PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

EFEITO DO EXTRATO DE GUARANÁ (*Paullinia Cupana*) EM CULTURA DE CÉLULAS T24 DO CÂNCER DE BEXIGA

Aluna: Eliza Ribas da Silveira Flôres
Orientadora: Claudia Giuliano Bica
Coorientadoras: Fernanda Bueno Morrone
Ivana Beatrice Mânica Da Cruz

Dissertação submetida ao Programa de Pós-Graduação em Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

2014

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

EFEITO DO EXTRATO DE GUARANÁ (*Paullinia cupana*) EM CULTURA DE CÉLULAS T24 DO CÂNCER DE BEXIGA

Aluna: Eliza Ribas da Silveira Flôres
Orientadora: Claudia Giuliano Bica
Coorientadoras: Fernanda Bueno Morrone
Ivana Beatrice Mânica Da Cruz

Dissertação submetida ao Programa de Pós-Graduação em Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

2014

Catalogação na Publicação

Flôres, Eliza Ribas da Silveira
Efeito do extrato de guaraná (Paullinia Cupana) em cultura de células T24 do câncer de bexiga / Eliza Ribas da Silveira Flôres. -- 2014.
69 p. : il., graf., tab. ; 30 cm.

Dissertação (mestrado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Patologia, 2014.

Orientador(a): Profa. Dra. Claudia Giuliano Bica ; coorientador(a): Profa. Dra. Fernanda Bueno Morrone, Profa. Dra. Ivana Beatrice Mânica Da Cruz.

1. Guaraná. 2. Câncer de Bexiga. 3. Células T24. 4. Cafeína. 5. Quimioterápicos. I. Título.

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

Dedicatória

Dedico esta conquista a Deus, aos meus pais, ao meu marido, ao meu irmão, aos meus amigos e aos demais incentivadores deste trabalho.

AGRADECIMENTOS

A Deus, Aquele que me criou e que de forma incondicional sempre está ao meu lado me orientando e protegendo.

Aos meus queridos pais Marly Ribas da Silveira Flôres e Francisco Cândido da Silva Flôres, que sempre me incentivaram a trilhar o caminho do conhecimento alicerçando meus passos na ética e na sabedoria.

Ao meu amado marido, Ewerton Freitas Silva, por seu amor paciente, que soube compreender as minhas ausências e sempre me incentivou para atingir meus objetivos.

Ao meu irmão, Gustavo Ribas da Silveira Flôres, que mesmo com a distância, muito me serviu de inspiração, principalmente na oratória.

A minha família em geral, a qual amo muito. Obrigada pelo carinho e incentivo!

A minha orientadora, Profa. Dra. Claudia G. Bica que me acolheu de braços abertos na UFCSPA e que irrestritamente se dispôs a me ajudar nessa jornada dando conselhos profissionais e pessoais.

A minha coorientadora Profa. Dra. Ivana B. M. Cruz, que abriu as portas do mundo científico. Obrigada pelo incentivo, confiança e apoio absoluto em cada passo da minha trajetória, me atendendo até mesmo em sua própria casa.

A minha coorientadora Profa. Dra. Fernanda B. Morrone, pelo grande apoio em mim depositado.

A todos do Laboratório Biogenômica da UFSM (“minha família científica”), por todo apoio, em especial agradeço aos meus padrinhos de

casamento Francine C. Cadoná e Alencar K. Machado, os quais muito me auxiliaram mesmo quando distantes.

A todos os Professores da Universidade Federal de Ciências da Saúde de Porto Alegre/RS agradeço pelos conhecimentos compartilhados.

Aos funcionários do PPG, pelo grande apoio dedicado aos alunos, seja este de cunho profissional como também pessoal. Em especial agradeço as funcionárias Terezinha Stain, Rosalva Meurer, Kely Cristine Reiter, Maristela Pasin, Luciana Espencer e Isabel Cristina Moura, que muito me ajudaram, mesmo quando eu ainda estava em Santa Maria.

Aos colegas pelo companheirismo na realização das atividades propostas.

A todos os meus colegas do PPG-Patologia, em especial a Alana D. Ranzi, Giovana T. dos Santos, Stephanie D. Rocha, Munique Mendonça e Jaqueline L. Machado. Agradeço pela parceria na realização de atividades científicas e nos momentos de descontração.

A Fundação de Amparo à Pesquisa do Estado do Amazonas (FEPEAM), a Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), a Coordenação de Aperfeiçoamento de Pessoa de Nível Superior (CAPES) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), agradeço pelo apoio financeiro.

Em fim, agradeço a todos aqueles que de alguma forma, cooperaram para a realização deste trabalho.

No Norte cultivado,
No Sul estudado.
Sucesso em Maués e suas comunidades
Impressiona por suas propriedades:

Tem teobromina, tem!
Tem teofilina, tem!
Têm catequinas, também têm!

E de tanta cafeína
Até o café se humilha
Frente a esse fruto misterioso
Oh Guaraná Majestoso!

Guaraná Majestoso, de Eliza Ribas da Silveira Flôres.

SUMÁRIO

I. Lista de abreviaturas

ANVISA: Agência Nacional de Vigilância Sanitária.

ATCC: American Tissue Cell Culture.

Ca= Carcinoma Papilífero.

CTT: Carcinoma de Células Transicionais

DCFH-DA: Diacetato de 2 '-7'-diclorofluoresceína.

DNA: Ácido Desoxirribonucléico.

dsDNA: dupla fita de DNA.

EGCG: Epigalocatequina-3-galato.

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

NUPBPM: Neoplasia Urotelial Papilífera de Baixo Potencial de Malignidade

OMS: Organização Mundial da Saúde.

ROS: Espécies Reativas de Oxigênio.

SIPU: Sociedade Internacional de Patologia Urológica.

SUS: Sistema Único de Saúde.

II. Resumo

Introdução: Vários estudos têm indicado que compostos bioativos presentes em frutos e vegetais possuem atividade anticarcinogênica. Este é o caso das catequinas e cafeínas que são encontradas em alimentos como o chá verde, o café e o chocolate. Tais componentes também foram previamente identificados no guaraná [*Paullinia cupana* var. *sorbilis* (Mart.)]. Investigações *in vitro* mostraram que o extrato de guaraná, uma fruta amazônica rica em cafeína, tem atividade antitumoral em células de melanoma e no câncer hepático. Entretanto, se tal planta age sobre outras neoplasias como é o caso do câncer de bexiga ainda é uma questão a ser elucidada. **Objetivo:** avaliar o possível efeito anticarcinogênico do extrato de *Paulinia cupana* em linhagem T24 de células do câncer de bexiga. **Material e métodos:** células T24 foram expostas a diferentes concentrações de guaraná (3,8-30.000 µg/mL) para avaliar o efeito sobre a viabilidade e a taxa proliferativa com os quimioterápicos metotrexato, cisplatina e gencitabina como controles positivos. O efeito sobre a apoptose também foi avaliado a partir da quantificação de caspases (1, 3 e 8). Também foram avaliadas as espécies reativas de oxigênio (ROS) frente a esse extrato. **Resultados:** semelhante aos quimioterápicos, o guaraná apresentou um efeito hermético em células T24. Nas concentrações mais baixas do guaraná, ocorreu uma diminuição da viabilidade e da proliferação celular, e um aumento da atividade das caspases 1, 3 e 8 e dos níveis de ROS. Já nas concentrações mais elevadas do guaraná ocorreu justamente o contrário. **Conclusão:** os resultados descritos aqui sugerem que alimentos ricos em metilxantinas e cafeína como o guaraná poderiam apresentar um duplo efeito.

1. INTRODUÇÃO

1.1 Compostos Bioativos

Muitos compostos bioativos encontrados nos alimentos são responsáveis por efeitos benéficos aos indivíduos que os consomem. É crescente a procura investigativa pelo entendimento da atuação desses compostos no tratamento e prevenção de diversas doenças, entre elas destaca-se o câncer, doença que tem se tornado cada vez mais popular pela sua alta incidência.

Estudos epigenéticos têm demonstrado que compostos bioativos da dieta exercem efeitos anticâncer como é o caso da vitamina D3 que pode ter sua atividade parcialmente ligada a atuar como um agente quimiopreventivo e anticarcinogênico (Dace e cols., 1997; Guy e cols., 2004; Palmer e cols., 2003). Alguns polifenóis como as catequinas muito frequente em chás como no chá verde [catequina, epicatequina, (-)-epigalocatequina-3-galato (EGCG)] e os polifenóis do café (ácido clorogênico) também constituem uma excelente abordagem na prevenção do câncer e potencialmente na terapia anticâncer (Mittal e cols., 2003; Lu e cols., 2006; Fang e cols., 2007).

1.2 *Paullinia cupana* var. sorbilis (Mart.)

O guaraná [*Paullinia cupana* var*. sorbilis (*Mart.)] tem origem amazônica, sendo cultivada principalmente na região de Maués, cidade conhecida como *"A Capital do Guaraná".* A semente do guaraná é a parte utilizável do fruto dando

origem a um pó produzido através de processo de torrefação e moagem (Henman, 1982; Correia, 1984). Esse produto é consumido como um suplemento alimentar aprovado pelo Ministério da Saúde através da Agência Nacional de Vigilância Sanitária (ANVISA).

Amplamente conhecido no contexto de medicina popular, essa planta tem despertado a curiosidade de pesquisadores nas últimas décadas devido a suas propriedades.

1.2.1 Propriedades

O guaraná apresenta compostos bioativos como as metilxantinas e as catequinas, dentre as metilxantinas está presente à cafeína, a teobromina e a teofilina (Antonelli-ushirobira e cols., 2004).

A cafeína encontrada em 63 diferentes espécies de plantas está presente no guaraná três vezes mais do que no café, a qual é totalmente absorvida no trato intestinal sendo distribuída para todos os tecidos corporais uma vez que tem a capacidade de cruzar a barreira hemato-encefálica, testicular e barreira placentária (Tfouni e cols., 2007, Andrews e cols., 2007). É uma substância psicoativa mais usada no mundo, pois atua no sistema nervoso central, aumentando a vigilância e a pressão arterial (Mitchell e cols., 2011).

A teofilina encontrada principalmente no chá permite relaxamento da musculatura lisa dos brônquios, estimulação do sistema nervoso central, do músculo cardíaco e diurese. Já a teobromina apresenta atividade vasodilatadora, sendo utilizada como antiasmático e cardioestimulante (Belliardo e cols., 1985; Salvadore e cols., 1994). Além desses compostos, o

guaraná também apresenta teofilina, epicatequinas, taninos, catequinas, saponinas, pró-antocianinas e outros compostos (Salvadore e cols., 1994; Carlson e Tompson, 1998).

Dessa forma, a suplementação com guaraná age farmacologicamente sobre a modulação dos metabolismos: energético (Smith e Atroch, 2007), oxidativo-vascular, inflamatório, lipídico-glicêmico (Fukumasu e cols., 2008) e plaquetário (Bydlowski e cols., 1988).

Pelo fato de o guaraná apresentar essas propriedades, o mesmo vem sendo utilizado como afrodisíaco, antitérmico, analgésico e antidiarréico (Antonelli-ushirobira e cols., 2004).

Estudos prévios têm relatado o potencial antioxidante do guaraná (Mattei e cols., 1998; Basile e cols., 2005; Jimoh e cols., 2007). De acordo com o estudo de Basile e cols. (2005) o guaraná possui efeitos antioxidantes, já que diminui a peroxidação lipídica causada por agentes pró-oxidantes.

1.2.2 Guaraná e câncer

Poucos são os estudos que relacionam extratos da planta popularmente conhecida como guaraná [*Paullinia cupana* var. sorbilis (Mart.)] com algum tipo de neoplasia. Entretanto, cabe destacar os estudos de Fukumasu e colaboradores (2006a, 2006b, 2008, 2010) com suas pesquisas associando as propriedades do guaraná com algumas neoplasias.

Nesses estudos são relatados tanto efeitos protetores (antimutagênicos e antitumorais) do guaraná frente ao dano no DNA (Fukumaso e cols., 2006a), como a efeitos quimiopreventivos em fígados de ratos com

hepatocarcinogenese (Fukumasu e cols., 2006b). Posteriormente, foram identificados efeitos anticarcinogênicos através do bloqueio do crescimento de células tumorais de melanoma de pulmão (Fukumasu et al., 2008). Mais recentemente o guaraná foi identificado como um composto potencialmente terapêutico anticarcinogênico (Fukumasu e cols., 2010).

O guaraná também foi associado com a melhora da fadiga em pacientes com quadro de câncer de mama submetidos à quimioterapia sistêmica, visto que é um problema comum que pode impactar negativamente a qualidade de vida das pacientes (De Oliveira e cols., 2011).

Uma vez que o guaraná apresenta um conjunto de compostos ativos que podem ter efeitos antineoplásicos, investigações sobre este tema são de fundamental importância ainda mais em neoplasias que agravam o sistema de saúde como o câncer de bexiga.

1.3 Câncer de bexiga

1.3.1 Epidemiologia

O tumor de bexiga é uma das neoplasias mais comuns no mundo já ocupando o nono lugar de câncer mais comumente diagnosticado com alta taxa de incidência em países desenvolvidos e com uma maior representatividade em pessoas do gênero masculino (Ploeg e cols., 2009). Nos EUA, o câncer de bexiga é o quarto tumor de maior incidência em homens, sendo a nona causa de mortalidade por câncer em pessoas desse gênero, e o nono tumor de maior incidência em mulheres (Jemal e cols., 2005). Realidade semelhante é observada em outros países como no Brasil, onde se estima que

em 2012 surjam 8.900 casos novos de câncer de bexiga, sendo 6.210 em homens e 2.690 em mulheres, segundo dados do Instituto Nacional do Câncer (INCA). O Estado do Rio Grande do Sul tem uma taxa bruta estimada em 10,29 casos para cada 100 mil homens e de 3,76 casos para cada 100 mil mulheres. Já na capital, Porto Alegre, esse número passa para 16,40 em homens e 6,04 em mulheres, segundo o INCA.

Esses dados corroboram com o fato que, devido à alta incidência e complexidade no tratamento, são grandes os custos envolvidos com essa neoplasia (Dall'Oglio e cols., 2013). Essa condição é agravada devido ao grande número de recidivas, que acontecem em 43% nos portadores de tumores únicos e 73% nos portadores de tumores múltiplos sendo que pacientes com câncer de bexiga superficial recidivam de 50-75% no primeiro ano (Lopes e cols., 2013). Esse quadro de recidivas frequentes é responsável por onerar o Sistema Único de Saúde (SUS).

1.3.2 Fatores de risco e classificação

Alguns fatores de risco estão associados ao aparecimento desse tipo de neoplasia como gênero (masculino), idade, tipo de trabalho e o tabagismo que possui uma importante influência na ocorrência desse tipo de câncer (Kiriluk e cols., 2012), principalmente em homens onde se estima que o fumo esteja associado em 50% a 65% dos casos, enquanto para mulheres é de 20-30% (IARC, 2004; Dall'Oglio e cols., 2013). Além disso, são encontradas neoplasias secundárias como consequência de radioterapia pélvica e quimioterapia com

agentes irritantes à bexiga como a ciclofosfamida (Kumar e cols., 2008; Guimarães e Rosa, 2008).

