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Avaliação *in vitro* da atividade antiproliferativa de compostos isolados de espécies de *Hypericum* nativas do Sul do Brasil

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Porto Alegre, janeiro de 2013

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Dissertação apresentada ao Programa de Pós- Graduação em Medicina: Ciências Médicas, UFRGS, como requisito para obtenção do título de Mestre

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Dedicatória

Dedico esta dissertação a todos àqueles que acreditam no valor do conhecimento e fazem deste um instrumento de modificações positivas na sociedade.

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Epígrafe

“Conheça todas as teorias, domine todas as técnicas, mas ao tocar uma alma humana, seja apenas outra alma humana”

Carl Gustav Jung

Resumo

Devido ao grande avanço na descoberta de novos fármacos a partir de compostos naturais, tornou-se interessante avaliar o potencial antiproliferativo de moléculas isoladas de extratos de plantas. Este trabalho prioriza o estudo da atividade antitumoral de benzofenonas (carifenona A e carifenona B) e floroglucionóis (japonicina A e uliginosina B), isolados das espécies nativas do sul do Brasil, *Hypericum carinatum* e *Hypericum myrianthum*, respectivamente, bem como a associação destes com quimioterápicos utilizados na clínica. Os experimentos propostos foram realizados em modelos *in vitro*, utilizando diferentes tipos de linhagens tumorais humanas comercialmente disponíveis. Foi avaliado o efeito de diferentes doses destes compostos através de experimentos de viabilidade e sobrevivência celular, análise morfométrica nuclear (NMA) e citometria de fluxo. Na análise estatística foi utilizada a variância de uma via (ANOVA) seguida de teste post-hoc (Tukey). Os resultados foram expressos como média ± erro padrão da média (SEM), sendo valores de *P* menores do que 0,05 considerados significativos. Verificamos que nas linhagens de adenocarcinoma de ovário e colorretal e de glioblastoma (OVCAR-3, HT-29 e U-251) ocorreu uma diminuição significativa na viabilidade celular quando tratadas com a dose de 100μg/mL tanto de carifenona A como de carifenona B, enquanto os compostos japonicina A (50μg/mL) e uliginosina B (20μg/mL) só foram ativos na linhagem OVCAR-3. Dentre as associações com quimioterápicos, a única que apresentou efeito sinérgico foi a combinação de japonicina A e paclitaxel na linhagem OVCAR-3. A partir deste momento selecionamos a japoncina A para dar continuidade aos estudos. Este composto foi avaliado frente a outros tipos de linhagens tumorais, sendo ativa somente em células de adenocarcinoma de ovário e próstata (OVCAR-3 e PC-3). Na linhagem PC-3, a análise do ciclo celular demonstrou decréscimo da fase G1 e indução ao arraste da fase G2, assim como, através da técnica de NMA, foi verificado um aumento de células apoptóticas, quando as células foram tratadas com japonicina A. Estudos moleculares devem ser realizados para melhor entendimento do mecanismo de ação da japonicina A, composto que pode servir de modelo para o desenho de fármacos mais específicos para este tipo de neoplasia.

PALAVRAS-CHAVE: atividade antiproliferativa, floroglucinóis, benzofenonas, *Hypericum*, OVCAR-3, PC-3.

Abstract

Due to the great progress in the discovery of new drugs from natural compounds, it has become interesting to evaluate the antiproliferative activity of molecules isolated from plant extracts. This work emphasizes the study of antitumor activity of benzophenones (cariphenone A and cariphenone B) and phloroglucionols (japonicin A and uliginosin B), isolated from *Hypericum* species native to southern Brazil, *H. carinatum* and *H. myrianthum*, respectively, as well as their association with chemotherapeutic drugs used in the clinic. The proposed experiments were performed *in vitro* using commercially available cell lines. The effect of different doses of these compounds were evaluated via cell viability and survival assay, nuclear morphometric analysis (NMA) and flow cytometry. One way analysis of variance (ANOVA) followed by post hoc tests (Tukey) were utilized for statistical analysis. Results were expressed as mean ± standard error of the mean (SEM), and *P* values less than 0.05 were considered significant. We found that in ovarian, colorectal (adenocarcinoma) and glioblastoma cell lines (OVCAR-3, HT-29 and U-251) a significant decrease in cell viability occurred when these were treated with a dose of 100μg/mL of cariphenone A and B, while compounds japonicin A (50μg/mL) and uliginosin B (20μg/mL) were active only in OVCAR-3. Among the associations with chemotherapeutic agents, only japonicin A presented a synergistic effect with paclitaxel in the OVCAR-3 cell line. We then selected japonicin A for evaluation against other cell lines, but its effects were only observed in ovary and prostate adenocarcinoma cell lines (OVCAR-3 e PC-3). In PC-3, the cell cycle revealed a decreased in the G1 phase and induction of G2 arrest, the NMA showed an increase in apoptotic cells when cells were treated with japonicin A. More studies should be conducted to better understand the mechanisms of action of japonicin A, since this compound may serve as pharmacophore model for the design of more specific drugs to treat this tumor type.

KEYWORDS: antiproliferative activity, phloroglucinols, benzophenonas, *Hypericum*, OVCAR-3, PC-3.

Lista de ilustrações e tabelas

Lista de abreviatura e símbolos

APAF-1: Apoptotic Protease Activanting Factor1 (Fator 1 de Ativação Protease Apoptótica)

Bcl-2: B-cell lymphoma 2 (célula B de linfoma 2)

CDK: Cyclin-dependent kinase (Quinases Dependentes de Ciclinas)

FAK- Focal adhesion kinase (Quinase de Adesão Focal)

HP1: 6-isobutiril-5,7-dimetoxi-2,2-dimetil-benzopirano

HP2: 7-hidroxi-6-isobutiril-5-metoxi-2,2-dimetil-benzopirano

HP3: 5-hidroxi-6-isobutiril-7-metoxi-2,2-dimetil-benzopirano

INCA: Instituto Nacional de Câncer

LLA: Leucemia Linfoblástica Aguda

LLC : Leucemia Linfocítica Crônica

MTT: (3-(4,5-dimetiltiazol-2-yl)-2,5 difeniltetrazólio brometo)

OMS: Organização Mundial da Saúde

PSA: Prostate-Specific Antigen (Antígeno Prostático Específico)

TNFR : Tumor Necrosis Factor Receptor (Receptor de Fator de Necrose Tumoral)

Sumário

1.Introdução

A natureza é uma fonte muito atrativa na busca por novos fármacos devido a sua vasta diversidade química, encontrada em milhões de espécies de plantas, animais e organismos marinhos, selecionado por mecanismos evolutivos durante um período de milhões de anos.

Nas últimas décadas ocorreram avanços na compreensão da biossíntese dos produtos naturais, bem como na melhoria de processos de isolamento e caracterização destas substâncias, além de descobertas importantes na etiologia de muitas doenças, auxiliando a elucidação dos mecanismos de desenvolvimento e manutenção destas patologias, abrindo muitas portas para a implementação de pesquisas em indústrias e no meio acadêmico.

Os produtos naturais obtidos de plantas têm inspirado e fornecido uma série de compostos candidatos a utilização terapêutica, sendo uma importante fonte de novos fármacos contra o câncer. Existem vários exemplos bem sucedidos de medicamentos que atualmente são largamente utilizados na clínica contra vários tipos de tumores, como por exemplo, os alcalóides da Vinca (vincristina, vinblastina) no tratamento de leucemias agudas, linfomas, mieloma múltiplo e neuroblastoma; os taxanos (paclitaxel e docetaxel) usados em câncer de mama, ovário, pulmão e tumores de cabeça e pescoço; bem como, os derivados semissintéticos da camptotecina (topotecano e irinotecano) com principais indicações no câncer de ovário, pulmão e colorretal, derivados de *Catharantus roseus*, *Taxus brevifolia* e *Camptotheca acuminata*, respectivamente.

A obtenção de novos compostos promissores pode ser através de programas de rastreamento bioguiado ou pela busca ao acaso a partir da observação, descrição e investigação experimental de drogas utilizadas tradicionalmente e de suas atividades biológicas. Contudo, tem se verificado uma tendência no predomínio da utilização de substâncias isoladas em substituição aos extratos vegetais que apresentam alguma propriedade terapêutica comprovada e tenham seus constituintes ativos identificados. Isso se deve a vantagens como a constância da composição, ausência de qualquer outra substância ativa, além daquela determinante da atividade, e maior facilidade para o controle da qualidade em relação aos produtos de composição complexa e não conhecida.

Sendo o Brasil um país com uma vasta biodiversidade e o câncer uma patologia que, apesar de todo avanço nesta área, ainda necessita de agentes antineoplásicos mais efetivos, a

busca por moléculas promissoras presentes em espécies da flora nativa, torna-se um importante nicho de pesquisa.

2. Revisão da Literatura

2.1 O câncer

Conhecido há muitos séculos, o câncer foi inicialmente considerado uma doença dos países desenvolvidos e com grandes recursos financeiros. Há aproximadamente quatro décadas, a situação vem mudando, e a maior parte do ônus global do câncer pode ser observada em países em desenvolvimento, principalmente aqueles com poucos e médios recursos. Assim, nas últimas décadas, o câncer ganhou uma dimensão maior, convertendo-se em um evidente problema de saúde pública mundial. O câncer atualmente é uma das principais causas de morte no mundo, e segundo a Organização Mundial da Saúde (OMS) estima-se que, no ano 2030, podem-se esperar 27 milhões de casos incidentes, 17 milhões de mortes e 75 milhões de pessoas vivas, anualmente, com câncer.

No Brasil, as estimativas para o ano de 2012 serão válidas também para o ano de 2013 e apontam a ocorrência de aproximadamente 518.510 casos novos de câncer, incluindo os casos de câncer de pele não melanoma, reforçando a magnitude do problema do câncer no país. Entre as dez neoplasias de maior incidência no Brasil estão o câncer de próstata, ovário e colorretal e tumores do sistema nervoso central, no qual os gliomas tem grande destaque (Fig. 1).

Figura 1. Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2012 por sexo, exceto pele não melanoma, no Brasil*.

Localização primária	casos novos	percentual			Localização primária	casos novos	percentual
Próstata	60.180	30,8%	Homens	Mulheres	Mama Feminina	52.680	27,9%
Traqueia, Brônquio e Pulmão	17.210	8,8%			Colo do Útero	17.540	9,3%
Cólon e Reto	14.180	7,3%			Cólon e Reto	15.960	8,4%
Estômago	12.670	6,5%			Glândula Tireoide	10.590	5,6%
Cavidade Oral	9.990	5,1%			Traqueia, Brônquio e Pulmão	10.110	5,3%
Esôfago	7.770	4,0%			Estômago	7.420	3,9%
Bexiga	6.210	3,2%			Ovário	6.190	3,3%
Laringe	6.110	3,1%			Corpo do Útero	4.520	2,4%
Linfoma não Hodgkin	5.190	2,7%			Linfoma não Hodgkin	4.450	2,4%
Sistema Nervoso Central	4.820	2,5%			Sistema Nervoso Central	4.450	2,4%

*Números arredondados para 10 ou múltiplos de 10

Fonte: Estimativa 2012- Incidência de Câncer no Brasil- INCA

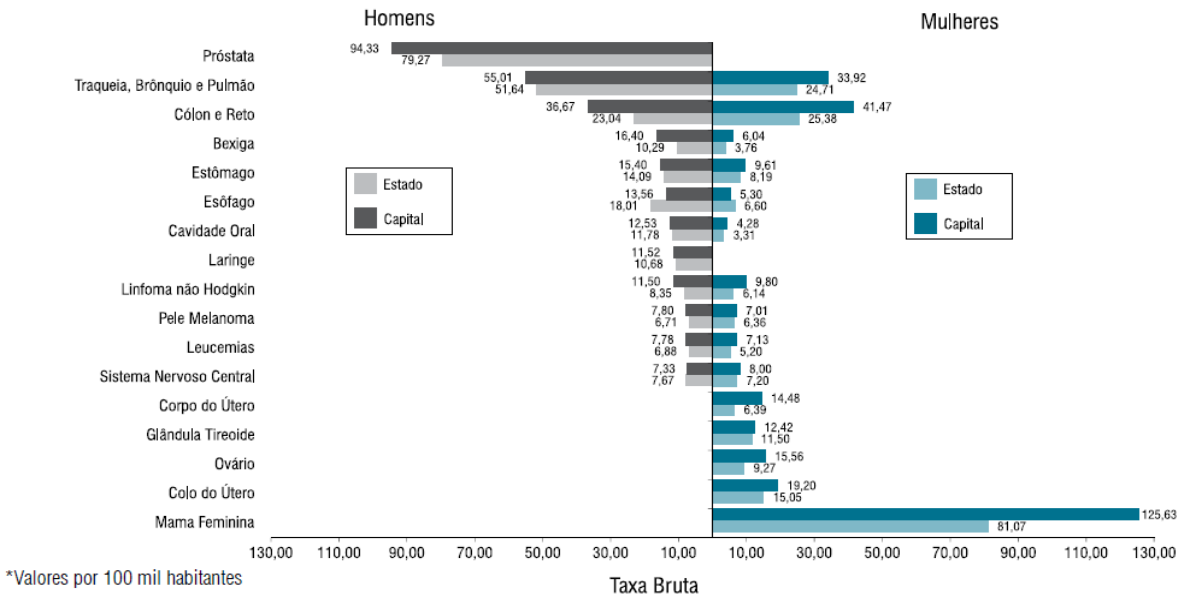
Estas neoplasias além da grande incidência têm como característica comum o bom prognóstico se tratados precocemente. Contudo, a maioria dos pacientes recebe o diagnóstico

em graus avançados da doença e já possuem perfis tumorais mais complexos. Também devido ao grande índice de resistência tumoral, isto torna o arsenal terapêutico para essas neoplasias insuficiente, sendo um vasto campo para o estudo de agentes mais efetivos e menos citotóxicos.

O câncer colorretal é o terceiro tumor maligno mais frequente no mundo em ambos os sexos, inclusive no Brasil (INCA, 2012). Além disso, é a principal causa de morte relacionada a neoplasias no mundo, quando tumores relacionados ao fumo são excluídos (Frattini et al., 2004; Schally et al., 2004). Durante o estágio pré-neoplásico há mudanças hiperplásicas ou displásicas que com o passar do tempo, formam um adenoma que pode levar ao aparecimento de um carcinoma com metástases (Sweetser et al., 2011). Por isso, a remoção dos pólipos (lesão precursora) está associada à redução da incidência de câncer colorretal. Infelizmente, na maioria dos casos, o câncer é detectado tardiamente, o que resulta em uma alta taxa de mortalidade (Davies et al, 2005; Ramsoekh et al., 2007; Castells, 2011).

No sexo masculino, segundo a última estimativa mundial, o câncer da próstata foi apontado como sendo o segundo tipo de câncer mais frequente em homens, com cerca de 915 mil casos novos no ano de 2008. No Rio Grande do Sul e capital (Fig.2) este câncer é o de maior incidência entre os homens.

Figura 2. Taxas brutas de incidência estimadas para 2012 por sexo, segundo Estado e capital brasileira*.



