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Uncaria tomentosa: ADJUVANTE NO TRATAMENTO DO CÂNCER DE MAMA

TESE DE DOUTORADO

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Santa Maria, RS, Brasil.

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Uncaria tomentosa: ADJUVANTE NO TRATAMENTO DO CÂNCER DE MAMA

Maria do Carmo dos Santos Araujo

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Bioquímica Toxicológica**.

Orientador: Profa. Dra. Maria Rosa Chitolina Schetinger

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Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada,
aprova a Tese de Doutorado

***Uncaria tomentosa*: ADJUVANTE NO TRATAMENTO DO CÂNCER DE MAMA**

elaborada por
Maria do Carmo dos Santos Araujo

como requisito parcial para obtenção do grau de
Doutor em Bioquímica Toxicológica

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Dedico este trabalho às pacientes que corajosamente enfrentaram a luta contra o câncer de mama e aceitaram gentilmente partilhar comigo este período das suas vidas.

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Não sei se a vida é curta ou longa para nós, mas sei que nada do que vivemos tem sentido, se não tocarmos o coração das pessoas.
Muitas vezes basta ser: colo que acolhe, braço que envolve, palavra que conforta, silêncio que respeita, alegria que contagia, lágrima que corre, olhar que acaricia, desejo que sacia, amor que promove.
E isso não é coisa de outro mundo, é o que dá sentido à vida. É o que faz com que ela não seja nem curta, nem longa demais, mas que seja intensa, verdadeira, pura enquanto durar. Feliz aquele que transfere o que sabe e aprende o que ensina.

Cora Coralina

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

Uncaria tomentosa: ADJUVANTE NO TRATAMENTO DO CÂNCER DE MAMA

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Data e local da defesa: Santa Maria, 24 de abril de 2013.

Unha de gato (*Uncaria tomentosa*) é uma planta medicinal que tem sido utilizada no tratamento de diferentes patologias, dentre elas, o câncer. Estudos demonstram seus efeitos na restauração do DNA celular; no aumento nas frações dos leucócitos; ação mieloestimulantee na produção de interleucinas, entre elas a IL1 e a IL6, além de apresentar propriedades antioxidantes, antiproliferativas e auxiliar na indução da apoptose. O câncer de mama é a neoplasia maligna mais comum entre as mulheres. Um dos tratamentos preconizados para essa patologia é os agentes quimioterápicos, cujos efeitos tóxicos incluem a leucopenia e a neutropenia, com elevado risco de infecções. Desta forma, intervenções farmacológicas capazes de reduzir ou prevenir os efeitos adversos podem ter um impacto substancial sobre o tratamento do câncer. Neste contexto, situaram-se os objetivos deste estudo: avaliar o efeito da *Uncaria tomentosa* como adjuvante no tratamento do câncer de mama através de um ensaio clínico randomizado e o efeito citotóxico e a indução de apoptose na linhagem de células do câncer de mama MCF-7. Os resultados demonstram que *Uncaria tomentosa*, utilizada na dose de 300 mg de extrato seco (Unha de Gato Herbarium®), por dia, é eficaz na recuperação de neutropenia induzida por quimioterapia em mulheres diagnosticadas com Carcinoma ductal invasivo estágio II, e também é capaz de restaurar o DNA celular. O extrato de *Uncaria tormentosa* exerceu uma atividade citotóxica em células MCF-7 associada à morte celular por apoptose através da ativação das Caspases 3/8, e não desencadeou mudanças no perfil de resposta das células em apoptose, permanecendo semelhante à doxorrubicina. Dessa forma, a *Uncaria tomentosa* pode ser uma opção benéfica como adjuvante no tratamento do câncer de mama.

Palavras chave: Câncer. Mieloproliferação. Apoptose. *Uncaria tomentosa.*

ABSTRACT

Doctorate's Thesis
Post graduation Program in Biological Sciences: Biochemestry and Toxicology
Federal University of Santa Maria

Uncaria tomentosa: ADJUVANT IN BREAST CANCER TREATMENT

AUTHOR: MARIA DO CARMO DOS SANTOS ARAUJO
ADVISOR: MARIA ROSA CHITOLINA SCHETINGER
Date and place of defense: Santa Maria, April 24th, 2013

Cat's Claw (*Uncaria tomentosa*) is a medicinal plant that has been used in the treatment of different diseases, among them, cancer. Studies show its effects in restoring the cellular DNA, in increasing leukocyte fractions, myelostimulant action, and in producing interleukins, including IL1 and IL6, besides presenting antioxidant, antiproliferative, and assisting in the induction of apoptosis. Breast cancer is the most common malignancy among women. One of the recommended treatments for this pathology is the chemotherapeutic agents whose toxic effects include leucopenia and neutropenia, with high risk of infections. Thus, pharmacological interventions that reduce or prevent adverse effects can have a substantial impact on the treatment of cancer. In this context, the objectives of this study are: to evaluate the effect of *Uncaria tomentosa* as an adjuvant for breast cancer treatment through a randomized clinical trial and cytotoxic effect, induction of apoptosis in MCF-7 cell lines of breast cancer. Results show that *Uncaria tomentosa,* used at a dose of 300mg of dried extract (Unha de Gato Herbarium®), per day, is effective in recovery from neutropenia induced by chemotherapy in women diagnosed with invasive ductal Carcinoma Stage II, and it is also capable of restoring the cellular DNA. Extracts of *Uncaria tomentosa* exerted a cytotoxic activity in MCF-7 cells associated with cell death by apoptosis through the activation of caspases 3/8, and did not trigger changes in the response profile of cells undergoing apoptosis, remaining similar to doxorubicin. Thus, *Uncaria tomentosa* may be a beneficial option as an adjuvant therapy for breast cancer.

Keywords: Cancer. Myeloproliferation. Apoptosis. *Uncaria tomentosa.*

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LISTA DE ABREVIATURAS E SIGLAS

B-CFC	*Células formadoras de colônias de basófilos*
CAT	Catalase
CFU-GM	Unidades Formadoras de Colonias de Granulócitos e Macrófagos
CSFs	Fatores Estimuladores de Colônia
CFC	Células Formadoras de Colônia
DNA	Ácido Desoxirribonucleico
EROS	Espécies Reativas de Oxigênio
E-CFC	Células formadoras de colônias de eosinófilo
IL	Interleucina
HER2	Human Epidermal growth factor Receptor 2
IMC	Índice de massa corporal
C-ERBB2	Human Epidermal growth factor Receptor 2
MG-CFC	Células formadoras de colônia megacariocítica, monocítica-granulocítica
RE	Receptor de Estrógeno
RP	Receptor de Progesterona
RNA	Ácido Ribonucleico
SOD	Superóxido Dismutase
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico

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INTRODUÇÃO

A *Uncaria tomentosa* (Willd.) DC. também conhecida como *Cat's claw*, *una de gato* ou unha de gato, é um cipó espinhoso, pertencente à família das Rubiaceae, que se desenvolve em florestas tropicais úmidas do Oriente e do Sul da América (NOWAKOWSKA et al., (2009). Por centenas de anos tem sido utilizada por diversas tribos amazônicas, como a Ashaninka, a Aguaruna e a Cashibo Shipibo, no tratamento de várias doenças, inclusive do câncer (REINHARD, 1999). Foi legitimada como planta medicinal na *1st International Conference*, em Geneva, no ano de 1994 (NOWAKOWSKA et al., 2009).

Essa planta apresenta grandes quantidades de constituintes químicos bioativos, como o ácido quínico, os alcalóides oxíndoles tetracíclicos e pentacíclicos, os triterpenos, os glicosídeos, as flavonóides e as procianidinas. (LAUS et al.; AKESSON et al., 2005).

Dentre os compostos farmacologicamente ativos, os alcalóides oxindólicos são reconhecidos como marcadores fitoquímicos, pois estão associados às diversas ações farmacológicas (HEITZMAN et al., 2005). Os principais alcalóides da *Uncaria tomentosa* estão demonstrados na figura 1.

Alcalóides Oxindólicos	**Alcalóides Pentacíclicos**	**Alcalóides Tetracíclicos**
	Pteropodina **Isopteropodina** **Especiofilina** **Uncarina F** **Mitrafilina** **Isomitrafilina**	**Rincofilina** **Isorincofilina** **Corinoxeina** **Isocorinoxeina**

Quadro 1 - Principais alcalóides presentes no extrato de Uncaria tomentosa.
Fonte: Adaptado de Heitzman et al. (2005).

As propriedades medicinais da *Uncaria tomentosa* vêm surpreendendo cada vez mais o meio científico. Os pacientes que utilizavam *Uncaria tormentosa* em conjunto com terapias tradicionais para o câncer, como a quimioterapia e a radioterapia, relataram menores efeitos adversos a essas terapias, como perda de

cabelo, perda de peso, náuseas, infecções secundárias e problemas de pele (KEPLINGER et al.,1999; RIVA et al., 2001). Estudos relataram que a *Uncaria tomentosa* pode ajudar na restauração do DNA celular, prevenir mutações e danos celulares causados por quimioterápicos (SHENG et al., 2001). Outros estudos comprovaram que os alcalóides oxindólicos pentacíclicos de *Uncaria tomentosa* modulam atividades no sistema imune, como a proliferação de linfócitos B e T normais, inibindo a proliferação de linfoblastos e induzindo as células endoteliais humanas a produzirem interleucinas (IL) entre elas a IL-1 e IL-6, que são capazes de estimular a proliferação/ maturação celular e ativam células T, atuam como fatores de diferenciação de células B. (WURM et al., 1998; AKESSON et al., 2003; ALEN-HALL et al., 2007).

Diferentes extratos de *Uncaria tomentosa* foram testados *in vitro* a fim de determinar sua atividade antioxidante. Os extratos alcoólicos e aquosos previnem a produção dos produtos de reação com o ácido tiobarbitúrico (TBARS) e consequentemente dano à membrana citoplasmática (lipídios) e DNA através da não formação de radicais livres (DESMARCHELIER et al., 1998; GONÇALVES et al., 2005). Pilarski e colaboradores (2006), demonstraram que o extrato alcoólico (etanol 50%) apresentava atividade antioxidante *in vitro* destacando um aumento da atividade da *superóxido dismutase* (SOD).

A atividade moduladora no sistema imune e a capacidade de induzir o aumento da reserva de precursores mieloides na medula óssea foram comprovadas por diferentes grupos de pesquisa. Estudos realizados por Sheng et al. (2000), utilizando o extrato de *Uncaria tomentosa* na dose de 5 mg/kg/dia por seis semanas consecutivas, comprovaram o aumento do número de leucócitos (SHENG et al., 2000). Estes mesmos autores utilizando um modelo de ratos avaliaram o tratamento com extrato aquoso de *Uncaria tomentosa* (C-MED 100®) na recuperação da leucopenia induzida pela quimioterapia (doxorrubicina), utilizando como controle positivo o Fator Estimulante de Colônia de granulócito (Neupogen®). O extrato aquoso recuperou todas as frações dos leucócitos proporcionalmente, o que sugere um efeito mieloestimulante direto (SHENG et al., 2000). O efeito mieloestimulante direto com aumento na reserva de precursores mieloides na medula óssea, em conseqüência da atividade biológica de citocinas liberadas (IL-1, IL-6 e CSFs), foi comprovado por Eberlin et al. (2005) através do modelo de ratos infectados com dose letal de *Listeria monocytogenes.* Em 2011, Farias e colaboradores confirmaram

esses resultados valendo-se de um modelo de neutropenia induzida por ifosfamida em camundongos, o que causou uma neutropenia grave e os bioensaios mostraram que o tratamento com *Uncaria tomentosa* aumentou significativamente as contagens de neutrófilos e que uma potência de 82,5% foi calculada em relação ao Filgrastim (rhG-CSF: fator de crescimento hematopoético humano que regula a produção e liberação dos neutrófilos funcionais da medula óssea) em doses correspondentes testadas (5 e 15 mg/dia de *Uncaria tomentosa* e 3 e 9 mcg/dia Filgrastim, respectivamente).

