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Efeitos antineoplásicos do extrato bruto e de diferentes frações de *Uncaria tomentosa* diante do tumor Walker-256

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Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná como requisito parcial para obtenção do título de Mestre em Farmacologia.

Orientadora: Profª. Drª. Alexandra Acco

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NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo –artigo para publicação– de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma revisão de literatura, objetivos do trabalho e um artigo científico abordando os experimentos realizados, com resultados e discussão, além de considerações finais. O artigo foi preparado seguindo formatação proposta por periódicos científicos internacionais.

Dedico este trabajo a

Walter Dreifuss
…mi Opa.
Aún con tanta distancia entre nosotros,
me enseñaste las maravillas de la ciencia
y me inspiraste la capacidad de asombro;

Carmen Rosa Serrano del Portal
…mi Mamamama.
Siempre a mi lado, con mucho cariño y orgullo,
me enseñaste a procurar la excelencia
y me inspiraste el amor por el trabajo.

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Siempre habrá más preguntas que respuestas...
Felizmente.

Anônimo

RESUMO

Objetivos: Este trabalho foi delineado para comparar os efeitos antineoplásicos de um extrato bruto hidroetanólico (BHE) de *Uncaria tomentosa* (UT) com os de duas frações dele derivadas: uma rica em alcaloides oxindólicos pentacíclicos (POA), preparada com clorofórmio (fração $CHCl_3$); e outra rica em substâncias antioxidantes, preparada com *n*-butanol (fração BuOH). O modelo escolhido para o estudo foi o tumor sólido Walker-256 em ratos.

Materiais e Métodos: Um extrato BHE de UT foi fracionado a fim de isolar os POA numa das frações ($CHCl_3$) e deixar a maioria das substâncias antioxidantes em outra fração (BuOH). Inocularam-se células da linhagem Walker-256 no membro pélvico direito de ratos Wistar machos, que foram em seguida tratados diariamente por 14 dias mediante gavagem com o extrato BHE (50 $mg.kg^{-1}$), suas frações (segundo o rendimento do processo de fracionamento) ou veículo (Controle, grupo C). Para alguns parâmetros foi adicionado um grupo basal (B), com indivíduos não portadores de tumor que apenas receberam veículo. Ao fim do tratamento foram avaliados os seguintes parâmetros: (a) massa e volume tumoral; (b) concentrações plasmáticas de ureia, alanina aminotransferase (ALT) e aspartato aminotransferase (AST); (c) parâmetros de estresse oxidativo, especificamente a atividade da catalase (Cat), superóxido dismutase (SOD), peroxidação lipídica (LPO), glutationa-S-transferase (GST) e glutationa reduzida (GSH); (d) aferição de TNF-α em homogenatos hepático e tumoral; (e) parâmetros metabólicos aferidos mediante perfusão hepática e (f) análises de sobrevivência.

Resultados e Discussão: Tanto o extrato BHE quanto sua fração BuOH reduziram extensamente o peso e volume tumoral, o que se deveu em parte à modulação da UT sobre o estresse oxidativo: a maioria dos sistemas enzimáticos antioxidantes aferidos no fígado foi reforçada, enquanto estes mesmos sistemas reduziram sua atividade no tumor. Os níveis plasmáticos da AST também foram reduzidos por estes tratamentos, indicando preservação da integridade celular. Os resultados da mensuração do TNF-α em homogenatos de fígado indicam um maior efeito antiinflamatório advindo do tratamento com o extrato BHE em comparação ao apresentado por sua fração BuOH. A fração $CHCl_3$ foi notavelmente ineficaz em todas as aferições. Os parâmetros de perfusão hepática mostraram diferenças metabólicas entre os grupos portadores de tumor e os grupos basais, porém não foram encontradas diferenças metabólicas expressivas entre os grupos portadores de tumor, tanto tratados quanto controle. Tanto o extrato BHE quanto sua fração BuOH incrementaram o tempo de sobrevivência dos animais portadores de tumor, em comparação ao grupo Controle.

Conclusões: Estes dados constituem uma demonstração *in vivo* da importância da modulação do estresse oxidativo como parte da atividade antineoplásica da UT, bem como ressaltam a falta de atividade dos POA isolados sobre a linhagem tumoral Walker-256. É provável que estes efeitos resultem de uma combinação sinérgica de substâncias presentes no extrato BHE de UT. Tais componentes também apresentaram certo grau de seletividade tecidual quando comparados os efeitos no fígado e no tumor, favorecendo a atividade antineoplásica.

Palavras-chave: *Uncaria tomentosa*, câncer, tumor Walker-256, estresse oxidativo.

ABSTRACT

Aim of the study: This study aimed to compare the anti-neoplastic effects of an *Uncaria tomentosa* (UT) brute hydroethanolic (BHE) extract with those of two fractions derived from it: one rich in pentacyclic oxindole alkaloids (POA), prepared with chloroform ($CHCl_3$ fraction), and another one rich in antioxidant substances, prepared with *n*-butanol (BuOH). The chosen model was the Walker-256 solid tumour in rats.

Materials and Methods: A BHE extract of UT was fractionated, isolating the POA in one fraction ($CHCl_3$) and most antioxidant substances in the other fraction (BuOH). Walker-256 cells were subcutaneously inoculated in the right pelvic limb of male Wistar rats. Daily gavage with the BHE extract (50 mg kg^{-1}), its fractions (as per the yield of the fractioning process) or saline solution (Control, Group C) was subsequently initiated, until 14 days afterwards. For some parameters, a baseline (B) group was added, consisting of non-tumour-bearing rats that received vehicle. At the end of treatment the following parameters were evaluated: (a) tumour volume and mass; (b) plasmatic concentration of urea, alanine aminotransferase (ALT) and aspartate aminotransferase (AST); (c) oxidative stress parameters: activity of catalase (Cat), superoxide dismutase (SOD), lipid peroxidation (LPO), glutathione-S-transferase (GST) and reduced glutathione (GSH); (d) measurement of TNF-α in hepatic and tumour homogenates; (e) metabolic parameters assessed by liver perfusion, and (f) survival analyses.

Results: Both BHE extract and its BuOH fraction successfully reduced tumour weight and volume, which was in part due to oxidative stress modulation by UT as most anti-oxidant enzymatic systems assessed in the liver were enhanced as a result of these treatments, while the same systems were decreased in the tumour. Plasmatic levels of AST were also reduced by these treatments, indicating a preservation of cellular integrity. TNF-α results in hepatic homogenates indicate a greater anti-inflammatory effect of the BHE extract when compared to that presented by its BuOH fraction. The $CHCl_3$ fraction was remarkably ineffective. Liver perfusion showed metabolic differences between baseline and tumour-bearing rats. However, the treatment with UT did not influence the metabolism. Both the BHE extract and its BuOH fraction increased the survival time of the tumour-bearing animals, as compared to control.

Conclusions: This data represents an in vivo demonstration of the importance of the modulation of oxidative stress as part of the anti-neoplastic activity of UT, as well as constitute evidence of the lack of activity of the isolated POAs in the primary tumour of the lineage Walker-256. It is probable that these effects are result of a synergic combination of substances. Some degree of locale selectivity was observed when liver and tumour tissues were compared, contributing to the UT antineoplastic effect.

Keywords: *Uncaria tomentosa*, cancer, Walker-256 tumour, oxidative stress

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LISTA DE ABREVIATURAS

ALT	Alanina aminotransferase
ANOVA	Análise de variância
AST	Aspartato aminotrasferase
BHE	Extrato bruto hidroetanólico
BSA	Albumina de soro bovino
BuOH	Butanol / Fração butanólica
Cat	Catalase
$CHCl_3$	Clorofórmio / Fração clorofórmica
COX-2	Ciclooxigenase 2
DMSO	Dimetil sulfóxido
DMSO-D_6	Dimetil sulfóxido na sua forma deuterada
DNCB	2,4-dinitroclorobenzeno
DPPH	2,2-difenil-1-picrilhidrazil (radical livre estável)
EtOH	Etanol
FOX-2	Segunda versão do método da oxidação ferrosa do xilenol laranja
GSH	Glutationa reduzida
GST	Glutationa-S-transferase
H_2SO_4	Ácido sulfúrico
HMBC	Correlação heteronuclear da ligação múltipla
HPLC	Cromatografía líquida de alta eficiência
HRP	Peroxidase do rábano
HSQC	Correlação heteronuclear de quantum-múltiplo
IC_{50}	Concentração inibitória 50%
IFN-γ	Interferon γ

IL-12	Interleucina 12
INCA	Instituto Nacional de Câncer
KCl	Cloreto de potássio
LDH	Lactato desidrogenase
LPO	Taxa de peroxidação lipídica
MHz	Megahertz
NaCl	Cloreto de sódio
NF-κB	Fator de transcrição nuclear κB
NIH	National Institutes of Health
NMR	Ressonância magnética nuclear
OMS	Organização Mundial da Saúde
OPD	o-fenilenediamina
PBS	Tampão fosfato-salino
POA	Alcaloides oxindólicos pentacíclicos
ROS	Espécies reativas de oxigênio
SAC	Sociedad Anónima Cerrada
SOD	Superóxido dismutase
TGF-β	Fator de crescimento tumoral β
TLC	Cromatografia em camada delgada
TMS	Tetrametilsilano
TNF-α	Fator de necrose tumoral α
UN	Nações Unidas
U SOD	Unidades de superóxido dismutase
UT	*Uncaria tomentosa*
UV	Ultra violeta

1. INTRODUÇÃO

1.1. O câncer e sua problemática atual:

Os processos neoplásicos constituem uma das maiores causas de mortalidade no mundo. Segundo dados da Organização Mundial da Saúde (OMS), no ano 2008 foram registrados quase 12,7 milhões de casos novos de câncer (excluindo o câncer de pele não melanoma e outros tipos não invasivos) e 7,6 milhões de óbitos por câncer no mundo, sendo os tipos histológicos mais letais aqueles originados nos pulmões, estômago, fígado, colo e mama. Estima-se também que estas cifras continuarão a aumentar, chegando aos 13,2 milhões de mortes no ano 2030. (GLOBOCAN, 2008). No entanto, dependendo dos fatores de risco individuais, 35% das mortes por câncer podem ser prevenidas (DANAEI et al., 2005).

O câncer de pulmão é o que acomete o maior número de pessoas no mundo: cerca de 1,2 milhão de novos casos, seguido pelos tumores malignos de cólon e reto, que a cada ano somam perto de 945 mil casos novos. Estimou-se que havia cerca de 2,4 milhões de pessoas vivas diagnosticadas como portadoras de neoplasia de cólon e reto entre 2000 e 2005. Estima-se também que ao longo do ano 2012 se diagnosticarão 177.980 casos novos dos seis principais tipos histológicos de câncer no Brasil, que são próstata, mama feminina, colo do útero, aparelho respiratório, cólon e reto, e estômago. Destes casos novos, quase 13 mil ocorrerão no estado de Paraná (INCA, 2011).

Todos os processos neoplásicos estão caracterizados pelo crescimento anormal e descontrolado de um ou mais grupos histológicos determinados. O desenvolvimento de um processo neoplásico começa por mudanças genéticas que acontecem em células previamente normais, levando à ativação de oncogenes, inibição de genes supressores de tumores e alteração de genes responsáveis pela síntese de microRNA (CROZE, 2008). O crescimento tumoral associado, seja este sólido ou líquido, deve apresentar características de malignidade, definidas como anaplasia, invasividade e potencial metastásico (KUMAR *et al.*, 2004). Durante o processo tumoral, as células neoplásicas devem também desenvolver diversos mecanismos

característicos, tais como a defesa contra a detecção e eliminação pelo sistema imunológico do hospedeiro, o recrutamento de vasos sanguíneos para obter nutrientes a despeito do hospedeiro, a desativação de limites programados do tempo de vida celular e intervalos de segurança entre a replicação do material genético, e a síntese de sinalizadores celulares de crescimento próprios, ignorando ao mesmo tempo sinais inibitórias do resto do corpo; tudo isto visando garantir a continuação da maquinaria de replicação e proliferação neoplásica (HAHN e WEINBERG, 2002).

Na atualidade o tratamento dos processos neoplásicos é motivo de árdua pesquisa em variados ramos, desde a epidemiologia até a aplicação de ensaios clínicos para avaliar e comparar o desempenho de novas estratégias de tratamento.

1.2. Medicina alternativa:

Na atualidade é vasto o uso das práticas alternativas da medicina pela população geral, em todos os níveis socioeconômicos. Efetivamente, existe um grande número de pacientes insatisfeitos pelo resultado final da atenção médica ocidental, o que os obriga a procurar um complemento em outras áreas, como a medicina tradicional ou a fitoterapia. Em muitos casos, inclusive, pacientes afetados por variados tipos de doenças procuram diretamente a atenção de curandeiros e xamãs, que estão socioculturalmente mais próximos às suas crenças e ideologias, e que utilizam medicinas derivadas de produtos naturais aplicando receitas semelhantes àquelas que por séculos têm formado parte da bagagem tradicional das culturas indígenas.

Uma pesquisa realizada em adultos moradores dos Estados Unidos, conduzida por órgãos oficiais, indicou que 74,6% da população pesquisada tinha usado em algum momento da sua vida alguma forma de medicina complementar ou alternativa, enquanto 62,1% tinha recorrido a ela nos 12 meses prévios à pesquisa. As terapias relacionadas à fitoterapia e produtos naturais ocuparam o primeiro lugar em popularidade entre as diferentes vertentes de medicina complementar e alternativa. Também se menciona que *"a maioria da popula*ção *pesquisada que usa alguma forma*

de medicina complementar ou alternativa pretende tratar e/ou prevenir (...) condições associadas à dor crônica ou recorrente" (BARNES *et al.*, 2004). Um recente estudo encontrou que esta situação resulta especialmente evidente no campo das terapias para doenças crônicas e inflamatórias (CLARKE *et al.*, 2008).

Um objetivo bem conhecido no estudo de plantas medicinais é o descobrimento de novos componentes bioativos. Existem novas e sólidas evidências que constatam que muitas terapias alternativas, incluindo os tratamentos com produtos naturais, têm propriedades imunomoduladoras através de uma variedade de mecanismos, tais como: efeitos antioxidantes, alteração de processos de sinalização celular (particularmente a via NF-κB), bem como expressão e inibição de citocinas e mediadores pro-inflamatórios. Em oposição a esta realidade, no entanto, existe uma notória escassez de estudos que descrevam e padronizem as propriedades desses produtos naturais de maneira que possam ser usadas em contextos ocidentais de atenção médica sob princípios baseados na evidência.