Fonte: Estimativa 2012- Incidência de Câncer no Brasil- INCA

O câncer de próstata frequentemente possui bom prognóstico se diagnosticado e tratado oportunamente, embora os métodos de rastreamento atuais, como o PSA, não demonstrem sucesso na redução da mortalidade (Gjertson e Albertsen, 2011). Isto é verdadeiro especialmente no câncer de próstata hormônio independente, no qual as células são reprogramadas para escapar da parada do ciclo celular e apoptose em condições andrógeno-empobrecido (Agus et al., 1999; Kim et al., 2011).

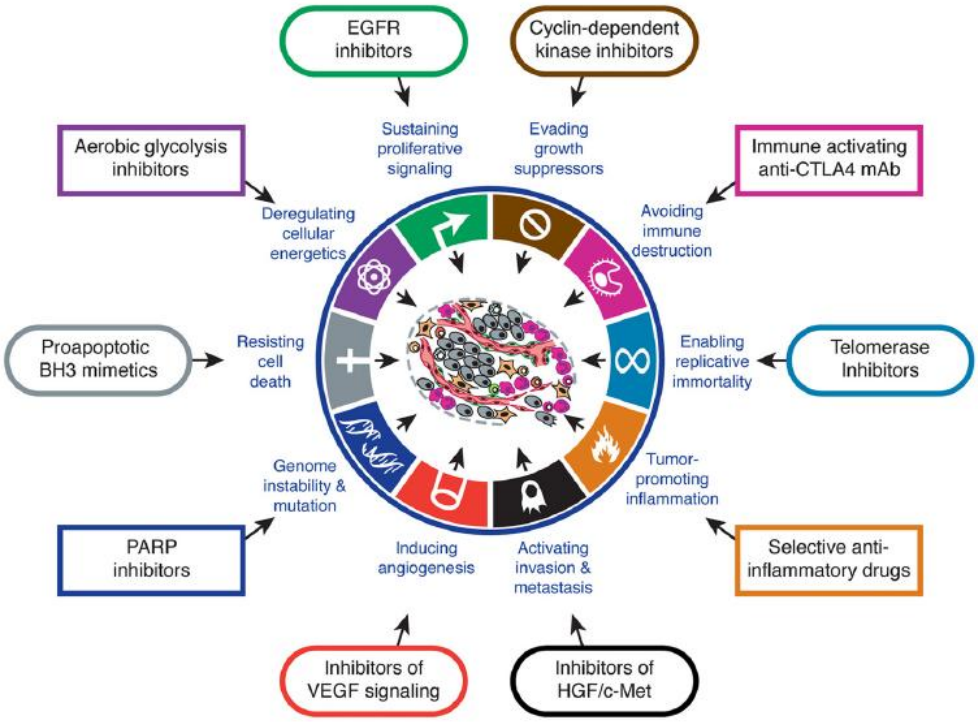
Já entre as mulheres, o câncer de ovário é o terceiro tumor ginecológico mais comum, porém o mais difícil de ser diagnosticado e o com as menores chances de cura entre os tumores do sistema reprodutivo feminino (INCA, 2012). Na fase inicial, o câncer de ovário não causa sintomas específicos, mas à medida que o tumor cresce, pode causar pressão, dor ou inchaço no abdômen que servem de alerta para que se procure um médico. Todavia nesses casos (cerca de75%), o tumor já se encontra em estágios muito avançados de grau III e IV. (Carvalho et al., 2011).

Outra malignidade com menor prevalência, mas de grande agressividade, são os gliomas cujo tratamento ainda é ineficiente sendo a sobrevida média de um ano após o diagnóstico (Osoba, 2000; Klein, 2001). Gliomas são tumores primários mais comuns no sistema nervoso central e correspondem à aproximadamente 70% de todos os tumores cerebrais, sendo que os glioblastomas multiformes são os mais comuns e mais malignos. Aproximadamente 60% dos pacientes com diagnóstico de glioblastoma possuem idade entre 55 e 74 anos, e a relação de incidência entre homens e mulheres é de 3:2 (Fisher et al., 2007; Brandes et al., 2008).

A ressecção cirúrgica, quando possível, acompanhada de radioterapia e quimioterapia é o tratamento padrão para estes tipos de neoplasia e requerem cuidadosa seleção de uma ou mais intervenção (Pavet, 2011). A tendência é que a quimioterapia assuma um papel cada vez maior, devido ao desenvolvimento de quimioterápicos mais seletivos e com menores efeitos prejudiciais (Vredenburgh et al. 2007, Sathornsumette e Rich, 2008). Uma das principais desvantagens da quimioterapia clássica são efeitos colaterais devido ao não direcionamento específico à células normais e cancerosas. Assim, nos últimos anos aumentou a busca pela descoberta de novos compostos alvo- específicos em células tumorais (Fig.3) e com o mínimo de efeitos tóxicos sobre as células normais, tornando o tratamento mais individualizado ao tipo de câncer e suas respectivas mutações (Pavet, 2011).

No entanto, terapias alvo-específicas têm limitações, entre as principais, está o potencial das células desenvolverem resistência aos mesmos, uma vez que o bloqueio de uma via, geralmente é compensada pela ativação de outras (Hanahan e Weinberg, 2011). Devido a natureza pleiotrópica dos tumores (Fig. 3) os agentes quimioterápicos são mais eficazes quando administrados em combinação. O uso de múltiplos fármacos com diferentes mecanismos de ação, agindo em diferentes etapas de uma mesma via, leva a maior porcentagem de morte celular e dificultam as células a tornarem-se resistentes. Mesmo que a probabilidade de desenvolvimento de uma mutação de resistência a um fármaco seja relativamente alta, o aparecimento simultâneo de mutações contra vários fármacos diferentes é menos provável. Outro benefício da associação de quimioterápicos é poder reduzir os efeitos adversos utilizando doses mais baixas e, alcançando um aumento global da morte celular (Golan et al., 2007).

Figura 3. Alvos terapêuticos das características de câncer.



Fonte: Hanahan e Weinberg (2011) Cell.

O conceito de combinação de quimioterápicos está sendo redefinido como um novo tratamento disponível e uma alternativa em muitas neoplasias que desenvolvem resistência. No final da última década, a disponibilidade de novos fármacos levou ao desenvolvimento de combinações que deram um substancial aumento na porcentagem de resposta em alguns tipos de tumores até recentemente considerados intratáveis (Galmarini et al., 2012). Estes incluem malignidades como o câncer de pulmão, o câncer de cólon avançado (Thatcher et al., 2005; von Weikersthalb et al., 2011) e a leucemia linfoblástica aguda (LLA), na qual combinações de quimioterápicos são curativas, com sobrevivência acima de 80%, embora cada agente possua uma pequena atividade individual (Pui e Evans, 1999; Crazzolara e Bendall, 2009).

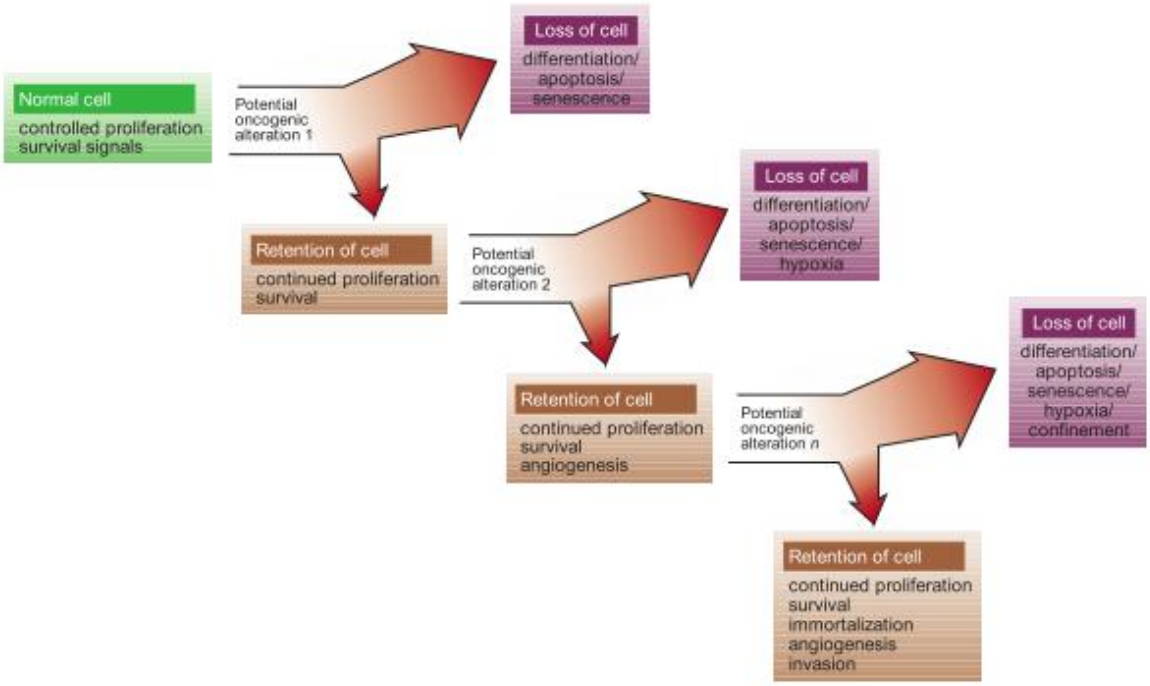
Porém, apesar de todo avanço nesta área, necessita-se de agentes antineoplásicos mais efetivos, uma vez que os tumores mais comuns em adultos são resistentes aos agentes neoplásicos disponíveis, sendo a maioria com ação limitada a tumores não-sólidos e com um pequeno impacto na sobrevida dos pacientes (Gibbs, 2000; Sukhai et al., 2011; Galmarini et al., 2012). Neste contexto, faz-se necessário incentivar a pesquisa de fármacos menos citotóxicos, mais seletivos e que atuem no bloqueio da proliferação celular por meio de mecanismos de fina modulação (Fig.3) em associação a outros agentes quimioterápicos.

2.2 Mecanismos de proliferação celular

Dentre a complexidade e idiopatia de cada tipo de câncer destacam-se “eventos críticos” que impulsionam a célula tumoral e sua progênie à expansão descontrolada e invasão. Nosso organismo possui diferentes mecanismo para bloquear alterações no DNA, de modo que respondemos ao dano do DNA através do reparo, controle de pontos de checagem do ciclo celular e indução a apoptose que, coletivamente, promovem a integridade genômica e suprimem a tumorigênese (Fig.4) (Evan e Vousden, 2001).

A evolução do câncer é mais complexa do que o acumulo simples e linear de mutações oncogênicas. Sinais proliferativos potencialmente oncogênicos são acoplados a uma variedade de inibidores dos processos de crescimento, tais como a indução a apoptose, diferenciação ou senescência, cada uma das quais limita a expansão clonal subsequente e evolução neoplásicas. A progressão do tumor ocorre apenas nos casos em que estes mecanismos inibitórios de crescimento, estão inibidos por mutações compensatórias (Hanahan e Weinberg, 2011).

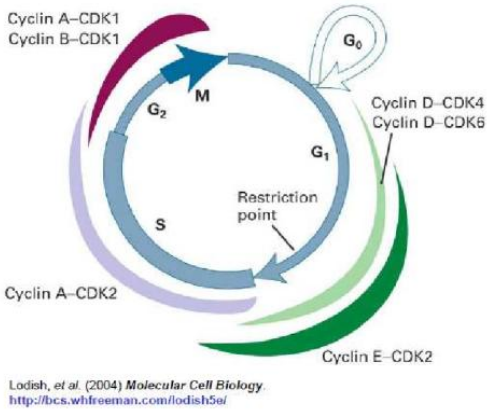
Figura 4. A evolução do câncer e mecanismos inibitórios do crescimento



Fonte: Evan e Vousden (2001) Nature.

Pontos de checagem do ciclo celular que controlam a ordem e a temporização das transições da divisão celular garantem que eventos críticos como a replicação do DNA e a segregação dos cromossomos seja completa e com alta fidelidade (Fig. 5.).

Figura 5. Ciclo celular



Fonte: Lodish et al. (2004) Molecular Cell Biology

Além disso, os pontos de checagem respondem ao dano através do arraste do ciclo celular, provendo tempo para reparo, e pela indução da transcrição de genes que facilitam o reparo, prevenindo a transmissão do dano para as células filhas. A perda destes pontos de checagem resulta em instabilidade genômica e tem sido relacionado à evolução de células normais em células cancerígenas. (Boucas et al., 2012).

Estas alterações em conjunto, resultam não somente em vantagens proliferativas, mas também, no aumento da suscetibilidade para acumular alterações genéticas adicionais que contribuem para a progressão tumoral e a aquisição de fenótipos mais agressivos. A importância dos defeitos em pontos de checagem no desenvolvimento do câncer pode prover importantes alvos para terapias. Tumores associados a defeitos no ciclo celular são frequentemente mediados por alterações na atividade de quinases dependentes de ciclinas (CDKs). Logo, estratégias terapêuticas que bloqueiam a atividade de CDKs podem promover benefícios terapêuticos contra certos tipos de neoplasias (Malumbres e Barbacid, 2009).

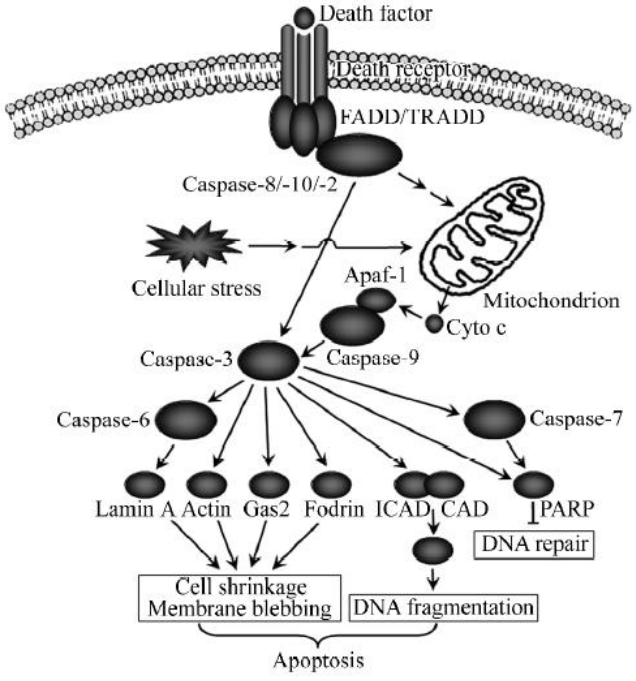
Outro mecanismo pelo qual os agentes antineoplásicos atuam na diminuição da proliferação celular é pelo processo de ativação da apoptose.(Wong, 2001). A apoptose é a morte celular programada que envolve a eliminação de determinada célula defeituosa. Ela ocorre normalmente durante o desenvolvimento e envelhecimento como forma de manutenção da população celular tecidual, além de ser um mecanismo de defesa em reações imunológicas ou quando células danificadas por alguma doença ou toxina precisam ser eliminadas. A apoptose é um processo coordenado e dependente da energia que envolve a ativação de proteases chamadas caspases (Fig.6). A ativação de caspases leva a uma complexa cascata de eventos iniciada por um estímulo específico que acaba por levar a morte celular (Elmore, 2007).

A via extrínseca de caspases é iniciada na membrana plasmática pela ativação de receptores de morte como os da família TNFR que se trimerizam e levam à ativação da caspase 8, que propaga o sinal de morte através do autoprocessamento intramolecular das caspases próximas.