Através de análises *in vitro* em células-tronco hematopoéticas (precursoras hHSPCs), obtidas a partir de sangue de cordão umbilical (SCU), concluiu-se que esse efeito ocorreu devido a proliferação de Unidades Formadoras de granulócitos e macrófagos (CFU-GM) (FARIAS et al., 2011).

As células sanguíneas originam-se na medula óssea a partir de células fonte com alto poder de diferenciação e capacidade de autorrenovação. Há dois tipos de células fonte: a célula totipotente e a célula multipotente ou pluripotente. As células multipotentes podem ser subdivididas em multipotente mieloide e multipotente linfoide, que irão dar continuidade à diferenciação para formação das células sanguíneas. Existe um tipo de Célula Formadora de Colônias (CFC) para cada tipo de linhagem de células sanguíneas. Essas células multipotentes mieloides diferenciam-se, também, em célula formadora de colônia megacariocítica, monocítica-granulocítica (MG-CFC), eosinofílica (E-CFC) e basofílica (B-CFC), a qual surge através de estímulos, como a IL-3, IL-1, IL-6 e GM-CSF (fatores estimuladores de colônias), conforme a figura 1 (ZAGO, 2004) Os agentes quimioterápicos são, em sua maioria, drogas com índices terapêuticos muito estreitos. A dose terapêutica é restrita pelos efeitos tóxicos, não seletivos, sobre os tecidos normais, e interfere no crescimento e na divisão celular, especificamente nas células de divisão rápida, como as células sanguíneas (EBERLIN, 2005). A neutropenia é o efeito mais observado, apresentando elevado risco de infecções (MANO 2008).

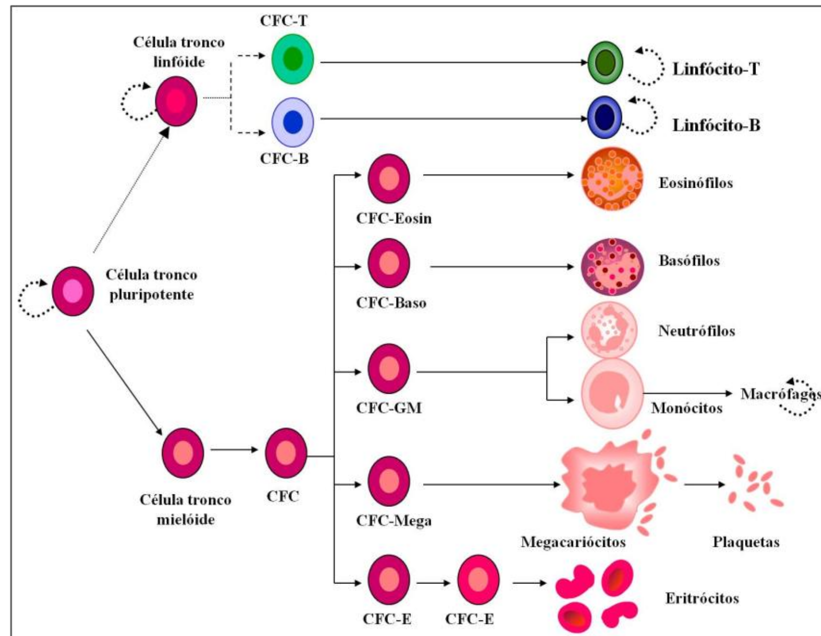

Figura 1 - Células pluripotentes com fatores estimuladores de colônias.
Fonte: www.lookfordiagnosis.com.

O câncer de mama é o tipo de câncer mais comum entre as mulheres e o segundo mais frequente no mundo, respondendo por 22% dos novos casos a cada ano. No Brasil a estimativa para ao no de 2012 foi de 52 casos/100 mil habitantes (BRASIL, 2008).

O prognóstico para as pacientes com diagnóstico de câncer de mama depende do estágio da doença, caraterísticas do tumor, idade, condições do paciente e do tratamento realizado. O estágio da doença inclui a localização do tumor primário, o envolvimento de linfonodos e as evidências de metástases. O sistema TNM (que inclui o tamanho do tumor primário, o envolvimento de linfonodos axilares e a evidência de metástases), é o método mais aceito para classificação de tumor de mama (Harris, 2000). Estágio I, tumor confinado na mama, ausente nos linfonodos; estágio II, tumor de mama com extensão axilar, lifonodos positivos; estágio III, tumor de mama envolvendo estruturas adjacentes, com linfonodos positivos; estágio IV, presença de sítios metastáticos a distância. (Harris, 1995; Lippman, 1999; Harris, 2000).

O carcinoma ductal e suas variantes representam 80% dos casos de tumores invasivos e a proporção de mulheres com tumores em estágios clínicos I e II aumentou de 41% para 65% na última década (DEVITA, 2008).

Avaliação prognóstica da neoplasia da mama também inclui a detecção de receptores hormonais de estrogênio e progesterona. Os receptores hormonais são proteínas especializadas, presentes em células mamárias, que ao se ligarem aos hormônios correspondentes desencadeiam uma série de eventos implicados com várias funções celulares, incluindo multiplicação celular e, consequentemente, o crescimento do tumor. A presença de ambos os receptores indica um estado funcional mais próximo da célula mamária normal, não tumoral e, portanto, são menos agressivos para o organismo. A falta de expressão do RP é um indicativo de menor diferenciação celular, sobretudo se esta negatividade estiver associada a altas taxas de proliferação celular. A ausência de ambos os receptores (RE e RP) já reflete um grau mais acentuado de perda de diferenciação e maior agressividade biológica. (UICC, 2006).

Os tratamentos preconizados, depois de feito o diagnóstico do câncer de mama, podem incluir cirurgia, radioterapia, quimioterapia e hormonioterapia. A quimioterapia é um tratamento sistêmico que pode ser uma terapia única ou uma combinação com múltiplos fármacos, que agem precisamente nas diferentes fases do ciclo do crescimento celular, inibindo a síntese de DNA, alterando as funções do DNA e do RNA, impedindo a divisão celular, levando, assim, à morte celular. O tratamento quimioterápico, em geral, utiliza um protocolo baseado na doxorrubicina a qual apresenta efeitos tóxicos não seletivos sobre os tecidos normais (BRASIL, 2004).

A doxorrubicina inibe a síntese de DNA e RNA através da intercalação entre os pares de bases do DNA, pela inibição da topoisomerase II (enzima responsável por modificações na estrutura espacial do DNA durante a sua duplicação e transcrição) e pelo impedimento estérico. Esta inibição resulta em ruptura das cadeias de DNA, levando a morte celular. (UICC, 2006).

O estresse oxidativo é um estado de desequilíbrio entre a produção de radicais livres, em particular as Espécies Reativas de Oxigênio (EROs) e a capacidade de defesa do organismo contra essas espécies, levando a um progressivo dano oxidativo (MANDA et al., 2009). Todos os componentes celulares são suscetíveis à ação das espécies reativas, porém, a membrana é um dos mais atingidos em decorrência da peroxidação lipídica, que acarreta alterações na estrutura e na permeabilidade das mesmas (HALLIWELL, 2007). A carbonilação de proteínas é outro exemplo de lesão biológica que pode ser promovida pelos radicais

livres. Seu aumento tem sido relacionado com muitas doenças, dentre elas o câncer (MAYNARD et al., 2009). As EROs podem agir na iniciação, na promoção e na progressão, ou seja, nos três estágios do desenvolvimento do câncer (BAKAN et al., 2003).

As mulheres acometidas por câncer de mama apresentam concentrações sanguíneas significativamente maiores de substâncias oxidadas, tais como os produtos derivados da oxidação de lipídeos, as proteínas e o DNA, se comparadas a mulheres que não possuem a doença. Há evidências de que o estado de estresse oxidativo é maior quanto maior for o grau de estadiamento da doença (GIBANANANDA et al., 2000; VALKO et al., 2006).

Para combater o câncer, o organismo vale-se de mecanismos de defesa naturais que o protegem das agressões impostas pelos diferentes agentes que entram em contato com suas diferentes estruturas. (HIDALGO et al., 2006).

Um processo fundamental para a manutenção e o desenvolvimento dos seres vivos é apoptose ou morte celular programada. Esse fenômeno biológico, além de desempenhar um papel importante no controle de diversos processos vitais, está associado a inúmeras doenças, inclusive ao câncer (HAIL, 2006).

As alterações morfológicas observadas na apoptose são consequência de uma sequência de eventos moleculares e bioquímicos específicos e geneticamente regulados (SARASTE, 2000). Dentre as alterações encontra-se a retração da célula, a perda de aderência com a matriz extracelular e as células vizinhas, a condensação da cromatina, a fragmentação internucleossômica do DNA e a formação dos corpos apoptóticos. (HAIL, 2006).

Muitas são as moléculas envolvidas no controle das vias de ativação da apoptose, como as proteínas antiapoptóticas e pró-apoptóticas, e as caspases. Desta forma, o processo de apoptose pode estabelecer-se por duas vias: a forma pela qual a célula responde à morte pode ser através do ambiente (via extrínseca/receptores de morte) ou por sinais internos, que induzem a ativação do processo apoptótico (via intrínseca/via mitocondrial). Independentemente da forma com que ela é iniciada, a apoptose resulta na ativação de uma classe específica de cisteína-proteases, as caspases (*cysteine-dependent aspartate-specificproteases*), presentes na sua forma inativa – pró-caspases, que clivam proteínas celulares, culminando na desestruturação celular (HENGARTNER, 2000).

Essas enzimas podem interagir com receptores de membrana ou moléculas adaptadoras que contenham domínios de morte (*death domain*) (BOATRIGHT et al., 2003). As caspases podem ser classificadas de acordo seu papel na apoptose, da seguinte forma: a caspases iniciadoras (ex. 8, 9) envolvidas na iniciação da cascata proteolítica, já as caspases efetoras (ex. 3, 7) responsáveis pela clivagem de substratos (RUPNARAIN et al., 2004, SHI, 2002).

A regulação da apoptose é mediada pela família Bcl-2, proteínas indutoras e repressoras de morte por apoptose. Os membros da família Bcl-2, como Bcl-2 e Bcl-XL, inibem a apoptose, prevenindo a liberação de citocromo c e são chamados de reguladores antiapoptóticos. Por outro lado, Bax, BID e Bad são proteínas pró-apoptóticas (HENGARTNER, 2000) (Figura 3).

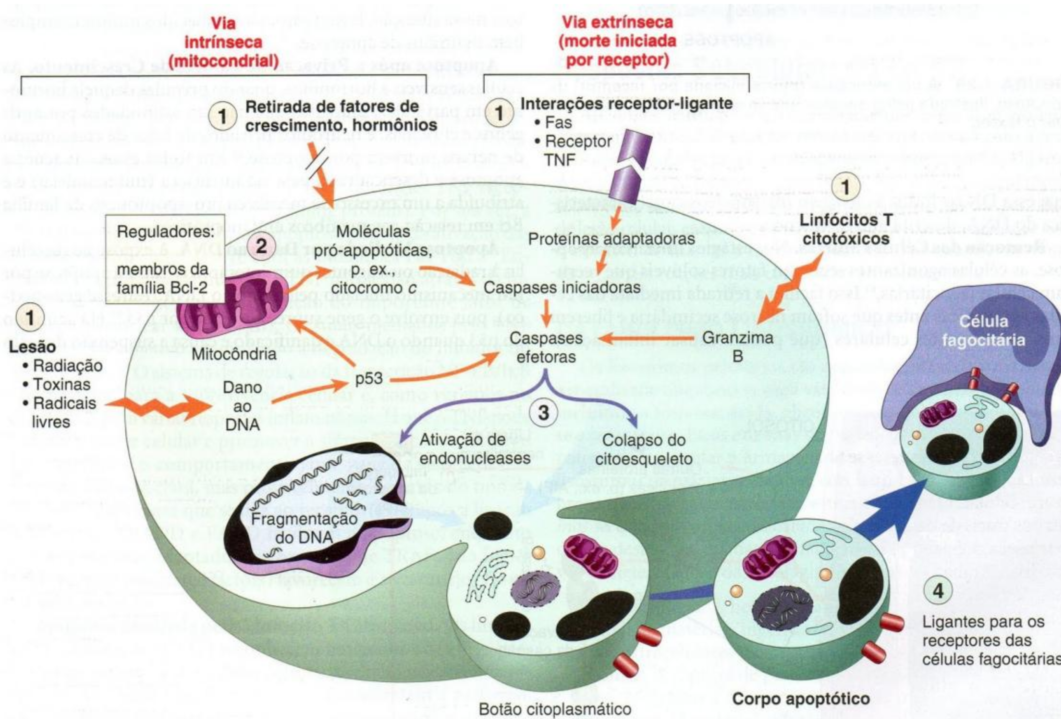

Figura 2 - Mecanismos gerais da apoptose.
Fonte: anatpat.blogspot.com

Sheng e colaboradores (1998, 2000) mostraram que os extratos de *Uncaria tomentosa* inibiram a proliferação de células tumorais *in vitro*, por meio de indução da apoptose, que foi demonstrada por características morfológicas e pela

fragmentação do DNA internucleossômico. Estudos *in vitro* apontam que a Unha de Gato inibiu diretamente o crescimento celular do câncer de mama em 90% (RIVA et al., 2001). Akesson et al. (2003) relataram que o extrato aquoso de *Uncaria tomentosa* inibiu, em ratos, a proliferação de linfócitos T e B. Já os estudos posteriores de Sheng et al. (2005) comprovam que o extrato de *Uncaria tomentosa* inibiu o crescimento celular sem causar morte celular, proporcionando, assim, melhores oportunidades de reparo de DNA, que resultaram na estimulação imunológica, na atividade anti-inflamatória e na prevenção do câncer.

