of time-dependent effects. The concentrations of 0.1, 1, and 10 μM of genistein and daidzein were selected for subsequent assays.

For the tested concentrations of genistein and daidzein, we did not observe mutagenic effects on HTCs because the number of micronuclei did not differ significantly from the controls. Many studies reported that genistein is able to induce DNA damage, though its mechanism is not known. It has been shown that genistein, daidzein and equol (a metabolite of daidzein) induced micronucleus formation on V79 cell lines. Genistein showed a clastogenic effect, and equol showed an aneugenic effect. Also, genistein has shown a pronounced genotoxic effect compared to daidzein (DI VIRGILIO *et al*., 2004). Genistein, but not daidzein, induced DNA strand breaks, and micronuclei in Chinese hamster V79 cells (Kulling and Metzler, 1997), chromosomal structural aberrations in human lymphocytes (Kulling *et al*., 1999), and in vitro formation of micronuclei in mouse splenocytes (Record *et al*., 1995). The required concentrations for genetic toxicity described in these studies ranged from 5 to 25 μM. However, when mice or humans are exposed to higher levels of dietary isoflavones, genetic damage is not observed (Record *et al*., 1995; Miltyk *et al*., 2003).

In contrast to these in vitro studies, we observed antimutagenicity against DXR or 2AA for genistein. This is in agreement with in vivo studies that have reported antimutagenic effects of genistein against direct and indirect mutagens and carcinogens in a bone marrow micronucleus test. (Polivkova *et al*., 2006, Pugalendhi *et al*., 2009). Antigenotoxic compounds can directly interact with genotoxins, inactivating them chemically (scavenging) or enzymatically (inhibiting the metabolic activation of pro-mutagenic compounds or inducing phase II enzymes) (Antunes and Araujo, 2000).

The use of DXR as an antitumor drug is due to its direct action on DNA. Different processes are responsible for the biological effects of DXR, such as through interactions with DNA topoisomerase II and the generation of free radicals and subsequent oxidative stress (Quiles *et al*., 2002). The proposed mechanism for antimutagenicity of genistein is related to its antioxidant properties. Genistein is a known antioxidant (Foti *et al*., 2005; Lee *et al*., 2004; Hernadez_Montes *et al*., 2006) and has been reported to inhibit oxidative stress and DNA damage induced by methylglyoxal (a reactive dicarbonyl compound) in human mononuclear cells (Wu and Shan, 2007). Another possible mechanism for the reduction of DNA damage by genistein is the stimulation of DNA repair. It was recently reported that genistein up-regulated the expression of genes involved in DNA damage repair in prostate tumor cells (Bhamre *et al*., 2010).

The genotoxic effect of 2AA depends upon metabolic activation to reactive intermediates on cytochrome P450 isoforms (CYP450), with involvement of mainly CYP1A2 isoenzymes (Jemnits *et al*., 2004). Genistein has been shown to modulate the CYP450 system, including CYP1A1 and CYP1A2 enzymes, and may induce phase-II enzymes that are involved in detoxification (Moon *et al*., 2006). To better understand the antimutagenic mechanism of genistein on indirect mutagens, we focused on the metabolic activation of phase II enzymes. We observed a time-dependent increase of total cytoplasmic GST activity for both isoflavones (increase was observed only for 12 hours of treatment, normalizing by 24 hours), but mRNA levels of GSTa2 isoenzymes were not differentially modulated by either genistein or daidzein after 12h of treatment. These results show that genistein and daidzein are able to increase GST activity by up-regulation of GST isoforms other than GSTa2.

The increase of total cytoplasmic GST activity found in our study may be responsible for the reduction of 2AA-mediated DNA damage by genistein. GST could increase the activity of the detoxification system, which may facilitate the elimination and inactivation of the active mutagens formed by metabolism of 2AA. However, this mechanism only partially explained the protective role of genistein because daidzein similarly induced GST activity in HTCs, but did not show an antigenotoxic effect. For in vivo assays, Wiegand et al. (2009) showed that GST activity was not affected by feeding a genistein-supplemented diet to rats, while mRNA levels of GSTa2 isoenzymes were significantly increased (plasma genistein concentration was 7.6 ± 1.0 μM).

From the chemical point of view, genistein and daidzein have strong similarities, except for the 5'-hydroxyl group; thus, they may perform their functions via similar pathways. Our results showed that genistein and daidzein similarly induce GST activity. However, genistein and daidzein exhibited different abilities to protect HTCs against DNA damage by DXR or 2AA. Previous studies have shown that the number and position of the hydroxyl group of the A ring effectively increased the radical scavenging activity of genistein (Choi et al., 2009). Thus, it is clear that several mechanisms may be involved in this antimutagenic process by genistein. Further investigation is needed to elucidate the mechanisms of cellular responses to soybean isoflavone exposure.

Acknowledgements

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6. ARTIGO 2

Analysis of expressed gluthatione-S-transferase (GST) and cytochrome P450 (CYP1A1) genes in human hepatoma cells (HepG2) exposed to genistein and daidzein

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Abstract

Flavonoids and other polyphenols (PPs) constitute a family of phenolic compounds present in fruits, vegetables and alcoholic beverages. These phytochemicals are associated with important biological properties, such as antioxidant activity and protective effects on cancer, cardiovascular and neurodegenerative disorders. The PPs are natural products and are generally recognized as "safe" for human consumption. However, PPs can interfere with xenobiotic metabolizing enzymes, including the induction or inhibition of cytochrome P450 (CYP) and phase II enzymes expression, resulting in pharmacological and toxicological effects. The objective of this study was to investigate the effect of two soy isoflavones (genistein and daidzein) on the expression of CYP1A1 and GST enzymes in human hepatoma cells (HepG2) by real-time PCR (RT-PCR). Cells were treated for 12 hours with genistein or daidzein at concentrations of 10 and 50 µM. This study showed genistein and daidzein as inhibitors and inducers of CYP1A1 gene expression, respectively in HepG2 cells. Further experiments are required to define the usual role of CYP1A1 for risk assessment because CYP1A1 activates environmental carcinogenic polycyclic hydrocarbons.

Keywords: HepG2 cells; genistein; daidzein; CYP1A1; GST.

Introduction

Genistein and daidzein are the most abundant proteins in soybean. They are bioactive compounds capable of protecting against carcinogenesis through biochemical mechanisms, such as anti-oxidative and anti-inflammatory properties, anti-proliferative activity, cell cycle arrest, induction of apoptosis, modulation of multidrug resistance and modulation of phase I and II metabolizing enzymes.[1-3] The CYP1 family of cytochrome P450 enzymes

consists of 1A1, 1A2 and 1B1 members that play an important role in the metabolism of many xenobiotic compounds. High expression and activity of CYP1A1 are associated with the risk of lung and colorectal cancer.[4-5] On the other hand, activation of detoxifying enzymes, such as UDP-glucuronyl transferase (UGT), glutathione-S-transferase (GST) and NAD(P)H:quinoine oxidoreductase (QR) result in the detoxification of carcinogens. This activation of detoxifying enzymes represents one mechanism of an anti-carcinogenic effect.[6] According to Manson *et al*.[7], the result of exposure to an environmental toxin in terms of acute or chronic toxicity largely depends on the balance between these two processes (phase I and II metabolizing enzymes).

Flavonoids have demonstrated the ability to inhibit CYP1 and increasing phase II enzymes of expression and/or activity, which is believed to be protective against cancer development.[8-12] However, the flavonoids also have been shown to induce CYP1A1 and CYP1B1 gene expression.[6] Although these two observations seem to be contradictory, flavonoids may be substrates for CYP1 enzymes and may result in the inhibition of tumor cell growth through the formation of conversion products that are more pharmacologically active.[13]

The present study was designed to examine whether genistein and daidzein, the most abundant phytoestrogens in soy, change the expression of metabolizing enzymes in the HepG2 cell line. We investigated the effects of these isoflavones on CYP1A1 and GST gene expression.

Material and Methods

Chemical Agents

Genistein (CAS # 446-72-0) and Daidzein (CAS # 486-66-8) were obtained from Acros Organics and dissolved in dimethyl sulfoxide obtained from Mallinckrodt Chemicals (DMSO) to prepare appropriate concentrations. The final DMSO concentration in the medium did not exceed 1%.

HepG2 cell culture conditions

Human hepatocellular liver carcinoma cells (HepG2), were obtained from the cell bank of Rio de Janeiro (RJCB–Brazil). The cells were grown as monolayer

cultures in 25 cm^2 culture flasks in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with the addition of 10% fetal bovine serum (FBS) (Gibco). The cultures were then incubated at 37°C in 5% CO_2.

MTT cytotoxicity assay

The cytotoxic effect of the isoflavones genistein and daidzein on human hepatocellular liver carcinoma HepG2 was determined by 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay based on the protocol previously described by Mossmann.[14] Briefly, cells were plated onto 24-well tissue culture dishes at a density of 2.0 x 10^5 cells/well in 500 µL medium. The cells were cultured for 24 hours at 37°C in 5% CO_2. The supernatant solution was removed and replaced with a new medium, without FBS, containing genistein or daidzein at 5, 10, 25, 50 and 100 µM. After the end of the culture period, 200 µL of culture medium plus MTT (0.5 mg/mL) was added to each well, and the mixture was incubated for 4 hour. The MTT solution was carefully decanted, and formazan was extracted from the cells with 200 µL of DMSO in each well. The absorbance was measured using a 96-well plate ELISA reader at a wavelength of 550 nm. Data were plotted as % relative cell survival rate = (Absorbance Test/Absorbance control) × 100. MTT assays were performed in triplicate. Data were analyzed by analysis of variance (ANOVA) followed by the Tukey's test.

Real-time quantitative PCR (RT-PCR)

The HepG2 cells (10^6/flask) were treated with genistein or daidzein at concentrations of 10 and 50 µM for 12 hours. Total RNA was prepared from genistein- or daidzein-treated and DMSO-treated HepG2 cells using Trizol® LS reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. RNA samples were incubated with DNase (1 U) (Invitrogen Life Technologies). The quantity and integrity of the RNA prepared from each sample were determined by spectrophotometer (BioPhotometer-Eppendorf) and by electrophoresis in 1% agarose gels, respectively. The cDNA synthesis was carried out in reactions containing (20 µl) total RNA (1 µg), 10 pmol/µL oligo dT primer (1µL), 10 mM dNTP (2µL), RNAseOUT (0.1µL) and reverse Transcriptase M-MLV (1µL) (Invitrogen life Technologies). The real-time RT-

PCR was performed in duplicate using Platinum® SYBR Green qPCR SuperMix-UDG (Invitrogen Life Technologies). Each primer at 0.4 µM and 2 µl of template cDNA was added to make a final volume of 20 µl. The reactions were developed in a PTC 200 DNA Engine Cycler using a Chromo4 Detection System (MJ Research). The oligonucleotides utilized in these experiments are in table 1.

Table 1. Oligonucleotide primers used for RT-PCR.