Na via intrínseca, células reagem em resposta a diferentes condições estressantes ou ao dano ao DNA e sofrem mudanças que aumentam a permeabilidade da membrana mitocondrial externa controlada pela família Bcl-2. A liberação de proteínas do espaço intermembrana para o citosol permite a formação do apoptossoma, um complexo ativador de caspases formado por

APAF-1, caspase- 9 e citocromo- c, resultando na ativação de caspases efetoras que são responsáveis pela degradação de estruturas celulares, culminando na morte celular (Delhalle et al., 2003; Fuchs e Steller, 2011).

Figura 6. Ativação da apoptose por caspases.



Fonte: Fan et.al. (2005) Acta Biochimica et Biophysica Sinica.

A compreensão deste processo é muito importante para o desenvolvimento de novas terapias que visam alterar os genes ligados a indução/inibição da apoptose com ferramentas da biologia molecular ou através de fármacos que modulem essas vias (Fleischer et al., 2006; Wong , 2011; Tao et al. ,2012).

Devido a desregulação na proliferação celular e a apoptose estarem no cerne do desenvolvimento tumoral, estes são dois óbvios alvos para a intervenção terapêutica em todos os tipos de câncer. Claramente há numerosos mecanismos pelos quais esses dois defeitos podem ocorrer (Martin e Elkon, 2004; Moore et al. 2010; Cho e Liang, 2011), e portanto o sucesso da terapia alvo irá depender das características moleculares de cada tumor (Gibin et al., 2012).

2.3 Agentes neoplásicos derivados de compostos naturais

O uso da medicina complementar e alternativa, representadas principalmente por plantas, tem grande apelo no mundo todo, especialmente entre os pacientes com câncer (Bell, 2010; Tan et al., 2011). No Brasil, segundo dados do INCA (2009) a utilização de plantas varia entre 30 a 75%. A vasta diversidade da flora encontrada em nosso país, aguça cada vez mais o interesse de pesquisadores na investigação de possíveis agentes anticancerígenos obtidos a partir de plantas nativas (Mans et al., 2000; Júnior et al., 2010).

Existem vários compostos derivados de plantas utilizados como agentes antineoplásicos, a ressaltar a vimblastina, vincristina, etoposídeo, teniposídeo, taxol, taxotere, topotecano, irinotecano e navelbine (Shoeb, 2006). O estudo de plantas na investigação de novos agentes terapêuticos no tratamento do câncer se concentra no isolamento de substâncias bioativas (Patel et al.., 2010) e na realização de testes *in vitro* visando entender o mecanismo de ação destas moléculas (Tsukamoto e Yokosawa, 2010; Fulda, 2010, Filippi-chiela et al., 2011), bem como, estudar a associação destas com outros antineoplásicos (Yue et al., 2008; Wang et al., 2009; Kanai et al., 2011) visando a geração de protocolos de tratamento mais eficazes, como no caso da associação demonstrada pelo paeonol, um componente fenólico, extraído a partir da casca da raiz de *Paeonia moutan* que exerce um efeito aditivo com a cisplatina em câncer de esôfago (Wan et al., 2008), e com o 5-fluoracil em linhagens celulares de câncer colorectal (Ji, 2005).

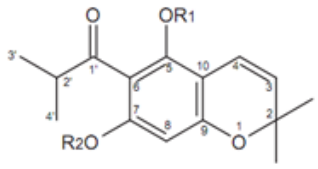
Vários estudos sugerem que membros da família Guttiferae parecem ser uma fonte valiosa de compostos citotóxicos (Ho, et al., 2002; Merza et al.,2005; Wabo et al., 2010) A família Guttiferae é composta por 25 gêneros, representados por cerca de 1000 espécies distribuídas em 6 subfamílias (Kielmeyeroideae, Calophytiloideae, Clusioideae, Moronoboideae, Lorostemonoideae e Hypericoideae), todas com representantes no Brasil (Barroso, 1978). A maioria das espécies pertence a apenas 3 gêneros: *Clusia, Garcinia e Hypericum*. As plantas da família Guttiferae apresentam uma ampla gama de constituintes, dentre eles os derivados de floroglucinol, benzofenonas e benzopiranos. Seus representantes são encontrados principalmente em regiões tropicais úmidas, exceto aqueles do gênero *Hypericum* que se desenvolvem nas zonas temperadas (Bennete e Lee, 1989).

O gênero *Hypericum* tem sido largamente pesquisado quanto a sua ação antitumoral (Momekov et al., 2008, Saad et al. 2011) sendo que várias espécies têm apresentado importantes atividades frente à diferentes linhagens tumorais (Mans et al., 2000). Este gênero

compreende cerca de 400 espécies amplamente distribuídas. Aproximadamente 20 espécies são nativas do sul do Brasil, pertencentes às seções Brathys e Trigynobrathys (Robson,1986).

Uma triagem realizada com 6 espécies de *Hypericum* nativas do sul do Brasil demonstrou um interessante resultado dos extratos hexânicos de todas as espécies avaliadas, frente as linhagens tumorais humanas de câncer colorretal, HT-29; de carcinoma de pulmão, H-460 e de glioblastoma, U-373 (Ferraz et al., 2005a). A partir deste estudo, a espécie *Hypericum polyanthemum* teve isolada de suas partes aéreas 3 benzopiranos (Ferraz et al., 2001), o 6-isobutiril-5,7-dimetoxi-2,2-dimetil-benzopirano (HP1), o 7-hidroxi-6-isobutiril-5-metoxi-2,2-dimetil-benzopirano (HP2) e o 5-hidroxi-6-isobutiril-7-metoxi-2,2-dimetil-benzopirano (HP3) sendo verificada sua atividade antitumoral frente as linhagens humanas H460, HT-29 e U-373, serem menos ativos do que o extrato hexânico (Ferraz et al., 2005b). Foi verificado então, que os benzopiranos agiam de maneira sinérgica entre si desencadeando uma parada no ciclo celular em G2/M (Grivicich et al., 2008). Como os benzopiranos (HP1, HP2 e HP3) não apresentam genotoxicidade nem atividade mutagênica *in vivo* (Ferraz et al., 2009), são moléculas promissoras para as etapas de estudos pré clínicos embora necessitem de uma avaliação mais detalhada do mecanismo de ação pelo qual exercem a inibição da proliferação celular.

Figura 7. Benzopiranos isolados da espécie *Hypericum polyanthemum*.



1 R^1=Me, R^2=Me

2 R^1=Me, R^2=H

3 R^1=H, R^2=Me

Fonte: Ferraz et al. (2005).Fitoterapia.

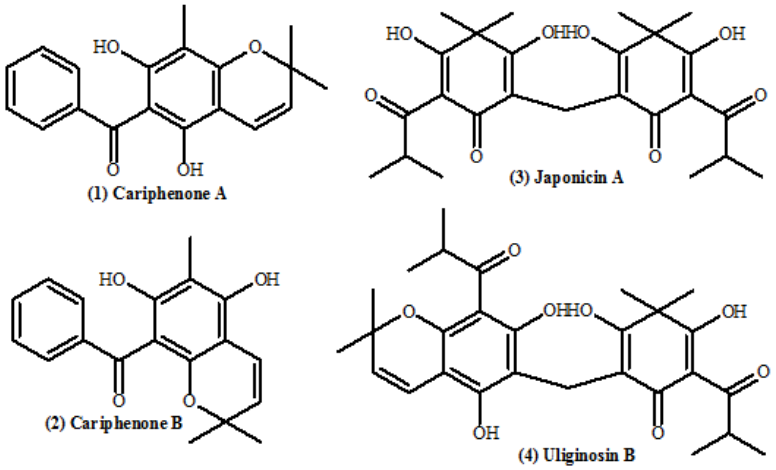
Já duas benzofenonas simples foram isoladas da espécie *Hypericum carinatum* (Fig.8.1), a carifenona A (Fig.9.1) e carifenona B (Fig.9.2), que apresentam atividade antioxidativa (Bernardi et al. 2005). Da triagem com espécies de *Hypericum* nativas do sul do Brasil, a espécie *Hypericum myrianthum* (Fig.8.2) foi a que apresentou melhores resultados de inibição em todas as linhagens testadas (Ferraz et al., 2005a). Entre os compostos já isolados desta espécie estão os floroglucinóis, japonicina A (Fig. 9.3.) e uliginosina B (Fig. 9.4.)

(Ferraz et al., 2002), sendo que, a última, em estudos recentes, demonstrou um potencial antidepressivo e antinoceptivo (Stein et. al., 2012; Stolz et al., 2012).

Figura 8. Flores das espécies *Hypericum carinatum* (1) e *Hypericum myrianthum* (2).

Fonte: fotos adaptadas cedidas por Gilsane von Poser.

Figura 9. Estrutura química das benzofenonas, carifenona A (1) e carifenona B(2) e dos floroglucinóis, japonicina A (3) e uliginosina B (4).



Fonte: Estruturas desenhadas pelo programa chemoffice2004.

Uma proeminente atividade antiproliferativa tem sido estabelecida por uma variedade de metabólitos secundários que possuem padrões complexos de prenilação, como os

floroglucinóis (Winkelmann et al., 2003), xantonas (Laphookhie et al., 2006; Suksamrarn et al., 2006), benzofenonas (Carroll et al., 2009; Martins et al., 2009), e depsidonas (Permanaa et al., 2005).

Dentre os floroglucionóis, a molécula de hiperatomarina obtida da espécie *Hypericum annulatum* M., se mostrou ativa frente a um painel de linhagens tumorais de leucemias, linfomas e tumores sólidos, apresentando também efeitos pró-apoptoticos (Momekov et al. 2008). Outro floroglucionol grande destaque é a hiperforina, presente na espécie *Hypericum perforatum* L. que demonstrou ser um importante indutor da apoptose (Schempp et al. 2002; Hostanska et al. 2003), especialmente em pacientes com leucemia linfocítica crônica (LLC), onde também exerceu um papel importante antimigratório e antiangiogênico. (Quiney et al, 2006 abc). Além disso, no estudo de Gey e colaboradores (2002) a hiperforina, assim como os floroglucinóis, gutiferona G e a aristoforina, apresentaram modulação negativa sob histonas desacetilases, permitindo que as células entrassem em apoptose quando submetidas a eventos de estresse ou quando houvesse dano ao DNA.

As benzofenonas também exibem uma significante atividade antitumoral *in vivo* e *in vitro* (Seo et al., 2002; Hsieh et al., 2003), porém o mecanismo pelo qual as benzofenonas poliisopreniladas inibem o crescimento e a proliferação celular é pouco compreendido. Estudos recentes sugerem que esses compostos causam uma redução na invasão e na sobrevida celular através da inibição de sinalização *downstream* mediada por proteína quinase FAK. (Liao et al., 2005).

O estudo da estrutura de benzofenonas tem demonstrado que benzofenonas para-metóxi substituídas foram avaliadas como inibidores do ativador mitógeno da proteina quinase p38α com alta eficácia e seletividade (Revesz et al., 2004), e benzofenonas amino e metóxi substituídas são relatadas ativas contra uma série de linhagens celulares tumorais humanas (Hsieh et al.., 2003), sendo que os derivados de benzofenonas polipreniladas tem sido apontados como indutores da ativação da apoptose caspase-mediada (Balasubramanyam et al., 2004).

Com essas considerações, o trabalho desta dissertação foi delineado segundo o objetivo principal e os objetivos secundários, listados a seguir.

3.Objetivos

3.1 Objetivo geral

Avaliar atividade antiproliferativa *in vitro* de compostos naturais isolados de espécies de *Hypericum* nativas do Sul do Brasil, bem como avaliar a eficácia da associação destes compostos e quimioterápicos tradicionalmente utilizados na clínica oncológica.

3.2 Objetivos específicos

Avaliar a atividade antiproliferativa *in vitro* das benzofenonas isoladas da espécie *Hypericum carinatum*, carifenona A e carifenona B, bem como a associação destas com quimioterápicos (paclitaxel, irinotecano e temozolamida) em linhagens celulares humanas de adenocarcinoma de ovário e colorretal (OVCAR-3 e HT-29) e de glioblastoma humano (U-251);

Avaliar a atividade antiproliferativa *in vitro* dos floroglucinóis isolados da espécie *Hypericum myrianthum*, japonicina A e uliginosina B, bem como a associação destas com quimioterápicos (paclitaxel, irinotecano e temozolamida) em linhagens celulares humanas OVCAR-3, HT-29 e U-251;

Realizar a triagem da atividade antiproliferativa *in vitro* do floroglucinol isolado da espécie *Hypericum myrianthum*, japonicina A, frente a um painel de linhagens tumorais;

Avaliar a atividade antiproliferativa *in vitro* do floroglucinol isolado da espécie *Hypericum myrianthum*, japonicina A, na linhagem celular humana de câncer de próstata (PC-3) pelos métodos de viabilidade celular e sobrevivência;

Avaliar os efeitos de diferentes doses do floroglucinol, japonicina A no ciclo celular da linhagem humana de câncer de próstata (PC-3);

Avaliar o dano celular na linhagem humana de câncer de próstata (PC-3) tratada com diferentes doses do floroglucinol, japonicina A, pela análise morfométrica nuclear (NMA) por marcação com DAPI.

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5. Artigos

5.1 Artigo 1: Antiproliferative activity of the dimeric phloroglucinol and benzophenone derivatives of *Hypericum* species native to southern Brazil, submetido à revista Anti- Cancer Drugs.

5.2 Artigo 2: Antiproliferative activity of Japonicin A a phloroglucinol derivatives of *Hypericum* species in the human prostate adenocarcinoma cell line PC-3, escrito nos moldes para submissão à revista Planta Medica.

Anti-proliferative activity of the dimeric phloroglucinol and benzophenone derivatives of *Hypericum* species native to southern Brazil

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Abstract

A large number of plants are known to possess strong antitumor properties. Previous studies verified the anti-proliferative activity of the extract and fractions of six species of *Hypericum* growing in southern Brazil. Currently, the *in vitro* anti-proliferative effects of two dimeric phloroglucinols (japonicin A and uliginosin B, isolated from *H. myrianthum*) and two benzophenones (cariphenone A and cariphenone B, isolated from *H. carinatum*) were investigated against three tumor cell lines (HT-29 - human colon carcinoma cells; U-251 - human glioma cell line and OVCAR-3 human ovarian carcinoma cells). Afterwards, different doses of these compounds were associated with citotoxic drugs commonly used as chemotherapy in the clinic. Cariphenone A and cariphenone B showed moderate anti-proliferative activity against all tumor cell lines at dose of 100µg/mL. Unlike the effects obtained by benzophenones, japonicin A and uliginosin B exerted anti-proliferative effect only in the OVCAR-3 cell line. Moreover, an important reduction in cellular proliferation of OVCAR-3 was demonstrated by the association of the sub-effective doses of japonicin A and the chemotherapeutic drug paclitaxel. These preliminary results provide a scientific basis to further pursue these compounds as potential combined therapy for certain tumor types.

Keywords: anti-proliferative activity, dimeric phloroglucinols, benzophenones, *Hypericum*.