1.3. *Uncaria tomentosa*:

O gênero *Uncaria*, reestruturado em 1978 por Risdale, é descrito como uma enredadeira lenhosa. É um arbusto com pedúnculos característicos que parecem ganchos curvos nos brotos laterais (Figura 1) e que são responsáveis pelo nome vulgar de muitas espécies deste gênero: "unha de gato" (RISDALE, 1978). Existem ao redor de 40 espécies neste gênero, apresentando uma distribuição pantropical. A maioria das espécies é encontrada no sudeste asiático, África e a região do Mediterrâneo. Na América do Sul existem duas espécies: *U. tomentosa* e *U. guianensis*. Muitos

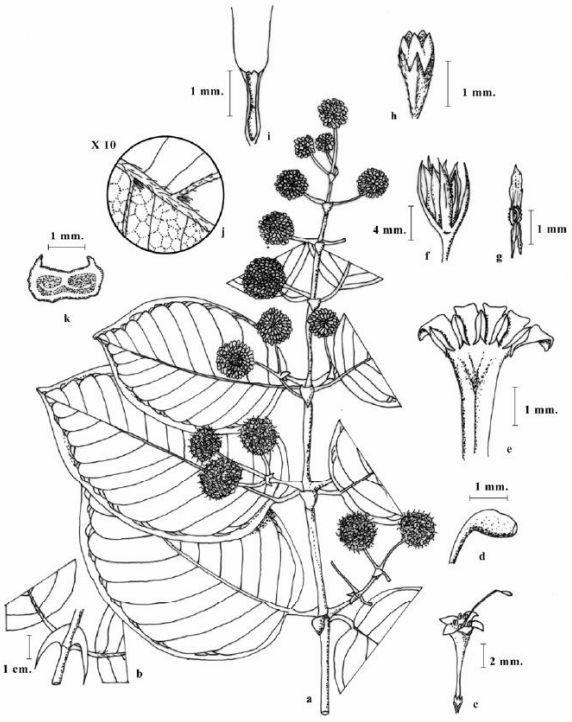

Figura 1. *Uncaria tomentosa*, evidenciando um dos seus ramos terminais e inflorescência. Notam-se os pedúnculos curvos característicos em formato de "unha de gato" (Zevallos-Pollito e Tomazello Filho, 2010).

preparados medicinais tradicionais usam espécies do gênero *Uncaria* para o tratamento de doenças variadas (CHANG *et al.*, 1989). A espécie *Uncaria tomentosa* ocorre na América Central e do Sul. Tem uma enorme importância etnofarmacológica, sendo amplamente utilizada por várias tribos e etnias da Amazônia. O povo Asháninka do território peruano, por exemplo, a considera uma planta sagrada, patrimônio exclusivo de xamãs, que a usam em rituais para favorecer a comunicação entre a dimensão física e espiritual do ser humano (KEPLINGER *et al.*, 1999). Seu modo de preparação terapêutico tradicional é o extrato aquoso da casca ou da raiz, e tem sido reportada como remédio ou coadjuvante no tratamento de enfermidades tão variadas como abscessos, alergias, artrite, asma, câncer, efeitos secundários produzidos por quimioterapias, contracepção, prevenção de doenças, febres, úlceras gástricas, hemorragias, processos inflamatórios, irregularidades menstruais e dismenorreia, recuperação puerperal, reumatismo, inflamação do trato urinário, infecções virais, feridas, entre outras (CERRI *et al.*, 1988; AQUINO *et al.*, 1991; RIZZI *et al.*, 1993; WURM *et al.*, 1998; LEMAIRE *et al.*, 1999).

Existe uma grande variedade de compostos biologicamente ativos em todo o gênero *Uncaria*; tradicionalmente a maioria de pesquisadores tem considerado mais importante o grupo dos alcaloides oxindólicos, e dentre estes, os tetracíclicos e pentacíclicos (STUPPNER *et al.*, 1992; LAUS e KEPLINGER, 1994; HEITZMAN *et al.*, 2005). Outros grupos presentes incluem saponíferas, taninos, triterpenos, vários glicosídeos do ácido quinóvico, entre outros (AQUINO *et al.*, 1989 e 1991). Muitos grupos de pesquisadores concentraram-se na caracterização da porção rica em alcaloides oxindólicos, resultando no descobrimento de substâncias como uncarina F, especiofilina, mitrafilina, pteropodina, isomitrafilina e isopteropodina (SANDOVAL *et al.*, 2002; KURAŚ *et al.*, 2006 e 2009; ALLEN-HALL *et al.*, 2007; PILARSKI *et al.*, 2006, 2007 e 2010). No entanto, a maioria de estudos farmacológicos realizados com *U. tomentosa* usou preparações de tipo “droga crua”, ou seja, extratos elaborados a partir de porções da planta maceradas numa variedade de diluentes como a água ou o etanol. Em contraposição, são poucos os estudos focados na bioatividade de compostos isolados específicos (PANIAGUA-PÉREZ *et al.*, 2009).

Os primeiros efeitos da *U. tomentosa* observados experimentalmente relacionam-se às suas propriedades anti-inflamatórias e antioxidantes. Já em 1998 sabia-se que extratos aquosos da casca da planta exibiam um notório efeito anti-inflamatório por meio da inibição da ativação do fator de transcrição tumoral NF-κB (SANDOVAL-CHACÓN *et al.*, 1998), observando-se também que estes efeitos estavam estreitamente relacionados com os efeitos antioxidantes da planta (MILLER *et al.*, 2001). Demonstrou-se que extratos metanólicos, tanto da raiz quanto da casca da planta, são capazes de sequestrar radicais livres, provavelmente devido ao alto teor de antioxidantes como proantocianidinas, triterpenos e outras saponinas (WIRTH e WAGNER, 1997). Também se observou uma redução em 76% na concentração do radical peroxinitrito. Foi sugerido que este poder antioxidante pode ser de muita utilidade contra doenças crônico-degenerativas como Alzheimer, artrite reumatoide e câncer (CHOI *et al.*, 2002).

Mediante sua inibição de NF-κB, *U. tomentosa* diminuiu a produção de nitritos e de TNFα, postulando-se que este efeito é independente da concentração dos alcaloides oxindólicos pentacíclicos presentes no extrato (SANDOVAL *et al.*, 2002). Outro estudo usou o modelo de edema de pata de camundongo induzido por carragenina para comparar a atividade anti-inflamatória de dois extratos de *U. tomentosa*: um hidroetanólico, preparado com uma mistura de etanol e água em proporção 4:1, e outro aquoso. O extrato hidroetanólico apresentou uma atividade anti-inflamatória consideravelmente maior, observando-se também maior supressão da expressão da via de transcrição NF-κB em comparação ao extrato aquoso (AGUILAR *et al.*, 2002). Nesse mesmo ano, realizou-se um ensaio clínico, no qual se administrou um extrato de *U. tomentosa* abundante em alcaloides oxindólicos pentacíclicos a pacientes com artrite reumatoide. Após um tratamento de 24 semanas, logrou-se reduzir em 53.2% a ocorrência de articulações comprometidas no grupo tratado, em comparação a 24.1% no grupo controle. (MUR *et al.*, 2002) Parece provável que a forte atividade anti-inflamatória de *U. tomentosa* seja resultado de uma combinação sinérgica de compostos (RIZZI *et al.*, 1993).

Por outro lado, observaram-se propriedades imunoestimulantes de *U. tomentosa*. Wagner e colaboradores empregaram provas de fagocitose para avaliar

seis compostos isolados da raiz desta planta, observando que quatro deles favoreceram significativamente a fagocitose (WAGNER *et al.*, 1985). Sandoval e colaboradores postularam que as atividades imunomoduladoras da *U. tomentosa* devem estar ligadas à sua capacidade em suprimir a síntese de TNFα, o que também estaria relacionado aos efeitos anti-inflamatórios da planta (SANDOVAL *et al.*, 2000). Åkesson e colaboradores administraram por via oral a camundongos um extrato aquoso da planta, observando um incremento no número de linfócitos B, T e NK, bem como granulócitos e linfócitos de memória, devido ao aumento na taxa de sobrevivência, que por sua vez poderia ser consequência da capacidade de *U. tomentosa* de proteger as células contra o estresse oxidativo e a ativação de NF-κB (ÅKESSON *et al.*, 2003).

Além das propriedades previamente mencionadas, estudos indicam que a *U. tomentosa* pode apresentar variados efeitos benéficos adicionais, como: (a) atividade antiviral de um glicosídio do ácido quinóvico extraído desta planta (AQUINO *et al.*, 1989); (b) efeito protetor contra fotomutagenicidade (WURM *et al.*, 1998); (c) notória atividade antinociceptiva sistêmica com doses que não interferiram com a atividade locomotora dos animais (JÜRGENSEN *et al.*, 2005); (d) atividade antimicrobiana *in vitro* sobre cepas de microrganismos isolados da cavidade bucal humana (CCAHUANA-VASQUEZ *et al.*, 2007); (e) atividade imunomoduladora e antiviral diante do dengue vírus-2 (REIS *et al.*, 2008), entre outros.

Por último são comentados alguns conhecimentos existentes acerca dos efeitos citotóxicos da *U. tomentosa*, com ênfase nos achados que poderiam nos orientar a considerá-la uma terapia efetiva para os processos neoplásicos. Os primeiros estudos destes efeitos foram realizados em tumores de linhagens hematológicas. Alguns extratos aquosos de *U. tomentosa* inibiram significativamente a proliferação de cepas celulares de leucemia humana (estirpe HL-60) bem como de linfoma B modificado (estirpe Raji), observando-se também fragmentação do material genético das células tumorais. Concluiu-se assim, que os extratos conseguiram induzir apoptose nas células neoplásicas, o que é altamente promissor (SHENG *et al.*, 1998). O primeiro estudo direto dos efeitos da *U. tomentosa* em modelos de tumor sólido *in vitro* utilizou extratos da casca da planta e frações cromatográficas na linhagem MCF7 de câncer de

mama humano. Os dados indicam que, além de propriedades antimutagênicas, os extratos e frações da *U. tomentosa* apresentaram atividade antiproliferativa direta: ao redor de 90% de inibição em concentrações de 100 mg/mL (RIVA *et al.*, 2001). Tratando células ativamente proliferantes com um extrato de *U. tomentosa* rico em alcaloides, observou-se uma efetiva inibição da divisão celular (mitose) sem originar alterações na estrutura genética das células (KURAŚ *et al.*, 2006). Posteriormente, a mesma equipe de investigação confirmou a importância dos alcaloides como responsáveis pelos efeitos observados, repetindo este ensaio e comparando uma fração rica em alcaloides com uma fração livre deles (KURAŚ *et al.*, 2009). Outro estudo reportou um importante efeito indutor de apoptose em células tumorais de leucemia humana, mediado pela modulação do estresse oxidativo, liberação do citocromo *c* e ativação da via das caspases, demonstrando pela primeira vez uma forte relação entre os efeitos antioxidantes e antineoplásicos da *U. tomentosa* (CHENG *et al.*, 2007).

Em vista dos promissores efeitos antineoplásicos dos extratos brutos da planta, começou-se a investigar frações e componentes isolados. De Martino e colaboradores estudaram comparativamente os efeitos antiproliferativos das frações solúveis em água e em *n*-butanol sobre três linhagens tumorais humanas *in vitro*. Seus dados indicam claramente um efeito indutor de apoptose da fração solúvel em *n*-butanol via ativação da caspase3 (DE MARTINO *et al.* 2006). Outro estudo testou isoladamente a atividade antitumoral de cinco alcaloides oxindólicos obtidos da *U. tomentosa* sobre células T de leucemia linfoblástica humana, sendo que quatro deles apresentaram inibição significativa na taxa de proliferação, tanto das células ativamente proliferantes quanto das detidas nas fases G0/G1 do ciclo celular (BACHER *et al*., 2005). Em concordância com isto, a equipe de Pilarski avaliou comparativamente extratos aquosos e alcoólicos de *U. tomentosa* contra a linhagem HL-60 de leucemia promielocítica, concluindo que os mais apropriados para uso padronizado são os hidroetanólicos, pois eles apresentam as maiores concentrações de alcaloides oxindólicos (PILARSKI *et al.*, 2007). Outro estudo observou que um componente da *U. tomentosa* chamado mitrafilina tem atividade antitumoral *in vitro* em linhagens celulares de neuroblastoma e glioma humanos. Reportou-se inibição na proliferação

de ambas as linhagens celulares em concentrações micromolares do princípio ativo, de maneira dose-dependente (GARCÍA PRADO *et al.*, 2007).

Embora estes numerosos resultados interessantes coloquem em evidência o novo e promissor potencial da *U. tomentosa* e seus componentes para o tratamento de processos neoplásicos, são poucas as publicações que reportem efeitos anticancerígenos da *U. tomentosa* em modelos tumorais *in vivo* (CABALLERO *et al*., 2005; FAZIO *et al.*, 2008). Recentemente, a nossa equipe testou os efeitos antioxidantes e antitumorais de um extrato hidroetanólico no tumor sólido Walker-256 em ratos, estabelecendo uma forte relação entre ambos os efeitos. Nossos dados indicam uma redução expressiva tanto em peso quanto em volume dos tumores, bem como significante alteração nos parâmetros de estresse oxidativo, tanto em tecido tumoral quanto hepático. Concluiu-se que *U. tomentosa* exerce seus efeitos mediante a modulação do estresse oxidativo associado a neoplasias (DREIFUSS *et al.*, 2010).

Devido à grande variedade de compostos concentrados nos extratos e frações usados na maioria dos estudos, existe um crescente interesse pelo teste de diferentes formas de preparação e extração da *Uncaria tomentosa* frente a diversas linhagens neoplásicas. Pilarski e colaboradores, por exemplo, testaram várias frações da planta preparadas com diferentes proporções de água e etanol e com diferente teor de alcaloides oxindólicos sobre nove linhagens tumorais *in vitro*, comparando seus resultados com o modelo *in vivo* de carcinoma pulmonar de Lewis em camundongos. Seus resultados foram muito interessantes: esperava-se que a fração com maior teor de alcaloides (mais de 50%) registrasse a maior atividade antitumoral. No entanto, ela exibiu uma atividade antitumoral irregular, com taxas de êxito diferentes entre as linhagens. Os pesquisadores propõem como possíveis explicações uma aparente seletividade da fração por algumas linhagens tumorais, baixa solubilidade em água dos alcaloides isolados e problemas para conseguir medições confiáveis da IC_{50} (PILARSKI *et al.*, 2010). A nossa equipe de investigação propõe ainda outra hipótese: nossas observações prévias demonstram a importância do estresse oxidativo em modelos de tumor sólido *in vivo* como o tumor Walker-256 (ACCO *et al.*, 2007; BASTOS-PEREIRA *et al.*, 2009; JUMES *et al.*, 2010), junto com o relevante sinergismo de componentes antioxidantes e citotóxicos nos efeitos antineoplásicos da *U. tomentosa* (DREIFUSS *et*

al., 2010). Parece provável que mediante o isolamento de uma fração do extrato rica em alcaloides, deixa-se de avaliar os efeitos antioxidantes de outros componentes do extrato, tais como taninos, proantocianidinas e triterpenos, o que explicaria em parte os recentes resultados de Pilarski e colaboradores.

1.4. O tumor Walker-256:

O tumor Walker-256 foi descoberto em 1928 como um tumor mamário espontâneo (EARLE, 1934). É um carcinosarcoma que tem sido usado em muitos estudos por ser facilmente transplantável, ser espécie-específico para ratos e ser de crescimento rápido, dependendo do número de células inoculadas (KWONG *et al.*, 1984). Tradicionalmente, é considerado ideal para estudos de caquexia e dor relacionados à neoplasia (GUAITANI *et al.*, 1982; BRIGATTE *et al.*, 2007). Depois de aproximadamente quatro dias, o tumor é palpável; aos oito dias apresenta um diâmetro de 20 a 30 mm e ao redor do 14° dia, os hospedeiros apresentam diminuição ponderal, anorexia e alterações metabólicas, terminando finalmente em morte ao redor do 15^{o} dia depois da inoculação, para muitos animais (MORRISON, 1972; CHO-CHUNG, 1974; KRAUSE *et al.*, 1979; OWEN, 1982; VARANI e PERONE, 1985; VICENTINO *et al.*, 2002a).

Vários processos celulares no desenvolvimento do tumor Walker-256 foram descritos, entre eles o ciclo da ureia (CORBELLO-PEREIRA *et al.*, 2004) e a expressão das citocinas TGF-β, IL-12, IFN-γ e TNF-α em duas variantes do tumor (PERROUD *et al.*, 2006). Ainda, este modelo tumoral foi utilizado anteriormente em estudos similares ao que nos propusemos a desenvolver. Mund e colaboradores, por exemplo, suplementaram ratos portadores do tumor com óleo de peixe, observando diminuição no peso do tumor, aumento na taxa de apoptose, diminuição da taxa de proliferação tumoral, diminuição da expressão de COX-2 e diminuição da concentração de prostaglandina E_2 (MUND *et al.*, 2007). No mesmo ano, membros da nossa equipe de pesquisa inocularam este tumor em ratos, aos quais se administrou também celecoxibe, medindo seus efeitos no metabolismo hepático com metodologia similar à

proposta no presente estudo (ACCO *et al.*, 2007). As características sistêmicas mais importantes desta linhagem tumoral estão esquematizadas na Figura 2.