Em decorrência desses fatores de risco, os tumores de bexiga podem variar de neoplasias epiteliais benignas que produzem projeções digitiformes ou verrucosas visíveis micro ou macroscopicamente, os papilomas, até tumores invasivos e metastáticos (Wood e cols., 2012). A camada mais interna que reveste o sistema urinário é o urotélio onde mais de 90% dos carcinomas de células transicionais são originados (Piazza e cols., 2001; Guimarães e Rosa, 2008; Guimarães, 2008; Kumar e Stricker, 2010). Apenas 5% dos tumores de bexiga são carcinomas de células escamosas (Kumar e cols., 2008) e 2% são adenocarcinomas (Guimarães, 2008). O estágio clínico dessa neoplasia é determinado pelo grau de invasividade, considerando o comprometimento de nódulos linfáticos regionais e a presença de metástases.

De maneira geral, o câncer de bexiga pode ser classificado em três tipos: carcinoma de células de transição (maioria dos casos e começa nas células do tecido mais interno da bexiga); carcinoma de células escamosas (afetam as células delgadas e planas que podem surgir na bexiga depois de infecção ou irritação prolongadas) e adenocarcinoma (que se inicia nas células glandulares e podem se formar na bexiga depois de um longo tempo de irritação ou inflamação).

Em 2004 foi proposto pela Sociedade Internacional de Patologia Urológica (SIPU) um novo sistema de classificação das neoplasias uroteliais (OMS, 2004) substituindo o sistema de classificação de 1973. A principal diferença nesse sistema consiste no agrupamento dos tumores ficando o grau I

como carcinoma urotelial papilífero de baixo grau e os graus 2 e 3 como carcinoma papilífero de alto grau (Figura 1).

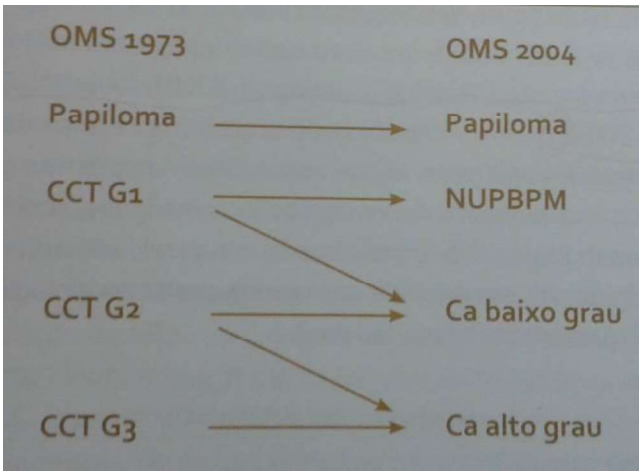

Figura 1: Comparação entre os sistemas de classificação de neoplasias uroteliais da Organização Mundial da Saúde, 1973 vs 2004. CTT= carcinoma de células transicionais, G= grau, NUPBPM = neoplasia urotelial papilífera de baixo potencial de malignidade, Ca= carcinoma papilífero (Dall'Oglio e cols., 2013).

Os tumores que surgem na bexiga urinária são classificados segundo o sistema TNM (Tumor, Nodo, Metástase) (Brasil, 2004) da *American Joint Committee on Cancer* (AJCC) (Figura 2, Tabela 1).

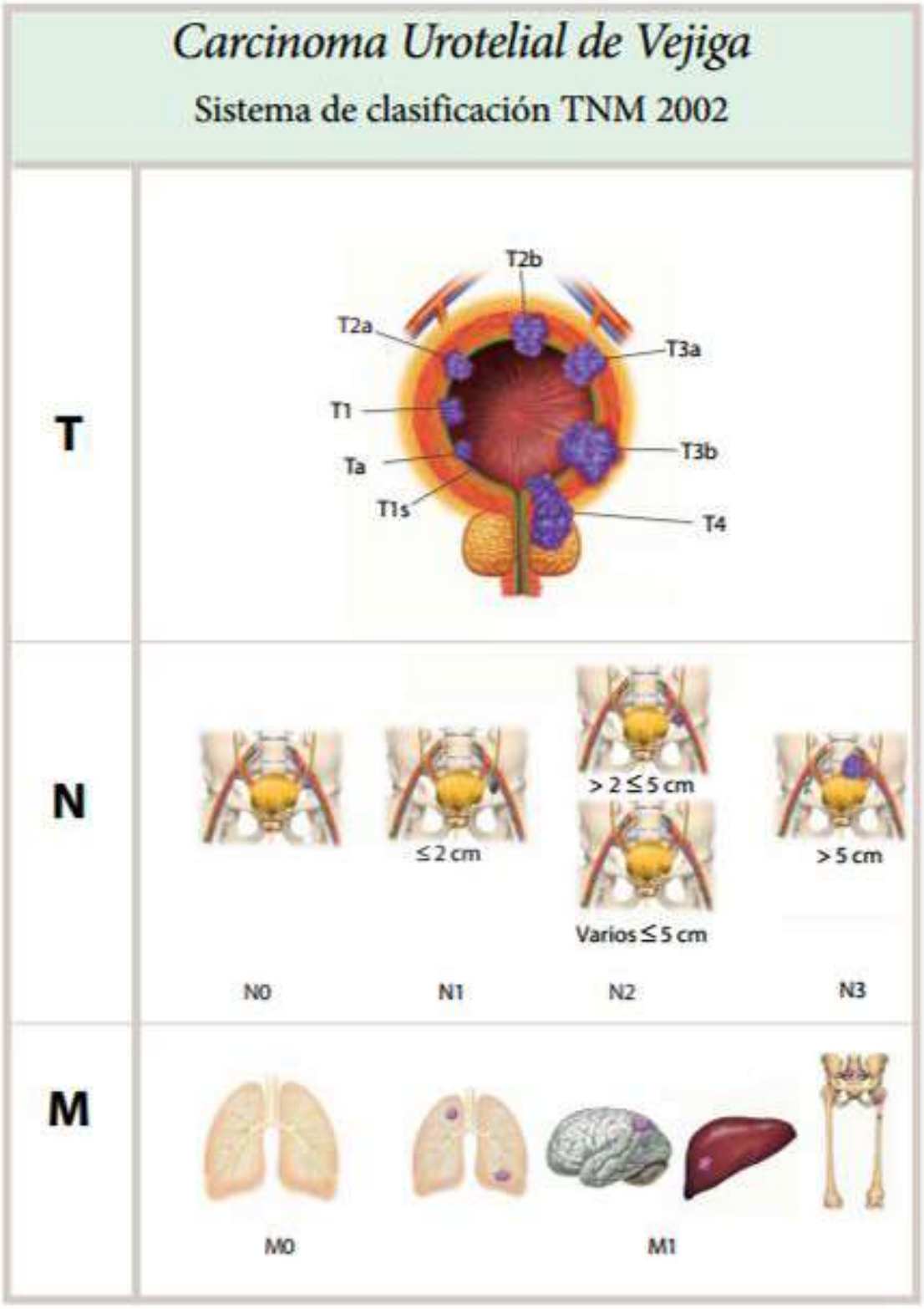

Figura 2: Classificação TNM (Pompeo e cols., 2007).

Tabela 1: Classificação TNM 2004.

Classificação TNM	
T	**Tumor Primário**
Tx	O tumor primário não pode ser avaliado
T0	Não há evidência de tumor primário
Ta	Carcinoma papilífero não invasivo
Tis	Carcinoma *in situ*: tumor plano
T1	Tumor que invade o tecido conjuntivo sub-epitelial
T2	Tumor que invade o músculo
T2a	Tumor que invade a musculatura superficial (metade interna)
T2b	Tumor que invade a musculatura profunda (metade externa)
T3	Tumor que invade tecido perivesical
T3a	Microscopicamente
T3b	Macroscopicamente (massa extravesical)
T4	Tumor que invade qualquer uma das seguintes estruturas: próstata, útero, vagina, parede pélvica ou parede abdominal
T4a	Tumor que invade próstata, útero ou vagina
T4b	Tumor que invade parede pélvica ou parede abdominal
N	**Linfonodos Regionais**
Nx	Os linfonodos regionais não podem ser avaliados
N0	Ausência de metástase em linfonodo regional
N1	Metástase, em um único linfonodo, com 2cm ou menos em sua maior dimensão
N2	Metástase, em um único linfonodo, com mas de 2cm até 5cm em sua maior dimensão, ou em múltiplos linfonodos, nenhum com mais de 5cm em sua maior dimensão
N3	Metástase em linfonodo com mais de 5cm em sua maior dimensão
M	**Metástase à distância**
MX	A presença de metástase à distância não pode ser avaliada
M0	Ausência de metástase à distância
M1	Metástase à distância

1.3.3 Cultura de células em câncer

O uso de linhagens para o cultivo celular tem sido amplamente utilizado em diversas pesquisas facilitando análises de propriedades biológicas e de processos biológicos que dificilmente poderiam ser realizados *in vivo*. Nesse

âmbito, destacam-se os estudos realizados com linhagens celulares neoplásicas, uma vez que são as principais ferramentas para estudos do câncer.

A partir de linhagens neoplásicas é possível compreender melhor sobre a carcinogênese, angiogênese e sobre a expressão gênica das células neoplásicas (Ross e Perou, 2001; Yamori, 2003; Kim e cols., 2004). Isso é fundamental no entendimento dos eventos biológicos das neoplasias.

O American Tissue Cell Culture (ATCC) é o principal banco privado de recursos biológicos que viabiliza a aquisição, autenticação, produção e desenvolvimento de linhagens celulares. Esse banco mantém cerca de 4.000 linhas de células que são de valor inestimável para a pesquisa em saúde pública, incluindo modelos de câncer, como HeLa, MCF7, HT29, entre outras.

No caso do câncer de bexiga, utiliza-se a linhagem T24 que é derivada de um tumor de grau 3 (T3, tumor que invade o tecido perivesical, segundo o sistema TNM), pouco diferenciada e com alta invasividade. Essas características possibilitam que a linhagem T24 seja usada como modelo de tumor invasivo de bexiga (Kim e cols., 2004; Ashfaque e cols., 2005).

1.4 Justificativa

Frente ao crescente avanço das neoplasias em nosso país busca-se cada vez mais novas fontes de tratamento, onde se destacam fontes exógenas provenientes da dieta. Nesse contexto insere-se o guaraná (*Paullinia cupana*) com um possível efeito antineoplásico em células de linhagem T24 podendo vir a contribuir na prevenção e tratamentos do câncer de bexiga. Destaca-se ainda a importância no estudo dessa neoplasia, uma vez que o câncer de bexiga é

uma das neoplasias que mais oneram o sistema de saúde devido as constantes recidivas.

1.5 Referências Bibliográficas

Antonelli-ushirobira TM, Yamaguti E, Uemura LM, Mello JCP. Controle de qualidade de amostras de *Paullinia cupana* H.B.K. var. sorbilis (Mart.) Ducke. Acta Farmaceutica Bonaerense. 2004;23(3):383-6.

Andrews KW, Schweitzer A, Zhao C, Holden JM, Roseland JM, Brandt M, et al. The caffeine contents of dietary supplements commonly purchased in the US: analysis of 53 products with caffeine-containing ingredients. Anal Bioanal Chem. 2007 Sep;389(1):231-9.

Basile A, Ferrara L, Pezzo MD, Mele G, Sorbo S, Bassi P, et al. Antibacterial and antioxidant activities of ethanol extract from *Paullinia cupana* Mart. J Ethnopharmacol. 2005 Oct;102(1):32-6.

Belliardo F, Martelli A, Valle MG. HPLC determination of caffeine and theophylline in *Paullinia cupana* Kunth (guarana) and Cola spp. Samples. Lebensm Unters Forsch. 1985;180:398-401.

Bittencourt LS, Machado DC, Machado MM, Dos Santos GFF, Algarve TD, Marinowic DR, et al. The protective effects of guaraná extract (*Paullinia cupana*) on fibroblast NIH-3T3 cells exposed to sodium nitroprusside. Food Chem Toxicol. 2013 Mar;53:119-25.

BRASIL, Ministério da Saúde. TNM: classificação de tumores malignos. 6. ed. Rio de Janeiro: INCA; 2004.

Bydlowski SP, Yunker RL, Subbiah MT. A novel property of an aqueous guaraná extract (*Paullinia cupana*): inhibition of platelet aggregation in vitro and *in vivo*. Braz J Med Biol Res. 1988;21(3):535-8.

Carlson M, Thompson RD. Liquid chromatographic determination of methylxanthines and catechins in herbal preparations containing guaraná. AOAC International. 1998;81:691-701.

Correia P. Dicionário das plantas úteis do Brasil. Rio de Janeiro: Imprensa Nacional; 1984.

Dace A, Martin-el Yazidi C, Bonne J, Planells R, Torresani J. Calcitriol is a positive effector of adipose differentiation in the OB 17 cell line: relationship with the adipogenic action of triiodothyronine. Biochem Biophys Res Commun. 1997;232:771-6.

Dall'Oglio M, Crippa A, Srougi M. Câncer de bexiga. São Paulo: Santos; 2013.

De Oliveira Campos MP, Riechelmann R, Martins LC, Hassan BJ, Casa FB, Del Giglio A. Guarana (*Paullinia cupana*) improves fatigue in breast cancer patients undergoing systemic chemotherapy. J Altern Complement Med. 2011;17:505-12.

Ellis EM. Reactive carbonyls and oxidative stress: potential for therapeutical intervention. Pharmacol. Ther. 2007 Jul;115(1):13-24.

Esposti MD. Measuring mitochondrial reactive oxygen species. Methods. 2002;26(4):335-40.

Fang M, Chen D, Yang CS. Dietary polyphenols may affect DNA methylation. J Nutr. 2007;137:223S-8S.

Fukumasu H, Avanzo JL, Heidor R, Silva TC, Atroch A, Moreno FS, et al. Protective effects of guarana (*Paullinia cupana* Mart. var. Sorbilis) against DEN-induced DNA damage on mouse liver. Food Chem Toxicol Int J Publ Br Ind Biol Res Assoc. 2006a;44(6):862-7.

Fukumasu H, Da Silva TC, Avanzo JL, De Lima CE, Mackowiak II, Atroch A, Moreno FS, et al. Chemopreventive effects of *Paullinia cupana* Mart var. sorbilis, the guaraná, on mouse hepatocarcinogenesis. Cancer Letters. 2006b;233(1):158-64.

Fukumasu H, Avanzo JL, Nagamine MK, Barbuto JA, Rao KV, Dagli ML. *Paullinia cupana* Mart var. sorbilis, guaraná, reduces cell proliferation and increases apoptosis of B16/F10 melanoma lung metastases in mice. Braz J Med Biol Res Rev Bras Pesqui Médicas e Biológicas Soc Bras Biofísica Al. 2008;41(4):305-10.

Fukumasu H, Latorre AO, Zaidan-Dagli ML. *Paullinia cupana* Mart. var. sorbilis, guarana, increases survival of Ehrlich ascites carcinoma (EAC) bearing mice by decreasing cyclin-D1 expression and inducing a G0/G1 cell cycle arrest in EAC cells. Phytother Res. No prelo 2010.