1. Introduction

Despite advances in diagnostic and treatment strategies, cancer remains of the most prevalent causes of death worldwide. Until the moment, surgery, radiotherapy and systemic chemotherapy still comprise the standard treatment for the majority of cancer types, requiring a careful selection of the intervention strategy. Because of the pleiotropic nature of the tumors, chemotherapeutic drugs are most effective when given in combination [1].

For many years, natural compounds have been a source and inspiration for new medicinal drugs. Anticancer agents first found in plants, such as taxol, vinblastine, vincristine, camptothecin (topotecan and irinotecan) and epipodophyllotoxin derivatives (etoposide) are in clinical use all over the world [2]. One of the plants currently being studied for its anti-proliferative activity is the *Hypericum* species [3]. According to previous reports on the anti-proliferative activity of six southern Brazilian species of *Hypericum*, the *n*-hexane fractions demonstrated good results [4]. In addition, the benzopyrans HP1, HP2 and HP3, which are structurally related to benzophenones, presented activity against the U-373 glioblastoma cell line [5]. In the lipophilic extracts of *H. myrianthum* and *H. carinatum*, one can detect dimeric phloroglucinols [6,7] and benzophenones [8], which are metabolites with anti-proliferative potential [9,10]. For this reason, this study aimed to evaluate the *in vitro* cytotoxic effects of the dimeric phloroglucinols japonicin A and uliginosin B, and the benzophenones cariphenone A and cariphenone B against different tumor cell lines. Whether these compounds enhance the activity of established chemotherapeutic agents was also evaluated.

2. Methods

2.1. Plant material

Blossoming aerial parts of *Hypericum carinatum* (Griseb) and *Hypericum myrianthum* (Cham. & Schlecht) were collected in the cities of Glorinha and Paraíso do Sul, respectively, in the state of Rio Grande do Sul, southern Brazil, between September and December, 2008. Plants were identified by Sérgio Bordignon (UNILASALLE, Canoas, Brazil). Voucher specimens were deposited in the herbarium of the Universidade Federal do Rio Grande do Sul. The Plant collection was authorized by the Conselho de Gestão do Patrimônio Genético (CGEN) and the Instituto Brasileiro do Meio Ambiente (IBAMA – 003/2008 P 02000.001717/2008‑60).

2.2. Extraction, fractionation and purification

All solvents were purchased from F. Maia (São Paulo, Brazil). Powdered aerial parts of the *Hypericum* species were thoroughly extracted with *n*-hexane. The fractions were combined, evaporated to dryness under reduced pressure (Rotavapor 8020 Fisatom®), treated with acetone and filtered off to remove waxes and insoluble impurities. The obtained fractions were then submitted to column chromatography on silica gel (70 – 230 Mesh, Merck, Darmstadt, Germany) using an *n*-hexane/ethyl acetate gradient system followed by preparative-TLC on silica gel GF 254 (Merck, Darmstadt, Germany) with hexane/dichloromethane (50:50) in accordance to Ferraz [6]. The benzophenones cariphenone A and cariphenone B were isolated from *H. carinatum*, while the dimeric phloroglucinols uliginosin B and japonicin A were isolated from *H. myrianthum*. The identity and purity of compounds were confirmed by ^{1}H-NMR (60 MHz) spectroscopy (Eft-60®, Anasazi Instruments) and compared with literature data [8].

2.3. *In vitro* anti-proliferative activity

2.3.1. Cell Culture and Treatment

Human glioblastoma cells (U-251), colorectal cancer cells (HT-29) and ovarian cancer cells (OVCAR-3) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The human glioblastoma cells were plated into 96 multi-well plates at a density of $5x10^3$ cells/well and maintained in Dulbecco's modified Eagle's growth medium (DMEM) (Gibco BRL, Carlsbad, USA). The others cells were plated at a density of $7x10^3$ cells/well and grown in RPMI-1640 medium (LGC, Brazil). Both media were supplemented with 10% (v/v) fetal bovine serum (FBS; Sorali, Campo Grande, Brazil) containing 2% (w/v) H-glutamine, 0.1 % fungizone, 0.125% gentamicin and 1% ampicillin. After 24 h, the cultures were treated with the benzophenones cariphenone A and cariphenone B at concentrations of 6.25, 25 and 100 µg/mL, and with the phloroglucinol derivative uliginosin B and japonicin A at concentrations of 0.5-50 µg/mL. The range of concentrations employed was chosen according to previous results for the lipophilic extracts in which the compounds are presented [4]. The samples were dissolved in DMSO, not exceeding a concentration of 0.5% (v/v), and further diluted with cell culture medium. All compounds were then used in association with paclitaxel, irinotecan and temozolamide. Cell viability was measured after 48 h. Cells were kept at a temperature of 37 °C, with minimum relative humidity of 95% and an atmosphere of 5% CO_2 in air.

2.3.2. Cell Proliferation

Cell viability was measured by 3-(4,5 dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich), which measures mitochondrial activity in viable cells, 48 h after the treatment. The cells were washed with Hank’s Balanced Salt Solution (Invitrogen,

São Paulo, Brazil), and 90 µl of DMEM or RPMI 1640 with 10 µl of the MTT solution at 5mg/mL solution were added to each well and incubated for 4 h at 37°C. The plate was left at room temperature until completely dried. At this point, dimethyl sulfoxide was added and the plate was read in an absorbance of 492 nm in a multi-plate reader.

2.4. Statistical Analysis

All experiments were performed in triplicates and the results are expressed as the mean ± standard error (SEM). The mean values for control cells were taken as 100%. The statistical significance of the results was determined by One-Way ANOVA followed by the Tukey post-hoc test when ANOVA *P* values were below 0.05. The SPSS program (Statistical Package for the Social Sciences®) software v.16.0 was used. *P* values <0.05 were considered statistically significant and the significance level of the results are represented by * (P <0.05), ** (P <0.01) and ***, (P<0,001) when compared to control.

3. Results and Discussion

The anti-proliferative activity of two benzophenones and two dimeric phloroglucinols was investigated against gliomas, colorectal and ovarian cancer cells. The results are presented in Table 1. Cariphenone A and cariphenone B promoted a reduction in cellular proliferation against all cancer lines at a dose of 100 µg/mL. In this concentration, the higher rates of inhibition were observed against U-251 (54 - 53%), HT-29 (53-37%) and OVCAR-3 (32-27%) cell lines, respectively.

Unlike the effects obtained with the benzophenones, the dimeric phloroglucinols japonicin A and uliginosin B exerted a significant effect only against the ovarian carcinoma cell line (OVCAR-3). At a dose of 50µg/mL, proliferation was reduced to 35-34% for both compounds.

The association of these natural compounds with the chemotherapeutic agents irinotecan, temozolamide and paclitaxel were also investigated. Both benzophenones (1-100µg/mL) and dimeric phloroglucinol derivatives (1-50µg/mL) failed to show a positive interaction with irinotecan (1-10µg/mL) and temozolamide (1-100µg/mL)**.** Nevertheless, an important reduction in cellular proliferation was observed in the OVCAR-3 cell line when it was treated in association with sub-effective doses of japonicin A and paclitaxel (1-10µg/mL; Fig.2).

Cariphenone A and cariphenone B (Fig. 1) belong to the group of the simple benzophenones which are not prenylated, and are considered rare because of their scarce presence in only a few species of *Hypericum* and *Garcinia* [8]. These benzophenones demonstrated moderate anti-proliferative activity (Table 1) when compared with hexane extracts from *H. carinatum* [4]. It is possible that the compounds act synergistically in the extract, producing a major effect. This profile action has already been observed, exemplified by the three benzopyrans (HP1, HP2 and HP3) isolated from *H. polyanthemum* [5]. Interestingly, both benzophenones investigated in this study (Table 1), as well as the benzopyrans, presented an important anti-proliferative response in glioblastoma cell lines [5]. The structural analysis of these metabolites revealed similarities between the benzopyran nucleus and an acyl radical, and this could justify the effectiveness against this tumor type.

In previous screenings with the *Hypericum* species native to southern Brazil, the hexane extract of *H. miryanthum* also showed the best inhibitory results against all tumor cells tested [4]. Among the compounds present in this fraction are the dimeric phloroglucinols japonicin A and uliginosin B [6,7] (Fig. 1). The phloroglucinols isolated from several species of this genus showed antimicrobial, antinociceptive and antidepressant potential besides cytotoxic activity; tumor suppression [9, 11-13]. was observed as well. In this context, we evaluated the anti-proliferative effects of japonicin A and uliginosin B; an interesting

therapeutic potential was verified, because of its low citotoxity and its selectivity for the OVCAR cell line (Table 1).

The association of natural compounds with chemotherapeutic agents has already has been demonstrated in other studies. The effect of paeonol, a phenolic component from the root bark of *Paeonia moutan*, for example, exerts an additive effect with cisplatin in esophageal cancer [14].

A major concern for cancer patients undergoing chemotherapy is the side effects caused by non-specific targeting of both normal and cancerous cells. Combination therapy is a common practice in cancer treatments. Besides aiming at different targets, this practice can achieve higher therapeutic effects and, consequently, reduce unspecific side effects and resistance to drugs [15]. In this study, a synergistic inhibitory effect on the viability of the human ovarian cancer cell line, OVCAR-3, was observed after combining sub-effective doses of paclitaxel with japonicin A. Therefore, the development of new drugs based on benzophenones or ploroglucinols scaffolds can be a useful alternative for the treatment of this disease.

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TABLES, FIGURES AND LEGENDS

Table 1.

Compounds	Dose (µg/mL)	Cell viability ± Standard deviation (%)		
		HT-29	OVCAR-3	U-251
Irinotecan	6	30 ± 4.3***	NT	NT
Paclitaxel	8	NT	58 ± 7.9**	NT
Temozolamide	100	NT	NT	40 ± 8***
	6	100 ± 3.9	100 ± 4.1	100 ± 10.6
	25	100 ± 9.7	96 ± 4.5	100 ± 7.6
Cariphenone A	100	47 ± 1.8**	68 + 1.4*	46 ± 2.0***
	6	100 ± 4.6	100 ± 5.1	100 ± 11.4
	25	100 ± 3.4	100 ± 6.4	97 ± 10
Cariphenone B	100	63 ± 3.1*	73 + 3.1*	47 ± 1.8***
	8	95 ± 9.2	91 ± 1.4	100 ± 1.9
	20	92 ± 9.7	78 ± 1.4	100 ± 2.7
Japonicin A	50	75 ± 3.4	65 ± 0.6**	95 ± 2.3
	8	100 ± 1.4	81 ± 1.0	100 ± 14.7
	20	100 ± 3.4	71 ± 1.2*	100 ± 9.6
Uliginosin B	50	85 ± 1.3	66 ± 0.8**	91 ± 8.3

NT = not tested

HT-29 = colorectal cancer cells

OVCAR-3 = ovarian cancer cells

U-251 = human glioblastoma cells

Table 1. Anti-proliferative activity . Anti-proliferative activity of the benzophenones and dimeric phloroglucinols isolated from *Hypericum* spp. P <0.05 *, P <0.01**, P <0.001 ***, compared to control.

Figure 1.

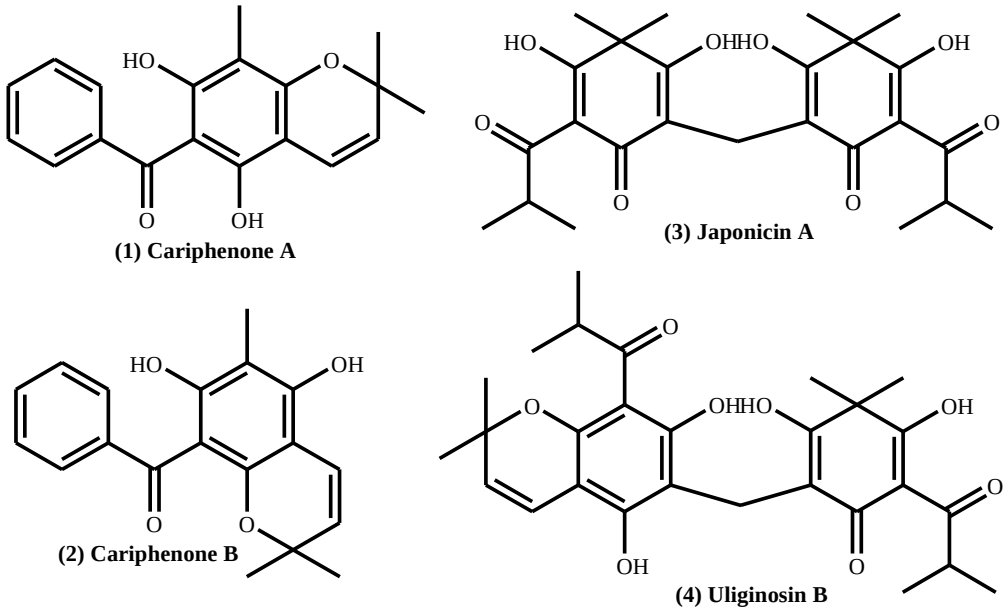

Figure 1. **Chemical structure.** Chemical structures of the benzophenones cariphenone A (1) and cariphenone B (2), and the dimeric phloroglucinols japonicin A (3) and uliginosin B (4).

Figure 2.

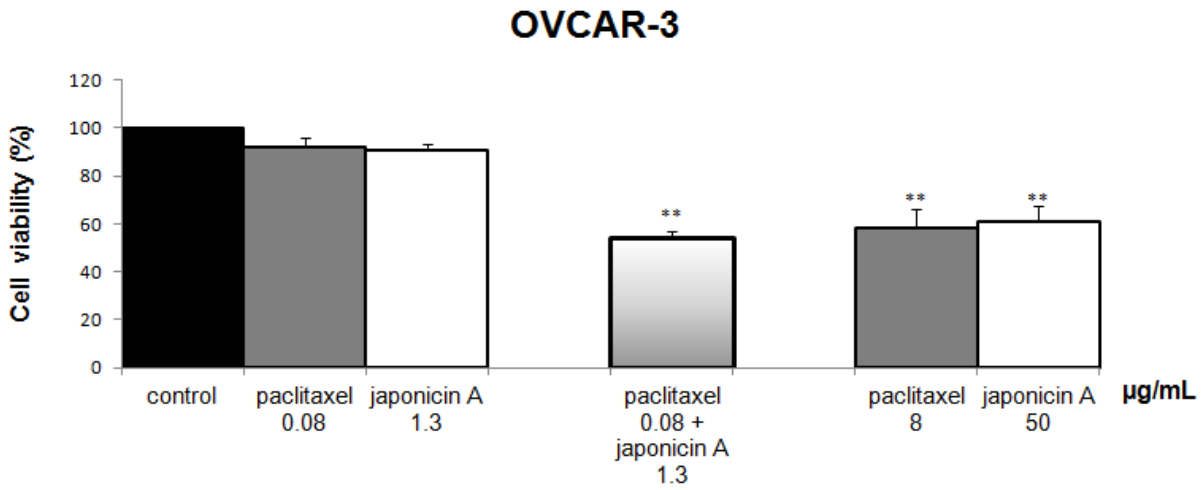

Figure 2. **Association compounds.** Anti-proliferative activity of associating sub-effective doses of japonicin A with paclitaxel, as measured by the MTT assay. *P* <0.01**, compared to control in OVCAR- 3 cell lines.