	Sequence	Size (bp)	NCBI	Ref.
GAPDH	Forward - 5'- GAA GGT GAA GGT CGG AGT C - 3' Reverse - 5'- GGA AGA TGG TGA TGG GAT TT - 3'	227	NM_002046	(15) with modifications
CYP1A1	Forward - 5'- TCA TCC CTA TTC TTC GCT ACC - 3' Reverse - 5'- CAG GAG ATA GCA GTT GTG AC - 3'	277	NM_000499	(16) with modifications
GSTP1	Forward - 5'- CAA TAC CAT CCT GCG TCA CC - 3' Reverse - 5'- GGA GAT GTA TTT GCA GCG GA - 3'	121	NM_000852	Present study

Reaction volumes contained: 10 µl Platinum SYBR Green Supermix (Invitrogen, CAS 11733-38), 0.5 µl forward and reverse primer, 7 µl DEPC H_2O and 2 µl of cDNA. The reaction conditions were as follows: 95ºC for 3 min., 40 cycles (95ºC for 30 s, 60ºC for 30 s, 72ºC for 20 s), 95ºC for 10 s and 40ºC for 1 min., followed by melting curve analysis to confirm amplification of the desired product. The calculated resulting relative expression of CYP1A1 and GSTP1 genes were normalized to relative GAPDH expression. The relative differences between control and treatment groups were calculated and expressed as relative increases, calculated according to Pfafll.[17]

Each experiment was performed in triplicate with two independent experiments. Data are expressed as mean values ± standard derivation (SD). The Pair Wise Fixed Reallocation Test was used and statistical significance was assumed at level $p<0.05$. REST (Relative Expression Software Tool–384, REST-384©-version 2 (Pfaffl et al., 2002)) software was used for statistical analysis. For the mRNA induction or repression studies, only levels above two-fold with statistically significant differences were seen as relevant.

Results

MTT cytotoxicity assay

Figure 1 shows the relative cell survival for the treatment of the Hep G2 cells in the presence of genistein and daidzein for 24 h. The chemical doxorubicin was used at a concentration of 3.7 μM. The survival of the cells in the treated groups was expressed as a percent of the control groups. Our results show the decline of Hep G2 cell growth in the presence of 100 μM genistein and daidzein cultures.

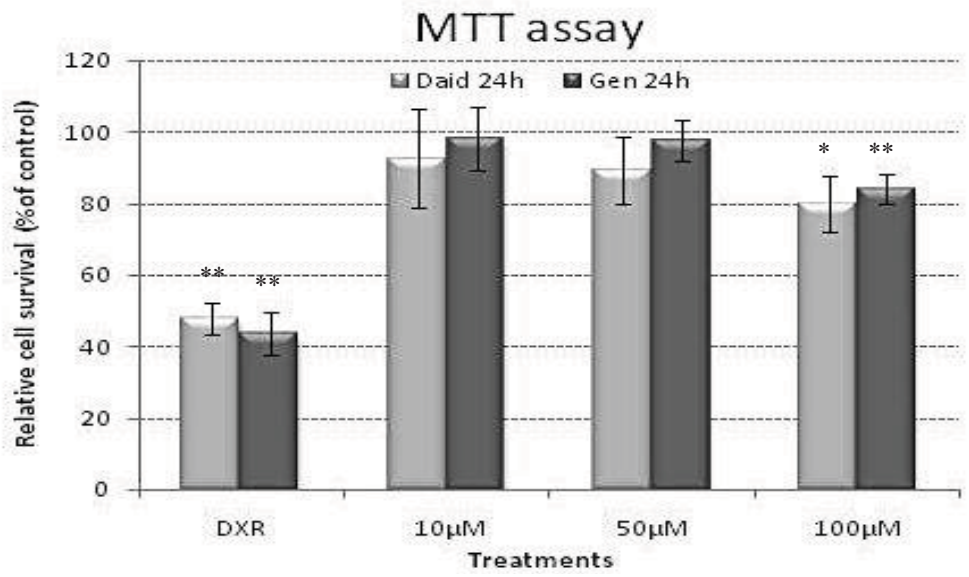

Figure 1. Relative cell survival determined by the cytotoxicity assay. Cells HepG2 were treated with different concentrations of genistein or daidzein (10 – 100 μM), and the cytotoxicity was determined using the MTT assay for 24 h. Data are represented as the mean ± S.D. of the triplicate experiments. The *asterisk* (**) indicates a significant difference (p<0.01) and (*) indicates a significant difference (p<0.05) between the group treated and the group control. The statistic analysis was performed using the absorbance data.

Real-time quantitative RT-PCR

As observed in Figure 2, treatment of HepG2 cells with 10 or 50 μM of daidzein for 12 h resulted in three- or five-fold increases, respectively in CYP1A1 mRNA compared to DMSO. These results were determined using RT-PCR. Genistein at 10 μM did not produce an effect on CYP1A1 transcription, whereas 50 uM of genistein significantly reduced CYP1A1 mRNA expression.

No significant changes in GST expression after exposure to genistein or daidzein at 10 or 50 µM were seen (Figure 3).

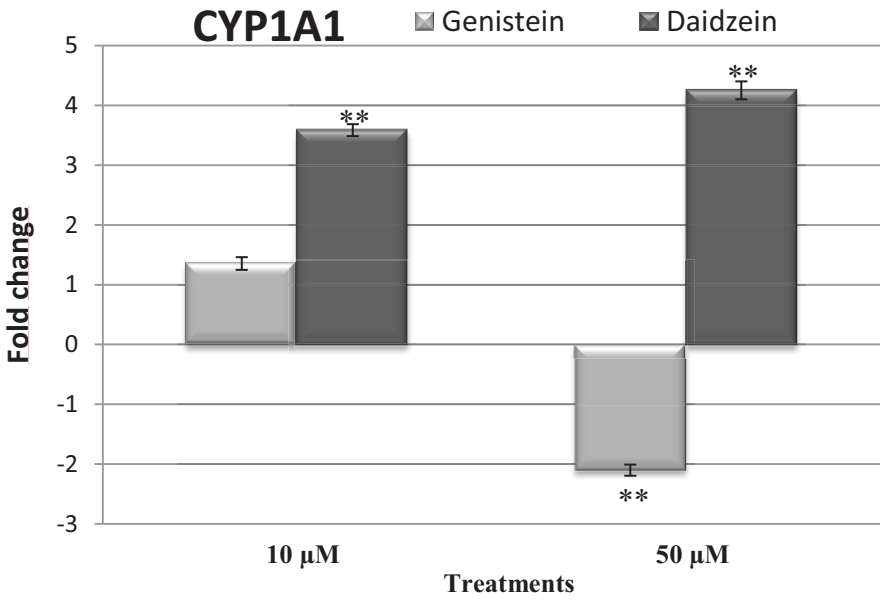

Figure 2. *CYP1A1* mRNA expression following a 12h exposure to genistein and daidzein in HepG2 cells. Data represents the means ± SD of the two experiments. Significant differences of genistein- and daidzein-induced effects and control values were calculated according to the Pfaffl method[17]. The (*) indicates a significant difference (p<0.003) and (*) indicates a significant difference (p<0.001) between the group treated and the group control.

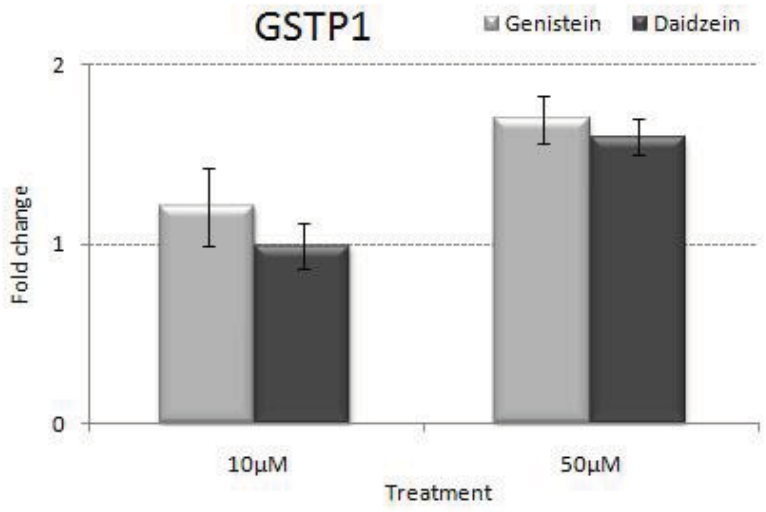

Figure 3. *GSTP1* mRNA expression following a 12 h exposure to genistein and daidzein in HepG2 cells. Data represents the means ± SD of the two experiments. Significant differences of genistein- and daidzein-induced effects and control values were calculated according to the Pfaffl method (2001).

Discussion

The human hepatocellular carcinoma HepG2 cells are a highly differentiated cell line that have retained the activity of a number of phase I and phase II enzymes which play an essential role in the activation and detoxification of promutagens and procarcinogens.[18] Making use of this in vitro model, the present study was designed to investigate whether the flavonoids, genistein and daidzein, effected the expression of the cytochrome P450 phase I (CYP1A1) and II (GSTP1) enzymes in the Hep G2 cell line. The genistein and daidzein non-cytotoxic concentrations (10 and 50 μM) were selected from a preliminary MTT assay.

Our study showed that isoflavones display differential effects on CYP1A1 gene expression. Addition of the genistein in the culture medium at 50 μM decreased *CYP1A1* gene expression about two-times compared to the control. Because the induction of CYP1 is associated with the activation of many carcinogens and numerous compounds that exert their genotoxic and carcinogenic effects only after metabolic activation, genistein-mediated inhibition of CYP1A1 activity may contribute to the mechanism by which these soybean isoflavones protect against carcinogenesis. This supposition is supported by Chan & Leung,[19] who reported that genistein inhibits CYP1A1 enzyme activity and expression by polycyclic aromatic hydrocarbons (PAHs) induced in human breast cancer cell line (MCF-7). Additionally, Shertzer *et al*.[20] demonstrated that in the mouse hepatoma cell line (hepa1), genistein prevents the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-dependent induction of CYP1A1 activity in a concentration-dependent manner.

In contrast with the genistein effect on *CYP1A1* expression in our study, daidzein produced a dose-dependent increase in *CYP1A1* mRNA expression of about three and four times compared to the control. In this context, if we accept the current model, CYP1A inducers would be detrimental effects upon individuals and might increase the human risk of cancer development by way of metabolic activation of PAHs and other chemicals to ultimate carcinogens or reactive toxic intermediates.

However, an in vivo study demonstrated that pre-treatment with daidzein significantly reduced the increase in CYP1A1 gene expression by 7,12-dimethylbenz[α]anthracene (DMBA) in a dose-dependent manner.[21] Moreover,

many flavonoids, such as galangin, curcumin and quercetin have been found to either stimulate an increase in CYP activity or induce *CYP1* expression, or both. They also antagonize the same expression induced by DMBA.[22-24] Additionally, recent literature indicates that these flavonoids are linked with a diminished risk of cancer[6,25] by inhibiting the proliferation of tumor cells.[26,27,28,29] Androutsopoulos *et al*.[13] showed that aromatic hydroxylation reactions of dietary flavonoids catalyzed by CYP1 enzymes promote anti-proliferative activity toward breast cancer cells. It seems that the more active compounds are those that produce a larger number of CYP1-metabolites. Similarly, Atherton *et al*.[30] observed that the metabolism of daidzein in cultured MCF-7 cells (CYP1A1/A2-mediated), enhances the pharmacological and cytotoxic properties of the parental compound. Although the effects of flavonoids on cell growth and proliferation have been extensively studied in recent decades, only recently have the substrate-like interactions with CYP1 enzymes been explored. The induction of CYP1A1 mRNA by daidzein in our study may indicate that daidzein is a natural inducer of this metabolic pathway in the HepG2 cell line.