Guimarães JLM, Rosa DC. Rotinas em oncologia. Porto Alegre: Artmed; 2008.

Guimarães JRQ. Manual de oncologia. 3. ed. São Paulo: BBS; 2008.

Guy M, Lowe LC, Bretherton-Watt D, Mansi JL, Peckitt C, Bliss J, et al. Vitamin D receptor gene polymorphisms and breast cancer risk. Clin Cancer Res. 2004;10:5472-81.

Henman A. Guarana (*Paullinia cupana* var. sorbilis): ecological and social perspectives on an economic plant of the central Amazon basin. J Ethnopharmacol. 1982;6:311-38.

IARC Working group on the evaluation of carcinogenic risks to humans. Tobacco smoke and involuntary smoking. IARC Monog Eval Carcinog Risks Hum. 2004;83:1-1438.

Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. CA Cancer J Clin. 2005;55:10-30.

Jimoh FO, Sofidiya MO, Afolayan AJ. Antioxidant properties of the methanol extracts from the leaves of *Paullinia pinnata*. J Med Food. 2007;10:707-11.

Kim JB, Stein R, O'Hare MJ. Three-dimensional in vitro tissue culture models of breast cancer – a review. Breast Cancer Res Treat. 2004;85:281-9.

Kiriluk KJ, Prasad SM, Patel AR, et al. Bladder cancer risk from occupational and environmental exposures. Urol Oncol. 2012;30:199-211.

Kumar V. Robbins patologia básica. 8. ed. São Paulo: Elsevier; 2008.

Kumar V, Stricker TP. Neoplasia. In: Kumar V, Abbas AK, Fausto N, Aster J. Robbins and Cotran pathologic basis of disease. 8th ed. Philadelphia: Saunders/Elsevier; 2010. Chapter 7.

Lopes A, Chammas R, Iyeyasu H. Oncologia para a graduação. 3. ed. São Paulo: Lemar; 2013.

Lu Q, Qiu X, Hu N, Wen H, Su Y, Richardson BC. Epigenetics, disease, and therapeutic interventions. Ageing Res Rev. 2006;5:449-67.

Mattei R, Dias RF, Espínola EB, Carlini EA, Barros SB. Guarana (*Paullinia cupana*): toxic behavioral effects in laboratory animals and antioxidant activity *in vitro*. Journal Ethnopharmacology. 1998;60(2):111-6.

Mittal A, Piyathilake C, Hara Y, Katiyar SK. Exceptionally high protection of photocarcinogenesis by topical application of (−)-epigallocatechin-3-gallate in hydrophilic cream in SKH-1 hairless mouse model: relationship to inhibition of UVB-induced global DNA hypomethylation. Neoplasia. 2003;5:555-65.

Memon AA, Chang JW, Oh BR, Yoo YJ. Identification of differentially expressed proteins during human urinary bladder cancer progression. Cancer Detect Prev. 2005 Jan;29(3):249-55.

Palmer HG, Sanchez-Carbayo M, Ordonez-Moran P, Larriba MJ, Cordon-Cardo C, Munoz A. Genetic signatures of differentiation induced by 1alpha, 25-dihydroxyvitamin D3 in human colon cancer cells. Cancer Res. 2003;63:7799-806.

Piazza GA, Thompson WJ, Pamukcu R, Alila HW, Whitehead CM, Liu L, et al. Exisulind, a novel proapoptotic drug, inhibits rat urinary bladder tumorigenesis. Cancer Res. 2001 May;61:3961-8.

Ross DT, Perou CM. A comparison of gene expression signatures from breast tumors and breast tissue derived cell lines. Dis Markers. 2001;17:99-109.

Salvadori MC, Rieser EM, Ribeiro Neto LM, Nascimento ES. Determination of xanthines by high-performance liquid chromatography and thin-layer chromatography in horse urine after ingestion of Guaraná powder. Analyst. 1994 Dec;119(12):2701-3.

Santa Maria A, Lopez A, Diaz MM, Muñoz-Mingarro D, Pozuelo JM. Evaluation of the toxicity of guarana with *in vitro* bioassays. Ecotox Environ Safe. 1998;39(3):164-7.

Smith N, Atroch AL. Guarana's journey from regional tonic to aphrodisiac and global energy drink. Evid Based Complement Alternat Med. 2010 Sep;7(3):279-82.

Tfouni SAV, Camargo MCR, Vitorino SHP, Menegário TF, Toledo MCF. Contribution of guaraná powder (*Paullinia cupana*) as a source of caffeine in the diet. Rev Nutrição. 2007;20:63-8.

Yamori T. Panel of human cancer cell lines provides valuable database for drug discovery and bioinformatics. Cancer Chemother Pharmacol. 2003;52:74-9.

Wood Jr DP. Urothelial tumors of the bladder. In: Wein AJ, Kavoussi LR, Novick AC, et al. Campbell-Walsh urology. 10th ed. Philadelphia: Elsevier Saunders; 2012. p. 2309-34.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar o efeito do extrato de *Paullinia cupana* em células de câncer de bexiga (linhagem T24) comparando com quimioterápicos utilizados na terapêutica desta neoplasia.

2.2 Objetivos específicos

Em células comerciais de câncer de bexiga (linhagem T24) tratadas com extrato aquoso de *Paullinia cupana*:

- Determinar a viabilidade das células de linhagem T24 submetidas a diferentes concentrações do extrato de *Paullinia cupana*;
- Determinar a proliferação celular das células de linhagem T24 submetidas a diferentes concentrações do extrato de *Paullinia cupana*;
- Comparar o efeito anticarcinogênico com quimioterápicos utilizados na terapêutica do câncer de bexiga;
- Investigar a ocorrência de indução da cascata apoptótica;
- Investigar o efeito do extrato sobre a formação de Espécies Reativa de Oxigênio nas células T24.

3. ARTIGO CIENTÍFICO EM INGLÊS

GUARANÁ EXTRACT (*Paullinia cupana*), AN AMAZONIAN FRUIT RICHEST IN CAFFEINE ON HUMAN BLADDER CANCER CELL LINE

Eliza Ribas de Silveira Flôres[1], Ivana Beatrice Mânica da Cruz[2], Fernanda Bueno Morrone[3], Alana Duranski Ranzi[1], Francine Carla Cadoná[2], Alencar Kolinski Machado[2], Pauline Ledur[2], Stephanie Damasceno Rocha[1], Marta Maria Medeiros Frescura Duarte[4], Túlio M. Graziottin[5], Claudia Giuliano Bica[1].

[1] Department of Basic Health Sciences, Federal University of Health Sciences of Porto Alegre, Porto Alegre, Brazil. Sarmento Leite, 245, Porto Alegre/RS, Brazil. Zip Code 90050-170

[2] Health Sciences Center, Federal University of Santa Maria, Av Roraima 1000, Prédio 19, Santa Maria, Brazil. Zip Code 97105900

[3] Pontifical Catholic University of Rio Grande do Sul, Faculty of Pharmacy. Av Ipiranga, 6681. Prédio 94, Zip Code 90619-900, Porto Alegre/RS, Brazil. Zip Code 90619-900

[4] Lutheran University of Brazil, Santa Maria, Brazil. Br 287 - Km 252 - Trevo Maneco Pedroso. Zip Code 97.020-001

[5] Department of Urology, Federal University of Health Sciences of Porto Alegre,. Sarmento Leite, 245, Porto Alegre/RS, Brazil. Zip Code 90050-170

Correspondence to: Claudia Giuliano Bica, Department of Basic Health Sciences, Federal University of Health Sciences of Porto Alegre, Porto Alegre, Brazil. Sarmento Leite, 245, Porto Alegre/RS, Brazil. Zip Code 90050-170. Email: claudia@ufcspa.edu.br

ABSTRACT

The potential effect of foods richest in caffeine on bladder carcinogenesis is not conclusive. For this reason, an *in vitro* study was performed to evaluate antitumoral activity of guaraná, an Amazon fruit commonly used in energy drinks. T24 bladder cancer cells were exposed to a broad range of guaraná concentrations and the chemotherapics methotrexate, cisplatin and gemcitabine used as positive controls to evaluate the effect on viability, proliferation, apoptosis and intracellular reactive oxygen species levels. Results showed that lowest guaraná concentrations decreased the cellular viability and proliferation. However, higher guaraná concentrations presented a procarcinogenic activity. The results described here suggest that guaraná extracts presented a dual effect on T24 bladder cells viability. Despite the methodological limitations (just *in vitro* protocols), we believe that the results presented here are relevant since suggest that consumption of food and beverage rich in caffeine and other bioactive molecules as guaraná present a cytotoxic effect against bladder cancer cells.

.

Key-words: Caffeine, catechins, T24, anticarcinogenic, chemotherapics.

Introduction

Similar to green tea and coffee, guaraná (*Paullinia cupana* Kunth var. sorbilis, Mart. Ducke), an Amazonian fruit used to produce energetic beverages is very rich in caffeine and polyphenol molecules such as catechin and (-)-epigallocatechin-3-gallate (EGCG) (Belliardo et al. 1985; Smith and Atroch 2010; Schimpl et al. 2013). Studies using *in vitro* models showed important biological effects of guaraná including antibacterial and antifungal activities (Basilea et al. 2013), cytoprotective effect of dopaminergic neuroblastoma SH-SY5Y cell exposed to rotenone that is a neurotoxic molecule, (De Oliveira et al. 2011) as well as in NH3-T3 embryonic fibroblast cells exposed to higher nitric oxide levels (Bittencourt et al. 2013). Guaraná lower effect on LDL oxidation (Portella et al. 2013) as well as on peripheral blood mononuclear cells (PBMCs) proinflammatory cytokines (Montano et al. 2012) were also described.

Antitumoral proprieties were also related to guaraná treatment of *in vitro* and *in vivo* experimental models (Fukumasu et al. 2006; Fukumasu et al. 2008; Fukumasu et al. 2011). This is the case of bladder cancer, which is common in men (Jemal et al. 2010) and it presents high recurrence rate (Wood 2012). Previous studies, as performed by Philips et al. (2009) described *in vitro* antitumor effect of green tea and its main polyphenol compounds in malignant bladder cell lines. On the other hand, some studies suggested that caffeine intake could be a risk factor of bladder cancer. However, data from case-control and cohort studies did not provide conclusive evidences (Zhou et al. 2012).

Due to the epidemiological relevance, we performed here an *in vitro* study that evaluated the potential antitumor activity of guaraná extract against commercial T24 bladder cell line. The study evaluated potential guaraná effect

on cell viability and proliferation, comparing the results obtained from cells treated with guaraná extract to cells exposed to three chemotherapeutic drugs (methotrexate, cisplatin, and gemcitabine). These chemotherapic are commonly used in bladder cancer treatment.

Material and methods

Analytical reagents and chemicals

Analytical grade chemicals and reagents were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). The bladder cancer T24 cell line was obtained from American Type Culture Collection (ATCC®). The RPMI 1640 medium culture, fetal bovine serum, heat-inactivated horse serum, penicillin, and streptomycin were purchased from Gibco (Grand Island,NY, USA); Vacutainer® by BD Diagnostics (Plymouth, UK) cytokines kits to ELISA immunoassay by Biomyx Technology (San Diego, CA, USA).

Guaraná source and extract preparation

Guaraná powder was supplied by EMBRAPA, Amazônia Ocidental (Agropecuary Research Brazilian Enterprise), a non-profit Brazilian governmental sector that offers technical support to guaraná production in the state of Amazonas. The bioactive compounds present in guaraná powder used in the study were previously determined and described at Bittencourt et al. (2013). The extract presented 12.240 mg/g of caffeine, 6.733 mg/g of theobromine, 4.336 mg/g total catechins. The concentration of condensed tannin was 16 mg/g. To perform the *in vitro* assay described here, the lyophilized extract was diluted with distilled water and prepared at a concentration of 200 mg/mL. The mixture was infused for 7 min by boiling,

centrifuging (1500 rpm, 15 min) and filtering. The solution was sterilized by filtration (0.20 µM), diluted with distilled water and added to the final concentrations of 3.8, 4.5, 6.0, 10, 30, 100, 300, 1000, 3000, 10000 and 30000 µg/mL in cell culture medium.

Cell culture conditions

The T24 cell line used as an experimental model was cultured in RPMI 1640 medium supplemented with 10% FBS, 100 Units/mL penicillin, 100 µg/mL streptomycin, and 2mM L-glutamine at 37°C under a humidified 5% CO_2 and 95% air at one atmosphere. All assays were performed at least in triplicate with cells in exponential growth after adhesion to the bottom of the plates. To test guaraná anticarcinogenic effect, the T24 cells/well (2×105) were seeded into 12-well plates for cell adherence with three wells for each concentration level. T24 cells were also exposed to three chemotherapy drugs used in bladder cell clinical therapy as positive controls: methotrexate, cisplatin and gemcitabine at 3.8, 4.5, 6.0, 10 and 30 µg/mL concentrations. These concentrations were chosen from a previous *in vitro* study that described antitumoral activity of these chemoteraphics on T24 bladder cancer cells (Yoon et al. 2011).

Antitumoral assays

Guaraná effect on cell viability was observed after a 24-hour exposition and the effect on cell proliferation was determined after a 72-hour exposition. Both analyses were made by using three methods: MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, lactate dehydrogenase (LDH) activity and double-strand DNA (dsDNA) concentration in the supernatant and into the cells. Briefly, MTT was dissolved in phosphate buffered saline at 5

mg/ml, and added to each well and incubated for 4 hours. To dissolve formazan crystals, further incubation with 200 µL of DMSO was performed. The absorbance at 570 nm was measured with a microplate reader. The cell viability observed in each treatment was expressed as a percentage of the control absorbance value. Since the MTT assay presents some limitations that can underestimated the anti-proliferative effects of extracts rich in polyphenols (dos Santos Montagner et al. 2010), before the analysis, the cells were centrifuged at 3000 rpm for 3 minutes, the supernatant discarded and the cells were resuspended in PBS buffer (dos Santos Montagner et al. 2010). For the same reason, the analysis of LDH was determined. This is a soluble enzyme located in the cytosol that is released into the surrounding culture medium upon cell damage or lysis, processes which occur during both apoptosis and necrosis (Haslam et al. 2000).

An additional test was used to confirm the cytotoxic effect of guaraná extracts on bladder cancer cells viability from analysis of cell-free DNA concentrations measured by fluorimetric method using DNA PicoGreen® dye with a similar protocol described by Ha et al. (2011) and Jobim et al. (2014). After 24 and 72 hours of guaraná and chemotherapy drugs exposition, the supernatants were collected (5 µl of each well). The PicoGreen (Molecular Probes) assay was done according to protocol supplied by the manufacturer. PicoGreen dye was diluted 1:200 with TE buffer (10mM Tris–HCl, 1mM EDTA, pH 7.5) and incubated with plasma DNA in the dark at room temperature for 5 min. To minimize photobleaching effects, time for fluorescence measurement was kept constant for all samples. Fluorescence emission of PicoGreen alone (blank) and PicoGreen with double stranded DNA (dsDNA) were recorded at

528 nm using an excitation wavelength of 485 nm at room temperature (25°C). A standard curve was generated by using double-stranded (ds) lambda DNA provided by the manufacturer. The fluorescence emission measurement of each medium sample of cell culture was an average of eight wells of independent measures and the corresponding standard deviation is indicated in the data. The dsDNA concentration in culture medium represents the presence of death cell degradation compounds. Thus, results were expressed as a percentage of fluorescence values in relation to negative control group treatment. Percentage values higher than control group indicated cytotoxic effect of guaraná extracts.

