Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós Graduação em Ciências Biológicas: Bioquímica

EFEITOS DO γ-ORIZANOL E EXTRATO HIDROALCÓLICO DE *Thuya occidentalis* SOBRE LINHAGENS DE CÂNCER DE PRÓSTATA RESPONSIVAS E NÃO-RESPONSIVAS A ANDRÓGENOS

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Orientadora: Prof. Dra. Fátima Costa Rodrigues Guma

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito para obtenção do grau de Doutor em Bioquímica.

Porto Alegre

2015

CIP - Catalogação na Publicação

Hirsch, Gabriela Elisa
Efeitos do γ-orizanol e extrato hidroalcólico de Thuya occidentalis sobre linhagens de câncer de próstata responsivas e não-responsivas a andrógenos / Gabriela Elisa Hirsch. -- 2015.
161 f.

Orientadora: Fátima Teresinha Costa Rodrigues Guma.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2015.

1. Câncer de Próstata. 2. Gama-orizanol. 3. Thuya occidentalis. I. Guma, Fátima Teresinha Costa Rodrigues, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

“A ciência nunca resolve um problema sem criar pelo menos outros dez”.

(George Bernard Shaw)

AGRADECIMENTOS

A Deus, por estar sempre comigo me dando força para continuar!

Aos meus amados pais, Roseli e Paulo Hirsch, pelo exemplo de caráter, dedicação e força; por me incentivarem sempre a estudar e buscar meus sonhos, me apoiando e amparando em todos os aspectos, pessoal, emocional, financeiro..., e me incentivando quando tinha vontade de desistir, sempre enfatizando que os resultados que tanto almejo ainda virão!

Aos meus irmãos, Leandro e Danusa, pelo apoio, incentivo e dedicação!

Ao meu sobrinho e afilhado, Miguel, por ter me oferecido tanto carinho, mesmo inconsciente, me confortando e deixando a jornada menos difícil.

Ao Sergio, pelo companheirismo, apoio, paciência, compreensão, motivação e intensa dedicação despendidos em todos os momentos enquanto estivemos juntos.

Às minhas amigas e colegas do tempo da faculdade, Karina, Karine, Aline, Juliana, Camila, Jaqueline e Charlise por estarem ao meu lado desde o começo e me incentivarem a não desistir.

Aos meus colegas, mas acima de tudo, amigos, Priscila, Moema, Cassiane, Franciele e Lucas, por ouvirem minhas reclamações, enxugarem minhas lágrimas, me apoiarem e sugerirem soluções para os milhares de problemas que surgiram ao longo desta jornada, tornando essa caminhada mais leve e feliz.

Em especial, a minha mais recente “melhor amiga”, Mariana Parisi, por estar sempre ao meu lado nos piores momentos, me ouvindo chorar e reclamar da vida quase todos os dias quando nada dava certo; por me apoiar a tentar todas as possibilidades profissionais possíveis; por participar ativamente para a

construção deste trabalho realizando muitos experimentos e me explicando mil vezes a mesma coisa porque não tinha paciência e atenção suficiente para entender, me orientando e ajudando sempre! E por estar ao meu lado também nos melhores momentos, nos inúmeros *"happys"* prolongados que fizemos juntas, pelas muitas risadas, sempre parceira pra qualquer programação. Com certeza deixou o percurso mais aceitável e muito mais feliz!

Ao Leo, que além de amigo e colega, participou da orientação deste trabalho, dando sugestões (desde experimentos até revistas...) para melhorá-lo, ajudando na realização dos experimentos que muitas vezes não deram em nada e sem reclamar, por me ensinar a usar o Photoshop e múltiplos outros programas, sempre me socorrendo nas minhas dúvidas; e acima de tudo, me apoiando quando me desesperava e achava que não teria solução! Obrigada pela amizade e pelo conhecimento passado, és um exemplo de pesquisador!

À Florencia Barbé-Tuana, pela sua orientação e seu apoio e pela disposição e dedicação dedicados ao meu trabalho, importantíssimos para que pudesse concluí-lo!

À minha orientadora, Drª Fátima Guma, por me oferecer as oportunidades e a confiança para aprender e desenvolver este trabalho e acreditar em mim; pelo modelo de caráter na simplicidade e companheirismo, sempre interessada na vida dos seus alunos, tanto pessoal quanto profissional. Por apoiar as minhas ideias, entender e aceitar as minhas revoltas e desesperos, me acalmar nos momentos difíceis; e sempre lutar por mim e por todos seus alunos, e por ser este exemplo profissional e pessoal a ser seguido.

Aos demais colegas de laboratório 21, Patrícia, Elvira, Franciele, Ketlen, Leticia, Nevton, Mariana Ilha, Sílvia, Barbara, Tatiana e Vanessa. Vocês foram fundamentais para o trabalho ser divertido além de produtivo.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão da bolsa para desenvolvimento desse trabalho.

Para todos os funcionários que contribuem com muita competência para o excelente andamento do departamento e do nosso trabalho aqui, da portaria aos técnicos.

À todos que colaboraram de forma direta ou indireta para a execução desse trabalho.

Obrigada!!!

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PARTE I

I.1. RESUMO

O câncer de próstata é a segunda causa de morte entre homens no Brasil. É tipo um câncer de crescimento lento, podendo levar anos para o tumor atingir 1 cm^3, porém, em alguns casos ele pode se espalhar pelo corpo, sendo o osso o principal sítio de metástase. No estágio de desenvolvimento do câncer conhecido como metástase, o principal tratamento consiste em terapia de restrição andrógena, levando as células prostáticas a pararem de proliferar, uma vez que elas crescem em resposta a presença de hormônios andrógenos, como a diidrotestosterona e testosterona. Porém, em alguns casos, as células proliferam mesmo na ausência de andrógenos e isto se deve a diversos fatores que, em geral, estão associados a mutações no receptor andrógeno e/ou alterações no metabolismo andrógeno. Quando isto acontece, os tratamentos disponíveis são menos efetivos e costumam falhar. Porém, estudos sugerem que o γ-orizanol, um fitoesterol extraído do óleo do farelo do arroz; e extratos amplamente utilizados na medicina popular, como o extrato hidroalcólico de *Thuya occidentalis*, poderiam atuar inibindo o desenvolvimento e progressão do câncer de próstata. Neste estudo, com o uso de abordagens bioquímicas e de biologia molecular, foi demonstrado que o tratamento com γ-orizanol diminui a viabilidade e biomassa celular em cultura, associado ao aumento da morte celular por apoptose e/ou necrose, em linhagens celulares responsivas (LNCaP) e não-responsivas a andrógenos (PC3 e DU145), além de aumentar a pERK1/2 em células LNCaP e DU145. O γ-orizanol também foi capaz de bloquear o ciclo celular em G2/M nas células PC3 e LNCaP e em G0/G1 nas células DU145. Estes efeitos foram ainda acompanhados por uma redução da expressão do gene e proteína caveolina-1- uma importante molécula envolvida no aumento da agressividade do câncer de próstata, e também, na progressão da doença para o fenótipo andrógeno resistente - nas células não-responsivas a andrógenos, e do gene PCGEM1 - gene específico da próstata regulado por andrógeno - nas células LNCaP e DU145. Ainda, γ-orizanol também mostrou capacidade de regular vários miRNAs - pequenas moléculas de RNA não codificantes de proteínas - envolvidos no controle de funções associadas ao desenvolvimento, progressão e invasão no câncer de próstata, como o miR16-1, miR19b-2, miR24b-1, miR24b-2, miR99a, miR133a-5p, miR182-5p, miR198 e miR222. O extrato hidroalcólico de *Thuya occidentalis* reduziu a viabilidade e biomassa celular nas linhagens responsiva (LNCaP) e não-responsivas (DU145 e PC3) a andrógenos, além de induzir parada do ciclo celular na fase G0/G1 nas células DU145 e aumentar a morte celular por apoptose e/ou necrose em todas as linhagens. Da mesma forma que o γ-orizanol, este extrato reduziu a expressão da caveolina-1 nas linhagens não-responsivas a andrógenos. Trabalhos anteriores mostram que o monoterpeno α-tujona é o principal composto ativo do extrato de *Thuya occidentalis*. Por cromatografia gasosa acoplada a detector de massas foi mostrada a existência de 0,0016 µg de α-tujona na dose de extrato usada neste estudo. No entanto, o tratamento com 0,0016µg de α-tujona foi efetivo somente sobre linhagem LNCaP, não tendo efeito sobre as outras linhagens estudadas, reforçando a hipótese da diferença de sensibilidade entre as linhagens responsivas e não responsivas a andrógeno e mostrando a contribuição de outros componentes do extrato nos efeitos observados neste estudo. Concluindo, estes resultados demonstram que tanto γ-

orizanol como o extrato de *Thuya occidentalis* podem vir a ser agentes terapêuticos promissores no tratamento de câncer de próstata, não só por inibirem o crescimento celular, mas também e principalmente pela possibilidade de induzirem a recuperação da sensibilidade a andrógenos, aumentando as possibilidades de tratamento da doença.

Palavras-chaves: γ-orizanol, *Thuya occidentalis*, câncer de próstata, PCGEM1, caveolina-1, miRNA.

I.2. ABSTRACT

Prostate cancer is the second cause of death among men in Brazil. It is a slow-growing cancer and it may take years for tumor to reach 1 cm^3, but in some cases it can spread throughout the body and the bone is the main site of metastasis. At this cancer stage known as metastasis, the principal treatment involves anti-androgen therapy, leading to prostate cells stop proliferating, because they grow in response to presence of androgens such as testosterone and dihydrotestosterone. However, in some cases, the cells can proliferate even in the absence of androgens and this fact occurs due to many factors and they are generally associated with mutations in the androgen receptor and/or alterations in androgen metabolism. In this stage, the treatments available are less effective and usually fail. However, studies suggest that γ-oryzanol, a phytosterol extracted of rice bran oil; and extracts widely used in folk medicine, as *Thuya occidentalis* hidroalcolic extract, could act inhibiting the development and progression of prostate cancer. In this study, using molecular biology and biochemical approaches we showed that γ-oryzanol treatment was able to decrease cell viability and biomass in culture, and this fact was linked to increased cell death by apoptosis and/or necrosis in androgen responsive (LNCaP) and unresponsive (DU145 and PC3) prostate cancer cell lines, besides increasing pERK1/2 in LNCaP and DU145 cells. γ-oryzanol was also able to cause cell cycle arrest at G2/M phase in LNCaP and PC3 cells and at G0/G1 phase in DU145 cells. These effects were also accompanied by a reduction in caveolin-1 gene and protein expression - an important molecule related to high aggressiveness in prostate cancer and also in the progression of the disease to androgen resistant phenotype - in androgen unresponsive cells, and also PCGEM1 gene - a prostate specific gene regulated by androgens - in LNCaP and DU145 cells. γ-oryzanol also showed ability to regulate several miRNAs - small non-coding RNA molecules - involved in the control of many functions associated with the development, progression and invasion of prostate cancer, such as miR16-1, miR19b-2, miR24b-1, miR24b-2, miR99a, miR133a-5p, miR182-5p, miR198 and miR222. *Thuya occidentalis* hidroalcolic extract also reduce cell viability and biomass in androgen responsive (LNCaP) and unresponsive (DU145 and PC3) cells, in addition to inducing cell cycle arrest at G0/G1 phase in DU145 cells and to increase apoptosis and/or necrosis cell death in all cell lines. The same way that γ-oryzanol, this extract reduced the caveolin-1 expression in androgen unresponsive prostate cancer cells. Prior studies showed that the monoterpene α-thujone is the main active compound in the *T. occidentalis* extract. By gas chromatography coupled to mass detector it was showed the existence of 0.0016 µg of α-thujone in extract dose used in this study. However, the treatment with 0.0016 µg of α-thujone was effective only on LNCaP cell line, having no effect on the other studied lines, supporting the hypothesis of difference in sensitivity between responsive and unresponsive cell lines and showing the contribution of other components in the effects caused by the extract, observed it this study. In conclusion, these results demonstrate that both γ-oryzanol as *T. occidentalis* extract may become promising therapeutic agents in treatment of prostate cancer, not only inhibit cell growth but also and manly by the possibility of inducing the recovery of androgen sensitivity, increasing the treatment chances of treatment this disease.

Keywords: γ-orizanol, *Thuya occidentalis*, prostate cancer, PCGEM1, caveolin-1, miRNA.

I.3. LISTA DE ABREVIATURAS

DHT – *dihidrotestosterona*

PSA - *Antígeno Prostático Específico*

CPRC - *Câncer de Próstata Resistente a Castração*

mCPRC - *Câncer de Próstata Resistente à Castração Metastático*

SHBG - *Globulina de Ligação de Hormônios Sexuais*

Cav-1 - *Caveolina-1*

mRNA - *RNA mensageiro*

ncRNA - *RNA não-codificante*

sncRNA - *pequenos RNAs não-codificantes*

lncRNA - *longos RNAs não-codificantes*

miRNA - *microRNAs*

PCGEM1 - *Gene específico da próstata regulado por andrógeno 1*

INCA - *Instituto Nacional do Câncer*

GS - *Escore de Gleason*

ERK1/2 - *Cinase regulada por sinal extracelular*

pERK1/2 - *ERK1/2 fosforilada*

IAP - *Proteínas Inibidoras de Apoptose*

CaP – Câncer de Próstata

SRB – Sulforodamina B

u.m.a. – Unidades de Massa Atômica

I.4. INTRODUÇÃO

I.4.1. Câncer de próstata

O número de casos de câncer de próstata tem crescido dramaticamente nas últimas duas décadas (Deng, He et al. 2014), sendo a segunda causa de morte entre os homens no Brasil, atrás apenas do câncer de pele não-melanoma ((INCA) 2014). É o sexto tipo de câncer mais comum no mundo e o mais prevalente entre os homens. Segundo o Instituto Nacional do Câncer (INCA), 13.129 pessoas morreram em decorrência do câncer de próstata no Brasil em 2011, e no Rio Grande do Sul foram 1.032 óbitos por neoplasia de próstata. A região Sul do Brasil é a mais afetada no país, com cerca de 91 casos a cada 100 mil habitantes. Além disso, estima-se que em 2015 serão diagnosticados 68.800 novos casos da doença no país ((INCA) 2014).

O câncer de próstata está intimamente relacionado ao envelhecimento, acometendo principalmente homens acima de 50 anos de idade. Além da idade, os principais fatores de risco para o desenvolvimento do câncer de próstata são a raça, etnia (mais prevalente em negros) e histórico familiar (Stacewicz-Sapuntzakis and Bowen 2005).

Em geral, o câncer de próstata é um câncer de crescimento lento, levando até quinze anos para atingir 1 cm^3, e em seus estágios iniciais costuma ser assintomático. Porém, pode se desenvolver rapidamente, espalhando-se por outros órgãos e levando a morte ((INCA) 2014). A medida que o tumor cresce, sintomas como sangue na urina, dor ou sensação de queimação ao urinar, fluxo

urinário fraco ou incapacidade de urinar, e micção frequente (especialmente à noite), podem começar a aparecer (Thompson, Thrasher et al. 2007).

O diagnóstico do câncer de próstata é feito pelo exame clínico (toque retal) e pela dosagem da PSA sérica, que em 80% dos casos se eleva de maneira significativa e pode sugerir a existência da doença (Gleason 1988; Gleason 1992; Heidenreich, Bastian et al. 2014). O diagnóstico é reforçado pelos exames histopatológicos, já que na maioria dos casos os tumores de próstata apresentam uma forte correlação entre a aparência histológica e sua expressão clínica (Gleason 1992).

Para o prognóstico de evolução da doença, tem sido largamente utilizado o método histológico denominado *Gleason Grading System*, que é baseada na estrutura anátomo-patológica, ou seja, na disposição organizada ou anárquica das células glandulares da próstata. O patologista observa, ao microscópio, o pedaço biopsiado da próstata e classifica-o segundo a morfologia e disposição das células glandulares e vários aspectos da arquitetura do tecido, que indicam o nível de diferenciação celular. Verificando os tipos morfológicos predominantemente encontrados, classifica-se o tecido numa escala de 1, bem diferenciado, a 5 conforme a diferenciação é perdida. Esta análise é realizada em duas regiões do tecido, o primeiro e o segundo padrão mais abundantes na amostra. A soma dos dois valores é que irão compor o *Gleason Score* (GS) do tumor do paciente. A soma obtida das duas referências mais vistas, quando igual ou superior a sete, merece atenção especial (Gleason 1988).

Amostras de pacientes que apresentam GS de 2 a 4 indicam pouca agressividade e um risco mínimo de morte por câncer de próstata, amostras com valores de GS de 5 a 6 apresentam baixa agressividade com um bom

prognóstico, amostras com GS de 8 a 10 são muito agressivas e o prognóstico é ruim, e finalmente tumores com valor de GS 7 indicam tumores de agressividade intermediária e prognóstico bastante heterogêneo, de difícil predição (Albertsen 1998; Stacewicz-Sapuntzakis and Bowen 2005).

O tratamento do câncer de próstata depende deste estagiamento clínico. A doença localizada, confinada no órgão é curável e normalmente cirurgia, radioterapia ou até mesmo uma observação vigilante (em algumas situações especiais - pacientes de baixo risco) são recomendados (Freeman, Yang et al. 2012). Quando a doença passa a não ser mais localizada, ou seja, atinge órgãos próximos como vesículas seminais, uretra e bexiga (localmente avançada) ou órgãos distantes (metastática) (Lynch and Lynch 1996; Gomes 2008), o tratamento de privação andrógena passa a ser o tratamento padrão e pode ou não ser associado a outras estratégias (Freeman, Yang et al. 2012). Para doença localmente avançada, radioterapia ou cirurgia em combinação com tratamento hormonal tem sido utilizada como tratamento de escolha. Para doença metastática, o tratamento de eleição é somente a privação andrógena (Stacewicz-Sapuntzakis and Bowen 2005; Madan and Arlen 2013).

As células prostáticas proliferam em resposta a hormônios andrógenos (Toledo-Pereyra 2001), e isso se deve a presença de receptores andrógenos no núcleo das células prostáticas, como pode ser observado na Figura 1 (Agoulnik, Vaid et al. 2006). A testosterona circula no sangue ligada a albumina ou globulina de ligação de hormônios sexuais (SHBG) e é convertida em dihidrotestosterona (DHT) pela enzima 5-α-redutase tipo 2, localizada principalmente nas células estromais, atuando de modo autócrino e parácrino, por difusão, nas células epiteliais subjacentes. A DHT é um metabólito 10 vezes mais potente que a

testosterona, e é um mediador crítico do crescimento prostático. A DHT liga-se aos receptores andrógenos nucleares e ativa a transcrição de fatores de crescimento mitógenos nas células estromais e epiteliais, ativando a sua proliferação (Lindzey, Kumar et al. 1994; Kaplan, McConnell et al. 2006). Assim, a terapia de restrição andrógena é baseada na administração de medicamentos que reduzem os níveis de andrógenos endógenos ou inibem a ligação da DHT na parte C-terminal do receptor andrógeno inativando temporariamente a proliferação das células prostáticas (de Bono, Logothetis et al. 2011; Scher, Fizazi et al. 2012; Culig and Santer 2013; Rajan, Sudbery et al. 2014) com o objetivo de inibir a progressão da doença localmente avançada ou metastática (Heinlein and Chang 2004).

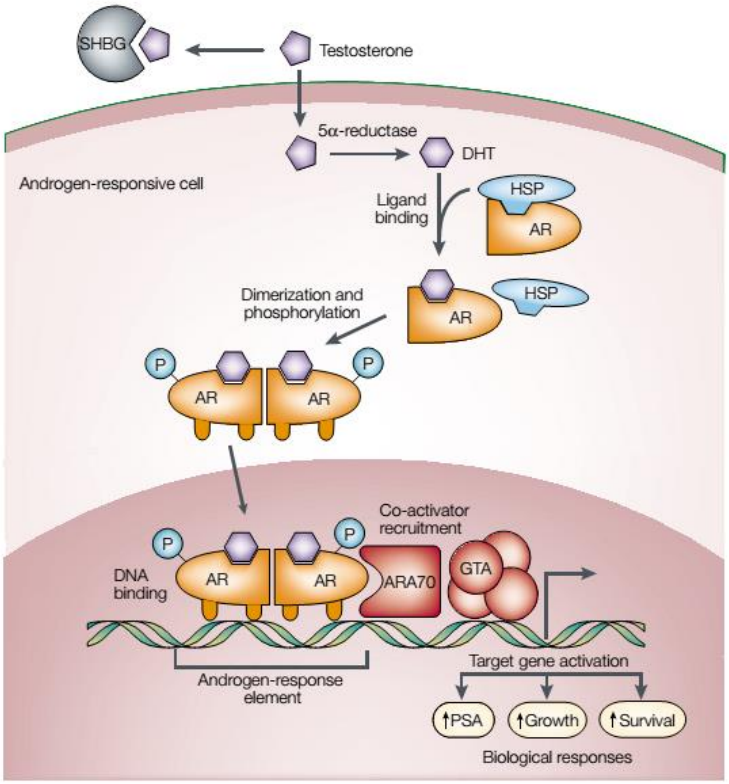

Figura 1. Mecanismo de ação da testosterona sobre o receptor andrógeno. (Adaptada de Feldman, B.J. e Feldman, D., 2001 (Feldman and Feldman 2001)).

Como a maioria dos tumores de próstata precisam de andrógenos para seu crescimento e sobrevivência, com a terapia de restrição andrógena o tumor metastático inicial pode entrar em remissão e ser até eliminado. Porém, algumas subpopulações destas células podem resistir ou tornarem-se resistentes a terapia antiandrógena e o tumor volta a crescer (Ganguly, Li et al. 2014). Em geral, essas células não são dependentes de andrógeno circulante, porém, precisam de andrógenos para sobreviver ou da ativação do receptor andrógeno, e em geral, isso ocorre devido a alterações na sinalização do receptor andrógeno e/ou do metabolismo andrógeno, como por exemplo: hipersensibilidade do receptor andrógeno devido à superexpressão ou amplificação do receptor resultando em resposta alterada aos antiandrógenos; mutação no receptor andrógeno tornando-o sensível a outros hormônios como progesterona, estrógeno, andrógeno da adrenal, além de antiandrogênicos; ativação independente do ligante do receptor andrógeno devido ao aumento das vias de transdução de sinal ativados por fatores de crescimento oncogênicos e citocinas via receptores de tirosina cinase; conversão de andrógenos da adrenal em diidrotestosterona e testosterona; além de síntese intraprostática de andrógenos causado pelo aumento da expressão de enzimas esteroidogênicas como a CYP17 (Feldman and Feldman 2001; Pienta and Bradley 2006; Bonkhoff and Berges 2010). Assim, as células prostáticas passam a proliferar na presença de concentrações muito baixas de testosterona na circulação ou outros hormônios, e o tratamento não é mais eficaz (Grossmann, Cheung et al. 2013).

O tratamento do câncer de próstata avançado ou metastático evoluiu muito nas últimas décadas, melhorando consideravelmente a expectativa de vida dos pacientes nesta fase da doença. No início deste século, a sobrevida esperada dos

homens diagnosticados com câncer de próstata metastático resistente à castração (mCPRC) não ultrapassava 12 meses (Caffo 2015). Após a introdução de novas drogas para o tratamento de câncer como o docetaxel (introduzido na prática clínica no ano de 2004), e de drogas mais recentes como o cabazitaxel, além das drogas inibidoras da biossíntese androgênica como o acetato de abiraterona (Zytiga®) e enzalutamida (Xtandi®), o cenário do tratamento do mCPRC mudou drasticamente com aumento da sobrevida para de cerca de 3 anos. Contudo, estes medicamentos costumam funcionar somente por um curto período de tempo nos tumores resistentes à terapia antiandrógena (Ganguly, Li et al. 2014; Zobniw, Causebrook et al. 2014) e, ainda, ao mesmo tempo em que surgem novas drogas para o tratamento do câncer, também ocorrem mudanças na biologia da doença, e novos mecanismos de resistência surgem e são comuns a quase todas as drogas (Caffo 2015).

Os benefícios da terapia de restrição andrógena são bem estabelecidos para o tratamento do câncer de próstata localmente avançado e metastático. Entretanto, esta é uma terapia adjuvante no tratamento da doença e que não leva à cura, e com a progressão, em geral, a doença acaba tornando-se resistente à castração (Pagliarulo, Bracarda et al. 2012). Ainda, o tratamento de restrição andrógena tem o objetivo de inibir a produção endógena de testosterona, o que causa uma série de efeitos indesejáveis como: perda de libido, osteoporose, fadiga, perda de massa magra, anemia, ginecomastia, além de efeitos metabólicos associados a mudanças no perfil lipídico e aumento do risco de desenvolvimento de resistência à insulina, diabetes, síndrome metabólica (Jeong 2001; Flaig and Glode 2008; Leahy 2008; Nobes, Langley et al. 2009; Saylor and Smith 2013) e alterações cardiovasculares (Saigal, Gore et al. 2007; Efstathiou,

Bae et al. 2009; Van Poppel and Tombal 2011; Roayaei and Ghasemi 2013), fatores que tornam o tratamento difícil.

A maioria dos pacientes, inicialmente, respondem a terapia de privação andrógena, porém a progressão para o câncer de próstata resistente à castração (CPRC) ocorre em 10 a 20% dos casos, cerca de 2 a 3 anos após o início do tratamento (de Bono, Logothetis et al. 2011; Kirby, Hirst et al. 2011; Scher, Fizazi et al. 2012; West, Kiely et al. 2014) e pode levar à morte (de Bono, Logothetis et al. 2011). Depois do fracasso da terapia hormonal, atualmente ainda não existem muitas estratégias de tratamento bem sucedidas disponíveis, tornando a doença, em muitos casos, incurável, sendo este um grande problema oncológico (Scott, Menon et al. 1980; Eisenberger, Blumenstein et al. 1998, Pagliarulo, Bracarda et al. 2012). Em geral, pacientes neste estágio da doença (andrógeno-independente) morrem em um período médio de 40 semanas após a recidiva (Mahler and Denis 1992).

I.4.2 Linhagens de câncer de próstata

A maior parte das mortes causadas por câncer de próstata ocorrem devido a progressão para o distúrbio metastático (Coleman 2006) e cerca de 85-90% (Hess, Varadhachary et al. 2006) (de Bono, Logothetis et al. 2011) dos homens com mCPRC têm metástases ósseas, sendo este o único sítio em 86% dos pacientes (Hess, Varadhachary et al. 2006). O osso é sítio mais comum de metástase neste tipo de câncer (Coleman 2006; Hess, Varadhachary et al. 2006; Briganti, Suardi et al. 2014; Deng, He et al. 2014), e isso se deve a fatores ainda não bem estabelecidos, porém, acredita-se que está relacionado ao ambiente bioquimicamente e fisiologicamente favorável que o osso oferece (Fidler and Kripke 1977), como TGF-β, quimiocinas e citocinas, que funcionariam como

quimioatrativos para as células de câncer de próstata colonizarem o osso (Figura 2) (Festuccia, Bologna et al. 1999; Teicher and Fricker 2010; Salazar, Castellan et al. 2013). Metástases em tecidos moles são menos comuns, atingindo somente cerca da metade dos pacientes (de Bono, Logothetis et al. 2011).

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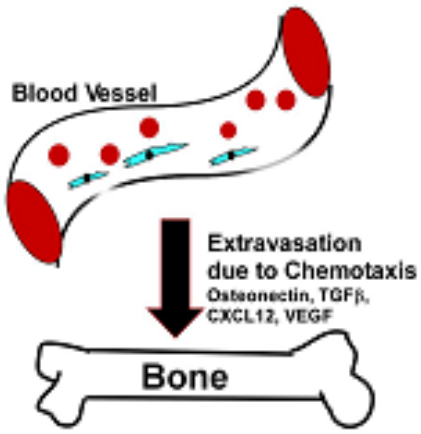

Figura 2. Mecanismo de desenvolvimento de metástase óssea de câncer de próstata. (Adaptada de Ganguly, S.S., Li, X. e Miranti, C.K., 2014 (Ganguly, Li et al. 2014)).

A linhagem celular PC3 é derivada de uma metástase em osso a partir de um adenocarcinoma de próstata (Kaighn, Narayan et al. 1979). É uma linhagem celular muito útil para avaliação de mudanças bioquímicas no câncer de próstata avançado e também para medir a resposta a agentes quimioterápicos, principalmente durante a metástase, pois apresenta alto potencial metastático e agressividade (Kaighn, Narayan et al. 1979; Jarrard, Blitz et al. 1994). Além disso, é uma linhagem de câncer de próstata andrógeno-independente, ou seja, mesmo na ausência de diidrotestosterona a linhagem celular é capaz de proliferar (Jarrard, Blitz et al. 1994), sendo importante na mimetização de situações onde o paciente não responde mais ao tratamento de privação andrógena.

A linhagem celular DU145 também é derivada de uma lesão metastática, porém, localizada no cérebro (Stone, Mickey et al. 1978). Assim como a linhagem de câncer de próstata PC3, esta linhagem celular também é andrógeno-independente (Stone, Mickey et al. 1978), porém apresenta potencial metastático (moderado), menor do que a anterior (Keer, Gaylis et al. 1991).