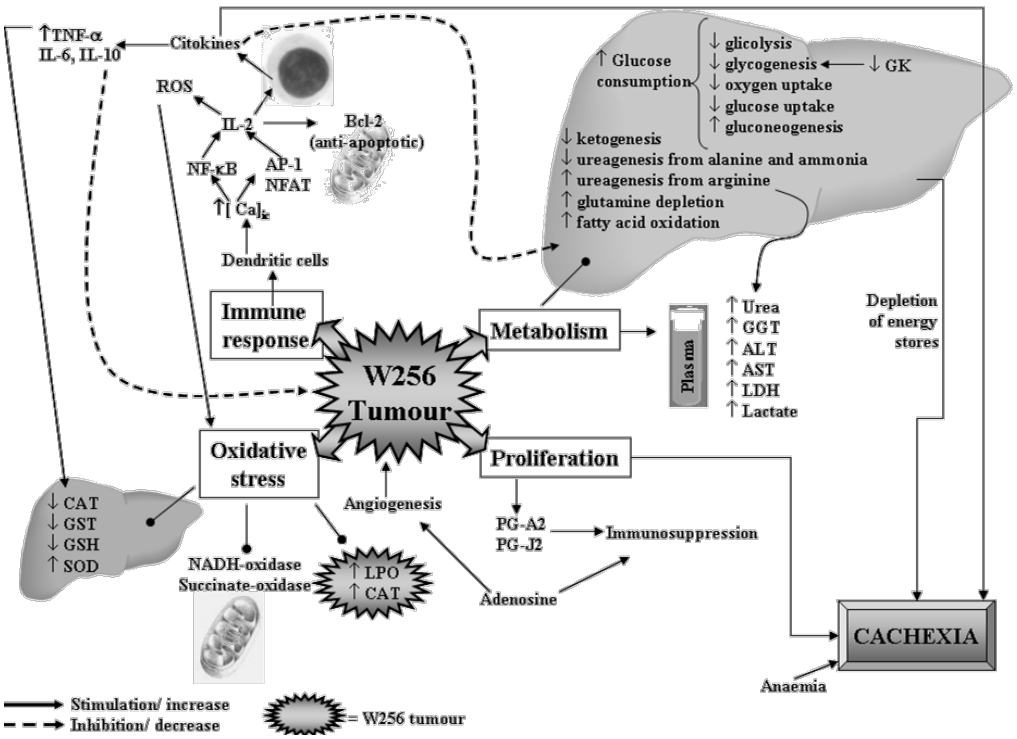

Figura 2. Principais alterações e distúrbios causados pela inoculação do tumor Walker-256 em ratos (Acco *et al.* 2012).

2. OBJETIVOS

2.1. Objetivo:

Estudar o crescimento tumoral e as alterações metabólicas provocadas pela inoculação do tumor Walker 256 no tecido celular subcutâneo de ratos, e pelo tratamento subsequente com um extrato bruto hidroetanólico de *Uncaria tomentosa*, comparando estes resultados com os obtidos após tratamento com duas frações derivadas deste extrato bruto: a primeira contendo as substâncias solúveis em *n*-butanol e a segunda contendo as substâncias solúveis em clorofórmio.

2.2. Objetivos específicos:

- Fracionar e caracterizar o extrato bruto hidroetanólico de *Uncaria tomentosa* em duas frações de conteúdo e composição distintos;
- Avaliar o crescimento e desenvolvimento tumoral em ratos inoculados com o tumor Walker-256 mediante a análise das suas massas e volumes, bem como a porcentagem de supressão tumoral ao final do período de tratamento com *U. tomentosa* (extrato e frações);
- Avaliar parâmetros de estresse oxidativo diante do tumor e do tratamento, através da medida da atividade hepática e tumoral das enzimas catalase e superóxido dismutase, bem como a taxa de peroxidação lipídica e a concentração da glutationa reduzida tanto em tumores quanto em fígados, e a atividade da glutationa-S-transferase em tecido hepático;
- Avaliar se os tratamentos com *Uncaria tomentosa* diante do tumor alteram parâmetros hepáticos (ALT e AST) bem como a concentração da ureia, através de bioquímica plasmática;
- Investigar o envolvimento do mediador inflamatório TNF-α em homogenatos de tecido hepático e tumoral, e avaliar o efeito do tratamento com *Uncaria tomentosa* nas concentrações deste marcador;

- Avaliar parâmetros de metabolismo mediante perfusão hepática em grupos tratados e não tratados com *Uncaria tomentosa*;
- Avaliar o impacto do tratamento com o extrato bruto e as frações de *Uncaria tomentosa* na sobrevida dos ratos portadores de tumor.

Title

Uncaria tomentosa exerts extensive anti-neoplastic effects against the Walker-256 tumour by modulating oxidative stress and not by alkaloid activity

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Abstract

This study aimed to compare the anti-neoplastic effects of an *Uncaria tomentosa* (UT) brute hydroethanolic (BHE) extract with those of two fractions derived from it. These fractions are choroformic ($CHCl_3$) and *n*-butanolic (BuOH), rich in pentacyclic oxindole alkaloids (POA) and antioxidant substances, respectively. The cancer model was the subcutaneous inoculation of Walker-256 tumour cells in the pelvic limb of male Wistar rat. Subsequently to the inoculation, gavage with BHE extract (50 $mg.kg^{-1}$) or its fractions (as per the yield of the fractioning process) or vehicle (Control) was performed during 14 days. Baseline values, corresponding to individuals without tumour or treatment with UT, were also included. After treatment, tumour volume and mass, plasma biochemistry, oxidative stress in liver and tumour, TNF-α level in liver and tumour homogenates, hepatic metabolism and survival rates were analysed. Both the BHE extract and its BuOH fraction successfully reduced tumour weight and volume, and modulated anti-oxidant systems; while the BuOH fraction reduced the hepatic production of ammonia and increased its production of pyruvate. The hepatic TNF-α level indicate a greater effect from the BHE extract as compared to its BuOH fraction. Importantly, both the BHE extract and its BuOH fraction increased the survival time of the tumour-bearing animals. Inversely, the $CHCl_3$ fraction was ineffective on all parameters. These data represent an in vivo demonstration of the importance of the modulation of oxidative stress as part of the anti-neoplastic activity of UT, as well as constitute evidence of the lack of activity of isolated POAs in the primary tumour of this tumour lineage. These effects are possibly resulting from a synergic combination of substances, most of them with antioxidant properties.

Introduction

Uncaria tomentosa is a vine native to the Peruvian Amazon Basin [1] that is biologically active especially as antiinflammatory, immunonomodulant and anti-oxidant agent. For the last couple of decades, researchers have experimented several types and methods of extraction to observe a wide array of pharmacological properties, including limitation of the epithelial cell death in response to oxidant stress [2], amelioration of the oedema via inhibition of cyclooxygenase-1 and -2 [3], cytoprotection by means of free radical scavenging, reduction of oxidative stress and direct inhibition of the TNF-α production [4-6], to name a few.

Most of these researchers have attributed the biological effects of *U. tomentosa* to the pentacyclic oxindole alkaloids present in this plant. This idea is supported by the apparently restricted occurrence of these alkaloids within this genus [7], as well as by studies performed with alkaloids isolated from the plant rather than with brute extracts. Indeed, reference to the use of alkaloids isolated from *U. tomentosa* goes as far back as to 1985, when Wagner and colleagues [8] observed that four out of six oxindole alkaloids present in this plant caused a pronounced enhancement of phagocytosis, both *in vitro* and *in vivo*. Likewise, studies with several of these isolated alkaloids yielded reports on their antioxidant, immunomodulant [9] and even anti-neoplastic properties [10,11]. Other studies, however, have demonstrated that compounds different than the oxindole alkaloids may be at least partially responsible for the observed effects. Among the most cited such substances are triterpenes [12], quinovic acid glycosides [13], and others hitherto not identified [14]. Yet a third hypothesis, albeit older, suggests that the anti-inflammatory properties of UT may be related to a synergic combination of compounds [7,15].

Currently, growing attention is being paid to the anti-neoplastic potential of *U. tomentosa*. Indeed, various extracts and compounds derived from this plant have been found to alter or downright inhibit the growth and proliferation of several different tumour lineages including human neuroblastoma and glioma [11], HL60 promyelocytic leukaemia [16,17] and MCF7 breast cancer [18], among others. Also, our team of researchers published one of the first studies of the anti-neoplastic properties of *U. tomentosa* on a solid tumour *in vivo*. In addition to observing an important reduction of the tumour mass and volume as a result of the treatment with a hydroethanolic extract of the plant, we noted a remarkable modulation of the oxidative stress caused by the neoplastic process, both in the liver and in the tumour of the subjects [19]. Our conclusion was that the modulation of the redox processes would probably play a pivotal role in the anti-proliferative effects of the plant, perhaps via alteration of one or more metabolic pathways.

It is only logical to assume that all these anti-neoplastic properties should be due to the pentacyclic oxindole alkaloids, which have been shown to exert the anti-

inflammatory properties mentioned above, especially when considering cancer as a chronic inflammatory disease [20,21]. Thus, the present study aimed to evaluate the anti-neoplastic effects of two different fractions obtained from a hydroethanolic extract of *U. tomentosa*: one composed roughly of pentacyclic oxindole alkaloids and triterpene glycosides (chloroformic fraction, $CHCl_3$), and the other composed of most of the substances other than alkaloids present in the original extract, namely, phenolic glycosides and other anti-oxidant substances (*n*-butanolic fraction, BuOH). Additionally, we compared the effects of both fractions to those of the original hydroethanolic extract. The chosen *in vivo* tumour model was the Walker-256 (W256) carcinosarcoma in rats, which was the same model used in our previous experiments.

Materials and Methods

Botanic material, extraction and chemical analyses

All botanic material was kindly provided by the Peruvian Heritage SAC. It consisted on a brute hydroethanolic (BHE) extract of the bark of *U. tomentosa* prepared by decoction using a mixture of ethanol and water in the proportion of 7:3 for one hour at 20°C, and subsequently dried by atomization until the obtaining of a fine powder. Total alkaloid content was ascertained at 5.03% by means of high-performance liquid chromatography (HPLC) according to methods described elsewhere [22].

To achieve further fractionation, 20 g of this BHE extract were dissolved in ethanol-H_2O 1:1 and successively extracted with chloroform and *n*-butanol (3 x 150 mL for each solvent). After solvent removal under reduced pressure, the fractions in chloroform (labelled $CHCl_3$) and butanol (labelled BuOH) were obtained, with a yield of 1.9 and 9.5 g, respectively. These fractions were examined by analytical thin layer chromatography (TLC) using silica gel 60 PF_{254} precoated plates (Whatman) and several solvent systems ($CHCl_3$:MeOH 1:1). The spots were revealed by exposure under $UV_{254/366}$ light, spraying with the Dragendorff alkaloid-marking reagent and with 5% (v/v) H_2SO_4 in ethanol (EtOH) solution, followed by heating on a hot plate.

HPLC fingerprints analyses of the $CHCl_3$ and BuOH fractions were performed on a Waters HPLC equipped with a 2998 photodiode array detector. For all analyses a Waters X-Terra C18 column (250 x 4.6 mm, particle size 5 um) was used. The $CHCl_3$ fraction was analysed according the method proposed by Ganzera *et al.* [23]. The eluent for the BuOH fraction consisted of H_2O with 1% of acetic acid (A) and methanol (B), applied in a linear gradient from 80:20 (A:B) to 100 (B) over 70 min at a flow rate of 1 $mL.min^{-1}$. The column effluent was monitored at 254 nm.

Nuclear magnetic resonance (NMR) spectra 1D ($_1$H, $_{13}$C) and 2D (HSQC, HMBC) of the $CHCl_3$ and BuOH fractions were recorded on a Bruker AC 200 and/or Avance 400 spectrometer, observing ^{1}H at 200 or 400 MHz and ^{13}C at 50 or 100 MHz. The solvent used was DMSO-D_6, with tetramethylsilane (TMS) as internal reference.

In vitro free radical-scavenging activity

The reactivity of the *U. tomentosa* BHE extract and both its resulting fractions with the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured by means of an adaptation of the method of Chen and colleagues [24]. The system consisted of 250 µL of a methanolic solution of DPPH (1 mg in 25 mL), which was then combined with 750 µL of six crescent test solutions of each product. For the BHE extract we tested concentrations within the interval of 1 – 300 $\mu g.mL^{-1}$, whereas for its fractions we tested extrapolated concentrations as per the yield of the fractioning process. The decrease in absorbance was measured after 5 minutes. A solution of the reducing agent ascorbic acid (50 $\mu g.mL^{-1}$) was used as positive control and distilled water was used as negative control. Considering possible colour shifts in the solutions due to naturally occurring pigments in the tested substances, we included measurements of the absorbance of each solution prior to the addition of DPPH, which were subtracted when calculating the final values.

Tumour cells, handling and inoculation

Walker-256 tumour cells were kindly donated by Prof. Dr. Luiz Cláudio Fernandes, from the Physiology Department of the Federal University of Paraná. Their maintenance was carried out by weekly passages through intraperitoneal (IP) inoculation according to Vicentino and colleagues [25]. After five to seven days, the animal would appear emaciated and with vast ascitic signs. At this point, it was submitted to euthanasia and its ascitic fluid collected and centrifuged for 10 min at 1126 ×*g* at 4 °C. The supernatant was discarded, and the precipitate was re-suspended in 1.0 mL of PBS (16.5 mM of phosphate, 137 mM of NaCl and 2.7 mM of KCl). The viability of tumour cells was assessed by the Trypan blue exclusion method in a Neubauer chamber. Finally, around 10^7 Walker-256 cells were injected subcutaneously in the right pelvic limb of each animal.

Animal handling, experimental design, sample collection and ethical issues

Male Wistar rats weighing 180 – 250 g were obtained from the Central Animal House of the Federal University of Paraná (Curitiba, Brazil). Housing conditions

included temperature of 22 ± 1 °C, constant 12-hour light-dark cycles and free access to standard laboratory food (Nuvital®) and tap water. No other experiments were conducted in these animals prior to those of the current research.

We performed a pre-clinical trial in this animal model. Treatment began 1 day after the subcutaneous inoculation of 10^7 tumour cells in the right pelvic limb of the subjects, and continued for 14 days. All three treatment products were dissolved daily in 6 mL of vehicle. The selected doses, which were administered daily by gavage, were 50 $mg.kg^{-1}$ for the BHE extract (Group BHE) and extrapolated doses for both fractions as per the yield of the fractioning process (approximately 23.75 $mg.kg^{-1}$ and 4.75 $mg.kg^{-1}$ for groups BuOH and $CHCl_3$, respectively). Animals of the control group (Group C) received a similar volume of vehicle. For some parameters, a Baseline group (Group B) was added, which was composed of non-tumour-bearing individuals that received vehicle during 14 days. The number of individuals (*n*) in each of these groups was 4-6.

Following the 14-day treatment, all animals were anesthetized with intraperitoneal injection of ketamine (Quetamina®, Vetnil, Louveira, Brazil), in a dose of 60 $mg.kg^{-1}$, and xylazine (Kensol®, König, Santana de Parnaíba, Brazil), in a dose of 7.5 $mg.kg^{-1}$. Blood samples from the inferior cava vein were obtained for biochemical assays; subsequently, animals underwent euthanasia by diaphragmatic puncture. All primary tumours were removed and had their biophysical parameters measured as described below. Finally, tumour and liver samples were collected and stored at −70 ^{o}C for further analyses.