Martino et al. (2006) investigaram o possível mecanismo proapoptótico e os efeitos citostáticos do extrato aquoso de *Uncaria tomentosa* em três diferentes linhagens de células tumorais: SAOS (*Células de osteosarcoma humano*), MCF7 (celulas de adenocarcinoma de mama humano) e HeLa (células de carcinoma cervical humano). O extrato foi fracionado em n-butanol e água. Os dados obtidos mostram claramente uma indução de apoptose, pela fração solúvel em n-butanol através da ativação de caspase 3 (MARTINO et al., 2006). Bacher et al. (2005) investigaram os efeitos antiproliferativos e apoptoticos de alcalóides oxindólicos purificados (Isopterodina, Pterodina, Isomitraphylina, Uncarine F, Mitraphylina), obtidos a partir da *Uncaria tomentosa* em células de leucemia linfoblástica humana (CCRF-CEM-C7H2). Os alcalóides Pterodina e Uncarine F apresentaram um potente efeito antiproliferativo com indução de apoptose. Posteriormente, Pilarski e colaboradores (2010) estudaram a propriedade anticâncer de extratos de *Uncaria tomentosa* com diferenças quantitativa em suas composições usando modelos *in vivo* e *in vitro* com diferentes linhagens de células cancerígenas. Os resultados comprovam que o extrato alcóolico de *Uncaria tomentosa* inibiu o desenvolvimento de células tumorais, entre elas, as MCF-7.

Os efeitos mieloestimulantes, antioxidantes, antiproliferativos e de reparos no DNA são atribuídos à *Uncaria tomentosa*, porém, nenhum dos estudos consistiu em ensaios clínicos. Neste contexto, situam-se os objetivos do estudo aqui presente: - utilizando comprimidos de *Uncaria tomentosa* Herbarium® como adjuvante no tratamento do câncer de mama, avaliou-se seu efeito mieloestimulante, sua ação nos sistemas oxidante e antioxidante, bem como sua interferência nos parâmetros hematológicos e imunológicos nas diferentes fases de tratamento do câncer. Após, através de um estudo *in vitro,* avaliou-se o efeito citotóxico e a indução de apoptose

do extrato de *Uncaria tomentosa* em linhagem celular MCF-7 de adenocarcinoma de mama.

Como objetivo geral, este estudo pretendeu:

- Avaliar a eficácia de *Uncaria tomentosa* como adjuvante no tratamento de câncer de mama considerando seus efeitos mieloproliferativos e citotóxicos.

Como objetivo específico do Artigo 1, teve:

- avaliar a efetividade da *Uncaria tomentosa* quando utilizada como adjuvante no tratamento do câncer de mama, nos parâmetros hematológicos, imunológicos, estresse oxidativo e danos ao DNA

Já o Manuscrito 2 teve por objetivos específicos:

- analisar o efeito citotóxico do extrato de *Uncaria tomentosa* na linhagem célular de câncer de mama (MCF-7);
- verificar a indução da apoptose por diferentes concentrações do extrato de *Uncaria tomentosa* nas células MCF-7;
- verificar a interação do extrato de *Uncaria tomentosa* e doxorrubicina com as células MCF-7.

ARTIGOS

Na sequencia são apresentados o artigo1 e o manuscrito 1 que contem os resultados dos estudos realizados. São eles:

1- *Uncaria tomentosa* - Adjuvant Treatment for Breast Cancer: Clinical Trial. Evidence-Based Complementary and Alternative Medicine (2012), Volume 2012, Article ID 676984, doi:10.1155/2012/676984.

2- Cytotoxicity and induction of apoptosis in MCF-7 cells by *Uncaria tomentosa*

ARTIGO 1

***Uncaria tomentosa* - Adjuvant Treatment for Breast Cancer: Clinical Trial.** Evidence-Based Complementary and Alternative Medicine (2012), Volume 2012, Article ID 676984, doi:10.1155/2012/676984.

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Abstract

Breast cancer is the most frequent neoplasm affecting women worldwide. Some of the recommended treatments involve chemo- therapy whose toxic effects include leukopenia and neutropenia.

This study assessed the effectiveness of *Uncaria tomentosa* (Ut) in reducing the adverse effects of chemotherapy through a randomized clinical

trial. Patients with Invasive Ductal Carcinoma—Stage II, who underwent a treatment regimen known as FAC (Fluorouracil, Doxorubicin, Cyclophosphamide), were divided into two groups: the UtCa received chemotherapy plus 300mg dry Ut extract per day and the Ca group that only received chemotherapy and served as the control experiment. Blood samples were collected before each one of the six chemotherapy cycles and blood counts, immunological parameters, antioxidant enzymes, and oxidative stress were analyzed. *Uncaria tomentosa* reduced the neutropenia caused by chemotherapy and was also able to restore cellular DNA damage. We concluded that Ut is an effective adjuvant treatment for breast cancer.

1. Introduction

Breast cancer is the most frequente neoplasm affecting women world wide, both in terms of incidence and mortality. The disease is more common in developed countries with its highest incidence being observed in the United Kingdom, Australia, USA, and Canada. From invasive tumors, ductal carcinoma and its variants represent 80% of cases [1] and the proportion of women with tumors in clinical stages I and II increased from 41% to 65% in the last decade [1, 2]. About 70% of breast cancers express Estrogen hormone receptors and/or Progesterone receptor [3]. These markers along with the HER-2 receptor (c-erbB2) provide information about the tumor and how it might respond to different treatments [4].

Chemotherapy is among the recommended treatments for breast cancer, which can be a single or combination therapy with multiple drugs. Chemotherapy drugs have very narrow therapeutic indexes in terms of nonselective toxic effects on normal tissues, with neutropenia being the most frequently observed adverse reaction, which increases the risk of infections [5]

Pharmacological interventions that reduce or prevent adverse effects may have a substantial impact on cancer treatment. According to World Health Organization (WHO), 80% of the population use medicinal plants as alternative or complementary procedures for the treatment of their diseases. [6]

Studies have reported the use of herbal medicines in cancer patients to minimize the effects of chemotherapy. *Uncaria tomentosa* (Ut or Cat's Claw) is

a medicinal herb that has been used in the treatment of different diseases including cancer. Patients who use Cat's Claw along with traditional cancer therapies, such as chemotherapy and radiation, reported fewer adverse effects to those therapies [7]. *Uncaria tomentosa* helps in the restoration of cellular DNA, preventing mutations and cell damages caused by chemotherapy drugs [8]. It modulates the activity in the immune system, such as the proliferation of normal T and B lymphocytes [9], also modulating certain cytokines, including IL-1 and IL-6, TNF-α [10]. In addition, it has antioxidant properties [11]. Its direct myelostimulating effects, through myelopoiesis stimulation and Colony-Stimulating Factors (G-CSF) [8, 12], seem to be a beneficial option to minimize the risks associated with neutropenia.

Numerous reports present a theoretical understanding of *Uncaria tomentosa* action mechanisms, but none of these studies consisted of clinical trials. Thus the objectives of this study are situated in this context, which consisted of a clinical trial using *Uncaria tomentosa* Herbarium tablets, as adjuvant treatment for breast cancer.

2. Methods

2.1. Design and Patients. A randomized interventional study was performed. It was carried out with 40 patients who had undergone complete breast cancer resection, which was histologically diagnosed as Invasive Ductal Carcinoma—Stage II [2], and who were going to begin adjuvant chemotherapy with Doxorubicin-based scheme for six cycles, at the Santa Maria University Hospital, Brazil.

Patients were randomly divided into two groups: the Ca Ut group, which was treated with six cycles chemotherapy + Ut and the cancer group (Ca), which only received six cycles of chemotherapy, according to the date treatment was started, as follows: the first patient who agreed to participate in the study was included into the CaUt group, the second, into the Ca group, and, thus, successively, until the end.

For the control group were invited to participate healthy women, classified by clinical trial, with similar age of the patients and that did not receive any medication in the last 30 days or have chronic disease.

Patients were part of the study during 6 chemotherapy cycles, of 21 days each. Medication dosage in the CaUt group was as follows: FAC (Fluorouracil, Doxorubicin, and Cyclophosphamide) and 3 tablets of Ut (Unha de Gato Herbarium), daily, from day 2 to day 21. The dose of Ut was similar to that used in previous studies, with 250–350mg C- MED-100, in aqueous Ut extracts [13].

The calculation to estimate the sample size required for randomized clinical trial was performed according to Greenberg et al. [14], with constant significance level (α) of 5%, and statistical power of 90% (β 10%), using as reference the studies of Sheng et al. [15].

The Human Ethics Committee of the Santa Maria University Hospital, Brazil, approved the present study and informed consent was obtained from all participants (protocol number: 0169.0.0242.000-07.). All subjects were invited to participate and were informed in detail about the design of this study through a Statement of Consent signed by the researcher and participants. They were informed that they could be selected randomly for the Ca or UtCa group.

2.2. Materials. Each tablet of Unha de Gato Herbarium contained 100mg of dry *Uncaria tomentosa* extract. Biological materials used in the tablets were derived from plants in their natural habitat. The *Uncaria tomentosa* extract was prepared by Ultra-turrax Extraction (Biotron, Kinematica AG) from ground bark (Centroflora) using 70% ethanol (Dipalcool). The HPL Canalysis of the Ut dry extract presents 2.57% pentacyclic oxindole alkaloids (POAs) content, which was calculated with reference to external calibration curves of mitraphylline. The extract analysis showed absence of tetracyclic oxindole alkaloids in the sample, allowing its use for therapeutic and research purposes in accordance with the U.S. Pharmacopeia.

2.3. Sample Collection. Blood was collected into citrate, EDTA, heparin Vacutainer tubes, without any anticoagulants, before chemotherapy and after each of the 6 cycles.

2.4. Biochemical Parameters. A COBAS INTEGRA system was used for the quantitative determination of the blood chemical constituents, and data were acquired through a COBAS INTEGRA 400 Plus apparatus (USA).

2.5. Hemograms. Blood samples were analyzed using a Pentra apparatus (France). The lowest values were confirmed by observation of slides, using a MayGr¨unwald-Giemsa Stain and optical microscopy.

2.6. CD3+, CD4+, and CD8+ Cells. Samples were collected in EDTA and analyses were performed using a three-color fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson Biosciences, United States) and a Multiset soft- ware (Becton Dickinson). FITC-conjugated anti-CD4, PE- conjugated anti-CD8, and PerCP-conjugated anti-CD3 were used. Immune subpopulations were measured as a percent- age of the total CD3+ cell number.