Apesar de ambas serem andrógeno-independentes e originadas de lesões metastáticas, elas apresentam características fenotípicas e comportamentos em cultura diferentes e o uso das mesmas é importante para representar as diferenças entre os tumores encontrados na clínica oncológica.

Já a linhagem celular de câncer de próstata LNCaP, que também foi estabelecida a partir de uma lesão metastática de adenocarcinoma prostático humano, caracteriza-se por apresentar receptores andrógenos no citosol e núcleo das células, além de ser responsiva a andrógenos, ou seja, o crescimento celular é modulado pela presença de diidrotestosterona *in vitro*, o que estimula a produção de PSA (Horoszewicz, Leong et al. 1983), representando a fase do câncer de próstata onde a proliferação e sobrevivência celulares ainda são depende de andrógenos circulantes. É uma linhagem importante para a avaliação da fase sensível a andrógenos da doença.

I.4.3. γ-orizanol

O arroz é o segundo cereal mais cultivado no mundo e o Brasil é o nono maior produtor mundial deste cereal (Brasil 2014). Além disso, o arroz é um alimento essencial para mais da metade da população mundial, sendo que cerca de 3 bilhões de pessoas consomem cerca de 100 kg por ano (Nguyen and Ferrero 2006; Hu, Pan et al. 2012). No entanto, o processo de polimento do arroz produz

resíduos de baixo valor comercial, como farelo de arroz (Parrado 2006), e este produto é uma importante fonte de fitoquímicos com propriedades benéficas para a saúde, tais tocoferóis, tocotrienóis e γ-orizanol (Xu and Godber 1999).

O γ-orizanol foi inicialmente caracterizado como um composto isolado obtido a partir do óleo de arroz, e devido ao nome científico do arroz ser *Oryza sativa* e de conter um grupo hidroxila em sua estrutura, a substância foi convenientemente chamada de orizanol (Graf 1992). Porém, estudos posteriores revelaram que o γ-orizanol não é um composto simples e sim uma mistura de ésteres do ácido ferúlico (Graf 1992) que são formados por esterificação de um grupo hidroxila de esteróis (campesterol, estigmasterol ou β-sitosterol) ou álcoois triterpênicos (cicloartanol, cicloartenol, 24-metilenocicloartanol, ciclobranol) com a carboxila do ácido ferúlico (Diack and Saska 1994; Bucci, Magri et al. 2003; Yu, Nehus et al. 2007; Imsanguan, Roaysubtawee et al. 2008; Lu, Chen et al. 2011; Jeng, Shih et al. 2012).

O γ-orizanol é uma substância caracterizada como um pó branco ou levemente amarelo, cristalino, insípido, com pouco ou nenhum odor e os principais compostos que o compõem são cicloartenil ferulato, 24-metileno-cicloartanil ferulato, campesteril ferulato, β-sistoteril ferulato e cicloartanil ferulato (Evershed 1998), mas inclui também outros componentes menores como estigmastenil ferulato, campestenil ferulato e sitostanil ferulato (Xu and Godber 1999), pois dependendo da técnica cromatográfica utilizada, diferentes componentes têm sido identificados (Figura 3).

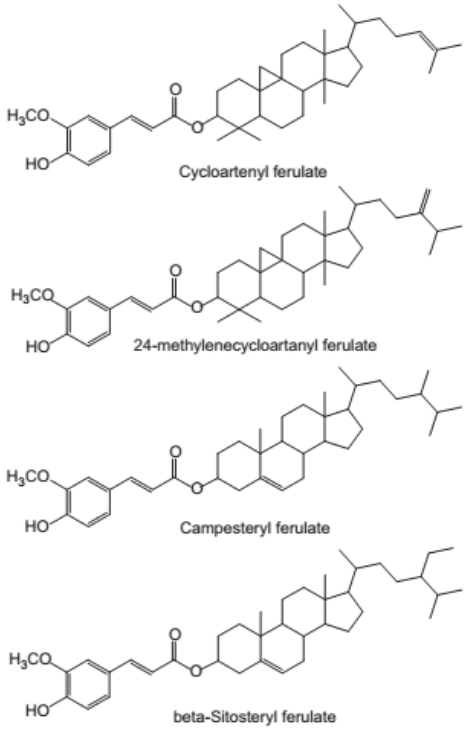

Figura 3. Estrutura dos principais componentes do γ-orizanol. (Adaptada de Chotimarkorn, C. e Ushio, H., 2008 (Chotimakorn 2008)).

Este composto é encontrado principalmente no farelo do arroz bruto e seu conteúdo varia entre 1 % a 2 % (Yu, Nehus et al. 2007; Tuncel and Yilmaz 2011; Huang and Ng 2012; Jeng, Shih et al. 2012). Os componentes do γ-orizanol também foram isolados de grãos de milho, trigo e cevada (Seitz 1989), porém o óleo de farelo de arroz é a fonte natural mais acessível para recuperação deste composto (Das, Chaudhuri et al. 1998).

A atividade antioxidante do γ-orizanol pode ser atribuída principalmente ao ácido ferúlico. Este, esterificado com esteróis de plantas, como é o caso do γ-orizanol, tem aumento do seu potencial antioxidante promovendo acesso molecular a componentes hidrofóbicos que são mais suscetíveis à destruição celular oxidativa (Graf 1992).

Segundo o IRGA (Arroz 2015), o γ-orizanol possui diversos efeitos benéficos à saúde podendo ser utilizado pelas indústrias de fármacos para

formulações de medicamentos capazes de prevenir e combater diversas doenças. Dentre os efeitos do γ-orizanol, encontramos: neuromodulação, atividade de remoção de radicais livres, atividade antiúlcera (Lerma-Garcia, Herrero-Martinez et al. 2009; Ghatak 2011), controle da glicemia e do metabolismo lipídico (Chotimakorn 2008; Son, Rico et al. 2010; Son, Rico et al. 2011). Ainda, recentemente alguns trabalhos tem demonstrado que o γ-orizanol de arroz pigmentado exerce atividade anticarcinogênica e inibe o crescimento de células de câncer (Banjerdpongchai, Wudtiwai et al. 2013; Zeng, Yang et al. 2013; Summart and Chewonarin 2014).

I.4.4. *Thuya occidentalis*

A *Thuya occidentalis* (*T. occidentalis*) é uma árvore nativa da Europa (British Herbal Pharmacopoeia 1983) comumente conhecida como Arbor vitae ou cedro branco (Chang, Song et al. 2000) e é utilizada na medicina popular para tratamento de catarro dos brônquios, enurese, cistite, psoríase, carcinoma do útero e próstata, amenorréia e reumatismo (Shimada 1956; British Herbal Pharmacopoeia 1983; Baran 1991; Offergeld, Reinecker et al. 1992). Hoje, é usada principalmente na homeopatia como tintura-mãe ou diluição (Homöopathisches Arzneibuch 1985; Homöopathisches Arzneibuch 2003). Como tintura, é popularmente utilizada como um agente para cauterização no tratamento de papilomas e condilomas (verrugas causadas por vírus HPV) (Valsa, Felzenszwalb et al. 1990). Já o extrato é usado popularmente no tratamento da cistite e hipertrofia prostática nos homens senis e na incontinência urinária em mulheres (Pozetti 1980; Pizsolitto 1982; Leal 1986). Estudos realizados para avaliação dos efeitos adversos associados ao uso deste composto revelaram que

eles raramente ocorrem e em geral são de gravidade leve a moderada (Naser 2005).

De acordo com Hänsel et al. (Hänsel 1994), a erva seca contém 1,4 - 4% de oléo essencial, sendo que 60% do óleo é composto de uma cetona monoterpênica conhecido como tujona (Chang, Song et al. 2000; Naser 2005). A quantidade de tujona varia conforme o método de extração, sendo que o maior rendimento é obtido por destilação (Tegtmeier 1994; Naser 2005). Este óleo é encontrado em duas formas isoméricas, a α-tujona e a β-tujona. Em geral, na natureza, encontramos uma mistura dos dois isômeros, com predominância maior da forma alfa (Sondermann 1962; Traud 1983; Lachenmeier 2006), sendo este também o isômero com maior atividade (Hold, Sirisoma et al. 2000; Czyzewska and Mozrzymas 2013).

A tujona é o principal agente tóxico encontrado numa bebida popularmento conhecida, o Absinto, e possui atividade convulsiva no sistema nervoso central, agindo principalmente como bloqueador não competitivo do receptor inibitório $GABA_A$-R (Hold, Sirisoma et al. 2000; Czyzewska and Mozrzymas 2013). Porém, devido aos métodos de extração usados para a produção das tinturas e extratos obtidos a partir de *T. occidentalis*, a quantidade de tujona nos extratos que seriam injeridas pelo paciente estão bastante abaixo do considerado seguro para saúde (36 mg), segundo estudos de segurança e eficácia, e até mesmo abaixo do que é encontrado nas bebidas alcólicas (Naser 2005).

Além do óleo essencial, recentemente, outros compostos bioativos foram encontardos, como as cumarinas (ácido *p*-cumárico) e flavonódes ((+/-)-catequina, (-)-galocatequina, campferol, campferol-3-*O*-α-ramnosídeo,

mearnsitrina, miricetina, miricitrina, procianidina B-3, prodelfinidina, quercetina e quercitrina (Chang, Song et al. 2000).

I.4.5 Caveolina-1

A caveolina-1 (Cav-1) é uma proteína de membrana de 22 kDa, sendo o principal componente estrutural da caveola, que são invaginações da membrana plasmática relacionadas com o transporte molecular, adesão celular e com as vias de transdução de sinal (Harder and Simons 1997; Simons and Ikonen 1997; Shaul and Anderson 1998; Smart, Graf et al. 1999). São abundantes nas células musculares lisas, adipócitos, epitélios e endotélios (Harder and Simons 1997). Por outro lado, a Cav-1 tem mostrado ser um supressor do crescimento celular em linhagens específicas (Koleske, Baltimore et al. 1995; Engelman, Wykoff et al. 1997; Lee, Reimer et al. 1998; Suzuki, Suzuki et al. 1998; Bender, Reymond et al. 2000) e funcionaria como um gene de supressão tumoral (Engelman, Wykoff et al. 1997). Porém, análises genéticas específicas demonstram que a Cav-1 poderia atuar como um gene pró- ou anti-apoptótico, dependendo do seu grau de expressão (Shinoura, Yoshida et al. 1999).

Diversos autores têm relatado uma relação entre os níveis elevados de Cav-1 e o câncer de próstata (Yang, Galbiati et al. 1998), estando positivamente correlacionados com Escore de Gleason (GS) (Yang, Truong et al. 1999; Tahir, Frolov et al. 2006). Estudos demonstraram que a expressão de Cav-1 está aumentada em células de câncer de próstata metastático de camundongos e humanas (Nasu, Timme et al. 1998; Yang, Truong et al. 1998; Yang, Truong et al. 1999). Além disso, a diminuição dos níveis de Cav-1 através da supressão da expressão do gene Cav-1 (transfecção com cDNA antisense) levaram ao reestabelecimento da sensibilidade a andrógenos *in vivo* e *in vitro,* para células de

câncer de próstata, e a superexpressão de Cav-1 poderia converter as células de câncer de próstata responsivas a andrógenos em células não-responsivas a andrógenos (Nasu, Timme et al. 1998), sugerindo que este gene estaria relacionado com o desenvolvimento de metástase, assim como ao desenvolvimento da resistência à terapia de supressão andrógena que ocorre com a progressão da doença (Yang, Truong et al. 1999). Além disso, alguns estudos também mostram que a Cav-1 teria um papel importante na resistência de vários tipos de cânceres a múltiplos agentes antineoplásicos (Lavie, Fiucci et al. 1998; Yang, Galbiati et al. 1998).

Os mecanismos pelo qual a expressão aumentada da Cav-1 leva ao desenvolvimento do câncer de próstata parecem estar relacionados à supressão da apoptose induzida por *c-MYC* (Timme, Goltsov et al. 2000), um oncogene importante no desenvolvimento de diversos tipos de câncer (Cole and McMahon 1999; Levens 2002), e também a hipersensibilização do receptor andrógeno a testosterona (Yang, Truong et al. 1998; Yang, Addai et al. 2000). Estudos anteriores revelam que as células epiteliais normais de próstata possuem níveis baixos ou indetectáveis de Cav-1, e que com a progressão da doença para fase metastática a expressão de Cav-1 aumenta significativamente (Yang, Truong et al. 1998). Possivelmente, a terapia de restrição andrógena selecionaria as células que apresentam maior expressão de Cav-1 e que seriam resistentes a terapia de restrição andrógena, fazendo com que a doença passasse a não responder mais a restrição da testosterona. Essa seleção causada pela testosterona envolve, provavelmente, mecanismos dependentes da ativação do receptor andrógeno (Hu, Lam et al. 2001; Li, Yang et al. 2001).

Ainda, os níveis de Cav-1 séricos encontrados em homens com câncer de próstata são significativamente maiores do que em homens com hiperplasia prostática benigna (Yang, Truong et al. 1999). Esses níveis elevados também estão relacionados ao aumento do risco de recorrência da doença após a prostatectomia radical (Nasu, Timme et al. 1998; Yang, Truong et al. 1999; Tahir, Frolov et al. 2006). O aumento dos níveis séricos da Cav-1 ocorre devido ao aumento da expressão da proteína Cav-1, que é secretada, e exerce efeitos parácrinos ou autócrinos, permitindo que mais células de câncer de próstata se tornem resistentes a estímulos pró-apoptóticos, característica esta que é muito importante para o desenvolvimento de metástases (Figura 4) (Mouraviev, Li et al. 2002).

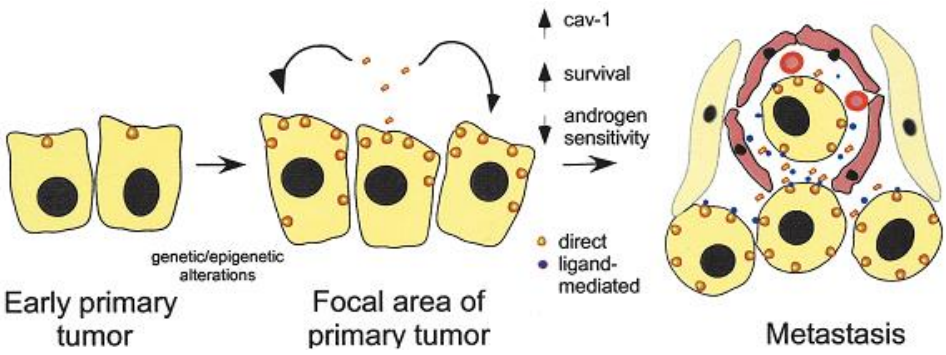

Figura 4. Mecanismo de ação da Cav-1 no desenvolvimento de metástase e resistência andrógena no câncer de próstata. (Adaptada de Mouraviev et al., 2002 (Mouraviev, Li et al. 2002)).

Todos esses mecanismos sugerem a importância da Cav-1 no desenvolvimento de metástases no câncer de próstata, além de estar envolvida na progressão da doença para a fase hormônio-refratária, onde os tratamentos disponíveis são menos eficazes (Scott, Menon et al. 1980; Eisenberger, Blumenstein et al. 1998).

I.4.6 Genes de RNAs não-codificantes

Uma das maiores descobertas da biologia moderna foi que somente aproximadamente 2% do genoma humano codifica proteínas, mas que cerca de 90% do genoma é transcrito, mostrando que o transcriptoma humano é mais complexo do que se imaginava e grande parte dele deve ser formado por genes de RNAs não-codificantes (ncRNA) (Stein 2004; Birney, Stamatoyannopoulos et al. 2007; Costa 2010; Ponting and Belgard 2010; Gibb, Brown et al. 2011).

Ao invés de expressar RNAs mensageiros (mRNA) que codificam proteínas, esses RNAs produzem transcritos com função regulatória, catalítica ou estrutural (Eddy 1999; Erdmann, Barciszewska et al. 2001; Erdmann, Barciszewska et al. 2001). Os ncRNAs são divididos em dois grandes grupos baseados no tamanho do transcrito: pequenos RNAs não-codificantes (sncRNA) - possuem entre 18-200 nucleotídeos; e longos RNAs não-codificantes (lncRNA) - possuem de 200 nucleotídeos a, aproximadamente, 100 quilobases (kb) (Eddy 2001).

A classe dos sncRNAs é formada por uma gama de bem documentadas espécies de RNA como, por exemplo, os pequenos RNAs nucleolares (snoRNAs) e nucleares (snRNAs), pequenos RNAs de interferência (siRNA), microRNAs (miRNAs), entre outros (Lewin 1982; Walter and Blobel 1982; Tilghman 1999; Franke and Baker 2000; Kelley and Kuroda 2000; Avner and Heard 2001; Eddy 2001). Dentre estes, podemos destacar a grande importância dos miRNAs no desenvolvimento de patologias como o câncer (Chang, Lin et al. 2002; Ambros 2004; Kidner and Martienssen 2005; Iorio and Croce 2009; Fabbri 2010; Liao, Yu et al. 2010; Farazi, Spitzer et al. 2011; Ting, Lipson et al. 2011).

O primeiro miRNA descrito foi o *Lin4* (do inglês *lineage-deficient-4*), descoberto em 1993, e associado à regulação do desenvolvimento larval em

Caenorhabditis elegans (Kim 2005). Os miRNAs exercem seus efeitos regulatórios ligando-se à região 3' não traduzida do mRNA alvo para reprimir a sua tradução ou levar a sua degradação (Bartel 2004; Filipowicz, Jaskiewicz et al. 2005; Sontheimer and Carthew 2005). Este mecanismo permite a redução dos níveis proteicos de genes-alvo, raramente afetando o nível de expressão transcricional. Esta regulação pós-transcricional exercida pelos miRNAs depende do grau de complementaridade com o mRNA alvo, podendo ocorrer por dois mecanismos distintos, como ilustrado na Figura 5: (i) inibição do complexo de tradução proteica ou por (ii) degradação do mRNA através de RNAses tipo III. O pareamento dos miRNAs de modo imperfeito com o mRNA acarreta a inibição da tradução do mRNA alvo, sendo este o mecanismo principal de atuação dos miRNAs em mamíferos. Em função dos miRNAs possuírem sequências pequenas e poderem ser ativos sem a necessidade de pareamento completo, um único miRNA pode regular muitos mRNAs alvo, além de cooperarem no controle de um único mRNA. Dessa forma os miRNAs constituem uma enorme e complexa rede regulatória da sinalização celular (Wiemer 2007).

Alterações na expressão de miRNAs vêm sendo relacionadas a diversos tipos de câncer e à modulação da expressão de genes envolvidos com a proliferação e sobrevivência de células tumorais. Por exemplo, os miRNAs miR15 e miR16 regulam negativamente a expressão de *Bcl-2*, um oncogene anti-apoptótico que se apresenta superexpresso em diversos tipos de câncer humanos, incluindo leucemias e linfomas (Cimmino, Calin et al. 2005). Por outro lado, alguns miRNAs exercem ação oncogênica. MiR155 apresenta expressão aumentada em linfomas e células de câncer de mama, sugerindo que possa agir como oncogene (Kluvier 2005). A superexpressão dos miR221 e miR222 está

envolvida no aumento do processo de crescimento independente de andrógenos e formação de colônias, além de induzir mudança no ciclo celular na fase G1-S, em células de câncer de próstata responsivas a andrógenos LNCaP (Sun, Yang et al. 2009). Ainda, o silenciamento desses dois miRNAs em células PC3 (linhagem celular de câncer de próstata não-responsiva a andrógenos) causou redução da tumorogênse e formação de colônias nesta linhagem, por suprimir $p27^{Kip1}$ (Mercatelli, Coppola et al. 2008; Sun, Yang et al. 2009). MiR125b também está envolvido no surgimento de câncer de próstata hormônio refratário (Epis, Giles et al. 2009). A superexpressão do miR125b causa redução da expressão de *Bak1* (gene pró-apoptotico da família do *Bcl*-2), estimulando o crescimento de células LNCaP (Shi, Xue et al. 2007; DeVere White, Vinall et al. 2009). Além desses, diversos outros miRNAs estão envolvidos no desenvolvimento, progressão e resistência andrógena no câncer de próstata, demonstrando sua importância nesta patologia.

Em contraste aos miRNAs, os lncRNAs são transcritos semelhantes ao mRNA (Cheng, Kapranov et al. 2005; Wu, Kim et al. 2008), porém, geralmente seu nível de expressão é menor do que a dos genes codificadores de proteínas (Bono, Yagi et al. 2003; Babak, Blencowe et al. 2005; Ramskold, Wang et al. 2009; Guttman, Garber et al. 2010) e estes costumam ser tecido específicos (Mercer, Dinger et al. 2008). O genoma humano gera mais de 10,000 moléculas de lncRNA (Schmitt and Chang 2013) e trabalhos recentes sugerem que a expressão aberrante dos lncRNAs contribui consideravelmente para a tumorogênese (Huarte and Rinn 2010).

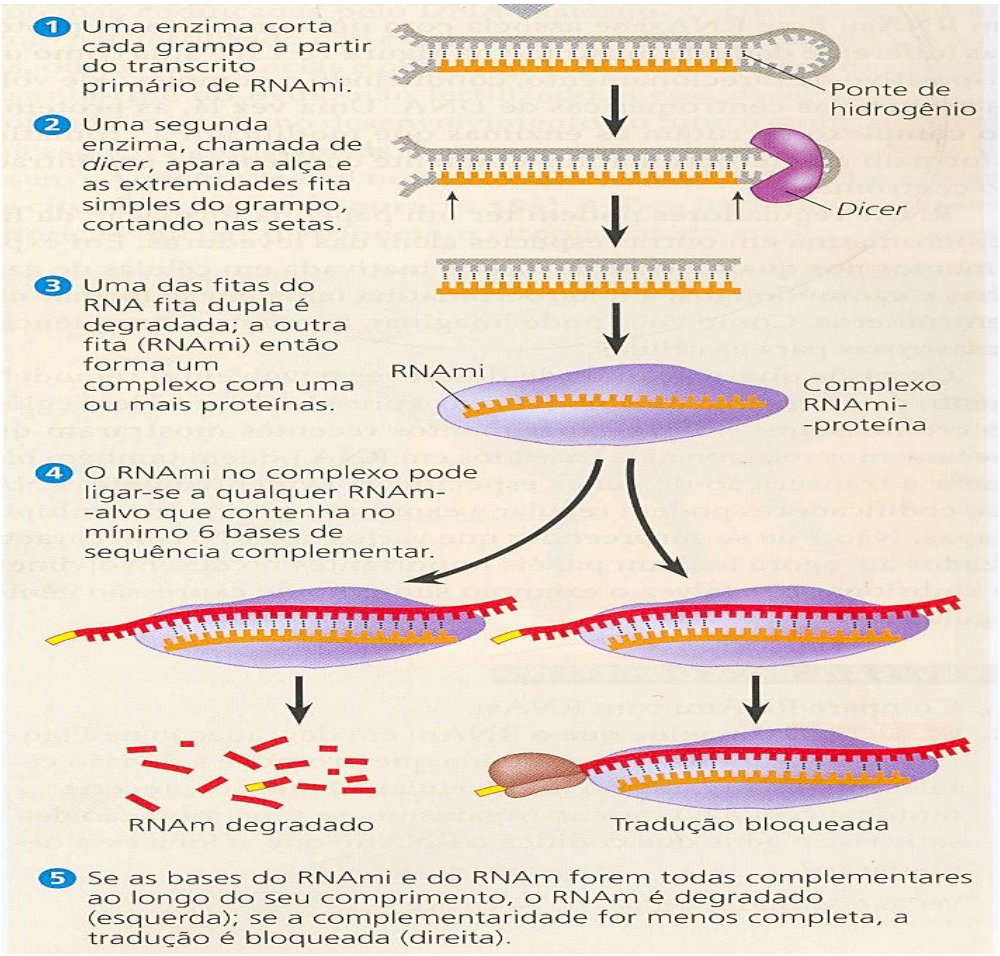

Figura 5. Mecanismo de regulação dos miRNAs (RNAmi) sobre mRNA alvo. (Adaptada de CAMPBELL, N.A. et al., 2010 (Campbell 2010)).

Muitos desses lncRNAs são expressos numa variedade de tipos de câncer, porém, alguns estão associados a um único tipo de câncer, como, por exemplo, o gene específico da próstata regulado por andrógeno PCGEM1 (*prostate cancer gene expression marker 1*) (Bussemakers, van Bokhoven et al. 1999; Fu, Ravindranath et al. 2006; Szell, Bata-Csorgo et al. 2008; Gupta, Shah et al. 2010; Chung, Nakagawa et al. 2011).

O gene PCGEM1 contém 1643 nucleotídeos e está intimamente relacionado ao risco aumentado de desenvolvimento do câncer de próstata (Bussemakers, van Bokhoven et al. 1999; Fu, Ravindranath et al. 2006), além de estar associado ao desenvolvimento da doença resistente a terapia de castração

(Yang, Lin et al. 2013). A superexpressão dele em linhagens celulares de câncer de próstata resultou em aumento do crescimento celular e da formação de colônias, suportando a ideia de que os lncRNA são importantes no desenvolvimento do câncer (Petrovics, Zhang et al. 2004). Além disso, trabalhos anteriores mostram que pacientes com câncer de próstata possuem expressão aumentada deste gene (Prensner, Sahu et al. 2014) e que inibição do mesmo bloqueou o crescimento de células de câncer de próstata responsivas e não-responsivas a andrógenos. Este último fato ressalta a importância do PCGEM1, o qual pode ser um importante alvo de novas drogas para tratar esta patologia, principalmente na fase resistente a castração, onde não há muitas alternativas de terapias curativas eficazes disponíveis atualmente (Schmitt and Chang 2013; Yang, Lin et al. 2013).

I.5. OBJETIVOS

Conforme descrito acima, o câncer de próstata é a segunda causa mais comum de morte entre os homens no Brasil, o sexto tipo de câncer mais comum no mundo e o mais prevalente entre os homens. Em geral, a morte desses pacientes está associada ao desenvolvimento de doença metastática ((INCA) 2014) e, nesse caso, o tratamento de eleição é a restrição andrógena (Stacewicz-Sapuntzakis and Bowen 2005; Madan and Arlen 2013). Porém, 10-20% dos pacientes que usam este tratamento tornam-se tolerantes a restrição hormonal, e neste estágio não existem muitas alternativas de terapias curativas disponíveis (de Bono, Logothetis et al. 2011; Kirby, Hirst et al. 2011; Scher, Fizazi et al. 2012; West, Kiely et al. 2014). Além disso, a terapia de restrição andrógena é longa e causa uma série de efeitos colaterais bastante desagradáveis ao paciente, podendo levar a outros problemas de saúde relacionados ao seu uso, que

também podem acabar levando o paciente à morte. Assim, a busca de novas abordagens terapêuticas com menos efeitos indesejáveis e mais eficazes para o tratamento desta patologia são de extrema importância.

Portanto, os objetivos da presente tese foram avaliar os efeitos do γ-orizanol e do extrato hidroalcólico de *Thuya occidentalis* em linhagens celulares de câncer de próstata responsivas (LNCaP) e não-responsivas a andrógenos (DU145 e PC3) sobre os seguintes parâmetros:

a) Proliferação, viabilidade, morte celular e progressão do ciclo celular em células de câncer de próstata, após o tratamento com o γ-orizanol e com extrato de *Thuya occidentalis*.
b) O padrão de expressão do gene e da proteína Caveolina-1 após o tratamento com o γ-orizanol e com extrato de *Thuya occidentalis*.
c) O padrão de expressão do gene conhecido como biomarcador do câncer de próstata, PCGEM1 após o tratamento com o γ-orizanol.
d) O efeito do γ-orizanol na modulação da expressão de microRNAs relacionados ao câncer de próstata.

PARTE II

II.1 γ-ORYZANOL REDUCES CAVEOLIN-1 AND PCGEM1 EXPRESSION, MARKERS OF AGGRESSIVENESS IN PROSTATE CANCER CELL LINES

(Artigo publicado no periódico *The Prostate*)

γ-Oryzanol Reduces Caveolin-1 and PCGEM1 Expression, Markers of Aggressiveness in Prostate Cancer Cell Lines

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BACKGROUND. Prostate cancer is a leading cause of death among men due to the limited number of treatment strategies available for advanced disease. γ-oryzanol is a component of rice bran, rich in phytosterols, known for its antioxidant, anti-carcinogenic and endocrinological effects. It is known that γ-oryzanol may affect prostate cancer cells through the down regulation of the antioxidant genes and that phytosterols have anti-proliferative and apoptotic effects. There are evidences showing that some of the components of γ-oryzanol can modulate genes involved in the development and progression of prostate cancer, as caveolin-1 (Cav-1) and prostate specific androgen-regulated gene (PCGEM1).

METHODS. To determine the effects of γ-oryzanol on prostate cancer cell survival we evaluated the cell viability and biomass by MTT and sulforhodamine B assays, respectively. Cell death, cell cycle and pERK1/2 activity were assessed by flow cytometry. The changes in gene expression involved in the survival and progression of prostate cancer *cav-1* and *PCGEM1* genes were evaluated by quantitative real time reverse transcriptase polymerase chain reaction (RT-PCR) and cav-1 protein by immunofluorescence followed by confocal microscopy analysis.