All experimental protocols using animals were performed following the recommendations of Brazilian Law 6638 (05/11/1979) for the scientific management of animals and the “Principles of Laboratory Animal Care” (NIH Publication 85 - 23, revised in 1985). The Institutional Animal Ethics Committee of Federal University of Paraná revised and approved all procedures of this study, issuing the certificate number 501.

Biophysical parameters measurement

All animals had their weight measured every other day during the treatment period. After fourteen days, all tumours were removed and weighted in an analytical balance. Tumour volume was calculated by means of the measure of its diameters, according to Mizuno *et al*. [26], using the formula $V\ (cm3) = 4\pi\ /\ 3.a2.(b/2)$, where *a* is the lesser diameter and *b* is the greater diameter, in cm. Furthermore, we assessed the inhibitory effect, when appropriate, using the following formula: $Tumour\ Suppression\ (\%) = (1 - T/C)$, where *T* is the average of the volumes of the tested group and *C* is the average volume of the control group.

Plasma biochemical assays

Plasma samples were obtained after blood centrifugation at 3000 ×*g* for 10 minutes. These samples were used for determination of plasmatic urea, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by means of commercial kits (Labtest Diagnostica®, Lagoa Santa, Brazil) using the automating device Cobas Mira, of Roche Diagnostics.

Oxidative stress parameters

i. Determination of the lipid peroxidation rate

Lipid peroxidation (LPO) rate was measured by the FOX-2 method [27]. This technique determines lipid hydroperoxide synthesis during peroxidation. Tumour and liver samples were homogenized at 25,000 rpm and then dissolved in methanol (1:2 ratio). Finally, they were centrifuged at 5000 ×*g* for 5 minutes at 4 °C and the supernatant was placed in the presence of the reagent xylenol orange. The absorbance of the ensuing reaction was measured at 560 nm in a spectrophotometer model Ultrospec 4300 pro. The results were expressed in nmol.mg.protein^{-1}.

ii. Determination of the activity of the enzymes catalase, superoxide dismutase and glutathione-S-transferase

For the biochemical analyses of these enzymes, liver and tumour samples were homogenized in phosphate buffer at pH 6.5. Catalase (Cat) activity was measured according to Aebi [28]. The reaction was examined at 240 nm in a spectrophotometer during 60 seconds for the liver samples and 180 seconds for the tumour samples. This difference in the time of the reaction was due to the much lower Cat levels in tumour tissue when compared to liver tissue.

Superoxide dismutase (SOD) activity was measured in both tissues by the ability of this enzyme to inhibit pyrogallol auto-oxidation, according to the method described by Gao and colleagues [29]. The reaction was performed in a 96-well microplate and examined at 440nm. The amount of enzyme that inhibited the reaction by 50% (IC_{50}) was defined as one unit of SOD, and the enzyme activity was expressed in units of SOD per milligram of total protein (U SOD.mg protein^{-1}).

The activity of glutathione-S-transferase (GST) in the liver was measured following the method of Habig and colleagues [30], which is based on the capacity of this enzyme to conjugate the substrate 2,4-dinitrochlorobenzene (DNCB) with glutathione in its reduced form, forming a tioether that can be measured by the increase in absorbance at 340 nm. Enzyme levels were expressed as nmol.minute^{-1}.mg of protein^{-1}.

iii. Determination of the reduced glutathione levels

Reduced glutathione (GSH) levels were measured by the method described by Sedlak and Lindsay [31]. Tumour and liver tissue were diluted in phosphate buffer 0.1 M (pH 6.5) in the proportion of 1:10. Subsequently, 250 µL of the homogenate were mixed with trichloroacetic acid (200 µL of 12.5% purity) and kept in ice for 30 min in order to allow protein precipitation. The supernatant was separated by centrifugation at 13,750 ×*g* for 10 min at 4 °C. Then, 30 µL of the clear supernatant was mixed with 270 µL of phosphate buffer 0.1 M (pH 8.5) and 5 µL of 5,5'-dithiobis-(2-nitrobenzoic acid) in methanol. The absorbance of the reaction solution was measured at 415 nm in a microplate reader, using reduced glutathione as external standard.

iv. Quantification of proteins

The quantification of proteins in liver and tumour samples was performed according to the method designed by Bradford [32]. The reaction was examined at 595 nm in a microplate reader, using bovine serum albumin (BSA) as protein standard.

Measurement of the inflammatory cytokine TNF-α

The measurement of this cytokine was performed in the liver and tumour homogenates by ELISA method, following the manufacturer's instructions (R&D Systems). A plate of 96 wells was coated with 100 µL of purified anti-mouse TNF-α antibody (1 $\mu g.mL^{-1}$) and incubated overnight at 4°C. In the following day, recombinant murine TNF-α standard (31.5–2000 $pg.mL^{-1}$) and samples were added into their corresponding wells and the plate was incubated overnight at 4°C. After this period, 100 µL of biotinylated anti-mouse TNF-α was added to each well in an amount of 50 $ng.mL^{-1}$. The plates were then incubated for 2 h at room temperature. Following the removal of the unbound antibody-enzyme reagent, 100 µL of streptoavidin–horseradish peroxidase (HRP) solution was added to the wells, which were then incubated for 20 min. Afterwards, 100 µL of the substrate solution containing hydrogen peroxide and o-phenylenediamine (OPD; Sigma-Aldrich Corporation) was added to the wells for colour development. The enzyme reaction yields a yellow product that turns orange in the presence of 50 µL of the stop solution (H_2SO_4 1 M), which was added to each well after 25 min. The optical density was determined using a microplate reader at 450 nm.

Liver perfusion and metabolism parameters

We inoculated tumour cells as previously described and treated the subjects with vehicle, BHE extract or BuOH fraction for 14 days. Baseline individuals were not

inoculated with Walker-256 cells, and received vehicle during this period. The number of individuals (*n*) in each group was 3 to 6, and these animals were subjected to these experiments only. All rats were fasted for approximately 18 hours prior to the experiments.

The used perfusion technique is described elsewhere [33]. The perfusion fluid was the Krebs-Henseleit-bicarbonate buffer (pH 7.4) containing 0.025% bovine serum albumin and saturated with an oxygen–carbon dioxide mixture (95:5%). This fluid was pumped through a temperature-regulated (37 °C) membrane oxygenator prior to entering the liver via a cannula inserted in the portal vein. For each individual experiment the perfusion flow was kept constant and adjusted between 29 and 30 $mL.min^{-1}$. After a stabilization perfusion period of 10 minutes, 1.25 mM alanine (a gluconeogenic, ammoniagenic and ureagenic substrate) was infused for an additional 40 minutes. For the analytical procedures, samples of the effluent perfusion fluid were collected at two-minute intervals during the whole perfusion time (50 minutes) and analysed for glucose, lactate, pyruvate, urea and ammonia. All these metabolites were assayed employing standard enzymatic procedures [34]. The oxygen concentration in the outflowing perfusate was monitored continuously employing a Teflon-shielded platinum electrode adequately positioned in a Plexiglas chamber at the exit of the perfusate [35]. Metabolic rates were calculated from input–output differences and the total flow rates, and referred to the wet weight of the liver.

Survival analysis

In order to ascertain the possible influence of the treatment with *U. tomentosa* on the overall survival rate of the tumour-bearing animals, we conducted a survival analysis employing different individuals as those used for all other experiments. Subcutaneous inoculation of the Walker-256 cells and the configuration of treatment groups and doses were performed as previously described. Treatment period lasted 30 days instead of 14 days in order to fully observe the survival times for each treatment group, whereupon all surviving animals were subjected to euthanasia by means of anaesthesia with ketamine and xylazine, as described previously, followed by decapitation.

The Kaplan-Meier statistical method was used, and survival curves were compared using the logrank test. This test generates a *p* value that verifies the null hypothesis, which in turn states that all curves are equal.

Additionally, a correlation was constructed between the survival rate (%) after 30 days of treatment and the tumour weight (g) after 14 days of treatment, which was analysed by linear regression.

Statistical analysis

Unless otherwise noted, statistical analyses were performed using one-way analysis of variance (ANOVA) with post hoc Neumann–Keuls multiple comparison testing, in the Graph Pad Prism program version 5.0. Hepatic metabolic production was analysed by two-way ANOVA followed by Bonferroni's test, using perfusion time and treatment as variables. Differences were considered significant when $p<0.05$.

Results

Chemical analyses

TLC analyses showed that the $CHCl_3$ and BuOH fractions were mixtures. Compounds were revealed with UV_{254} light and H_2SO_4 solution in both fractions, but only the $CHCl_3$ fraction provided a positive test with the Dragendorff alkaloid-specific reagent.

The BuOH fraction was found to be constituted by a complex mixture of components, as shown in its HPLC chromatogram (Figure 1A). Comparison with previously reported HPLC chromatogram of polar extracts of *U. tomentosa* [36] suggests that BuOH fraction contain proanthocyanidins and phenolic compounds, as well as caffeic acid and derivatives.

The ^{1}H NMR spectrum of the BuOH fraction (Supplementary Figure 1) showed several multiplets at δ 3.0-4.0, along with doublets at δ 4.3-5.5, suggesting the presence of sugars. We also observed signals of aromatic protons (6.5-8.0 ppm) and a broad singlet at δ 8.90, characteristic of a hydroxyl group of phenols or carboxylic acids. Two doublets at δ 7.35 (*J* 1.0 Hz) and δ 5.15 (*J* 5.7 Hz) were associated with 7-deoxyloganic acid [37], which is a rare iridoid illustrated in the figure 2C. In agreement, the ^{13}C NMR spectrum of this fraction (Supplementary Figure 2) showed several peaks assignable to oxygenated carbons (62-82 ppm), anomeric carbons (92-105 ppm), aromatic carbons (115-150 ppm) and carbonyl groups (169 ppm). The presence of 7-deoxyloganic acid was confirmed by correlations observed in the heteronuclear single quantum coherence (HSQC) spectrum (Supplementary Figure 3) and heteronuclear multiple bond correlation (HMBC) spectrum (Supplementary Figure 4) experiments. Furthermore, the doublet at δ 5.15 in the ^{1}H NMR spectrum showed correlation with a carbon at δ 96.2 (C-1) in the HSQC spectrum and cross-peaks with carbons at δ 33.8 (C-5), 34.8 (C-8), 99.0 (anomeric carbon of glucose) and 150.8 (C-3) in the HMBC spectrum. In addition, the doublet at δ 7.35 showed correlation with a carbon at δ 150.8 (C-3) in the HSQC spectrum and cross-peaks with carbons at d 33.8 (C-5), 96.4 (C-1), 112.1 (C-4) and 169.1 (COOH). In conclusion, the BuOH fraction is constituted of 7-deoxyloganic acid, along with proantocyanidins and phenolic glycosides.

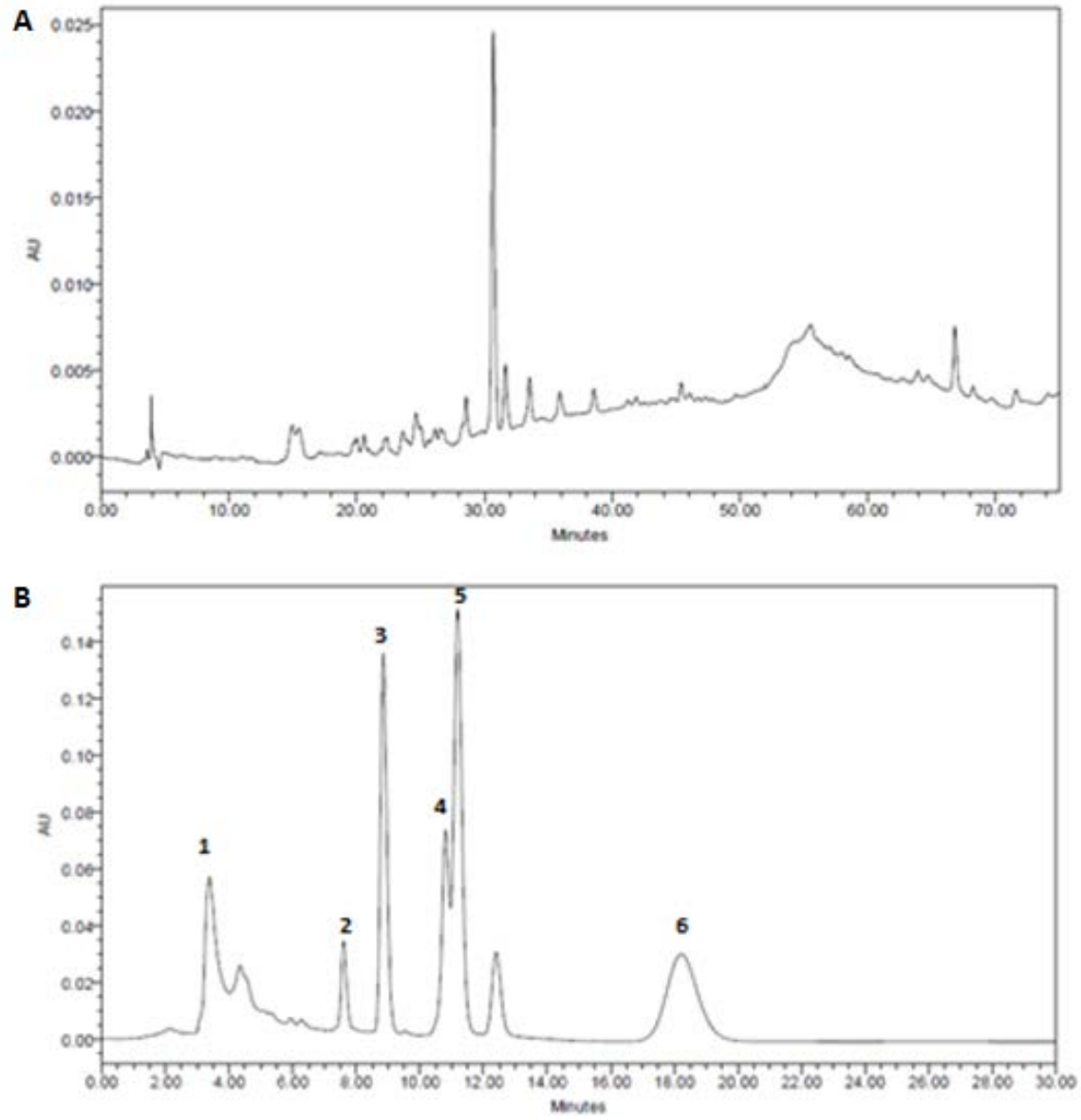

Figure 1. (A): Chromatogram of the BuOH fraction of *U. tomentosa*. This profile is very similar to that which was previously reported [36] for an extract containing phenolic compounds, including proanthocyanidins in the region between 50 and 60 minutes. **(B):** Chromatogram of the $CHCl_3$ fraction of *U. tomentosa*. The following compounds were tentatively identified as 1- speciophylline; 2- uncarine F; 3- mitraphyline; 4- isomitraphyline; 5- pteropodine and 6- isopteropodine by comparison with the HPLC profile reported by Ganzera *et al*. [23] and the one performed by Peruvian Heritage SAC .

The chromatogram of the $CHCl_3$ fraction (Figure 1B) exhibited a profile consistent with the presence of pentacyclic oxindole alkaloids. The compounds were tentatively identified as speciophylline, uncarine F, mitraphyline, isomitraphyline, pteropodine and isopteropodine by comparison with chromatograms previously reported [23] and an analytical report of Peruvian Heritage S.A.C (data not shown). Other unidentified peaks were also observed.