We also have studied the effect of genistein and daidzein on the expression of the phase II enzyme, GSTP1. GSTs constitute a complex supergene family that collectively metabolize chemotherapeutic drugs, carcinogens and environmental pollutants.[31] Activation of phase II enzymes by flavonoids resulted in detoxification of carcinogens and represented an effective strategy for reducing susceptibility to carcinogens.[6]

In our study, there were no significant changes in GST expression after exposure to genistein or daidzein at 10 or 50 μM in HepG2 cells. The lack of effect on GSTP1 expression by genistein and daidzein in our study differed from the results of Steiner *et al.*[12], which showed that genistein induces the activity and expression of GSTP1 in human mammary non-neoplastic MCF-10A cells. Differences that may be responsible for these contrasting results may be the source of the tissue used in the studies because cytosolic GSTs show tissue- and cell-specific isoform expression.[32]

This study showed that genistein and daidzein act as inhibitors and inducers of CYP1A1 mRNA expression, respectively in cultured HepG2 cells. It is remarkable that genistein and daidzein, which exhibit structural similarity, can have such differing effects. Although many reports emphasized potential

chemopreventive effects of flavonoids in humans, the present results with the human HepG2 cell line showed that more experimental data are required to define the usual role of CYP1A1. Moreover, we reinforced the idea that HepG2 provides an excellent model for investigation of the phase I enzymes CYP1A1.

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7. Artigo 3

The effects of genistein and daidzein on cell proliferation kinetics in HT29 colon cancer cells: the expression of *CTNNBIP1* (β-catenin), *APC* (adenomatous polyposis coli) and *BIRC5* (survivin)

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The effects of genistein and daidzein on cell proliferation kinetics in HT29 colon cancer cells: the expression of *CTNNBIP1* (β-catenin), *APC* (adenomatous polyposis coli) and *BIRC5* (survivin)

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Abstract

Several phytochemicals and micronutrients that are present in fruits and vegetables are known to exert cancer chemopreventive effects in a number of organs, including the colon. Soybean isoflavonoids have received significant attention due to their potential anticarcinogenic and antiproliferative effects and possible role in many signal transduction pathways. Here, we provide experimental evidence that genistein could reduce the proliferation of the human colon adenocarcinoma grade II cell line (HT-29) at concentrations of 25 – 100 μM. Similarly, daidzein is only capable of inhibiting the growth of HT-29 at concentrations of approximately 100 μM. In order to elucidate the mechanisms that underlie the anti-tumor effects of genistein and daidzein, we examined the influence of genistein and daidzein on the expression of *Adenomatous polyposis coli (*APC), β-catenin and survivin via real-time RT-PCR. The obtained data indicate that neither genistein nor daidzein effect APC or survivin expression when cells were treated with 10- or 50-μM concentrations. The expression of β-catenin was suppressed by genistein at a dose of 50 μM; however, the effects of daidzein were not the same. These data suggest that the down-regulation of β-catenin by genistein may constitute an important determinant of the suppression HT-29 cell growth and may be exploited for the prevention and treatment of colon cancer.

1. Introduction

Dietary chemoprevention agents have received significant attention in the area of cancer research. It is estimated that one third of all cancers could be prevented simply by eating a healthy diet, maintaining an ideal body weight, and participating in regular physical activity (American Cancer Society, 2009). Because of the association of Japanese and Chinese diets with lower rates of many forms of cancer, such as breast, prostate, and colon cancer, investigators have assumed that this phenomenon is due to soy food consumption (Birt *et al*.,

2001). Isoflavones are a class of organic compounds that have been found in large quantities in the soybean. In various experimental models, isoflavones have exhibited properties that suggest that they reduce the risk of certain cancers, heart disease, and osteoporosis and relieve symptoms of menopause (Klein & King, 2007). In addition to their variety of health-promoting activities, flavonoids themselves are believed to have little to no toxicity and have a long history of human consumption, making them excellent candidates for chemopreventive agents (Moon *et al*., 2006); however, the specific mechanisms of action of isoflavonoids, with respect to cancer prevention, are not clear and appear to be varied, complementary, and/or overlapping (Birt *et al*., 2001).

It is now well accepted that the Wnt-signaling pathway plays an important role in the control of cell growth and apoptosis (Polakis, 2000). *Adenomatous polyposis coli (*APC) is a component of the Wnt signaling pathway and is best known for its ability to down-regulate β-catenin and its consequent effects on transcriptional regulation (Polakis, 2000). In addition, APC has been shown to control survivin expression via β-catenin–Tcf4 signaling in colon cancer cells (Zhang *et al*., 2001). Mutations in the APC gene are an early event in colorectal tumorigenesis and can be detected in a majority of colorectal tumors, although the overall frequency of β-catenin mutations is quite low (Polakis, 2000). Recent studies have demonstrated that genistein confers protection from breast cancer through its post-transcriptional regulation of E-cadherin expression, leading to the sequestration of β-catenin in the membrane and the inhibition of β-catenin transcriptional activity (Su & Simmen, 2009). Additionally, genistein has been shown to inhibit survivin expression in malignant B cells (Mansour *et al*., 2004) and prostate cancer cells (Suzuki *et al*., 2002). Survivin is overexpressed in the tumors of the lungs, breast, colon, stomach, esophagus, pancreas, bladder, uterus, ovaries, large-cell non-Hodgkin's lymphoma, leukemias, neuroblastoma, melanoma and non-melanoma skin cancers (Altieri, 2001); however, survivin was found to be absent or in low concentrations in most terminally differentiated normal tissues (Pennati *et al*., 2007). Survivin is an anti-apoptotic protein that is also involved in mitotic checkpoint control and growth factor-induced apoptosis in human cancer cells. The overexpression of survivin by neoplasms correlates with more aggressive behavior, decreased responsiveness to chemotherapeutic

agents, and shortened survival when compared to cancers that are survivin-negative (Fukuda & Peleus, 2006).

In this study, we analyzed the effect of genistein and daidzein on cell proliferation kinetics in colon cancer HT29 cells in vitro. We sought to determine if genistein and daidzein could inhibit the growth of HT-29 cells and the possible involvement of β-catenin, APC and survivin gene expression.

2.Materials and methods

2.1. Chemical Agents

Genistein (CAS nº 446-72-0) and Daidzein (CAS nº 486-66-8) were obtained from Acros Organics and were dissolved and diluted in dimethyl sulfoxide (DMSO, Mallinckrodt Chemicals) so as to prepare the appropriate concentrations. The final DMSO concentration in the medium did not exceed 1%. Doxorubicin (DXR, Adriblastina Pharmacia) was diluted in phosphate buffered saline (PBS). The concentrations of genistein and daidzein that were used in the present study were 10 and 50 μM, which is consistent with typical procedures in the literature, and can be considered to be realistic average intestinal concentrations (Sergent et al., 2009). As a positive control, doxorubicin was used at a concentration of 0.05 μg/mL.

2.2. HT29 cell culture conditions

The human colon adenocarcinoma grade II cell line (HT-29) was obtained from the cell bank of Rio de Janeiro (RJCB – Brazil). The cells were grown as monolayer cultures in 25-cm^2 culture flasks in DMEM medium (Gibco) that had been supplemented with 10% fetal bovine serum (FBS, Gibco) (Gibco, BRL). The cultures were maintained in a humidified incubator at 37°C and 5% CO_2.

2.3. Cell proliferation kinetic assay

The cells (3.5×10^4) were seeded on 6-well tissue culture dishes and cultured for 24, 48, 72 and 96 h in a medium that contained genistein or daidzein at 10, 25, 50 and 100 μM and doxorubicin at 0.1 μM. The number of cells was counted every 24 h. The cells were trypsinized (0.1%) and quantified using a

hemacytometer chamber with 0.4% trypan blue (Gibco, BRL) (Trypan blue/cell suspension v/v). The cell proliferation kinetics was estimated by exponential regression. The cell numbers were determined in triplicate.

2.4. Real-time RT-PCR

The HT-29 cells (1 x 10^6/flasks) were pre-incubated for 24 h and subsequently exposed to genistein and daidzein at 10 and 50 µM concentrations for 12 hours. Total RNA was prepared from genistein, daidzein or DMSO-treated HT-29 cells using TRIZOL® LS reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. The RNA samples were incubated with DNase (1U) (Invitrogen Life Technologies). The quantity of the RNA prepared from each sample were determined by spectrophotometric analysis (Bio Photometer - Eppendorf), and the RNA integrity was analyzed by 1% agarose gel electrophoresis. The cDNA synthesis was carried out in reactions containing (20 µl) total RNA (1 µg), 10 pmol/µL oligo dT primer (1µL), 10 mM dNTP (2µL), RNAseOUT (0.1µL) and reverse Transcriptase M-MLV (1µL) (Invitrogen life Technologies). The real-time RT-PCR was performed using Platinum® SYBR Green qPCR SuperMix-UDG (Invitrogen Life Technologies). A total of 0.4 µM of each primer and 2 µL of template cDNA were added to a final reaction volume of 20 µL. The reactions were carried out in a PTC 200 DNA Engine Cycler using a Chromo4 Detection System (MJ Research). The oligonucleotides that were utilized in these experiments are listed are in table 1.

The reaction conditions were performed as follows: 95ºC for 3 min, 40 cycles (95ºC for 30s, 60ºC for 30s and 72ºC for 20 s), 95ºC for 10s and 40ºC for 1 min, followed by melting curve analysis at a temperature between 50 to 90ºC. The data were normalized to the *GAPDH* housekeeping gene. The relative differences between the HT-29 control (DMSO) and treatment groups (genistein or daidzein) were calculated according to the Pfaffl method (2001).

Each experiment was performed in triplicate in two independent experiments. Data are expressed as the average values ± standard derivation (SD).

Table 1. Oligonucleotide primers used for RT-PCR.

	Sequence	NCBI/Size	Ref.
GAPDH	Forward - 5'- GAA GGT GAA GGT CGG AGT C -3'	NM_002046	Weglarz et al., 2006
	Reverse - 5'- GGA AGA TGG TGA TGG GAT TT -3'	227 bp	with modifications
Survivin	Forward - 5'- AGC CCT TTC TCA AGG ACC AC -3'	NM_001168.2	Present
	Reverse - 5'-TGG CTC GTT CTC AGT GGG GCA GT-3'	125 bp	study
β-catenin	Forward – 5'- CCT ATG CAG GGG TGG TCA AC - 3'	NP_064633	Present
	Reverse – 5'- CGA CCT GGA AAA CGC CAT CA - 3'	95 bp	study
APC	Forward - 5'- AAA GCG CCA TGA TAT TGC ACG GTC – 3'	M 74088	Present
	Reverse – 5'- TGT TTG CTG TGC TCA CGT TTC CAG -3'	93 bp	study

2.5. Statistical analyses

The cell proliferation kinetics was estimated by exponential regression as described by Weisstein, <http://mathworld.wolfram.com/LeastSquaresFittingExponential.html>. Statistical analyses were performed via analysis of the variance ANOVA followed by the Dunnett test. REST (Relative Expression Software Tool - 384 = REST-384 © - version 2) (Pfaffl et al., 2002) software was used for the statistical analysis. For the mRNA induction or repression studies, only levels of greater than 2-fold with statistically significant differences were considered to be relevant.