Original Paper…… Planta Medica

Antiproliferative activity of Japonicin A a phloroglucinol derivatives of *Hypericum* speciess in the human prostate adenocarcinoma cell line PC-3

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Abstract

In an *in vitro* screening with *Hypericum* species native to southern Brazil, the species *Hypericum miryanthum* showed the best inhibition results in all cell lines tested. In lipophilic extracts of *H. myrianthum* large quantities of dimeric phloroglucinols are found such as, japonicin A and uliginosin B. Some compounds belonging to classes of phloroglucinols are known for their antiproliferative activity. Prostate cancer is the second leading cause of cancer mortality and the most prevalent among men. It is kwon that some prostate cancer cells are reprogrammed to escape cell cycle arrest and apoptosis under androgen-depleted conditions. Thus, targeting the escape programs of tumor cells can be a promising strategy for treating androgen-independent prostate cancer. In this study, we investigated the antiproliferative effects of japonicin A, a dimeric phloroglucionol isolated from *H. myrianthum*, on prostate cancer using the PC-3 prostate cancer cell line as an *in vitro* tool. Japonicin A presented a dose-dependent activity in PC-3 cells reducing their proliferative potential and survival capability. Therefore, japonicin A may be a useful chemical moiety to serve as a lead molecule for anticancer drug development. In addition, an effort to understand the mechanisms underlying the actions of japonicin A can provide insight into novel therapeutic target discovery.

Key words: phlorogucinol, japonicin A, *Hypericum myrianthum*, PC-3 cell line.

Introduction

The Guttiferae is a large family with more than 1000 species. The genus *Hypericum* presents 494 species accommodated in 36 taxonomic sessions [1-2]. In southern Brazil, the representatives tend to accumulate compounds with phloroglucinol substitution patterns, [3] besides flavonoids and phenolic acids [4]. In *Hypericum* genus many phloroglucinol derivatives have been isolated. We know among ploroglucionols that the molecule of hiperatomarin obtained from *H. annulatum* demonstrated pro-apoptotic effect in a panel of tumor cell lines such as leukemias, lymphomas and solid tumors [5]. Furthermore, studies with another ploroglucinol, hyperforin, present in the species *H. perforatum*. have proven to be an inducer of apoptosis [6-7], especially in patients with chronic lymphocytic leukemia (CLL). Hyperforin also plays an important role in preventing the migration of tumor cells and in inhibiting angiogenesis [8-10]. Moreover, the study of guttiferona G and aristoforin, phloroglucinol derivatives isolated from plant species belonging to Guttiferae family, showed downregulation of histone deacetylases allowing the cells to enter apoptosis when subjected to stress or when suffering DNA damage[11].

When screening with *Hypericum* species native to southern Brazil the species *H. miryanthum* showed the best inhibition results in all strains tested [12].The lipophilic extract of *H. myrianthum* contains dimeric phloroglucinols[3,13-14]. Among the compounds of this kind are already isolated the ploroglucinols, japonicin A and uliginosin B have already been isolated [3], and the latter, in recent studies, showed a potential antidepressant and antinoceptive effect [15-16].

According to reports on the antiproliferative activity of *Hypericum* species native to southern Brazil, this study aimed to evaluate the *in vitro* cytotoxic effect of japonicin A one of the major phloroglucinol derivatives present in the lipophilic extracts of *H. myrianthum*, on PC-3 prostate cancer cell lines and evaluate its probable mechanism of action, which still remains poorly understood.

Materials and methods

Plant material

Blossoming aerial parts of the *Hypericum myrianthum* Cham. & Schlecht. were collected in Paraíso do Sul in Rio Grande do Sul, southern Brazil, between September and

December, 2008. Plants were identified by Sérgio Bordignon (UNILASALLE, Canoas, Brazil). Voucher specimens were deposited in the herbarium of Universidade Federal do Rio Grande do Sul. The Plant collection was authorized by the Conselho de Gestão do Patrimônio Genético (CGEN) and the Instituto Brasileiro do Meio Ambiente (IBAMA – 003/2008 P 02000.001717/2008- 60).

Extraction, fractionation and purification

Powdered aerial parts of the *Hypericum* speciess were thoroughly extracted with *n*-hexane. The fractions were combined, evaporated to dryness under reduced pressure, treated with acetone and filtered off to remove waxes and insoluble impurities. The obtained fractions were then submitted to column chromatography on silica gel using an *n*-hexane/ethyl acetate gradient system followed by preparative-TLC on silica gel with hexane/dichloromethane (50:50) in accordance to Ferraz et al., 2002[3]. Additionally, the purified compounds japonicin A from *H. myrianthum* were compared with patterns and proton and carbon resonance was conducted to confirm the identity.

***In vitro* antiproliferative activity**

Cell Culture and Treatment

The human prostate adenocarcinoma cell line, PC-3 was obtained from American Type Culture Collection (Rockville, Maryland, USA). The PC-3 cells were plated into 96 multiwell plates (TPP) at a density of de $3x10^3$ cells per well and maintained in Dulbecco's modified Eagle's medium : Nutrient Mixture F-12 (Ham) (1:1) (D-MEM/F-12,Gibco, Scotland, UK) supplemented with 10%(v/v) fetal bovine serum (FBS; Sorali, Campo Grande, Brazil) containing 0.1 % fungizone, 0.125% gentamicin and 1% ampicillin. [17-19]. After 24 h, the cultures were treated with the phloroglucinol derivative 4 - [[2,6-dihydroxy-3,3-dimethyl-5-(2-methylpropanoyl)-4-oxocyclohexa-1,5-dien-1-yl]methyl]-3,5-dihydroxy-6,6-dimethyl-2-(2 methylpropanoyl) cyclohexa-2,4-dien-1-one, japonicin A, at a concentration range of 0.0-200 µM . The range concentration employed was chosenaccording with previous results (not show).The samples were dissolved in DMSO, not exceeding a DMSO concentration of 0.5% (v/v), and further diluted with cell culture medium .Cell viability was measured after 48 h, 72h and 96h. Cells were kept at a temperature of 37 °C, at a minimum relative humidity of 95% and an atmosphere of 5% CO_2 in air.

Cell proliferation and survival assay

For trypan blue cell counting, the medium was removed and cells were washed with Hanks` Balanced Salt Solution (Invitrogen), 100 µL of 0,25% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a hemocytometer. Cell viability was measured by 3-(4,5 dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich), which measures mitochondrial activity, 48 h after the treatment. The cells were washed with Hank's Balanced Salt Solution (Invitrogen, São Paulo, Brazil), and 90 µl of DMEM plus 10 µl of MTT 5mg/ml solution was added to each well and then incubated for 4 h at 37°C. The plate was left at room temperature until it was completely dry. Dimethyl sulfoxide (DMSO) was added and the absorbance was read in 492 nm in a multiplate reader.

Cell survival was measured with a colony formation assay. PC-3 cells were seeded into 6-well plates (500 cells/well) after being treated with japonicin A for 48h. After incubation for 7-10 days, the cells were fixed with 70% ethanol and counterstained with 0,5% crystal violet. The colony counting was performed using Image J, a digital method for counting colonies [20], in which other parameters like Total Area and Avarage Size also taken into consideration. All parameters ware divide from control values and converted to percentages.

Flow cytometric analysis of cell cycle phase distribution

The human prostate cancer cell line PC-3 ($3x10^3$) was seeded in a 10 cm^3 culture plate and incubated for 24 h, after which the medium was removed and replaced with fresh mediumcontaining 8, 15, 25, 40, 70, 120 or 200 µg/mL of japonicin A and incubated for another 48 h. Then, cells were washed with PBS, and 0.25% trypsin- EDTA was added to detach cells for collection with PBS. Next, cells were centrifuged at 1500 rpm at (4°C) for 5 min, and the supernatant was collected in PBS containing chilled 70% ethanol was added to fix cells, and the cell suspension was centrifuged at 1500 rpm (4°C) for 5 min, followed by removal of the supernatant. The cell pellet was, washed three times with PBS, after which propidium iodide (PI) buffer (0.1%Triton X-100, 0.2 µg/mL RNase A, and 20 µg/mL PI) was added . After 30 minute incubation - 25°C cells were analyzed in a flow cytometer (Guava).

Nuclear morphometric analysis

The PC-3 cells treated for 72h with japonicin A had nuclei stained with DAPI (49,6-diamino-2-phenylindole) and pictures were taken on a fluorescent inverted microscope. Images were analyzed with the Image J program with the plugin NII. This tool evaluates the size and regularity of adhered cells in culture. These measurements, namely aspect, area box, radius ratio and roundness, were combined into a single nuclear irregularity index (NII). Normal nuclei are used to set the parameters for a given cell type, and different nuclear phenotypes are separated in an area versus NII plot [21].

Statistical analysis

All experiments were performed in triplicate and the results expressed as mean ± standard error (SEM). The statistical significance of the results was determined by one way analysis of variance (ANOVA) followed by the Tukey post hoc test, using software for statistical calculations SPSS (Statistical Package for the Social Sciences) 16.0 ®. P values <0.05 were considered statistically significant and the significance level represented by *, P <0.05, ** P <0.01 and ***, P <0.001 compared to control.

Results

The PC-3 cells showed the best results when screening a panel tumor cells lines (Tab.1S) We saw a reduction in PC-3 cell proliferation (Fig.2 and Fig.3) and in survival (Fig.4) in a dose-dependent manner, having the most pronounced effect in 72h when compared to 48h; this effect could be reverted in 96h when treatment doses were low. Cell cycle analysis in PC-3 cells treated for 72h with japonicin A were performed via flow cytometry. Japonicin A appeared to decrease G1 phase and induce an arrest in G2 (Fig.5) when cells were treated with high doses (200μM) of japonicin A. This effect was similar to the effect elicited by doxorubicin (10μM) when compared to control, although not statistically significant and NMA analysis, showed an increased in the apoptotic population 72h after treatment (Fig.2S).

Discussion

Prostate cancer is the second leading cause of cancer mortality and the most prevalent among men [22]. Timely diagnosis and treatment provide high cure rates, however resistance to chemotherapy is a significant barrier to the effective management of prostate cancer.

Unequivocal evidence has been gathered to consider that the androgen receptor axis is the most critical pathway for the progression of prostate cancer [23] and is known that some prostate cancer cells are reprogrammed to escape from cell cycle arrest and apoptosis under androgen-depleted conditions [24-25].

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair, thus preventing their transmission to the resulting daughter cells. Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells into cancer cells [26-27]. When taken together these alterations, result not only in proliferative advantages but also in increased susceptibility to the accumulation of additional genetic alterations that contribute to tumor progression and acquisition of more aggressive phenotypes [28]. The importance of checkpoint defects for the development of cancer provides important targets for therapy. In this context, japonicin A appears to restore the capacity of the PC-3 cell cycle to respond to damage, decreasing G1 phase and inducing an arrest in G2 (Fig.5) when cells were treated with high doses (200μM). Furthermore, the NMA analysis, showed an increase in the apoptotic population 72h after treatment. Thus, targeting the escape programs of these tumor cells can be a promising strategy for treatment of androgen-independent prostate cancer.

Despite the high doses at which japonicin A shows to be effective, it harbors a specificity for independent hormonal prostate cancer (Fig. 1S), which is more aggressive and is presents in most cases of resistance to chemotherapy. Therefore, this compound may present a useful chemical moiety to serve as a lead molecule for anticancer drug development, especially when looking at pharmacophore models. Since japonicin A is a relatively simple substance, this facilitates the process of molecular modeling and synthesis. In addition, an

effort to understand the mechanisms of action underlying the results of japonicin A treatment can provide insight into novel therapeutic target discoveries.

Acknowledgement

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Legends for figures

Fig. **1** Chemical structure of japonicin A isolated from *Hypericum myrianthum*

Fig **2**. PC-3 cell viability when treated with different doses of japonicin A analyzed in 48, 72 and 96h by the MTT assay.

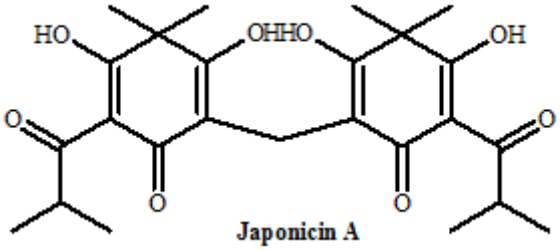
Fig. **3**. PC-3 cell viability when treated with different doses of japonicin A analyzed in 48, 72 and 96h with Trypan blue.

Fig.**4**. Survival of PC-3 cells after treatment with different doses of japonicin A, as measured by the colony formation assay.

Fig.**5a.** Representative cell cycle histograms of PC-3 cells treated with japonicin A and analyzed in 72h. **A**.control, **B**. doxorrubicin 10µM, **C**.japonicin A 200 µM, **D**. japonicin A 120 µM, **E**. japonicin A 70 µM, **F**. japonicin A 40 µM, **G**. japonicin A 25 µM, **H**. japonicin A 14 µM and **I**. japonicin A 8 µM.

Fig.**5b.** Graphic representation of PC-3 cell cycle analysis treated with different doses of japonicin A analyzed in 72h.