RESULTS. We found that γ-oryzanol decreases cell viability and culture biomass by apoptosis and/or necrosis death in androgen unresponsive (PC3 and DU145) and responsive (LNCaP) cell lines, and signals through pERK1/2 in LNCaP and DU145 cells. γ-oryzanol also appears to block cell cycle progression at the G2/M in PC3 and LNCaP cells and at G0/G1 in DU145 cells. These effects were accompanied by a down regulation in the expression of the cav-1 in both androgen unresponsive cell lines and *PCGEM1* gene in DU145 and LNCaP cells.

CONCLUSION. In summary, we used biochemical and genetics approaches to demonstrate that γ-oryzanol show a promising adjuvant role in the treatment of prostate cancer. *Prostate 9999: 1–15, 2015.* © 2015 Wiley Periodicals, Inc.

KEY WORDS: **γ-oryzanol; prostate cancer cell lines; survival genes; metastasis; androgen resistance**

INTRODUCTION

Androgen deprivation is the first choice of treatment for metastatic prostate cancer (PCa) in United States. However, 10–20% of patients diagnosed with prostate cancer develop resistance to anti-androgen therapy 2–3 years after treatment inception [1–4]. Tumor cell phenotype switch is not completely understood and much more effort has been focused on alterations regarding signaling of androgen receptor and/or alterations in the androgen metabolism [5–7].

Grant sponsor: Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS- Edital Pesquisador Gaucho—n° 1952-2551/13).

Conflicts of interest: The authors declare that they have no competing interests.

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Received 8 September 2014; Accepted 9 December 2014
DOI 10.1002/pros.22960
Published online in Wiley Online Library (wileyonlinelibrary.com).

As metastatic prostate cancer circumvents androgen ablation therapy through progression, prostate cancer is classified as being hormone-refractory or in a castrate-resistant prostate cancer stage. At this advanced pathologic stage, therapeutic options are limited and conventional chemotherapy fails to prolong survival [1] for longer than 40 weeks [8].

Rice is probably the most consumed cereal in the world and Brazil is the ninth largest producer [9]. Rice processing generates several waste of low commercial value, such as rice bran [10] rich in different phytochemicals [11–13]. Among them, much attention has been given to γ-oryzanol due to its nutraceutical properties, composed of a mixture of various compounds, majorly ferulic acid esters of phytosterols (steryl ferulates) [14].

Phytosterols have anti-proliferative and apoptotic effects [15–16] which make them attractive biomolecules for cancer treatment. Components of γ-oryzanol modulate genes involved in the development and progression of prostate cancer, such as caveolin-1 (*Cav-1*) identified as a marker of aggressiveness in prostate carcinomas [17] and the prostate specific androgen-regulated gene PCGEM1 [18]. *Cav-1* has an important role in prostate cancer cells as its product, the caveolin-1 protein, co-localizes with androgen receptors within the caveolae domains and mediate androgen-dependent signals [19–20].

In this regard, recent data has shown that cav-1 can modulate downstream signals that affect the expression of genes implicated in unregulated cell growth [21]. Overexpression of *Cav-1* is a relevant feature of castrate-resistant prostate cancer and the elevated expression of the Cav-1 protein is a marker of poor prognosis in localized human prostate cancers. Ongoing studies looking for biomarkers had positively correlated Cav-1 expression with indicators of the aggressiveness of the disease, as the Gleason grade [22]. Besides *Cav-1* gene, another important gene involved in progression and development of prostate cancer is *PCGEM1* [18]. *PCGEM1* is transcribed into a non coding poly(A)$^+$ RNA of 1,643 nucleotides, found specifically in prostate and non-coding protein [23]. Recent studies have linked overexpression of *PCGEM1* gene to prostate cancer development [18,24]. In an androgen-responsive prostate cancer cell line, LNCaP, *PCGEM1* significantly augmented cell proliferation and colony formation, suggesting an important biological role in prostate tumorigenesis [23]. Furthermore, additional studies have suggested that the modulation of gene expression could be involved in the alteration of the regulation of cell death and cell cycle in prostate cancer [23–24].

Considering the fact that at the time of clinical diagnosis, most prostate cancers are an heterogeneous population of androgen-responsive and androgen unresponsive cells [25] and the already published properties of γ-oryzanol, the aim of this study was to examine the effect of γ-oryzanol in three different cell lines. LNCaP, which is an androgen responsive prostate cancer cell line and DU145 and PC3 as a cell culture model of androgen-unresponsive prostate cancer cell lines. Our results demonstrates an important role for γ-oryzanol in the regulation of an anti-proliferative response on androgen-responsive and androgen-unresponsive cell lines, namely LNCaP and DU145 or PC3, respectively, using a wide range of technical approaches, such as biochemistry, gene and protein expression and flow cytometry assays.

MATERIALS AND METHODS

Chemicals and Reagents

γ-oryzanol was obtained from Tokyo Chemical Industry Co., Ltd. (TCI America, Tokyo, Japan). The stock solution was prepared dissolving γ-oryzanol powder in ethanol to a final concentration of 6.6 mM. As the γ-oryzanol is insoluble in water, many options to solubilize it has been suggested and one option involves conversion to a cyclodextrin inclusion compound [18,25]. Then, γ-oryzanol was mixed in the culture medium (RPMI-1640) with the sterol carrier (2-hydroxypropyl)-β-cyclodextrin (β-CD) in a non-toxic form at molar ratio of 1:300 for all concentrations, as previously reported [18,26–27]. β-sitosterol and ferulic acid were purchased as powder from MP Biomedicals, LLC (Brazil) and diluted in RPMI-1640 medium supplemented with 10% FBS to a final concentration of 16 μM.

3-(4, 5-dimethyl)-2, 5-diphenyltetrazolium bromide (MTT), trypan blue, (2-hydroxypropyl)-β-cyclodextrin (β-CD), dimethylsulfoxide (DMSO), sulforhodamine B, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), paraformaldehyde, triton X-100 and HEPES buffer were obtained from Sigma Aldrich (St. Louis, MO).

RPMI-1640 medium, fetal bovine serum (FBS), propidium iodide (PI), Annexin-V FITC Conjugate Kit, TRIzol Reagent, RNAse A, SuperScript®-III RT First-Strand Synthesis SuperMix, Alexa Fluor 555, were purchased from Invitrogen (Grand Island, NY). Mouse anti-human cav-1 monoclonal antibody (clone 7C8) was purchased from Santa Cruz Biotechnology (CA). CaspACE, FITC-VAD-fmk In Situ Marker kit was purchased from Promega, Madison, and PE mouse anti-ERK1/2 (pT202/pY204) was purchased from BD Bioscience (San Jose, CA).

Cell Culture and Treatments

LNCaP (clone CRL-1740, ATCC, Manassas, VA, USA), DU145 (clone HTB-81, ATCC,) and PC3 (clone CRL-1435, ATCC,) prostate cancer cell lines were kindly provided by Dr. Mari C. Sogayar from São Paulo University (SP, Brazil), cultured in RPMI-1640 medium supplemented with 10% FBS and 2 g/L HEPES buffer, 1% penicillin, pH 7.4, under 37°C and 5% CO_2 conditions. The cells were plated (5×10^4/cm^2) in 12 or 24-well plates and cultured for 24 hr to reach 60–70% of confluence before treatment.

Experimental media was supplemented with β-sitosterol, ferulic acid + β-sitosterol or γ-oryzanol at final concentration of 16 μM. Optimum γ-oryzanol concentration of 16 μM was chosen after dose-response experiments produced results that were consistent with physiological levels for phytosterols [21]. DU145 and PC3 cells were treated with γ-oryzanol for 48 hr and LNCaP cells were treated for 24 hr. Each concentration treatment was done in triplicate. Routinely cultured cells were used as controls.

Colorimetric MTT Assay

MTT assay is based on the capacity of mitochondrial enzymes of viable cells to reduce the MTT yellow tetrazolium salt to purple formazan crystals [28]. This assay is used for the assessment of cytotoxicity, cell viability, and proliferation studies in cellular biology [29]. Pre-confluent prostate cancer cells were incubated with γ-oryzanol for 48 hr (DU145 and PC3 lines) and 24 hr (LNCaP line). Cells were then incubated with 1 mg/ml MTT for 2 hr at 37°C. Purple crystals were dissolved in DMSO. The absorbance was measured using a spectrophotometric microtiter plate reader (Spectra Max M5, Molecular Devices) at 570 and 630 nm.

Sulforhodamine B Assay

The assay was performed as previously described for Skehan et al. [30] with some modifications. After the treatment with γ-oryzanol, culture medium was removed, cells were washed three times with PBS and 500 μl PBS/4% paraformaldehyde was added. After 15 min, fixed cells were stained with SRB. Subsequently, the cells were washed with Milli-Q® water to remove unbound stain. The culture plates were air dried and protein-bound sulforhodamine was solubilized in 1% SDS. Absorbance was measured using a spectrophotometric microtiter plate reader (Spectra Max M5, Molecular Devices) at 560 nm. This absorbance was linearly proportional to the number of cells.

Trypan Blue Dye Exclusion Assay

As indicated, cells proliferation was also determined by trypan blue dye exclusion method. In this regard, treatment media was removed and cells were trypsinized. Aliquots of cell suspensions (100 μl) were incubated with the same volume of trypan blue (0.4% in PBS) for 5 min [31]. Finally, cells were transferred to a Neubauer chamber and counted by light microscope. Dead cells were defined as those stained with the dye.

Flow Cytometry Assays

Cell death in prostate cancer cell lines treated with γ-oryzanol was analyzed with Annexin-V/PI. Trypsinized cells were washed with PBS, suspended at a final concentration of 1×10^6 cells/ml in Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated (1×10^5 cells) with 5 μl of Annexin-V FITC for 15 min at room temperature in the dark. Before acquisition, PI was added (1 μg/ml).

For cell cycle analysis, cells were washed in PBS, suspended in 400 μl (1×10^6 cells/ml) of cell cycle solution (3.5 mM trisodium citrate, 0.5 mM tris, 0.1% Nonidet, 100 μg/ml RNAse A, 50 μg/ml PI) and incubated in the dark at room temperature for 15 min.

For pERK1/2 analysis, cells were fixed with 2% paraformaldehyde for 10 min at 37°C and subseqeuntly permeabilized in 90% methanol for 30 min on ice. Cells were washed twice in PBS and stained with PE mouse anti-ERK1/2 (pT202/pY204, BD biosciences) for 30 min.

Caspase activation in PC3 cells was analyzed using a FITC-VAD-fmk in Situ Marker kit (Promega). 1×10^6 cells were washed with PBS, suspended in 100 μl of staining solution containing 10 nM of fluorescein isothiocyanate conjugated of z-VAD-fmk (FITC--VAD-fmk) for 15 min at room temperature in the dark, before acquisition by flow cytometry.

Acquisition was performed using a FACSCalibur flow cytometer (BD Biosciences, USA). Data was analyzed using FCS Express 4 Flow Cytometry software (De Novo Softwares, USA) or CellQuest Pro software (Becton, Dickinson and Company, USA).

RNA Extraction, cDNA Synthesis and Real-Time PCR

Total RNA was isolated using the TRIzol Reagent following manufacturer's instructions. RNA was quantified using the BioPhotometer Plus (Eppendorf). 1.2 μg of total RNA was added to each cDNA synthesis reaction, using the SuperScript®-III RT

First-Strand Synthesis SuperMix (Invitrogen). Gene sequence information was collected from gene databases (www.ncbi.nlm.nih.gov and www.ensembl.org) and primers were designed using idt online software (www.idtdna.com). To analyze the effects of γ-oryzanol in expression genes involved in cell growth signaling, the following primers were designed: (a) *Cav-1*: sense 5′- ACCCTAAACACCTCAACGATG- 3′ and anti-sense 3′- CAGACAGCAAGCGGTAAAAC -5′ (amplicon product 108 bp); and (b) *PCGEM1*: sense 5′- TCTGCAACTTCCTCTAATTGGG - 3′ and anti-sense 3′- TGCTCACTTGATAAGGTCACG -5′ (amplicon product 150 bp). The *36B4* (acidic ribosomal phosphoprotein P0, RPLP0) primer (sense 5′-CAG CAAGTGGGAAGGTGTAATCC- 3′ and anti-sense 3′- CCCATTCTATCATCAACGGGTACAA -5′) (amplicon product 75 bp) was used as housekeeping gene for all relative expression calculations. qRT-PCR reactions were performed in triplicates and carried out in a StepOnePlus real-time PCR system (Applied Biosystems®, New York, NY, USA). The thermal cycling profile for *Cav-1* and *36B4* genes was an initial denaturation step at 94°C for 10 min followed by 40 cycles of 10 sec at 94°C, 15 sec at 60°C, 15 sec at 72°C and 35 sec at 60°C for data acquisition followed by a melting dissociation curve.

For *PCGEM1* detection, initial amplification was achieved in an end-point semi-quantitative (sqPCR) reaction carried out in a Veriti thermal cycler (*Veriti*™ *Thermal Cycler*, Applied Biosystems®). An aliquot of this amplification was then used as the starting material for qPCR carried out in a StepOnePlus real-time PCR system. Samples were normalized by the constitutive gene (36B4) and calibrated by the average of the ΔCT of the group itself. The specificity of amplification and absence of primer-dimers were confirmed using melting curve analysis at the end of each run. Results were analyzed by the ΔΔCT method [32].

Immunocytochemistry

Immunocytochemistry was performed as previously described [33]. Briefly, cells (DU145 and PC3 cell lines) were treated with 16 μM of γ-oryzanol for 48 hr, fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. After blocking for 2 hr with BSA 5% in PBS, cells were washed and incubated overnight with mouse anti-human cav-1 monoclonal antibody (1:250, clone 7C8 Santa Cruz Biotechnology, CA) at room temperature, followed by PBS washes and incubated with specific anti-mouse secondary antibody conjugated with Alexa Fluor 555 (1:1.000) (Invitrogen, Grand Island, NY) for 2 hr. In negative controls, reactions were performed by omitting the primary antibody.

Confocal images were collected using Olympus FV1000 laser-scanning confocal microscopy. Ten single confocal sections of 0.7 μM were taken parallel to the coverslip (*xy* sections) with an × 60 (numeric aperture 1.35) oil-immersion objective (Olympus, U plan-super-apochromat, UPLSAPO60XO). For each sample, images of twenty fields were acquired and processed with Olympus FluoView FV1000 software and the measurement of fluorescence intensity was performed using the imageJ software. Cav-1 fluorescence was measured after being excited by a 555 nm laser beam and emission scan collected at 565 nm. ImageJ software, a public domain Java Image processing program (http://rsb.info.nih.gov/ij/) was used to analyze the fluorescence intensity of the samples.

Statistical Analysis

Data were reported as mean ± standard deviations (SD). Results were analyzed by *t* test ($P < 0.05$). All analyses and graphical were performed using the statistical software GraphPad Prism 5 for Windows (GraphPad Software Inc., version 5.01). The same program was used to calculate the EC_{50} doses used in this work.

RESULTS

γ-Oryzanol Concentration and Dose Response

In order to define the correct concentration of γ-oryzanol used in this work, we performed dose-response proliferation curves and calculated the effective concentration (EC_{50}) values for each of the androgen responsive (LNCaP) and unresponsive (DU145 and PC3) prostate cancer cell lines used in this study. γ-oryzanol dependent cell proliferation was measured by the sulforhodamine B assay. As expected, the different prostate cancer cell lines showed different sensitivities to γ-oryzanol. Androgen responsive LNCaP (Fig. 1A) and unresponsive PC3 (Fig. 1C) prostate cancer cell lines depicted similar values of EC_{50}, 17.31 and 17.20 μM, respectively ($P < 0.05$), whereas DU145 cells were more sensitive to γ-oryzanol treatment with an EC_{50} value of 10.77 μM (Fig. 1B). In agreement with previous studies that showed that the physiological concentrations of phytosterols are in the μM range [14,26], we chose the dose of 16 μM of γ-oryzanol as treatment for the following experiments performed in this study.

Reasoning in the same way, chosen treatment times of 48 hr for DU145 and PC3 cells and 24 hr for LNCaP

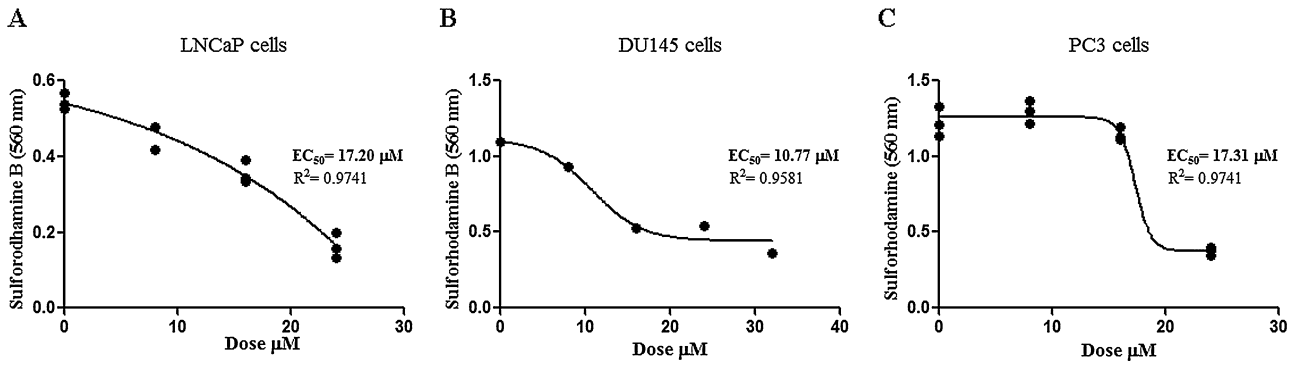

Fig. 1. Dose-response curve measured by sulforhodamine B proliferation assay in (A) LNCaP cells; (B) DU145 cells and (C) PC3 cells.

cells were based on experiments that showed similar anti-proliferative effects in the three lines used, supplemented with γ-oryzanol (16 μM) at different time points (data not shown).

Because γ-oryzanol is a mixture of various compounds, particularly of ferulic acid esters of phytosterols (steryl ferulates) and it is known that β-sitosteryl ferulate is one of the main components, we decided to test the anti-proliferative effects of ferulic acid and β-sitosterol in an independent way. Similar to incubation with γ-oryzanol, reduction of cell proliferation with 16 μM ferulic acid, 16 μM β-sitosterol or a combination of both, showed comparable results after 48 hr of treatment of DU145 or PC3 cells ($P > 0{,}05$) (Fig. 2A,B).

γ- Oryzanol Induces Cell Death Through Necrosis and/or Apoptosis

The effects of γ-oryzanol on cell viability in the prostate cancer cell lines were measured by the MTT assay and are presented in Figure 3. At 24 hr of treatment, γ-oryzanol was able to reduce the cell viability in the LNCaP androgen unresponsive cell line (Fig. 3A). When both, DU145 and PC3 androgen responsive cell lines, were treated with γ-oryzanol for 48 hr, there was also a decrease in cell viability (Fig. 3C,E).

Sulforhodamine B assays were used to estimate the consequences of γ-oryzanol treatment on culture biomass (Fig. 3B,D,F). Comparable with cell viability analysis, results obtained with the MTT assay were in agreement and showed a significant decrease in cell biomass in LNCaP or DU145 and PC3 culture treated cells (Figs. 3B,D,F).

With the intention of exploring the mechanism by which γ-oryzanol was acting, we decided to investigate cell death by flow cytometry using double staining with FITC-conjugated Annexin V and propidium iodide (PI). Even though LNCaP and DU145 cells are unresponsive and responsive androgen cell lines, they had similar behavior. In this regard, flow cytometry analysis revealed only PI staining in both cell lines, showing a γ-oryzanol dependent increase of positive necrotic cells (Fig. 4A–C). In DU145 and LNCaP cells, apoptotic cells were negligible.

Different from the other cell lines, after 48 hr of treatment of PC3 cells, γ-oryzanol was able to induce an increase in the percentage of apoptotic and late apoptotic cells, when compared to control cells

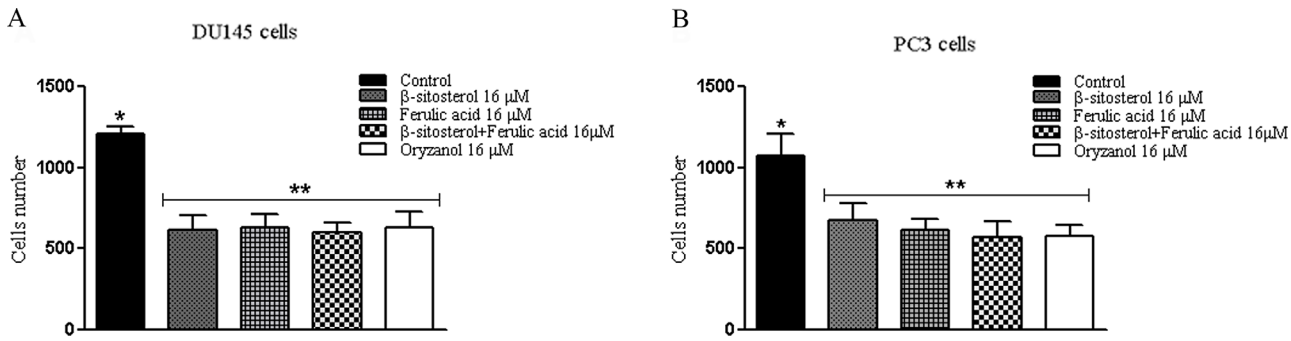

Fig. 2. Effects of 16 μM of β-sitosterol, ferulic acid, β-sitosterol + ferulic acid or γ-oryzanol in cells number after 48 hr of treatment in (A) DU145 and (B) PC3 cells.

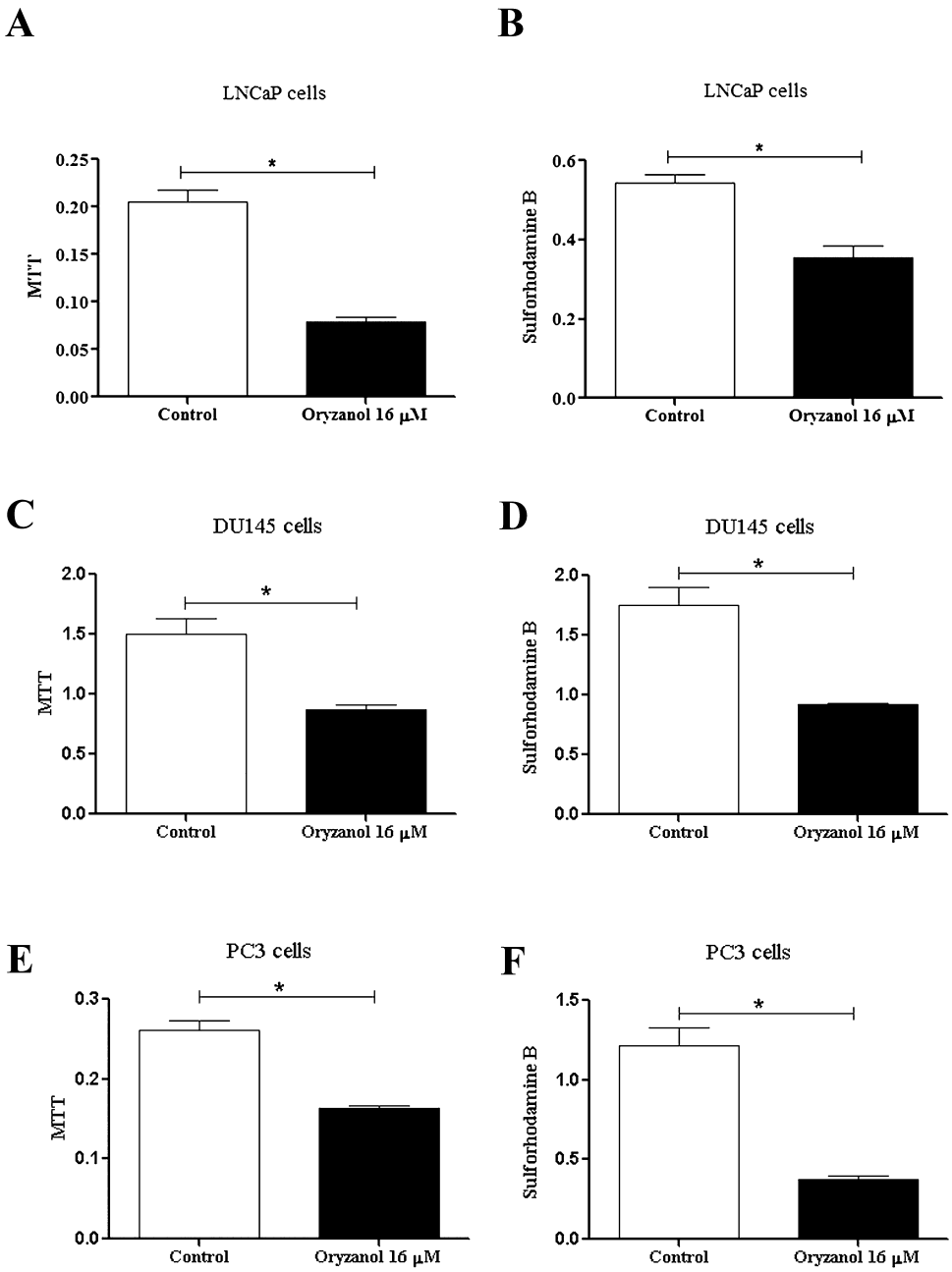

Fig. 3. Effects of γ-oryzanol on cell viability and biomass in androgen responsive and unresponsive prostate cancer cell lines. Cell viability measured by MTT assay (A) LNCaP cells, (C) DU145 cells, (E) PC3 cells and cell biomass by sulforhodamine B assay (B) LNCaP cells, (D) DU145 cells, (F) PC3 cells. Values are show as mean ± SD of three independent experiments. $^{*}P < 0.05$ versus untreated control cells.

(Fig. 4A,D), probably associated with caspase activation, as shown in Figure 4E.

γ- Oryzanol Induces Cell Cycle Arrest

We next determined the role of γ-oryzanol in the cell cycle arrest. In this regard, we quantified cellular DNA by univariate analysis following cell staining with PI. DNA content analysis revealed changes in the cell cycle of the three cell lines studied. LNCaP and PC3 cells resulted in arrest and accumulation of cells in G2/M phase and concomitant decrease in the S phase (Fig. 5A,C). The 48 hr treatment with γ-oryzanol also induced an increase in the percentage of DU145 cells in the G0/G1 phase (Fig. 5B).

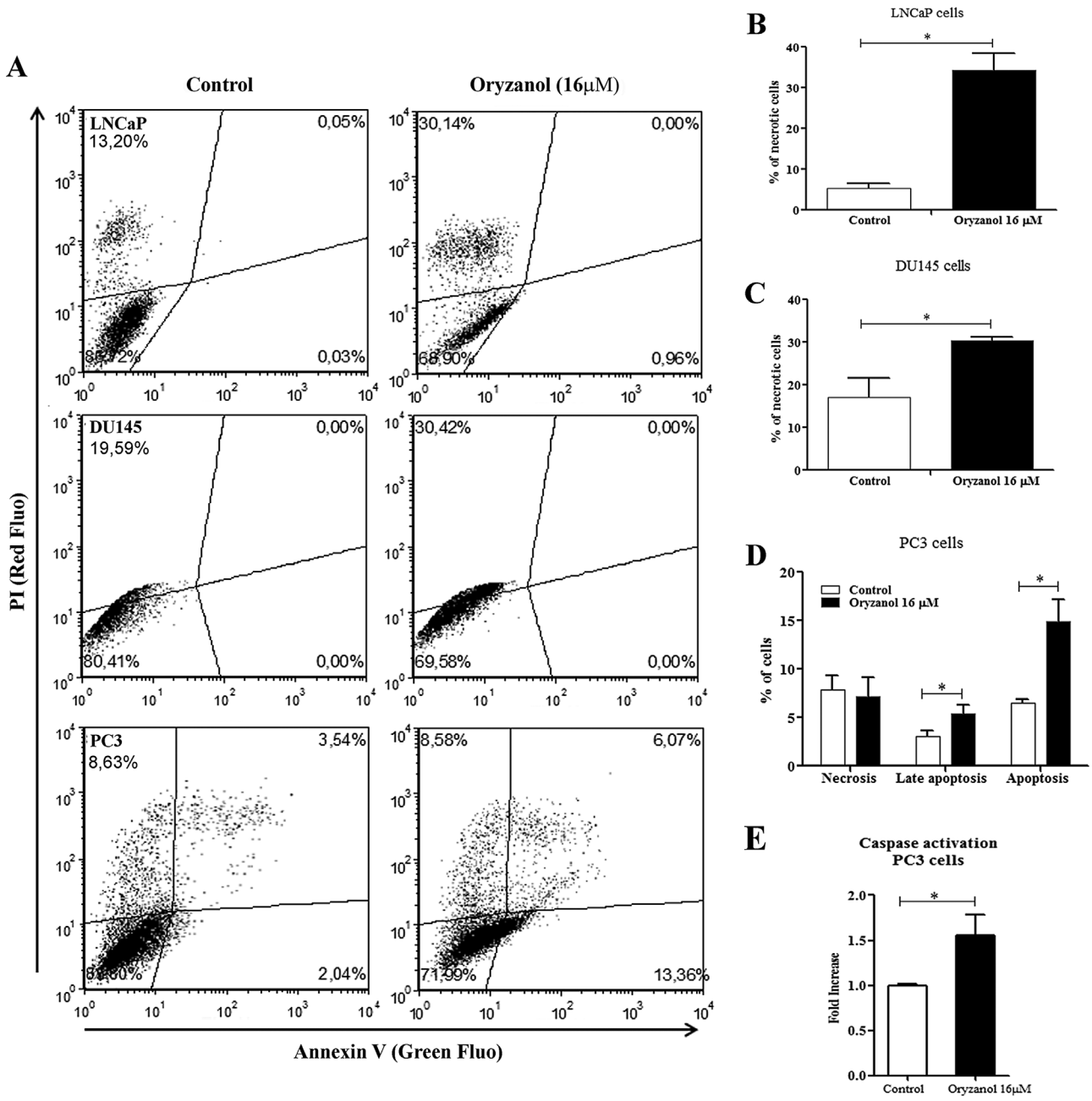

Fig. 4. Apoptotic and/or necrotic effects of γ-oryzanol in androgen responsive and unresponsive prostate cancer cell lines. (A) Dot plots graphics representative of cell death analysis by flow cytometry in LNCaP androgen responsive and DU145 and PC3 androgen unresponsive cell lines (Apoptotic cells: Annexin-V positive and PI negative; Late apoptotic cells: Annexin-V positive and PI positive; Necrotic cells: Annexin-V negative and PI positive); representative graphics of cell death in (B) LNCaP cells, (C) DU145 cells and (D) PC3 cells. (E) Detection of caspase activation using "CaspACE, FITC-VAD-fmk In Situ Marker" in PC3 androgen unresponsive cell line after treatment with γ-oryzanol. Values are show as mean ± SD of three independent experiments. $^{*}P < 0.05$ versus untreated control cells.