2.7. Interleukin 6 (IL-6). ELISA assays of IL-6 were carried out according to a previously published method [16], at room temperature in Microtiter 96-Well Plates (Nunc-Immuno Plate MaxiSorp) and optical densities (O.D.) at 490nm, which were determined using a Microplate Reader (Thermo Scientific Multiskan FC, Vantaa, Finland).

2.8. Single Cell Gel Electrophoresis (Comet Assay). The alkaline comet assay was performed as described by Singh et al. [17] in accordance with the general guidelines for use of the comet assay [18, 19]. Lymphocytes were suspended in 0.7 % low-melting-point agarose and phosphate-buffered saline (PBS) at 37◦C and placed on microscopic slides with a layer of 1% agarose. The slides were immersed in lysis solution at 4◦C for 1h and followed by electrophoresis at 25V, 300mA, for 40 min at steady temperature. The slides were thensilver-stained, as described by Nadin et al. [20]. Allsteps, from sample collection to electrophoresis, were conducted under yellow light to minimize the possibility of cellular DNA damage. One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration representing a damage index of 0%) to 400 (all cells with maximal migration, representing a damage index of 100%). The slides were analyzed under blind conditions by at least two different individuals [21].

2.9. Carbonylation of Serum Protein. The carbonylation of serum proteins was determined by a modified Levine's method [22]. The absorbance of the supernatant at 370nm was measured using a spectrophotometer. Carbonyl content was calculated using 22 × 103 mM−1 cm−1 as the molar extinction coefficient, and the results were expressed as nanomoles of carbonyl groups per milligram protein.

2.10. Determination of Lipid Peroxidation. Lipid peroxidation was estimated by measuring TBARS levels in plasma samples according to a modified method of Jentzsch et al. [23]. The concentration of malondialdehyde (MDA) was determined by measuring the absorbance at 532nm using a spectrophotometer. The results were expressed as nanomoles of MDA per milliliter of plasma.

2.11. Catalase (CAT) and Superoxide Dismutase (SOD) Activities. CAT activity was determined in accordance with a modified method of Nelson and Kiesow [24]. The change in absorbance at 240nm was measured for 2 min. CAT activity was calculated using the molar extinction coefficient (0.046 mM^{-1} cm^{-1}), and the results were expressed as picomoles of CAT per milligram of protein. SOD activity was determined based on the inhibition of the radical superoxide reaction with adrenaline as described by McCord and Fridovich [25]. SOD activity is determined by measuring the rate of adrenochrome formation, observed at 480nm, in a medium containing glycine-NaOH (50mM, pH 10) and adrenaline (1mM).

2.12. Statistics. Results are expressed as mean ± standard deviation. The statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Prism 5.0 Software Inc., USA) using the Student's t-test. P<0.05 was considered to represent a significant difference in all tests.

3. Results

All patients (40) included in the trial had Breast Cancer, Invasive Ductal Carcinoma—Stages II A or II B, according to the American Joint Committee on Cancer (AJCC) and the American Cancer Society (ACS) staging systems [2].

The general characteristics of patients and controls who participated in the study are described in Table 1.

Table 1 - Clinical characteristic of patients. It represents age, body mass index (BMI), total cholesterol levels, estrogen receptor (ER) and progesterone receptors (PR), as well as the HER-2 status in different groups.

Clinical parameters	*Control (n=20)*	*Ca (n=20)*	*UtCa (n=20)*
Age interval	32-79	32-71	40-75
Mean Age	56.5± 11.6	55.0± 9.7	54.4± 11.0
BMI	25.0± 1.93	27.27± 1.49	26.82± 5.03
Cholesterol levels	202.5± 1.90	238.9± 57.9	244.2± 44.5
Estrogen receptor status (ER)			
Positive	-	+14	+17
Negative	-	-6	-3
Progesterone receptor status (PR)			
Positive	-	+10	+11
Negative	-	-10	-9
HER-2 receptor status (HER2)*			
Positive	-	+2	+6
Negative	-	-16	-12

The results for ER, HER2, and REP are represented as positive and negative numbers for the expression of receptors by number of women, while other parameters are expressed as mean ± standard deviation. UtCa group: patients treated with chemotherapy +300 mg Uncaria tomentosa daily; Ca group: patients received chemotherapy; control group. *To HER-2 receiver only data were obtained from 18 patients.

To evaluate the effectiveness of Ut as adjuvant treatment for breast cancer, haematological parameters were used and analyzed (Table 2). At day zero, the results of the haematological parameters analyzed in the blood count.

Did not significantly differ among the control, the Ca and the UtCa greater reduction in the white blood cell (WBCs) and the neutrophil counts were observed in the Ca group along the treatment, differently from the UtCa group, which remained closely the reference values, obtained in the control group (Figure 1). Considering the lymphocytes number, a significant difference between the control group and the groups of patients with breast cancer, either treated or not with Ut in the chemotherapy cycles, was observed. ($P< 0.05$).

Monocytes number in patients with breast cancer (treated and not treated with Ut) at 5-6 chemotherapy cycles were higher than control group, but in the UtCa group, this increase was more strong (Table 2).

Table 2 - Leukocytes, neutrophils, lymphocytes, and monocytes levels in breast cancer patients before treatment and after 6 cycles of chemotherapy without *Uncaria tomentosa* supply (Ca group) or receiving 300 mg/day of *Uncaria tomentosa* (UtCa group).

Parameters	***cycles***			
(cells/mm³)	***0***	***1-2***	***3-4***	***5-6***
Leukocytes				
Control	6800±1458			
UtCa	6800±1458	7890±1615	6636±2578	5469±1626
Ca	6653±1158	6617±1504	4092±1047*#	3247±1117*#
Neutrophils				
Control	3510±1077			
UtCa	3496±1108	4335±1626	3937±1992	4016±1545
Ca	3588±1081	2663±1351*	2028±512*#	1083±368*#
Lymphocytes				
Control	2264±490,6			
UtCa	2276±503,3	2376±708,1	1627±578,7*	1411±596,6*
Ca	2177±453,3	2284±867,9	1460±512,5*	1208±395,1*
Monocytes				
Control	487,6±128,9			
UtCa	515,3±169	560±322	814,9±309#	817±444,6
Ca	541,6±161	526,6±154	654,1±310*#	500,9±226*#

Data expressed as mean ± standard deviation. *Represent difference significant between the Ca and UtCa groups, P < 0.05. #Represent difference significant of the control group, P < 0.05 (Student's t-test).

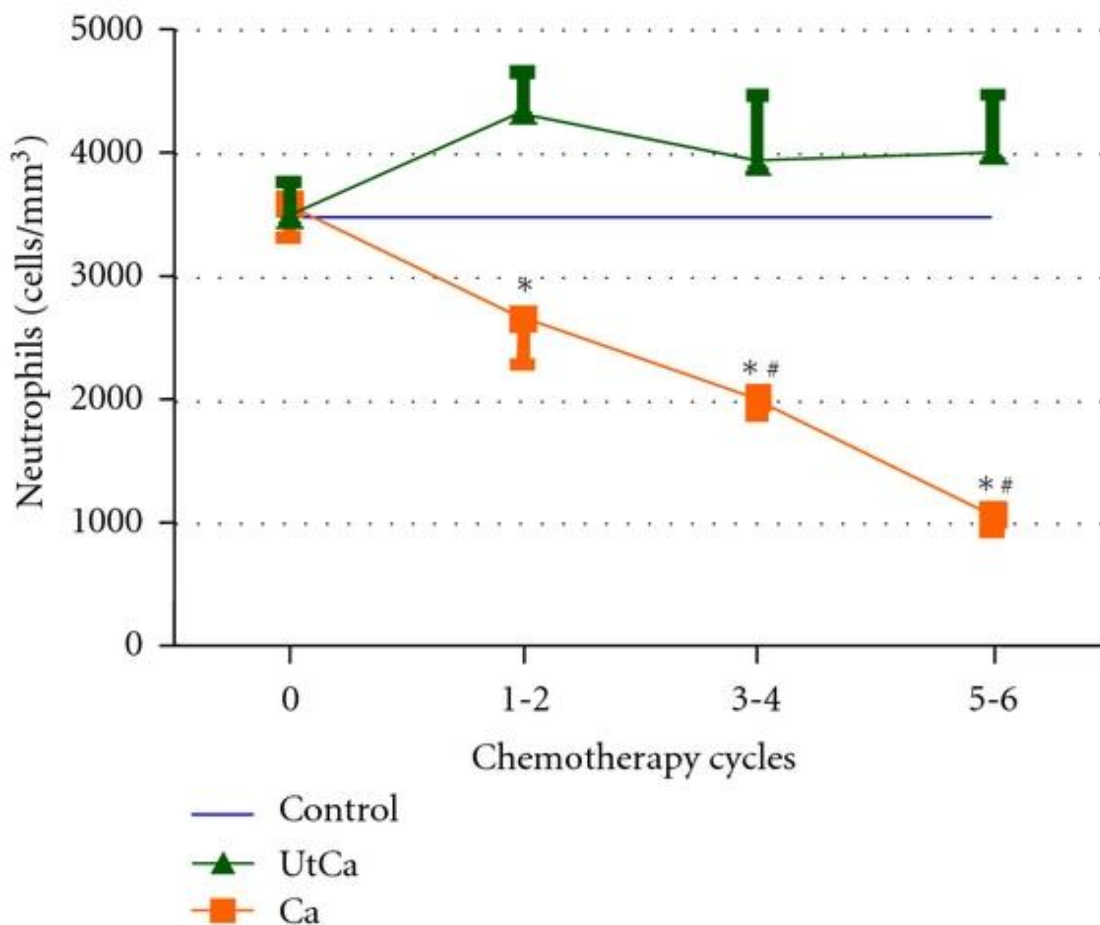

Figure 1 - Values neutrophil granulocytes in patients with breast cancer undergoing chemotherapy with (UtCa) and without (Ca) supplementation with Uncaria tomentosa and reference values (control). Data are expressed as mean±standard deviation.

To evaluate the immune response of patients with breast cancer, $CD4^+$ T cells, $CD8^+$ T cells (absolute count and ratio) and IL-6 levels were analyzed. During the chemotherapy treatment cycles, no significant difference was observed between groups. There was no difference between groups for any of the parameters analyzed (Table 3).

Table 3 - Immune status of breast cancer patients before treatment and after 6 cycles of chemotherapy without *Uncaria tomentosa* supply (Ca group) or receiving 300 mg/day of *Uncaria tomentosa* (CaUt group).

Parameters	***Group***	***Chemotherapy cycles***	
		0	**6**
$CD4^+$ T Cells Cells/µL	Ut Ca	1008.25 (379.21)	786.60. (310.49)
	Ca	1053.00 (620.81)	798.00 (366.14)
$CD8^+$ T cells Cells/µL	Ut Ca	568.81 (295.60)	459.87 (246.46)
	Ca	679.84 (273.22)	565.62 (231.05)
$CD4^+T/CD8^+T$ Ratio	Ut Ca	2.044 (0.62)	1.858 (0. 89)
	Ca	1.630 (0.69)	1.652 (0.49)
IL6 pg/mL	Ut Ca	3.4 (4.50)	2.1 (6.6)
	Ca	5.6 (5.53)	3.8 (7.312)

Data expressed as mean ± standard deviation; UtCa group: patients treated with chemotherapy + 300mg *Uncaria tomentosa* daily (n=20); Ca group: patients received chemotherapy (n=20).

No correlation between the IL-6, $CD4^+T/CD8^+T$ ratio and age, body mass index, and hormone receptor status was found (Data not shown).

Antioxidant defenses were analyzed by the activity of Superoxide Dismutase (SOD) and Catalase (CAT) compared to treatment cycles zero and six, as well as between the UtCa and the Ca groups. There were no statistically significant differences among groups. An increase in SOD enzyme when compared to treatment cycles zero and six for the group supplemented with *Ut* was observed, but that difference was not observed between the groups (UtCa = 11.53 U/ mg protein, Ca = 11.43 U/ mg protein,) or at the end of treatment (17.32 U/mg protein, 11.74 U/mg protein). Lipid Peroxidation was also estimated by the TBARS scale and the carbonylation of serum proteins, but there was no difference between groups (UtCa and Ca).