Apoptosis biomarkers analysis

Considering which chemotherapeutic drugs used as positive control in the present study, act on cell cycle S phase inducing apoptotic events that involve caspases 3 and 8 activation, the potential effect of guaraná on these caspases were also evaluated as well as the effect on caspase-1 levels (Yoon et al. 2011). The levels on caspase-1 levels, a proinflammatory molecule that also acts as tumor suppressor (Celardo et al. 2013) was also determined. The potential induction of T24 cells apoptosis cascade by guaraná exposition was investigated from determination of caspases 1, 3 and 8 levels. The caspases activities were evaluated by immunoassay following the manufacture instructions (BioVision, Mountain View, CA). All experiments were performed in triplicate.

Intracellular ROS levels determination

As guaraná is rich in antioxidant molecules (Klein et al. 2012), its potential anticarcinogenic effect probably involves oxidative metabolism

imbalance of cancer cells. To test the hypothesis, the intracellular ROS production of T24 bladder cancer cells was also analyzed. The ROS concentration was determined by using the non-fluorescent cell permeating compound 2′–7′-dichlorofluorescein diacetate (DCFDA) assay. In this technique the DCFDA is hydrolyzed by intracellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by cellular oxidants. After predetermined time exposure, the cells were treated with DCFDA (10 μM) for 60 minutes at 37°C. The fluorescence was measured at an excitation of 485nm and an emission of 520nm (Costa et al. 2013).

Statistical analysis

All results were expressed as a percentage of control group, and statistically compared by One way analysis of variance followed by *post hoc* Dunnet test. A p value of less than 0.05 was considered to be statistically significant.

Results and discussion

Considering that guaraná used in several energy drinks consumed in developed countries has some chemical similarities with coffee and tea in its nutritional matrix (Angelo et al. 2008), we performed an *in vitro* protocol to evaluate its potential effect on bladder cancer cells.

Guaraná extract showed dose-dependent effect on T24 cells viability concentration-dependent evaluated by MTT and DNA Picogreen assays. Results using MTT assay are presented in Figure 1 showing that from 3.8 μg/mL to 100 μg/mL, guaraná significantly decreased the cells viability.

The T24 cells viability observed at lowest guaraná concentrations was also compared to methotrexate, cisplatin and gemcitabine drugs. The results showed that guaraná extract presented similar viability curve observed in T24 cells treated with methotrexate being less effective to decrease the cell viability than cisplatin and gemcitabine (Figure 1b).

Figure 1 here

The antiproliferative effect of guaraná extract at lowest concentrations (3.8 - 100 µg/mL) was assayed and the results are show presented in Figure 2. As expected, T24 cells exposed to chemotherapics significantly decreased cellular proliferation at a 72-hour exposition. Similar antiproliferative effect was also found in T24 cells exposed to guaraná. However, in lower concentrations (< 10 µg/mL), guaraná effect was not so effective as chemotherapics. On the other hand, similar antiproliferative effect of guaraná and chemotherapic drugs was observed at 30µg/mL concentration.

Figure 2 here

Considering that data obtained from spectrophotometric and fluorimetric protocols (MTT and dsDNA Picogreen assays, respectively) could present some interferences in the results, a complementary analysis was performed to determine whether guaraná was able to change the caspases levels that are considered apoptosis biomarkers.

Therefore, potential guaraná effect on apoptotic cascade was evaluated considering the caspases 3 and 8 as well as caspase 1 level. Guaraná activated the caspase cascade since a significant increase in the concentrations of these molecules occurred after a 24-hour exposition in a similar way

observed in the T24 cells treated with gemcitabine, cisplatin and methotrexate (Figures 3). However, the level of caspases in cells exposed to guaraná extract showed lower values than those observed in cells treated with chemotherapic drugs.

Figure 3 here

An additional analysis evaluated whether guaraná caused change in intracellular ROS levels of T24 bladder cancer cells. A significant increase in ROS levels in the T24 bladder cancer cells exposed to different guaraná extract concentrations was observed when compared to controls. The increase in ROS levels was also similarly dose-dependent to those observed in experiments of caspases levels (Figure 4).

Figure 4 here

The results provided evidence that guaraná presents an important antitumoral effect on bladder cancer cells with decrease and increase viability of cancer cells at doses lower < 100 µg/mL.

Guaraná, as coffee and tea, have methylxanthines and polyphenols in its composition (Smith and Atroch et al. 2010). Methylxanthines, including caffeine, are integral part of everyday human food and drink consumption. Catechins are other group of substances also present in guaraná with important antioxidant and antitumoral activity (Yang and Wang 2011). Prevention of carcinogenesis with catechins, mainly epigallocatechin-3-gallate (EGCG), has been corroborated by animal and epidemiological studies (Yang and Wang 2011). Catechins inhibit cell proliferation, cell invasion, angiogenesis, metastasis, and stimulate apoptosis (Chen et al. 2011; Da Silva et al. 2010). These processes

occur by multiple biological pathways like growth factor-mediated, mitogen-activated protein kinase-dependent, ubiquitin/proteasome degradation, and gene expression (Chen et al. 2011).

The results described herein also showed that lower concentrations of guaraná extract, methotrexate, cisplatin, and gemcitabine increased caspases 1, 3 and 8 concentrations, indicating a route for cell apoptosis. Our results are related to previous *in vitro* studies of cisplatin and gemcitabine demonstrating cell cycle arrest and apoptosis of bladder carcinoma cell lines (Da Silva et al. 2010). Therefore, the possible mechanism of antitumoral effect of low concentrations of guaraná extract includes the apoptosis cascade induction.

Cellular apoptosis results in the activity of both initiator caspases (caspases 2, 8, 9, and 10) and executor caspases (caspases 3, 6, and 7), a class of enzymes which also includes inflammatory mediators (caspases 1, 4, and 5). So, detection of active caspase-3 in cells and tissues infers the occurrence of an apoptotic process (Fulda 2009). Caspase-8 also plays a key role in the regulation of programmed cell death in normal development. Disturbance of its function may contribute to cancer diseases (Fulda 2009). Caspase-8 is inactivated in a variety of human cancers, which may promote tumor progression and resistance to treatment (Fulda 2009). Therefore, caspase-8 represents a promising target to restore defective apoptosis programs; higher concentration of caspase-8 in low concentration guaraná treated T24 cancer cells, which may be considered a relevant result.

Despite caspase-1 playing a role in programmed cell death, the importance in the apoptotic process is still to be understood (Denes et al. 2012). Caspase-1 is more related to the inflammation process. Moreover, caspase-1

transforms the precursor of inflammatory cytokines interleukin-1-β and -18 into active mature peptides. Caspase-1 induces cell necrosis or pyroptosis in macrophages (Denes et al. 2012). However, our results cannot explain the specific role of caspase-1 in T24 cells. T24 bladder cells do not seem to produce caspase-1 cleaved interleukin-1, and apoptosis may occur as an event per se.

Low concentration of guaraná extracts in T24 cells culture induced an increase in ROS production. Intracellular ROS modulation has recently attained scientific interest due to its role in cellular homeostasis carcinogenesis. Oxidative metabolism imbalance caused by antioxidant compounds probably breaks the cancer cell homeostasis contributing to cell death, and explains cell mortality in cisplatin-treated T24 cells (Zhang et al. 2012). The effect of guaraná extracts in dose-dependent increase of ROS levels in our study are also in accordance with the increase of ROS species in T24 bladder cancer experiments with green tea extracts (Chen et al. 2011).

Conclusions

Overall, our results demonstrated that guaraná extracts present a dual effect on T24 bladder cells viability. Whereas lowest concentrations decrease tumor cell viability, highest concentrations show protective effect. Interestingly, lowest guaraná concentrations reproduce similar results achieved with chemotherapic drugs regarding cell viability, caspases activity and ROS levels. In spite of methodological limitations of the present study that was performed using just *in vitro* protocols, we believe that the results presented here are relevant since suggest that consumption of food and beverage rich in caffeine

and other bioactive molecules as guaraná present a cytotoxic effect against bladder cancer cells.

Acknowledgements

The authors are grateful for the help of the Laboratory of Biogenômica - Federal University of Santa Maria, RS, Brazil, and of the Laboratory of Applied Pharmacology - Catholic University of Porto Alegre, RS, Brazil. This research study was funded by the following research grants and fellowships: FAPERGS-PRONEN, CNPq, FAPEAM and CAPES.

Declarations of interest: The authors declare that they have no conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, the present work.

References

Angelo PC, Nunes-Silva CG, Brígido MM, Azevedo JS, Assunção EN, Sousa AR, Patrício FJ, Rego MM, Peixoto JC, Oliveira WP, Jr Freitas DV, Almeida ER, Viana AM, Souza AF, Andrade EV, Acosta PO, Batista JS, Walter ME, Leomil L, Anjos DA, Coimbra RC, Barbosa MH, Honda E, Pereira SS, Silva A, Pereira JO, Silva ML, Marins M, Holanda FJ, Abreu RM, Pando SC, Gonçalves JF, Carvalho ML, Leal-Mesquita ER, da Silveira MA, Batista WC, Atroch AL, França SC, Portom JI, Schneiderm MP, Astolfi-Filho S (2008) Brazilian Amazon Consortium for Genomic Research (REALGENE). Guaraná (*Paullinia*

cupana var. sorbilis), an anciently consumed stimulant from the Amazon rain forest: the seeded-fruit transcriptome. Plant Cell Rep 27:117–124

Basilea A, Riganob D, Contea B, Brunoc M, Rossellic S, Sorbod S (2013) Antibacterial and antifungal activities of acetonic extract from *Paullinia cupana* Mart. Seeds. Natural Product Research, 27(22):2084-2090

Belliardo F, Martelli A, Valle MG (1985) HPLC determination of caffeine and theophylline in *Paullinia cupana* Kunth (guarana) and Cola spp. samples. Z. Für Lebensm.-Unters. –Forsch 180:398–401

Bittencourt LS, Machado DC, Machado MM, Dos Santos GF, Algarve TD, Marinowic DR, Ribeiro EE, Soares FA, Barbisan F, Athayde ML, Cruz IBM (2013) The protective effects of guaraná extract (*Paullinia cupana*) on fibroblast NIH-3T3 cells exposed to sodium nitroprusside. Food Chemistry Toxicology 53:119-25

Celardo I, Grespi F, Antonov A, Bernassola F, Garabadgiu AV, Melino G, Amelio I (2013) Caspase-1 is a novel target of p63 in tumor suppression. Cell Death and Disease 23:4:e645

Chen D, Wan SB, Yang H, Yuan J, Chan TH, Dou QP (2011) EGCG, green tea polyphenols and their synthetic analogs and prodrugs for human cancer prevention and treatment. Adv Clin Chem, 53:155–177

Costa F, Dornelles E, Mânica-Cattani MF, Algarve TD, Souza Filho OC, Sagrillo MR, Garcia LF, da Cruz IB (2012) Influence of al16Ala SOD2 polymorphism on the in-vitro effect of clomiphene citrate in oxidative metabolism. Reprod Biomed Online 24:474-81

Da Silva GN, de Castro Marcondes JP, de Camargo EA, da Silva Passos Júnior GA, Sakamoto-Hojo ET, Salvadori DM (2010) Cell cycle arrest and apoptosis in TP53 subtypes of bladder carcinoma cell lines treated with cisplatin and gemcitabine. Exp Biol Med 235:814–824

De Oliveira DM, Barreto G, Galeano P (2011) *Paullinia cupana* Mart. var. Sorbilis protects human dopaminergic neuroblastoma SH-SY5Y cell line against rotenone-induced cytotoxicity. Human & Exp Toxicol. 30(9):1382-91

Denes A, Lopez-Castejon G, Brough D (2012) Caspase-1: is IL-1 just the tip of the ICEberg? Cell Death and Diseases 3:e338

dos Santos Montagner GF, Sagrillo M, Machado MM, Almeida RC, Mostardeiro CP, Duarte MM, da Cruz IB (2010) Toxicological effects of ultraviolet radiation on lymphocyte cells with different manganese superoxide dismutase Ala16Val polymorphism genotypes. Tox *In vitro* 24:1410-6

Fukumasu H, Avanzo JL, Heidor R, Silva TC, Atroch A, Moreno FS, Dagli ML (2006) Protective effects of guarana (*Paullinia cupana* Mart. var. Sorbilis)

against DEN-induced DNA damage on mouse liver. Food Chem Toxicol 44:862–867

Fukumasu H, Avanzo JL, Nagamine MK, Barbuto JA, Rao KV, Dagli ML (2008) *Paullinia cupana* Mart var. sorbilis, guaraná, reduces cell proliferation and increases apoptosis of B16/F10 melanoma lung metastases in mice. Braz J Med Biol Res 41:305–310

Fukumasu H, Latorre AO, Zaidan-Dagli ML (2011) *Paullinia cupana* Mart. var. sorbilis, guarana, increases survival of Ehrlich ascites carcinoma (EAC) bearing mice by decreasing cyclin-D1 expression and inducing a G0/G1 cell cycle arrest in EAC cells. Phytother. Res 25:11–16

Fulda S (2009) Caspase-8 in cancer biology and therapy. Cancer Lett 281:128–133

Ha TTN, Huy NT, Murao LA, Lan NT, Thuy TT, Tuan HM, Nga CT, Tuong VV, Dat TV, Kikuchi M, Yasunami M, Morita K, Huong VT, Hirayama K (2011) Elevated levels of cell-free circulating DNA in patients with acute dengue virus infection. Plos One 6:e25969

Haslam G, Wyatt D, Kitos PA (2000) Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. Cytotechnol 32:63-75

Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics. CA-Cancer J. Clin 60277–300.300

Jobim ML, Santos RC, Dos Santos Alves CF, Oliveira RM, Mostardeiro CP, Sagrillo MR, de Souza Filho OC, Garcia LF, Manica-Cattani MF, Ribeiro EE, da Cruz IB (2014) Antimicrobial activity of Amazon Astrocaryum aculeatum extracts and its association to oxidative metabolism,Microbiol Research, 169(4):314-23

Klein T, Longhini R, de Mello JCP (2012) Development of an analytical method using reversed-phase HPLC-PDA for a semipurified extract of *Paullinia cupana* var. sorbilis (guaraná). Talanta 88:502–506

Montano MA, Da Cruz IB, Duarte MM, Krewer Cda C, da Rocha MI, Mânica-Cattani MF, Soares FA, Rosa G, Maris AF, Battiston FG, Trott A, Lera JP. (2012) Inflammatory cytokines *in vitro* production are associated with Ala16Val superoxide dismutase gene polymorphism of peripheral blood mononuclear cells Cytokine. 60(1):30-3