γ- Oryzanol Signals Through the ERK 1/2 Pathway

In order to dissect the signaling mechanism of action of γ-oryzanol, we evaluated ERK1/2 activity through phosphorilation. In this regard, γ-oryzanol treatment was dependent on ERK1/2 activity in LNCaP and DU145 cells, where pERK1/2 activity was 0.31 and 0.57 times greater than control, respectively (Fig. 6A and B). No statistical difference was found in ERK1/2 activity in PC3 cells (Fig. 6A and B).

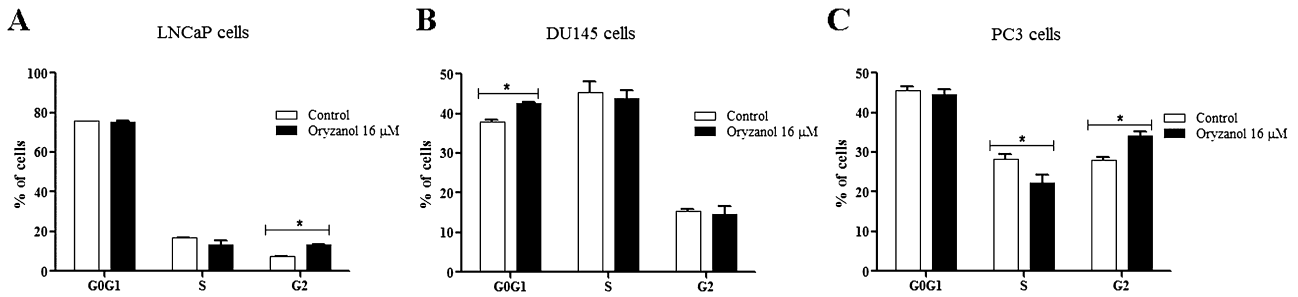

Fig. 5. Effects of γ-oryzanol on cell cycle progression in androgen responsive and unresponsive prostate cancer cell lines. Cell cycle analysis was analyzed by flow cytometry and stained with propidium iodide (A) LNCaP cells, (B) DU145 cells and (C) PC3 cells. Values are show as mean ± SD of three independent experiments. $^{*}P < 0.05$ versus untreated control cells.

γ- Oryzanol Downregulates the Expression of Genes Associated With Prostate Cancer Progression

Aberrantly overexpression of cav-1 is a predominant feature of prostate cancer and correlates with disease progression resulting in an increased severity and mortality of patients with prostate cancer. To determine if cav-1 expression was modulated after γ-oryzanol treatment, we analyzed cav-1 expression by confocal microscopy. As depicted in Figure 7, cav-1 protein expression was modulated and decreased in γ-oryzanol treated DU145 and PC3 cells. These results were confirmed at the mRNA level (Fig. 7F).

As already published, we didn't detect expression of *cav*-1 in LNCaP cells [22,34]. Besides cav-1, recent studies have also linked the overexpression of the non-coding protein *PCGEM1* gene to prostate cancer [24]. In our settings, LNCaP and DU145 treated cells had reduced *PCGEM1* gene expression (Fig. 8A, B). We couldn't detect *PCGEM1* expression in PC3 cells.

DISCUSSION

PCa is the leading cause of newly estimated cases among cancer in men and is responsible, in second place, for the increasing mortality associated with the

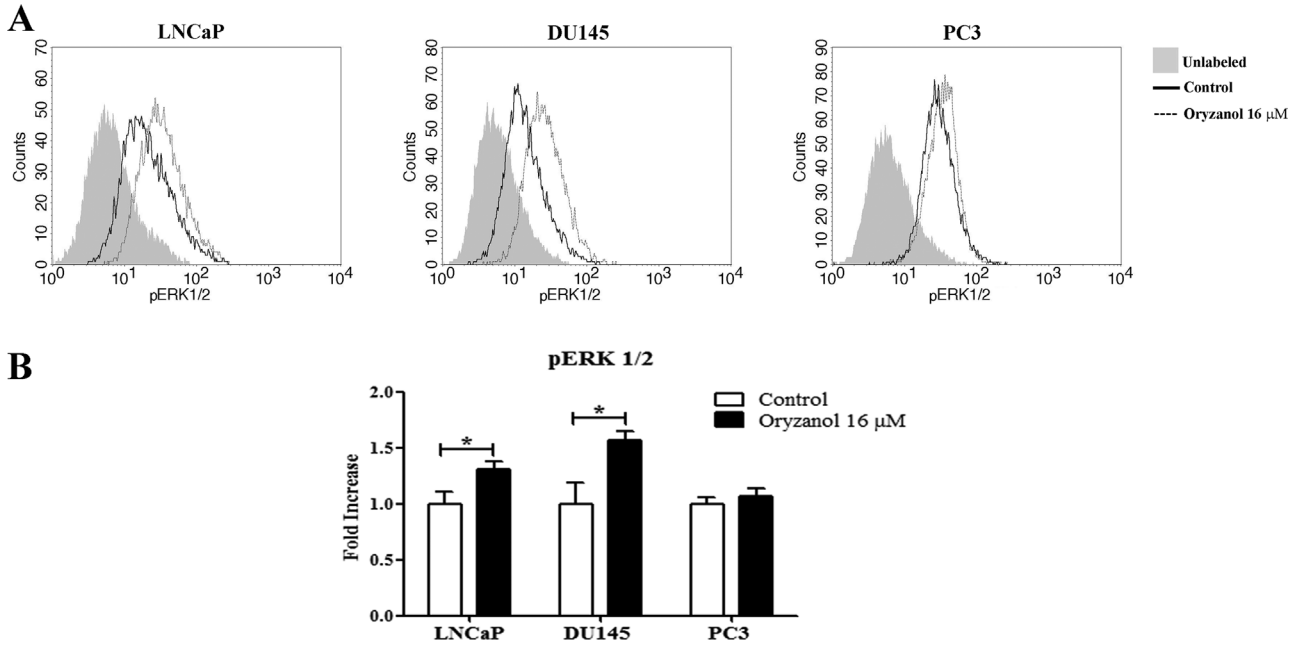

Fig. 6. Effects of 16 μM of γ-oryzanol in ERK1/2 activity measured by flow cytometry in androgen unresponsive and responsive prostate cancer cell lines. Histograms plots of pERK1/2 are shown in (A) LNCaP, DU145 and PC3 cells and (B) Fold increase of ERK1/2 activity in treated and control prostate cancer cell lines. Values are show as mean ± SD of three independent experiments $^{*}P < 0.05$ versus untreated control cells.

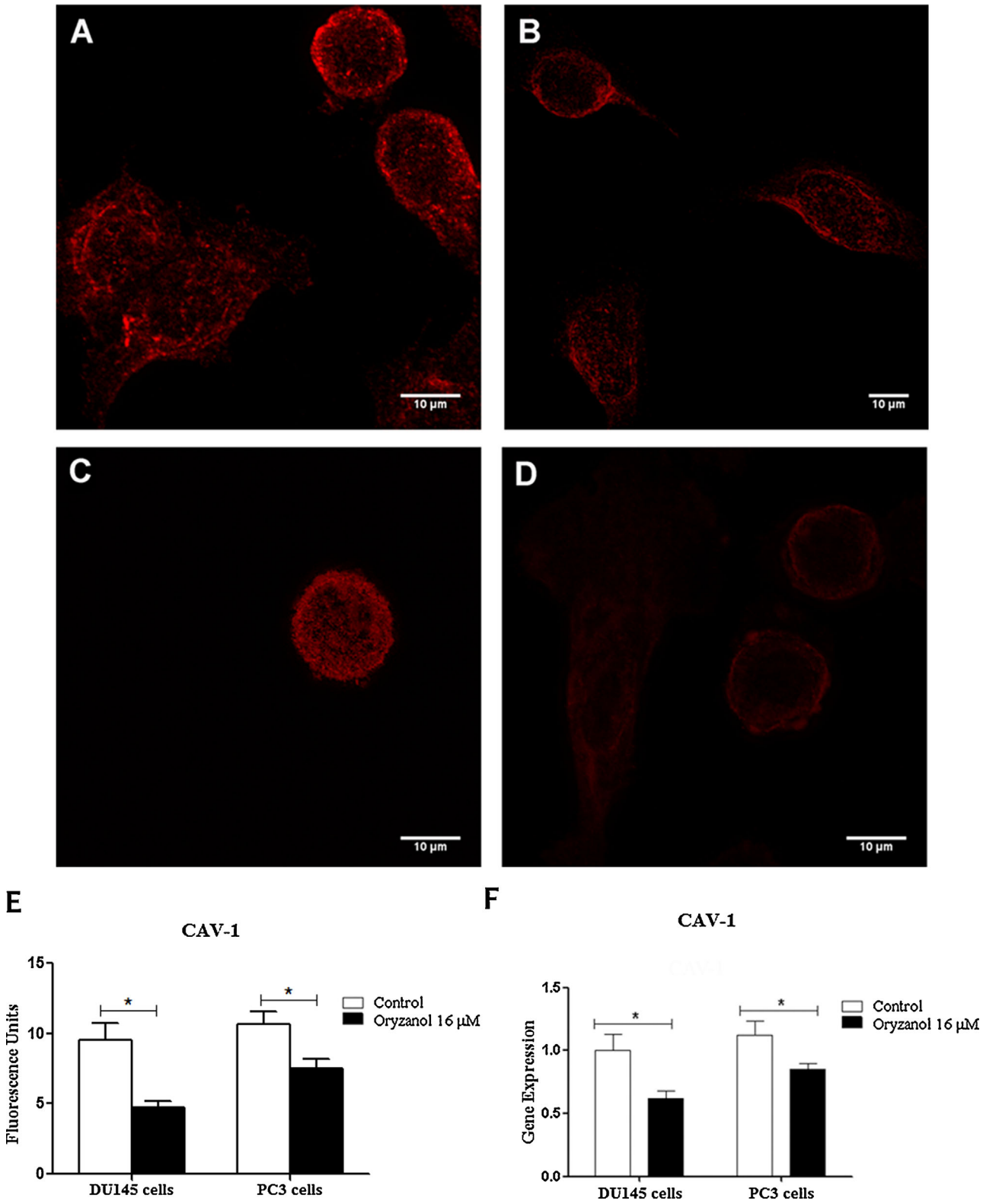

Fig. 7. Effects of 16 μM of γ-oryzanol on expression of Cav-1 mRNA and protein in androgen unresponsive prostate cancer cell lines. Images of confocal microscopy of Cav-1 protein content stained by immunocitochemistry in DU145 (A,B) and PC3 (C,D) cell lines. (A,C) are untreated cells and (B,D) are treated cells with 16 μM of γ-oryzanol; (E) Fluorescent intensity of confocal microscopy images of Cav-1 protein measured by ImageJ software and (F) qRT-PCR of Cav-1 gene expression normalized by the 36B4 constitutive gene. Values are show as mean ± SD of three independent experiments $^{*}P < 0.05$ versus untreated control cells. Scale bar = 10 μM.

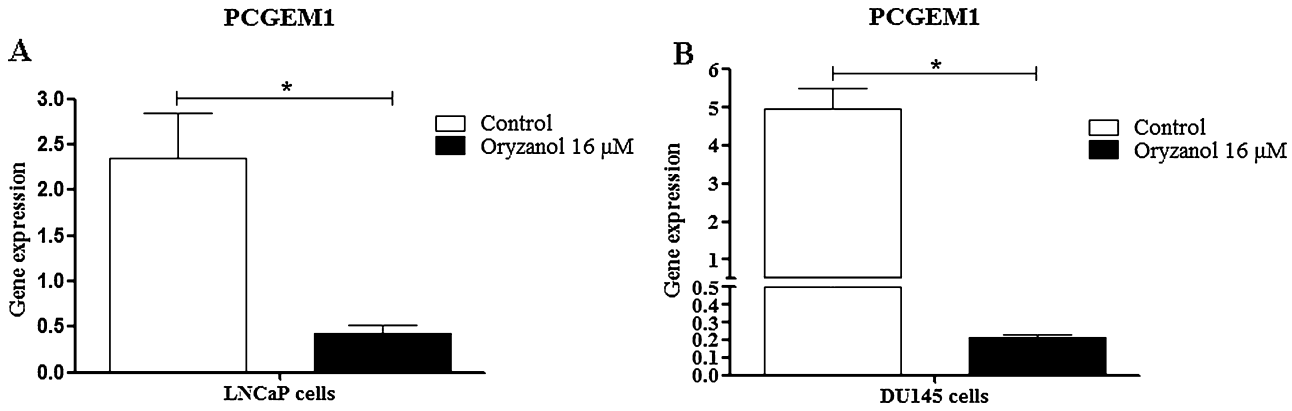

Fig. 8. Effects of 16 μM of γ-oryzanol on expression of *PCGEM1* mRNA in LNCaP androgen responsive PCa cells and DU145 androgen unresponsive PCa cells Gene expression was normalized by the 36B4 constitutive gene. Values are show as mean ± SD of three independent experiments $^{*}P < 0.05$ versus untreated control cells.

disease [35]. In Brazil is the second most common cancer among men only behind skin cancer non-melanoma. Brazilian National Institute of Cancer (INCA) estimates approximately 68,800 new cases by the end of 2014 [36]. Radical treatment, as curative resection of PCa is often not possible and androgen deprivation remains the cornerstone therapy for patients with advanced pathologic stages of the disease [36]. Androgen-ablative therapies are initially very effective because induce cell growth arrest and death in androgen-dependent tumor cells by apoptosis [37].

Unfortunately, PCa recurrence is highly prevalent occurring within 1–3 years of treatment. At these advanced pathological stages; PCa tends to be highly resistant to conventional cytotoxic agents [38]. While androgens are required for normal growth and development of prostate; they may also play part in prostate carcinogenesis acting either as initiators or promoters of cancer [39] by switching into a non-responsive androgen phase and worsening the prognosis of PCa. In this regard, although well established, the underlying mechanisms of phenotype modulation are not well characterized and still poorly understood.

In the present study we determined whether γ-oryzanol could be useful as a bioactive phytochemical agent or had any effect in androgen responsive and unresponsive cells. Our study demonstrated that γ-oryzanol treatment had anti-proliferative action, majorly by induction of necrosis and reduction of *cav-1* and *PCGEM1*, markers of aggressiveness in PCa.

γ-oryzanol is not a single compound but a mixture of steryl ferulates. Currently evidence shows that γ-oryzanol has anticarcinogenic activity [40] against breast, lung, liver and colorectal cancer through its ability to induce apoptosis, inhibit cell proliferation and alter cell cycle progression [45]. Moreover, its components, such as phytosterols, also act on prostate cancer cell lines downregulating the expression of *cav-1* and *PCGEM1*, a prostate specific gene [18,41].

Actually, it is known that the evolution of cancer is associated with deregulated cellular proliferation and suppressed cell death [42]. The concentration of γ-oryzanol used in this work was able to reduce cell viability and cell population in the three cell lines studied. We also demonstrated that the same concentration of ferulic acid and β-sitosterol were able to reduce the cell population in the same proportion as γ-oryzanol.

Much research has been conducted in order to develop anticancer agents that selectively kill cancer cells where the apoptotic process is the major route of death [43–44]. However, further studies had emerged showing that cell death by necrosis can also be a beneficial process. In this case, the inflammatory scenario could have a potential advantage as it stimulates an immune response that could increase the recruitment of immune system cells and augments, in some way, the efficacy of many drugs [45–46].

In DU145 and LNCaP prostate cancer cell lines, the treatment with γ-oryzanol significantly increased the percentage of cells by necrosis. However in the androgen unresponsive cell line PC3 there was an increase in the percentage of cells under apoptosis and late apoptosis. This result emphasizes the use of PC3 as a representative cell line of the most prevalent site of metastasis in prostate cancer, the bone [47–50], where few treatment alternatives are offered at this stage of disease [51–52].

The regulation of cell cycle is critical for growth and development of cancer cells. Our findings show

that the inhibitory effects of γ-oryzanol in cell growth occurs via induction of G0/G1 arrest in DU145 cells or G2/M arrest in LNCaP and PC3 cells, as similarly reported by others with the incubation with different phytosterols [18,26].

One of the pathways that is activated through cell cycle progression is ERK1/2 activation. Former studies showed that nontumorigenic prostate epithelial cells have high basal levels of ERK1/2 activity, that gradually decreases during progression of prostate cancer as cell proliferation increases [55–58]. Transient ERK1/2 activation can augment cell proliferation. Conversely the strong and sustained ERK1/2 activation can lead to a reduction of proliferation with concomitantly cell cycle arrest [58–60]. In our experiments we observed an increase of pERK1/2 activity in LNCaP and DU145 cells, suggesting that the inhibition of proliferation observed could be associated with a sustained increase in ERK1/2 activity and lately associated with cell cycle arrest, as previously reported [59,61]. pERK1/2 sustained activity induced by γ-oryzanol treatment could represent an important strategy for prostate cancer chemoprevention. We did not observe differences in pERK1/2 activation in PC3 androgen unresponsive γ-oryzanol treated cells, but we observed an increase in the percentage of cells under apoptosis.

Trying to dissect the mechanism of action of γ-oryzanol related to pERK1/2 activation and associated to apoptosis, we measured activation of caspases as central to cell death. In γ-oryzanol treated PC3 cells it was possible to detect an increase in the percentage of caspases activation, suggesting that the reduced viability and cell population observed in this cell line probably is, in part, associated with the increase of apoptosis death.

Heterogeneity, found in the different cells lines used in this study, has already been shown by others. Jayakumar et al. [68] studied the effects of ionizing radiation in DU145 and PC3 prostate cancer cells and found different responses. This fact has also been demonstrated by others showing that, although the cell lines are androgen unresponsive and originated from the same pathology, they show differential chemotherapeutic responses [68–70]. Indeed, small changes in treatment composition may also trigger different responses to similar lines. In this regard, steroids from *Commiphora mukul* although possessing similar structures, display different results on cell cycle arrest of PC3 cells [71]. PC3 cells treated with stigmata-5,22E-diene-3β,11α-triol (compound 1) significantly accumulates in the G0/G1 phase in a dose dependent manner. When treated with stigmata-5,22E-diene-3β,7α,11α triol (compound 2) PC3 cells were arrested at G2/M phase. It was also found that compound 2 induced an accumulation of cells in G2/M phase of cell cycle in DU145 and MCF-7, prostate and breast cancer cell lines, and had no impact on cell cycle progression of leukemia K562 cells. Interestingly, the compound 1 induction of PC3 cells G0/G1 phase arrest, was not accompanied by apoptosis. On the other hand, compound 2 promoted G2/M arrest and induced cell apoptosis in PC3 and DU145 cells. In light of our results and of the studies discussed here, we can conclude that the cancer chemopreventive mechanism of natural products depends of the compound and the cell type.

In general, prostate cancer shows locally restricted slow development. However, cancer can spread throughout the body and in some cases, after metastasis, tumor cells can adapt to an environmental deprived of androgens and proliferate [1–4]. For the reasons mentioned above, the inhibition caused by treatment with γ-oryzanol in androgen unresponsive prostate cancer lines is very important as there are no successful treatments available at this cancer stage actually [51–52]. Thus, the reduction in cell viability and biomass associated with increased cell death by apoptosis and/or necrosis and cell cycle arrest observed in DU145 and PC3 cell lines suggest that γ-oryzanol may be promising for the development of cancer treatment in castration-resistant stage.

Many factors are associated with the transformation of prostate cells in androgen-resistant cells and the majority of studies show that there are, in fact, changes in androgen metabolism and/or in the androgen receptor signaling [5–7]. Among them, many studies show that *Cav-1*, a vital component of the caveolae [21,72] is important for cancer progression as it has been identified as a marker of aggressiveness in prostate carcinomas [17]. It is suggested that *Cav-1* hypersensitizes the androgen receptor, suggesting that it is involved in the androgen resistance process exhibited by prostate cells during disease progression [20]. A study conducted by Lu et al. [20] reinforces the important role of *Cav-1* in androgen resistance. They demonstrated that *Cav-1* and androgen receptor forms a complex in the presence of androgens acting in androgen-dependent signaling. Furthermore, they also suggest that *Cav-1* could hypersensitize the androgen receptor, even in the presence of very low concentrations of androgens [20]. This fact is confirmed by Nasu et al. [73], which demonstrated that suppression of *Cav-1* expression in mouse prostate cancer cell lines converted androgen-insensitive cells in androgen-sensitive cells again. Subsequently, the overexpression of *Cav-1* appears to be associated with castration resistant disease and metastatic prostate cancer lines have high expression of the gene and protein Cav-1 compared to normal

epithelial cells. Moreover, studies have demonstrated that overexpression of *Cav-1* is associated with reduced capacity of apoptosis, corroborating the progression of metastatic prostate cancer [22,74].

On the other hand, in prostate cancer cells, *Cav-1* overexpression has been proposed to be stage specific. In this way at early stages of the disease absence of *Cav-1* promotes proliferation and survival, whereas at later stages, upregulated expression correlates with invasiveness, metastasis and multidrug resistance [75]. Currently, several works have reported that *Cav-1* gene can act as a proto-oncogene in prostate cancer and that the increased expression is associated with increased aggressiveness and metastatic potential of prostate tumor [73,76], besides being a marker of poor prognosis of prostate cancer [22]. Furthermore, there is evidence showing that reduction in *Cav-1* expression can transform androgen unresponsive cells in androgen responsive cells [34,74,76], as previously mentioned. Some studies associated with phytosterols demonstrated that the anti-proliferative effects of those substances are associated with alterations in expression of growth signaling genes, including *Cav-1*.

Treatment with γ-oryzanol induced a decrease of Cav-1 gene and protein expression in both androgen unresponsive prostate cancer cell lines (DU145 and PC3). Yang et al. [17] evaluated the expression of Cav-1 in patients with prostate cancer and found that 25% expressd Cav-1 protein. Even more, these patients had a tendency to develop lymph node metastases, showing a clear relationship between these two factors. Yet, at least 70% of these patients died of cancer. This fact highlights the importance of *Cav-1* in the progression of prostate cancer, besides being correlated with recurrence of this cancer after radical prostatectomy [17,74]. Furthermore, previous studies showed that modest expression of *Cav-1* leads to increased cell viability, a fact that contributes to the progression to metastasis [77–78]. Thus, our data highlights the increase in Cav-1 gene and protein expression in androgen unresponsive cell lines, which is probably related to the increase of cell survival and that reduced expression of this gene and protein may contribute to the increase in cell death.

Unlike androgen unresponsive cell lines, we did not found expression of *Cav-1* gene in LNCaP cells, fact which corroborates the observation made for others authors that studied this gene expression in LNCaP cells [22,34]. This result can be explained by the fact that at early stages of the disease loss of *Cav-1* promotes proliferation and survival [75].

Furthermore, other genes such as *PCGEM1* are also involved in prostate cancer. *PCGEM1* is prostate-specific non-coding functional RNA gene [23]. The high expression of *PCGEM1* gene also has been correlated with cell growth in human prostate cancer, suggesting its potential as biomarker for high-risk prostate cancer [23,79]. Increased expression of *PCGEM1* gene is related to increased cell proliferation and colony formation, an indication that the altered expression of this gene contribute to the development and/or progression of prostate cancer in androgen independent cells [23]. Furthermore, the use of an siRNA *PCGEM1* by He et al. [80] inhibited cell growth and reduced cell migration and colony formation in LNCaP cells, confirming that the key role of this gene in prostate cancer associated to hyperproliferation and (or) metastasis [23]. Yet, *PCGEM1* gene is also overexpressed in castration resistance prostate cancer. Yang et al. [81] demonstrated that *PCGEM1* gene can be involved in activation of the androgen receptor in the absence of its specific ligand. In this regard, a proposed mechanism for *PCGEM1* action would be through interaction with the androgen receptor and enhancing of its expression. Ligand-dependent and ligand-independent androgen receptor activation could lead to prostate cancer cell through proliferation [81–82]. In this study, we observed decreased expression of the gene *PCGEM1* in DU145 and LNCaP cells, but no expression was detect in PC3 cells. Ifere et al. [18] also found that phytosterols treatment decrease the *PCGEM1* expression in DU145.

Fu et al. [24] studied the *PCGEM1* expression in LNCaP cell culture model and observed that the increased of expression of this gene inhibit the experimental induced apoptosis, contributing to the development of the carcinoma. Thus, the reduction of *PCGEM1* gene expression could be seen as an important feature observed in the development of anticancer drugs.

In addition to the differences found in this study between androgen unresponsive and responsive prostate cancer cell lines, we also observed differences between DU145 and PC3 cells. Both lines are not responsive to androgens and are metastatic [83–86]. However, they originate from different organs: PC3 cells are prostatic adenocarcinoma and they originate from a metastasis of bone [85]. On the other hand, DU145 cells are derived from a human prostatic carcinoma and originated from a brain metastasis [83]. Moreover, they have different metastatic potentials: PC3 cells have higher metastatic potential than DU145 cells (moderate potential) [87]. Furthermore, these cell lines exhibit phenotypic characteristics and behavior different in culture [83,85], explained by the different responses exhibited after γ-oryzanol treatment, as exemplified in other reports [18].

CONCLUSION

In summary, in this study we demonstrate that treatment with γ-oryzanol caused a decrease in *PCGEM1* (DU145 and LNCaP cells) and *Cav-1* gene expression (DU145 and PC3 cells), as well as a reduction in Cav-1 protein content in androgen unresponsive prostate cancer cell lines. We also showed that γ-oryzanol effectively reduced cell viability and culture biomass in all prostate cancer cell lines. In PC3 cells, this fact was probably caused by the increase of necrosis or apoptosis death by caspase activation, and in LNCaP and DU145 cells probably associated to ERK1/2 activation and necrosis death, by cycle arrest in G0/G1 or G2-phase for DU145 cells or PC3 and LNCaP cells, respectively. We also observed that the cell lines exhibited different behaviors towards γ-oryzanol treatment and this finding can probably be explined by distinct intrinsic characteristics present in the different prostate cancer cell lines studied. Thus, we conclude that γ-oryzanol could be seen as a promising phytosterol for the treatment of prostate cancer, even when the disease is already resistant to anti-androgen treatment.

ACKNOWLEDGMENTS

The authors acknowledge to Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) for research fellowships and to Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS-Edital Pesquisador Gaucho—n° 1952-2551/13) for financial support.

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II.2 γ-ORYZANOL ALTERS MicroRNA EXPRESSION PROFILES IN ANDROGEN RESPONSIVE AND UNRESPONSIVE PROSTATE CANCER CELLS

(Manuscrito a ser submetido para o periódico Molecular Cancer)

γ-ORYZANOL ALTERS MicroRNAs EXPRESSION PROFILE IN ANDROGEN RESPONSIVE AND UNRESPONSIVE PROSTATE CANCER CELLS

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ABSTRACT

MicroRNAs (miRNAs) are a large family of non-coding RNAs with ~22 nt in length and they exert regulatory effects by binding to the 3' UTR of mRNA target in humans, inhibiting the protein synthesis through translationally repressed target mRNAs. Many studies suggest that aberrant expression of miRNAs is involved in the development of many types of cancer, including prostate cancer. Some natural products have been shown to be effective in controlling the growth and metastasis in various cancers and γ-oryzanol seems to be promising in this regard. Here, we tested this phytosterol as a potential antitumor agent in androgen unresponsive (PC3 and DU145) and responsive (LNCaP) prostate cancer cell lines, and evaluate its ability to alter miRNAs involved cellular functions related with development and progression of prostate cancer using for this RT-qPCR analysis. Our results showed that many miRNAs were changed after treatment with 16 µM γ-oryzanol in three prostate cancer lines, suggesting its potential use in the treatment of this pathology.