Fig. 1



Japonicin A

Fig.2

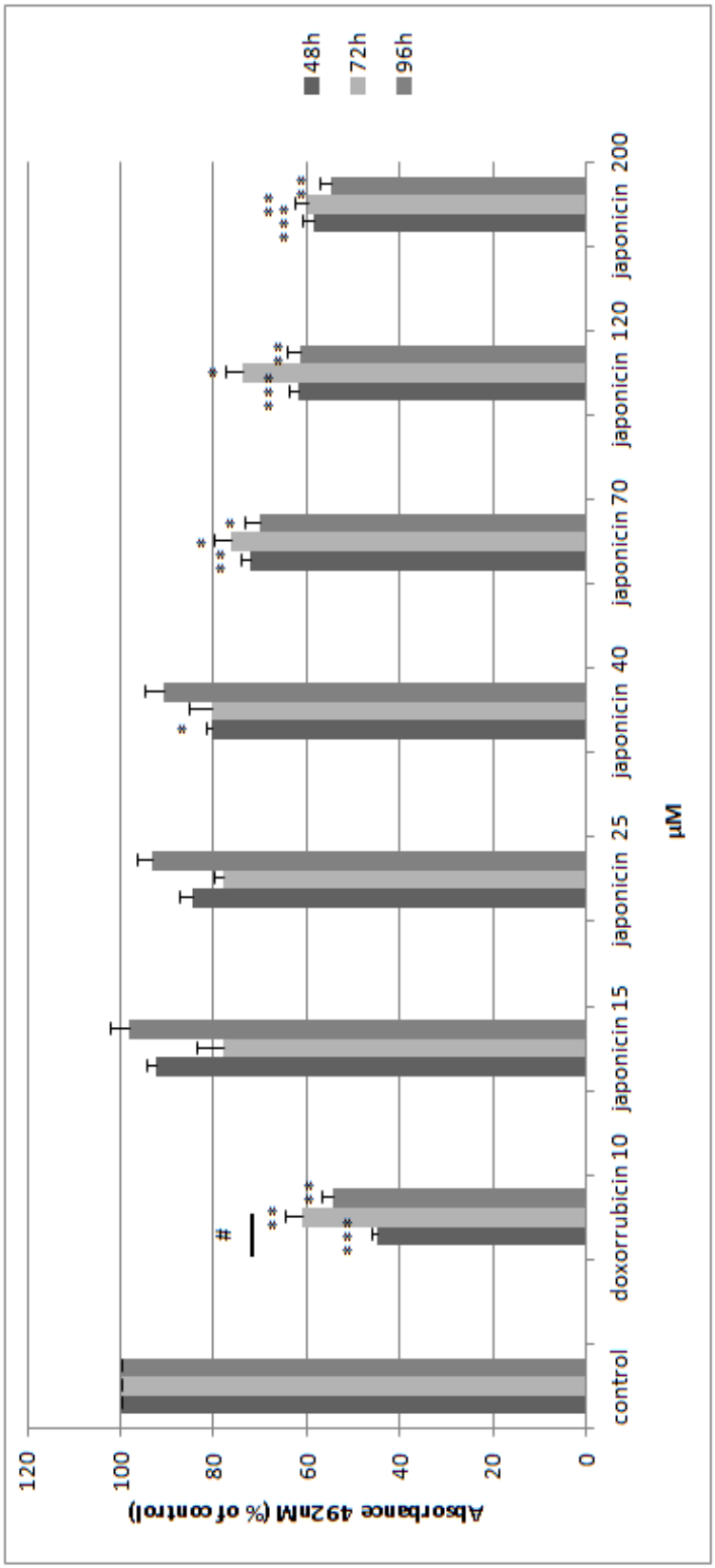

48h
72h
96h
Absorbance 492nM (% of control)
120
100
80
60
40
20
0
control
doxorrubicin 10
japonicin 15
japonicin 25
japonicin 40
japonicin 70
japonicin 120
japonicin 200
μM

Fig 3

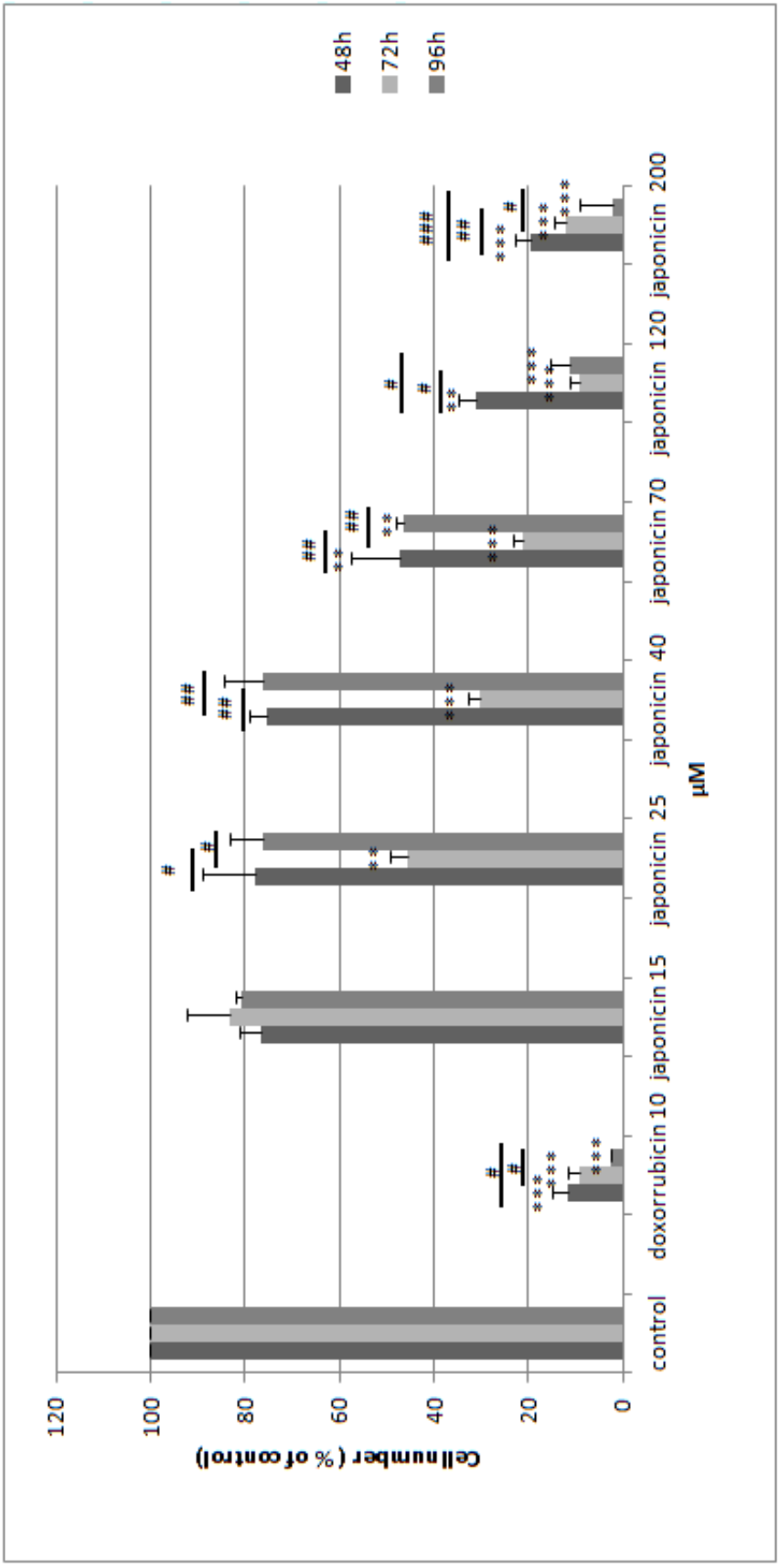

48h
72h
96h
Cell number (% of control)
120
100
80
60
40
20
0
control
doxorrubicin 10
japonicin 15
japonicin 25
japonicin 40
japonicin 70
japonicin 120
japonicin 200
μM

Fig 4

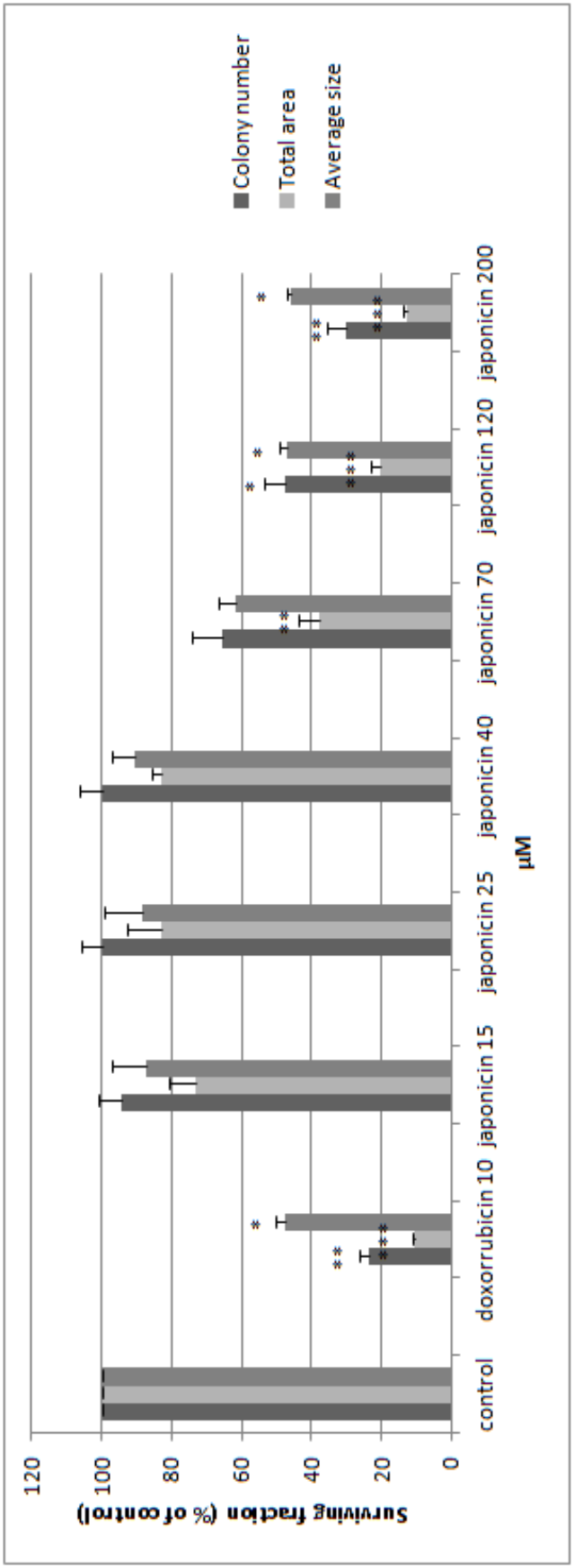

Colony number
Total area
Average size
Surviving fraction (% of control)
120
100
80
60
40
20
0
control
doxorrubicin 10
japonicin 15
japonicin 25
japonicin 40
japonicin 70
japonicin 120
japonicin 200
μM

Fig. 5a

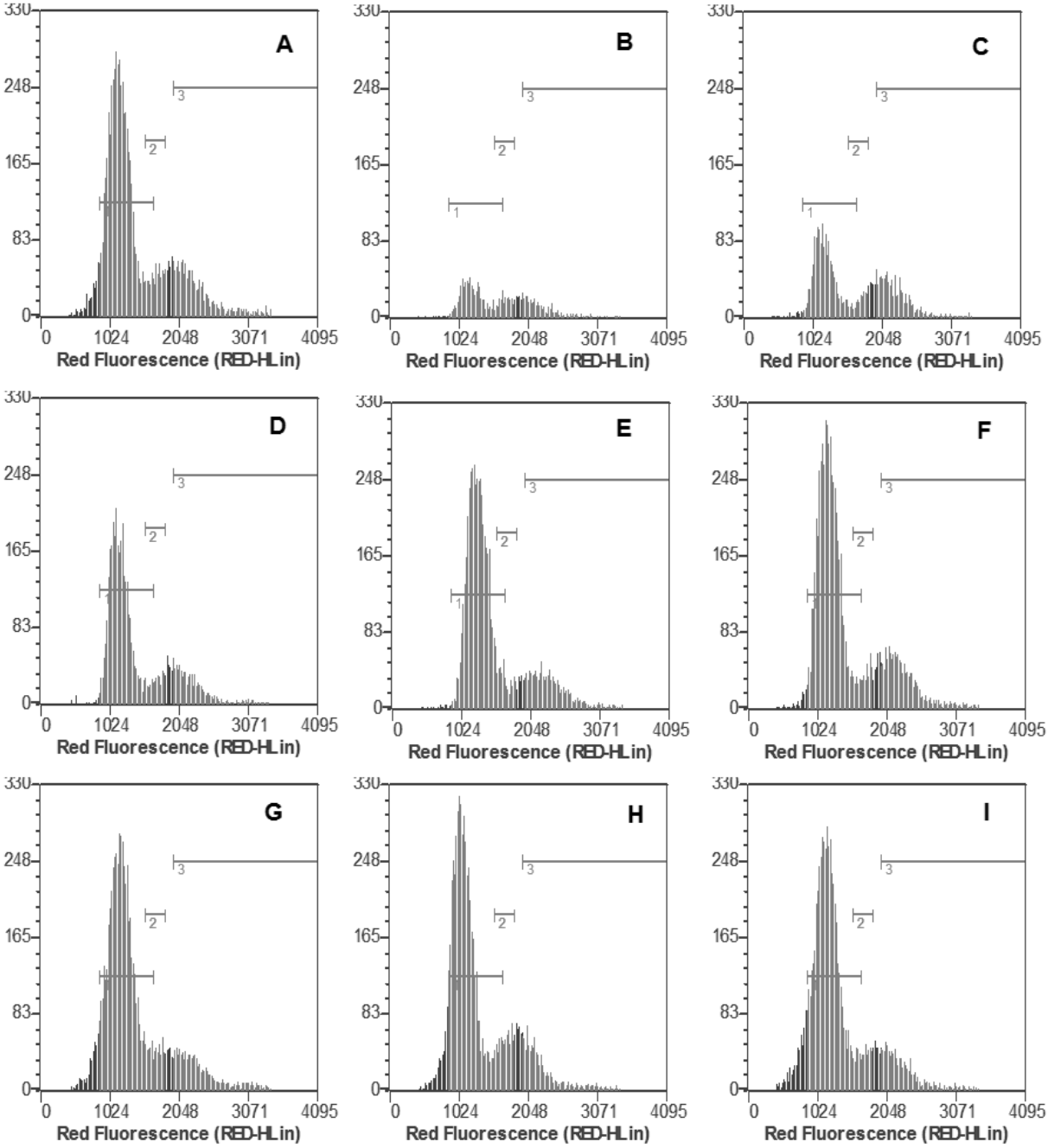
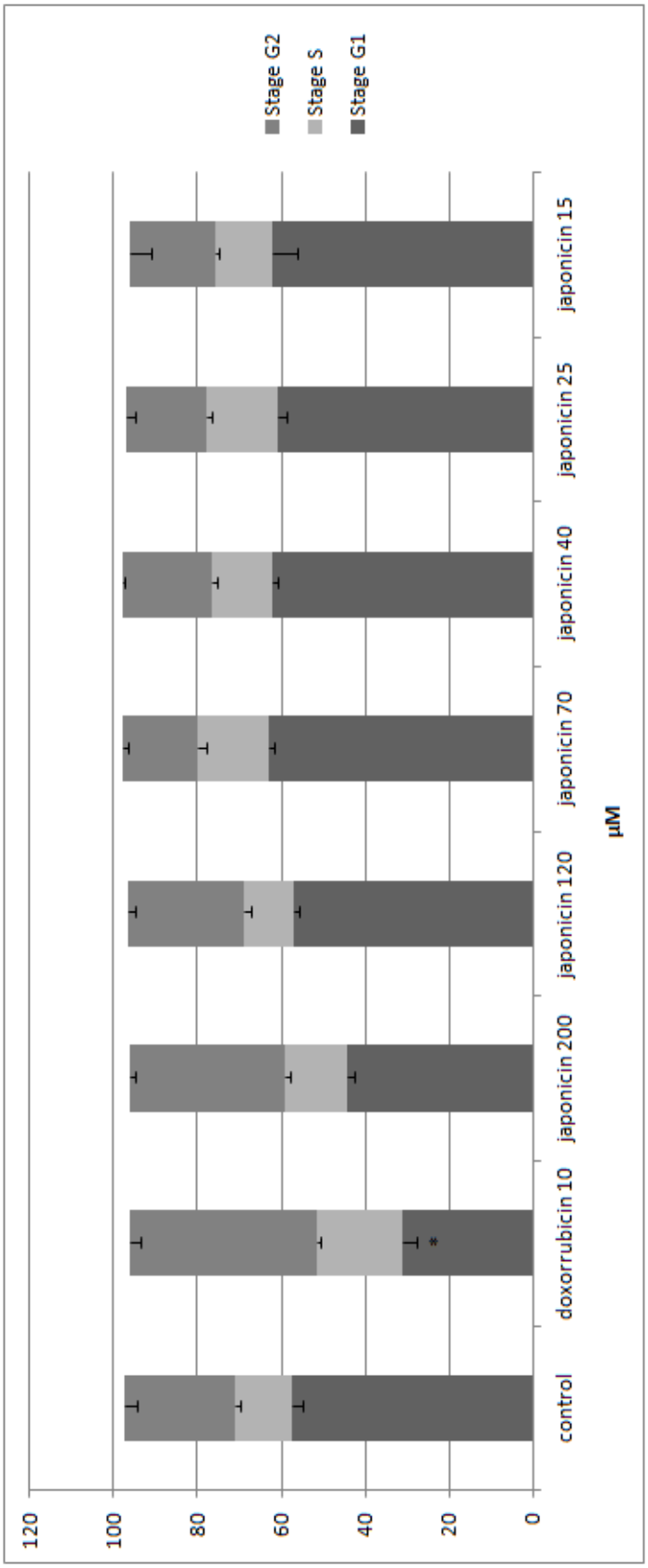