3. Results

3.1. Cell proliferation kinetic assay

The exposures of the HT-29 cells to genistein and daidzein at 0, 10, 25, 50 and 100 μM for 24, 48, 72 and 96 hours is illustrated in figures (1 and 2) and tables (2 and 3). The HT-29 cells that were exposed to genistein showed decreased in the number of viable cells, except for genistein at 100 μM. At 96 hours, we observed reductions of 15, 48 and 97% when the cells were treated with 25, 50 and 100 μM of genistein, respectively. At 10 μM of genistein, no alteration of HT-29 cell proliferation was observed. Figure 2 depicts the results that were obtained when HT-29 cells were exposed to different concentrations of

daidzein. Daidzein did not inhibit the proliferation kinetics at concentrations ranging from 10 or 25 μM. The HT-29 cells exhibited a decline of 48% viability when exposed to 100 μM of daidzein, and the observed reduction in growth was dose- and time-dependent. The cell viability of HT-29 cells was unaffected (≥ 97%) by 10 – 100 μM concentrations of genistein or daidzein after 72 hours ($p > 0.05$) and was ≥ 93% after 96 h at all of the tested concentrations ($p > 0.05$).

Table 2. Cell proliferation kinetics after 24, 48, 72 and 96 h of genistein-treated HTC cell. Data are means ± standard deviations from three independent experiments for HT-29.

	24 h	48 h	72 h	96 h
CTRL	7.4 ± 0.7	17.4 ± 2.0	58.0 ± 7.9	125.9 ± 17.3
DOXO 0.1 μM	4.6 ± 0.7**	3.6 ± 1.2**	40.9 ± 0.9**	2.5 ± 0.4**
GEN 10 μM	6.3 ± 1.0	14.2 ± 1.8	49.3 ± 9.2	125.3 ± 13.7
GEN 25 μM	5.4 ± 0.7*	14.0 ± 1.0*	40.1 ± 5.5*	107.2 ± 10.7
GEN 50 μM	3.7 ± 0.6**	9.6 ± 1.2**	26.6 ± 6.5**	66.4 ± 6.5**
GEN 100 μM	4.0 ± 0.8**	4.2 ± 0.6**	6.9 ± 1.9**	4.3 ± 0.3**

*P<0.05. **P<0.01

Table 3. Cell proliferation kinetics after 24, 48, 72 and 96 h of daidzein-treated HTC cell. Data are means ± standard deviations from three independent experiments for HT-29.

	24 h	48 h	72 h	96 h
CTRL	7.4 ± 0.7	19.0 ± 2.7	59.3 ± 5.0	122.0 ± 3.1
DOXO 0.1 μM	4.6 ± 0.3**	4.7 ± 1.4**	5.1 ± 0.6**	2.4 ± 1.2**
DAID 10 μM	7.4 ± 0.5	19.0 ± 0.3	64.1 ± 5.3	117.0 ± 5.8
DAID 25 μM	7.0 ± 1.3	16.5 ± 1.2	60.1 ± 7.9	115.9 ± 7.4
DAID 50 μM	7.0 ± 0.8	14.2 ± 0.8*	54.3 ± 1.6	115.4 ± 13.4**
DAID 100 μM	6.5 ± 0.3	11.4 ± 2.3**	41.7 ± 7.8**	63.0 ± 18.7**

*P<0.05. **P<0.01

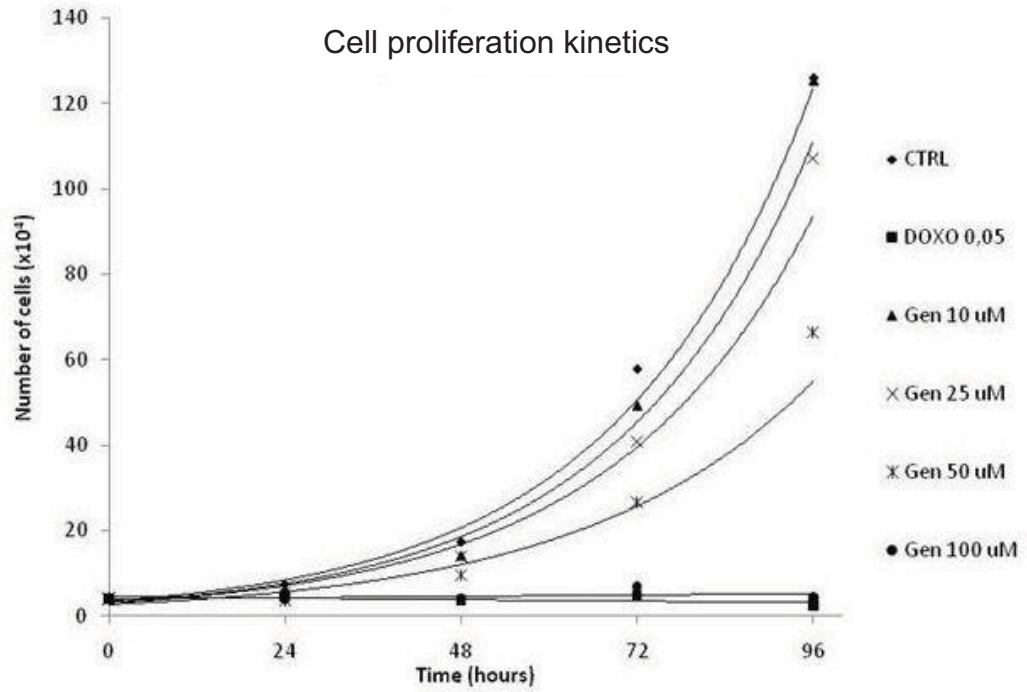

Figure 1. Effects of genistein on HT-29 cell proliferation kinetics.

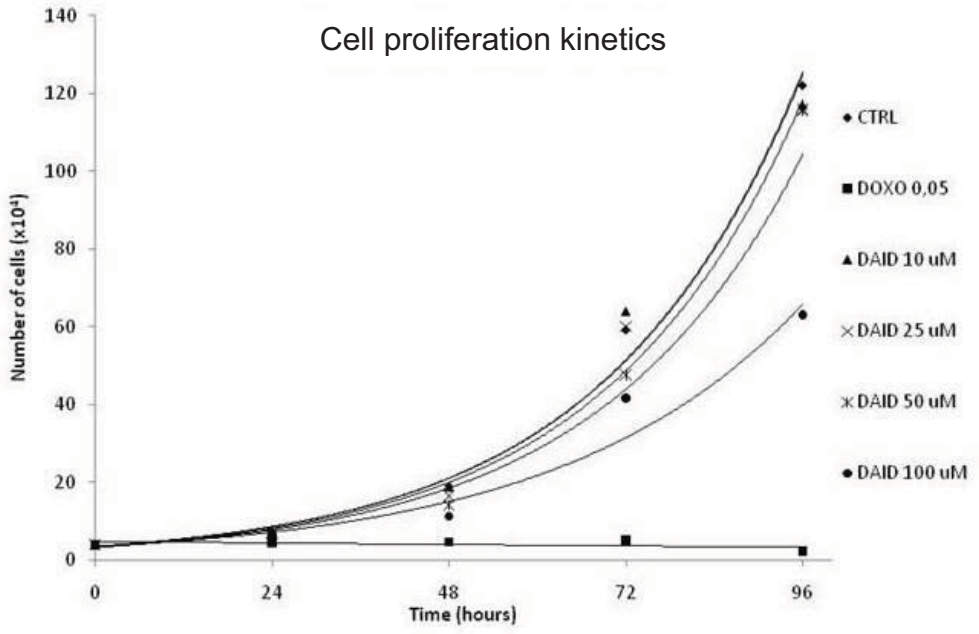

Figure 2. Effects of daidzein on HT-29 cell proliferation kinetics

3.2. Real-time RT-PCR

Based on the data that were obtained from the cell proliferation kinetics assay, we selected the concentrations of 10 and 50 μM of genistein and daidzein in the analysis of *APC,* β-catenin and survivin participation for when HT-29 cells are exposed to flavonoids. Data from the real-time RT-PCR indicated that the β-catenin transcript levels were 2.4-fold lower in cells that were treated with 50 μM of genistein (Figure 3). The HT-29 cells exposed to 10 μM of genistein did not show any significant difference. The transcript levels for the APC and survivin genes analyzed, but no significant differences were observed (Figure 4 and 5). When HT29 cells were exposed to 10 and 50 μM of daidzein, there were no significant differences in the transcript levels of β-catenin, APC and survivin (Figure 3, 4 and 5*).*

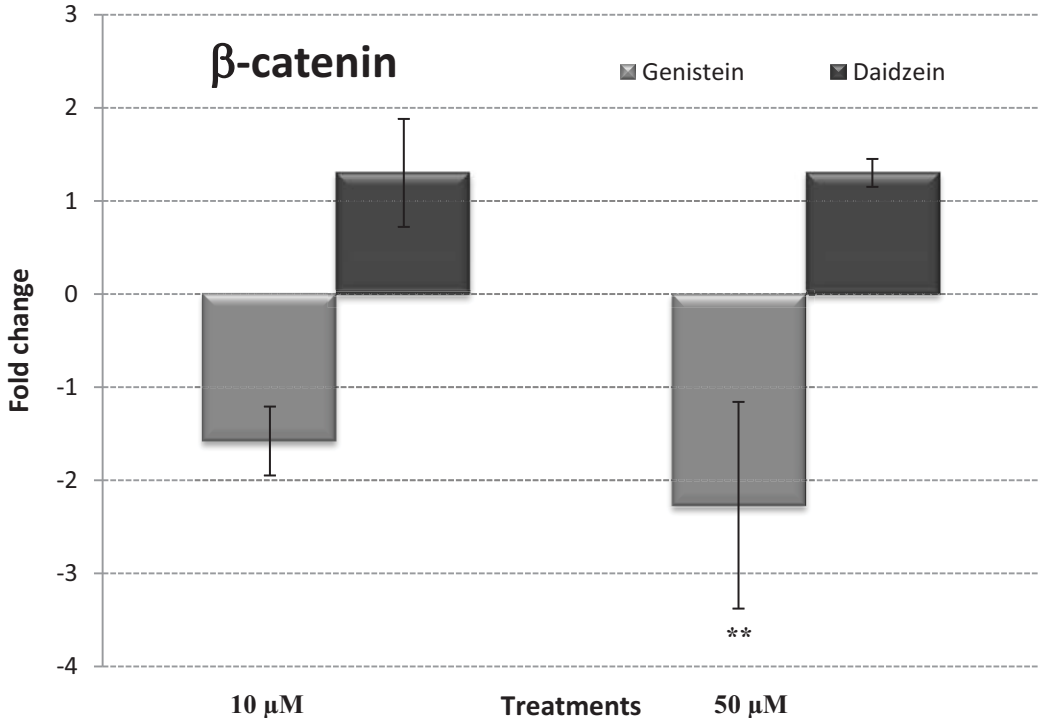

Figure 3. β-catenin mRNA expression after HT-29 cells were exposed to genistein and daidzein for 12 hours. The data represent the means ± SD of two experiments. Significant differences between genistein- and daidzein-induced effects and the control values were calculated according to the Pfaffl method (2001).