The ^{1}H NMR spectrum of the $CHCl_3$ fraction (Supplementary Figure 5) showed signals due the presence of several types of protons, such as aliphatic (0.7-3.0 ppm), oxy-aliphatic (3.0-5.0 ppm), olefinic (5.5 ppm), aromatic (6.8-7.5 ppm) and protons on heteroatoms (10.3-10.5 ppm). Accordingly, the ^{13}C NMR spectrum (Supplementary Figure 6) exhibited more than one hundred peaks, including some signals attributable to carbonyl groups (166-210 ppm). Comparison with spectral data of compounds previously isolated from *U. tomentosa* [13,38-41] led us to conclude that the fraction $CHCl_3$ is a complex mixture of oxindole alkaloids and triterpene glycosides. The presence of these classes of compounds was confirmed by detailed analysis of selected regions of the NMR 1D and 2D spectra (Supplementary Figures 7 and 8), which also revealed the presence of 7-deoxyloganic acid [37]. Thus, the presence of oxindole alkaloids (Figure 2A) was easily confirmed in the ^{1}H NMR spectrum by multiplets in δ 10.3-10.5, which can be assignable to protons attached to nitrogen. These protons did not show correlations in the HSQC spectrum, but showed cross-peaks with carbons at δ 55.9 and 56.6 (C-7), 133.6 and 133.8 (C-8) and 141.5 and 141.9 (C-13) in the HMBC spectrum. In the ^{1}H NMR spectrum we also observed several singlets at δ 0.7-1.0 that are characteristic of triterpenes. In the HSQC spectrum the signals at δ_H 0.76, 0.96 and 5.52 showed correlation with signals of carbons at δ_C 16.8, 28.1 and 128.5, respectively. The last value suggests a triterpene with an urs-12-ene skeleton, which could correspond to quinovic acid and its derivatives (Figure 2B), which are triterpenes of the oleanane or ursane type that are found free or attached to sugars, and are very common in *U. tomentosa*. HMBC correlations were observed between the proton assignable to H-24 (δ 0.96, s), C-3 (δ 88.4), C-4 (δ 39.1), C-5 (δ 55.6) and C-23 (δ 16.9). The chemical shift of C-3 is compatible with the presence of a sugar attached through an ether linkage in this position. Indeed, a doublet at δ 4.16 (7.2 Hz), which can be assignable to an anomeric proton, showed correlation in the HSQC spectrum with a signal of carbon at δ 105.7 and a cross-peak with C-3 in the HMBC spectrum. Finally, 7-deoxyloganic acid (Figure 2C) was deduced from a doublet at δ 5.15 in the ^{1}H NMR spectrum, which showed the same correlations observed in the spectra of the BuOH fraction.

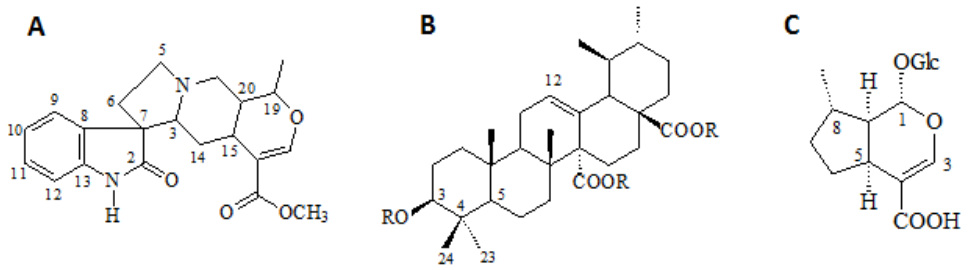

Figure 2. (A): Structure of the major alkaloids of *U. tomentosa*. The individual compounds differ in configuration at C-3, C-7, C-15, C-19 and C-20 [42]. **(B):** Structure of quinovic acid (R = H) and its glycosides (R = several sugars), as well as **(C):** of 7-deoxyloganic acid, all of them isolated from *U. tomentosa*.

In vitro free radical-scavenging activity

The antioxidant effect of the BHE extract of *U. tomentosa*, as well as of both its fractions, was assessed *in vitro*. A statistically significant antioxidant activity was observed in all tested concentrations of the BHE extract, this effect being similar to that of the positive control (ascorbic acid). Likewise, the BuOH fraction exhibited satisfactory free radical-scavenging of DPPH starting from the concentration of 5 $mg.mL^{-1}$. However, no tested concentration of the $CHCl_3$ fraction was successful in neutralizing DPPH. These results are shown in the Figure 3.

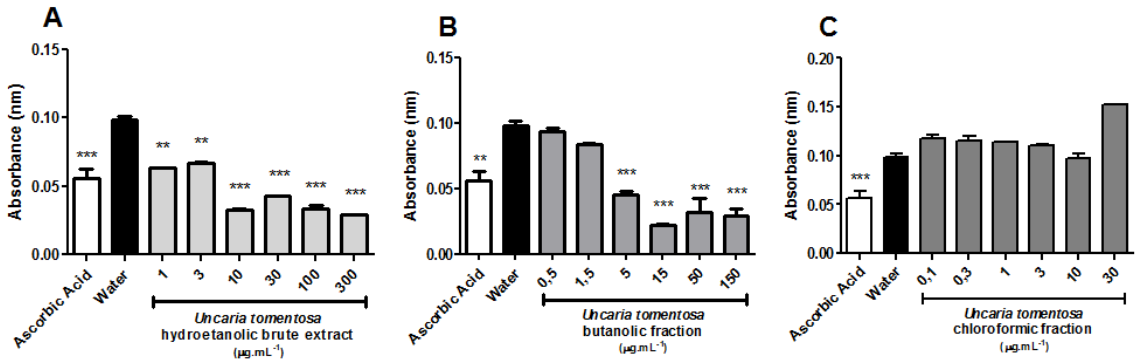

Figure 3. Assessment of the free radical-scavenging activity of the BHE extract of *U. tomentosa* **(A)**, as well as its two resulting fractions: BuOH **(B)** and $CHCl_3$ **(C)** at various concentrations. Negative control was distilled water and positive control was ascorbic acid (50 $\mu g.mL^{-1}$). Symbols: $^{**}p<0.01$ and $^{***}p<0.001$ as compared to the negative control.

Biophysical parameters of the tumours

To assess the anti-neoplastic effect of *U. tomentosa* we measured some tumour biometric parameters. Tumour mass of the control group at the end of the treatment was 24.67 ± 3.87 g. Treatments with the BHE extract and BuOH fraction had a tumour mass suppression of 52% and 49%, respectively, as compared to control ($p<0.01$). On the other hand, the $CHCl_3$ fraction presented a tumour mass of 24.74 ± 3.2 g, which was similar to that of the control group (Figure 4A). Tumour volume results were quite similar to those of tumour mass, exhibiting an evident difference between the control and BHE or BuOH treated groups. The control group showed a tumour volume of 73.8 ± 17.32 cm^3 at the end of the treatment, while the BHE extract- and BuOH fraction-treated group presented less than 50% of this volume (35.12 ± 16.87 cm^3 and 31.35 ± 14.93 cm^3, respectively), both results being statistically significant ($p<0.01$). Still, the group that received the $CHCl_3$ fraction presented a tumour volume similar to that of the control group (Figure 4B). The tumour volume suppression rates calculated for the BHE extract and BuOH fraction groups as compared to the control group were 52% and 58% respectively.

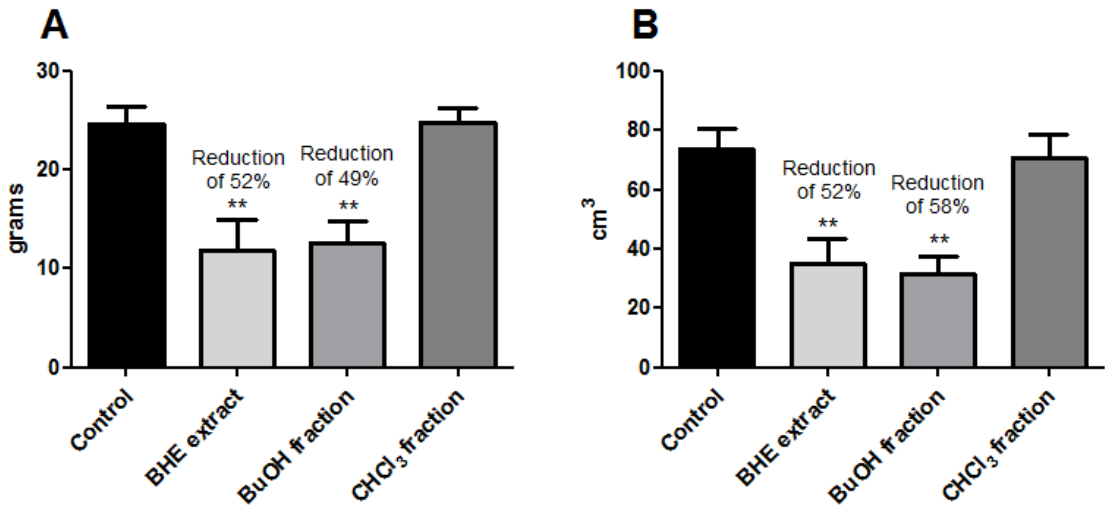

Figure 4. Tumour mass **(A)** and volume **(B)** in Walker-256 tumour-bearing rats treated with BHE *Uncaria tomentosa* extract or its two fractions (BuOH and $CHCl_3$). Symbols: ***p*<0.01 as compared to the control group.

Plasma biochemical assays

The plasmatic enzymes ALT and AST were measured as parameters that indicate cellular integrity, especially that of hepatocytes. No statistically significant differences were found for ALT values amongst control and all three treatment groups (BHE extract and its BuOH and $CHCl_3$ fractions). However, treatment with the BHE extract or its BuOH fraction did significantly reduce AST levels (Table 1) in an attempt to reverse the tumour-induced increase of this enzyme. An increase in plasmatic urea was observed in the rats treated with the $CHCl_3$ fraction, as compared to baseline, control and BHE extract groups (Table 1).

Table 1: Biochemical parameters measured in the plasma of Walker-256 tumour-bearing rats treated during 14 days with BHE *U. tomentosa* extract or its two fractions BuOH and $CHCl_3$.

Parameter	Experimental Groups					Units
	Baseline	Control	*Uncaria tomentosa*			
			BHE extract	BuOH fraction	$CHCl_3$ fraction	
ALT	55.5 ± 5.3 **	43.4 ± 6.4	44.25 ± 5.1 $^{\#\#}$	31.7 ± 10 ** $^{\#\#\#}$ °	31.3 ± 5.5 * $^{\#\#\#}$ °	$U.L^{-1}$
AST	69.53 ± 4.9 ***	324.4 ± 41.2	183.8 ± 60 ** $^{\#}$	167.2 ± 69.9 ** $^{\#\#}$	288.5 ± 97.9 $^{\#\#\#}$	$U.L^{-1}$
Urea	40.8 ± 7.5	34.8 ± 3.4	39 ± 3.6	45.8 ± 4.8	54.5 ± 11.4 ** $^{\#}$ °	$mg.dL^{-1}$

Symbols: **p*<0.05, ***p*<0.01 and ****p*<0,001 as compared to the control group; $^{\#}$*p*<0.01, $^{\#\#}$*p*<0.01 and $^{\#\#\#}$*p*<0.001 as compared to the baseline group; °*p*<0.05 as compared to the BHE extract.

Oxidative stress in liver and tumour tissues

Having observed a satisfactory antioxidant *in vitro* effect, and considering the different composition of the *U. tomentosa* extract and its fractions, we deemed appropriate to measure oxidative stress parameters *in vivo*, both in hepatic and tumour tissue. Thereby, we assessed the activity of the enzymes superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (Cat), along with the levels of reduced glutathione (GSH) and the lipid peroxidation rate (LPO).

The experiments regarding SOD activity produced interesting results. In the hepatic tissue of the animals of the control group we found SOD levels of 6.36 ± 0.44 U SOD.mg of protein^{-1}, which were quite increased when compared to those of the baseline group (1.99 ± 0.36 U SOD.mg of protein^{-1}). Treatment with the BHE extract further increased them to 10.16 ± 1.81 U SOD.mg of protein^{-1} (p<0.001) and the BuOH fraction exhibited a significant increase as well, to 8.8 ± 0.28 U SOD.mg of protein^{-1} (p<0.001). However, a statistically significant difference was found among these two treatment groups. In opposition, treatment with the $CHCl_3$ fraction generated similar SOD values as those of the control group. Remarkably, we obtained opposite results regarding the activity of this enzyme in the tumour tissue. SOD activity in the tumours belonging to the rats of the control group was 10.5 ± 2.89 U SOD.mg of protein^{-1}, and treatment with the *U. tomentosa* BHE extract and its BuOH fraction significantly reduced these levels. However, the $CHCl_3$ fraction exhibited SOD levels similar to those of the control group. These results are presented in the figures 5A and 5B.

Similar behaviour was observed upon measuring Cat activity. In the liver samples of the control group, this enzyme was found in amount of 179.5 ± 48.92 µmol.min^{-1}.mg of protein^{-1}, which represents an important decrease when compared to baseline values (399.8 ± 47.65 µmol.min^{-1}.mg of protein^{-1}). Treatment with the *U. tomentosa* BHE extract increased Cat activity to 303.8 ± 62.37 µmol.min^{-1}.mg of protein^{-1} (p <0.05), constituting a full reestablishment of baseline levels as there is no difference between these groups. Nevertheless, both BuOH and $CHCl_3$ fractions failed to show a statistically significant increase in Cat activity. Most interestingly, results in the tumours are in opposition of those found in the liver, but with much lower values of Cat activity in this tissue. These results are outlined in the figures 5C and 5D.

Regarding LPO measurements, in the liver samples of the control group we found levels of 28.22 ± 6.7 nmol.mg of protein^{-1}. In the same way as what we observed with SOD, treatments with the *U. tomentosa* BHE extract and its BuOH fraction reduced significantly these levels (Figure 5E) in an attempt to return this parameter to baseline levels. As expected, however, the $CHCl_3$ fraction showed similar LPO levels as those of the control group (54.8 ± 9.22 nmol.mg of protein^{-1}). In the tumour we observed a similar pattern. While treatment with the BHE extract and its BuOH fraction