The protective effect of chemotherapy to extract *Ut* was evaluated by the Comet Assay.

In the start of the treatment (zero cycle), the Ca group and UtCa group showed no significant difference in the Comet assay index. However, in the

sixth cycle (end of the treatment) it was observed a significant decrease in the index test in the UtCa group, when compared to the Ca group (*P <0.05*).

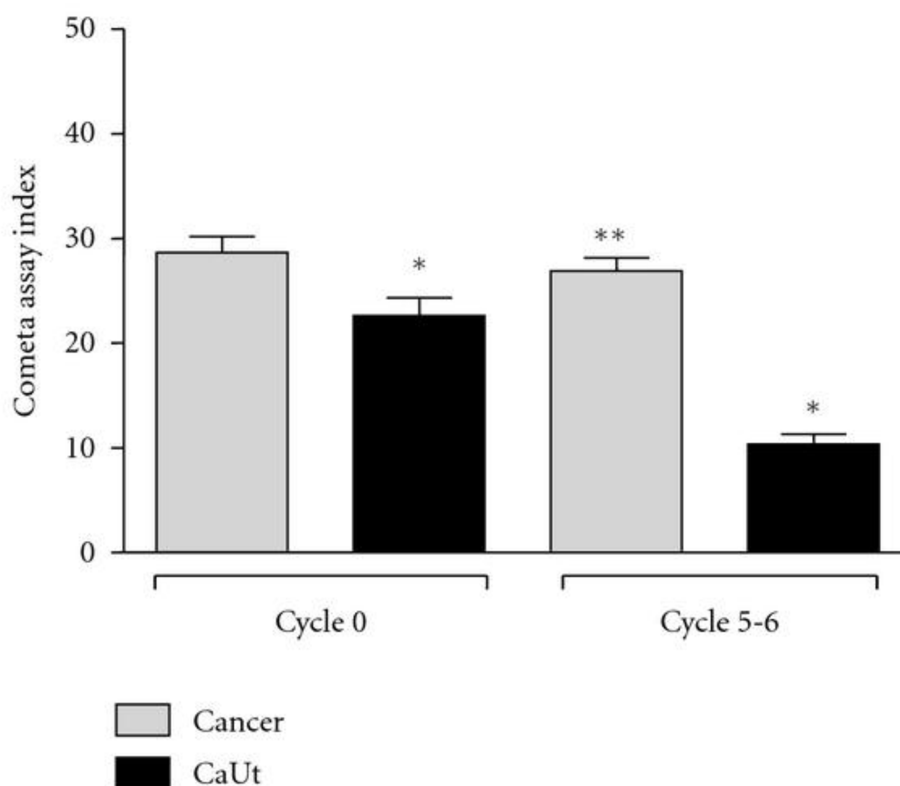

Figure 2. Index test of blood cells in patients with breast cancer treated and not treated with *Uncaria tomentosa.* * Represents significant difference between all groups (*P<0.05*). ** Represents significant difference between the UtCa group (cycle 0) and the UtCa group (cycle 5-6) *P <0.05* (Student's t-test).

4. Discussion

Uncaria tomentosa enables the stimulation of the immune system, increasing resistance to diseases when the body is immunosuppressed due to stress, malnutrition, or due to the effect of some medication.

Many herbal medicines are used for various purposes, in various combinations (along with allopathic and homeopathic, medicines, etc.) based on historical or personal evidences generally not being associated with any adverse effects [26]. Therefore, this study, through a randomized clinical trial, evaluated the efficacy of Ut as a complementary therapy to chemotherapy.

The cytotoxic effect of chemotherapeutic agents is not selective for neoplastic cells, being also harmful to other body cells. Hematopoietic suppression is the major complication limiting dosage of such cytostatic agents; neutropenia and thrombocytopenia are the most frequent ones [1].

Treatment should be discontinued when neutrophil count is below 500 cells/mm³ [27]. Thus, the success of the treatment process depends on the neutrophils content. Prevention of chemotherapy-induced neutropenia should be considered a clinical priority [28]. Once it is known that neutropenia predisposes to serious infections, often resulting in delays in treatment cycles and dose reductions. Treatment using a daily dose of 300mg dry Ut extract was effective in reducing the main chemotherapy effect, which is neutropenia. The effect of chemotherapy on blood cellstend to become more pronounced during treatment. However, our results show that in cycle six, which corresponds to the end of chemotherapy, the differences in the leukocytes and neutrophils counts were even more significant, as the group that was supplemented with Ut presented values twice as high of neutrophils when compared to the cancer group (without supplementation). In the group without supplementation, 67.89% of patients had neutropenia. Similarly, there was an increase in activated monocytes, as the activated precursors were common to both strains.

Our findings are corroborated by other studies that had already shown that the Ut extract has a stimulating effect on growth and differentiates the CFU-GM from mice bone marrow and spleen, using the model for listeriosis [29]. Increased leukocytes numbers were also detected using Ut aqueous extract for six consecutive weeks in volunteers [13]. The recovery of leukocytes was also observed in mice using a model for chemotherapy-induced leukopenia (Doxorubicin) using Granulocyte Colony-Stimulating Factor (Neupogen®) as a positive control [15].

Our group confirmed these results using a model for ifosfamide-induced neutropenia in mice, which caused a severe neutropenia. Bioassays showed that treatment with Ut significantly increased neutrophils counts, and a power of 85.2% was calculated in relation to Filgrastim (rhG-CSF) at the corresponding doses tested (5 and 15mg/day of Ut, and 3 and9mcg/day Filgrastim, resp) [13]. Through *in vitro* assays in human hematopoietic stem precursor cells (hHSPCs) obtained from umbilical cord blood (UCB), we reach the conclusion that this

effect happened due to proliferation of Forming Units-Granulocyte-Macrophage (CFU-GM) [13].

In this study, no differences were observed in lymphocyte counts between groups, either supplemented or not with Ut, over the chemotherapy cycles; however, its counts presented decrease due to chemotherapy when compared to the control group. These differences were not observed in the CD4+ and CD8+ subpopulations.

This paper reports the effects of different *Uncaria tomentosa* extracts. The aqueous extract that has the highest concentration of quinic acid and low concentrations of oxindole alkaloids, being related to immunomodulatory properties is mediated by cytokines such as TNF-α [30]. Clinical studies using 20 mg/day of *Uncaria tomentosa* extract for 2 to 5 months in patients with HIV, receiving no other therapy, showed an increase in total peripheral lymphocytes without significant changes in the proportion of CD4+ and CD8+[31]. Healthy volunteers receiving 350 mg of aqueous Ut extract for 8 weeks showed leukocytosis, with a tendency to higher proliferation of lymphocytes [15]. In an animal model, using aqueous extract, which has the highest concentration of quinic acid and low concentrations of oxindole alkaloids, an increase in lymphocytes was also observed [15, 32].

Furthermore, alcoholic extracts and/or pentacyclic oxindole alkaloids have higher myeloproliferative effects [33, 34]. Other studies have shown that the increase in the lymphocyte counts happens due to increased survival rates rather than proliferation [32].

Thus, changes observed in lymphocytes are associated with the chemically active components defined as quinic and bioactive acid esters in vivo, as quinic acid present in the aqueous extract used by authors. In our study, hydroalcoholic extract was used.

High levels of circulating IL-6 are associated with worse survival rates for patients with metastatic breast cancer, being correlated to the extent of the disease [30].

The patients who comprised our sample did not present a negative progression during the treatment cycles, that is, there was no occurrence of relapses or increased lesion extent, which could lead to an increase in IL-6.

Different *Uncaria tomentosa* extracts were tested *in vitro* in order to determine their antioxidant activity. Aqueous and alcoholic extracts prevent the production of reaction products with thiobarbituric acid (TBARS) and, therefore, damage the cytoplasmic membrane (lipids) and DNA by the nonformation of free radicals [35, 36] among the evaluated parameters of oxidative stress, such as SOD, CAT, TBARS, and carbonylated proteins.

Women with breast cancer present an increase in blood concentrations of oxidized substances, such as products derived from lipids peroxidation, proteins, and DNA [32, 33]

The only observed differences were in the SOD enzyme between groups, either with or without supplementation with Ut. These results were also found in an animal model, where an increase in the activity of this enzyme [10] was perceived. In a study on women with breast cancer, SOD activity showed a significant increase regardless of clinical stage and menopausal status [31]. There is evidence that the state of oxidative stress is higher than the greater degree of the disease stage is [37, 38].

Similar to results found in IL-6, the fact that all patients in the study had Stage II cancer may explain the results found.

The ability of doxorubicin to bind itself to the cell membrane lipid can affect a variety of cellular functions. The reaction of the doxorubicin enzymatic reduction by a variety of oxidase, reductase, and dehydrogenases genes generates ROS and, thus, may result in damage to DNA and proteins, triggering apoptosis [39, 40]

The performance of antioxidants in vivo depends on the types of free radicals formed, where and how these radicals are generated, and what are the doses for optimal protection. So it is entirely possible for an antioxidant to act as a protector in any given systems, but it is also possible for it to fail to protect, or even increase lesions induced in other systems or tissues. Thus, the use of antioxidants in cancer treatment is controversial.

Ambrosone and colleagues [41] observed that women having breast cancer with genotypes that result in higher levels of ROS had better survival rates than those with genotypes associated with lower generations of ROS. Such results indicate that an increased oxidative stress may increase the effects of chemotherapy and/or radiotherapy, resulting in proved treatment

efficacy and, thus, better survival rates. The overexpression of SOD is associated with better survival rates for patients diagnosed with colorectal cancer [42].

Cleveland and Kastan suggest that a promising treatment for some types of cancer could happen by increasing ROS levels and inhibiting SOD levels [43, 44]. Other authors report that the overexpression of SOD has presented resistance to doxorubicin [43, 44], but not 5-fluorouracil in gastric cells [41]. Another study on breast cancer cells showed an increased resistance to Adriamycin with the intracelular level of glutathione (GSH) [45].

The protective effect of Ut on DNA was observed during the breast cancer cycles of treatment, by the Comet test analysis.

Doxorubicin has its own mechanism of action related to itsbinding tothe DNA and the inhibition of nucleic acid synthesis. Studies have shown that aqueous Ut extracts present DNA repairing activities [15]. Mammone et al. showed the ability to modulate *Uncaria tomentosa* and repair of DNA in human skin and organ cultures [46]. In the present study, the results of comet test suggest that Ut had a protective effect of the DNA during the treatment cycles. However, it is necessary for other studies to confirm these effects.

5. Conclusions

Uncaria tomentosa, used at dose of 300mg dry extract per day, is effective in the recovery from neutropenia induced by chemotherapy in women diagnosed with Invasive Ductal Carcinoma—Stage II. It is also able to restore cellular DNA. Thus, it is a effective adjuvant treatment in reducing adverse chemotherapy effects.

Conflict of Interests

All authors deny any conflict of interests.

Acknowledgments

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MANUSCRIPT 1

Cytotoxicity and induction of apoptosis in MCF-7 cells by *Uncaria tomentosa*

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ABSTRACT

Several biological activities have been described for different constitutions of the extracts of *Uncaria tomentosa*: immunomodulatory, myeloestimulan*t*, cellular DNA restoration, antiproliferative and ability to induce apoptosis.

Breast cancer is the most common malignancy among women. A widely used model for the *in vitro* study of breast cancer, with positive estrogen expression is cell line MCF-7.

Previous studies with *Uncaria tomentosa* suggest potential anticancer activity but the mechanisms involved are controversial in the literature and not yet completely understood. Thus this study examined the cytotoxic effect and apoptosis induction in different concentrations of the extract of *Uncaria tomentosa* in the lineage of adenocarcinoma (MCF-7).

Data obtained in this study demonstrate that the extract *Uncaria tomentosa* extract with a content of 2.57% pentacyclic oxindole alkaloid exerted a cytotoxic activity in MCF-7 cells associated with apoptotic cell death

through activation of Caspases 3/8. The effects of *Uncaria tomentosa* did not trigger changes in the mechanism of action of doxorubicin. *Uncaria tomentosa* can be an adjuvant anticancer agent in the treatment of breast cancer.