A
B
C
D
E
F
G
H
I
330
248
165
83
0
0
1024
2048
3071
4095
Red Fluorescence (RED-HLin)
1
2
3

Fig.5b


Stage G2
Stage S
Stage G1
120
100
80
60
40
20
0
control
doxorrubicin 10
japonicin 200
japonicin 120
japonicin 70
japonicin 40
japonicin 25
japonicin 15
μM
*

Supporting information

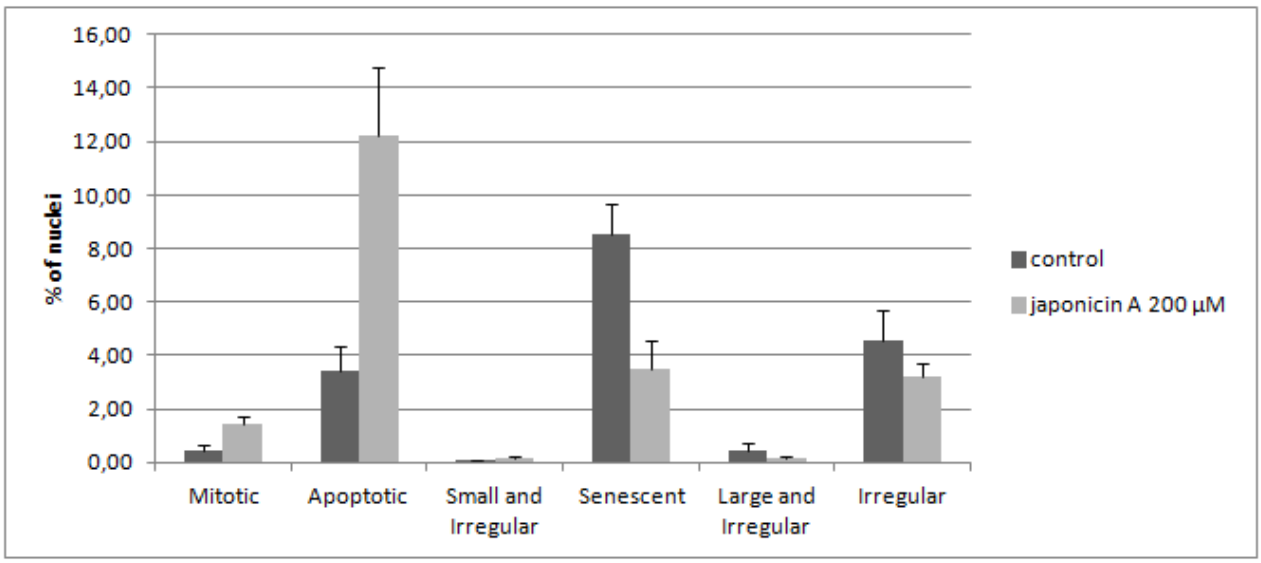
Tab.1S. Screening of antiproliferative activity of japonicin A in a panel of human cell lines by MTT assay in 48 hours.

Table 1. Screening of viability in cell lines treated 48h with Japonicin A by MTT assay.

Cell type	Names	Description	Activity (200 µM)
Normal	*	Stem cell from human deciduous teeth	n.d.
Tumor	U-251	Human glioblastoma	n.d.
	U-138	Human glioblastoma	n.d.
	D-283	Human medulloblastoma	n.d.
	SHSY5Y	Human neuroblastoma	n.d.
	SK-ES	Human anaplastic osteosarcoma or Ewing's sarcoma	n.d.
	HT-29	Human colorectal adenocarcinoma	n.d.
	OVCAR	Human ovarian adenocarcinoma	200
	PC-3	Human prostatic adenocarcinoma	< 200

n.d.= not detected
*stem cell from primary culture
The japonicin A activity was compared to untreated control of each cell lines

Fig 2S. Nuclear morphometric analysis of PC-3 cells treated 72h with 200µM of japoncin A



6.Considerações finais

As substâncias avaliadas neste trabalho são compostos majoritários extraídos de plantas de *Hypericum* nativas do sul do Brasil (*H. carinatum* e *H. myrianthum*), cujos extratos haviam sido testados quanto a sua atividade antiproliferativa *in vitro*.

Esses compostos pertencem a classe das benzofenonas (carifenona A e carifenona B) e dos floroglucionóis (japonicina A e uliginosina B) que apresentam estruturas promissoras em estudos relacionados a citotoxicidade e diminuição da viabilidade celular tumoral.

Avaliamos a atividade destas quatro substâncias em três diferentes tipos de linhagens tumorais (HT-29, OVCAR-3 e U-251), bem como a associação de doses inefetivas destes compostos e de quimioterápicos e verificamos que todos os compostos apresentavam uma moderada atividade antiproliferativa.

As benzofenonas (carifenona A e carifenona B) na dose de 100μg/mL demonstraram atividade em todas as linhagens (OVCAR-3 < HT-29 <U-251) avaliadas, contudo o extrato de *H. carinatum* apresentou uma atividade mais pronunciada segundo o estudo de Ferraz e colaboradores (2005a), isto pode ser atribuído a possível efeito sinérgico das substâncias presentes no extrato, assim como foi demonstrado no trabalho de Grivicich e colaboradores (2008), no qual os benzopiranos isolados de *H. polyanthemum* agiam sinergicamente na linhagem avaliada de glioblastoma humano. As semelhanças estruturais entre a classe dos benzopiranos e benzofenonas pode estar relacionado ao maior efeito que ambos os compostos desta classe apresentaram na linhagem de glioblastoma humano.

A combinação dos compostos apresentou como principal resultado a associação positiva entre doses inefetivas da substância japonicina A e do quimioterápico placitaxel, resultando na diminuição significativa da viabilidade celular na linhagem OVCAR-3. A utilização de compostos que agem por mecanismos diferentes ou ainda que inibem diferentes pontos de uma mesma via, reduzem a possibilidade de resistência ao tratamento, além de propiciarem a utilização de doses menores de cada composto o que minimiza os efeitos adversos, contudo aumenta na taxa de mortalidade de células malignas.

A partir dos resultados obtidos nesta primeira parte do trabalho em que todos os composto apresentavam uma moderada atividade antiproliferativa, optamos por investigar a aparente seletividade apresentada pela japonicina A. Neste momento avaliamos a atividade

deste composto frente um número maior de linhagens tumorais. Verificamos que japonicina A foi ativa somente nas linhagens OVCAR- 3 e PC-3. Contudo, demos continuidade aos estudos utilizando a linhagem PC-3 para uma melhor avaliação do efeito da japonicina A, uma vez que este composto nesta linhagem foi ativo de maneira dose-resposta e em doses mais baixas do que na linhagem OVCAR-3.

A linhagem PC-3 é originária de adenocarcinoma de próstata grau IV, neoplasia hormônio-independente conhecida por sua alta agressividade e baixa resposta aos quimioterápicos. Estudos apontam a depleção hormonal como um dos fatores para a presença de defeitos nos pontos de checagem do ciclo celular bem como da inativação da apoptose, presentes neste tipo de neoplasia. Contudo a japonicina A obteve um importante efeito na diminuição da viabilidade e sobrevivência desta linhagem, bem como, alterou o ciclo celular, fato este que pode estar relacionado a um aumento da apoptose.

Apesar da moderada atividade antiproliferativa destes compostos, são moléculas simples, o que facilita o processo de síntese e modelagem molecular, podendo ser utilizadas como modelos de estudos farmacofórios. Especialmente o composto japonicina A, que demonstrou seletividade pela linhagem OVCAR-3, na qual também apresentou uma interessante associação em doses inefetivas com o quimioterápico paclitaxel; e na linhagem PC-3 na qual apresentou um importante efeito dose-resposta.

7.Anexos

7.1 Confirmação envio do manuscrito a revista Anti-Cancer Drugs

Anti-Cancer Drugs

Anti-proliferative activity of the dimeric phloroglucinol and benzophenone derivatives of native Hypericum species of southern Brazil

--Manuscript Draft--

Manuscript Number:	ACD-2571
Full Title:	Anti-proliferative activity of the dimeric phloroglucinol and benzophenone derivatives of native Hypericum species of southern Brazil
Article Type:	Short Communication
Section/Category:	Pre-Clinical Report
Keywords:	anti-proliferative activity; dimeric phloroglucinols; benzophenones; Hypericum.
Corresponding Author:	Ana Lucia Abujamra Children´s Cancer Institute Porto Alegre, Rio Grande do Sul BRAZIL
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First Author Secondary Information:	
Order of Authors:	Amanda Valle Pinhatti
	Francisco Maikon Corrêa de Barros
	Caroline Brunetto de Farias
	Gilberto Schwartsmann
	Gilsane Lino von Poser
	Ana Lucia Abujamra
Order of Authors Secondary Information:	

7.2 Normas Anti-Cancer Drugs

SCOPE

Anti-Cancer Drugs is devoted to the rapid publication of innovative investigations on therapeutic agents against cancer and methods to diminish adverse effects of drugs. The journal publishes peer reviewed articles on anti-cancer drug design, drug delivery, pharmacology and chemotherapy evaluation.

Submissions

Authors are strongly encouraged to submit their manuscripts through the web-based tracking system at http://www.editorialmanager.com/acd. The site contains instructions and advice on how to use the system. Authors should NOT in addition then post a hard copy submission to the editorial office, unless you are supplying artwork, letters or files that cannot be submitted electronically, or have been instructed to do so by the editorial office. Should authors have difficulty in uploading files contact the Editor, Dr Mels Sluyser at e-mail: m.sluyser@planet.nl in the first instance, but do not send your files.

Double spacing should be used throughout the manuscript, which should include the following sections, each starting on a separate page: Title Page, abstract and keywords, text, acknowledgements, references, individual tables and captions. Margins should be not less than 3 cm. Pages should be numbered consecutively, beginning with the Title Page, and the page number should be placed in the top right hand corner of each page. Abbreviations should be defined on their first appearance in the text; those not accepted by international bodies should be avoided.

Authors are invited to list up to four potential reviewers, including their full addresses, telephone and fax numbers, and e-mail addresses.

PRESENTATION OF PAPERS

Title Page

The Title Page should carry the full title of the paper and a short title to be used as a ‘running head’ (and which should be so identified). The first name, middle initial and last name of each author should appear. If the work is to be attributed to a department or institution, its full name should be included. Any disclaimers should appear on the Title Page, as should the

name and address of the author responsible for correspondence concerning the manuscript and the name and address of the author to whom requests for reprints should be made. Finally, the Title Page should state conflicts of interest and source of funding, and when none state “none declared”.

Abstracts

The second page should carry a structured abstract of no more than 250 words(temos 190). The abstract should state the Objective(s) of the study or investigation, basic Methods (selection of study subjects or laboratory animals; observational and analytical methods), main Results (giving specific data and their statistical significance, if possible), and the principal Conclusions. It should emphasise new and important aspects of the study or observations.

Key Words

The abstract should be followed by a list of 3–10 keywords or short phrases which will assist the cross-indexing of the article and which may be published. When possible, the terms used should be from the Medical Subject Headings list of the National Library of Medicine (http://www.nlm.nih.gov/mesh/meshhome.html).

Text

Full papers of an experimental or observational nature may be divided into sections headed Introduction, Methods (including ethical and statistical information), Results and Discussion (including a conclusion), although reviews may require a different format.

Acknowledgements

Acknowledgements should be made only to those who have made a substantial contribution to the study. Authors are responsible for obtaining written permission from people acknowledged by name in case readers infer their endorsement of data and conclusions.

References

References should be numbered consecutively in the order in which they first appear in the text. They should be assigned Arabic numerals, which should be given in brackets, e.g. [17], [17, 19-23]. References should include the names of all authors when six or fewer; when

seven or more, list only the first six names and add et al. References should also include full title and source information. Journal names should be abbreviated as in MEDLINE (NLM Catalog, http://www.ncbi.nlm.nih.gov/nlmcatalog).

Articles in journals

Standard journal article:

Ravaud A, Bello CL. Exposure–response relationships in patients with metastatic renal cell carcinoma receiving sunitinib: maintaining optimum efficacy in clinical practice. Anticancer Drugs 2011; 22:377–383.

More than six authors:

Umemura S, Yamane H,Yano T, Hoshika T, Shiote Y, Takigawa N, et al. Synchronous double primary lung cancers with different response to pemetrexed. Anticancer Drugs 2011; 22:473–476.

Supplements:

Gianni AM, Berinstein NL, Evans PAS, López-Guillermo A, Solano C. Stem-cell transplantation in non-Hodgkin's lymphoma: improving outcome. Anticancer Drugs 2002; 13 (suppl): S35–42.

Books

Book:

DeVita VT, Hellman S, Rosenberg SA. Cancer: principles & practice of oncology, 7th edn. Philadelphia: Lippincott Williams & Wilkins, 2005.

Chapter in a book:

Restifo NP, Wunderlich JR. Cancer Immunology. In: DeVita VT, Hellman S, Rosenberg SA, eds. Cancer: principles & practice of oncology, 7th edn. Philadelphia: Lippincott Williams & Wilkins, 2005: 139-161.

Personal communications and unpublished work should not feature in the reference list but should appear in parentheses in the text. Unpublished work accepted for publication but not

yet released should be included in the reference list with the words ‘in press’ in parentheses beside the name of the journal concerned. References must be verified by the author(s) against the original documents.

Tables

Each table should be typed on a separate sheet in double spacing. Tables should not be submitted as photographs. Each table should be assigned an Arabic numeral, e.g. (Table 3) and a brief title. Vertical rules should not be used. Place explanatory matter in footnotes, not in the heading. Explain in footnotes all non-standard abbreviations that are used in each table. Identify statistical measures of variations, such as standard deviation and standard error of the mean.

Be sure that each table is cited in the text. If you use data from another published or unpublished source, obtain permission and acknowledge the source fully.

Illustrations

A) Creating Digital Artwork

1. Learn about the publication requirements for Digital Artwork: http://links.lww.com/ES/A42

2. Create, Scan and Save your artwork and compare your final figure to the Digital Artwork Guideline Checklist (below).

3. Upload each figure to Editorial Manager in conjunction with your manuscript text and tables.

B) Digital Artwork Guideline Checklist

Here are the basics to have in place before submitting your digital artwork:

- Artwork should be saved as TIFF, EPS, or MS Office (DOC, PPT, XLS) files. High resolution PDF files are also acceptable.

- Crop out any white or black space surrounding the image.

- Diagrams, drawings, graphs, and other line art must be vector or saved at a resolution of at least 1200 dpi. If created in an MS Office program, send the native (DOC, PPT, XLS) file.