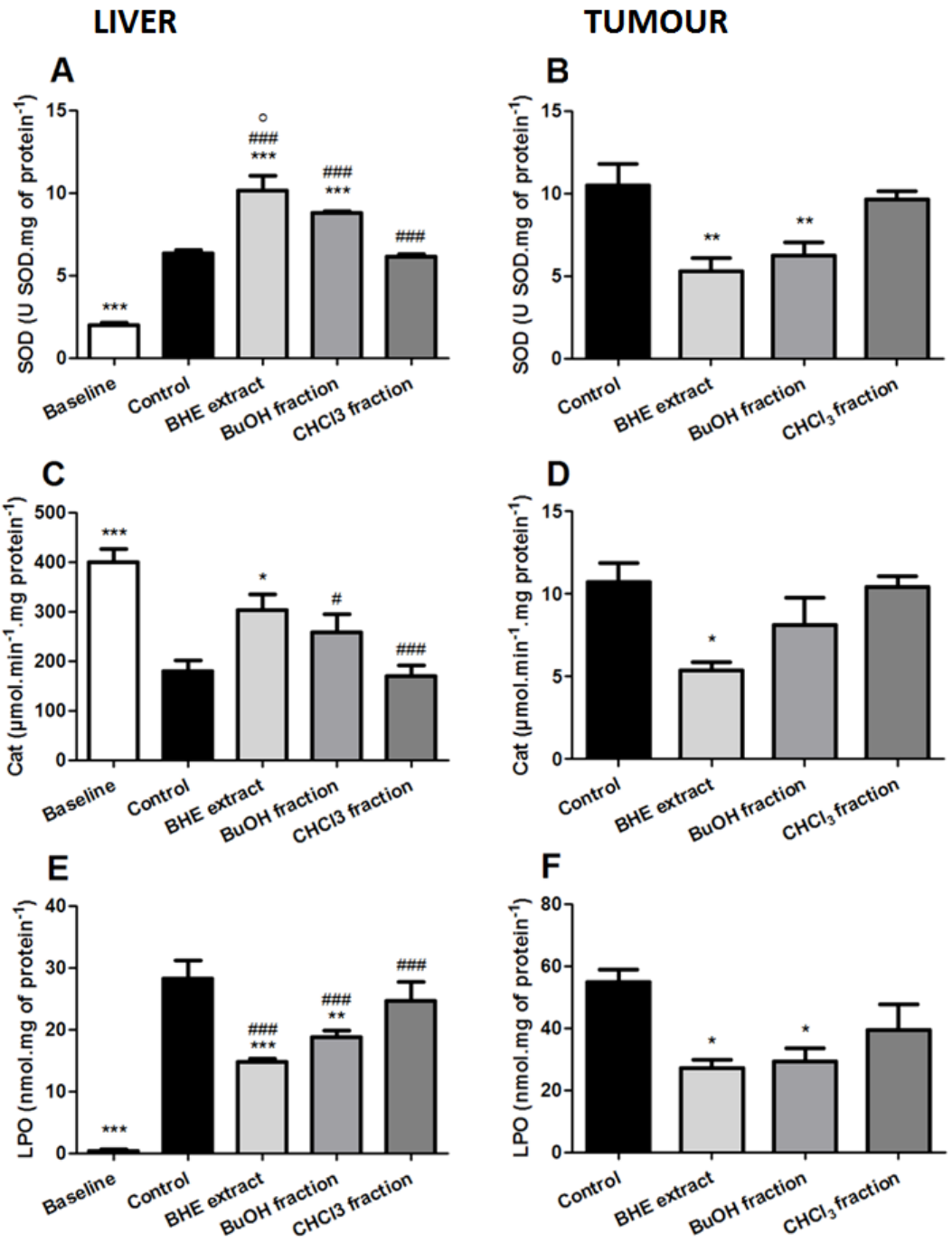

Figure 5. Main oxidative stress parameters measured in W256 tumour-bearing rats treated with *U. tomentosa* BHE extract or its two fractions (BuOH and $CHCl_3$): SOD activity in the liver **(A)** and tumour **(B)**; Cat activity in the liver **(C)** and tumour **(D)** and LPO rates in the liver **(E)** and tumour **(F)**. Symbols: **p*<0.05; ***p*<0.01 and ****p*<0.001 as compared to the control group; $^{\#}p$<0.05 and $^{\#\#\#}p$<0.001 as compared to the baseline group; °*p*<0.05 as compared to the BuOH fraction.

reduced LPO levels in a statistically significant manner, the $CHCl_3$ fraction did not alter this parameter (Figure 5F).

No statistically significant differences were found between the experimental groups regarding GST activity and GSH levels in the liver. Yet, treatment with the BHE extract did reduce GSH levels in tumour tissue when compared to the control group (p<0.05). Numeric values of these results are presented in the Table 2.

Table 2. GSH levels in liver and tumour tissue, as well as GST activity in the liver of W256 tumour-bearing rats treated with *U. tomentosa* BHE extract or its two fractions (BuOH and $CHCl_3$).

Parameter	Experimental Groups					Units
	Baseline	Control	*Uncaria tomentosa*			
			BHE extract	BuOH fraction	$CHCl_3$ fraction	
Liver GSH	589.3 ± 170.2 ***	103.0 ± 13.8	109.8 ± 7.82 ###	96.48 ± 6.21 ###	96.57 ± 6.63 ###	nmol.mg ptn^{-1}
Tumour GSH	(does not apply)	22.44 ± 2.39	13 ± 1.47 *	18.78 ± 6.86	24.8 ± 4.56	nmol.mg ptn^{-1}
Liver GST	0.6 ± 0,07 ***	1.35 ± 0.15	1.24 ± 0.38 ###	1.21 ± 0.24 ###	1.17 ± 0.31 ###	nmol.min^{-1}.mg ptn^{-1}

Symbols: *p<0.05 and ***p<0.001 as compared to the control group, respectively; ###p<0.001 as compared to the baseline group.

TNF-α measurement in the liver and tumour tissue

Since tumour development is characterized by an intense inflammatory reaction, TNF-α levels in the liver and tumour homogenates were measured as an inflammation marker. As the Figure 6 illustrates, the group treated with the BHE extract showed a TNF level of 8.68 ± 0.33 pg.mg of protein^{-1}, which is less than half of the value corresponding to the control group: 19.98 ± 1.61 pg.mg of protein^{-1}. Treatment with the BuOH fraction also achieved a statistically significant decrease in TNF-α, yet this decrease was not as pronounced as that of the BHE extract, and there exists statistical difference between these two groups as well. Finally, treatment with

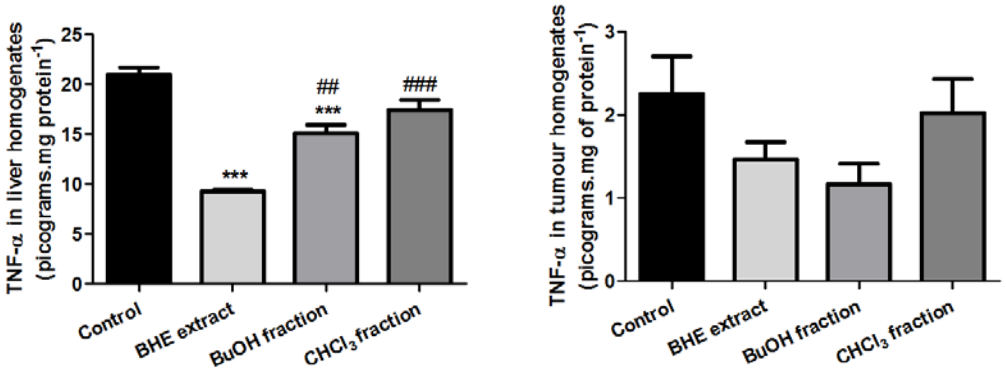

Figure 6: Levels of the inflammatory cytokine TNF-α in the hepatic **(A)** and tumour **(B)** homogenates of Walker-256 tumour-bearing rats treated with *U. tomentosa* BHE extract or its two fractions (BuOH and $CHCl_3$). Symbols: ***p<0,001 as compared to the control group; ##p<0.01 and ###p<0.001 as compared to the BHE extract-treated group.

the $CHCl_3$ fraction achieved a TNF-α level similar to those of the control group: 16.50 ± 2.46 pg.mg of protein^{-1}. Despite evident tendencies, no statistically significant differences were found among the experimental groups regarding the TNF-α level on the tumour homogenates.

Liver perfusion

Having observed an apparent lack of significant biological activity of the $CHCl_3$ fraction (at least when confronted with the W256 tumour model), we decided to drop it out of the liver perfusion experiments and work with the BHE extract and BuOH fraction only. Evaluated parameters included oxygen consumption, and production of glucose, pyruvate, lactate, ammonia and urea as a result of the hepatic metabolism of alanine. All of tumour-bearing rats presented a significantly smaller oxygen consumption (approximately -0.62 μmol.min.g^{-1}) than the baseline rats since the beginning of the perfusion (data not shown). Also, the tumour-bearing rats produced higher amounts of ammonia and pyruvate than the baseline group, which produced only a subtle amount of both metabolites, as shown in figure 7. The metabolic changes in the alanine pathways induced by the tumour (control group) were barely modified by the treatment with *U. tomentosa* preparations (Table 3).

Table 3: Influence of the Walker-256 tumour and treatment with a BHE of *U. tomentosa* or its BuOH fraction on the liver metabolism of the substrate alanine.

Metabolite	Experimental Groups				Units
	Baseline	Control	*Uncaria tomentosa*		
			BHE extract	BuOH fraction	
Glucose production	0.176 ± 0.019	0.152 ± 0.013	0.100 ± 0.033	0.178 ± 0.016	μmol·min^{-1}·g^{-1}
Urea production	0.102 ± 0.025	0.087 ± 0.036	0.064 ± 0.024	0.117 ± 0.031	μmol·min^{-1}·g^{-1}
Ammonia production	0.007 ± 0.005 **	0.162 ± 0.004	0.085 ± 0.025	0.117 ± 0.025 *	μmol·min^{-1}·g^{-1}
Pyruvate production	0.014 ± 0.0008	0.069 ± 0.012 ##	0.112 ± 0.008 #	0.069 ± 0.023 #	μmol·min^{-1}·g^{-1}
Lactate production	0.054 ± 0.023	0.096 ± 0.026	0.09 ± 0.022	0.092 ± 0.017	μmol·min^{-1}·g^{-1}
Oxygen consumption	0.237 ± 0.04	0.226 ± 0.016	0.227 ± 0.072	0.171 ± 0.046	μmol·min^{-1}·g^{-1}

Data represents the mean ± mean standard errors of the alterations caused by alanine 1.25 mM infusion in 3-6 experiments. These alterations were calculated as [final values at the end of the alanine infusion period] – [basal rates before alanine infusion]. One-way variance analysis was followed by Neumann–Keuls test. Symbols: **p<0.01 as compared to the Control group; #p<0.05 and ##p<0.001 as compared to the Baseline group.

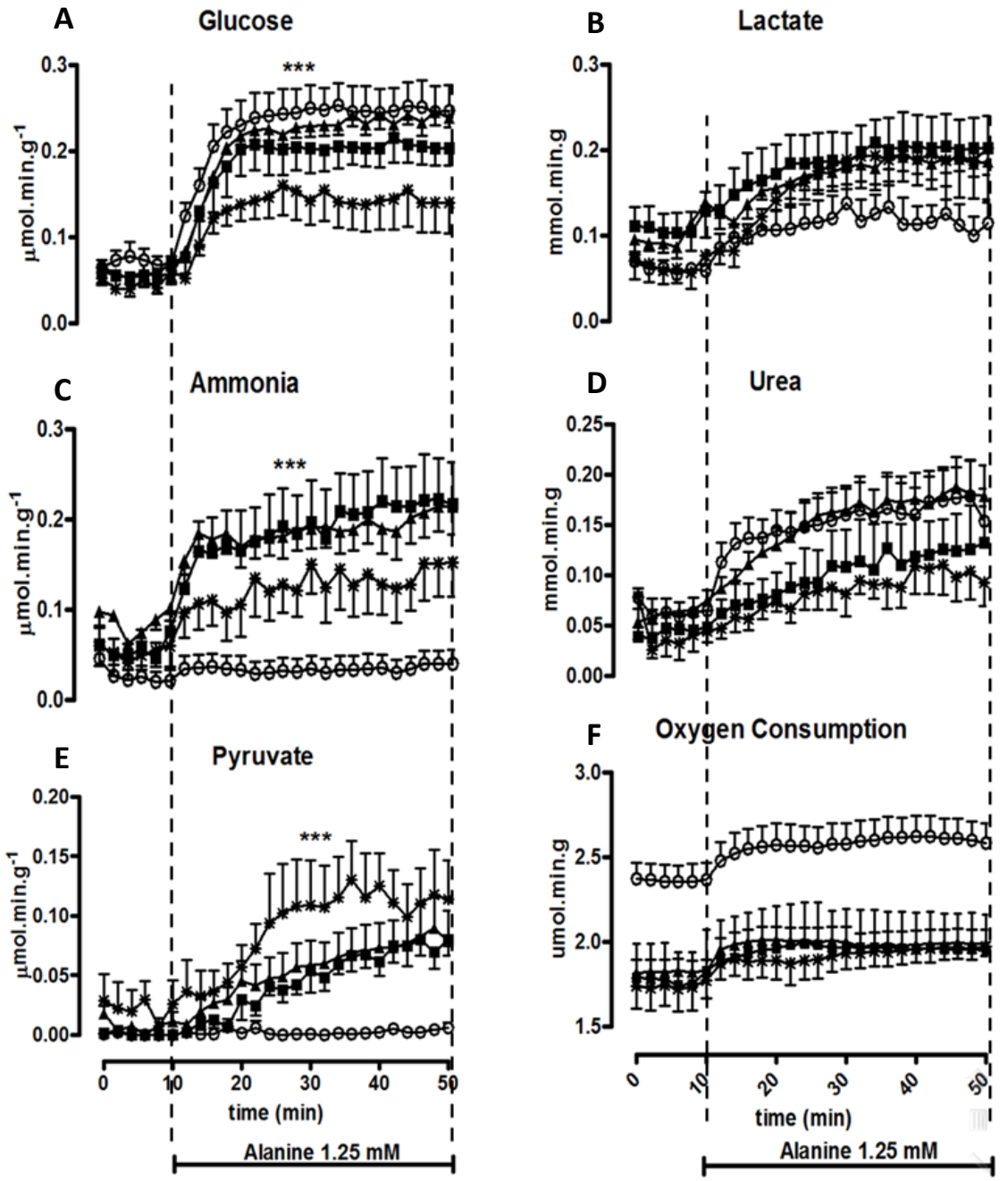

Figure 7. Time courses of hepatic glucose **(A)**, lactate **(B),** ammonia **(C)**, urea **(D)** and pyruvate **(E)** productions, as well as hepatic oxygen consumption **(F)** after 14 days of treatment with BHE *U. tomentosa* extract or its two fractions BuOH and $CHCl_3$ in Walker-256 tumour-bearing rats as well as in baseline rats. Zero time corresponds to the time at which sampling of the outflowing perfusate was initiated after oxygen consumption stabilization. Alanine infusion was started at 10 min. Data points are the mean ± mean standard errors of 3 to 6 experiments. Symbol: ***p<0.001 comparing the treatments by two-way ANOVA followed by Bonferroni's test.

Survival analysis

Our survival analysis revealed statistically significant differences between the studied groups. While all the tumour-bearing rats treated with the BHE extract survived the entire observation period (30 days), no individual belonging to the control and $CHCl_3$ groups remained alive at the end of the trial. Regarding the BuOH fraction, we obtained a survival rate of 66.67%, seeing that two out of six individuals died within the treatment period. These results are illustrated in the figure 8A. A positive correlation was observed between the survival rate and the tumour weight of each tumour-bearing rat (Figure 8B).

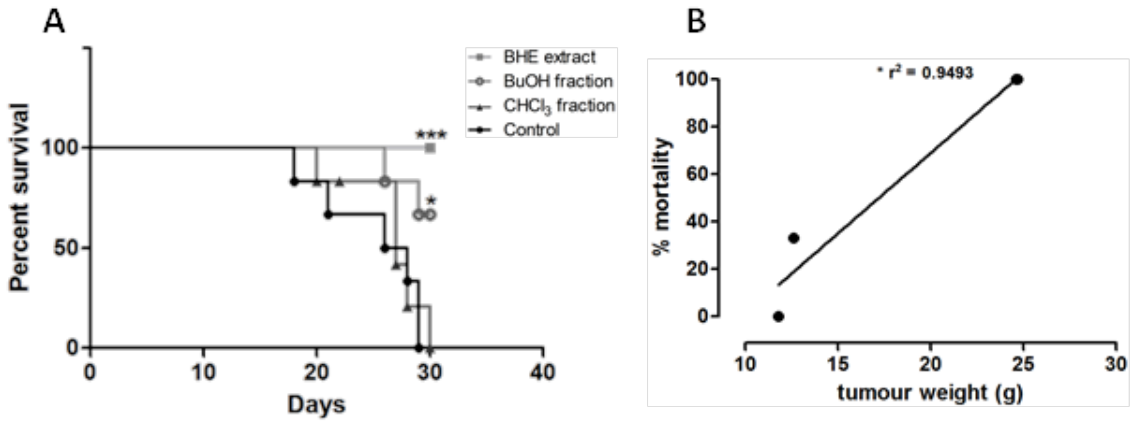

Figure 8. (A): Survival analysis of W256 tumour-bearing rats treated with *U. tomentosa* BHE extract or its two fractions (BuOH and $CHCl_3$) during 30 days. Data is expressed as percentage of survival as per the logrank (Mantel-Cox) test. **(B):** Correlation between tumour weight (g) and survival rate (%) analysed by linear regression. Symbols: *p<0.05 and ***p<0.001 as compared to the control group.