1 INTRODUCTION

Uncaria tomentosa (Ut) is a medicinal plant that grows as a climbing shrub with compound leaves, opposite and oval. Its name was inspired by the similarity of its spines with cat's nails. In its chemical composition were identified more than 50 chemical constituents including indole and oxindole alkaloids, polyphenols (flavonoids, proanthocyanidins, tannis), glycosides tripenos derivates from quinic acid, and quinovic and saponins (HEITTZMAN et al., 2005). The oxindole alkaloids are recognized as phytochemicals of this species due to their association with various pharmacological actions (HEITTZMAN et al., 2005; KEPLINGER et al., 1999).

Different biological activities have already been described for extracts of *Uncaria tomentosa:* restoration of cellular DNA; preventing mutations and cell damage cause by chemotherapy; modulating the activity of the immune system (WURM et al., 1988; ALEN-HALL et al., 2007); myeloestimulante direct effect, i.e. through stimulation of myelopoiesis and colony-stimulating factors (G-CSF) (SHENG et al., 2000; EBERLIN et al., 2005; FARIAS et al., 2012); antiproliferative effect on cell lines (SHENG et al., 1998; RIVA et al., 2001), as well as studies *in vitro* have demonstrated that *Uncaria tomentosa* inhibited about 90%, the growth of a lineage cell of breast cancer, MCF-7 (RIVA et al., 2001).

Akesson et al, 2003, report that the aqueous extract of *Uncaria tomentosa* inhibited proliferation of T and B lymphocyted in mice. In 2005, Sheng et al. demonstrated that the aqueous extract of *Uncaria tomentosa* inhibited cell growth without cell death, thus providing better opportunities for DNA repair. Subsequent studies have shown that the extract was able to induce apoptosis and inhibit proliferation in tumor cells *in vitro* such as lymphoblast leukaemic acute, human skin, human ostesarcoma cell line,human breast carcinoma cell line, (BACHER et al., 2005; MAMMONE et al., 2006; MARTINO et al., 2006; PILARSKI et al., 2010).

Morphological changes observed in the apoptotic process are the result of a series of molecular events and specific biochemical and, genetically regulated and divided into three phases: initiation, effector, and degradation (HENGARTNER, 2000). The initiation phase is dependent on the type of apoptotic stimulus received by the cell through endogenous or surface receptors with death domains (e.g., oxidative stress, errors in DNA, and ionic inbalance and presence of cytokines). In the effector phase there is the activation of the Caspases cascade (cysteine-dependent aspartate-specific proteases), the initiator Caspases among them, caspase-8 and caspase-9 that cleave inactive pro-forms of effector caspases group, activating them. Thus, the effector caspases such as caspase-3 and caspase-7 cleave other proteins resulting in apoptosis process (KAM; FERCH, 2002; WONG, 2001). At the stage of degradation, there is completion of the apoptotic process with decreased cell volume, chromatin condensation, cariorex, and any budding membranes, forming apoptotic bodies (GREEN; KROEMER, 1998; HAIL et al., 2006).

An important factor in the apoptosis way is a family of Blc-2 genes encoding two protein classes: those with anti-apoptotic activity (e.g. Bcl-2 and Bcl-xl) and with pro-apoptotic activity (e.g. Bid and Bax). These proteins may be associated with membranes of organelles or into the extracellular cytosol (extrinsic way/death receptors) and intracellular (intrinsic way/mitochondrial way. (BORNER, 2003). Some studies support the hypothesis of the central role of apoptosis in tumorogenesis. Since malignant cells are characterized by reduced ability to undergo apoptosis in responsive to various stimuli (HANAHAN & WEINBERG, 2000; OKADA et al., 2004; MORRISON et al., 2008).

Previous studies with *Uncaria tomentosa* suggest potential anticancer activity but the mechanisms involved are controversial in the literature and not yet completely understood. A widely used model for the *in vitro* study of breast cancer with positive estrogen expression is cell line MCF-7. Thus this study examined the cytotoxic effect and apoptosis induction by different concentrations of the extract of *Uncaria tomentosa* in the lineage of adenocarcinoma (MCF-7), in an attempt to corroborate previous studies, and

therefore allow Ut can be used as an additional strategy for the treatment of breast cancer.

2 MATERIALS AND METHODS

2.1 Uncaria tomentosa

Uncaria tomentosa extract was gently donated by Herbarium Botanic Laboratory. The *Uncaria tomentosa* extract has a content of 2.57% pentacyclic oxindole alkaloids. The concentrations of each POA were as follows: speciophylline – 0.26%; uncarine F – 0.07%; mitraphylline – 0.80%; isomitraphylline – 0.40%; uncarine C – 0.46%; and uncarine E – 0.58%.

2.2 Cell Cultures

For biological tests with the extract it was used cell line breast adenocarcinoma (MCF-7) cells acquired from the bank of cells from the Federal University of Rio de Janeiro (BCRJ). Cells were cultured and frozen in liquid nitrogen for storage. Subsequently they were grown in culture in Eagle's medium, modified according to Dulbecco (DMEM) (Sigma Chemical Co., Italy) plus HEPES, penicillin, streptomycin, sodium bicarbonate, and fetal calf serum (FCS, Cultilab). Cells were cultured in bottles and stored at 5% CO_2 at the temperature of 37 °C until the formation of cell monolayer, after, the cell bottles were subjected to trypsinization. Cultures were monitored by cell counting and viability using trypan blue and examined by microscopy.

2.3 Treatment of MCF-7 line

For tests, MCF-7 cells were incubated with increasing concentrations of hydroalcoholic extract of *Uncaria tomentosa* (250, 500, 750, 1000 µg/mL) for 48 and 72 hours, according results of Pilarski et al. (2010) and Diaz et al. (2010). For each test was included positive control (Doxorubicin, Eurofarma, SP, final

concentration of 2 mg/ml), and negative control (untreated cells) in accordance with Rusetskaya et al, (2009).

2.4 Cytometric analysis of apoptosis/necrosis flow by Anexina V

Cells (1.0 x 10^6cell/well) were cultured in 12-well plates by 48 and 72 hours and treated with predetermined concentrations to test the cytotoxicity of Ut extract. After treatment, the cells were trypsinzed, transferred to sterile eppendorfes and centrifugated for 10 minutes. Cells were washed with PBS and centrifuged again. To test for Annexin V, it was used the apoptosis detection kit for Annexin V – FITC labeled (Alexis, Lausen, Switzerland). Next, the cells were resuspended with 400 µL of ligation buffer, 5 µL of Annexin V conjugated with FITC, and 5 µL Aminoactinomycin D (7-AAD). The reaction was incubated for 5 minutes at room temperature under protection from light. The intensity of fluorescence (FITC and 7AAD) was evaluated using the equipment BD FACScalibur analyzer, USA.

2.5 Assay of caspase 3, 8 activities.

2.5.1 Pro Caspase 3 Human ELISA – Enzyme-Linked Immunoabsorbent Assay ABCAM®

The kit is an *in vitro* enzyme-linked immunosorbent assay for the accurate quantitative measurement of pro-form of caspase 3 protein in human cell and tissue lysates. The assay employs an antibody specific to pro-form of caspase 3 protein coated onto well plate strips. Samples are pipetted into the wells and pro-form of caspase 3 present in the sample is bound to the wells by the immobilized antibody. The wells are washed and an anti-caspase 3 detector antibody is added. After washing away unbound detector antibody, HRP-conjugated label specific for the detector antibody is pipetted into the wells. The wells are again washed, a TMB substrate solution is added to the wells and blue color develops in proportion to the amount of captured pro caspase 3. The developing blue color is measured at 600 nm. Optionally the reaction can be stopped by adding hydrochloric acid which changes the color from blue to

yellow and the intensity can be measured at 450 nm. Results are expressed in µg/ml.

2.5.2 Pro Caspase 8 Human ELISA Enzyme-Linked Immunosorbent Assay ABCAM ®

Human Caspase-8 present in the sample or standard binds to antibodies adsorbed to the microwells. The detection antibody binds to human Caspase-8 captured by the first antibody. Following incubation unbound detection antibody is removed during a wash step. Anti-rabbit-IgG-HRP is added and binds to the Detection Antibody. Following incubation unbound anti-rabbit-IgG-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of human Caspase-8 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. Results are expressed in ng/ml.

2.5.6 Statiscs

Results are expressed as mean ± standard deviation. One-way ANOVA with Bonferroni multiple comparisons was used for statistical analysis. $P<0.05$ was considered to represent a significant difference in all tests.

3 RESULTS

The figure 1 shows the effect of *Uncaria tomentosa* extract on cell viability of MCF-7. The cell line was treated with different concentrations of the extract Ut (250, 500, 750 and 1000 µg/mL) for a period of 48 and 72 hours. In 48 hours, it was observed that there was a decrease in cell viability; however this reduction was most obvious in treatment during 72 hours. The highest concentrations of *Uncaria tomentosa* extract corresponde to lower rates of cell viability. The lowest viability was seen for concentrations of 750 µg/mL (30 % and 22, 2%) and 1000 µg/mL for 72 hours respectively.

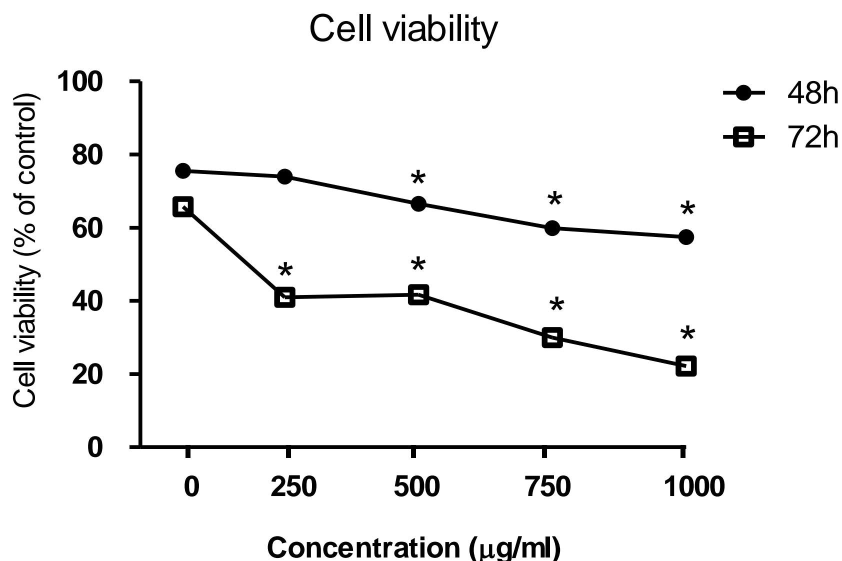


Figure 1 - Cell viability of MCF-7 cell detected with Anexina-V. The effects of Ut in different concentrations on human breast cancer cells are shown. Cells were cultured at different concentrations of *Uncaria tomentosa* for 48 h and 72h. Ut was found to decrease breast cancer cells viability at concentrations of 500, 750, and 1000 µg/ml in breast cancer cells in 48h, and 250, 500, 750, and 1000 in 72h. Data are presented as mean±SEM. One-way ANOVA with Bonferroni multiple comparisons was used for statistical analysis. $p<0.05$, compared to the control group.

Many chemical compounds can inhibit the growth of tumor cells but not all of them can trigger apoptosis. To determine whether *Uncaria tomentosa* indicates apoptosis in MCF-7 cells it was performed the analysis byt cytometry with Annexin V – FITC conjugated 7AAD using doxorubicin as a positive control. The results are shown in figure 2.