Portella RL, Barcelos RP, Da Rosa EJ (2013) Guaraná (*Paullinia cupana* Kunth) effects on LDL oxidation in elderly people: an *in vitro* and *in vivo* study. Lipids in Health and Disease. 8(12):12

Philips BJ, Coyle CH, Morrisroe SN, Chancellor MB, Yoshimura N (2009) Induction of apoptosis in human bladder cancer cells by green tea catechins. Biomed Res (Tokyo, Japan) 30:207–215

Schimpl FC, da Silva JF, Gonçalves JF, Mazzafera P (2013) Guarana: Revisiting a highly caffeinated plant from the Amazon. J Ethnopharmacol 150(1):14-31

Smith N, Atroch AL (2010) Guaraná's Journey from Regional Tonic to Aphrodisiac and Global Energy Drink. J Evid Based Complementary Altern Med 7:279-282

Wood JrDP (2012) Urothelial Tumors of the Bladder. In: Campbell-Walsh urology. Edited by AJ Wein, LR Kavoussi, AC Novick, et al. 10th ed. Philadelphia, PA: Elsevier Saunders pp2309–2334

Yang CS, Wang H (2011) Mechanistic issues concerning cancer prevention by tea catechins. Mol Nutr Food Res 55:819–831

Yoon CY, Lee JS, Kim BS, Jeong SJ, Hong SK, Byun SS, Lee SE (2011) Sunitinib malate synergistically potentiates anti-tumor effect of gemcitabine in human bladder cancer cells. Korean J Urol 52:55–63

Zhang MMN, Long Y-T, Ding Z (2012) Cisplatin effects on evolution of reactive oxygen species from single human bladder cancer cells investigated by scanning electrochemical microscopy. J Inorg Biochem 108:115–122

Zhou Y, Tian C, Jia C (2012) A dose-response meta-analysis of coffee consumption and bladder cancer. Prev Med 55:55:14–22

Legends

Figure 1 T24 bladder cancer cells viability after 24 hours (data are presented as % of control) measured by dsDNA supernatant concentration; (A) guaraná extract at different concentrations (µg/mL); (B) viability comparison of T24 cells exposed to guaraná chemotherapics at different concentrations (3.4 – 30 µg/mL). The treatments were compared by analysis of variance followed by post hoc Dunnet test. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Figure 2 T24 bladder cancer cells proliferation after 72 hours among guaraná extract at different concentrations and chemotherapic drugs (data are presented as % of control) measured by MTT assay. The groups were compared by analysis of variance followed by post hoc Dunnet test. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Figure 3 Caspase levels of T24 bladder cells exposed to different concentrations of guaraná extracts and chemotherapic drugs (data are presented as % of control) measured by immunoassays. The caspase levels of each group were compared by analysis of variance followed by post hoc Dunnet test. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Figure 4 Reactive oxygen species (ROS) concentration of T24 bladder cells exposed to different concentrations of guaraná extracts and chemotherapic drugs (data are presented as % of control) measured by fluorimetric DCFH-DA assay. The groups were compared by analysis of variance followed by post hoc Dunnet test. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Figures

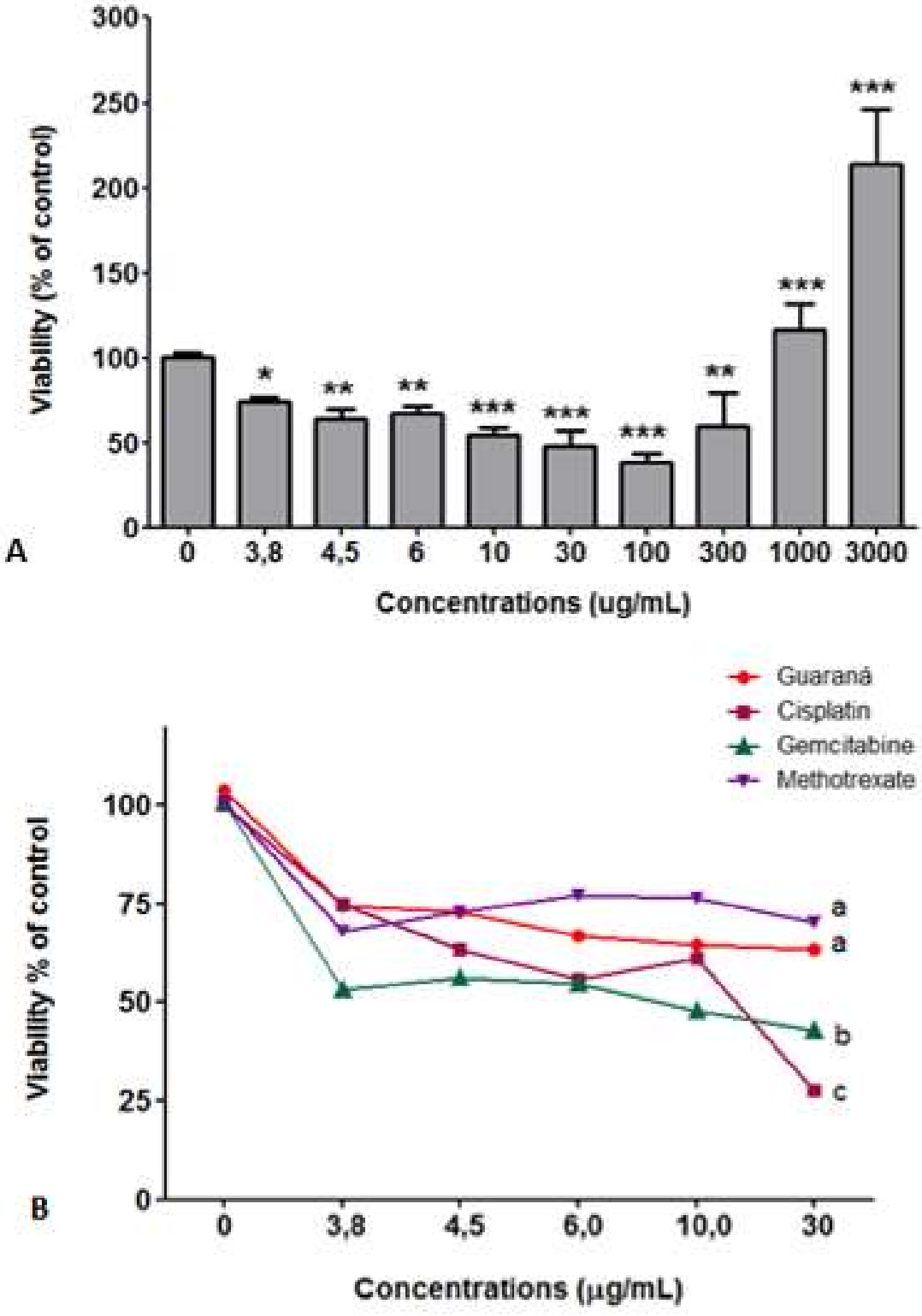

Figure 1

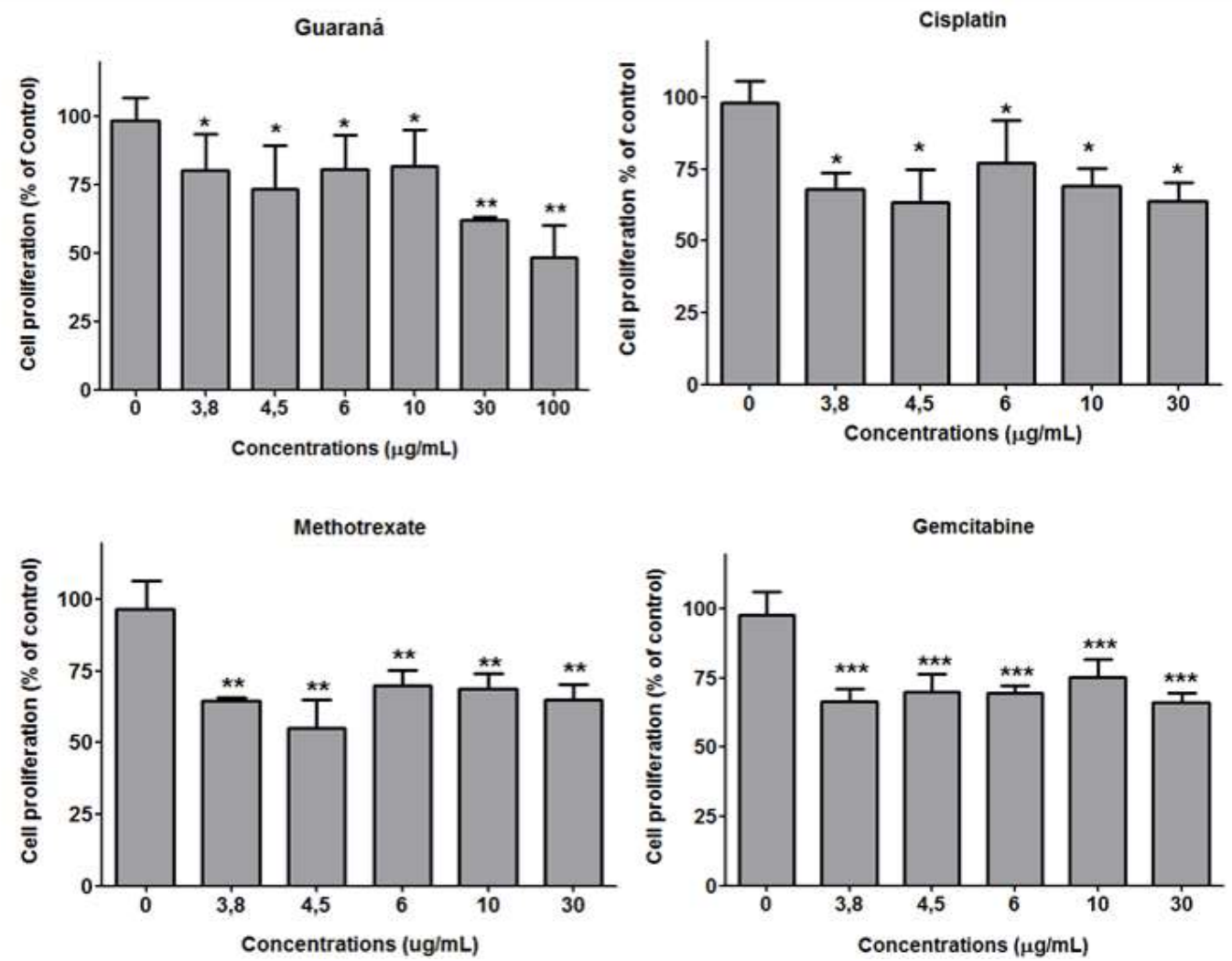
Guaraná
Cell proliferation (% of Control)
100
75
50
25
0
*
*
*
*
*
**
**
0
3,8
4,5
6
10
30
100
Concentrations (µg/mL)
Cisplatin
Cell proliferation % of control
100
75
50
25
0
*
*
*
*
*
0
3,8
4,5
6
10
30
Concentrations (µg/mL)
Methotrexate
Cell proliferation (% of control)
100
75
50
25
0
**
**
**
**
**
0
3,8
4,5
6
10
30
Concentrations (ug/mL)
Gemcitabine
Cell proliferation (% of control)
100
75
50
25
0
***
***
***
***
***
0
3,8
4,5
6
10
30
Concentrations (µg/mL)

Figure 2

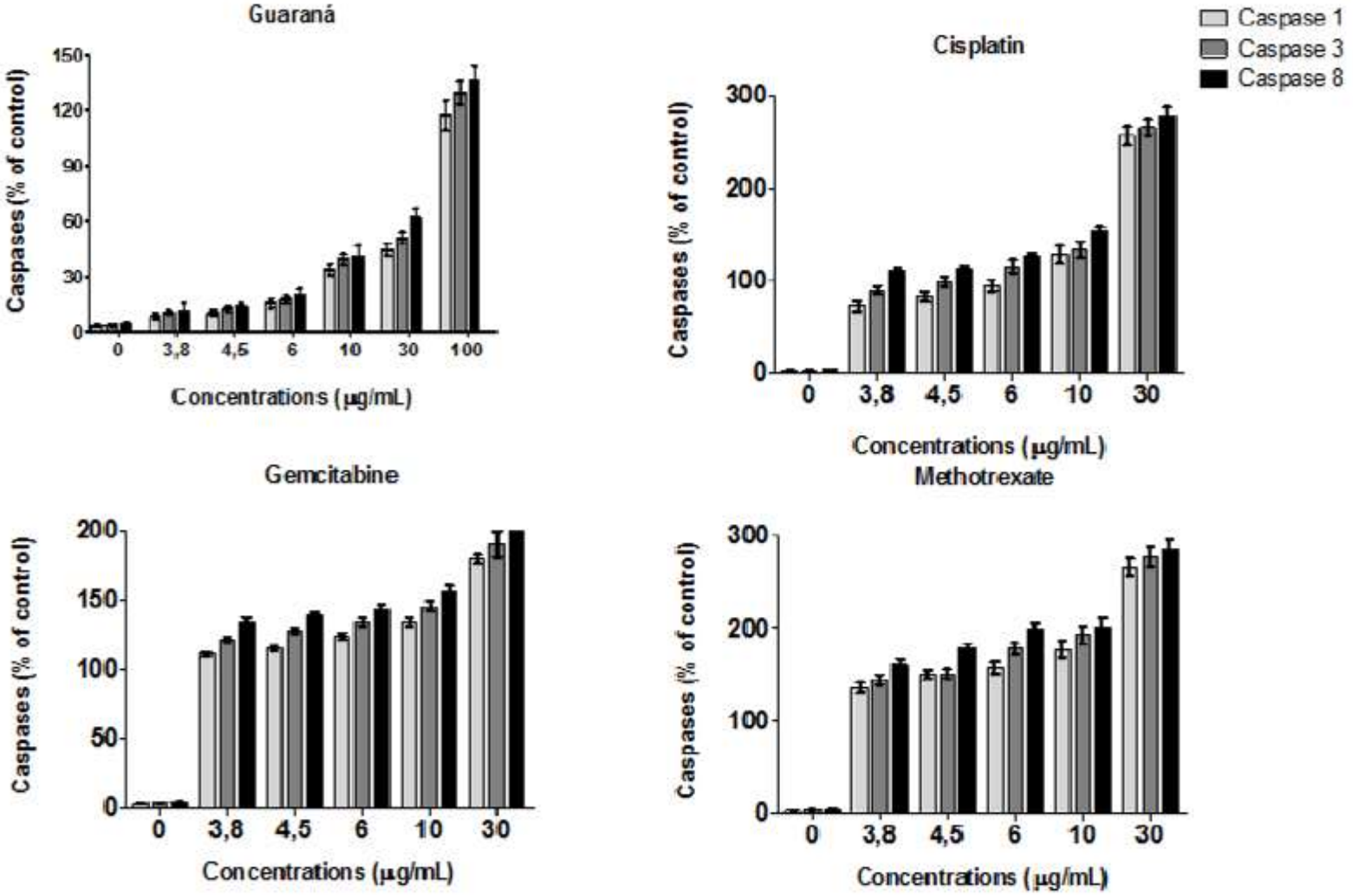
Guaraná
Caspases (% of control)
150
120
90
60
30
0
0
3,8
4,5
6
10
30
100
Concentrations (µg/mL)
Caspase 1
Caspase 3
Caspase 8
Cisplatin
Caspases (% of control)
300
200
100
0
0
3,8
4,5
6
10
30
Concentrations (µg/mL)
Gemcitabine
Caspases (% of control)
200
150
100
50
0
0
3,8
4,5
6
10
30
Concentrations (µg/mL)
Methotrexate
Caspases (% of control)
300
200
100
0
0
3,8
4,5
6
10
30
Concentrations (µg/mL)