Keywords: γ-oryzanol, microRNA, prostate cancer, metastasis, cancer therapy.

1. INTRODUCTION

MicroRNAs (miRNAs) are a large family of non-coding RNAs with ~22 nt in length (ranging 19-25 nt) (Ambros, Bartel et al. 2003), which regulate the translation and/or degradation of messenger RNAs target (Ambros 2004; Bartel and Chen 2004; Stefani and Slack 2008). They exert regulatory effects by binding to the 3'UTR of mRNA target, where they can repress mRNA translation or cause the degradation of mRNA (Bartel 2004; Filipowicz, Jaskiewicz et al. 2005; Sontheimer and Carthew 2005). Thus, there is a reduction in protein levels of target genes, but rarely transcriptional expression levels are affected (Wiemer 2007).

The regulatory mechanism of miRNA is very complex because they are very small sequence and may be active without the need for complete pairing (Wiemer 2007), so a single miRNA can bind and regulate multiple mRNA targets and one mRNA targets can be bind and regulated by several different miRNAs (Lewis, Shih et al. 2003). They act by two modes to regulate the gene expression, varying depending on the miR complementarity with the mRNA target: pairing with mRNA targets by precise or nearly precise complementary, causing direct cleavage and destruction of the target mRNA through a mechanism involving the RNA interference (RNAi) machinery - most common mechanism in plants (Rhoades, Reinhart et al. 2002; Tang, Reinhart et al. 2003); or pairing with mRNA targets by imprecisely complementary causing inhibition of protein synthesis through translationally repressed target mRNAs that remains associated with ribosomes - most common in animals (Olsen and Ambros 1999; Seggerson, Tang et al. 2002).

MiRNA are a huge and complex regulatory network of cell signaling and they are implicated in the regulation of many biological processes, including muscle, cardiac, neural, and lymphocyte development, or the regulation of both the innate and adaptative immune responses (Tili, Michaille et al. 2007; Tili, Michaille et al. 2008). They are also involved in pathological processes such as inflammation, auto-immune diseases and cancer (Tili, Michaille et al. 2007; Tili, Michaille et al. 2008; Sonkoly and Pivarcsi 2009; Tili, Croce et al. 2009; Iborra, Bernuzzi et al. 2012). Recent studies have demonstrated that miRNAs participate in the regulation of target genes involved in growth, differentiation and apoptosis in carcinoma cells (Kloosterman and Plasterk 2006; Iorio and Croce 2009; Baranwal and Alahari 2010). The expression of several of these target genes are altered in cancer and they are intimately involved in the development and progression of many types of human cancers, including prostate cancer, acting as oncogenes or tumor suppressors (Cimmino, Calin et al. 2005; Vrba, Jensen et al. 2010).

Prostate cancer (PCa) is the second leading cause of death among men in Brazil and the sixth most prevalent in the world ((INCA) 2014). Despite being a slow growing disease, tumor can spread rapidly through the body causing metastasis ((INCA) 2014). The main treatment in this step of disease is anti-androgen therapy (Stacewicz-Sapuntzakis and Bowen 2005; Madan and Arlen 2013), however, many patients become resistant to the therapy (Ganguly, Li et al. 2014) and has a relapse within 2 to 3 years after initiation of treatment (de Bono, Logothetis et al. 2011; Kirby, Hirst et al. 2011; Scher, Fizazi et al. 2012; West, Kiely et al. 2014). In this stage there are no successful treatment strategies currently available and the disease become incurable (Scott, Menon et al. 1980; Eisenberger, Blumenstein et al. 1998).

Recent studies have shown that γ-oryzanol, a phytosterol extracted from rice bran (Yu, Nehus et al. 2007; Tuncel and Yilmaz 2011; Huang and Ng 2012; Jeng, Shih et al. 2012), has anti-carcinogenic activity (Banjerdpongchai, Wudtiwai et al. 2013; Zeng, Yang et al. 2013; Summart and Chewonarin 2014), including in PCa (Hirsch, Parisi et al. 2015). Furthermore, it has been shown a great relationship between the development, invasion and metastasis of prostate cancer and the change in the expression of miRNAs (Shi, Xue et al. 2007; Lu, Liu et al. 2008; Musiyenko, Bitko et al. 2008). So, the aim of this study was to examine the effect of γ-oryzanol on the expression of different miRNAs involved in PCa using three different cell lines - androgen responsive and unresponsive.

2. MATERIALS AND METHODS

2.1 Cell culture and γ-oryzanol treatment

LNCaP androgen responsive prostate cancer cell line (clone CRL-1740, ATCC, Manassas, VA, USA), DU145 (clone HTB-81, ATCC,) and PC3 (clone CRL-1435, ATCC,) androgen unresponsive prostate cancer cell lines were kindly provided by Dr Mari C. Sogayar from São Paulo University (SP, Brazil). The cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 2g/L HEPES buffer, 1% penicillin, pH 7.4, under 37ºC and 5% CO_2 conditions and they were plated ($5x10^4/cm^2$) in 6-well plates and cultured for 24 h to reach 60-70% of confluence before treatment.

Experimental media was supplemented with 16 μM of γ-oryzanol and the sterol carrier (2-hydroxypropyl)-β-cyclodextrin (β-CD) in a nontoxic form at molar ratio of 1:300 for all concentrations for 24 h (LNCaP) or 48 h (DU145 and PC3). Each concentration treatment was done in sextuplicate and routinely cultured cells were used as controls.

2.2 RNA extraction, cDNA synthesis and Real-Time PCR

2.2.1 RNA extraction and Stem-loop RT primers

Total RNA was extracted using the TRIzol Reagent following manufacturer instructions. The oligonucleotides corresponding to the N-loop primer, universal primer and miRNAs specific primers (Table 1) were designed as described by Chen et al. (2005) (Chen and Lodish 2005) and synthesized by Sigma Aldrich (St. Louis, MO, USA).

2.2.2 cDNA synthesis

The universal cDNA of each sample was synthesized from total RNA using the specific primer miRNA-loop. For each reaction, 10 µL of total RNA, 3 µL of 10 µM specific miRNA-loop primer and 2µL of water were mixed and heated at 70° C for 5 minutes. The mixture was immediately placed and kept in an ice bath. Then, 3 µL 10X PCR buffer, 2 µL 5mM dNTPs, 1 µL 200U/µL MMLV-reverse transcriptase and 9 µL of water was added and mixed. The final volume of 30 µL was incubated for 30 minutes at 16° C, 30 minutes at 42° C and 5 minutes at 85° C. cDNAs were diluted 10x and kept as a stock solution. For PCR reactions, cDNAs were diluted 100x.

2.2.3 *Real-time qPCR*

The expression of miRNAs was quantified using RT-qPCR reactions. The reactions were performed in a final volume of 10 µL, 5 uL compound of 100x diluted cDNA, 0.2 µL of each forward (5 µM) and universal (5 µM) primers, 1 µL 1x PCR buffer, 0.6 µL 50mM $MgCl_2$, 0.2 µL 5mM dNTPs, 0.75 µL of water, 0.5 µL SybrGreen 100X and 0.05 µL 5U/µL Taq Platinum DNA polymerase (Invitrogen). The RT-qPCR conditions were 94°C for 5 minutes followed by 40 cycles at 95°C for 15 seconds, 60°C for 10 seconds, 72°C for 15 seconds. Then, the samples

were heated from 55° to 99°C with an increase of 0.1°C/second to acquire the denaturing curve of the amplified products. Relative quantifications of amplified products were made by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using the Applied Biosystem 7500 Real-time PCR with the SDS software. SYBR-green (Molecular Probes) was used to detect amplification and estimate C_t values and to determine specificity of amplicons by denaturing curves and melting temperatures (T_m). miR133a-3p and miR21 were used as internal reference genes to normalize the expression of the miRNAs, based on data obtained from geNorm softwere.

2.3 Statistical analysis

Data were reported as mean ± standard deviations (SD). Results were analyzed by t test ($p<0.05$). All analyses and graphical were performed using the statistical software GraphPad Prism 5 for Windows (GraphPad Software Inc., version 5.01).

3. RESULTS AND DISCUSSION

PCa is the most common cancer among men and one of the leading causes of cancer death, behind non-melanoma skin cancer (Jemal, Siegel et al. 2006; INCA 2015). PCa in early stage is curable, however as the disease progresses and generates more advanced tumors, treatment becomes difficult and generally only tumors before metastasis can be successfully treated (Loberg, Logothetis et al. 2005; Pienta and Bradley 2006).

MiRNAs, small non-coding RNA molecules (Ambros, Bartel et al. 2003), appear to control basic cell functions how proliferation and apoptosis (Bartel 2004; Care, Catalucci et al. 2007). Those functions generally are unregulated in tumorigenesis process (Evan and Vousden 2001; Shi, Xue et al. 2007). In addition, recent studies have shown that many miRNAs are deregulated in PCa

and they are closely related to the development, invasion and metastasis of this disease (Shi, Xue et al. 2007; Lu, Liu et al. 2008; Musiyenko, Bitko et al. 2008). Furthermore, it has increasingly arisen natural products that show efficacy in controlling the growth and metastasis of various cancers, such as curcumin, isoflavones, resveratrol, epigallactocatechin-3-gallate (EGCG) and γ-oryzanol (Khan, Afaq et al. 2008; Hirsch, Parisi et al. 2015). Hirsch et al. (Hirsch, Parisi et al. 2015) showed that γ-oryzanol was able to inhibit the proliferation, to reduce cell biomass and to induce apoptosis in androgen responsive and unresponsive PCa cell lines and this effects could be mediated by miRNAs regulation. So, we tested this hypothesis in this study.

In fact, treatment with 16 μM of γ-oryzanol was able to cause alteration in the expression of several miRNAs. The miR16-1 this often decreased in cancer and it is involved in several tumors characteristics, such as survival, proliferation and invasion (Cimmino, Calin et al. 2005; Esquela-Kerscher and Slack 2006; Bonci, Coppola et al. 2008). In advanced PCa, this miRNA expression is significantly reduced and previous studies suggest that the reduction of miR16-1 expression increased proliferation in untransformed PCa cells, but the reconstitution of miR16-1 expression induced grow arrest and apoptosis, probably associated to reduction of *BCL-2,* an anti-apoptotic gene (Bonci, Coppola et al. 2008). The treatment with γ-oryzanol was also able to increase expression of this miRNA in PC3 cells, how can see in Figure 1C. This result corroborates an earlier study that says γ-oryzanol caused increased of apoptosis and growth reduction in same cell line (Hirsch, Parisi et al. 2015).

A peculiar feature of miRNAs is the fact that many of its genes are aligned in the genome, forming *clusters* (Hayashita, Osada et al. 2005). In this case, a

group of genes form a single primary transcript which will yield many mature miRNAs after processing (Lee, Jeon et al. 2002). *MiR 17-92 cluster* encodes six different miRNAs (miR17, miR18a, miR19a, miR20a, miR19b and miR92), including miR19b (Mendell 2005). γ-oryzanol treatment significantly reduced the miRNA 19b-2 expression in androgen unresponsive PCa cell (DU145 and PC3), but no effect was observed in LNCaP androgen responsive PCa cell line (Figure 1).

Several evidences suggesting the importance of the *miR 17–92 cluster* in cell transformation and tumorigenesis and this *cluster* have been considered potentially oncogenic (Hayashita, Osada et al. 2005; Mendell 2005). It overexpression has been observed in a wide range of human solid tumors such as breast, colon, lung, pancreatic, stomach cancers and even prostate (Volinia, Calin et al. 2006). Studies suggesting that this *cluster* can regulate multiple cell processes and it can participate to malignant transformation because it causes a reduction in cell death and rapid cell proliferation, besides to increase angiogenesis (He, Thomson et al. 2005; O'Donnell, Wentzel et al. 2005; Dews, Homayouni et al. 2006; Ventura, Young et al. 2008). The *cluster miR17-92* is also involved in the modulation of transforming growth factor-β (TGF-β) and its activation can results in the development of many human tumors, interfering with cell cycle arrest and apoptosis when overexpressed in malignant cells (Petrocca, Visone et al. 2008).

Quin et al. (Qin, Shi et al. 2010) showed that reduced miR24 expression decreases cell proliferation and increases apoptosis in PCa cells caused by caspase-8 activation, an important feature to be considered in cancer treatment. In this study, we observed that γ-oryzanol treatment was able to reduce the

expression of both miR24b-1 in PC3 cells and in miR-24b-2 expression in LNCaP cells (Figure 1C and A, respectively). A previous study by our group also showed an increased in caspases activation and in apoptosis in PC3 cells, but no change was observed in LNCaP cells, confirming the role of this miRNA in apoptosis and highlighting once again the potential of phytosterol in advanced PCa treatment (Hirsch, Parisi et al. 2015). However, more studies are necessary to elucidate the relationship of this miRNA with the results observed previously.

Some miRNAs have been linked to progression of PCa, among them we can highlight the miR99a and miR222. MiR99 family (including miR99a) was decreased in human PCa tissue, more aggressive and metastatic prostate tumors when compared with normal prostate, emphasizing their importance in the development and progression of PCa (Porkka, Pfeiffer et al. 2007; Sun, Lee et al. 2011). Moreover, a study by Sun et al. (Sun, Lee et al. 2011) showed that miR99a had reduced expression as the PCa progresses and that the increased expression of this miRNA in PCa cells caused a growth arrest associated with the regulation of the androgen receptor, showed by prostate-specific antigen (PSA) decrease (Sun, Lee et al. 2011). However, a recent study conducted by Sun et al. (2014) showed that introduction of the miR 99a/let7c/125b-2 *cluster* was not able to significantly inhibit the growth of LNCaP cells in the absence of androgen, suggesting that this control in cell proliferation is specifically associated with the presence of androgens. The phytosterol γ-oryzanol reduced miR99a expression in androgen responsive (LNCaP) and unresponsive (DU145) PCa cells (Figure 1A and B, respectively), however, a previous study conducted by our group using the same dose of γ-oryzanol was able to reduce cell proliferation in both DU145 and LNCaP cell lines (Hirsch, Parisi et al. 2015), suggesting the control of proliferation

by miR99a is complex and dependent of other factors, such as presence of androgens quoted earlier by Sun et al. (2014).

In addition miR99a, increased expression of miR222 is also related to the progression of hormone-refractory PCa phase. Comparison of an aggressive PC3 PCa cell line to LNCaP PCa cell line showed that miR222 is more expressed in PC3 cells and that it upregulation promoted cell proliferation through targeting a cell-cycle regulator p27 (Galardi, Mercatelli et al. 2007). Galardi et al. showed that the overexpression of this miR causing increased of cell cycle progress from G1 to S phase, probably related to block the translation of $p27^{Kip1}$ mRNA by miR222, causing a decrease in it protein level (Galardi, Mercatelli et al. 2007). Here, we did not observe any significant differences in the level of expression in PC3 and LNCaP cell lines ($p=0.2613$), however the γ-oryzanol treatment reduced miR222 expression in both cell lines. This fact may be related to cell cycle arrest observed in these same lines in a previous study conducted by our group (Hirsch, Parisi et al. 2015), but the arrest occurred in G2/M phase and more studies are needed to verify the relationship of this result with the change in miR222 expression after treatment with 16 μM of γ-oryzanol.

Downregulation of miR133a leads to the development of various cancers and it overexpression is associated to reduction of cell proliferation, migration and invasion in bladder and esophageal carcionomas, suggesting that it could act as a tumor suppressor (Chiyomaru, Enokida et al. 2010; Kano, Seki et al. 2010). Caveolin-1 (Cav-1) is also associated with the development of various types of cancer, besides has been identified as a marker of aggressiveness in prostate carcinoma (Yang, Truong et al. 1999). Cav-1 has predicted binding sites for miR133a (Nohata, Hanazawa et al. 2011) and γ-oryzanol treatment caused a

reduction of miR133a expression in LNCaP androgen responsive PCa cells (Figure 1A). However, a previous study conducted by our group examined Cav-1 expression in LNCaP cells and any expression was detected (Hirsch, Parisi et al. 2015), a fact that corroborates previous studies (Thompson, Timme et al. 1999; Freeman, Yang et al. 2012). This fact reinforces the previous suggestion that miR133a could be targeting Cav-1 mRNA (Nohata, Hanazawa et al. 2011), which could be involved in the lack of Cav-1 expression in LNCaP cells. In addition to prior results conducting by our group, this found suggests that the reduction of miR133a expression by γ-oryzanol treatment probably was not able to significantly increase the expression of Cav-1 in LNCaP cells, to the point of its being detected. However, more research needs to be conducted to confirm this hypothesis.

Another miRNA involved in prostate cancer is the miR182. Some authors have shown that this miRNA is upregulated in PCa compared to normal tissues (Ambs, Prueitt et al. 2008; Schaefer, Jung et al. 2010). In addition, a relationship between the expression profile of miR182-5p and Gleason score (GP), a histological method widely used to provide prognostic PCa (Gleason 1988), has been demonstrated (Tsuchiyama, Ito et al. 2013). MiR182-5p has been shown as useful marker for high grade PCa (Tsuchiyama, Ito et al. 2013). In addition, the overexpression of miR182 is also involved in the control of zinc homeostasis in PCa, and it acts decreasing labile zinc pools and reduced zinc uptake, demonstrating this miR could act a regulator of zinc homeostasis (Mihelich, Khramtsova et al. 2011). Zinc is an important nutrient that acts as a protector in the development of PCa and it concentration is lower in PCa compared to normal tissue (Mihelich, Khramtsova et al. 2011). Thus, the overexpression of miR182 in this pathology suggests a linkage to malignancy (Schaefer, Jung et al. 2010;

Mihelich, Khramtsova et al. 2011). However, others studies show that miR182 suppresses cell proliferation in other types of human cancers, as gastric and lung adenocarcinoma cells (Sun, Fang et al. 2010; Zhang, Liu et al. 2011; Kong, Bai et al. 2012) and reduce *in vitro* invasion of PCa cells (Rasheed, Teo et al. 2013), suggesting that this miR also could act as a tumor suppressor (Peng, Li et al. 2013). Peng et al. (Peng, Li et al. 2013) also showed anti-proliferative effects in PC3 PCa cells and they suggested the miR182 as a possible mediator of this effect. In this work we observed a decrease in this miRNA expression, but in other PCa lines (LNCaP and DU145) (Figure 1 A and B).

Overexpression of miR198 was also correlated with high grade tumors (GP≥8) in PCa (Walter, Valera et al. 2013). Ye et al. (Ye, Li et al. 2013) studied miR198 and they showed that it probably targets Livin mRNA, a protein that bind to certain caspases to suppress apoptosis and it is upregulated in a variety of cancers (Kim, Alvarado et al. 2005; Ye, Li et al. 2013). Furthermore, it was shown an inverse correlation between miR198 and Livin. But γ-oryzanol treatment reduced the expression of this miRNA in PC3 androgen unresponsive PCa cells, how can see in Figure 1C. However, a previous study conducted by our group found increase in apoptosis in PC3 cells when they were treated with γ-oryzanol (Hirsch, Parisi et al. 2015). Furthermore, other studies show that this miRNA is able to inhibit migration and invasion of hepatocellular carcinoma cells and the overexpression of this miR was found only in hormone-refractory PCa (Porkka, Pfeiffer et al. 2007; Tan, Li et al. 2011), suggesting that the reduction in this miRNA expression in androgen independent PCa cells may be beneficial. Yet, some researchers have reported that Livin can act as both anti-apoptotic and pro-apoptotic factor (Nachmias, Lazar et al. 2007; Abd-Elrahman, Hershko et al.

2009) and that high Livin expression may be a good prognostic to patient survival (Choi, Hwang et al. 2007; Gordon, Mani et al. 2007). Ye et al. (Ye, Li et al. 2013) also suggests that miR198 can influence other mRNA targets beyond Livin, but more studies are needed to elucidate the role of miR198 in PCa.

In conclusion, in this work we showed that miRNA profiling may be useful in characterizing molecular signatures of PCa. Furthermore, these findings have therapeutic implications and may be exploited for future development of drugs more effective to treatment of PCa, especially in hormone-refractory phase, where there are few effective treatments currently and γ-oryzanol seems to be promising in this regard.

Acknowledgements

G. E. Hirsch is the recipient of a fellowship from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazil). This work was also supported by FAPERGS (Edital Pesquisador Gaucho – nº 1952-2551/13) (PqG n°1009169).

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FIGURES

Table 1 MiR primers sequences

Gene	Primer sequences
MiR16-1	Forward - 5' CCA GTA TTA ACT GTG CTG CTG A 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACC ACT CAG CA 3'
MiR16-2	Forward - 5' CCA ATA TTA CTG GTC CTC TTT A 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACC ACT AAA GC 3'
MiR19a	Forward - 5' TGT GCA AAT CTA TGC AAA ACT GA 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAG TT 3'
MiR19b-1	Forward - 5' AGT TTT GCA GGT TTG CAT CCA GC 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG CTG GA 3'
MiR19b-2	Forward - 5' AGT TTT GCA GGT TTG CAT TTC A 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT GAA AT 3'
MiR21	Forward - 5' TAG CTT ATC AGA CTG ATG TTG A 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAA CA 3'
MiR23a	Forward - 5' ATC ACA TTG CCA GGG ATT TCC 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GAA AT 3'
MiR23b	Forward - 5' ATC ACA TTG CCA GGG ATT ACC 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GTA AT 3'
MiR24-1	Forward - 5' TGC CTA CTG AGC TGA TAT CAG T 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CTG AT 3'
MiR24-2	Forward - 5' TGC CTA CTG AGC TGA AAC ACA G 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TGT GT 3'
MiR99a	Forward - 5' AAC CCG TAG ATC CGA TCT TGT G 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ACA AG 3'
MiR125b-1	Forward - 5' ACG GGT TAG GCT CTT GGG AGC T 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GCT CC 3'
MiR125b-2	Forward - 5' TCA CAA GTC AGG CTC TTG GGA C 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG TCC CA 3'
MiR133a-5p	Forward - 5' AGC TGG TAA AAT GGA ACC AAA T 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA TTT GG 3'
MiR133a-3p	Forward - 5' TTT GGT CCC CTT CAA CCA GCT G 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC AGC TG 3'
MiR182-5p	Forward - 5' TTT GGC AAT GGT AGA ACT CAC ACT 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GTG TG 3'
MiR182-3p	Forward - 5' TGG TTC TAG ACT TGC CAA CTA 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT AGT TG 3'
MiR198	Forward - 5' GGT CCA GAG GGG AGA TAG GTT C 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG AAC CT 3'
MiR205-5p	Forward - 5' TCC TTC ATT CCA CCG GAG TCT G 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC AGA CT 3'
MiR205-3p	Forward - 5' GAT TTC AGT GGA GTG AAG TTC 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG AAC TT 3'
MiR222	Forward - 5' AGC TAC ATC TGG CTA CTG GGT 3' Loop Primer - 5' GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CCC AG 3'

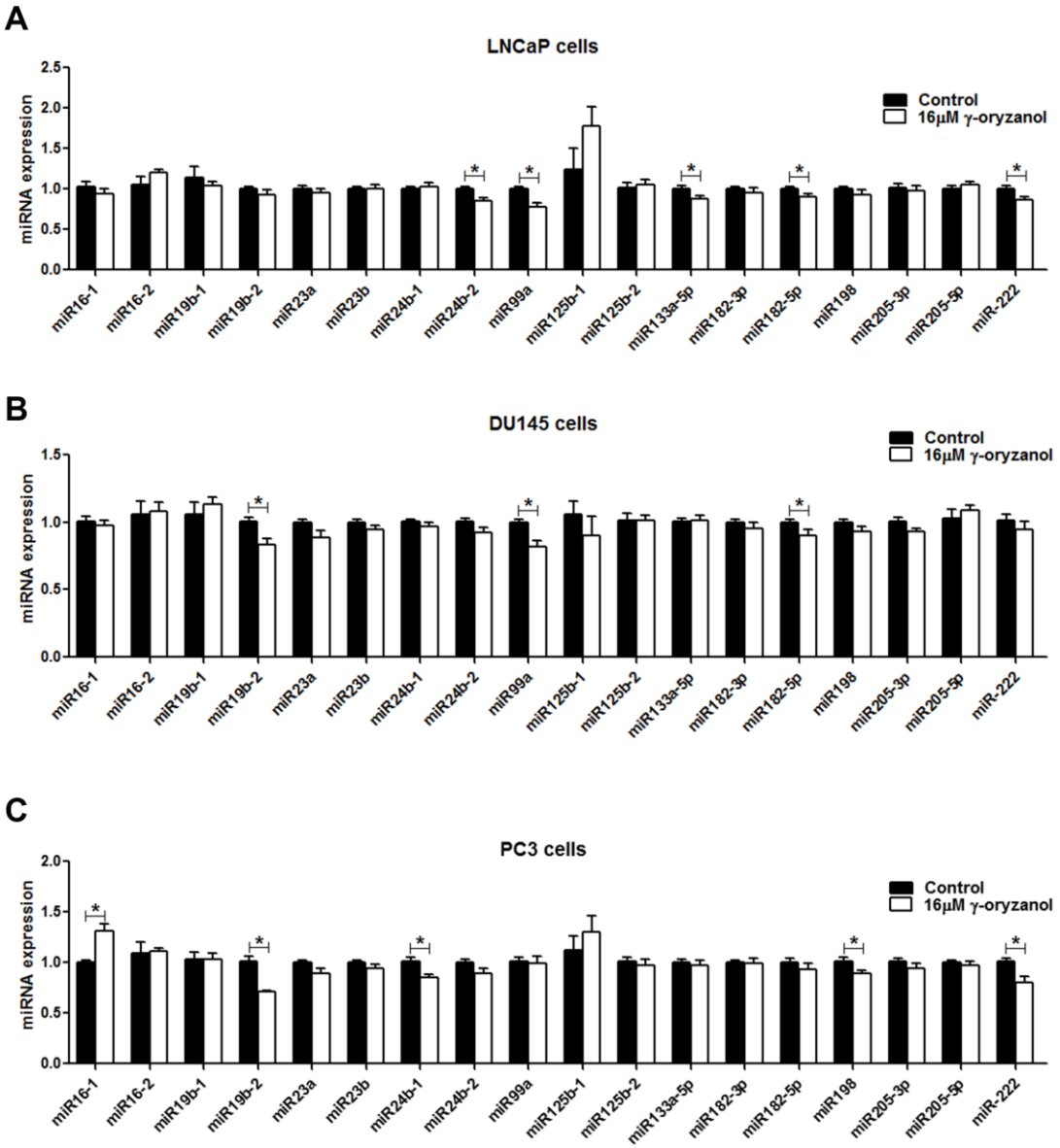

Figure 1 The effect of 16 μM γ-oryzanol on miRNAs expression in (A) LNCaP androgen responsive PCa cells and (B) DU145 and (C) PC3 androgen unresponsive PCa cells. Similar symbols indicate which groups are statistically different ($p \leq 0.05$). Data is expressed as mean ± SD ($n = 6$).

II.3 *Thuya occidentalis* extract inhibits the expression of Caveolin-1, a marker of prostate cancer aggressiveness

(Manuscrito a ser submetido ao periódico Food and Chemical Toxicology)

***THUYA OCCIDENTALIS* EXTRACT INHIBITS THE EXPRESSION OF CAVEOLIN-1, A MARKER OF PROSTATE CANCER AGGRESSIVENESS**

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ABSTRACT

Prostate cancer is the most common cancer among men and the second leading cause of death. Multimodality treatment accomplished by surgical resection and androgen deprivation are the treatments of choice. Still, prostate cancer recurrence is highly prevalent, underlying unexplored molecular mechanisms. Caveolin-1 is a protein component of the caveolae plasma membranes, and it has been identified as a marker of aggressiveness in prostate carcinomas promoting progression from androgen responsive to androgen unresponsive in the metastatic phenotype. The reduction of the caveolin-1 expression has been suggested to be an important therapeutic target in prostate cancer by being able to transform prostate cancer cells susceptible to anti-androgen therapy again. Here we used a *Thuya occidentalis* (*T. occidentalis*) extract as a potential antitumor agent in androgen responsive (LNCaP) and unresponsive (PC3 and DU145) prostate cancer cell lines. Large variations in drug responses were observed between the androgen responsive and unresponsive cell lineages. *T. occidentalis* showed highest cytotoxicity on androgen responsive LNCaP cells; in these cells 1h of treatment with *T. occidentalis* results in effects similar to these found after 24 h in androgen unresponsive cells. *T. occidentalis* treatment decreased cell viability and biomass by apoptosis and/or necrosis cell death in DU145, PC3 and LNCaP cells. In DU145 was also observed cell cycle arrest in G0/G1 phase. The expression of the caveolin-1 protein was down regulated in both androgen unresponsive cells. Anti-proliferative and cytotoxic effects indicate that *T. occidentalis* has potential as adjuvant therapy for prostate cancer. We understand that the most interesting and important result of this study

seem to be the inhibitory effects on the Caveolin-1expression in androgen unresponsive cells which can be related to reducing the metastatic potential.