- Photographs, radiographs and other halftone images must be saved at a resolution of at least 300 dpi.

- Photographs and radiographs with text must be saved as postscript or at a resolution of at least 600 dpi.

- Each figure must be saved and submitted as a separate file. Figures should not be embedded in the manuscript text file.

Remember:

- Cite figures consecutively in your manuscript.

- Number figures in the figure legend in the order in which they are discussed.

- Upload figures consecutively to the Editorial Manager web site and enter figure numbers consecutively in the Description field when uploading the files.

- References to figures and tables should be made in order of appearance in the text and should be in Arabic numerals in parentheses, e.g. (Fig. 2).

- If hard copies are submitted they should have a label pasted to the back bearing the figure number, the title of the paper, the author's name and a mark indicating the top of the figure. Illustrations should be presented to a width of 82 mm or, when the illustration demands it, to a width of 166 mm.

- Photomicrographs must have internal scale markers.

- If photographs of people are used, their identities must be obscured or the picture must be accompanied by written consent to use the photograph.

- If a figure has been published before, the original source must be acknowledged and written permission from the copyright holder for both print and electronic formats should be submitted with the material. Permission is required regardless of authorship or publisher, except for documents in the public domain.

- Figures may be reduced, cropped or deleted at the discretion of the editor.

- Colour illustrations are acceptable but authors will be expected to cover the extra reproduction costs (for current charges, contact the publisher).

Legends for illustrations

Captions should be typed in double spacing, beginning on a separate sheet of paper. Each one should have an Arabic numeral corresponding to the illustration to which it refers. Internal scales should be explained and staining methods for photomicrographs should be identified.

Units of measurement

Measurements of length, height, weight, and volume should be reported in metric units (metre, kilogram, or litre) or their decimal multiples. Temperatures should be given in degrees Celsius. Blood pressures should be given in millimetres of mercury.

All haematologic and clinical chemistry measurements should be reported in the metric system in terms of the International System of Units (SI). Editors may request that alternative or non-SI units be added by the authors before publication.

Abbreviations and symbols

Use only standard abbreviations. Avoid abbreviations in the title and abstract. The full term for which an abbreviation stands should precede its first use in the text unless it is a standard unit of measurement.

Offprints

Offprints may be purchased using the appropriate form that will be made available with proofs. Orders should be sent when the proofs are returned; orders received after this time cannot be fulfilled.

7.2 Normas Planta Medica

Format of Manuscripts

Original Papers. Original papers are research articles describing original experimental results. Thematerial should be arranged in the order: Title Page/ Abstract /Keywords/Abbreviations / Introduction/ Materials and Methods/Results / Discussion /Acknowledgements/ References / Figure Legends/ Tables/ Structural Formulas/ Figures.

Results and Discussion sections may appear as two separate parts or as a combined "Results and Discussion" section. No subheadings are allowed within this section. The normal length of the main text of an Original Paper, excluding references, tables, figures and figure legends, is about 3,000 words. In exceptional and well justified cases longer manuscripts may be accepted. When submitting such manuscripts, authors should provide a justification statement, giving compelling reasons for the length of the paper.

Preparation of Manuscripts

In addition to the Guidelines, authors should consult the sample manuscript (for Original Papers) or the sample letter at http://mc. manuscriptcentral.com/plamed → Instructions and Forms, or at www.thieme.de/fz/plantamedica prior to preparing their contribution. Commonly used text processors should be used for preparation of the manuscripts.

For submission of all manuscripts, follow the instructions of the online submission system. Before submission, prepare the cover letter, and keep ready all information on the manuscript (title, full name and affiliation of all authors, abstract, name of all files to be submitted). The author submitting themanuscript will be corresponding author.

The Title Page must contain the title of the manuscript, the full names referenced by numerical superscripts with affiliation and addresses of all authors, and thefull address of the corresponding author.

The Abstract should contain brief information on purpose, methods, results and conclusion (without subheadings).

The Keywords should include the scientific name and family of the plant(s) or other organism(s) investigated. 4–6 keywords should be listed.

Abbreviations should generally be used sparingly. Standard abbreviations such as m.p., b.p., K, s, min, h, μL, mL, μg, mg, g, kg, nm, mm, cm, ppm, mmol, HPLC, TLC, GC, UV, CD, IR,MS, NMR can be used throughout themanuscript. Non-standard abbreviations must be defined in the text following their first use. Provide a list of all nonstandard abbreviations after the keywords. Define all symbols used in equations and formulas. If symbols are used extensively, provide a list of all symbols together with the list of abbreviations.

The Introduction should state the purpose of the investigation and relate to current knowledge in the specific topic addressed.

Materials and Methods. Specific details about test materials and test compounds, instrumentation and experimental protocols should be given here. This section should contain sufficient details so that others are able to reproduce the experiment(s). Purity (%) of all reference and standard compounds should be mentioned, as well as the method howit was determined. Previously reported methods should be referenced only. Suppliers for major equipment, cell lines, chemicals, biochemical reagents and major disposables should be indicated.

Documentation of plants and other organisms or starting materials. Use the correct scientific nomenclature. For plants, the Index Kewensis (electronic Plant Information Centre ePIC, Royal Botanic Gardens, Kew, UK: http://www.kew.org/epic), and/or the Inter- national Code of Botanical Nomenclature (www.bgbm.fu-berlin.de/ iapt/nomenclature/code/tokyo-e/default.htm) should be followed. Give the scientific name (in italics), the author of this name and the family. Indicate who identified the material. The manuscriptmust include references to voucher specimens of the plants (deposited in a major regional herbarium) or the material examined including their registration number(s). It should be mentioned which plant parts have been used.

Description of the preparation of extracts and isolation of compounds. The kind and amount of starting material, solvents and extraction methods must be indicated. The description of chromatographic systems should contain the quantitative information that allows the reader to repeat the work. Column dimensions, stationary phase, particle size, mobile phase composition, flow rate, sample amount, and elution volumes (or retention times, k' values) of fractions should be given. E. g.: "MPLC on silica gel (40–63 μm; 2 × 50 cm), MeOH/EtOAc 8: 2, 3mL/min; tR of 1: 60–70 mL, 2: 120–140 mL, 3: 145–175 mL; detection of eluates by

TLC (SiO2, MeOH/H2O 9:1; Dragendorff reagent), Rf 1: 0.35, 2: 0.55, 3: 0.73).” When using gradients the volumes of solvents should be presented; fractions should be defined by their elution volume. Similar information is necessary for HPLC, GLC, DCCC,MLCC and all other methods of purification. Figures of chromatograms will only be accepted if they are essential for understanding themethods or the results described. GC identifications of constituents of essential oils must be supported by retention indices on a polar and an apolar column. Identification by GC‑MS is preferred.

Physico-chemical characterisation of compounds. Data provided for new compounds should enable an unambiguous identification of the substance and have to appear in the following order, if available: visual appearance, chromatographic mobility in TLC, GC, or HPLC, mp, UV‑vis, specific optical rotation, CD, IR, 1H‑NMR, 13C‑NMR, low resolution MS, high resolution MS, elemental analysis. Note that for specific optical rotation [α]D temp, the symbol c is defined as mass of substance (in g) in 100 mL of solution. For specific optical rotation no unit should be specified; the “degree” symbol “°” should not be used. In case of spectroscopic work on known substances refer, if possible, to published data; the manuscript should then contain the following indication: Copies of the original spectra are obtainable from the corresponding author. Such original spectra and/or spectral assignments can be provided as Supporting Information (see below), as well as structural formula outlining NMR spectral correlations, MS fragmentations, etc. IR, NMR, mass, and UV spectra should normally not be given in the manuscript as figures, but only if the listing of characteristic signals is not sufficient.

Chemical nomenclature used should be based on the systematic rules adopted by Chemical Abstracts and IUPAC. Trivial names should be avoided unless they are definitely advantageous over the corresponding systematic names. Trivial names are not accepted for close analogues and derivatives of known compounds. For reference drug substances the INN names should be used.

X‑Ray crystallographic data must include a line drawing of the structure, a perspective drawing, and a discussion of bond lengths and angles. A supplement describing full details of the structure and methods and means of its determination in a form suitable for deposition must be submitted to the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (fax: + 44 (0)1223 33 60 33 or e-mail: deposit@ccdc.cam.ac.uk). Deposition of th e data has to be prior to submission of the manuscript, and appropriate

reference has to be made in the Materials and Methods section, including the deposition number.

Analytical studies. Key data on method validation must be provided and should typically include information on specificity, linearity, limit of detection, limit of quantification, accuracy, precision, intermediate precision, and some robustness studies. Information on the purity of reference compounds, and on the methods used for the determination of purity must be given. Recoveries of extraction and sample pre-purification steps have to be indicated. Adequate statistical treatment of data is required. For more information regarding validation issues, prospective authors should also refer to ICH guidelines. Analytical studies of a routine nature will not be considered for publication.

Pharmacological investigations. Planta Medica will only considermanuscripts in which conclusions are based on adequate statistics that incorporate the appropriate tests of significance, account for the type of data distribution and are based on the number of experimental observations required for the application of the respective statistical method. In each case positive controls (reference compounds) should be used and the dose/activity dependence should be shown. When working with experimental animals, reference must be made to principles of laboratory animal care or similar regulations, and to approval by the local ethical committee. The approval number and the corresponding date must be provided. Pharmacological investigations of extracts require detailed extract characterisation. This includes botanical characterisation of plant material, solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). The drug to extract ratio (DER) must be given. Chromatographic profiling (e.g. HPLC profile with a reference compound recorded at different wavelengths) should be carried out, with at least the major peaks identified, or qualitative and quantitative information on active or typical constituents should be provided. Altogether the phytochemical standardization of an extract and/or fraction(s) require state-of-the-artmethods.

Clinical studies. Studies reporting on plant preparations tested in humans will be accepted for review and publication. Clinical studies must be designed, implemented and analyzed in a manner to meet current standards of randomised controlled trials. For guidelines see the following reviews: Begg C et al. JAMA 1996; 276: 637–639 and Altmann DG. BMJ 1996; 313: 570–571. Reference must be made to approval of the study by the local ethical committee. The approval number and the corresponding date must be provided. All methods

and variables used in a trial should be described; the data must be based on adequate statistics. Herbal medicinal products used must be characterised as described above for pharmacological investigations.

Biological screening. Papers dealing with the biological screening of a meaningful number of extracts of plants or other organisms can be considered for publication in Planta Medica. Identification of the material should properly be documented, and preparation of the extracts should clearly be described (see above, sections 4.6.1 and 4.6.2). Biological activities should be reported by listing IC50 values, or a dose-response relationship should be shown by using at least two test concentrations. Positive controls (reference compounds) should be included. Results should be presented in a concise format, and the discussion should be kept to a minimum. 4.7. Results should be presented in a concise manner. Tables and figures should be presented in a manner which maximises clarity and comprehension. The Discussion should provide an interpretation of the data and relate them to existing knowledge. Subtitles are only admitted in exceptional cases.

Acknowledgements should list persons who made minor contributions to the investigation and organisations providing support.

References should be numbered in the order in which they are cited in the text, using arabic numbers between square brackets, e.g. [1]; for multiple references, e.g. [1–3] or [1,2,5].The list of references should be arranged consecutively according to the numbers in the text. Use Index Medicus abbreviations for journal titles. Authors bear complete responsibility for the accuracy of the references. The following examples illustrate the format for references:

a) Journals

Trute A, Nahrstedt A. Separation of rosmarinic acid enantiomers by three different chromatographic methods and the determination of rosmarinic acid in Hedera helix. Phytochem Anal 1996; 7: 204–208 Article in press without doi:

Lim EK, Ashford DA, Hou B, Jackson RG, Bowles DJ. Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. Biotech Bioeng, in press Article in press with doi:

Lim EK, Bowles DJ. A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J, advance online publication 8 July 2004; doi: 10.1038/sj.emboj.7600295

b) Books

Citation to complete book:

Mabberley DJ. The plant book, 2nd edition. Cambridge: Cambridge University Press; 1997: 520–521

Citation to article within a book:

Lechtenberg M, Nahrstedt A. Cyanogenic glycosides. In: Ikan R, editor. Naturally occurring glycosides. Chichester: Wiley & Sons; 1999:147–191

Lorberg A, Hall MN. TOR: the first ten years. In: Thomas G, Sabatini DM, Hall MN, editors. TOR – target of rapamycin. Heidelberg: Springer Verlag; 2004: 1–18

Multi-volume books and encyclopedias:

Warren SA. Mental retardation and environment. In: International encyclopedia of psychiatry, psychology, psychoanalysis and neurology, Vol. 7. New York: Aesculapius Publishers; 1977: 202–207 Pharmacopoeia of China, Part 1. Beijing: People's Health Press; 1977: 531–534

c) PhD and Diploma Theses Dettmers JM. Assessing the trophic cascade in reservoirs: the role of an introduced predator [dissertation]. Columbus: Ohio State University; 1995

d) Patents Cookson AH. Particle trap for compressed gas insulated transmission system. US Patent 4554399; 1985

e) Conference Paper Okada K, Kamiya Y, Saito T, Nakagawa T, Kaawamukai M. Localization and expression of geranylgeranyldiphosphate synthases in Arabidopsis Arabidopsis thaliana. Annual Meeting of the American Society of Plant Physiologists, Baltimore, MD; 1999.

f) Electronic Sources

Agatep R, Kirkpatrick RD, Parchaliuk DL,Woods RA, Gietz RD. Transformation of S. cerevisiae by the lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol. Technical tips online. Available at http://research.bmn.com/tto. Accessed September 22, 2005.

If no author is given, the title is used as the first element of the citation.

If reference is made to papers submitted or in press, authors are requested to add a file of the manuscript or galley proof to the online submission. Avoid references to unpublished personal communications.

Structural formulas should be prepared with ChemDraw® or a similar program using the following settings: bond lengths 0.508 cm, bond width 0.021 cm, bold bond width 0.071 cm, bond spacing 18% of length, hash spacing 0.088 cm, atom labels Helvetica 10, compound

numbers Helvetica 10 bold. These settings correspond to American Chemical Society document settings preset in Chem-Draw®. The configuration of all stereocenters present should be indicated; use of bold and dashed lines rather than solid and dashed wedges is recommended. The formulas should be integrated into the manuscript file (see above: 2. Submission of Manuscripts). They will be reproduced without reduction and the charts should be prepared with maximum widths of up to 8.0 cm for single column print and up to 17 cm for double column print.

Supporting Information: To keep articles as concise and at the same time as informative as possible, authors are strongly encouraged to submit part of their tables and figures as Supporting Information.

The following type of data will be preferentially published as Supporting Information rather than in the print article: High-resolution halftone and colour illustrations, spectra, chromatograms, structural drawings outlining NMR correlations, experimental procedures of secondary importance, tables summarising data that are non-quintessential but useful to the understanding of an article. Tables, figures and text provided as Supporting Information must be referred to in the manuscript as follows: (Table 1S, Supporting Information, etc.).

The cover page for Supporting Information must contain the title of the manuscript, names and affiliations of all authors, and the full address of the corresponding author. Legends for Figures and Tables must appear directly on the respective figure pages. Pages have to be numbered consecutively. Supporting Information has to be submitted as a separate file.

Supporting Information is published on the journals homepage at http://www.thieme-connect.de/ejournals/toc/plantamedica.