Discussion

Uncaria tomentosa has been widely studied and acclaimed as a powerful antioxidant and immunomodulant agent. Currently, considerable attention is being granted to its anti-neoplastic potential as well. Various preparation methods and administration regimes have been tested against tumour lineages *in vitro* [10,16,18,43] and *in vivo* [19], with promising results. Our current efforts follow the wake of some investigators, that experimented with fractions and isolated compounds of *U. tomentosa* extracts with the aim to attribute the observed pharmacological activities to a specific substance or group of substances [10,11,17,44].

The chemical composition of *U. tomentosa* has been extensively investigated. Nowadays, it is known that this species accumulates alkaloids, triterpenes and other classes of compounds, including phenolic glycosides, flavonoids and proanthocyanidins [8,13,36,37,39-41,45]. The content of individual compounds is variable, according to the origin of the plant, the season of collection and the part of the plant studied [38,46].

Among the most abundant alkaloids found in this plant, there is a mixture of pentacyclic oxindole stereoisomers that differ in configuration at chiral centres in the positions 3, 7, 15, 19 and 20 (Figure 2A). In attempting to explain the anti-neoplastic effects of *U. tomentosa*, researchers have demonstrated interest in this group of substances, based on previous observations that gave them credit for its anti-inflammatory activity [8,9]. However, it is important not to overlook the fact that cancer is a vast group of diseases that may vary substantially among them. Hence, the number of possible clinical scenarios is equally diverse, with different outcomes among tumour lineages, individuals affected, and even throughout the various locales affected by the neoplastic process in each individual [47].

Pilarski and colleagues [48] demonstrated awareness of this wide range of possible deviations by cross-testing extracts of *U. tomentosa* of different alkaloid content against several tumour lineages, both *in vivo* and *in vitro*. Interestingly, their results suggest that preparations with greater alkaloid concentrations do not necessarily achieve the best anti-neoplastic effects. As possible explanations for their results, the authors suggest an apparent selectivity of the fractions with higher alkaloid levels for some tumour lineages, low water solubility of isolated alkaloids and issues regarding the IC_{50} measures. Taking into account the previous observations of our research team, including the importance of oxidative stress in the W256 *in vivo* tumour model [49-51], and the relevant synergism between antioxidant and cytotoxic components in the anti-neoplastic effects of *U. tomentosa* [19], we propose yet another hypothesis: *U. tomentosa* fractions that are alkaloid-rich, but otherwise devoid of most other substances present in the original extract, may perhaps leave out and disregard the antioxidant effects of such other substances, along with the beneficial synergic effects that they could be providing. Consequently, in the present study we sought to put this last hypothesis to the test by evaluating the anti-neoplastic effects of an *U. tomentosa* fraction composed roughly of alkaloids ($CHCl_3$) and a fraction composed of most other antioxidant substances (BuOH), finally comparing these fractions to the original BHE extract containing all groups of compounds.

According to our chemical analyses, the $CHCl_3$ fraction contains alkaloids, triterpenes and 7-deoxyloganic acid, which also was found in the BuOH fraction, along with antioxidant substances such as phenolic glycosides and anthocyanidins. These observations are further supported by the DPPH results, which point out the BHE extract as the most effective free radical scavenger. Significant antioxidant activity in the BuOH fraction was detected, while the $CHCl_3$ fraction had no antioxidant activity whatsoever. The results of this assay are in accordance to the aforementioned composition of these three substances and are adequate for the testing of our hypothesis.

Expressive reduction in tumour weight and volume was observed in W256 tumour-bearing rats treated with both BHE extract and BuOH fraction. This restriction of tumour development was directly related with increase in the survival rate, as shown in the figure 8B. All (100%) and two thirds (66%) of the rats treated with the BHE extract and BuOH fraction, respectively, survived for the tested period (30 days), while none of the animals treated with the $CHCl_3$ fraction nor those who received vehicle (control group) survived. These results clearly show that by fractioning *U. tomentosa* extracts, active compounds that present anti-neoplastic effects are also separated, as well as constitute evidence of the importance of the oxidative stress modulation as part of the action mechanisms of this plant.

The results obtained with the *in vivo* oxidative stress parameters that we assessed also confirmed these findings. SOD and LPO measures in the liver and tumour tissue demonstrated a major beneficial effect of the *U. tomentosa* BHE extract and BuOH fraction, while the $CHCl_3$ fraction consistently produced negligible results, similar to those of the control group. It is noteworthy that the BHE extract was significantly more successful than its BuOH fraction in heightening SOD levels, which were already increased in the control group as compared to the baseline group as a natural reaction to the considerable oxidative stress caused by the neoplastic process. Regarding Cat measures in both tissues, only the BHE extract achieved a statistically significant effect as compared to control, effectively increasing hepatic Cat levels to similar values as those of the baseline group. Despite the evident lower levels of GSH in tumour tissue found in the BHE extract and BuOH fraction-treated groups as compared to control, only those of the BHE extract were statistically significant. This is a favourable result, taking into account the important role that GSH plays in such cellular processes as the transport of amino acids, the synthesis of proteins and DNA, and even cellular detoxification [52]. Consequently, it seems appropriate to establish that the biological activities of *U. tomentosa* are indeed enhanced by the synergic action of its various components, at least regarding the analysed parameters.

It is remarkable to acknowledge that *U. tomentosa* seems to exert a degree of selectivity over its site of action, an observation which supports our previous findings [19]. Indeed, in various parameters we observed that the BHE extract (and sometimes even its BuOH fraction) had different, if not opposite effects in the liver and tumour tissue. Even more important is the fact that this selectivity resulted in an apparent protection of the hepatocytes, which came along with a simultaneous attack to the neoplastic cells. This most intriguing and favourable behaviour was observed for the SOD enzymatic system, which is responsible for the conversion of the superoxide anion to oxygen and hydrogen peroxide. It was suggested that the Walker-256 tumour-bearing rats present an increased amount of superoxide anion through the respiratory chain [50], promoting an oxidative cascade that could lead to necrosis [53,54]. Thus, the increase in SOD observed in the hepatic tissue is beneficial, because it suggests an

adequate response to this superoxide excess. Conversely, treatment with *U. tomentosa* resulted in a reduction of the SOD levels in the tumour tissue, which in turn constitutes an advantageous result as well, as it indicates that the tumour cells are being left more susceptible to the effects of the superoxide anion. This apparent selectivity was also observed for Cat levels. The importance of Cat in the neoplastic scenarios has long been appreciated, as various studies have demonstrated that increased activity of this enzyme in tumour cells constitute a protection mechanism against cell death induced by reactive oxygen species (ROS), and that by inhibiting this enzyme, the neoplastic cells become once more vulnerable to this outcome [55,56]. Our findings indicate that Cat levels were reduced in the tumour tissue and increased in the liver, both these results occurring simultaneously and due to the treatment with the *U. tomentosa* BHE extract. Indeed, these results establish quite opposite scenarios in both studied tissues; we may be witnessing a protective effect on the liver associated with a further exposure of the tumour cells to ROS-induced necrosis/apoptosis, especially by means other than the increase of lipid peroxidation (see below). Moreover, according to Valko *et al*. [58], the lower Cat activity induced by various tumours has been attributed to an increase in TNF-α level, which reduces hepatic Cat activity [58,59]. Interestingly, our results show that the BHE extract expressively reduced the TNF-α level on the liver homogenates of tumour-bearing rats. Such results may have contributed to a recovery of the hepatic Cat activity in these animals.

Treatment with the *U. tomentosa* BHE extract and its BuOH fraction successfully reduced the lipid peroxidation (LPO) rates in the tumour as well as in the liver. As we did not observe the aforementioned locale selectivity for this parameter, we can only assume that the substances responsible for that particular behaviour work upon somewhat restricted pathways. The reduction of LPO in the liver may indicate greater protection of the cell membrane integrity, as it is formed by poli-unsaturated fatty acids highly susceptible to oxidative stress-induced lipid peroxidation. This particular effect in the liver is further supported by the reduction observed in plasmatic AST in the individuals treated with both the BHE extract and its BuOH fraction, as comparing to control, bringing this parameter closer to baseline values. As this enzyme belongs in the intracellular medium of hepatocytes, high plasmatic levels commonly imply death and lysis of these cells or increase in its membrane permeability at the very least [60]. The unaltered ALT values, along with the differences among groups regarding AST values, suggest that the effects of *U. tomentosa* are not restricted to the liver, given that one major difference between both transaminases is that while ALT is restricted to the cytosol of hepatocytes, AST may be found in various other tissues, such as myocardium, skeletal muscle, kidneys, brain, pancreas, lungs and blood cells [61]. Regarding the reduction of LPO levels in the tumour as a result of treatment with the BHE extract and its BuOH fraction, it may be

explained by GSH consumption, as demonstrated by the lesser GSH levels in the tumour tissue of the subjects of these two groups.

The mensuration of the cytokine TNF-α in liver homogenates shows an anti-inflammatory activity of both the BHE extract and its BuOH fraction. Interestingly, however, we observed greater TNF-α reduction as a result of the former treatment, leading us to conclude that, regarding this parameter, the BHE extract is indeed more effective than its BuOH fraction. On the whole, reduction of this cytokine is bound to ameliorate some hallmarks of this tumour, such as cachexia [62]. While the same tendencies were observed in the tumour homogenates, no statistically significance was found upon comparison of the experimental groups.

Confirming the similarity in the plasma urea concentration among the groups, the hepatic production of urea did not differ between the groups. It seems that all alanine present in the liver of baseline rats, characterized by the absence of tumour, was converted in glucose, ammonia and lactate. Only a minimal hepatic production of ammonia and pyruvate was observed in those animals. However, the hepatic metabolism of alanine was quite modified by the presence of the tumour. Previous data on liver perfusion showed that the W256 tumour-bearing animals present significant alterations in a wide range of hepatic metabolic pathways, especially in ammoniagenesis, ureagenesis and gluconeogenesis [63]. Our data shows that treatment with *U. tomentosa* did not expressively modify the hepatic metabolism of alanine. However, the BHE extract-treated rats tended to produce less ammonia and glucose and more pyruvate, when compared to other tumour-bearing groups. Both pyruvate and lactate are products of the same enzyme (lactate dehydrogenase - LDH), and we have already reported that the W256 tumour reduces the plasmatic levels of this enzyme, while treatment with BHE extract ameliorates this reduction [19]. Thus, the BHE extract may be causing a shift in the LDH equilibrium towards pyruvate production at the hepatocytes. The most expressive metabolic influence of the BHE extract as compared to the BuOH fraction can be explained, partially at least, by the complex composition of BHE extract, with concomitant effects of stimulator and inhibitor compounds.

On the whole, our results support our initial hypothesis, which may be in part explained by the complex composition of BHE extract. Numerous substances seem to be acting synergically along with the oxindole alkaloids or even independently of them. More studies are required in order to evaluate the degree of participation of these and other substances in the mechanisms by which *U. tomentosa* exerts its anti-neoplastic effects.

Conclusions

This paper confirms the expressive anti-neoplastic, anti-oxidant and metabolic activity of *Uncaria tomentosa* preparations. Brought together, our results constitute evidence that the oxindole alkaloids present in this plant are not the sole substances responsible for its biological effects, at least when tested against the primary tumour of the Walker-256 lineage *in vivo*. Modulation of oxidative stress appears to be of utmost importance in thwarting the neoplastic process triggered by this tumour, which is probably achieved by means of a combined and synergic activity from different classes of chemical compounds existing in the brute hydroethanolic extract of this plant. The anti-neoplastic effects produced in this manner seem even more appealing when considering its anti-oxidant and metabolic effects in the liver.

Henceforth, we deem appropriate to perform new experiments, testing the BHE extract and its fractions against different neoplastic scenarios, both *in vitro* and *in vivo*. It would also be interesting to evaluate oxidative stress parameters in other tissues than the liver, in an attempt to further explore the apparent selectivity that *U. tomentosa* seems to exert over its locale of action, and thus achieve a better understanding of the overall therapeutic potential of this plant.

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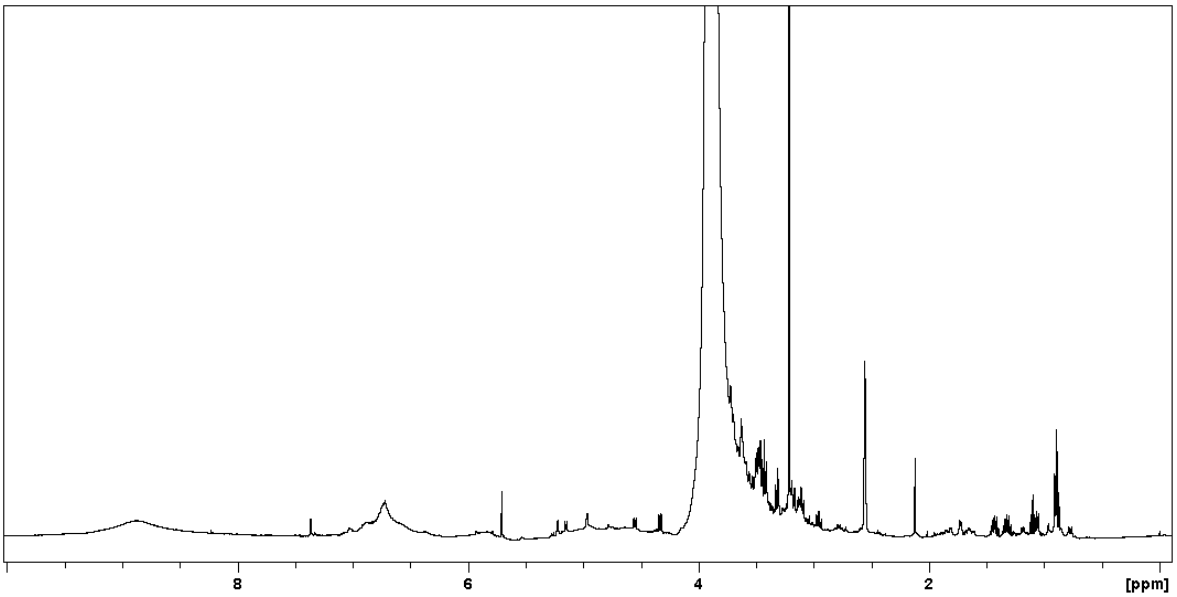
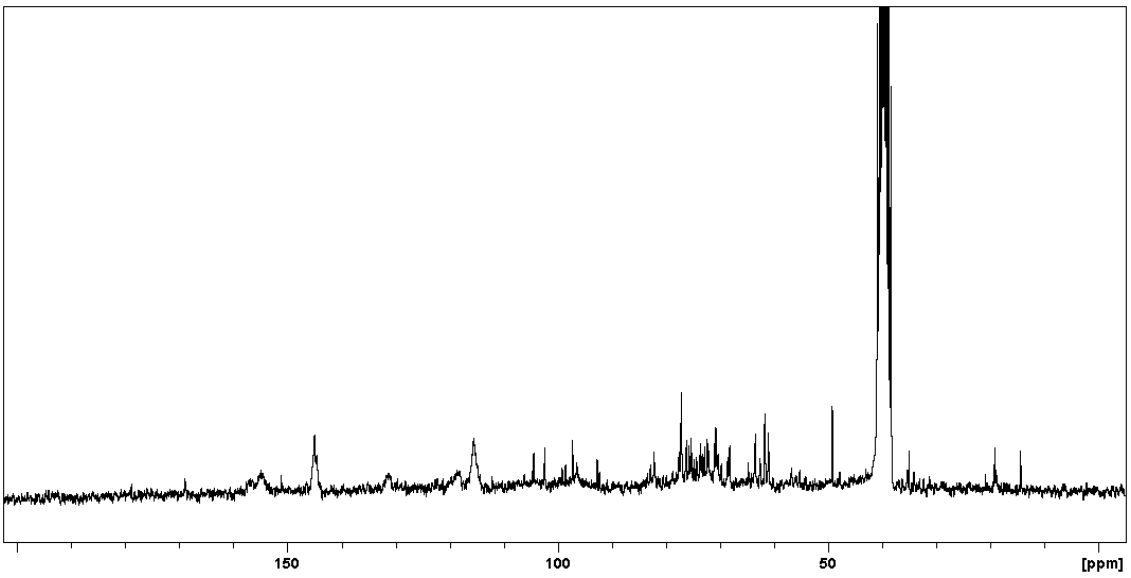
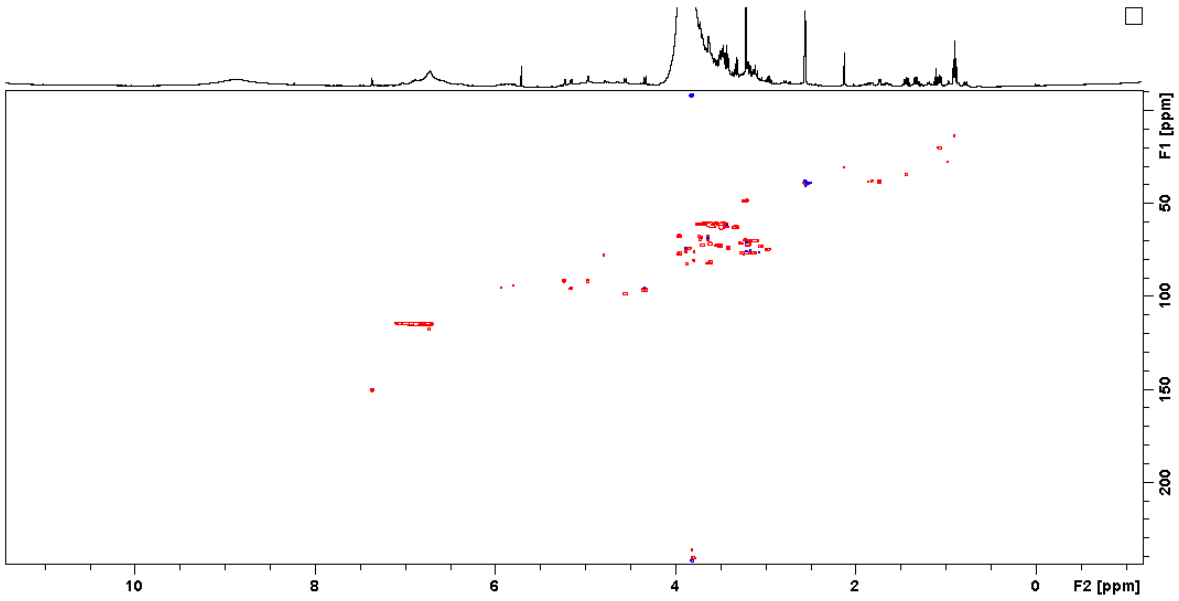
Supplementary Information