Keywords

Androgen resistance; caveolin-1; cell cycle; prostate cancer; *Thuya occidentalis* extract; viability.

1. INTRODUCTION

Prostate cancer (PCa) ranks second among the leading causes of cancer deaths in men, surpassed only by lung (Siegel, Naishadham et al. 2013).

In Brazil, prostate cancer is the most frequent cancer in men in all regions of the country, excluding non-melanoma skin cancers (INCA). The National Cancer Institute (INCA) has recorded 12,778 deaths from prostate cancer in 2010, and estimated over 68,800 new cases of the disease for 2014 (INCA 2015).

PCa occurs mainly in men aged over 65 years old and, in general, is a slow-growing tumor, taking about 15 years to reach one cubic centimeter . However, some of these tumors can grow quickly, spreading through the body and leading to death (Siegel, Naishadham et al. 2013).

Androgens are required for the normal growth and development of prostate, but they may also play a role in prostate carcinogenesis by acting either as initiators or promoters of cancer (Craft, Chhor et al. 1999). Radical surgery or radiotherapy are indicated for patients with localized cancer (1984; Gittes 1991), but this option often not possible, and androgen deprivation remains the cornerstone therapy for patients with advanced pathologic stages of the disease (INCA 2013). The main treatment for prostate cancer consists of androgen deprivation therapy, however, it can cause various metabolic changes as changes in lipid profile, increased risk of the development of insulin resistance, diabetes, metabolic syndrome and cardiovascular changes (Choi and Kam 2015). In addition to the inconvenience caused by androgen deprivation therapy, this therapies are initially very effective because cause cell growth arrest and cell death induction in androgen-dependent tumor cells by apoptosis (Berges, Furuya et al. 1993), however, PCa recurrence is highly prevalent generally occurring

within 1 to 3 years of treatment, in an independent androgen phase. Worsening the prognosis, at advanced pathological stages PCa tends to be highly resistant to conventional cytotoxic agents such as cisplatin (Mukhopadhyay, Bueso-Ramos et al. 2001), emphasizing the search for new strategies for the treatment of PCa.

In the normal hormone-sensitive tissue, the androgen receptor is largely localized in the nucleus, but in the absence of androgen, the androgen receptor is more present in the cytoplasm. This receptor can form complexes with many proteins, including caveolin-1 (cav-1) (Freeman, Yang et al. 2012). Cav-1 is a vital component of the caveolae (El-Yazbi, Cho et al. 2006) and it has been identified as a marker of aggressiveness in prostate carcinomas (Yang, Truong et al. 1999) promoting progression into the metastatic phenotype. The association of cav-1 and the androgen receptor is largely androgen-dependent. The enforced expression of cav-1 was found to potentiate androgen-dependent signaling, while the down-regulation of cav-1 inhibited androgen signaling. Thus, it is believed that cav-1 can hypersensitize androgen receptor and circumvents hormonal suppression (Lu, Schneider et al. 2001).

Phytotherapy appears as an important alternative for the treatment of various disorders in many cultures and there is evidence demonstrating that bioactive compounds present in many plants have important contributions in this aspect. *Thuya occidentalis (T. occidentalis)* is a plant commonly known as Arbor vitae or white cedar (Chang, Song et al. 2000). It was first identified in Canada (Millspaugh 1974) and it is used in folk medicine to treat bronchial catarrh, enuresis, cystitis, psoriasis, uterine carcinomas, amenorrhea and rheumatism (Shimada 1956; Baran 1991; Offergeld, Reinecker et al. 1992). Today, it is mainly used in homeopathy as mother tincture or dilution ((HAB) 1985; (HAB) 2003). *T.*

occidentalis is rich in bioactive compounds such as flavonoids and coumarins (Chang, Song et al. 2000) , however, few studies showing the effect of this plant are found in the literature.

In general, prostate cancer is made up of a heterogeneous population of androgen responsive and androgen unresponsive cells (Kenji 1985). Furthermore, 10-20% of patients using anti-androgen therapy (main treatment used in metastatic phase) become tolerant to hormone restriction and in this stage there was no curative therapy available (de Bono, Logothetis et al. 2011; Kirby, Hirst et al. 2011; Scher, Fizazi et al. 2012; West, Kiely et al. 2014). Therefore, in this work we use cell lines that represent both cell populations found in different clinical stages of diagnosis to evaluate the efficacy of *T. occidentalis* treatment. LNCaP is an androgen sensitive metastatic cellular model (Horoszewicz, Leong et al. 1983) while DU145 and PC3 are androgen insensitive metastatic cellular models (Stone, Mickey et al. 1978; Kaighn, Narayan et al. 1979). Thus, in this study, we evaluate the anti proliferative and cytotoxic effects of *T. occidentalis* extract on prostate cancer cell lines, and also investigated the treatment effect on the expression of cavelin-1 for its relationship with the metastatic potential of prostate cancer.

2. MATERIAL AND METHODS

2.1 Chemicals and Reagents

T. occidentalis extract was obtained as a tincture in local market, a volume of 1.5 mL was aliquoted and dried in a vacuum centrifuge concentrator until complete evaporation of the ethanol. The dried samples were stored at -20º C. Immediately before use, *T. occidentalis* dried extract was diluted again in 1.5 mL of culture medium and the final concentration of *T. occidentalis* hidroalcolic extract was 0.05 mL of extract/mL of RPMI-1640 medium supplemented with 10% FBS.

α-thujone, 3-(4,5-dimethyl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), sulforhodamine B (SRB), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), paraformaldehyde, triton X-100 and HEPES buffer were obtained from Sigma Aldrich (St. Louis, MO, USA).

RPMI-1640 medium, fetal bovine serum (FBS), propidium iodide (PI), Annexin-V FITC Conjugate Kit, TRIzol Reagent, RNAse A, SuperScript®-III RT First-Strand Synthesis SuperMix, anti-mouse secondary antibody conjugated with Alexa Fluor 555, were purchased from Invitrogen (Grand Island, NY, USA). Mouse anti-human cav-1 monoclonal antibody (clone 7C8) was purchased from Santa Cruz Biotechnology (CA, USA).

2.2 Cell culture and treatments

Prostate cancer cell lines androgen-responsive LNCaP (clone CRL-1740, ATCC, Manassas, VA) and androgen-unresponsive DU145 (clone HTB-81, ATCC) and PC3 (clone CRL-1435, ATCC) were cultured in RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 2g/L HEPES buffer, pH 7.4, under 37ºC and 5% CO_2 conditions. Cells were seeded at 5 x 10^4 cells/cm^2 in 12 or 24-well plates and cultured for 24h to reach 60-70% of confluence before treatment.

T. occidentalis extract (0.05 mL/mL) or α-thujone (0.0016 µg/mL) were diluted in culture medium just before use for 24 hours (androgen unresponsive cells) or 1 hour (androgen responsive cells). Treatments were done in triplicate. For comparison, cells grown in the absence of extract were used as normal controls.

2.3 Colorimetric MTT assay

Cells were incubated with 1 mg/mL MTT for 2 hours at 37ºC. Formazan purple crystals were dissolved DMSO (Sigma) and absorbance was measured using a spectrophotometric microtiter plate reader (Spectra Max M5, Molecular Devices) at 570 nm and 630 nm.

2.4 Sulforhodamine B assay

The assay was performed as previously described for Skeahan et al. (1990) (Skehan, Storeng et al. 1990) with minor modifications. After treatment with *T. occidentalis*, the culture medium was removed; the cells were washed three times with PBS and incubated with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes. Fixed cells were stained with SRB (0.4%, 45 minutes) and subsequently, washed with tipe I water (milli-Q®) to remove unbounded SRB. The culture plates were air dried and protein-bound SRB was solubilized in 1% SDS. Absorbance was measured with a 96 wells plate reader at 560 nm.

2.5 Flow cytometry assays

Cell death analysis was performed by FITC Annexin V and propidium iodide (PI) Kit (Invitrogen, Carlsbad, CA, USA). The androgen unresponsive and responsive cell lines were washed with PBS and resuspended at $1x10^6$ cells/mL in Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 10^5 cells/mL were incubated with 5 µL of Annexin-V FITC and 1 µg/mL of propidium iodide (PI), mixed and incubated for 15 minutes at room temperature in the dark. Four hundred microliters of binding buffer were added to each tube and cells were acquired in the FL1-H and FL3-H channels.

Cell cycle entry and progression characterized by changes in DNA content were analyzed by flow cytometry. Androgen unresponsive cells DU145 and PC3 ($1x10^6$ cells/mL) were washed in PBS, resuspended in cell cycle solution (3.5 mM

trisodium citrate, 0.5 mM tris, 0.1% Nonidet, 100 µg/mL RNAse A, 50 µg/mL propidium iodide) and incubated in the dark at room temperature for 15 minutes.

Data acquisition and analysis was performed using a FACSCalibur™ flow cytometer (BD Bioscience, San Jose, CA, USA). Data was analyzed using FCS Express 4 Flow Cytometry software (De Novo Softwares, USA).

2.6 RNA extraction, cDNA synthesis and Real-time PCR

To determine the effects of *T. occidentalis* treatment on the expression of cav-1 of prostate cancer lines, total RNA was isolated using TRIzol® (Invitrogen, Carlsbad, CA, USA). RNA was quantified using the BioPhotometer Plus (Eppendorf, Hamburg, Germany). cDNA synthesis was done using the SuperScript®-III RT First-Strand Synthesis SuperMix Specific primers were designed for Cav-1 (NM_001753.4) and 36B4 using the IDT Design Software (Integrated DNA technologies Inc., USA). Cav-1 sense 5´-ACCCTAAACACCTCAACGATG- 3´ and anti-sense 3´-CAGACAGCAAGCGGTAAAAC- 5´ (amplicon product 108 bp) or 36B4 (acidic ribosomal phosphoprotein P0, RPLP0), which encodes for an acidic phosphoprotein, as the endogenous control previously described, sense 5´-CAGCAAGTGGGAAGGTGTAATCC-3´ and anti-sense 3´-CCCATTCTATCATCAACGGGTACAA -5´ (amplicon product 75 bp) (Cawthon 2002) for normalization. All PCR reactions were performed in triplicate and carried out in a StepOnePlus™ real-time PCR thermal cycler (Applied-Biosystem, New York, NY, USA). The thermal cycling profile for cav-1 and 36B4 genes began with an initial denaturation step at 94°C for 10 min to activate the enzyme (Invitrogen) followed by 40 cycles of 10 seconds at 94°C, 15 seconds at 60°C, 15 seconds at

72°C and 35 seconds at 60°C for data acquisition followed by a melting dissociation curve.

All samples were normalized by the constitutive gene (36B4) and calibrated by the average of the ΔCT of the group itself. The specificity of amplification and absence of primer-dimers were confirmed using melting curve analysis at the end of each run and then, all results were analyzed by the ΔΔCT method (Livak and Schmittgen 2001).

2.7 Immunocytochemistry

Immunocytochemistry was performed as described previously (Gomes, Paulin et al. 1999). Briefly, treated cells (DU145 and PC3) were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. After blocking for 2 hours with BSA (Sigma) 5% in PBS, cells were washed and incubated overnight with mouse anti-human cav-1 monoclonal antibody (1:250, clone 7C8 Santa Cruz Biotechnology, CA, USA) at room temperature, followed by PBS washes and incubated with specific secondary antibody anti-mouse conjugated with Alexa Fluor 555 (1:1.000) (Invitrogen) for 2 hours. Negative controls reactions were performed by omitting the primary antibody. No reactivity was observed when the primary antibody was excluded. Confocal images were acquired using an Olympus FV1000 laser-scanning confocal microscopy. Ten single confocal sections of 0.7 µm were taken parallel to the coverslip (*xy* sections) with an x 60 (numeric aperture 1.35) oil-immersion objective (Olympus, U plan-super-apochromat, UPLSAPO60XO). For each sample, images of ten fields were acquired and processed with Olympus FluoView FV1000 software. Cav-1 fluorescence was measured after being excited by a 559 nm laser beam and emission scan collected at 565 nm. Analysis of

fluorescence intensity was performed using the Image J software, a public domain Java Image processing program (http://rsb.info.nih.gov/ij/).

2.8 Analysis of α-thujone by Gas Chromatography Mass Spectrometry (GC-MS)

For the determination of α-thujone in 0.05 mL of *T. occidentalis* extract /mL of culture medium of a chromatographic analysis was carried out. The volatile compounds of *T. occidentalis* extract were extracted by microextraction technique in solid phase in the headspace of the sample (SPME-HS). 100 µL of the extract was added to 900 µL of culture medium and 300 mg of NaCl in 4 mL vial which was performed using the fiber Car/PDMS (Supelco, 10 mm) at a 50° C under stirring with a 5 min for equilibration, and after 30 min of extraction. The SPME fiber was desorbed in the injector (230° C for 1 min Splitless mode) of a gas chromatograph coupled to a mass spectrometer (GC/MS, Shimadzu QP2010 Plus). The separation of the volatiles compounds was performed in a fused silica capillary column ZBWax Plus (60 m/0.25 mm, 0.25 µm) using helium as carrier gas. The temperature ramp initiated at 35° C for 5 min, and after a ramp is triggered with a gradient of 2° C/min until 80° C and 4° C/min until 210° C, keeping in isotherm for 5 min. The interface between the gas chromatography, mass spectrum detector and the ionization source was maintained at 230° C. The quadrupole mass analyzer was operated in scan mode monitoring the masses between 35 to 350 u.m.a. The identification of the compounds is carried out by comparing the retention times of the analyte and the mass spectra with authentic standards. Quantitation was performed using calibration curve with authentic standard (α-thujone).

2.9 Statistical analysis

Data were reported as mean ± standard deviation (SD). Results were analyzed by t-test ($p<0.05$). All analyses and graphical were performed using the statistical software GraphPad Prism 5 for Windows (GraphPad Software Inc., version 5.01).

3. RESULTS

3.1 *T. occidentalis* concentration and dose response

In order to select the dose of *T. occidentalis* used in this study, a dose-response proliferation curve was performed using different concentrations of *T. occidentalis* extract which ranged 0.025 mL/mL to 0.1 mL/mL (mL of extract/mL of culture medium) (Fig 1A) in DU145 cells. The effective concentration (EC_{50}) values were evaluated by sulforhodamine B assay. From the calculation of the EC_{50} of this curve (EC_{50}=0.04387), the dose 0.05 mL/mL of *T. occidentalis* extract was chosen and used to perform all other tests for this lines. Yet, after the establishment of treatment dose, the time treatment was assessed using the SRB test. It was not possible to observe a significant reduction in cell biomass on DU145 cells treated with 0.05 ml/ml of *T. occidentalis* extract after 24 hours of treatment (Fig. 1B). So, both androgen unresponsive cell lines (DU145 and PC3) were exposed to 24 hours of treatment.

However, the LNCaP androgen responsive cell line showed greater sensibility to treatment then androgen unresponsive cell lines and then, to evaluating the effects of *T. occidentalis* extract in this cell line, a time curve was performed using the same dose of other lines (0.05 mL/mL). It was tested four different times: 1, 2, 3 and 4 hours (Fig 1C). We observed a reduction relative to control of approximately 50% (1.2293 to 0.6069) of the absorbance measured by

SRB test with 1 hour of treatment. Therefore, the LNCaP cell line was treated for 1 hour with 0.05 mL/ml of *T. occidentalis* extract in all tests in this work.

3.2 Differential effect of *T. occidentalis* extract in prostate cancer cell lines LNCaP, PC3 and DU145

3.2.1 Effects of *T. occidentalis* extract in androgen responsive prostate cancer cell line LNCaP

The results show that the cell viability measured by MTT assay was significantly decreased in the LNCaP cells treated with 0.05 mL/mL of *T. occidentalis* extract (Fig. 2A). Furthermore, it was also observed a reduction in the culture biomass measured by SRB assay (Fig. 2B).

The *T. occidentalis* extract was highly cytotoxic when incubated with LNCaP and it was able to reduce the cell metabolism and protein content (Fig. 2A and B), as mentioned earlier. To verify if this cytotoxic effect was related to an apoptotic mechanism, LNCaP cells were also assessed by Annexin V and PI staining, using FACS analysis (Fig. 2C and D). No differences in the number of cells undergoing apoptosis and late apoptosis were observed in LNCaP after 1 hour of treatment with *T. occidentalis* extract. However, we could observer and significantly increase in the percentage of necrotic cells (Fig. 2C). This is consistent with the observation of the cytotoxic effect of *T. occidentalis* observed in the MTT and SRB assays and suggests a higher susceptibility nature of LNCaP, when compared to the androgen unresponsive cell lines used in this work DU145 and PC3.

3.2.2 *T. occidentalis* extract alters cell growth and interferes with cell viability in androgen unresponsive prostate cancer cell lines (DU145 and PC3).

T. occidentalis extract effect on cell viability of androgen unresponsive cell lines PC3 and DU145 was assessed by the MTT assay. The results show that cell viability was significantly diminished on DU145 and PC3 cells treated with 0.05 mL/mL of *T. occidentalis* extract (Fig. 3A and B).

Similar results were obtained when cell biomass was assessed by absorbance of Sulforhodamine B. The cytotoxic effect of *T. occidentalis* was confirmed in both androgen unresponsive cell lines by the significant reduction in cell biomass, observed in Fig. 3C and D.

To confirm that *T. occidentalis* extract mediated cell death in an apoptotic dependent way, treated and control cells were examined by Annexin V and PI staining, using FACS analysis. Cells single positive for Annexin V or PI, were considered apoptotic or necrotic, respectively. Cells double positive for Annexin V and PI were considered in late apoptosis. We observed a significant increase in necrosis and apoptosis when cells were treated with *T. occidentalis* extract in DU145 and PC3 cells. Yet, there was no significant difference in the number of cells in late apoptosis in both cell lines (Fig. 3E and F). These results indicate that death observed in androgen unresponsive cell lines treated with *T. occidentalis* extract may have been caused by necrosis and apoptosis.

3.2.3 *T. occidentalis* extract modifies the cell cycle in androgen unresponsive prostate cancer cells

Cell cycle progression in prostate cancer cells treated with *T. occidentalis* extract was analyzed by flow cytometry, suggesting that the effects of *T. occidentalis* extract on cell proliferation could be due to its actions on cell cycle (Fig. 4). We observed an increase in the number of DU145 cells in G0G1-phase and reduction in the G2-M phase (Fig. 4A) when they were treated with 0.05

mL/mL of extract. However, we did not observe differences in the cell cycle of PC3 cells for the dose tested of *T. occidentalis* extract (Fig. 4B).

3.2.4 *T. occidentalis* extract diminishes expression of cav-1 protein in androgen unresponsive prostate cancer cells

We wanted to evaluate whether *T. occidentalis* extract would interfere with the expression of the cav-1 protein. However, as described by others (Thompson et al., 1999), it was not possible to detect expression of cav-1 gene in androgen-responsive cell line LNCaP. As it was not observed gene expression of cav-1 in LNCaP cell line, only the androgen unresponsive prostate cancer cell lines were analyzed for immunocytochemistry.

In this regard, both cell lines tested, DU145 and PC3, showed a decrease in cav-1 expression (Fig. 5E), when cells were treated with the *T. occidentalis* extract, indicating that indeed there was also a decrease in the amount of protein cav-1 compared to control (Fig. 5). The decreased Cav-1 expression was confirmed at the RNA level (Fig. 5F).

3.3 α-thujone reduces cell biomass in LNCaP cells

Some authors have suggested that the α-thujone could be the main active compound present in *T. occidentalis* (Biswas, Mandal et al. 2011; Siveen and Kuttan 2011). Thus, the amount of this monoterpene in *T. occidentalis* extract was quantified using a GC-MS analysis. It was found 0.0016 µg of α-thujone in each 1 mL of treatment medium. So, we wonder if this compound could have the capacity to reduce the cell biomass in prostate cancer cell lines using SRB assay. In this regard, α-thujone concentraction was able to significantly reduce the cell biomass in LNCaP androgen responsive cells, but no significant differences were found in DU145 and PC3cell lines.

4. DISCUSSION

Ethnobotanical approaches have become a successful strategy in order to identify new bioactive compounds found in higher plants, and occurs when the scientific community take advantage of the information collected from traditional communities along with studies done in the laboratory (Oliveira FC 2010). Brazil is the country with the highest plant diversity on the planet and around 65% of the plant species have not been analyzed yet for their antitumor potential (de Melo, Santos et al. 2011). In this regard, *T. occidentalis*, a native plant from Europe, is widely used in folk medicine in Brazil for bronchial catarrh, enuresis, cystitis, psoriasis, uterine carcinomas, amenorrhea and rheumatism (Shimada 1956; Baran 1991; Offergeld, Reinecker et al. 1992), with few studies showing its effects in the treatment of cancer diseases (Wustenberg, Henneicke-von Zepelin et al. 1999; Kohler, Bodinet et al. 2002; Teuscher 2004; MacLaughlin, Gutsmuths et al. 2006). Thus, in the present study we aimed to perform *in vitro* experiments with the *T. occidentalis* extract and different prostate cancer cell lines in order to analyze its potential antitumor activity.

Prostate cancer grows slowly, and can take 15 years for the tumor reach one cubic centimeter (Siegel, Naishadham et al. 2013). In general, in the early stages the tumor is confined to the prostate and surgical treatment is recommended (Freeman, Yang et al. 2012). However, with the progression of the disease can reach near prostate organs such as the bladder, urethra and seminal vesicles; or spread to distant organs, causing metastasis (Lynch and Lynch 1996; Gomes 2008; INCA 2015). At this stage, the main treatment is anti-androgen therapy (Cooperberg, Grossfeld et al. 2003; Hoimes and Kelly 2010; Mottet, Bellmunt et al. 2011) and the androgen signaling suppression inhibits, temporarily,

the natural progression of prostate cancer (Heinlein and Chang 2004) and tumor appear to enter remission (Ganguly, Li et al. 2014). However, one of the main hurdles of prostate cancer is the ability of these cancer cells to switch into a resistant androgen phenotype. Current literature shows that time and exposure to anti-androgen therapy induces a mutation in the androgen receptor, leading to a change in the regulation of prostate cancer cell growth (Waxman and Ngan 2008). The underlying mechanism of the phenotype switch is still poorly understood. Nevertheless, many factors are responsible for the androgen-independent androgen receptor activation in prostate cancer cells, including androgen receptor gene amplification (Linja, Savinainen et al. 2001; Haapala, Kuukasjarvi et al. 2007); intratumoral synthesis of androgen in the castration condition (Montgomery, Mostaghel et al. 2008; Locke, Fazli et al. 2009); increased androgen sensitivity due to somatic mutations and/or alterations in the sensitivity to other steroid hormones (Li, Cavasotto et al. 2005; Bergerat and Ceraline 2009), among others.

Earlier studies have defined a critical role for caveolin-1 in androgen signaling. Cav-1 gene expression is upregulated in metastatic cell lines (Nasu, Timme et al. 1998). Moreover, cav-1 expression positively correlates with indicators of aggressiveness, and its immunocontent is correlated with poor clinical prognosis in human prostate cancer (Tahir, Yang et al. 2001; Freeman, Yang et al. 2012). Whether these effects are important in the transformation of androgen responsive to androgen unresponsive cells is not well characterized. However, cav-1 is able to hypersensitize the androgen receptor at very low hormone concentrations. Importantly, recent studies have implicated cav-1 signaling in ligand-independent receptor activation (Lu, Schneider et al. 2001).

Interestingly, our study demonstrates that the *T. occidentalis* extract led to a marked reduction in the expression of cav-1 (at gene and protein level) on two different prostate cancer androgen unresponsive cell lines, DU145 and PC3. Another study demonstrated a protective effect cav-1 dependent, showing that cav-1 diminution was sufficient to convert androgen unresponsive cells to an androgen responsive phenotype (Nasu, Timme et al. 1998). Moreover, additional data suggest that high cav-1 levels can induce the progression of cancer cells into an advanced stage (Williams, Hassan et al. 2005). These facts support the idea that cav-1 could be used as a biomarker for prostate cancer prognosis and androgen management. Nowadays, metastasis resistant to conventional therapies represent the major cause of death from cancer (Senetta, Stella et al. 2013). In this way, the decrease of cav-1 expression in the androgen unresponsive cell lines DU145 and PC3 caused by treatment with *T. occidentalis* extract could represent an important adjuvant therapy for the treatment of prostate cancer, reducing its metastatic potential, besides being an important target for developing new drugs to treat this pathology, mainly in castration-resistant phase, where there is few effective curative therapies currently available.

In addition to observed genetic alterations, the development and progression of cancer is also based on an imbalance between proliferation and cell death. Deregulated cell growth is associated with the loss of the ability of cells to undergo apoptosis, leading to the development of cancer (Bruggers, Fults et al. 1999). Thus, the induction of cell death and inhibition of cell proliferation are key players in the treatment of prostate cancer.

Although the mechanisms of cav-1 downregulation remains to be elucidated, our data demonstrate that *T. occidentalis* treatment was able to induce

apoptosis and necrosis in DU145 and PC3 androgen unresponsive prostate cancer cell lines. This is consistent with the observation done by Biswas and coworkers (Biswas, Mandal et al. 2011) showing that *T. occidentalis* tincture induced apoptosis in a melanoma cell line. Furthermore, *in vivo* studies done on the anti-metastatic activity of the *T. occidentalis*, demonstrated the inhibition of lung metastasis induced by B16F-10 melanoma cells in a *T. occidentalis* treated mouse model and stimulation of the immune system (Sunila and Kuttan 2006) .

In agreement with previous results, *T. occidentalis* treatment also decreased DU145 and PC3 cell viability (MTT assay) and biomass (SRB assay). The combination of biomass and cell metabolism reduction indicates a decrease in cell number when compared to control cells, showing an inhibitory effect on prostate cancer cell growth.

We also observed that *T. occidentalis* extract caused the arrest of DU145 cells in G0G1 phase, but did not cause alterations in the cell cycle of PC3 cells. Indeed, many cancer treatments such as gamma radiation and chemotherapeutic drugs induce cell cycle arrest and consequently cell death by apoptosis. The effect of cell cycle arrest shown by *T. occidentalis* extract in DU145 cells suggests that our treatment may have inhibited the progression of cancer cells by artificially imposing a cell cycle checkpoint. In response to DNA damage, checkpoints arrest the cell cycle in order to provide time for DNA repair that happens before S phase (G1-S checkpoint). However, if cell damage is severe, genes involved in apoptotic signaling can be activated leading to cell death (Owen-Schaub, Zhang et al. 1995; Gottlieb and Oren 1998; Vermeulen, Van Bockstaele et al. 2003). Further studies are necessary to elucidate which genes are involved in cell cycle arrest in our model.

LNCaP, the androgen responsive cell line, had a complete different behavior and exhibited a strong cytotoxic effect after treatment. *T. occidentalis* extract was able to reduce cell viability and culture biomass after only 1 hour of treatment. Cell death was mainly dependent on necrosis with no increase in apoptosis when compared to control cells. LNCaP accumulated data showed a higher sensibility after treatment when compared to the other androgen unresponsive prostate cancer cell lines used in this study, DU145 and PC3. Additionally, previous reports demonstrate that androgen responsive LNCaP cells express androgen receptors but do not express detectable amounts of cav-1 (Nasu, Timme et al. 1998; Thompson, Timme et al. 1999) . In our current study, we also did not detect the expression of cav-1 in LNCaP cells. This finding can be associated with the different effects caused by the *T. occidentalis* extract observed in LNCaP cells. In this regard, many studies suggest that the interaction between the androgen receptor and cav-1 increased expression could be a determinant player in the process of metastasis of prostate cancer by transforming androgen sensitive cells into androgen insensitive cells (Nasu, Timme et al. 1998; Lu, Schneider et al. 2001). In this case, one likely explanation for the difference in behavior found in LNCaP cells could be that the absence of cav-1 expression in LNCaP cells highlight the important role of cav-1 in resistance to anti-androgen therapy in prostate cancer and could be related to higher sensibility to treatment with *T. occidentalis* extract in LNCaP cell line, but more studies are needed to elucidate this hypothesis.

According to Hänsel et al. (1994) (Hänsel 1994), *T. occidentalis* dried herb contains 1.4 to 4% of essential oil, and 60% of the oil consists of a monoterpene ketone known as thujone (Chang, Song et al. 2000; Naser 2005). This oil is found

in two isomeric forms, the α-thujone and β-thujone. In general, in nature, there is a mixture of two isomers, with a predominance of the alpha form (Sondermann 1962; Traud 1983), which is also the isomer with the highest activity (Hold, Sirisoma et al. 2000; Czyzewska and Mozrzymas 2013). Because of this, we hypothesize that α-thujone could be responsible for some of the effects observed when prostate cancer cells were treated with the *T. occidentalis* extract. Then, we quantified this monoterpene in the extract using a GC-MS analysis and we found 0.032 µg of α-thujone /ml of extract that match to 0.0016 µg of α-thujone/ml of treatment medium. When we tested this amount of oil on the cell lines, we observed a reduction in cell biomass (SRB assay) only in LNCaP cells. However, experiment with larger doses of α-thujone have been effective in reducing the cell biomass in cultures of DU145 and PC3 cells (data not shown). The effect in the biomass reduction observed only in the LNCaP androgen responsive cell line corroborates previous results that show the androgen responsive cell line is more sensitive to treatment that androgen unresponsive cells (DU145 and PC3). In addition, other components of the extract can also be influencing the reduction of viability observed, however further studies on this extract composition and its possible effects on the prostate cancer cell lines must be conducted to confirm this theory.