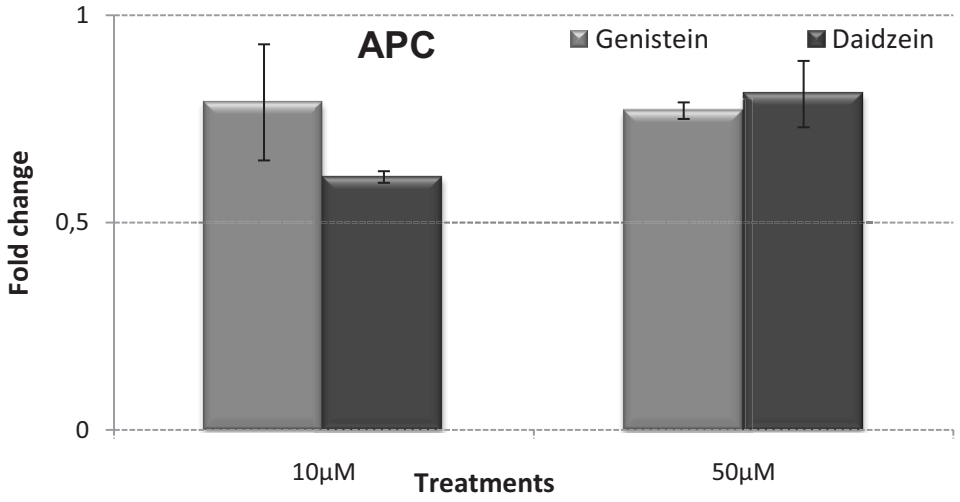

Figure 4. APC mRNA expression after HT-29 cells were exposed to genistein and daidzein for 12 hours. The data represent the means ± SD of two experiments. Significant differences between genistein- and daidzein-induced effects and the control values were calculated according to the Pfaffl method (2001).

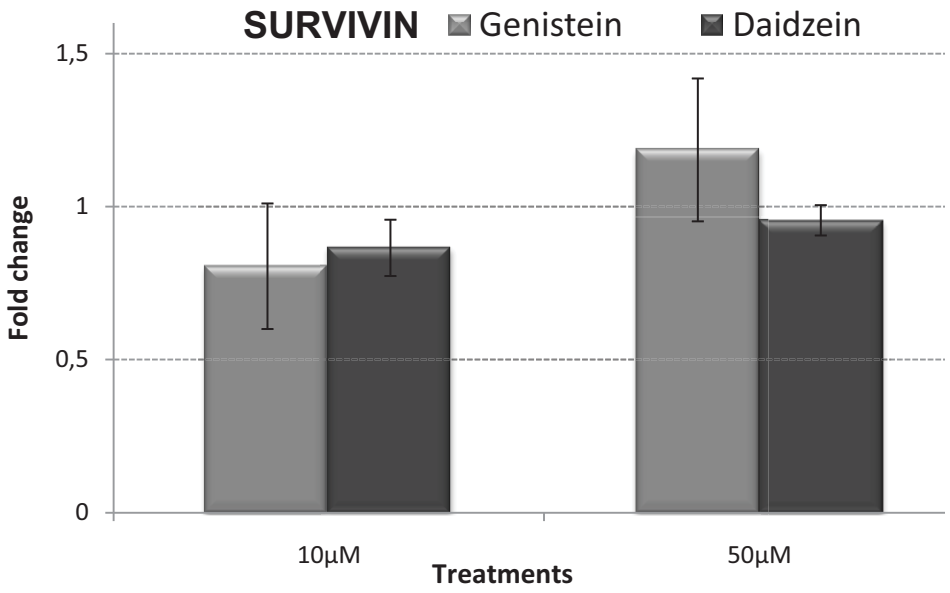

Figure 5. Survivin mRNA expression after HT-29 cells were exposed to genistein and daidzein for 12 hours. The data represent the means ± SD of two experiments. Significant differences between genistein- and daidzein-induced effects and the control values were calculated according to the Pfaffl method (2001).

4. Discussion

Flavonoids inhibit the growth of human colorectal cancer cell lines (Kanadaswami, 2005) and suppress the formation and development of azoxymethane (AOM)-induced colonic aberrant crypt foci (Birt *et al*., 2001). The possible biological mechanisms by which flavonoids exert their anticarcinogenesis effects include their antioxidative and anti-inflammatory properties, antiproliferative activity and cell-cycle arrest, induction of apoptosis, modulation of multidrug resistance and modulation of phase I and II metabolizing enzymes (Ren *et al*., 2003; Kanadaswami, 2005, Banerjee *et al*., 2008). In addition, flavonoids can affect the Wnt signaling that is involved in tumor development by their regulation of cell proliferation via β-catenin (Polakis, 2000).

In the present study, we have demonstrated that the exposure of HT-29 cells to genistein and daidzein results in a dose- and time-dependent inhibition of cell proliferation. Genistein is a more effective inhibitor of cell proliferation than daidzein. Genistein was observed to exert inhibitory effects on HT-29 cells after 24 hours of culture at concentrations ranging from 25 to 100 μM, whereas the inhibitory effect of daidzein occurred at 72 hours after administration at a concentration of 100 μM. These results are consistent with a previous study by Kuntz *et al*. (1999) in HT-29 and Caco-2 cells that were screened with 36 flavonoids. The authors observed that the reduced cell proliferation effect is dependent of the flavonoid tested and the cell type. Others have shown that genistein inhibits the growth and proliferation of MCF-7 (Chen *et al*., 2003) and both of the B16 and K1735M2 murine melanoma cell lines (Wang *et al*., 2007) at concentrations of 50 and 60 μM, respectively; however, in other cell types, even low doses can cause growth inhibition. The treatment of HepG2 cells for 48 h with 4, 8, 12 and 16 μM of genistein resulted in the inhibition of cell proliferation (Chodon *et al*., 2007). Daidzein significantly decreased the proliferation of MCF-7 and MDA-MB-453 cells after 24 h at concentrations ranging from 10 and 1 μM, respectively (Choi *et al*., 2008). In the HeLa cell line, genistein inhibited growth at a concentration of 20 μM, whereas daidzein had no influence on growth (Wang *et al*., 2007).

Notably, in our study, high concentrations of genistein resulted in the complete inhibition of cell proliferation but did not induce any sign of cytotoxicity, as was seen in the viability assay, suggesting that the inhibition of cell proliferation is mediated by changes in the cell cycle progression and/or the induction of apoptosis. In order to investigate the reduction in the proliferation of the HT-29 cells that have been exposed to genistein and daidzein, we analyzed the expression of the genes β-catenin and APC, which are involved in cell growth by signaling the Wnt pathway that is commonly activated in colon cancer. The activation of this pathway promotes the accumulation of β-catenin in the nucleus, resulting in the transcriptional activation of specific target genes for cellular proliferation (c-Myc) and the consequent progression of cancer (Narayan & Roy, 2003; Sarkar, 2009). In this work, there was a decrease in the expression of β-catenin in HT-29 cells that were treated with 50 μM of genistein, indicating a reduction in cell proliferation; however, HT-29 cells show mutations in the APC gene that participates, through a multiprotein complex, in the regulation of β-catenin expression (Yang *et al*., 2004). These mutations almost always result in a truncated protein product with abnormal functioning (Fearnhead *et al*., 2001). Because of this fact, we analyzed the APC gene expression; however, as expected, no alterations in the APC gene expression were found, confirming that the Wnt pathway is under regulation of other genistein-dependent intracellular pathways. Several reports exist concerning the reduction of cell growth due to the effect of isoflavones on the inactivation of Wnt pathway genes (Lee *et al*., 2005; Park *et al*., 2005a; Park *et al*., 2005b; Li *et al*., 2008). The results from in vitro assays that examined the effects of the flavonoids quercetin, naringenin and flavanone on *β*-catenin/Tcf signaling in AGS gastric and SW480 colon cancer cells have previously been published (Lee *et al*., 2005; Park *et al*., 2005a; Park *et al*., 2005b), and their data suggest that the different investigated flavonoids efficiently inhibit β-catenin/Tcf signaling, although a common mechanism by which flavonoids inhibit Tcf activity remains to be established. Li *et al*. (2008) found that isoflavone upregulated the expression of GSK-3β, enhanced GSK-3β binding to *β-catenin*, and increased the phosphorylation of *β-catenin*, suggesting that isoflavone could inhibit prostate cancer cell growth by the inactivation of Wnt signaling.

Others have also reported that isoflavone could inhibit the expression of the Wnt signaling components sfrp2 and Wnt5a in rat mammary epithelial cells (Su *et al*., 2007). Our results demonstrate that genistein decreased β-catenin expression, and this reduction may, consequently, lead to a decline in the levels of cytoplasmic β-catenin that is independent of the cytosolic degradation of β-catenin by the multiprotein complex (APC-GSK-3β-axin). Because HT-29 cells contain no intact APC protein, we can conclude that genistein might exert its antiproliferative effects on the HT-29 cell line, at least in part, via the down-regulation of β-catenin.

Another possible mechanism for the antiproliferative activity of the flavonoids may be mediated by the inhibition of survivin, which is a member of the inhibitor of the apoptosis protein (IAP) family that has been implicated in the control of cell division. Previous studies have demonstrated that survivin is overexpressed in colon cancer, and its overexpression is considered to participate in the development and progression of cancer by suppressing apoptosis and regulating cell division (Altieri, 2001). The down-regulation of survivin also results in a significant inhibition of tumor growth of human gastric carcinoma cells and human pancreatic cancer Patu8988 cells in vitro (Miao *et al*., 2007; Shen *et al*., 2010). We, therefore, examined whether genistein and/or daidzein could inhibit survivin expression in HT-29 cells. We found no significant effect of genistein or daidzein on the expression of survivin in any of the tested doses, suggesting that the inhibition of cell growth shown here is not due to an inhibition of survivin.

In summary, we have analyzed β-catenin, APC and survivin gene expression in HT-29 colon cancer cells that were exposed to genistein and daidzein. Genistein altered the expression of β-catenin, which is involved in the control of the cell cycle. Our results reveal a novel molecular mechanism wherein genistein can exert its inhibitory effects on colon cancer cells. The down-regulation of β-catenin by genistein may constitute an important determinant of the suppression HT-29 cell growth and may be exploited for the prevention and treatment of colon cancer.