Figure 3

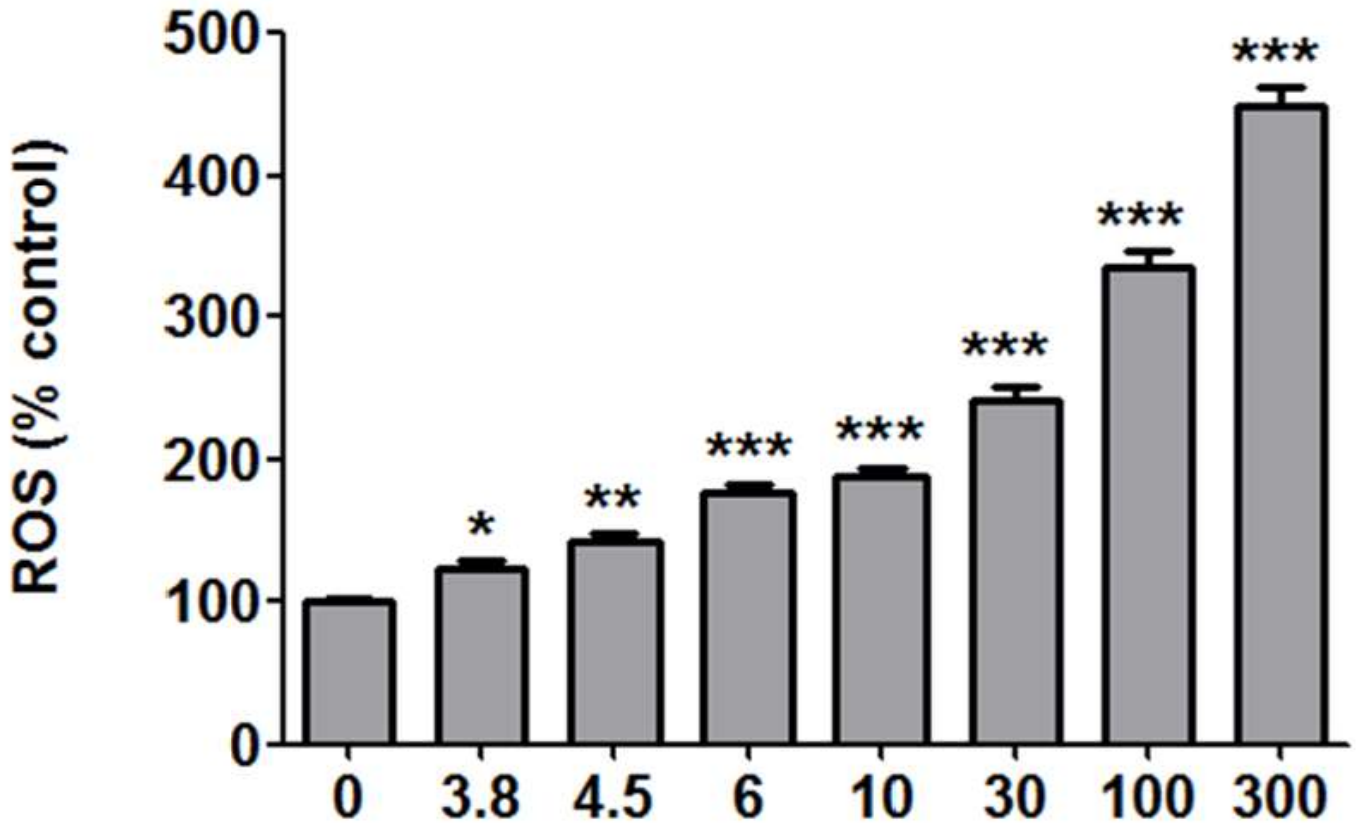

Figure 4

4. CONSIDERAÇÕES FINAIS

Este trabalho deixa algumas perspectivas para a continuação dos estudos relacionados à utilização do guaraná em quadros de neoplasias de bexiga já que pouco se sabe sobre os efeitos do extrato de *Paullinia cupana* nessa patologia. Assim, destaca-se a necessidade de investigar quais possíveis vias de ação do guaraná frente ao câncer de bexiga a fim de auxiliar na elaboração de medidas preventivas relacionadas com mudança do hábito de vida, emprego de quimioprevenção e alertar a população quanto aos efeitos maléficos do abuso de consumo do guaraná.

5. ANEXOS

Anexo 1: Parecer do Comitê de Ética em Pesquisa da UFCSPA

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Efeito antitumoral do extrato aquoso de Paullinia cupana H.B.K, Sapindaceae em células do câncer de bexiga

Pesquisador Responsável Cláudia Giuliano Bica **Parecer 1669/12**

Data da Versão 29/03/2012 | **Cadastro 955/12** | **Data do Parecer 17/05/2012**

Grupo e Área Temática III - Projeto fora das áreas temáticas especiais

Objetivos do Projeto
Avaliar o efeito antitumoral de células de linhagem T24 submetidas a diferentes concentrações do extrato de Paullinia cupana.

Sumário do Projeto
Dissertação de mestrado

Itens Metodológicos e Éticos	Situação
Título	Adequado
Autores	Adequados
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não
Aprovação no país de origem	Não necessita
Local de Realização	Outro (citar no comentário)
Outras instituições envolvidas	Sim
Condições para realização	Adequadas

Comentários sobre os itens de Identificação

As células tumorais foram obtidas por doação do Laboratório de Cultura Celular da PUC. O estudo será desenvolvido nas dependências do Laboratório de Biogenômica da Universidade Federal de Santa Maria

Introdução	Adequada

Comentários sobre a Introdução

Objetivos	Adequados

Comentários sobre os Objetivos

Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total Local
Cálculo do tamanho da amostra	Não necessário (pesquisa qualit
Participantes pertencentes a grupos especiais	Não
Seleção eqüitativa dos indivíduos participantes	Não se aplica
Critérios de inclusão e exclusão	Ausentes
Relação risco- benefício	Não se aplica
Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Nâo utiliza
Monitoramento da segurança e dados	Adequado
Avaliação dos dados	Adequada - qualitativa
Privacidade e confidencialidade	Não se aplica
Termo de Consentimento	Não se aplica
Adequação às Normas e Diretrizes	Não

Comentários sobre os itens de Pacientes e Métodos

Cronograma	Adequado
Data de início prevista	07/2012
Data de término prevista	12/2013
Orçamento	Adequado
Fonte de financiamento externa	Agência de fomento

Comentários sobre o Cronograma e o Orçamento

Referências Bibliográficas	Adequadas

Comentários sobre as Referências Bibliográficas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

Anexo 2: Autorização de acesso e de remessa de amostra de componente do patrimônio genético nº 010547/2013-4

CNPq
Conselho Nacional de Desenvolvimento Científico e Tecnológico

3905612739716199

AUTORIZAÇÃO DE ACESSO E DE REMESSA DE AMOSTRA DE COMPONENTE DO PATRIMÔNIO GENÉTICO nº 010547/2013-4

O CONSELHO NACIONAL DE DESENVOLVIMENTO CIENTÍFICO E TECNOLÓGICO - CNPq, credenciado pelo Conselho de Gestão do Patrimônio Genético (CGEN/MMA), por meio da Deliberação CGEN n° 246, de 27 de agosto de 2009, para autorizar instituições nacionais, públicas ou privadas, que exerçam atividades de pesquisa e desenvolvimento nas áreas biológicas e afins, a acessar e remeter amostras de componente do patrimônio genético para fins de pesquisa científica sem potencial de uso econômico, neste ato representado pelo seu Diretor de Ciências Agrárias, Biológicas e da Saúde, nos termos da Portaria CNPq nº 161/2010, autoriza a instituição abaixo qualificada a acessar e remeter amostras de componentes do patrimônio genético.

Instituição: UNIVERSIDADE FEDERAL DE SANTA MARIA - UFSM

CNPJ: 955.917.640/0001-05

Representante Legal: IVANA BEATRICE MANICA DA CRUZ

Cargo/Função: Docente - Departamento de Morfologia -Centro de Ciências da Saúde - UFSM

CPF: 331.322.400-87 **RG:** 8017462709

Projeto: ESTUDOS IN VITRO E IN VIVO DOS EFEITOS BIOATIVOS PRESENTES EM FRUTOS AMAZÔNICOS

Coordenador do Projeto: Ivana Beatrice Mânica Da Cruz

CPF: 331.322.400-87 **RG:** 8017462709 - ssp / RS

Finalidade do projeto: Este projeto irá realizar estudos in vitro e in vivo para avaliar a atividade biológica (antioxidante, antimicrobiana, anticolinesterásica, hipolipimiante, hipotensiva, antiobesogênica, hipoglicêmica, anticarcinogênica, no metabolismo e comportamentos circadianos) de frutos nativos da região amazônica.

Amostras a serem acessadas:

Espécie(s): frutos das seguintes famílias botânicas serão investigados: Arecaceae,Sapindaceae, Lecythidaceae, Myrtaceae, Clusiaceae, Solanaceae, Malvaceae e Fabaceae.

Tipo de material/quantidade de amostras: Serão coletados frutos, em um total máximo de 50 kg

Local de depósito de subamostra: NÃO INFORMADO

Equipe do projeto: IVANA BEATRICE MANICA DA CRUZ / CPF 331.322.400-87
EULER ESTEVES RIBEIRO / CPF 000.678.812-20
MARIA FERNANDA MANICA RIZZI CATTANI / CPF 983.518.260-49
MARIA IZABEL DE UGALDE MARQUES DA ROCHA / CPF 243.430.990-91
CRISTINA DA COSTA KREWER / CPF 805.802.890-87
MARTA MARIA MEDEIROS FRESCURA DUARTE / CPF 428.275.150-91
MICHEL MANSUR MACHADO / CPF 936.104.590-34
MICHELE RORATO SAGRILLO / CPF 926.721.830-15
OLMIRO CEZIMBRA DE SOUZA FILHO / CPF 322.916.710-49
ALENCAR KOLINSKI MACHADO / CPF 007.046.370-06
ARAE RIGAO DE OLIVEIRA / CPF 023.215.180-65
CINTIA CORTE REAL RODRIGUES / CPF 014.581.210-38
CLARICE PINHEIRO MOSTARDEIRO / CPF 457.318.370-15
ELIZA RIBAS DA SILVEIRA FLORES / CPF 023.464.970-45
FERNANDA BARBISAN / CPF 022.159.910-03
FRANCINE CARLA CADONA / CPF 005.561.340-36
GREICE FRANCIELE FEYH DOS SANTOS MONTAGNER / CPF 005.505.050-67
IVO EMILIO DA CRUZ JUNG / CPF 989.567.010-91
LUIZ FILIPE MACHADO GARCIA / CPF 003.418.820-70

RAUL MOREIRA OLIVEIRA / CPF 022.226.840-99

Validade da Autorização: 06/08/2013 a 06/08/2017

A instituição acima qualificada deverá enviar ao CNPq, por meio da Plataforma Carlos Chagas, relatório anual sobre o andamento do projeto de pesquisa, nos termos do Decreto nº. 4.946/2003.

Esta autorização está vinculada às informações, declarações e termos de compromisso firmados pelo coordenador do projeto e pelo representante legal, constantes do Processo n° 010547/2013-4. Atividades de acesso aos conhecimentos tradicionais associados, de acesso e de remessa de componente do patrimônio genético com finalidade comercial, aplicação industrial, bioprospecção ou desenvolvimento tecnológico não estão autorizadas.

Caso seja identificado uso econômico de produto ou processo, passível ou não de proteção intelectual, originado das amostras de componente do patrimônio genético acessado no âmbito desta autorização, a instituição beneficiada se compromete a adotar as providências cabíveis, nos termos da legislação vigente, junto ao CGEN/MMA.

A remessa de amostra de componente do patrimônio genético deverá ser precedida da assinatura do Termo de Transferência de Material (TTM) ou do Termo de Responsabilidade para Transporte de Amostra de Componente do Patrimônio Genético (TRM). Para a remessa de componente do patrimônio genético para instituição sediada no exterior, deverá ser solicitada ao Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA, por meio de formulário específico e mediante a apresentação de TTM ou TRTM, licença de exportação complementar a autorização de remessa, mormente quando se tratar de remessa de espécies constantes nos Anexos da Convenção sobre o Comércio Internacional das Espécies da Flora e Fauna Selvagens em Perigo de Extinção (Cites).

Brasília, 18 de Julho de 2013

Paulo Sergio Lacerda Beirao

Diretor de Ciências Agrárias, Biológicas e da Saúde

Para visualizar a versão digital da Autorização de Acesso e de Remessa de Amostra de Componente do Patrimônio Genético, V.Sa. poderá utilizar a ferramenta disponibilizada pelo CNPq para esse fim na página http://servicosweb.cnpq.br/visualizador/ e informar o número do protocolo 3905612739716199 para recuperá-la do banco de dados do CNPq, ou poderá selecionar o arquivo salvo em seu computador (em formato PKCS7). V.Sa. pode também usar outro aplicativo disponível no mercado capaz de reconhecer arquivos no padrão PKCS7 para fazer a visualização e extração do documento.

Anexo 3: Protocolos experimentais

Preparo do extrato de Guaraná:

Para realização de todos os experimentos o guaraná em pó foi fornecido pela EMBRAPA Oriental (Empresa Brasileira de Pesquisa Agropecuária), localizado na Amazônia Ocidental em Maués, Amazonas - Brasil.

Inicialmente, um extracto hidro-alcoólico de *P. cupana* foi produzido a partir de meio padrão de álcool e água (70:30) a 100 mL de fluido de extração preparado a uma concentração de 300 mg / mL. Após 21 dias de extração, a preparação foi centrifugada a 3000 rpm durante 10 min e o sobrenadante foi isolado .

A solução resultante foi liofilizada para determinar xantina e catequina composições por meio de análise cromatográfica, tal como descrito em Bittencourt e cols (2013).

O extrato da amostra de guaraná foi filtrada através de um filtro de 0,45 mM para um frasco de auto-amostrador para análise. As condições de HPLC foram: taxa de fluxo, 1 ml / minuto, fase móvel A , 0,1% H3PO4 em água, fase móvel B e , com 100 % de ACN . O sistema de cromatografia foi calibrado com, pelo menos, uma curva padrão de cinco pontos em cada conjunto de amostras analisado.

A partir destes resultados, o composto de guaraná foi preparado para ser adicionado ao meio de cultura. O extrato liofilizado foi diluído em água destilada e preparada a uma concentração de 200 mg/ml. A mistura foi infundida durante 7 min por ebulição, centrifugadas (1500 rpm, 15 min) e filtrado. A solução foi esterilizada por filtração (0,20 mM) antes de se adicionar ao meio de cultura.