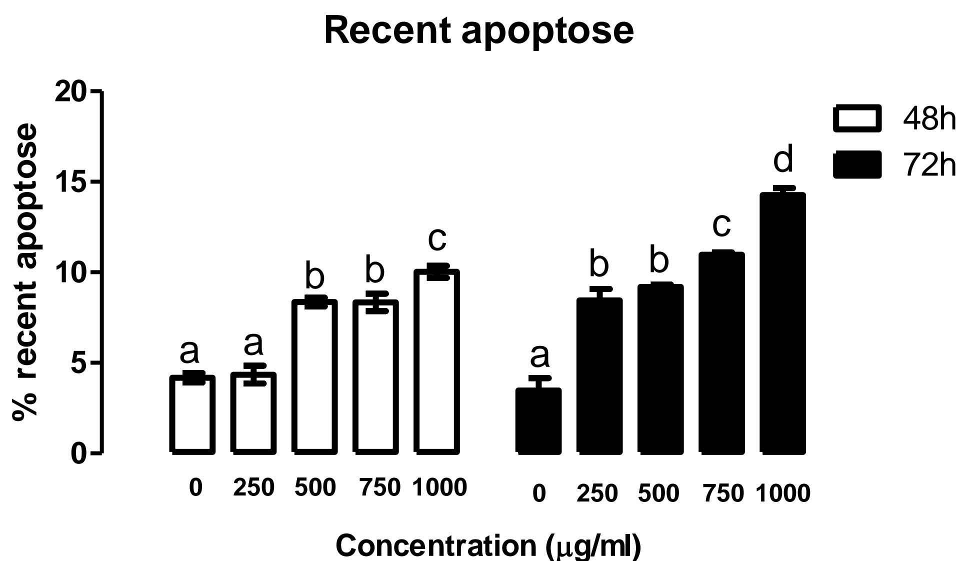


Figure 2 - Flow cytometry demonstrated the stages of cell apoptosis in MCF-7 breast cancer cells at different time points and for different concentrations of Ut. Panels show the percentages of different apoptotic stages in cells incubated with different concentrations of *Uncaria tomentosa* for 48 and 72 hours. Data are presented as the mean ±SEM. One-way ANOVA with a Bonferroni multiple comparisons was used for statistical analysis.

MCF-7 cells treated with different concentrations of *Uncaria tomentosa* in contact with the apoptotic agents expressed significantly higher indices for Annexin V-FITC-positive, 7-AAD-negative (Annexin V-FITC (+) 7AAD (-)) when compared to control (doxorubicin) which expressed higher indices for Annexin V-FITC-posivite-7AAD-positive (Annexin V-FITC (+) 7AAD (+)) compatible with late apoptosis (Data not shown).

Uncaria tomentosa extract at concentrations of 500, 750 and 1000 µg/mL recent induced apoptosis in MCF-7 cells during the incubation period of 48 hours. However, significant differences were observed in the induction of apoptosis in recent concentrations of 250, 500, 750 and 1000µg/mL in 72h, compared with the control. Higher values are observed in the concentration of 1000µg/mL (10.96%).

To confirm the apoptotic effect of the stratum Ut on MCF-7 cells it was investigated the concentration of proteins caspase 3 and caspase 8 (Figure 3).

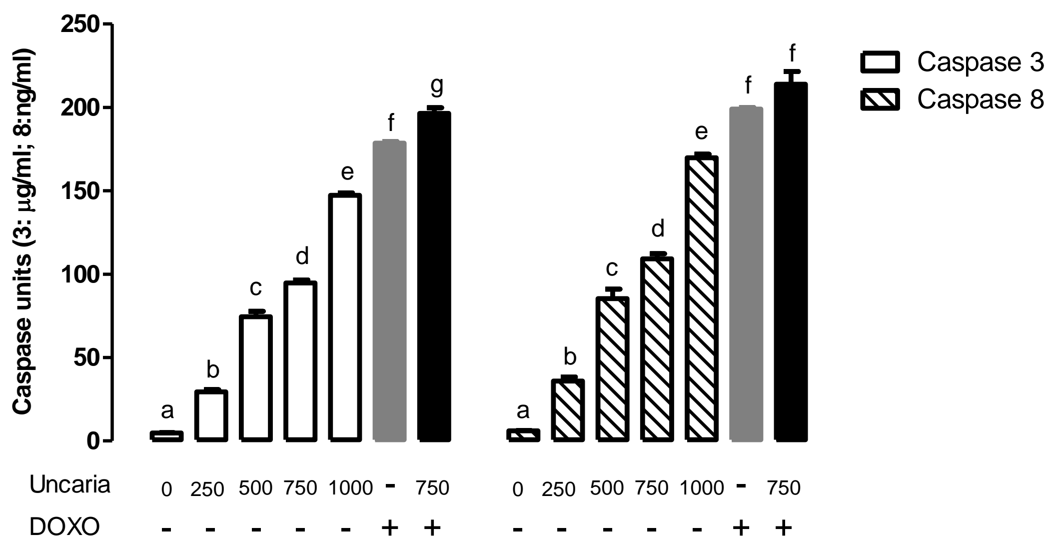
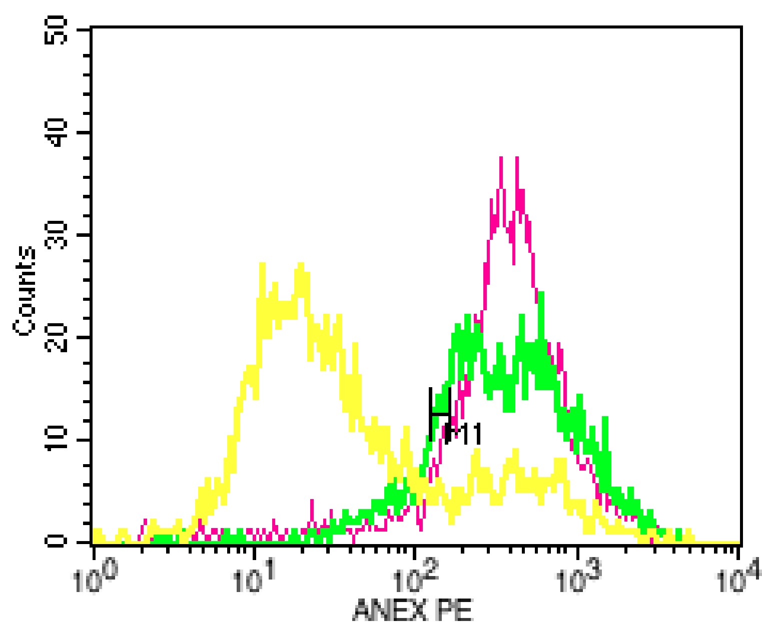


Figure 3 - Caspase 3 and 8 levels of extracts from control and 250, 500, 750 and 1000 µg/ml Ut treated human breast cancer cell cultures were detected with caspase activity by ELISA. The results indicate that the levels of caspase 3 and caspase 8 were upregulated by *Uncaria tomentosa* treatment. Greay and Black Bars represent doxorrubicin treatment. Different letters represent significant difference between groups. The ONE-WAY ANOVA was used for statistical analysis. Compared to the control group, $p<0.05$.

According to figure 3, the concentration of caspases 3 and 8 at different concentrations of *Uncaria tomentosa* in 48 hours with doxorubicin as a positive control, one can observe that the concentration, of both caspases, increases as the concentration of *Uncaria tomentosa* extract rises comparing to the negative control (only cells).

MCF-7 cells treated concomitantly with doxorubicin and 750 µg/ml of *Uncaria tomentosa* showed a slight increase in concentrations of caspase 3 and 8 (196.47 µg/ml and 211.9 ng/ml for caspase 3 and 8 respectively) compared to positive control, doxorubicin (178.5 µg/ml and 199 ng/ml, for caspase 3 and 8, respectively).

Using the average concentration of 750 µg/ml of *Uncaria tomentosa* extract it was verified by Annexin V-FTIC assay, the possibility of interference the activity of doxorubicin (Figure 4).



□ negative control; □ doxorubicin; □ doxorubicin + Ut 750 µg/ml

Figure 4 - Intencidade fluorescence marking the interference in the activity of doxorubicin and doxorubicin + MCF-7 treated with 750 µg/ml UT.

Results show that the extract of *Uncaria tomentosa* does not interfere with the action mechanism of doxorubicin, confirmed by Annexin V-FITC assay. Cells treated with doxorubicin and 750 µg/ml *Uncaria tomentosa* did not cause changes in the response profile of cells undergoing apoptosis, remaining similar to doxorubicin (control).

4 DISCUSSION

Many medicinal plants are being studied as anticancer agents among, them *Uncaria tomentosa.* This study examined the cytoxicity effect and induction of apoptosis by *Uncaria tomentosa* strain in breast adenocarcinoma (MCF-7). Susceptibility to apoptosis is a prerequisite to successful cancer treatment.

The pro apoptotic effect of *Uncaria tomentosa* in different tumor cell lines has been reported by several authors. Cheng et al. (2006) in HL-60 cell lines of human promyelocytic leukemia observed induction of caspase 3 and

procaspase 8 activity. Furthermore, apoptosis was accompanied by up-regulation of Bax, and negative regulation of expression Bcl-X (L). Rinner et al. (2008) when evaluating the anti-proliferative effects and pro apoptotic fractions obtained from *Uncaria tomentosa* in cells of medullary thyroid carcinoma (MTC) observed an inhibition of mitochondrial enzyme activity accompanied by an increase in the expression of caspase3/7 and the fraction of bcl-2. Garcia et al. (2007), examined the mitraphylline antitumor effects, a pentacyclic oxindole alkaloid of *Uncaria tomentosa* on human glioma and neuroblastoma cell lines. Micromolar concentrations of mitraphylline inhibited the growth of both cell lines studied, in a dose-dependent manner.

Pilarski et al. (2010) studied the anticancer property of *Uncaria tomentosa* extracts with quantitative differences in their compositions using models *in vivo* and *in vitro* with different strains of cancer cells. The results show that the alcoholic extract of *Uncaria tomentosa* inhibited the development of tumor cells, among them, MCF-7.

Diaz et al. (2010) have described an IC50 ranging from 25 to 1000 µg / ml depending on the preparation of the extract *Uncaria tomentosa* and cell lines utilized. Based on the results of these authors were determined the concentrations used in the tests (250, 500, 750, and 1000 ug / ml). In the results in concentration of 750 µg / ml of extract *Uncaria tomentosa* observed cell death in around 40%, so the tests were performed with concentrations above and below 750µg/mL.

To investigate whether the decrease in cell viability was caused by apoptosis or other mechanism, it was held a test with Annexin V-FITC conjugated to 7AAD, using doxorubicin as positive control. The Annexin V behaves as an extrinsic protein of plasma membrane and had the ability to bind to membrane phospholipids through their negative charges in the presence of calcium ions. The exposure of phosphatidylserine is one of the first events that occur on the surface of a cell undergoing apoptosis. The translocation of phosphatidylserine present on the inner face to the outer face of the cell membrane, and the binding of Annexin V is a clear indication of apoptosis (MOCHIZUKI et al., 2003). By using Annexin V-FITC labeled are measured levels of phosphatidylserine exposed on the outer cell membrane that were

associated with Annexin, indicating early apoptosis. To indicate late apoptosis, 7AAD is associated.

As noted in the assays of this study, the cell death process offers lower rates for late/necrosis apoptosis, but showed signs of early apoptosis. The responses of premature cell death were dose/time dependant when compared to the control. These results are in agreement with Martino et al. (2006), where there was an increased percentage of inhibition in MCF-7 cell growth in 48 and 72 hours according to the increase of the dose of Ut extract used. For 120mg/ml extract was observed 16%, 42% inhibition of cell growth 48h and 72h respectively 240mg/ml extract showed 30% and 74% inhibition at 48h, 72h respectively.

The apoptotic phenomenon is induced by a cascade of molecular events that can be started in different ways, culminating in the activation of caspases (Stransser, et al., 2006). According to Waxman and Schwartz (2003), the mechanism of activation of caspases is an essential step for apoptosis. Once activated, an initiator caspase cleaves others, in sequence, until it generates an executor caspase. This caspase destroys the proteins essential to the cell, activates toxic proteins or destroys proteins that protect the cell from apoptosis. Being caspases 3 and 8 one of the main effectors of apoptosis.