In the current study we used three different cell lines which exhibited different phenotypic characteristics and behavior in culture (Stone, Mickey et al. 1978; Kaighn, Narayan et al. 1979). These differences could explain the dissimilar responses observed in our study towards *T. occidentalis* extract treatment. DU145 and PC3 cell lines are useful in investigating the biochemical changes in advanced phase of prostate cancer and in assessing their response to chemotherapeutic

agents. Both prostate cancer cell lines DU145 and PC3 are androgen insensitive and metastatic (Stone, Mickey et al. 1978; Kaighn, Narayan et al. 1979), but they originate from distinct organs. DU145 prostate cancer cell line was derived from a human prostatic carcinoma metastasis to the brain (Stone, Mickey et al. 1978) while PC3 was derived from a human prostatic adenocarcinoma metastatic to the bone (Kaighn, Narayan et al. 1979). Although both lines are metastatic, DU145 cells have moderate metastatic potential compared to PC3 cells, which have high metastatic potential (Keer, Gaylis et al. 1991). The LNCaP cell line was also established from a metastatic lesion of human prostatic adenocarcinoma, however this model is androgen responsive and high-affinity specific androgen receptors are present in the cytosol and nuclear fractions of cells in culture and in tumors. Moreover, 5-alpha-dihydrotestosterone modulates cell growth of LNCaP *in vitro* (Horoszewicz, Leong et al. 1983) .

We also observed different responses when the three cell lines (androgen responsive and unresponsive cells) were treated with *T. occidentalis* extract. This differential behavior of prostate cancer cell lines to the treatment with the same substance has already been observed in other studies (Stone, Mickey et al. 1978; Kaighn, Narayan et al. 1979; Ifere, Barr et al. 2009), confirming our results. Moreover, in a previous study conducted by our group, we evaluating the effects of the phytosterol γ-oryzanol on LNCaP androgen responsive and DU145 and PC3 androgen unresponsive prostate cancer cell lines, and different behavior profiles were also found (Hirsch, Parisi et al. 2015). This found could be related to the fact of the cell lines originating from different organs and they present different metastatic potential, aggressiveness and androgen sensitivity.

To our knowledge, this is the first study that demonstrates the effects of *T. occidentalis* extract in responsive and unresponsive prostate cancer cell lines. In conclusion, our studies have confirmed the anti-proliferative effect of *T. occidentalis* extract and induction of citotoxicity that led to cell death by apoptosis and necrosis or cell cycle arrest, as can be seen briefly in Figure 7. In addition, the treatment also was able to reduce cav-1 protein content and gene expression in androgen unresponsive DU145 and PC3 cells. This negative feedback mechanism may be an important component for prostate cancer treatment, as caveolin-1 promotes androgen receptor-α ligand-independent signaling (Lu, Schneider et al. 2001), one of the main factors leading to resistance of prostate cancer to androgen ablation therapy.

Our results, examining the *in vitro* effect of *T. occidentalis* antiproliferative action, show a promising adjuvant role in the treatment of prostate cancer. However, further studies are necessary in order to establish the real therapeutic possibilities of *T. occidentalis* extract and the molecular mechanisms by which these effects operate.

5. ACKNOWLEDGEMENTS

The authors acknowledge to Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) and to Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS) for financial support. We are also grateful to Centro de Microscopia Eletrônica (CME) of UFRGS and specially to Henrique B. Biehl for the technical assistance in the confocal analyses.

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FIGURES

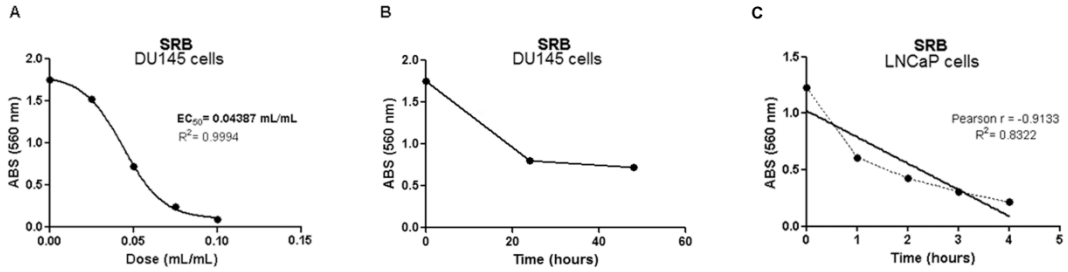

Figure 1. (A) Dose curve of the anti-proliferative effects evaluated by sulforhodamine B assay using different concentrations of *T. occidentalis* extract: 0.000 mL/mL, 0.025 mL/mL, 0.050 mL/mL, 0.075 mL/mL and 0.1 mL/mL (mL of tinture/mL of culture medium) and (B) time curve of the anti-proliferative effects evaluated by sulforhodamine B assay in androgen unresponsive prostate cancer cell line DU145; (C) Time curve of the anti-proliferative effects evaluated by sulforhodamine B assay using different treatment times with 0.050 mL/mL of *T. occidentalis* extract in androgen responsive prostate cancer cell line LNCaP: 1 hour, 2 hours, 3 hours and 4 hours.

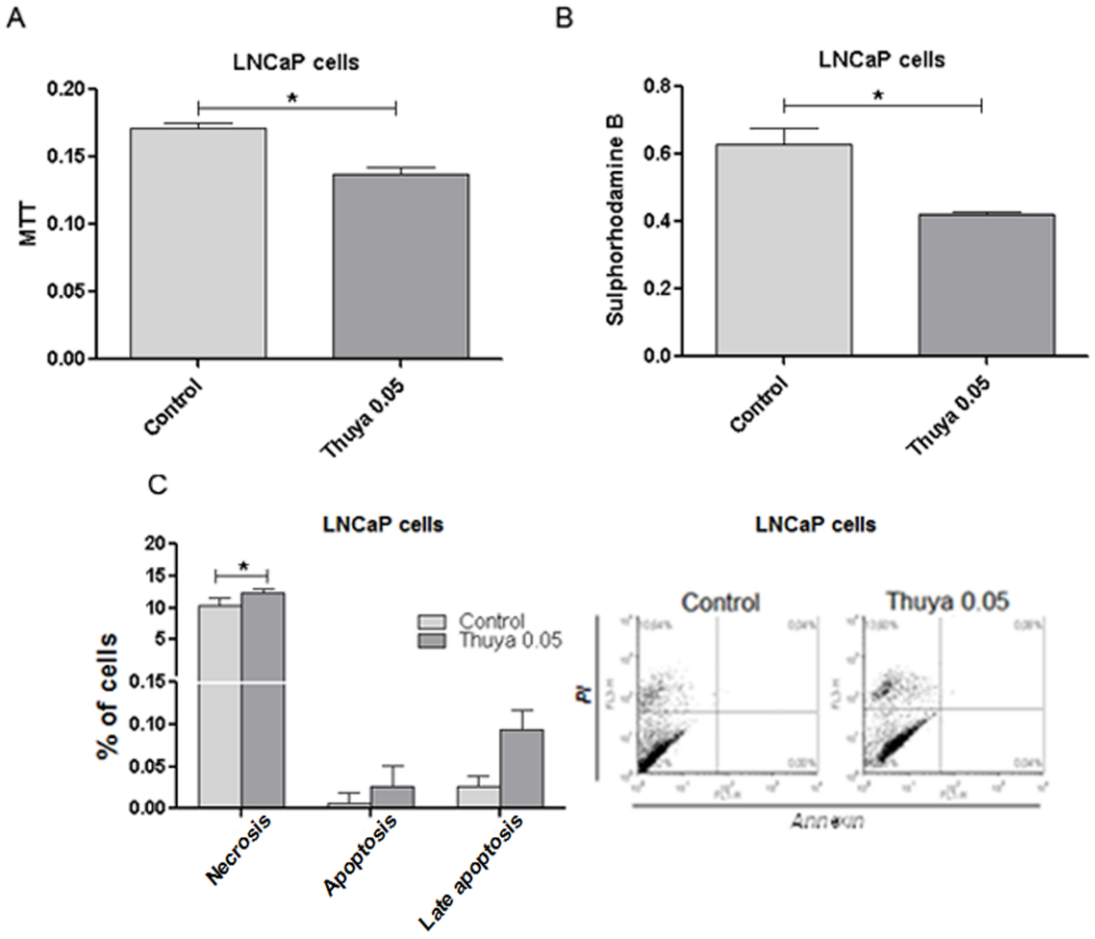

Figure 2. Effects of *T. occidentalis* hidroalcolic extract on viability, biomass and cell death in androgen responsive prostate cancer cell line LNCaP (A) Cell viability measured by MTT assay, (B) cell biomass measured by sulforhodamine B assay, (C) Apoptotic and necrotic effects measured by Anexinn V and PI and representative histogram of death cell by apoptosis and necrosis of LNCaP cells. Values are show as mean ± SD of three independent experiments. *p<0.05 vs. untreated control cells.

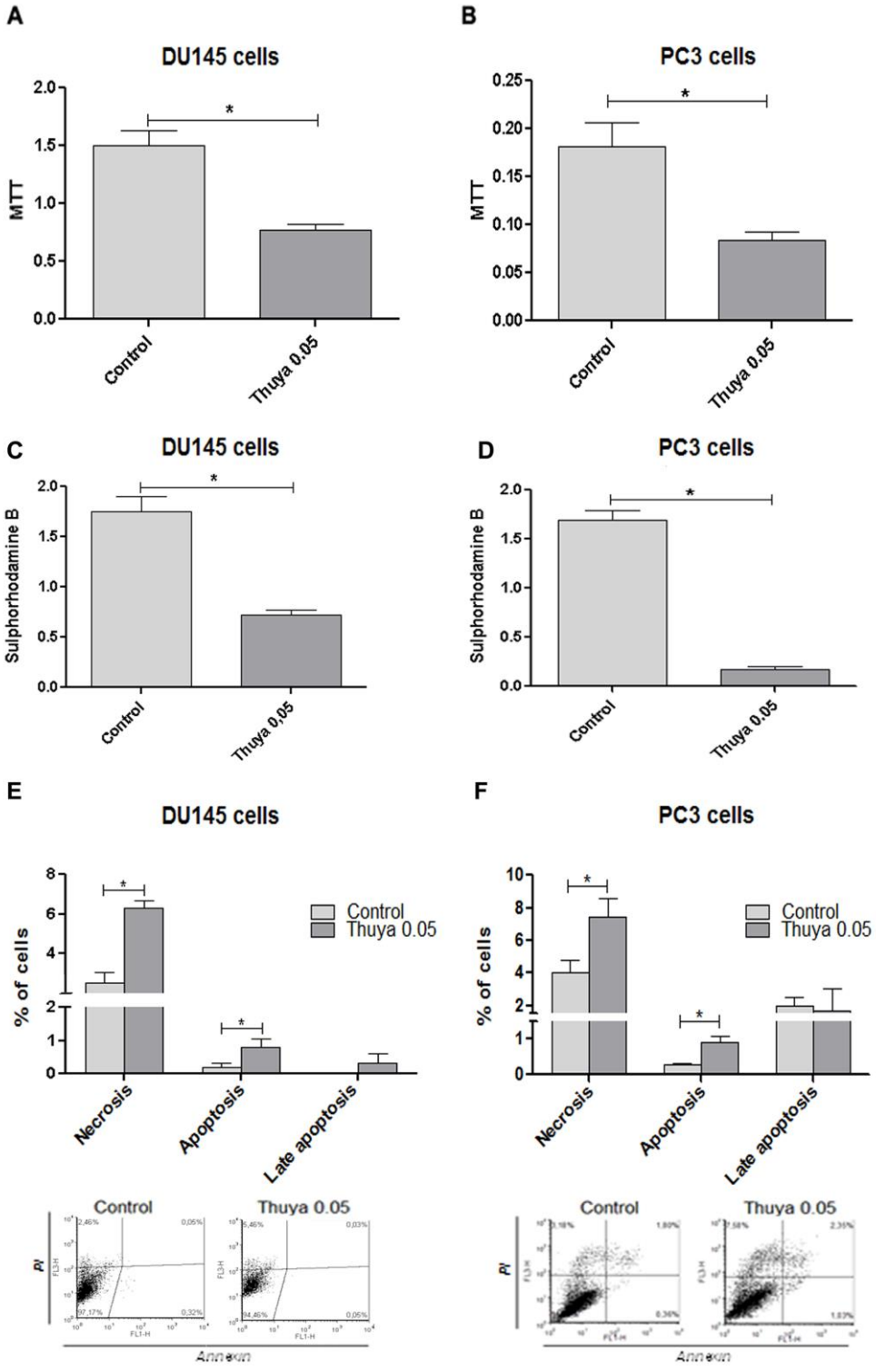

Figure 3. Effects of *T. occidentalis* hidroalcolic extract on cell viability, cell biomass and cell death by apoptosis and necrosis in androgen unresponsive prostate cancer cell lines (DU145 and PC3). Cell viability measured by MTT assay (A and B); cell biomass was measured by sulforhodamine B assay (C and D); and cell death by apoptosis and necrosis by flow cytometry (E and F). Values are show as mean ± SD of three independent experiments. *p<0.05 vs. untreated control cells.

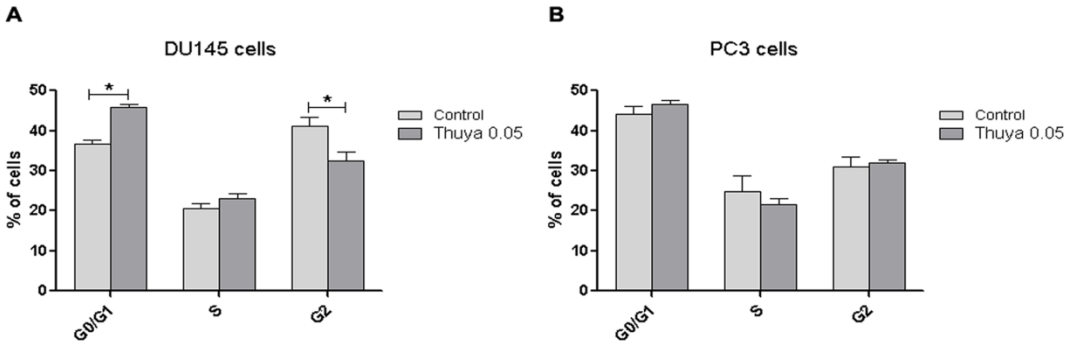

Figure 4. Effect of *T. occidentalis* hidroalcolic extract on cell cycle progression in androgen unresponsive prostate cancer cell lines (DU145 and PC3). (A) DU145 androgen unresponsive cell line and (B) PC3 androgen unresponsive cell lines. Values are show as mean ± SD of three independent experiments. $^{*}p<0.05$ vs. untreated control cells.

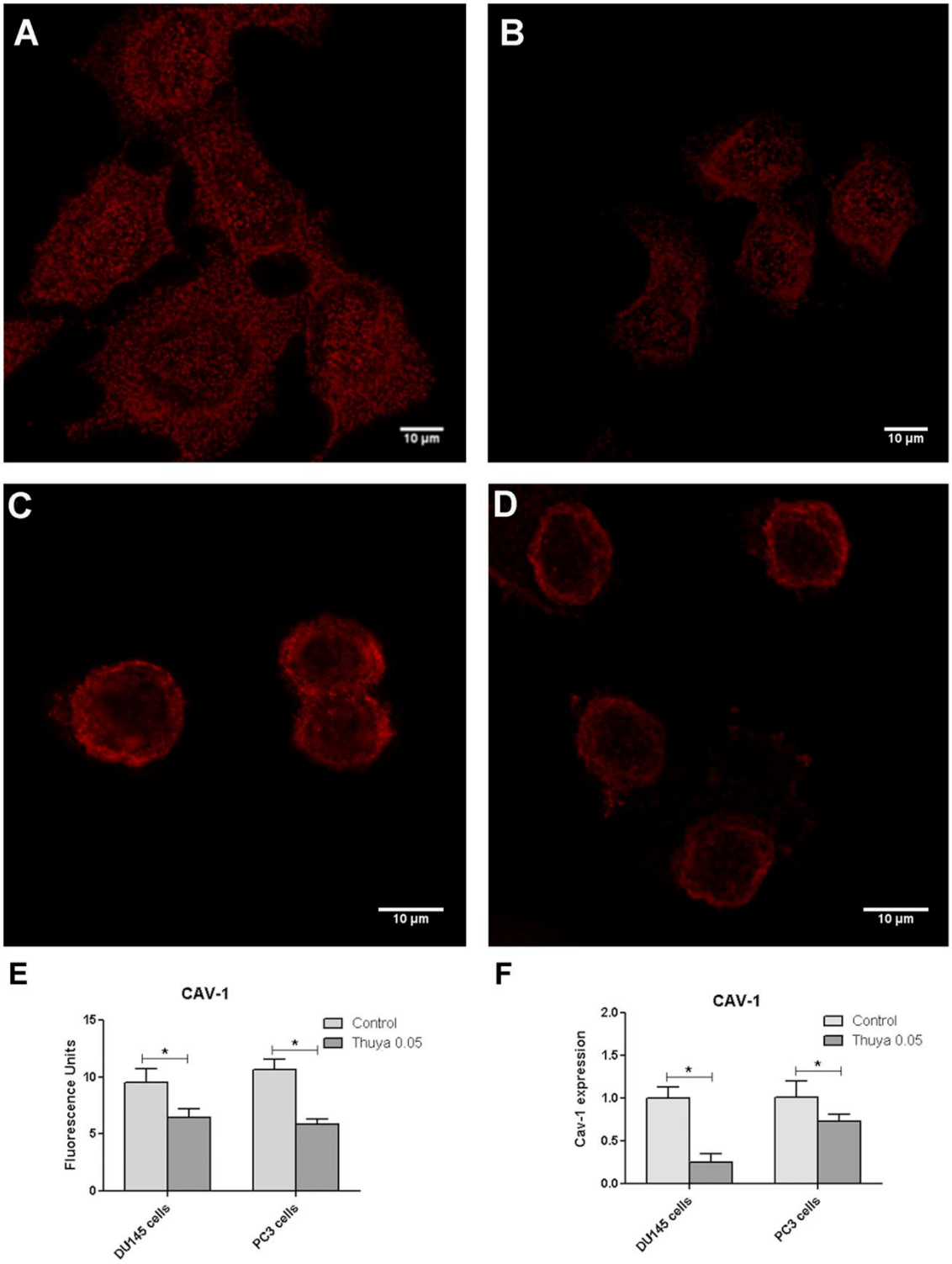

Figure 5. Effects of *T. occidentalis* extract in content protein and mRNA of cav-1 in androgen unresponsive prostate cancer cell lines (DU145 and PC3). Images of DU145 (A and B) and PC3 (C and D) androgen unresponsive cell lines. A and C are untreated cells and B and D are treated cells. (E) Fluorescence intensity quantification of cav-1 protein. (F) Expression of cav-1 mRNA. Values are show as mean ± SD of three independent experiments. $^{*}p<0.05$ vs. untreated control cells. Scale bar = 10 µm

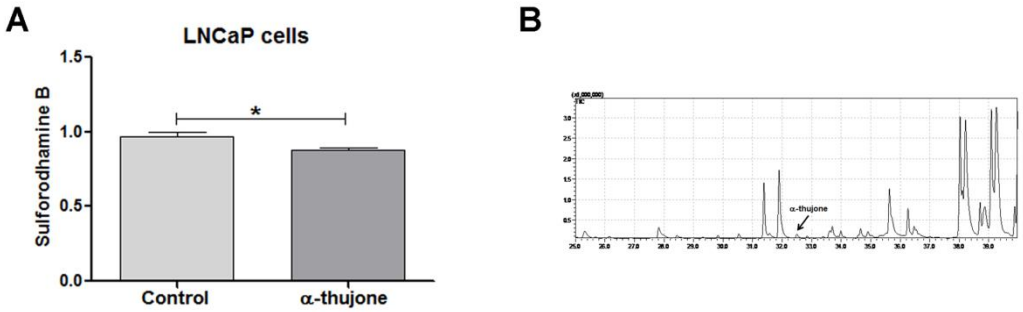

Figure 6. (A) Effects of α-thujone on biomass cell in LNCap androgen responsive cell line and (B) chromatogram of α-thujone analysis by Gas Chromatography Mass Spectrometry. Values are show as mean ± SD of three independent experiments. $^{*}p<0.05$ vs. untreated control cells.

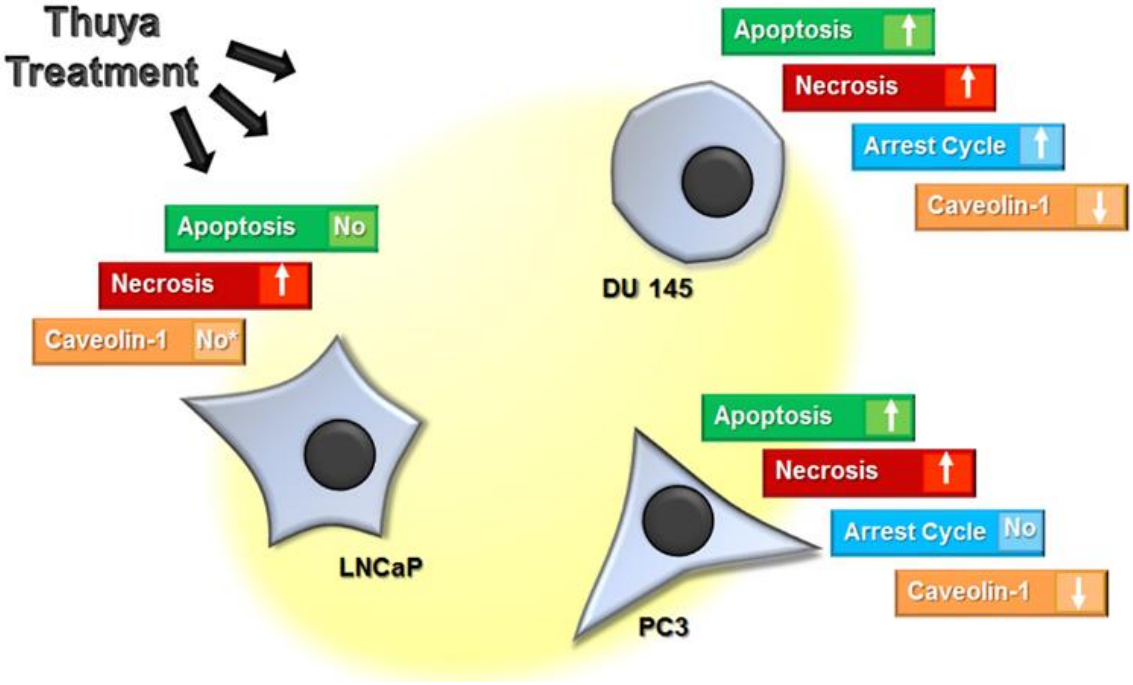

Figure 7. Schematic illustration showing an overall view of the androgen responsive and unresponsive prostate cancer cell lines after 24 or 48 hours of cell culture, respectively. For a detailed explanation of this figure, see text. *T. occidentalis* extract was able to reduce cell viability and biomass by apoptosis and/or necrosis cell death in LNCaP (androgen responsive) and DU145 and PC3 (androgen unresponsive) cells. These effects were accompanied by a cell cycle arrest in G0G1 phase in DU145 and down regulation in the expression of the caveolin-1 gene and protein in both androgen unresponsive cells. However, we could not detect any expression of caveolin-1 in LNCaP androgen unresponsive cells (*).

PARTE III

III.1 DISCUSSÃO

O câncer de próstata é uma doença amplamente estudada em diversos modelos biológicos, devido a sua alta prevalência e mortalidade em todo o mundo ((INCA) 2014; Deng, He et al. 2014). Além disso, é uma doença progressiva e a medida que evolui para formas mais agressivas, o tratamento se torna bastante difícil e muitas vezes, ineficaz (Grossmann, Cheung et al. 2013). Cerca de 86% dos homens com câncer de próstata são diagnosticados inicialmente com doença localizada e a taxa de sobrevivência relativa nesses casos é bastante alta, cerca de 5 anos em aproximadamente 100% dos casos (Keating, O'Malley et al. 2006). Porém, a mortalidade relacionada ao câncer de próstata também é elevada. Só no Rio Grande do Sul foram 1.032 óbitos por neoplasia de próstata em 2011, e 13.129 homens morreram em decorrência desta neoplasia no Brasil neste mesmo ano ((INCA) 2014). Este alto índice de mortalidade é atribuído a dois fatores: ao tratamento e a progressão da doença para fase hormônio refratária (Keating, O'Malley et al. 2006).

Como o prognóstico para o câncer de próstata em geral é favorável, as decisões sobre os tipos de tratamento são particularmente importantes, uma vez que alguns deles podem causar uma série de efeitos adversos e complicações que acabam por afetar o estado de saúde geral e a qualidade de vida do paciente, muitas vezes mais do que o próprio câncer de próstata em si (Keating, O'Malley et al. 2006). A terapia de restrição andrógena (castração) é o tratamento de eleição para doença avançada ou metastática (Stacewicz-Sapuntzakis and Bowen 2005; Freeman, Yang et al. 2012; Pagliarulo, Bracarda et al. 2012; Madan and Arlen 2013) e tem o objetivo de inibir a produção endógena de testosterona, e como as

células prostáticas proliferam em resposta a presença de andrógeno, essas células param de crescer (de Bono, Logothetis et al. 2011; Scher, Fizazi et al. 2012; Culig and Santer 2013; Rajan, Sudbery et al. 2014). Porém, outras funções do organismo também são afetadas pela ausência deste hormônio e os principais efeitos colaterais observados são perda de libido, osteoporose, fadiga, perda de massa magra, anemia, ginecomastia, além de efeitos metabólicos associados a mudanças no perfil lipídico e aumento do risco de desenvolvimento de resistência à insulina, diabetes, síndrome metabólica (Jeong 2001; Flaig and Glode 2008; Leahy 2008; Nobes, Langley et al. 2009; Saylor and Smith 2013), e alterações cardiovasculares (Saigal, Gore et al. 2007; Efstathiou, Bae et al. 2009; Van Poppel and Tombal 2011; Roayaei and Ghasemi 2013). Muitas das patologias associadas ao uso dessa terapia acabam sendo responsáveis pelos óbitos observados para esta doença.

Além dos problemas relacionados à terapia de castração, outro problema associado ao tratamento do câncer de próstata envolve a progressão para fase hormônio refratária. Em geral, inicialmente as células prostáticas respondem a queda da testosterona causada pela terapia antiandrógena e o tumor regride e pode até ser eliminado (Ganguly, Li et al. 2014). Entretanto, com o passar dos anos algumas das células podem se tornar resistentes a privação androgênica e o tumor volta a crescer e, em geral, pacientes nesta situação vem a óbito em cerca de 40 semanas após a recidiva (Mahler and Denis 1992; de Bono, Logothetis et al. 2011; Kirby, Hirst et al. 2011; Scher, Fizazi et al. 2012; West, Kiely et al. 2014). Ainda, muitos tumores avançados desenvolvem novos mecanismos de resistência às drogas disponíveis atualmente e são comuns a quase todas elas (Caffo 2015), tornando o tratamento do câncer de próstata ainda mais complexo.

Diante dos inúmeros problemas atribuídos ao uso das terapias disponíveis atualmente para tratar o câncer de próstata e da ausência de tratamento eficaz para cura da doença na fase mais avançada, têm aumentado o número de estudos buscando alternativas eficazes para o tratamento desta patologia. Associado a isso, cada vez mais produtos naturais tem mostrado efeitos benéficos no controle do crescimento e metástase em diversos tipos de câncer. Entre eles podemos destacar a curcumina, as isoflavonas, o resveratrol e as catequinas (Khan, Afaq et al. 2008).

O γ-orizanol é um fitoquímico extraído a partir do farelo do arroz, formado por uma mistura de ésteres do ácido ferúlico (Graf 1992) com diferentes esteróis (campesterol, estigmasterol ou β-sitosterol) ou álcoois triterpênicos (cicloartanol, cicloartenol, 24-metilenocicloartanol, ciclobranol) (Diack and Saska 1994; Bucci, Magri et al. 2003; Yu, Nehus et al. 2007; Imsanguan, Roaysubtawee et al. 2008; Lu, Chen et al. 2011; Jeng, Shih et al. 2012). Já a *Thuya occidentalis* é uma árvore nativa da Europa (1983) comumente utilizada na medicina popular para tratamento de diversas patologias (Naser 2005), principalmente na forma de homeopatia, seja como tintura-mãe ou como diluição ((HAB) 1985; (HAB) 2003).