Preparo da cultura celular e tratamentos:

A linhagem de células T24 foi cultivada em meio RPMI 1640 suplementado com 10% de FBS, 100 unidades/ml de penicilina, 100 ug / ml de estreptomicina e 2 mM de L-glutamina, a 37 ° C sob 5% de CO_2 umidificada e 95% ar atmosférico.

Para testar o efeito anticancerígeno guaraná, as células T24/poços (2 x 10^5) foram semeadas em placas de 12 poços para a aderência de células com três poços para cada nível de concentração. Todos os ensaios foram realizados pelo menos em triplicata, com células em crescimento exponencial, após adesão ao fundo dos poços. Os extratos de guaraná foram diluídos em água destilada e foram adicionados nas concentrações finais de 3,8, 4,5, 6,0, 10, 30, 100, 300, 1000, 3000, 10000 e 30000 µg/mL.

Além disso, as células T24 também foram expostas a três drogas de quimioterapia utilizadas no tratamento do câncer de bexiga. Dessa forma, foram testadas como controlo positivo: o metotrexato, a cisplatina e a gencitabina em 3.8, 4.5, 6.0, 10 e 30 ug/ml de concentração. Estas concentrações foram escolhidas tendo como base um estudo *in vitro* que foi realizado com esses quimioterápicos e a linhagem T24 (Yoon e cols., 2011).

Análises de viabilidade e proliferação celular:

O efeito sobre a viabilidade celular de guaraná foi observada após 24 horas de exposição e o efeito sobre a proliferação foi observada após 72 horas.

A análise de MTT foi determinada por [3 - (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio] ensaio, a lactato desidrogenase (LDH) e o conteúdo de DNA de cadeia dupla no sobrenadante e nas células (Wang e cols. 2010).

Em suma, o MTT foi dissolvido em solução salina tamponada com fosfato a 5 mg / ml, e adicionada a cada poço e incubou-se durante 4 horas. Para dissolver os cristais de formazan, foi realizada uma incubação adicional com 200 mL de DMSO. A absorvância foi medida com um leitor de microplacas a 570 nm. A viabilidade celular observada em cada tratamento foi expressa como uma percentagem do valor de absorvência do controle. Uma vez que o ensaio MTT apresenta algumas limitações que podem subestimados os efeitos antiproliferativos de extratos ricos em polifenóis, antes da análise, as células foram centrifugadas a 3000 rpm durante 3 minutos, o sobrenadante descartado e as células foram ressuspensas em tampão de PBS.

Pela mesma razão, a análise de LDH foi determinada. Esta é uma enzima solúvel localizada no citoplasma que é libertada para o meio de cultura circundante a um dano celular ou lise, os processos que ocorrem na apoptose e necrose (Haslam e cols. 2000). A atividade da LDH foi medida utilizando a técnica colorimétrica, seguindo as instruções do fabricante (Labtest CO, São Paulo, Brasil).

Um teste adicional foi feito a fim de confirmar o efeito citotóxico do extrato de guaraná em células de câncro de bexiga. A viabilidade foi medida a partir da análise das concentrações de DNA livre presentes nas células. Essa medição foi feita pelo método fluorimétrico usando corante de DNA PicoGreen ® com um protocolo análogo descrito por Ha e cols. (2011).

As células T24 tratadas com extrato de guaraná foram analisados para observar a potencial indução de determinação da apoptose das caspases 1, 3 e 8 níveis. As atividades das caspases foram determinadas por imunoensaio

seguindo as instruções do fabricante (BioVision, Mountain View, CA). Todos os experimentos foram realizados em triplicata.

Como guaraná é rico em antioxidante molecules seu potencial efeito anticancerígeno provavelmente envolve desequilíbrio do metabolismo oxidativo das células cancerosas. Para testar essa hipótese, também foram analisadas as espécies reativas de oxigênio intracelulares (ROS) de produção de células de câncer de bexiga T24. A concentração de ROS foi determinada usando a célula não fluorescente que permeia composto diacetato de ensaio de 2'-7'-diclorofluoresceína (DCFDA). Nesta técnica, o DCFDA é hidrolisado por esterases intracelulares para DCFH, que está retido dentro da célula. Esta molécula não fluorescente é então oxidado para fluorescente diclorofluoresceína (DCF) por oxidantes celulares. Após o tempo de exposição designado, as células foram tratadas com DCFDA (10 fiM) durante 60 minutos a 37 ° C. A fluorescência foi medida a uma excitação de 485nm e uma emissão de 520 nm.

Anexo 4: Normas da Revista Científica

International Journal of Food Sciences and Nutrition
Instructions for Authors

About the Journal
Aims and Scope
The primary aim of this journal is to integrate food science with nutrition. Topics covered include:

- impact of nutritional science on food product development
- nutritional implications of food processing
- bioavailability of nutrients and non nutrients
- nutritional quality of novel foods
- role of the human microbiota in nutrition
- food-nutrient interactions
- use of biotechnology in food science/nutrition
- food acceptability and dietary selection
- nutritional and physiological aspects of food
- dietary requirements and nutritive value of food
- food toxicology

- behavioural and consumer science.

The Journal accepts *in vivo*, as well as epidemiological studies in humans. *In vitro* or animal studies are not suitable for publication unless clearly necessary to establish safety or mechanisms of action of nutrients or non nutrients in humans.

Manuscript Submission

All submissions should be made online at ***International Journal of Food Sciences and Nutrition**'s **ScholarOne*** site. New users should first create an account. Once a user is logged onto the site, submissions should be made via the Author Center. If you experience any problems with your submission or with the site, please contact ScholarOne support through the „get help now" link.

All submissions to the journal must include full disclosure of all financial, consulting, and personal relationships that could be viewed as presenting a potential conflict of interest.
Please see our full Declaration of Interest Policy for further information.

Manuscript Preparation

File preparation and types

Manuscripts are preferred in Microsoft Word format (.doc files). Documents must be double-spaced, with margins of one inch on all sides. Tables and figures should not appear in the main text. Specific instructions for their submission are given below. References should be given in Harvard style (see References section for example).

Manuscripts should be compiled in the following order: title page; abstract; key words; main text; acknowledgments; declaration of interest statement; appendices (as appropriate); references; tables with captions (on separate pages); figures; figure captions (as a list).

Cover Letter

A letter of submission from the corresponding author is required. The cover letter must report any existing financial arrangement between an author and a company whose product figures prominently in the submitted manuscript and a statement about any author"s work being concurrently published or reviewed that is relevant to the review of the manuscript being submitted to The Journal. The cover letter must also include the following statements:

- That the corresponding author and all of the authors have read and approved the final submitted manuscript

- That no portion of the work has been or is currently under consideration for publication elsewhere
- That no portion of the manuscript, other than the abstract, has been previously published or posted in the Internet.

Title Page

A title page should be provided comprising the manuscript title plus the full names and affiliations of all authors involved in the preparation of the manuscript. One author should be clearly designated as the corresponding author and full contact information, including phone number and email address, provided for this person.

Key Words

Three to six key terms that are not in the title should also be included on the title page. The keywords will assist indexers in cross indexing your article.

Abstract

All reviews should start with an abstract of 150 or fewer words, summarising the central core of knowledge that is the focus of the paper. It should be written in an informative style permitting its use, without revision, by abstracting services, give essential details of research findings without further reference to the text, and avoid generalisations and nonessential information.

Main Text

The article types considered by the *International Journal of Food Sciences and Nutrition* are as follows:

- Research Papers
- Brief Communications
- Comprehensive Reviews (max 1 per issue)
- Commentaries
- Gap-bridging research

Research papers

These are complete studies dealing with one of the covered topics. The body of the article should include the following distinct sections: introduction; methods; results; discussion; conclusions. Please do not merge these sections.

Introduction: This section should state the relevance and background to the study, and its rationale and purpose.
Methods: This section should include only information that was available at the time the plan or protocol for the study was being written. Please identify the methods, apparatus and procedures in sufficient detail to allow others to reproduce the results, and describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results.
Results: Present your results in logical sequence in the text, tables, and illustrations.
Discussion: This should include implications of the findings and their limitations, with reference to all other relevant studies and the possibilities these suggest for future research.
Conclusions: This must summarize the main paper. Ensure that extrapolations are reasonable and that conclusions are justified by the data presented, and indicate if the study design can be generalized to a broader study population.
Declaration of interest section: Please see below ("Acknowledgments and Declaration of Interest Sections") for more information.

Comprehensive reviews

These articles are intended to be full-length critical appraisals of topics that would be of long-term archival value. Emphasis should be placed on late-breaking advances. The body of a

review article should be a comprehensive, scholarly evidence-based review of the literature, accompanied by critical analysis and leading to reasonable conclusions. Wherever appropriate details of the literature search methodology should be provided, i.e. the databases searched, the search terms and inclusive dates, and any selectivity criteria imposed.
Wherever possible, use primary resources, avoiding “Data on File”, “Poster” or other unpublished references.

Letters to the Editor

Letters to the Editor will be considered for publication subject to Editor approval and provided that the content relates to articles published in *International Journal of Food Sciences and Nutrition*. Letters should be received shortly after publication of the original work in question. Each letter will be submitted to the author of the original paper in order that any reply may be published simultaneously with the letter.

Commentaries

Commentaries should be knowledge-based or consensus-type articles (e.g. working group statement) expressing objective opinions, experiences or perspectives on an important area related to food science or nutrition.

Brief Communications

Brief Communications are short research articles intended to present exciting findings that will have a major impact in food science and nutrition. Brief Communications are limited to 3,500 words, including the abstract, introduction, materials and methods, results, discussion, references and figure legends. The total word count must be listed on the title page.
Additionally, Brief Communications may include no more than 3 figures and/or tables, which together may occupy no more than one full page. Brief communications should be organized as described for Research papers and the abstract, introduction, and discussion must adhere to the word limits listed for Research papers. Brief communications will be immediately rejected if they do not conform to these guidelines,.

Gap-bridging research

Gap-bridging research articles cover extremely recent topics, with the main aim of indicating to the reader what new research is needed to increase the scientific knowledge in specific areas of food science and nutrition. This article type is specifically linked with the evaluation and approval of “health claims” by public institutions and should be the inspiration and basis for future research.

Acknowledgments and Declaration of Interest Sections

Acknowledgments and declaration of interest sections are different, and each has a specific purpose.

Acknowledgments Section

The Acknowledgments section details special thanks, personal assistance, and dedications. Contributions from individuals who do not qualify for authorship should also be acknowledged here. Acknowledgments should be included in a separate headed section at the end of the manuscript preceding any appendices, and before the Declaration of Interest Section. Please do not incorporate acknowledgments into notes or biographical notes. Some authors may elect not to include any acknowledgments.

Declaration of Interest Section

The Declarations of Interest Section should disclose any financial, consulting, and personal relationships with other people or organizations that could influence (bias) the author‟s work. Within this section also belongs disclosure of scientific writing assistance (use of an agency or

freelance writer), grant support and numbers (including NIH/Wellcome-funded papers), and statements of employment.
All declarations of interest must be outlined under the heading „Declaration of Interest" after the text. When submitting a paper via ScholarOne Manuscripts, the „Declaration of Interest" field is compulsory.

Please see our full Declaration of Interest Policy for further information.

References

References should be given in the Harvard style. Citation in the text is by author and date (Smith, 2001). The list of references appears alphabetically by primary author"s last name. Examples:

- *Journal:* Iyengar BS, Dorr RT, Remers WA. (2004). Chemical basis for the biological activity of imexon and related cyanaziridines. J Med Chem 47:218–223.

- *Book:* Vyas SP, Khar RK. (2001). Targeted and Controlled Drug Delivery. New Delhi, India: CBS Publisher and Distributor.

- *Contribution to a Book:* Chandrasekaran SK, Benson H, Urquhart J. (1978). Methods to achieve controlled drug delivery: The biochemical engineering approach. In: Robinson JR, ed. Sustained and Controlled Release Drug Delivery Systems. New York: Marcel Dekker, 557–593.

- *Electronic Resources*: Lin A-S, Shibano M, Nakagawa-Goto K, Tokuda H, Itokawa H, Morris-Natschke, SL, Lee K-H, (2007). Cancer Preventive Agents. 7. Antitumor-Promoting Effects of Seven Active Flavonolignans from Milk Thistle (*Silybum marianum*) on Epstein - Barr virus Activation. Pharm Biol [Online] Available at: http://www.informahealthcare.com/doi/abs/10.1080/13880200701585592. Accessed on 12 April 2009

Periodical abbreviations should follow the style given by Index Medicus.

Tables

Tables should be used only when they can present information more efficiently than running text. Care should be taken to avoid any arrangement that unduly increases the depth of a table, and the column heads should be made as brief as possible, using abbreviations liberally. Lines of data should not be numbered nor run numbers given unless those numbers are needed for reference in the text. Columns should not contain only one or two entries, nor should the same entry be repeated numerous times consecutively. Tables should be grouped at the end of the manuscript on separate pages. If Tables are in Microsoft Word format, they can be submitted at the end of the text in the same file as the text. However, if any of the Tables are in formats other than Microsoft Office, such as in Excel, the Tables must be submitted separately.

Expression of food composition data

Data expression of the reported compositional data
• Always give a precise description of the data denominator, examples:
• per 100 g fresh weight of edible food
• per 100 g dry matter of edible food
• per g protein of edible food
• per g total lipid of edible food
• per 100 g fresh weight of total food (edible and inedible food)
• per 100 g dry matter of total food
• per g protein of total food
• per g total lipid of total food

• If data are not expressed „per 100 g fresh weight of edible food", additional data are needed to be reported so that values can be calculated as per 100 g edible portion:

• Edible conversion factor needs to be reported if data are expressed per total food.
• Water (or dry matter) content per 100 g fresh food is needed if data are expressed per percentage or g dry matter.
• Lipid content per 100 g fresh food is needed if data are expressed per percentage or g fat/lipid, or the total fatty acids (FA) content is provided per 100 g edible portion.
• Protein content per 100 g fresh food is required if data are expressed per percentage or g protein.
• Expression such as „individual AA in g per 100 g" should be avoided, as it is not clear whether they refer to „per 100 g edible portion" or „per 100 g protein".
• When the sum of FAs percentage totals 100, it can be assumed that all FAs are reported; if the sum is below 100, it should be stated that not all FAs were reported, or it could mean that FAs are expressed in relation to total lipids.
• For FAs, it should be stated explicitly if they are expressed as FA or fatty acid methyl ester (FAME); data expressed in FAME need to be transformed through the Sheppard factor to fatty acid content. This is important especially for short chain FAs, as it changes the values considerably. For long-chain FAs, the difference would be less than 5 percent.
• In some cases, two fat values are reported, e.g. using the Soxhlet or Folch methods; it should be clearly stated if FA data are reported in relation to the value obtained by the Soxhlet or Folch methods.
• In some cases, inconsistencies were found in the data presentation between tables and the texts, e.g. the text refers to FAME while the table refers to FA.
• The use of the unit ppm should be avoided; it is preferable to use mg/kg or mcg/g. In general, for food composition purposes, the use of g/100g or mg/100g or mcg/100g is recommended.
• If data are presented in a figure/graph, it would be useful to mention the related values in the text.