O'Donovan et al. (2001) studied the expression of caspase 3 in breast tumors at the mRNA level by RT-PCR. Caspase 3 mRNA was found in 72.4% of breast carcinomas. Martino et al. (2006) demonstrated that *Uncaria tomentosa* induces apoptosis via activation of caspase 3.

In the analysis made in this study, a significant increase in Caspase 3 and 8 activity at all concentrations of the *Uncaria tomentosa* extract was found, suggesting therefore that the response is dose dependent.

5 CONCLUSION

Data obtained in this study demonstrate that *Uncaria tomentosa* extract exerted a cytotoxic activity in MCF-7 cells associated with apoptotic cell death through activation of Caspases 3/8. The effects of Ut did not trigger changes in the mechanism of action of doxorubicin. Further studies are needed on the

mechanisms involved. *Uncaria tomentosa* can be an adjuvant anticancer agent in the treatment of breast cancer.

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CONFLICT OF INTERESTS

All authors deny any conflict of interests.

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DISCUSSÃO

Uncaria tomentosa, adjuvante no tratamento do câncer de mama

O efeito citotóxico dos agentes quimioterapêuticos não é seletivo para células neoplásicas, sendo também prejudicial para outras células do organismo. O tratamento quimioterápico deve ser interrompido quando a contagem de neutrófilos for inferior a 500 células/mm^3, pois a neutropenia predispõe o paciente à infecções e, muitas vezes, resulta em atrasos no ciclo do tratamento e em reduções de dose (UICC, 2006). Assim, o sucesso do tratamento depende do percentual de neutrófilos.

Esta pesquisa, através de um estudo randomizado, comprovou que o tratamento com uma dose diária de 300 mg de extrato seco de *Uncaria tomentosa* foi eficaz para reduzir a neutropenia. Os efeitos da quimioterapia em células do sangue tendem a tornarem-se mais centuados durante o tratamento. No entanto, os nossos resultados mostraram que, no decorrer dos ciclos, até o término da quimioterapia, o grupo que foi suplementado com *Uncaria tomentosa* apresentou valores duas vezes mais altos de neutrófilos em relação ao grupo sem suplementação, considerando-se também que, no grupo sem suplementação, 67,9% dos pacientes apresentaram neutropenia. Do mesmo modo, verificou-se um aumento nos monócitos. Estes resultados são compatívéis com os estudos de Sheng et al. (2000), em que a recuperação dos leucócitos foi observada em ratos submetidos a um modelo para a leucopenia, induzido pela quimioterapia (doxorrubicina) e que utilizou um fator estimulador de colônias de granulócitos (Neupogen®) como um controle positivo. Farias e colaboradores (2011) utilizaram um modelo para indução da neutropenia com ifosfamida em camundongos e, após serem tratados com *Uncaria tomentosa* observou-se um aumento significativo no percentual de neutrófilos dos camundongos.

No presente estudo não foram observadas diferenças relacionadas aos linfócitos entre os grupos, suplementados ou não com *Uncaria tomentosa,* ao longo dos ciclos de quimioterapia. No entanto, uma redução foi constatada ao se comparar esse dois grupos com o grupo controle. Tais resultados podem ser atribuídos a um efeito ocorrido somente sobre os precursores mieloides e, a partir deles, pode-se constatar que a diminuição dos linfócitos relaciona-se aos efeitos da quimioterapia**.**

Neste estudo, utilizou-se o extrato hidroalcoólico, que, segundo Gimenez e colaboradores (2010) e Pilarski e colaboradores (2010), apresenta maiores efeitos mieloproliferativos, antiproliferativos de células tumorais. Já Sandoval et al. (2000) afirmam que o extrato aquoso de *Uncaria tomentosa* apresenta alta concentração de ácido quínico e baixas concentrações de alcalóides oxindole, tendo, dessa forma, sua ação direcionada às propriedades imunomoduladoras mediadas por citocinas como a TNFα (SANDOVAL et al., 2002). Em relação às análises nas células $CD4^{+}$+, $CD8^{+}$ e de IL-6 circulante não foram observadas alterações.

Os níveis elevados de IL-6 circulante estão associados a piores taxas de sobrevivência em pacientes com câncer de mama metastático e a estágios mais avançados da doença, segundo Sandoval (2000). No decorrer do estudo, todas as pacientes que compuseram a amostra apresentaram respostas satisfatórias ao tratamento, sem ocorrência de recidivas, logo, mantendo-se estáveis. Esse fato pode contribuir para a estabilidade nos níveis de IL-6. Não houve correlação entre IL-6, *status* dos receptores $CD4^{+}$ T/CD8 e a idade, o índice de massa corporal e os receptores hormonais.

Mulheres com câncer de mama apresentam um aumento das concentrações sanguíneas das substâncias oxidadas, tais como produtos derivados de peroxidação de lipidios, oxidação de proteínas e DNA (AKESSON et al., 2000; GIMENEZ et al., 2010). Foram analisados os seguintes parâmetros relacionados ao estresse oxidativo: SOD, CAT, TBARS e carbonilação de proteínas. Foram observadas diferenças na enzima SOD entre os grupos, com ou sem suplementação com *Uncaria tomentosa*. Em um estudo realizado com mulheres diagnosticadas com câncer de mama, a atividade da SOD mostrou um aumento significativo, independentemente do estágio clínico e do status da menopausa (SANDOVAL, 2000). Outros autores afirmam que o estado de estresse oxidativo é maior quanto maior for o grau de estadiamento da doença (GIBANANANDA et al., 2000; VALKO et al., 2006). Todas as pacientes participantes do estudo, quanto ao estadiamento, foram caracterizadas na Fase II e não apresentaram alterações clínicas no decorrer do estudo, fato que pode explicar os resultados encontrados.

O efeito protetor da *Uncaria tomentosa* no DNA foi observado durante os ciclos do tratamento do câncer de mama pelo teste do cometa. Estudos têm demonstrado que os extratos aquosos de *Uncaria tomentosa*, apresentam atividade

de reparação do DNA (SHENG, 2000, MAMMONE et al., 2006). No entanto, outros estudos fazem-se necessários para confirmar tais efeitos.

A formação das neoplasias se dá pelo desequilíbrio que há entre a proliferação celular (ciclo celular) e a apoptose (morte celular programada). Drogas antineoplásicas impedem a proliferação celular através de diferentes mecanismos de ação, podendo atuar em elementos do citoesqueleto, prejudicando, assim, a divisão celular ou podem promover citotoxicidade capaz de destruir a célula por necrose ou apoptose (ROSALES-HERNANDEZ et al., 2009).

As propriedades da *Uncaria tomentosa,* tais como os efeitos antiproliferativos e a indução de apoptose, têm despertado o interesse de muitos pesquisadores nos últimos anos (CHENG et al., 2005; BACHER et al., 2005; PILARSKI et al, 2010). Muitas diferenças vêm sendo observadas nos resultados relatados pelos pesquisadores, estando relacionadas à linhagem celular estudada e à composição do extrato de *Uncaria tomentosa* utilizado. Um maior detalhadamento dos mecanismos de ação exercido sobre células tumorais pela *Uncaria tomentosa* também se faz necessário. Um modelo muito utilizado para o estudo *in vitro* do Adenocarcinoma de mama com expressão para estrógenos é a linhagem celular MCF-7. Desta forma este estudo analisou o efeito citotoxico e a indução da apoptose em células MCF-7 pela *Uncaria tomentosa.*

O efeito da *Uncaria tomentosa* sobre a viabilidade da linhagem celular de câncer de mama MCF-7 foi analisado pelo ensaio de Anexina-V. As células foram cultivadas em diferentes concentrações do extrato por 48h e 72h. Os resultados demonstraram que a viabilidade das células MCF-7 diminuiu para as concentrações de 500, 750 e 1000 µg/ml em 48h e em todas as concentrações, 250, 500, 750, 1000 µg/ml em 72h.

Muitos autores relatam o efeito antitumoral do extrato de *Uncaria tomentosa*, como, por exemplo, Garcia e colaboradores (2007), em células de neuroblastoma (SKN BE). Cheng e colaboradores (2007), através de um estudo realizado com células de leucemia promielocitica humana (HL-60), constataram que a indução da apoptose ocorreu devido à estimulação do flip-flop de fosfatidilserina, à liberação de citocromo c mitocondrial em citosol e à indução de atividade da caspase-3/8, de um modo dependente do tempo e de uma up regulação de Bax e regulação negativa de BCL-X(L).

Rinner et al. (2009) ao avaliarem os efeitos antiproliferativos e pró-apoptóticos de frações obtidas a partir de *Uncaria tomentosa* em células de carcinoma medular da tireóide (MTC), observaram uma inibição na atividade enzimática mitocondrial acompanhada de um aumento na expressão de caspase3/7 e na fração de bcl-2. Gimenez et al. (2010), em células de sarcoma humana Ewing (MHH-ES-1) e Pilarski et al. (2010) em células de câncer de mama (MT-3 células) constataram que a *Uncaria tomentosa* induziu apoptose via ativação da caspase 3.

Muitos compostos químicos podem inibir o crescimento de células tumorais, mas nem todos eles podem desencadear a apoptose. Os resultados do presente estudo estão de acordo com resultados de estudos anteriores. Em nossos resultados, células MCF-7, tratadas com diferentes concentrações de *Uncaria tomentosa* em contato com os agentes apoptóticos, expressaram índices significamente maiores para Anexina V-FITC-positivos, 7 AAD-negativas (anexina V-FITC (+) 7AAD (-) se comparadas ao controle (doxorrubicina) que expressou maiores índices para anexina V-FITC-positivos, 7AAD-positivos (anexina V-FITC (+) 7AAD (+), compatível com apoptose tardia.

Os dados obtidos no presente trabalho comprovam claramente uma indução de apoptose pela ativação das caspases 3 e 8 de uma forma dose dependentes. A concentração de ambas as caspases, aumenta à medida que se eleva a concentração do extrato de *Uncaria tomentosa* comparando-se ao controle negativo (somente células).

CONCLUSÕES

- O tratamento com uma dose diária de 300 mg de extracto seco de *Uncaria tomentosa* foi eficaz na recuperação de neutropenia induzida por quimioterapia em mulheres diagnosticadas com carcinoma ductal invasivo Fase II.
- Verificou-se um aumento dos monócitos no grupo suplementado.
- Um décrescimo relacionado aos efeitos da quimioterapia foi observado nos percentuais de linfócitos entre os grupos suplementados ou não, quando comparado ao controle.
- O tratamento com Ut não alterou o perfil imunológico, avaliado pela contagem de T CD4 + e CD8 + e IL6.
- Na análise do estresse oxidativo e das defesas antioxidantes, avaliados pelos níveis de TBARS, proteína carbonil, enzimas SOD e catalase, foram somente observadas diferenças na enzima SOD, entre os grupos suplementados ou não com *Uncaria tomentosa*.
- Os resultados do ensaio Cometa sugerem que *Uncaria tomentosa* exerceu um efeito protetor no DNA durante os ciclos de tratamento do câncer. No entanto, são necessários outros estudos para a confirmação de tais efeitos.
- O extrato de *Uncaria tomentosa* exerceu uma atividade citotóxica em células MCF-7 associado à morte celular por apoptose através da ativação das caspases 3/8.
- Os efeitos da *Uncaria tomentosa* não desencadearam mudanças no mecanismo de ação da doxorrubicina. Porém, mais estudos fazem-se necessários para o devido esclarecimento acerca dos mecanismos envolvidos nessa interação.

Tendo em vista o acima exposto, é possível afirmar que o uso de *Uncaria tomentosa,* como adjuvante no tratamento do câncer de mama, é eficaz, o que não anula a pertinência do desenvolvimento de outras pesquisas sobre o assunto.

PERSPECTIVAS FUTURAS

Estudos subsequentes serão realizados para: (i) analisar a expressão de membros a família Bcl-2, (ii) ampliar os dados referentes à reparação do DNA e sobre os efeitos da *Uncaria tomentosa* no mecanismo da apoptose,(iii) verificar se os efeitos também são observados em células de câncer de mama que não expressam receptores para estrógenos, (iv) verificar o efeito da *Uncaria tomentosa* nos receptores purinérgicos das células MCF-7.

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