Acknowledgements

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8. CONCLUSÕES GERAIS

Os resultados obtidos no presente trabalho permitiram constatar que:

A genisteína:

- apresentou atividade antimutagênica em células de hepatoma de rato (HTC) quando associada a agentes indudores de danos ao DNA de ação direta (Doxorrubicina) e indireta (2-aminoantraceno);
- induziu um aumento de atividade das enzimas citoplasmáticas GSTs em células de hepatoma de rato (HTC) de forma tempo dependente;
- reduziu significativamente o transcrito referente ao gene CYP1A1 em células de hepatoma humano (HepG2);
- apresentou atividade inibitória de proliferação celular em carcinoma de cólon humano (HT-29),
- reduziu significativamente o transcrito referente ao gene β-catenina em carcinoma de cólon humano (HT-29),

A daidzeína:

- induziu um aumento de atividade das enzimas citoplasmáticas GSTs em células de hepatoma de rato (HTC) de forma tempo dependente;
- aumentou o transcrito referente ao gene CYP1A1 em células de hepatoma humano (HepG2);
- não teve efeitos sobre os demais parâmetros analisados

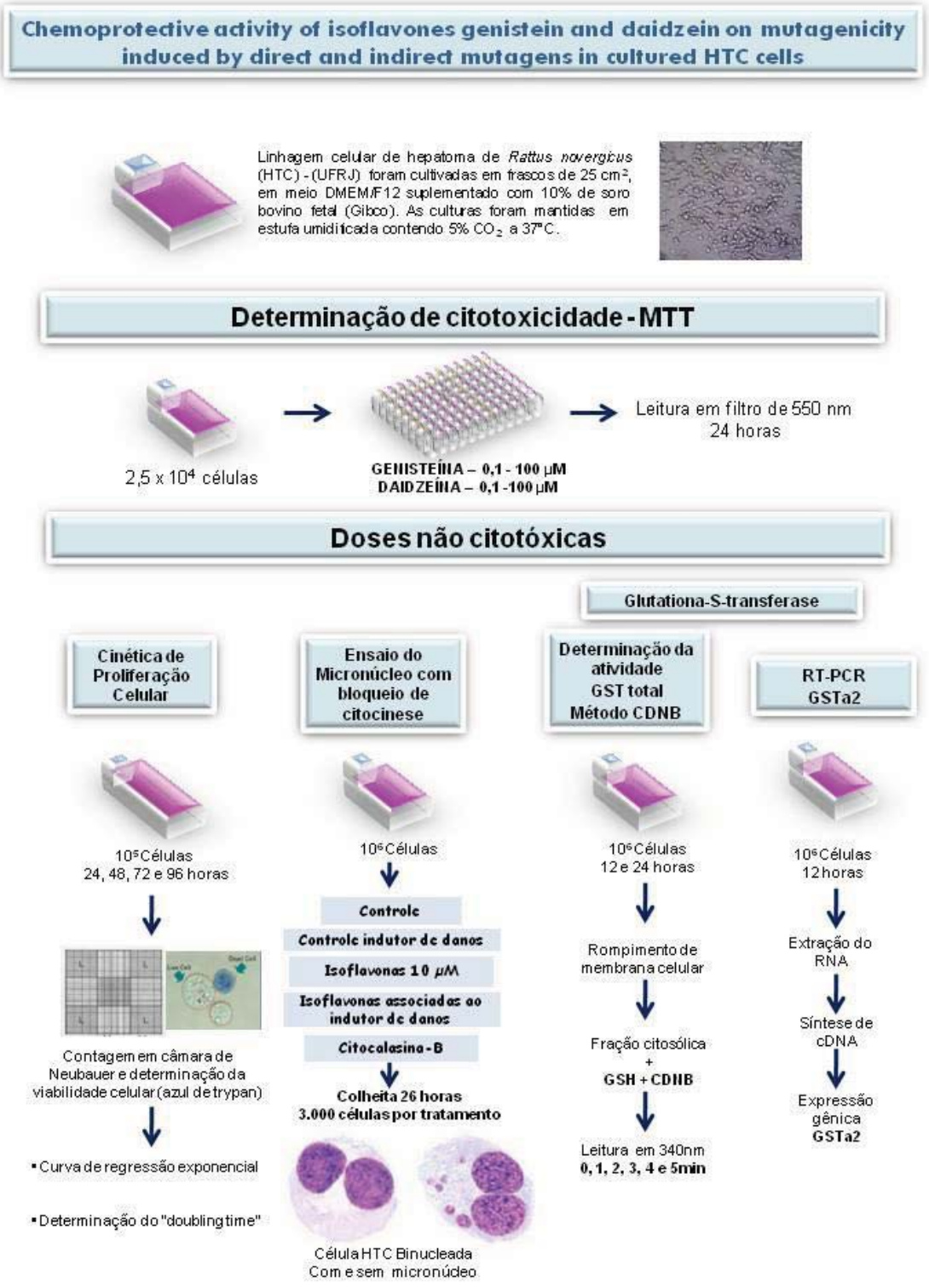
Estes resultados em conjunto e somados a resultados da literatura, mostram que especialmente a genisteína apresenta grande potencial a ser explorado para tratamento e prevenção de câncer.

Atenção especial deve ser dada ao efeito da daidzeína sobre o gene CYP1A1 em linhagem de hepatoma humano (HepG2). Apesar da maioria dos estudos indicarem uma ação deletéria de aumentada expressão de CYP1A1, estudos recentes sugerem que esta enzima pode ter importante papel na quimioprevenção, se opondo ao conceito previamente estabelecido. Outras pesquisas devem ser conduzidas para elucidar o papel de CYP1A1 na célula.

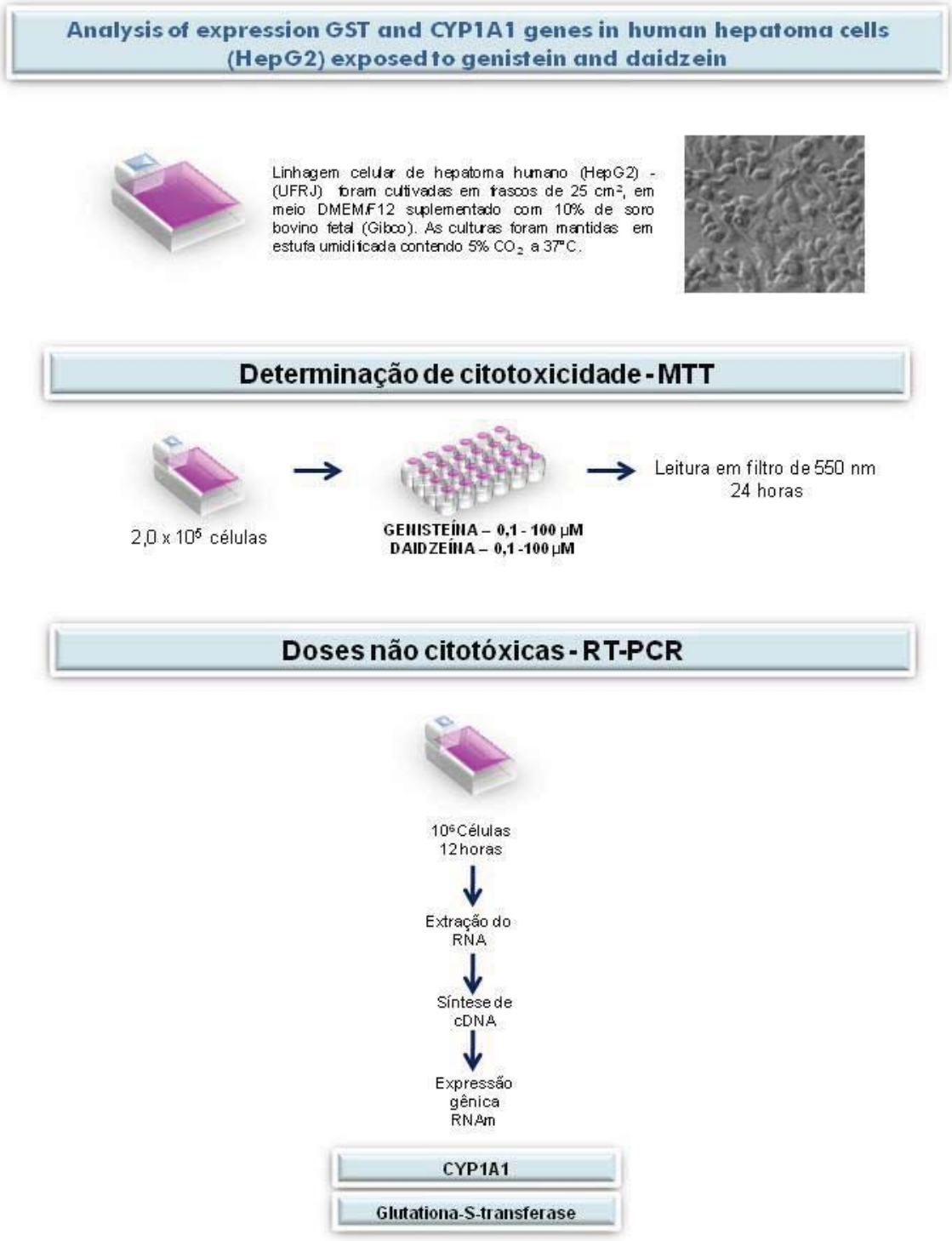
9. ANEXOS

9.1. Delineamento experimental

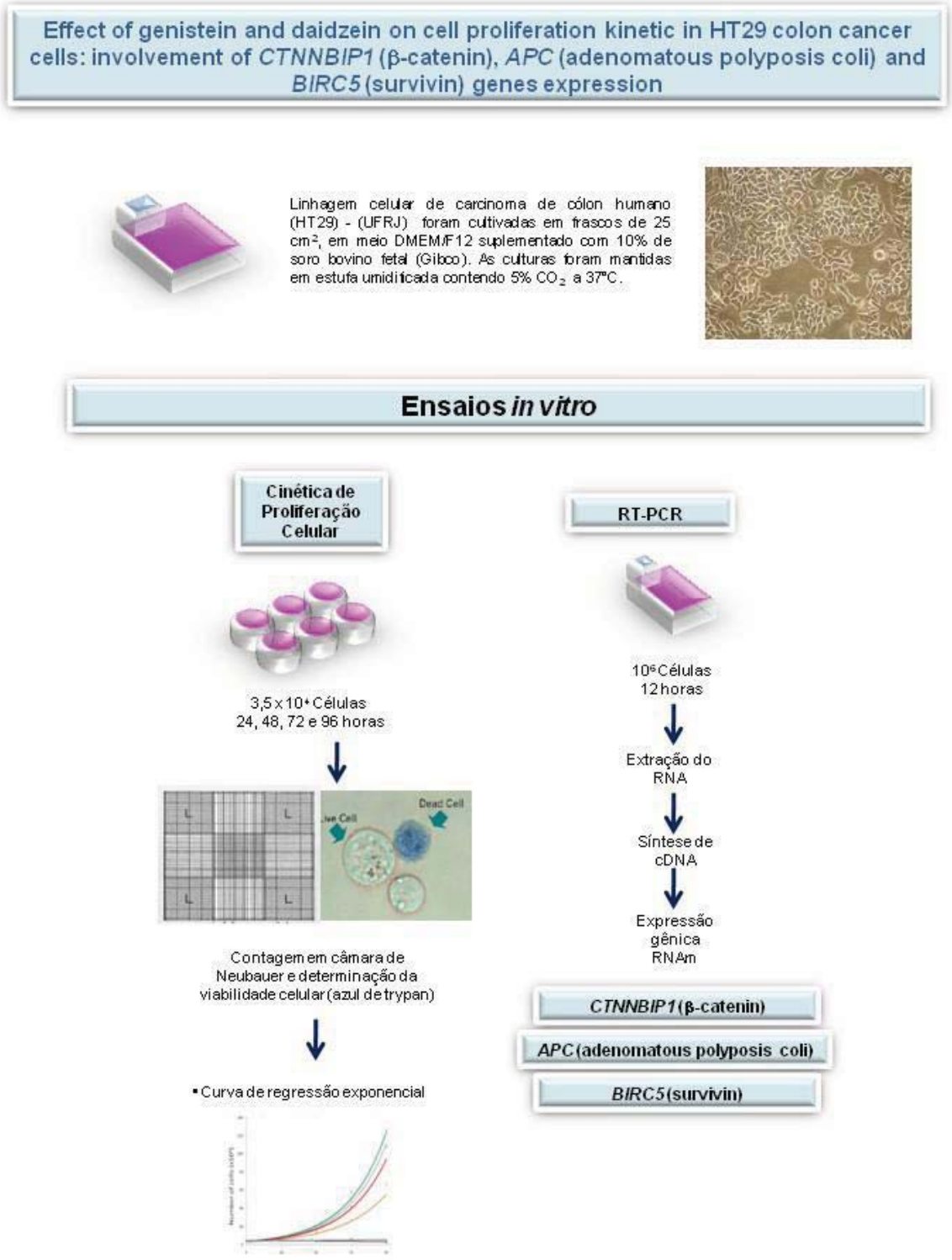
9.1.1. Delineamento experimental I.