Precision of data description (food, sampling, analytical method)
• It is important to describe food properly, always indicate:
• If with or without inedible part (and describe inedible part since refuse/waste depends on tradition/culture and personal preference). Examples: was the fish whole, or were fish fillets analysed with or without skin, including bones, visible fat removed)? Or for fruit: was it analysed with/without skin and with/without kernels?
• If raw or cooked (and described cooking method).
• If fresh or transformed (e.g. dried/sun-dried, frozen, salted, smoked, canned etc.).
• Food parts, e.g. leaf, tuber, root, meat cut, fillet, with or without skin or visible fat, from which animal, fat content, etc.
• For more information on food description, please see Module 3 of the Food Composition Study Guide (which can be downloaded from the INFOODS website: (http://www.fao.org/infoods/publications_en.stm).
• Indicate the sample collection site (e.g. country, region, etc.) as well as important sampling details (e.g. season, samples number per analytical sample, covering shops, markets, market share, etc.).
• Number of samples (n) should correspond to the number of individual samples analysed; however, „n" is often used in misleading manner, i.e. to express the analytical replicates number or the food item number initially sampled, but then analysed as composite sample.
• The component analytical method needs to be described correctly, especially if different methods are providing significantly different results (please see Module 4.b of the Food Composition Study Guide).
• Conversion factors and definitions should be indicated either in the text or as foot notes in table, e.g. nitrogen conversion factors, fatty acid conversion factors, energy conversion factors or vitamin conversion factors when used in vitamin equivalents (existing for niacin, vitamin E, vitamin D, vitamin A and folate. In these cases, the vitamin equivalent formula should be provided as well).

Examples for consistency checks
• The sum of proximates should ideally total 100. (Suggested acceptable ranges are 97-103 (Greenfield and Southgate, 2003) or 95-105. Proximates are: water, protein, fat, carbohydrates, dietary fibre (except if total carbohydrates are used), alcohol and ash.
• The sum of individual amino acids (AA) should be similar to the protein content.
• The sum of fatty acid (FA) should not be lower than the total lipid content.

Illustrations

Illustrations (line drawings, halftones, photos, photomicrographs, etc.) should be submitted as digital files for highest quality reproduction and should follow these guidelines:

- 300 dpi or higher
- Sized to fit on journal page
- EPS, JPG, TIFF, or PSD format only
- All illustrations should be submitted as separate files, not embedded in the text
- Legends or captions for figures should be listed on a separate page, double spaced

For information on submitting animations, movie files and sound files or any additional information including indexes and calendars please click here.

For information on colour figures and charges please click here.

Notes on Style

Style

Abbreviations and nomenclature
For abbreviations and nomenclature, authors should refer to The Proceedings of the Nutrition Society (1972: 31, 239-247) reproduction of "Conversion Factors and Nomenclature in Nutritional and Food Sciences". All vitamin names should adhere to these standards. Please provide scientific names (and references where applicable) for animals, microorganisms, and plants, including the full generic name at first mention. Full names should also be provided in captions, section heads, and keywords. All enzymes should be accompanied by its EC number.

Units/ Mathematical Modelling of Nutritional Processes
Data, including dosage forms, enzyme activity, and dietary ingredients, should be provided in SI units only, and energy values should be provided in both, kilocalories and kilojoules. Vitamin measurements must use the units mg, not IU. In all, units are year, month, week, d, h, min, s, kg, g, mg, mg, litre, ml, ml, fl. Please spell out the word "liter" whenever possible. Temperatures should be reported in degrees celsius.

Statistics
All methods of statistical analysis (and their software packages) should be described in the text. Both confidence intervals (CI) or significance assessments (provide p-values) are acceptable; standard deviation should only be used in instances in which emphasis needs to be given on varying individual values.

Solutions and Concentrations
Please use Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences. London: The Royal Society, 1972 for style requirements. Analytes in blood and other body fluids should be reported as molar concentrations (e.g. mmol/l).

Footnotes
Footnotes are not to be used except for designation of the corresponding author of the paper or current address information for an author (if different from that shown in the affiliation). Information concerning grant support of reviews should appear in a separate Declaration of Interest section at the end of the paper. Acknowledgments of the assistance of colleagues or similar notes of appreciation belong in a separate Acknowledgments section.
Footnotes to tables should be typed directly below the table and are indicated by the following symbols: * (asterisk or star), † (dagger), ‡ (double dagger), ¶ (paragraph mark), § (section mark), || (parallels), # (number sign). Reinitialize symbol sequence within tables.

General Style
Authors are asked to take into account the diverse audience of the journal. Please avoid the use of terms that might be meaningful only to a local or national audience, and do provide a clear explanation where this is unavoidable.

1. Plant classifications should follow the International Code of Botanical Nomenclature guidelines, put forth by the International Association for Plant Taxonomy. These guidelines can be found here.
2. Elements and chemicals should use standard Mendelian classifications.
3. When using a word which is or is asserted to be a proprietary term or trademark, authors must use the symbol ® or ™. Alternatively, a footnote can be inserted using the following wording: This article includes a word that is or is asserted to be a proprietary term or trademark. Its inclusion does not imply that it has acquired for legal purposes a non-proprietary or general significance, nor is any other judgement implied concerning its legal status.
4. Authors should write in clear, concise UK English. If English is not your native language, please ensure the manuscript has been reviewed by a native speaker. **Please note: extensive rewriting of the text will not be undertaken by the editorial staff.** For a list of English language writing services Informa Healthcare recommends, please click here.
5. "US" is preferred to "American", "USA" to "United States", and "UK" to "United Kingdom".
6. Upper case characters in headings and references should be used sparingly, e.g. only the first word of paper titles, subheadings and any proper nouns begin upper case; similarly for the titles of papers from journals in the references and elsewhere.
7. All acronyms for national agencies, examinations, etc., should be spelled out the first time they are introduced in text or references. Thereafter the acronym can be used if appropriate, e.g. "The work of the Assessment of Performance Unit (APU) in the early 1980s …" and subsequently, "The APU studies of achievement …", in a reference "(Department of Science [DS] 1999a)". Acronyms must not be used in the title of the manuscript.

Editorial Policies

Authorship

According to the International Committee on Medical Journal Ethics (ICMJE), an author is defined as one who has made substantial contributions to the conception and development of a manuscript. Informa Healthcare adheres to the ICMJE guidelines (http://www.icmje.org/#author), which state that "authorship credit should be based on all of the following: 1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; 2) drafting the article or advising it critically for important intellectual content; and 3) final approval of the version to be published"[1]. All other contributors should be listed in the Acknowledgements section.
All submissions are expected to comply with the above definition. Changes to the authorship list after submission will result in a query from the publisher requesting written explanation.

Redundant publications and plagiarism

The International Journal of Food Science and Nutrition considers all manuscripts on the strict condition that they have not been published already, nor are they under consideration for publication or in press elsewhere.
The International Journal of Food Science and Nutrition will not consider, in general, redundant publications, i.e. "publication of a paper that overlaps substantially with one already published in print or electronic media" (International Committee on Publication Ethics: http://www.icmje.org/publishing_4overlap.html). This rule applies whether the overlapping papers are from the same authors or not.
In addition, Informa has a strict policy against plagiarism. We define plagiarism as the use of extracts from another person's work that are not placed in quotation marks, without the permission of that person, and without acknowledgement to that person (using the appropriate reference style), with the result that your article presents these extracts as original to you. By submitting your work to an Informa Healthcare journal, you warrant that it is your original work, and that you have secured the necessary written permission from the appropriate copyright owner or authority for the reproduction of any illustration or other material.
Paraphrasing, defined as "taking portions of text from one or more sources, crediting the author/s, but only changing one or two words or simply rearranging the order, voice (i.e., active vs. passive) and/or tense of the sentences" by the US Office on Research Integrity (http://ori.dhhs.gov/education/products/plagiarism/7.shtml), will not be accepted if found to be

used extensively and to make up large sections of the manuscript. Extensive paraphrasing will not be deemed original work and will be viewed as redundant publication and/or plagiarism.
Informa Healthcare adheres to the Code of Conduct and Best Practice Guidelines set forth by the Committee on Publication Ethics (COPE). As per these guidelines, failure to adhere to the above conditions will result in the editor and Informa publishing an appropriate correction, a statement of retraction, or enacting a withdrawal of the article. In extreme cases (e.g. when an article is retracted), offending authors will be reported to their institution‟s Ethics committee and may be banned from submitting to Informa Healthcare journals in the future. Please refer to the COPE website for the detailed workflows for handling allegations of plagiarism or redundant publication (http://www.publicationethics.org/resources/flowcharts).

Peer Review

All manuscripts will be subjected to confidential peer review by experts in the field and on the basis of reviewers‟ feedback; papers will be accepted unconditionally, accepted subject to revision or rejected. Authors are required to submit the names of two to three recommended referees in the article‟s area of study. These referees should not work at the same institution or in the same lab group as the authors, nor should they have published with the authors within the last two years.

Ethics and Consent

- Do not use patients' names, initials, or hospital numbers, especially in illustrative material. Identifying information should not be published in written descriptions, photographs, and pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written informed consent for publication. Informed consent for this purpose requires that the patient be shown the manuscript to be published.
- To comply with FDAAA legislation, Informa Healthcare requires trial registration as a **condition of publication for all studies involving clinical trials**. Trial registration numbers should be included in the abstract, with full details provided in the methods section.
- Papers including clinical trials must be conducted with approval by human subject committees. All manuscripts, except reviews, must include a statement in the Introduction or Methods section that the study was approved by an Investigational Review Board (Human Studies Committee or Ethics Committee). Authors who do not have formal ethics review committees should include a statement that their study followed principles in the Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm).
- When a product has not yet been approved by an appropriate regulatory body for the use described in the manuscript, the author must specify that the product is not approved for the use under discussion or that the product is still under investigation.

Further information on Ethics and Consent can be found by clicking here

Copyright

It is a condition of publication that authors assign copyright or license the publication rights in their articles, including abstracts, to Informa UK Ltd. This enables us to ensure full copyright protection and to disseminate the article, and the Journal, to the widest possible readership in print and electronic formats as appropriate. Authors may, of course, use the article elsewhere after publication without prior permission from Informa UK Ltd., provided that acknowledgement is given to the journal as the original source of publication, and that Informa Healthcare is notified so that our records show that its use is properly authorised. Authors retain a number of other rights under the Informa UK Ltd. rights policies documents.
Authors are required to sign an agreement for the transfer of copyright to the publisher. All accepted manuscripts, artwork, and photographs become the property of the publisher. A copyright agreement form can be downloaded by corresponding authors of accepted manuscripts with proofs. This should be signed and returned to Informa Healthcare.
Authors are themselves responsible for obtaining permission to reproduce copyright material from other sources.

Further information on Permissions can be found by clicking here.

Declaration of Interest

It is the policy of all Informa Healthcare, to adhere in principle to the Conflict of Interest policy recommended by the ICMJE. All authors must disclose any financial and personal relationships with other people or organizations that could influence (bias) their work. It is the sole responsibility of authors to disclose any affiliation with any organization with a financial interest, direct or indirect, in the subject matter or materials discussed in the manuscript (such as consultancies, employment, paid expert testimony, honoraria, speakers bureaus, retainers, stock options or ownership, patents or patent applications or travel grants) that may affect the conduct or reporting of the work submitted. All sources of funding for reviews are to be explicitly stated. If uncertain as to what might be considered a potential conflict of interest, authors should err on the side of full disclosure.

Declarations of Interest should be stated at the point of submission (within the manuscript, after the main text, under a subheading "Declaration of interest", and within the appropriate field on the journal's ScholarOne site). Manuscript submission cannot be completed unless a declaration of interest statement is included.

Declaration of Interest statements will be made available to reviewers and will appear in the published article. If any potential conflicts of interest are found to have been withheld following publication, the journal will proceed according to COPE guidance.
The intent of this policy is not to prevent authors with any particular relationship or interest from publishing their work, but rather to adopt transparency such that reviewers, editors, the publisher, and most importantly readers can make objective judgements concerning the work product.

NIH/Wellcome Public and Open Access Policies

In consideration of the National Institutes of Health (NIH) and Wellcome Public and Open Access Policies, Informa Healthcare acknowledges that the broad and open dissemination of NIH/Wellcome-funded research results may benefit future scientific and medical research. Because we value the current and future contributions our journals make to the scientific body of knowledge, we have made certain that our policies accommodate those authors who wish to submit to PubMed Central.
As part of our author services program, Informa Healthcare will deposit to PubMed Central (PMC) and UK PubMed Central (UKPMC) author manuscripts reporting NIH or Wellcome Trust funded research.
This service will help authors to comply with the NIH and Wellcome Trust revised 'Public Access Policy' and „Open Access Policy", respectively.

NIH policy
NIH-funded authors must submit to PMC, or have submitted on their behalf, at the point of acceptance, their peer-reviewed author manuscripts, to appear on PMC no later than 12 months after final publication.
Click here for more information.

Wellcome Trust policy
Wellcome-funded authors must submit to UKPMC, or have submitted on their behalf, at the point of acceptance, their peer-reviewed author manuscripts, to appear on UKPMC no later than 6 months after final publication.
Click here for more information.

Informa Healthcare will deliver to PMC/UKPMC the final peer-reviewed manuscript, which was accepted for publication and that reflects any author-agreed changes made in response to the peer review. We will also authorise the author manuscript"s public access posting 12 months (NIH) or 6 months (Wellcome Trust) after final publication in print or electronic form (whichever is the sooner). Following the deposit, authors will receive further communications from the NIH

Manuscript Submission System/UK Manuscript Submission System with respect to the submission.

Under our Author Rights policy, authors also have the right to post their version of the submitted author manuscript (pre-print), or their version of the final published article (post-print) on their personal or institutional web site. Post-print web postings are subject to an embargo of 12 months. Please note that authors should not post manuscripts directly to PMC/UKPMC or other third party sites for any systematic external distribution by a third party (e.g., to a listserv or database connected to a public access server).

Additional Information

Proofs

Usual practice will involve corresponding authors receiving email notification with a password and web address from which to download a PDF. Hard copies of proofs will not be mailed. To avoid delays in publication, corrections to proofs must be returned within 48 hours, by electronic transmittal, fax or mail. Authors will be charged for excessive correction at this stage of production. If authors do not return page proofs promptly, the Publisher reserves the choice to either delay publication to a subsequent issue or to proceed to press without author corrections. The Publisher reserves the right to proceed to press without submitting page proofs to the author.

Reprints

Each corresponding author will receive a PDF file of the final version of their article. Reprints of individual articles are available for order at the time authors review page proofs. A discount on reprints is available to authors who order before print publication. Copies of the journal can be purchased at the author"s preferential rate of $25/£15 per copy.

Further information on Reprints can be found by clicking here.

Colour figure charges

Any figure submitted as a colour original will appear in colour in the Journal"s online edition free of charge. Print copy colour reproduction will only be considered on condition that authors bear the associated costs. The charge for an article with colour is US $575. There are no charges for non-colour pages.

Contact the publisher

Click here for contact details for the Publisher.