UNIVERSIDADE FEDERAL DO PARANÁ

KAUÊ MARCEL DE OLIVEIRA

AVALIAÇÃO DOS EFEITOS ANTITUMORAIS E HEPÁTICOS DE POLISSACARÍDEOS EXTRAÍDOS DO TUCUM-DO-CERRADO (*Bactris setosa* Mart) EM CAMUNDONGOS

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

Orientadora: Profa. Dra. Alexandra Acco

CURITIBA
2022

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP) UNIVERSIDADE FEDERAL DO PARANÁ
SISTEMA DE BIBLIOTECAS – BIBLIOTECA DE CIÊNCIAS BIOLÓGICAS

Oliveira, Kauê Marcel de
Avaliação dos efeitos antitumorais e hepáticos de polissacarídeos extraídos do tucum-do-cerrado (*Bactris setosa* Mart) em camundongos / Kauê Marcel de Oliveira. – Curitiba, 2022.
1 recurso on-line : PDF.

Dissertação (Mestrado) – Universidade Federal do Paraná, Setor de Ciências Biológicas, Programa de Pós-Graduação emFarmacologia.
Orientadora: Profa. Dra. Alexandra Acco.

1. Antineoplásicos. 2. Arecaceae. 3. Polissacarídeos. I. Acco,Alexandra., 1972-. II. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Farmacologia. III. Título.

Bibliotecária: Giana Mara Seniski Silva CRB-9/1406

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIÊNCIAS BIOLÓGICAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO FARMACOLOGIA - 40001016038P0

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **KAUÊ MARCEL DE OLIVEIRA** intitulada: **Avaliação dos efeitos antitumorias e hepáticos de polissacarídeos extraídos do tucum-do-cerrado (*Bactris setosa* Mart) em camundongos**, sob orientação da Profa. Dra. ALEXANDRA ACCO, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 22 de Agosto de 2022.

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23/08/2022 15:49:04.0
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Esta dissertação tem os principais resultados apresentados na forma de artigo científico, sendo constituída, portanto, por introdução, um artigo científico e considerações finais. Este formato está de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná.

Dedico este trabalho aos meus pais, ao meu irmão, à minha avó, à minha madrinha e à minha prima por todo apoio, paciência, auxílio e compreensão comigo durante todo esse período.

AGRADECIMENTOS

Agradeço à minha mãe, que é meu exemplo de vida, por sempre acreditar em mim e me dar suporte mesmo quando eu estive perdido e confuso. Sem o seu amor, paciência e compreensão eu jamais teria conseguido. Obrigado por absolutamente tudo. Agradeço ao meu pai por sempre fazer eu enxergar que sou uma pessoa esforçada,mesmo quando achei que não era. Obrigado por me ensinar a ser uma pessoa com caráter e determinação.

Agradeço ao meu irmão, por ser meu melhor amigo e ser meu fã número um. Quem fala que sou o seu espelho, porém é você que se tornou o meu. Agradeço à minha prima por também ser minha melhor amiga e literalmente minha "irmã de outra mãe"; você foi essencial nessa minha jornada.

Agradeço à Mama, minha avó, que é nada menos que essencial em minha vida. Nasceu com o dom de ser a melhor avó que pode existir e educou seus netos de maneira única. Sou eternamente grato pela sua existência. Agradeço também à minha madrinha por sempre me fazer rir quando eu não tive vontade e nem forças, por todo o suporte e torcida.

Agradeço àquela que quando entrei no laboratório todos se referiam como "mãe científica" e eu não entendia muito bem, inclusive achava um tanto quanto exagerado. Hoje entendo que não é exagero intitular a minha orientadora, Professora Dra. Alexandra Acco, desta forma, é pouco. Você é minha inspiração profissional e pessoal. Muito obrigado por todos os ensinamentos, conselhos, conversas. Se um dia eu for 1% do que você é como "mãe científica", já estarei realizado.

Agradeço aos meus colegas e amigos do Laboratório de Farmacologia & Metabolismo, por deixarem sempre o ambiente agradável e trabalharmos em sintonia. Agradeço também aos amigos e professores dos demais laboratórios do Departamento pelas ajudas, colaborações e momentos únicos de descontração.

Agradeço às minhas colegas que viraram minhas amigas e exemplos: Carol, obrigado por mesmo longe sempre estar ao meu lado me auxiliando nos experimentos, me consolando e aconselhando na minha vida pessoal; Claudinha, obrigado pela paciência, por me mostrar que ter calma é a chave pra tudo e por me trazer de volta à vida real quando eu levemente me desesperava; Clau, obrigado por absolutamente tudo, por ser a pessoa que eu corro pedir conselho quando algo dá errado, que me ensinou praticamente tudo o que eu sei. Sem vocês eu não seria o aprendiz de cientista que sou hoje.

Agradeço a todos os colaboradores e co-autores deste trabalho. Vocês permitiram que todas as etapas fossem cumpridas.