9.1.2. Delineamento experimental II.



9.1.2. Delineamento experimental III.



9.2. Ensaio de citotoxicidade – MTT

9.2.1. Resultados de absorbância (550nm) e porcentagem de viabilidade celular obtidos através do ensaio MTT em células **HTC** (hepatoma de rato) tratadas com diferentes doses de genisteína após em 24 horas de cultivo. Resultados de absorbância foram submetidos a análise de variância (ANOVA), seguido do teste Tukey.

Genisrteína 24 horas	Absorbância 550nm	DV	% do controle	DV
Controle	1,09	0,03		
DXR 10µg/mL	0,53	0,02	48,37	2,81
Genisteína 0,1 µM	1,06	0,03	96,62	2,95
Genisteína 1 µM	1,06	0,01	96,74	3,18
Genisteína 10 µM	1,06	0,03	97,01	1,91
Genisteína 50 µM	0,76*	0,03	69,88*	1,58
Genisteína 100 µM	0,77*	0,03	70,33*	4,23

*p<0,001

9.2.2. Resultados de absorbância (550nm) e porcentagem de viabilidade celular obtidos através do ensaio MTT em células **HTC** (hepatoma de rato) tratadas com diferentes doses de daidzeína após 24 horas de cultivo. Resultados de absorbância foram submetidos a análise de variância (ANOVA), seguido do teste Tukey.

Daidzeína 24 horas	Absorbância 550nm	DV	% do controle	DV
Controle	1,11	0,03		
DXR 10µg/mL	0,53	0,04	47,52	5,18
Daidzeína 0,1 µM	1,04	0,01	93,41	2,50
Daidzeína 1 µM	1,08	0,02	97,05	2,75
Daidzeína 10 µM	1,09	0,08	98,00	7,96
Daidzeína 50 µM	0,97*	0,03	84,21*	3,44
Daidzeína 100 µM	0,59*	0,03	53,13*	3,49

*p<0,001

9.2.3. Resultados de absorbância (550nm) e porcentagem de viabilidade celular obtidos através do ensaio MTT em células **HepG2** (hepatoma humano) tratadas com diferentes doses de genisteína após em 24 horas de cultivo. Resultados de absorbância foram submetidos a análise de variância (ANOVA), seguido do teste Tukey.

Genisrteína 24 horas	Absorbância 550nm	DV	% do controle	DV
Controle	0,98	0,038		
DXR 1µg/mL	0,43	0,04	43,73	5,95
Genisteína 5 µM	0,97	0,01	99,13	4,50
Genisteína 10 µM	0,96	0,05	98,06	9,04
Genisteína 25 µM	0,93	0,02	95,09	5,91
Genisteína 50 µM	0,96	0,02	97,70	5,76
Genisteína 100 µM	0,83**	0,03	84,19**	4,19

**p<0,01, *p<0,05

9.2.4. Resultados de absorbância (550nm) e porcentagem de viabilidade celular obtidos através do ensaio MTT em células **HepG2** (hepatoma humano tratadas com diferentes doses de daidzeína após 24 horas de cultivo. Resultados de absorbância foram submetidos a análise de variância (ANOVA), seguido do teste Tukey.

Daidzeína 24 horas	Absorbância 550nm	DV	% do controle	DV
Controle	0,900	0,082		
DXR 1µg/mL	0,428	0,044	47,73	4,62
Daidzeína 5 µM	0,866	0,056	96,58	7,74
Daidzeína 10 µM	0,825	0,045	92,57	13,81
Daidzeína 25 µM	0,801	0,061	89,50	9,92
Daidzeína 50 µM	0,797	0,011	89,13	9,48
Daidzeína 100 µM	0,716*	0,076	79,78*	7,82

**p<0,01, *p<0,05

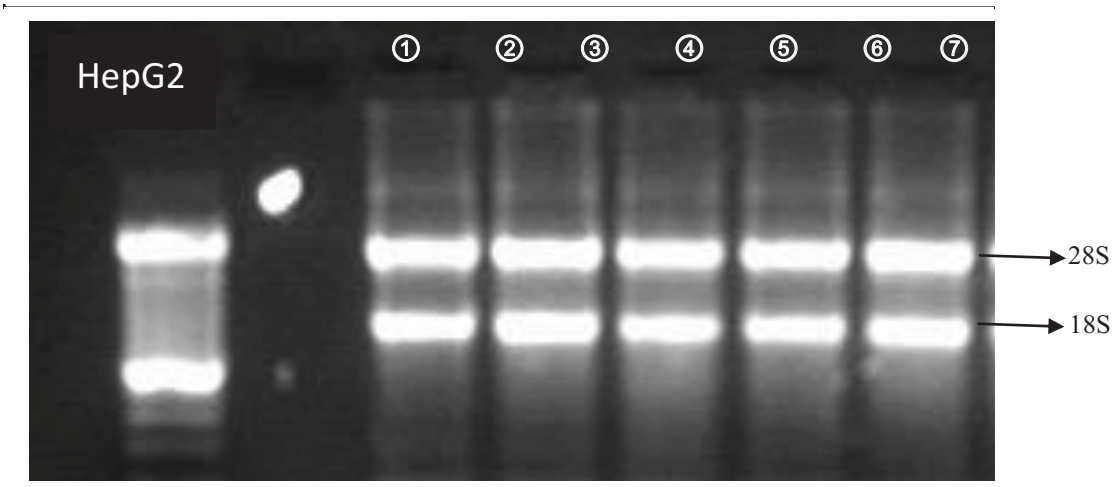
9.3. Ensaios de cinética de proliferação celular

9.3.1. Resultados de proliferação celular em células HTC (hepatoma de rato) tratadas com genisteína e daidzeína após 24, 48, 72 e 96 horas de cultivo. A cinética de proliferação celular foi estimada por regressão exponencial através da fórmula $Y=A\ln e^{BX}$, descrita por Weisstein <http://mathworld.wolfram.com/LeastSquaresFittingExponential.html>

	24h	48h	72h	96h	R^2
Controle	20,00 ± 2,78	47,83 ± 1,94	95,58 ± 9,22	191,00 ± 13,21	0,998
Genisteína 10μM	20,00 ± 1,88	42,66 ± 2,65	88,08 ± 7,87	159,75 ± 20,38	0,998
Daidzeína 10μM	19,08 ± 2,13	39,91 ± 2,76	95,33 ± 14,67	174,08 ± 23,38	0,997

9.4. RT-PCR em tempo real

9.4.1. Imagem representativa para avaliação da integridade do RNA. RNA total isolado de células HepG2.



9.4.2. Quantificação de RNA por espectrofotometria ($A_{260/280}$).

9.4.2.1. Em células de hepatoma de rato (HTC)

Tratamento	Repetição 1		Repetição 2	
	µg/ml	Razão	µg/ml	Razão
Controle	1815	2,09	1416	2,04
Genisteína 10 µM	1403	2,04	1432	2,02
Daidzeína 10 µM	1752	2,03	1884	2,04

9.4.2.2. Em células de hepatoma humano (HepG2)

	Repetição 1		Repetição 2	
Tratamento	µg/ml	Razão	µg/ml	Razão
1 - Controle	1729	2,03	2508	2,05
2 - Gen 5 uM	1452	1,98	1728	2,03
3 - Gen 10 uM	1296	1,98	490	1,91
4 - Gen 50 uM	1290	1,95	1844	2,01
5 - Daid 5 uM	1329	1,98	759	1,98
6 – Daid 10 uM	1256	1,97	1525	1,99
7 – Daid 50 uM	1511	2,01	344	1,95

9.4.2.3. Em células de adenocarcinoma humano (HT-29)

Tratamento	R1		R2	
	µg/ml	Razão	µg/ml	Razão
Controle	2420	2,04	2761	2,00
Genisteína 5 µM	2146	2,00	2069	1,99
Genisteína 10 µM	1977	2,01	2492	2,00
Genisteína 50 µM	2965	2,02	3253	1,99
Daidzeína 5 µM	2600	2,01	2706	2,01
Daidzeína 10 µM	2788	2,01	2941	1,99
Daidzeína 50 µM	2685	2,02	1978	1,98

9.4.3. Resultados detalhados do RT-PCR em tempo real.

9.4.3.1. Resultados detalhados do RT-PCR em tempo real do gene GSTa2 em células de hepatoma de rato HTC. Os dados representam a média aritmética em triplicata para cada repetição (R). Calculado de acordo com método de Pfaffl (2001).

Controle x Genisteína 10µM - R1							
		Controle	Genisteina	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiência	1,88	1,84	1,86			
	Ct	15,20	15,33		-0,13	0,92	
β-actina	Eficiência	1,93	1,77	1,85			0,78
	Ct	11,65	11,39		0,26	1,18	

Controle x Genisteína 10µM R2							
		Controle	Genisteina	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiência	1,92	1,87	1,90			
	Ct	14,48	15,05		-0,57	0,69	
β-actina	Eficiência	1,89	1,79	1,84			0,83
	Ct	10,72	11,01		-0,29	0,84	

Controle x Daidzeína 10µM R1							
		Controle	Daidzeína	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiência	1,88	1,88	1,88			
	Ct	15,20	15,75		-0,55	0,71	
β-actina	Eficiência	1,93	2,00	1,97			0,81
	Ct	11,65	11,86		-0,21	0,87	

Controle x Daidzeína 10µM R2							
		Controle	Daidzeína	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiência	1,92	1,88	1,90			
	Ct	14,48	14,68		-0,20	0,88	
β-actina	Eficiência	1,89	1,90	1,89			1,01
	Ct	10,72	10,92		-0,21	0,88	

9.4.3.2. Resultados detalhados do RT-PCR em tempo real do gene CYP1A1 em células HepG2. Os dados representam a média aritmética em triplicata para cada repetição (R). Calculado de acordo com método de Pfaffl (2001).