As ações do γ-oryzanol e do extrato hidroalcólico de *Thuya occidentalis* sobre diversas patologias, inclusive em alguns tipos de cânceres, foram a base para a hipótese que justifica o objetivo principal desta tese. Tanto o γ-oryzanol, como o extrato hidroalcólico de *Thuya occidentalis* poderiam atuar favoravelmente na prevenção ou no tratamento do câncer de próstata por interferirem em mecanismos bioquímicos e moleculares que resultam em inibição do crescimento celular, porém, poucos estudos sobre a ação desses compostos no câncer de próstata estão disponíveis.

No Capítulo I da segunda parte desta tese, foram descritos os efeitos do γ-orizanol sobre linhagens celulares de câncer de próstata responsivas e não-responsivas a andrógenos. Nós observamos que o tratamento com 16 μM de γ-orizanol foi capaz de diminuir significativamente a viabilidade e biomassa celular nas três linhagens celulares (LNCaP, DU145 e PC3), provavelmente associado ao aumento da apoptose na linhagem não-responsiva a andrógeno PC3, confirmada pelo aumento da ativação de caspases; ou da necrose nas linhagens LNCaP e DU145 responsiva e não-responsiva a andrógenos, respectivamente.

As caspases, uma família de proteases, desempenham um papel central na regulação da apoptose (Kitanaka and Kuchino 1999). Elas são divididas em duas categorias conhecidas como caspases iniciadoras (caspase-2, -8, -9 e -10) e caspases efetoras (caspase-3, -6 e -7) (Riedl and Shi 2004; Tait and Green 2010). No citosol de células normais, as caspases aparecem como zimogênios inativos, associadas com moléculas de Proteínas Inibidoras de Apoptose (IAP). Quando a célula recebe um estímulo pró-apoptótico, as caspases iniciadoras são ativadas, e estas por sua vez, medeiam a ativação de outras caspases, resultando em alterações bioquímicas e biofísicas que geram apoptose (Taylor, Cullen et al. 2008; Parrish, Freel et al. 2013; McCracken and Allen 2014). Como esse processo geralmente está desregulado no desenvolvimento do câncer (Fisher 1994), o aumento da ativação das caspases acompanhada pelo aumento da morte celular por apoptose nas células PC3 após o tratamento com γ-orizanol representa uma importante estratégia do desenvolvimento de terapias para essa patologia.

Além disso, o tratamento com γ-orizanol também bloqueou a progressão do ciclo celular através da indução da parada do ciclo em G0/G1 na linhagem DU145 ou em G2/M nas linhagens LNCaP e PC3. A regulação do ciclo celular é crítica

para o crescimento e desenvolvimento dos tumores (Collins, Jacks et al. 1997; Park and Lee 2003) e uma das vias que pode ser envolvida na progressão do ciclo celular é a via da ERK1/2, um membro da família das proteínas cinases ativadas por mitógeno (MAPK) (Meloche and Pouyssegur 2007). Em geral, a fosforilação da ERK1/2 (pERK1/2) ocorre em resposta a um estímulo mitogênico, e a relação entre a ativação da ERK1/2 (pERK1/2) e proliferação celular é complexa e dependente de muitos fatores, como a duração do estímulo, por exemplo (Agarwal 2000). Estudos tem demonstrado que a ativação transiente da ERK1/2 pode causar aumento da proliferação celular, e de fato, esse mecanismo ocorre em diversos tumores e tem sido considerado benéfico (Sah, Eckert et al. 2002; Steinmetz, Wagoner et al. 2004). Porém, estudos anteriores também mostraram que a ativação forte e sustentada da ERK1/2 reduz a proliferação celular por causar parada do ciclo celular (Agarwal 2000; Xiao and Singh 2002; Albrecht, Clubbs et al. 2008). Além disso, células epiteliais de próstata normalmente mostram altos níveis basais de atividade da ERK1/2 e durante a progressão do câncer esses níveis tendem a diminuir, causando aumento da proliferação celular (Paweletz, Charboneau et al. 2001; Malik, Brattain et al. 2002; Ghosh, Malik et al. 2003). Nossos estudos mostraram um aumento de 0.31 e 0.51 vezes na pERK1/2 nas linhagens celulares LNCaP e DU145, após o tratamento com γ-orizanol por 24 e 48 horas, respectivamente. Este fato justificaria a parada do ciclo celular e redução da proliferação observadas nas mesmas linhagens e poderia representar uma importante estratégia no tratamento do câncer de próstata.

Além das mudanças bioquímicas encontradas nas células de câncer e que contribuem para o seu desenvolvimento e progressão, fatores genéticos também

estão envolvidos e são importantes no câncer de próstata. A caveolina-1 (Cav-1), por exemplo, é um componente vital das caveolas (Zhuang, Kim et al. 2005; Daniel, El-Yazbi et al. 2006) e está intimamente relacionada ao aumento da agressividade em carcinomas da próstata (Yang, Truong et al. 1999). Estudos tem demonstrado que o aumento da expressão da Cav-1 seria capaz de hipersensibilizar o receptor andrógeno e contribuir significativamente para a progressão da doença para a fase hormônio refratária (Lu, Schneider et al. 2001). Como citado anteriormente, nesta fase, o tratamento do câncer se torna muito difícil e em geral, é ineficaz (Grossmann, Cheung et al. 2013). A redução da expressão da Cav-1 surge então, como uma importante estratégia para o tratamento da doença e estudos anteriores confirmam que a redução da expressão da Cav-1 foi capaz de tornar células não-responsivas a andrógenos em responsivas novamente (Nasu, Timme et al. 1998), corroborando a nossa hipótese. De fato, no Capítulo I desta tese nós mostramos que o tratamento com γ-orizanol reduziu a expressão do gene e da proteína Cav-1 nas linhagens não-responsivas a andrógenos (DU145 e PC3), fator este que também poderia estar contribuindo para a redução da sobrevivência observada nessas células após o tratamento.

Outro alvo molecular importante no câncer de próstata é o gene específico da próstata, PCGEM1 (Petrovics, Zhang et al. 2004). Este gene faz parte de um grupo de genes recentemente postos em evidência, os genes de RNA não-codificantes de proteínas (Petrovics, Zhang et al. 2004). Esses genes, ao invés de expressarem RNAs mensageiros (mRNA) que codificam proteínas, produzem transcritos com função regulatória, catalítica ou estrutural (Eddy 1999; Erdmann, Barciszewska et al. 2001; Erdmann, Barciszewska et al. 2001) e estão

amplamente envolvidos com o desenvolvimento de neoplasias (Huarte and Rinn 2010). Assim como a Cav-1, a alteração no padrão de expressão do gene PCGEM1 também está relacionada com a progressão do câncer de próstata para fase hormônio refratária e metastática (Yang, Lin et al. 2013), além de aumentar o risco de desenvolvimento deste tipo de neoplasia em homens (Bussemakers, van Bokhoven et al. 1999; Fu, Ravindranath et al. 2006). Ainda, o gene PCGEM1 também tem relação com as alterações encontradas no receptor andrógeno responsáveis pela resistência ao tratamento antiandrogênico (Yang, Lin et al. 2013) e, assim como a Cav-1, ele poderia ser um importante alvo molecular para o desenvolvimento de novas drogas para tratar essa patologia. Neste sentido, o γ-orizanol parece ser promissor, pois foi capaz de reduzir a expressão do gene PCGEM1 nas células LNCaP e DU145.

Outra classe de genes de RNA não-codificantes de grande importância no câncer são os miRNAs (Chang, Lin et al. 2002; Ambros 2004; Kidner and Martienssen 2005; Iorio and Croce 2009; Fabbri 2010; Liao, Yu et al. 2010; Farazi, Spitzer et al. 2011; Ting, Lipson et al. 2011). Os miRNAs são pequenas moléculas de RNA com aproximadamente 22 nucleotídeos que exercem seus efeitos regulatórios ligando-se à região 3’ não traduzida do RNA mensageiro alvo para reprimir a sua tradução ou levar a sua degradação (Bartel 2004; Filipowicz, Jaskiewicz et al. 2005; Sontheimer and Carthew 2005), reduzindo assim os níveis proteicos de genes-alvo, raramente afetando o nível de expressão transcricional deles (Wiemer 2007). Diversos estudos tem demonstrado que esses miRNAs estão envolvidos no controle de funções celulares como a proliferação e apoptose (Bartel 2004; Care, Catalucci et al. 2007) e que sua expressão encontra-se desregulada no câncer de próstata, estando intimamente relacionados ao seu

desenvolvimento, invasão e metástase (Shi, Xue et al. 2007; Lu, Liu et al. 2008; Musiyenko, Bitko et al. 2008). Como no Capítulo I nós observamos que o γ-orizanol foi capaz de modular diversas funções celulares e moleculares em células de câncer de próstata responsivas e não-responsivas a andrógeno, no Capítulo II nós estudamos o efeito deste fitoquímico sobre alguns miRNAs.

Diversos miRNAs tiveram suas expressões alteradas após o tratamento com γ-orizanol e diferentes padrões de expressão foram encontrados para eles.

Exemplos de miRNAs com expressão reduzida no câncer de próstata são miR16-1, miR99a e miR133a. O miR16-1 normalmente tem sua expressão reduzida na doença avançada e está relacionado com aumento da proliferação em células não-responsivas a andrógenos. Por outro lado, o aumento da expressão deste miRNA causa aumento da apoptose e parada do ciclo celular (Bonci, Coppola et al. 2008). A redução da expressão do miR99a também está correlacionado com o desenvolvimento do câncer de próstata e seu padrão de expressão é reduzido em tumores metastáticos e mais agressivos em relação a células normais de próstata (Porkka, Pfeiffer et al. 2007; Sun, Lee et al. 2011). Além disso, o aumento da expressão deste miRNA também resulta em parada do crescimento das células de câncer de próstata (Sun, Lee et al. 2011). O miR99a/let7c/125b-2 *cluster* tem sua transcrição reprimida pela ativação do receptor andrógeno por andrógenos. No entanto, a superexpressão do *cluster* não causou inibição da proliferação em células LNCaP na ausência de andrógenos (Sun 2014).

A redução da expressão do miR133a está associada ao desenvolvimento de muitos tipos de câncer, e o aumento da sua expressão leva a redução na proliferação, migração e invasão celular no câncer de próstata (Chiyomaru,

Enokida et al. 2010; Kano, Seki et al. 2010). Além disso, estudos anteriores sugerem que o mRNA da Cav-1, um importante marcador de agressividade do câncer de próstata (Yang, Truong et al. 1999), é um dos alvos do miR133a (Chiyomaru, Enokida et al. 2010; Kano, Seki et al. 2010). O tratamento com 16 µM de γ-oryzanol reduziu a expressão do miR133a na linhagem de câncer LNCaP, porém, nosso estudo anterior não foi capaz de identificar expressão nem da proteína e nem do gene da Cav-1 nesta linhagem celular. Mais estudos são necessários para comprovar a relação entre a expressão do miR133a e a ausência de Cav-1 nas células LNCaP, porém a redução deste miRNA após o tratamento com o γ-orizanol não foi capaz de induzir a expressão da Cav-1, como mostrado no Capítulo I.

γ-orizanol também aumentou a expressão do miR161 em células PC3 não-responsivas a andrógenos, o que poderia justificar o aumento da apoptose e parada do ciclo celular observados nesta linhagem no Capítulo I. Já nas linhagens LNCaP e DU145, o tratamento com este fitoquímico reduziu a expressão do miR99a, o que segundo trabalhos já citados, poderia estar relacionado com aumento de proliferação celular. No entanto, no Capítulo I nós mostramos que o γ-orizanol reduziu a proliferação em todas as linhagens, contrariando os resultados de Sun (2011).

Alguns miRNAs mostram expressão aumentada no câncer de próstata. A redução da expressão do miR24 está relacionada ao aumento da apoptose e redução da proliferação celular no câncer de próstata (Qin, Shi et al. 2010). Qin e colaboradores (Qin, Shi et al. 2010) mostraram que, provavelmente, estes efeitos estão associados ao aumento da ativação da caspase-8. Além dele, a superexpressão do miR222 também está correlacionada com o aumento da

agressividade no câncer de próstata, causando aumento da progressão do ciclo celular da fase G1 para S (Galardi, Mercatelli et al. 2007). Um estudo anterior mostrou que este miRNA também se encontra mais expresso em células não-responsivas a andrógenos (PC3) do que em células menos agressivas, como LNCaP (Galardi, Mercatelli et al. 2007). Porém, neste trabalho nós não observamos diferença no padrão de expressão deste miRNA nestas duas linhagens celulares (p=0.2613). O γ-orizanol reduziu a expressão deste miRNA nessas células, o que pode estar relacionado com a parada do ciclo celular em G2 mostrada no Capítulo I.

O tratamento com γ-orizanol foi capaz de reduzir a expressão do miR24b-1 nas células não-responsivas a andrógenos PC3. Além disso, no Capítulo I nós mostramos que este fitoquímico aumentou a apoptose nessa linhagem celular, confirmada pela ativação de caspases, fato que corrobora estudos anteriores que sugerem que os efeitos benéficos do miR24 sobre o câncer de próstata estão associados ao aumento da ativação de caspases, como a caspase-8 (Qin, Shi et al. 2010).

Porém, alguns miR tem mostrado comportamento dualístico no câncer. O miR182-5p têm se mostrado um útil marcador de agressividade do câncer de próstata, uma vez que se correlaciona com o Escore de Gleason (GS) e sua superexpressão parece correlacionar-se com o aumento da malignidade nesta doença (maior GS) (Tsuchiyama, Ito et al. 2013). Porém, outros autores mostram que este miRNA causa supressão da proliferação celular em câncer de pulmão e gástrico (Sun, Fang et al. 2010; Zhang, Liu et al. 2011; Kong, Bai et al. 2012) e reduz o potencial de invasão do câncer de próstata *in vitro* (Rasheed, Teo et al. 2013). Estes resultados sugerem que o miR182 também poderia agir como um

supressor tumoral (Peng, Li et al. 2013). Já a expressão do miR98 tem correlação com câncer de próstata de alto risco (GS ≥ 8) (Walter, Valera et al. 2013), porém, ele se encontra superexpresso somente em células de câncer de próstata hormônio refratárias e estudos em células de carcinoma hepatocelular mostram que ele atua inibindo a migração e invasão (Porkka, Pfeiffer et al. 2007; Tan, Li et al. 2011). O tratamento com γ-orizanol diminuiu a expressão do miR182-5p nas linhagens de câncer de próstata LNCaP e DU145 e reduziu a expressão do miR198 na linhagem PC3. No Capítulo I, mostramos a inibição da proliferação e biomassa celular nas três linhagens de câncer. Logo, a redução da expressão do miR182-5p nas células LNCaP e DU145 e do miR198 nas PC3 pode ter relação com esses efeitos, sugerindo uma possível ação antitumoral do γ-orizanol mediada por miRNAs. Os efeitos sobre a expressão do miR182 estão de acordo o descrito por Peng, Li et al. (2013).

Outros miRNAs podem atuar na forma de clusters, onde um grupo de genes gera um único transcrito primário que após o processamento pode dar origem a vários miRNAs maduros (Lee, Jeon et al. 2002). Um dos clusters mais estudados e envolvidos no câncer é o *cluster miR-17-92*, que codifica seis diferentes miRNAs: miR17, miR18a, mir19a, miR20a, miR19b e miR92; e é considerado potencialmente oncogênico (Hayashita, Osada et al. 2005; Mendell 2005). O tratamento com 16 μM de γ-orizanol reduziu a expressão do miR19b-2 nas linhagens não-responsivas a andrógenos DU145 e PC3. Este seria um resultado interessante uma vez que este cluster e seus miRNAs estão envolvidos na redução da morte e ao estimulo da proliferação celular (He, Thomson et al. 2005; O'Donnell, Wentzel et al. 2005; Dews, Homayouni et al. 2006; Ventura, Young et al. 2008).

Assim, nós mostramos nos Capítulos I e II, que o γ-orizanol foi capaz de efetivamente reduzir a proliferação e biomassa celular nas três linhagens de câncer de próstata (LNCaP, DU145 e PC3), provavelmente associado ao aumento da atividade das caspases que gerou aumento de apoptose na linhagem não-responsiva a andrógenos PC3; e parada do ciclo celular e ativação da pERK1/2 nas linhagens LNCaP (responsiva a andrógenos) e DU145 (não-responsiva a andrógenos). Ainda, este fitoesterol reduziu a expressão do gene e proteína Cav-1, um marcador de agressividade no câncer de próstata, nas linhagens não-responsivas a andrógenos. Além do mais, γ-orizanol também mostrou ser capaz de regular a expressão de genes de RNA não-codificantes, como o lncRNA específico da próstata PCGEM1 e diversos outros miRNAs, importantes no desenvolvimento, progressão e invasão da doença. Esses resultados sugerem que este fitoesterol poderia ser um importante candidato para o desenvolvimento de novas drogas mais eficazes para o tratamento do câncer de próstata avançado, e poderia também ser importante no tratamento inicial, substituindo terapias bem estabelecidas para esta patologia, mas que causam uma série de efeitos colaterais para os pacientes que a usam.

O extrato hidroalcólico de *Thuya occidentalis* (*T. occidentalis*) também parece ser promissor para o tratamento desta patologia. Embora seja amplamente utilizado na medicina popular ((HAB) 1985; (HAB) 2003; Naser 2005), são raros os estudos sobre o efeito deste extrato no câncer de próstata, e este foi o assunto discutido no Capítulo III desta tese.

Como no caso do γ-orizanol, as células responsivas a andrógenos LNCaP parecem ter maior sensibilidade ao tratamento com *T. occidentalis* (0.05 mL extrato/mL de meio) do que as não-responsivas a andrógenos. Uma hora de

tratamento de células LNCaP com 0,05 mL extrato/mL de meio provocou a mesma diminuição na biomassa celular que 24 h de tratamento nas células DU145 e PC3. Este resultado parece ser compatível com a progressão da doença, pois na fase responsiva a andrógenos, representada pela linhagem LNCaP, o tumor parece ser mais sensível às drogas disponíveis do que na fase avançada (Caffo 2015).

O tratamento com *T. occidentalis* foi capaz de reduzir a proliferação e biomassa celular em todas as linhagens estudadas (LNCaP, DU145 e PC3). Nós também observamos aumento da necrose e/ou apoptose em todas as linhagens celulares, sugerindo que estes devem ser os principais mecanismos tóxicos ou associados a morte celular causados pelo tratamento com *T. occidentalis*.

Muitos estudos tem sugerido que morte por apoptose deve ser preferencialmente escolhida como mecanismo para matar seletivamente as células de câncer, durante o desenvolvimento de novas drogas (Lowe and Lin 2000; Brown and Attardi 2005). Porém, atualmente a morte por necrose também tem sido sugerida como um processo benéfico no tratamento desta doença. O quadro inflamatório associado a este tipo de morte celular tem a vantagem de estimular a resposta imune e alguns autores tem mostrado que o recrutamento das células do sistema imune poderia aumentar a eficácia das drogas anticâncer (Edinger and Thompson 2004; Okada, Adachi et al. 2004). Dessa maneira, a morte por necrose estimulada pelo extrato hidroalcólico de *T. occidentalis* seria um importante mecanismo a ser considerado no desenvolvimento de novas alternativas para o tratamento do câncer de próstata.

Na linhagem de câncer de próstata não-responsiva a andrógeno DU145 nós observamos parada do ciclo celular na fase G0/G1. Alterações no ciclo celular

estão claramente ligadas ao desenvolvimento de câncer (Collins, Jacks et al. 1997) e a frequente perda da correta regulação do ciclo celular tem sido alvo para possíveis intervenções terapêuticas (Chen, Sharma et al. 1999). Além disso, anormalidades nos pontos de checagem G1-S são importantes no desenvolvimento de tumores (Hartwell and Kastan 1994). Ainda, estas vias parecem ser determinantes para as respostas causadas por diversos tipos de agentes citotóxicos usados para tratar tumores e são sugeridas como alternativas para tratar de forma mais eficaz o câncer (Kastan and Bartek 2004). Assim, a parada do ciclo celular observada nessa linhagem celular é importante e sugere que a *T. occidentalis* teria potencial no tratamento do câncer de próstata.

Como discutido anteriormente no Capítulo I desta tese, a Cav-1 é um importante marcador de agressividade no câncer de próstata (Yang, Truong et al. 1999). Além disso, seu gene parece ser superexpresso na metástase (Nasu, Timme et al. 1998) e contribui com a progressão da doença para a fase hormônio refratária, correlacionando-se com prognóstico desfavorável nesta neoplasia (Tahir, Frolov et al. 2006; Freeman, Yang et al. 2012). O tratamento com extrato de *T. occidentalis* reduziu a expressão do gene e da proteína Cav-1 nas linhagens de câncer de próstata não-responsivas a andrógenos DU145 e PC3. A redução da expressão da Cav-1 seria capaz de transformar células não-responsivas a andrógenos no fenótipo responsivo novamente (Nasu, Timme et al. 1998). Além disso, uma vez que a alta expressão da Cav-1 pode favorecer a progressão da doença para estágios mais avançados (Williams, Hassan et al. 2005) e que o tratamento do câncer de próstata nessa fase costuma ser ineficaz (Scott, Menon et al. 1980; Eisenberger, Blumenstein et al. 1998), a *T. occidentalis* pode vir a ser uma alternativa interessante de terapia.

Porém, a *T. occidentalis* é um extrato e sendo assim, diversos compostos poderiam ser os responsáveis pelos efeitos observados após o tratamento. Alguns autores tem mostrado diversos efeitos associados a α-tujona - uma cetona monoterpênica presente no óleo essencial da *Thuya* (Chang, Song et al. 2000; Naser 2005, Hold, Sirisoma et al. 2000; Czyzewska and Mozrzymas 2013), e nós investigamos se este composto não estaria causando os efeitos antiproliferativos nas linhagens de câncer usadas neste trabalho. A análise por Cromatografia Gasosa acoplada a Detector de Massas detetectou a presença de 0.0016 µg de α-tujona/ml de meio de cultura e o teste dessa quantidade de α-tujona nas linhagens de câncer somente mostrou efeitos na LNCaP. Esses resultados corroboram os resultados anteriores que sugerem que esta linhagem é mais sensível ao tratamento do que as não-responsivas a andrógenos.

Em resumo, o extrato hidroalcólico de *T. occidentalis* também pareceu ser promissor no tratamento do câncer de próstata. Além disso, este estudo esclareceu alguns dos mecanismos pelos quais este extrato amplamente usado na medicina popular pode estar exercendo seus efeitos, podendo ser utilizado, no futuro, como uma nova estratégia de tratamento para esta patologia.

Nos três estudos conduzidos nesta tese, nós mostramos os efeitos do γ-orizanol e do extrato de *T. occidentalis* sobre linhagens de câncer de próstata metastáticas. O fato de não haver muitas alternativas de terapias curativas disponíveis para esta fase da doença justifica o uso destas linhagens, ainda mais na fase hormônio refratária, amplamente discutida no decorrer deste trabalho. E embora as três linhagens usadas sejam metastáticas, nossos resultados mostram comportamentos diferentes entre elas. Essa característica já foi relatada por outros autores (Stone, Mickey et al. 1978; Kaighn, Narayan et al. 1979; Ifere, Barr

et al. 2009) e pode ser explicada pelo fato dessas linhagens serem provenientes de metástases em órgãos distintos e apresentarem potencial metastático, agressividade e sensibilidade a andrógenos diferentes (Stone, Mickey et al. 1978; Kaighn, Narayan et al. 1979; Horoszewicz, Leong et al. 1980; Horoszewicz, Leong et al. 1983).

Portanto, apesar de mostrar efeitos promissores quanto ao tratamento do câncer de próstata, a necessidade de se entender melhor outros alvos de ação do γ-orizanol e do extrato de *T. occidentalis* nas linhagens de câncer de próstata responsivas e não-responsivas a andrógenos ou em outros modelos que representem outras fases da doença e também, em células normais de próstata, é evidente. Assim, outros estudos são necessários para obtermos respostas mais amplas que possibilitem a utilização do γ-orizanol e do extrato de *T. occidentalis* no tratamento desta patologia, principalmente no que abrange seus possíveis efeitos tóxicos. Porém, atualmente estão disponíveis muito poucos estudos sobre o efeito desses compostos no câncer de próstata e em outros tipos de câncer, e aqui nós pudemos mostrar que esses compostos se mostram promissores no tratamento desta patologia.

III.2 CONCLUSÕES

No presente trabalho, nós mostramos os efeitos do γ-orizanol e do extrato hidroalcólico de *Thuya occidentalis*, que indicam seu potencial como uma alternativa mais eficaz e com menos efeitos colaterais de tratamento para o câncer de próstata.

Em resumo, nesta tese nós demonstramos que o γ-orizanol causou redução da viabilidade e biomassa celular em cultura nas três linhagens de câncer de próstata. Na PC3, o tratamento com γ-orizanol causou aumento da morte celular por apoptose, acompanhado da ativação de caspases; enquanto na LNCaP (responsiva a andrógeno) e na DU145 (não-responsiva a andrógeno) só observamos aumento de necrose, porém este fato foi acompanhado de aumento da pERK1/2. Ainda, observamos parada do ciclo em G0/G1 (DU145) ou G2 (PC3 e LNCaP), além de redução da expressão do gene de RNA não-codificante PCGEM1. Diversos miRNAs também tiveram suas expressões alteradas após o tratamento com γ-orizanol. miR16-1, miR19b-2, miR24b-1, miR24b-2, miR99a, miR133a-5p, miR-182-5p, miR198 e miR222 são exemplos de miRNAs que tiveram a expressão modulada pelo tratamento com 16 μM de γ-orizanol e que tem funções relacionadas ao câncer de próstata, mostrando serem potencialmente benéficos para o câncer de próstata.

Já o tratamento com extrato de *Thuya occidentalis* mostrou ter efeito antiproliferativo nas três linhagens avaliadas neste estudo. Nós observamos aumento da morte celular por necrose e/ou apoptose em todas as linhagens celulares, acompanhado de parada do ciclo celular em G0/G1 na DU145, e detectamos a presença de 0,0016 μg da cetona monoterpênica α-tujona na dose de extrato usada neste estudo. O tratamento com essa quantidade de α-tujona foi

efetivo, porém, somente sobre linhagem LNCaP, reforçando a hipótese da diferença de sensibilidade entre as linhagens responsivas e não responsivas a andrógeno e mostrando a contribuição de outros componentes do extrato nos efeitos observados.

Associado a esses achados, tanto o γ-orizanol quanto o extrato de *Thuya occidentalis* reduziram a expressão do gene e da proteína Cav-1 nas linhagens de câncer de próstata não-responsivas a andrógenos (DU145 e PC3) e a redução da sua expressão poderia ser uma estratégia importante usada no desenvolvimento de novas terapias para tratar esta patologia, pois Cav-1 está associada ao desenvolvimento e progressão do câncer de próstata para fase metastática e hormônio refratária (Williams, Hassan et al. 2005; Tahir, Frolov et al. 2006; Freeman, Yang et al. 2012).

Em conjunto, considerando que a linhagens celulares utilizadas nesta tese são um modelo patológico de estudo amplamente usado, estes resultados sugerem que tanto o γ-orizanol quanto o extrato de *Thuya occidentalis* são candidatos promissores para o desenvolvimento de futuras terapias anticâncer, por atuarem na modulação de alvos moleculares, como os genes de ncRNA; ou modulando vias importantes em neoplasias, como ciclo celular, necrose, apoptose, entre outras. No entanto, testes em modelos que representem outras fases da doença e sobre a toxicidade desses compostos são relevantes. Além disso, mais estudos são necessários para avaliar os efeitos e mecanismos envolvidos na ação do γ-orizanol sobre os miRNAs avaliados neste estudo.

III.3 PERSPECTIVAS

Em relação ao apresentado nesta tese, ainda resta uma grande perspectiva de aprofundar o estudo sobre os efeitos do γ-orizanol e do extrato de *Thuya occidentalis* sobre o câncer de próstata, especialmente quanto aos seus efeitos tóxicos. Neste sentido, seus efeitos sobre as linhagens celulares não metastáticas e de próstata normal devem ser avaliados para sugerir seus potenciais usos como drogas no tratamento do câncer de próstata. Da mesma forma, aprofundar os estudos sobre os mecanismos moleculares envolvidos nos efeitos observados nesta tese e que determinam a ação das mesmas sobre a patologia tratada neste trabalho, surgem como outra perspectiva importante. Além destas metas, um estudo mais aprofundado dos miRNAs discutidos aqui deve ser realizado, com o objetivo de confirmar as hipóteses sugeridas anteriormente e procurando elucidar quais os mRNAs alvo afetados por esses miRNAs. Ainda, técnicas que envolvem o silenciamento e superexpressão dos miRNAs aqui citados devem ser utilizados para confirmar os efeitos dos mesmos sobre funções celulares relacionadas ao desenvolvimento, invasão e progressão do câncer de próstata. Quanto ao estudo do potencial do γ-orizanol e do extrato de *Thuya occidentalis* sobre o câncer de próstata, avaliar os efeitos dose-dependentes em células primárias e em um modelo animal faz-se necessário. Sob este aspecto, tanto os efeitos citotóxicos podem ser explorados/estudados para o tratamento de indivíduos com esta patologia, quanto os efeitos quimiopreventivos desses fitoterapicos podem ser avaliados em modelos animais que possam ser induzidos a desenvolver esta patologia.

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ANEXOS

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