Agradeço à CAPES e ao CNPq pelo auxílio financeiro.

RESUMO

Muitas plantas produzem compostos, tais como alcaloides, polifenóis, óleos essenciais e polissacarídeos, com efeitos farmacológicos em humanos e animais. O objetivo deste estudo foi investigar os efeitos antitumorais e metabólicos dos polissacarídeos que constituem as fibras alimentares solúveis do tucum-do-cerrado (*Bactris setosa*, TUC), utilizando o carcinoma de Ehrlich como modelo tumoral em camundongos. O modelo começou com a inoculação de 1 x 10^6 células de Ehrlich de forma subcutânea no membro pélvico direito dos animais. Camundongos fêmeas portadoras de tumores foram tratadas por via oral com 50 e 100 $mg.kg^{-1}$ de TUC ou veículo, uma vez por dia, ou com 1,5 $mg.kg^{-1}$ de metotrexato (MTX) via intraperitoneal, a cada 3 dias, ao longo de 21 dias. Ambas as doses de TUC foram capazes de reduzir o peso e o volume do tumor em comparação com o grupo veículo. No tecido tumoral dos grupos tratados, foram encontradas alterações nos marcadores de estresse oxidativo, com diminuição dos níveis de GSH e aumento dos níveis de LPO. Além disso, os biomarcadores de inflamação, NAG e NO, foram aumentados nos tecidos tumorais dos animais tratados com TUC. A análise histológica mostrou maior área de necrose e infiltração de leucócitosno microambiente tumoral dos grupos que receberam TUC. Os efeitos metabólicos dospolissacarídeos foram investigados pela mensuração de CYP total e glicogênio dos animais com tumor e pelo sistema de perfusão hepática *ex-vivo* em camundongos machos não portadores de tumor, em jejum, utilizando o lactato como precursor da gliconeogênese. Metabolicamente, as produções hepáticas de glicose e piruvato, o consumo de oxigênio hepático e a concentração total de CYP não foram modificadas pelo TUC. O glicogênio hepático, porém, mostrou-se reduzido em todos os grupos com tumor, entretanto, a maior dose de TUC foi capaz de recuperar parcialmente os níveis de glicogênio hepático dos animais. Assim, os polissacarídeos do tucum-do-cerrado apresentam efeitos antitumorais através da modulação do estresse oxidativo e da inflamação, sem induzir alterações metabólicas expressivas no fígado, o principal órgãoresponsável pelo metabolismo dos compostos orgânicos e xenobióticos.

Palavras-chave: câncer; carboidratos complexos, gliconeogênese; tucum-do-cerrado; inflamação; estresse oxidativo.

ABSTRACT

The plants produce several compounds, such as alkaloids, polyphenols, essential oils and polysaccharides, with pharmacological effects in humans and animals. The objective of this study was to investigate the antitumor and metabolic effects of the polysaccharides that constitute the soluble dietary fibers of tucum-do-cerrado (*Bactris setosa*, TUC), using Ehrlich carcinoma as a tumor model in mice. The model started with inoculation of 1 x 10^6 Ehrlich cells, subcutaneously, into the right pelvic limb of the animals. Tumor-bearing female mice were treated orally with 50 and 100 mg.kg^{-1} TUC or vehicle once daily, or with 1.5 mg.kg^{-1} methotrexate (MTX) via intraperitoneal, every 3 days, over 21 days. Both doses of TUCwere able to reduce tumor weight and volume compared to vehicle group. In the tumor tissue of the treated groups, changes in oxidative stress markers were found, with decreased GSH levels and increased LPO levels. In addition, the biomarkers of inflammation, NAG and NO, were increased in the tumor tissues of the TUC-treated animals. Histological analysis showed increased area of necrosis and leukocyte infiltration in the tumor microenvironment of the groups receiving TUC. The metabolic effects of polysaccharides were investigated by measuring total CYP and glycogen of thetumor-bearing animals, and by the *ex-vivo* liver perfusion system in fasted non-tumor- bearing male mice, using lactate as the precursor of gluconeogenesis. Metabolically, hepatic glucose and pyruvate productions, oxygen consumption and total CYP concentration were not modified by TUC. The hepatic glycogen dosage, however, was decreased in all tumor-bearing groups; however, the highest dose of TUC was able to recover partially the hepatic glycogen levels. Thus, the polysaccharides from tucum-do-cerrado present antitumor effects through modulation of oxidative stress and inflammation, without inducing expressive metabolic changes in the liver, the main organ responsible for the metabolism of organic compounds and xenobiotics.

Key words: cancer; complex carbohydrates; gluconeogenesis; tucum palm tree; inflammation; oxidative stress.