Controle x Genisteína 10 R1							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Cyp1a1	Eficiência	1,97	1,93	1,95			
	Ct	21,52	23,07		-1,55	0,35	
GAPDH	Eficiência	1,76	1,82	1,79			1,55
	Ct	15,20	17,74		-2,54	0,23	

Controle x Genisteína 10 R2							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Cyp1a1	Eficiência	1,89	1,98	1,93			
	Ct	21,18	21,44		-0,26	0,84	
GAPDH	Eficiência	1,86	1,78	1,82			1,17
	Ct	16,60	17,16		-0,56	0,72	

Controle x Daidzeína 10 R1							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Cyp1a1	Eficiência	1,97	1,86	1,92			
	Ct	21,52	20,02		1,50	2,66	
GAPDH	Eficiência	1,76	1,86	1,81			4,07
	Ct	15,20	15,92		-0,72	0,65	

Controle x Daidzeína 10 R2							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Cyp1a1	Eficiência	1,89	1,88	1,88			
	Ct	21,18	19,60		1,58	2,72	
GAPDH	Eficiência	1,86	1,79	1,83			3,11
	Ct	16,60	16,82		-0,22	0,88	

Controle x Genisteína 50 R1							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Cyp1a1	Eficiência	1,97	1,94	1,96			
	Ct	21,52	22,31		-0,79	0,59	
GAPDH	Eficiência	1,76	1,80	1,78			0,50
	Ct	15,20	16,05		-0,85	0,61	

Controle x Genisteína 50 R2							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Cyp1a1	Eficiência	1,89	1,87	1,88			
	Ct	21,18	22,10		-0,92	0,56	
GAPDH	Eficiência	1,86	1,72	1,79			0,47
	Ct	16,60	16,31		0,30	1,19	

Controle x Daidzeína 50 R1							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Cyp1a1	Eficiência	1,97	2,00	1,99			
	Ct	21,52	20,58		0,94	1,91	
GAPDH	Eficiência	1,76	1,77	1,76			3,76
	Ct	15,2	16,395		-1,195	0,508588681	

Controle x Daidzeína 50 R2							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Cyp1a1	Eficiência	1,89	1,86	1,88			
	Ct	21,18	18,40		2,78	5,73	
GAPDH	Eficiência	1,86	1,73	1,80			4,85
	Ct	16,60	16,32		0,28	1,18	

9.4.3.3. Resultados detalhados do RT-PCR em tempo real do gene GSTP1 em células HepG2. Os dados representam a média aritmética em triplicata para cada repetição (R). Calculado de acordo com método de Pfaffl (2001).

Controle x Genisteína 10 R1							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiência	1,79	1,81	1,80			
	Ct	26,43	27,83		-1,41	0,44	
GAPDH	Eficiência	1,91	1,90	1,90			1,34
	Ct	14,07	15,81		-1,74	0,33	

Controle x Genisteína 10 R2							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiencia	1,83	1,84	1,84			
	Ct	27,65	27,69		-0,03	0,98	
GAPDH	Eficiência	1,92	1,84	1,88			1,10
	Ct	15,01	15,19		-0,18	0,89	

Controle x Daidzeína 10 R1							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiencia	1,79	1,81	1,80			
	Ct	26,43	26,97		-0,54	0,73	
GAPDH	Eficiência	1,91	1,85	1,88			0,84
	Ct	14,07	14,30		-0,23	0,86	

Controle x Daidzeína 10 R2							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiencia	1,83	1,91	1,87			
	Ct	27,65	27,88		-0,22	0,87	
GAPDH	Eficiência	1,92	1,81	1,86			0,95
	Ct	15,01	15,15		-0,14	0,92	

Controle x Genisteína 50 R1							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiencia	1,79	1,78	1,79			
	Ct	26,43	26,30		0,13	1,08	
GAPDH	Eficiência	1,91	1,88	1,90			1,36
	Ct	14,07	14,44		-0,37	0,79	

Controle x Genisteína 50 R2							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiencia	1,83	1,78	1,81			
	Ct	27,65	26,30		1,36	2,23	
GAPDH	Eficiência	1,92	1,93	1,92			1,69
	Ct	15,01	14,59		0,42	1,32	

Controle x Daidzeína 50 R1							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiencia	1,79	1,85	1,82			
	Ct	26,43	26,29		0,14	1,09	
GAPDH	Eficiência	1,91	1,89	1,90			1,34
	Ct	14,07	14,39		-0,32	0,81	

Controle x Daidzeína 50 R2							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
GST	Eficiencia	1,83	1,85	1,84			
	Ct	27,65	26,29		1,36	2,30	
GAPDH	Eficiência	1,92	1,89	1,90			1,54
	Ct	15,01	14,39		0,62	1,49	

9.4.3.4. Resultados detalhados do RT-PCR em tempo real do gene β catenina em células HT29. Calculado de acordo com método de Pfaffl (2001).

Controle x Genisteína 10 R1							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
β catenina	Eficiencia	1,80	1,77	1,78			
	Ct	24,95	24,45		0,49	1,33	
GAPDH	Eficiência	1,80	1,96	1,88			0,62
	Ct	15,46	14,26		1,20	2,13	

Controle x Genisteína 10 R2							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
β catenina	Eficiencia	1,85	1,78	1,82			
	Ct	23,80	24,09		-0,30	0,84	
GAPDH	Eficiência	1,91	1,89	1,90			0,64
	Ct	14,51	14,10		0,41	1,30	

Controle x Daidzeína 10 R1							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
β catenina	Eficiencia	1,80	1,83	1,81			
	Ct	24,95	23,67		1,28	2,13	
GAPDH	Eficiência	1,80	1,79	1,80			1,26
	Ct	15,46	14,56		0,91	1,70	

Controle x Daidzeína 10 R2							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
β catenina	Eficiencia	1,85	1,97	1,91			
	Ct	23,80	23,58		0,22	1,15	
GAPDH	Eficiência	1,91	1,76	1,84			1,35
	Ct	14,51	14,78		-0,27	0,85	

Controle x Genisteína 50 R1							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
β catenina	Eficiencia	1,80	1,73	1,76			
	Ct	24,95	25,36		-2,31	0,27	
GAPDH	Eficiência	1,80	1,93	1,87			0,50
	Ct	15,46	15,02		0,44	1,32	

Controle x Genisteína 50 R2							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
β catenina	Eficiencia	1,85	1,76	1,80			
	Ct	23,80	24,64		-0,84	0,61	
GAPDH	Eficiência	1,91	1,86	1,89			0,39
	Ct	14,51	13,82		0,69	1,55	

Controle x Daidzeína 50 R1							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
β catenina	Eficiencia	1,80	1,79	1,79			
	Ct	24,95	23,61		1,34	2,18	
GAPDH	Eficiência	1,80	1,86	1,83			1,33
	Ct	15,46	14,64		0,82	1,64	

Controle x Daidzeína 50 R2							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
β catenina	Eficiencia	1,85	1,76	1,81			
	Ct	23,80	23,50		0,30	1,20	
GAPDH	Eficiência	1,91	1,86	1,89			1,30
	Ct	14,51	14,64		-0,13	0,92	

9.4.3.5. Resultados detalhados do RT-PCR em tempo real do gene APC em células HT29. Calculado de acordo com método de Pfaffl (2001).

Controle x Genisteína 10 R1							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
APC	Eficiencia	1,95	1,95	1,95			
	Ct	24,14	23,03		1,11	2,10	
GAPDH	Eficiência	1,91	1,91	1,91			0,69
	Ct	16,46	14,74		1,72	3,03	

Controle x Genisteína 10 R2							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
APC	Eficiencia	1,88	1,90	1,89			
	Ct	23,51	23,17		0,34	1,24	
GAPDH	Eficiência	1,86	1,97	1,91			0,89
	Ct	15,38	14,86		0,52	1,40	

Controle x Daidzeína 10 R1							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
APC	Eficiencia	1,95	1,89	1,92			
	Ct	24,14	23,98		0,16	1,11	
GAPDH	Eficiência	1,91	1,83	1,87			0,61
	Ct	16,46	15,50		0,95	1,82	

Controle x Daidzeína 10 R2							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
APC	Eficiencia	1,88	1,89	1,89			
	Ct	23,51	23,98		-0,47	0,74	
GAPDH	Eficiência	1,86	1,71	1,78			0,63
	Ct	15,38	15,09		0,29	1,18	

Controle x Genisteína 50 R1							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
APC	Eficiencia	1,95	1,90	1,92			
	Ct	24,14	22,95		1,19	2,18	
GAPDH	Eficiência	1,91	1,86	1,88			0,76
	Ct	16,46	14,80		1,66	2,84	

Controle x Genisteína 50 R2							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
APC	Eficiencia	1,88	1,90	1,89			
	Ct	23,51	22,95		0,56	1,42	
GAPDH	Eficiência	1,86	1,82	1,84			0,80
	Ct	15,38	14,42		0,96	1,79	

Controle x Daidzeína 50 R1							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
APC	Eficiencia	1,95	1,87	1,91			
	Ct	24,14	22,89		1,25	2,25	
GAPDH	Eficiência	1,91	1,82	1,86			0,87
	Ct	16,46	14,93		1,52	2,58	

Controle x Daidzeína 50 R2							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
APC	Eficiencia	1,88	1,87	1,88			
	Ct	23,51	22,89		0,62	1,48	
GAPDH	Eficiência	1,86	1,81	1,83			0,77
	Ct	15,38	14,30		1,08	1,92	

9.4.3.6. Resultados detalhados do RT-PCR em tempo real do gene Survivina em células HT29. Calculado de acordo com método de Pfaffl (2001).

Controle x Genisteína 10 R1							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Survivina	Eficiencia	1,89	2,00	1,95			
	Ct	23,43	22,21		1,22	2,26	
GAPDH	Eficiência	1,75	1,89	1,82			0,95
	Ct	17,88	16,44		1,44	2,37	

Controle x Genisteína 10 R2							
		Controle	Gen 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Survivina	Eficiencia	1,81	2,01	1,91			
	Ct	22,41	22,63		-0,22	0,87	
GAPDH	Eficiência	1,87	1,86	1,86			0,66
	Ct	17,18	16,74		0,44	1,31	

Controle x Daidzeína 10 R1							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Survivina	Eficiencia	1,89	1,98	1,94			
	Ct	23,43	22,46		0,97	1,90	
GAPDH	Eficiência	1,75	1,87	1,81			0,93
	Ct	17,88	16,68		1,20	2,04	

Controle x Daidzeína 10 R2							
		Controle	Daid 10	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Survivina	Eficiencia	1,81	1,88	1,85			
	Ct	22,41	22,25		0,16	1,10	
GAPDH	Eficiência	1,87	1,87	1,87			0,81
	Ct	17,18	16,68		0,50	1,36	

Controle x Genisteína 50 R1							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Survivina	Eficiencia	1,89	2,00	1,95			
	Ct	23,43	22,38		1,05	2,01	
GAPDH	Eficiência	1,75	1,82	1,79			1,36
	Ct	17,88	17,21		0,68	1,48	

Controle x Genisteína 50 R2							
		Controle	Gen 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Survivina	Eficiencia	1,81	1,90	1,86			
	Ct	22,41	22,17		0,24	1,16	
GAPDH	Eficiência	1,87	1,91	1,89			1,02
	Ct	17,18	16,98		0,20	1,14	

Controle x Daidzeína 50 R1							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Survivina	Eficiencia	1,89	1,86	1,88			
	Ct	23,43	22,18		1,25	2,20	
GAPDH	Eficiência	1,75	1,91	1,83			0,99
	Ct	17,88	16,57		1,32	2,21	

Controle x Daidzeína 50 R2							
		Controle	Daid 50	Media Eficiência	DeltaCt	$E^{\Delta Ct}$	Razão
Survivina	Eficiencia	1,81	1,97	1,89			
	Ct	22,41	21,77		0,64	1,50	
GAPDH	Eficiência	1,87	1,81	1,84			0,92
	Ct	17,18	16,37		0,81	1,63	