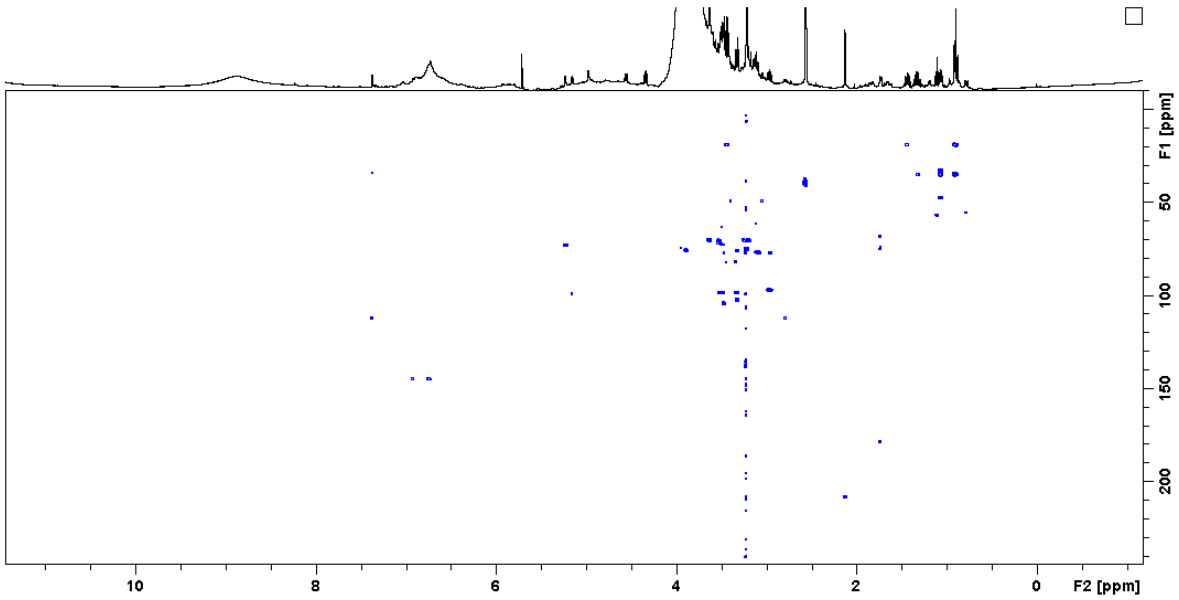
Supplementary Figure 1. ^{1}H NMR spectra of the BuOH fraction of *U. tomentosa* (400 MHz, DMSO-D_6).



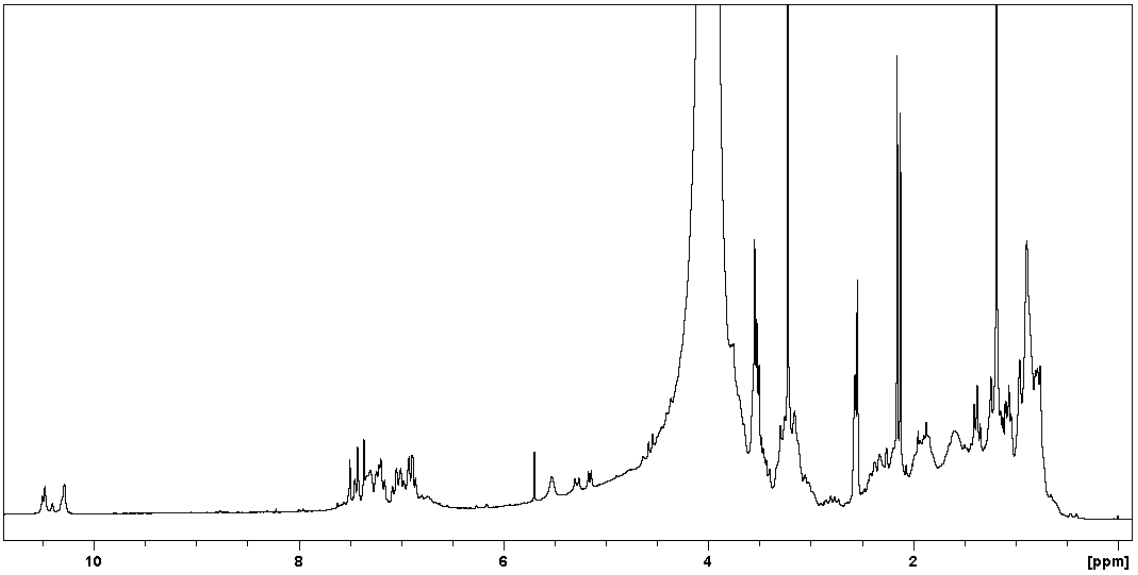
Supplementary Figure 2. ^{13}C NMR spectrum of the BuOH fraction of *U. tomentosa* (50 MHz, DMSO-D_6).



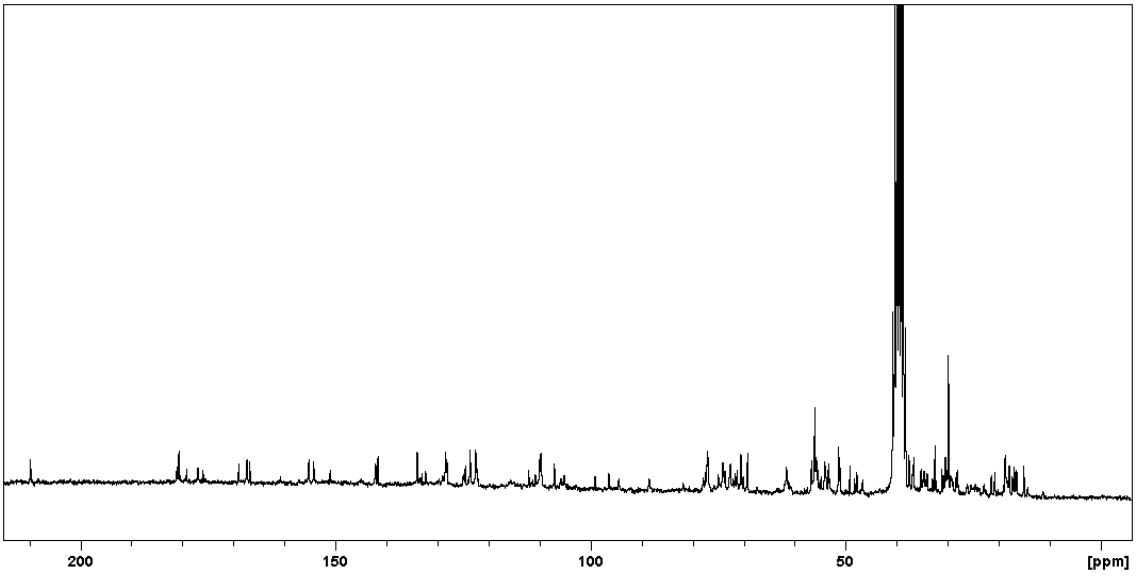
Supplementary Figure 3. HSQC spectra of the BuOH fraction of *U. tomentosa* (400 MHz, DMSO-D_6).



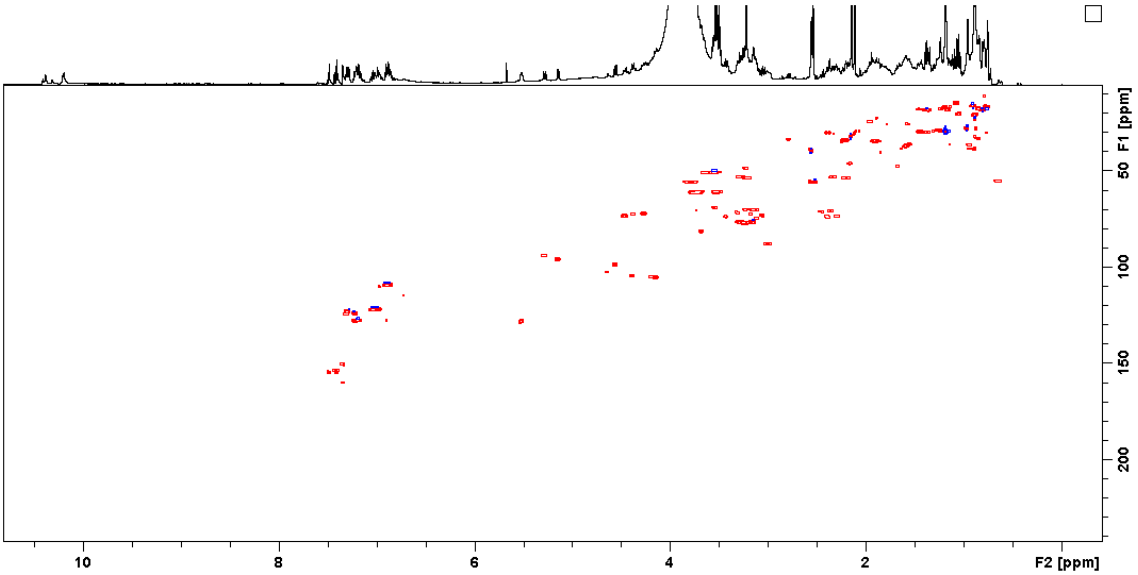
Supplementary Figure 4. HMBC spectra of the BuOH fraction of *U. tomentosa* (400 MHz, DMSO-D_6).



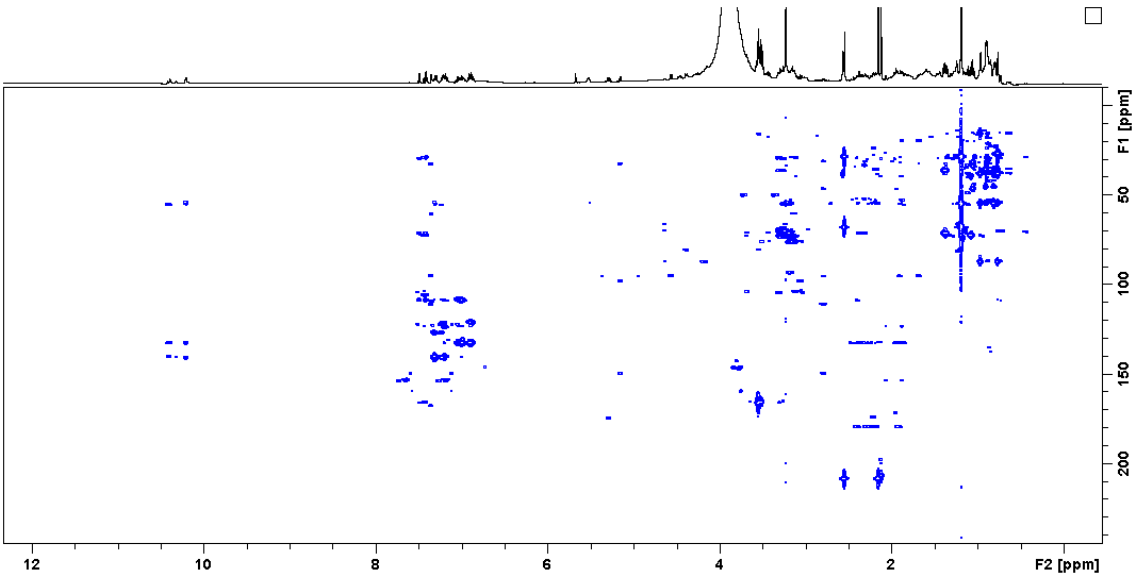
Supplementary Figure 5. ^{1}H NMR spectrum of the $CHCl_3$ fraction of *U. tomentosa* (200 MHz, DMSO-D_6).



Supplementary Figure 6. ^{13}C NMR spectrum of the $CHCl_3$ fraction of *U. tomentosa* (50 MHz, DMSO-D_6).



Supplementary Figure 7. HSQC spectra of the $CHCl_3$ fraction of *U. tomentosa* (400 MHz, DMSO-D_6).



Supplementary Figure 8. HMBC spectra of the $CHCl_3$ fraction of *U. tomentosa* (400 MHz, DMSO-D_6).

4. CONSIDERAÇÕES FINAIS

Diante dos resultados do presente trabalho, conclui-se que:

- A *U. tomentosa* possui intensa e interessante atividade anti-neoplásica;
- Os alcaloides oxindólicos pentacíclicos isolados não tiveram atividade no tumor primário deste modelo tumoral (Walker-256);
- A modulação do estresse oxidativo parece ser fundamental no mecanismo de ação da planta;
- Os efeitos de *U. tomentosa* provavelmente dependem da ação sinérgica de vários grupos de componentes;
- De maneira simultânea à atividade anti-neoplásica, a planta parece proteger tecidos vitais, como o fígado.

Perspectivas futuras:

- Testar o extrato BHE e suas frações em outros quadros neoplásicos, tanto *in vitro* quanto *in vivo;*
- Avaliar parâmetros de estresse oxidativo em outros tecidos que não o fígado;
- Mensurar e avaliar as possíveis metástases desenvolvidas pelo tumor, avaliando nelas os mesmos parâmetros do presente estudo.

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