LISTA DE FIGURAS DO ARTIGO

LISTA DE TABELAS DO ARTIGO

LISTA DE ABREVIATURAS

ALT: Alanina aminotransferase

ANOVA: Análise estatística de variância

AST: Aspartato aminotransferase

Bax: Bcl-2 associada à proteína X

Bcl-2: Linfoma 2 de células-B

CAR-T: Células T de receptor de antígeno quimérico

DNA: Ácido desoxirribonucleico

EDTA: Ácido etilenodiamino

EFSA: *European Food Safety Authority*

GSH: Glutationa reduzida

GPx: Glutationa peroxidase

GST: Gulationa-S-transferase

HE: Hematoxilina e eosina

HIF-1: Fator de hipóxia induzível

IL-1β: Interleucina-1 beta

IL-6: Interleucina-6

IL- 8: Interleucina-8

IL-12: Interleucina-12

LPO: Peroxidação lipídica

mAB: Anticorpo monoclonal

MIF: Fator inibidor da migração de macrófagos

MLKL: Proteína quinase de domínio de linhagem mista

MPO: Mieloperoxidase

mRNA: Ácido ribonucleico mensageiro

MTX: Metotrexato

NAG: N-acetil-β-D-glicosaminidase

NaOAc: Acetato de sódio

NaOH: Hidróxido de sódio

NF-κB: Fator nuclear Kappa B

NO: Óxido nítrico

O2•-: Ânion superóxido

OH•: Radical hidroxila

OMS: Organização Mundial da Saúde

p53: Proteína 53

SOD: Superóxido dismutase

RO•: Radical alcoxil

ROO•: Radical peroxil

ROOH•: Radical hidroperoxil

ROS: Espécies reativas de oxigênio

TUC: Polissacarídeos do tucum-do-cerrado

VEGF: Fator de crescimento vascular epidérmico

SUMÁRIO

1 REVISÃO BIBLIOGRÁFICA

1.1 Câncer: definição e incidência

O câncer é um dos grandes problemas de saúde pública no mundo e está entre as quatro principais causas de morte prematura por doenças (antes dos 70 anos) na maioria dos países. O envelhecimento populacional, associado aos vários fatores que desencadeiam o câncer, como sedentarismos e alimentação inadequada, faz com que a prevalência e mortalidade desta doença continuem a aumentar (BRAY et al, 2018). Uma das recentes estimativas, de 2020, mostra que ocorreram no mundo 19,3 milhões de novos casos de câncer e 10 milhões de óbitos no ano mencionado (World Health Organization, 2022).

Wongtrakoongate (2015) descreveu o câncer como sendo uma doença maligna caracterizada por mutações de células normais do organismo, levando ao crescimento e diferenciação anormais dessas células de forma descontrolada. Ou seja, segundo o INCA (2018), a formação desses tumores malignos ocorre devido a eventos químicos, biológicos ou até mesmo físicos, que causam danos ao nível do genoma. Há diversas causas externas que acarretam tais danos, como por exemplo o tabagismo, bem como a fumaça do cigarro, exposição a produtos químicos voláteis e a radiações. Também há fatores internos, como desequilíbrios hormonais, mutações gênicas e condições imunológicas. Entretanto, de 80 a 90% dos casos de câncer estão associados a causas externas.

Essa transformação de células normais em anormais origina, então, um funcionamento autônomo do novo tecido formado. As principais características destas células se referem à perda de determinadas funções especializadas, associadas à aquisição de novas e marcantes propriedades biológicas, como por exemplo, a autossuficiência em sinais de crescimento, a insensibilidade aos sinais de inibição do crescimento, evasão de apoptose, ilimitado potencial replicativo, angiogênese sustentada, invasão de tecido e, por fim, metástase (ZAIDMAN et al., 2005).

1.2 Carcinogênese e seus moduladores

O processo de carcinogênese pelo qual um tumor maligno é formado segue três estágios: iniciação, promoção e progressão. É por meios dessas fases que genes específicos sofrem mutações e passam a desempenhar seus papéis de maneira errônea em nível celular. Alguns desses genes específicos são os supressores de tumor, os proto-oncogenes e os genes de reparo do DNA, que normalmente são responsáveis por codificar proteínas envolvidas no controle do ciclo celular, na transdução de sinal e regulação de transcrição (TSAO et al., 2004). Além de mutações em nível de genes supressores de tumor, tem-se essencial envolvimento dos proto-oncogenes, que normalmente codificam proteínas que estimulam a divisão celular, inibem diferenciação celular e impedem a morte celular. Quando ativados (mutados) são denominados oncogenes, que irão aumentar a produção das proteínas, conduzindo a um aumento na divisão celular, redução da diferenciação celular, e inibição de morte celular, favorecendo o crescimento do tumor (CHIAL, 2008). Obviamente, para que estas células mutadas continuem vivas, o tumor necessita ser nutrido com oxigênio, aminoácidos, glicose e lipídeos, por meio de vasos sanguíneos (AL-ZOUGHBI et al., 2014). Para tanto, ocorre angiogênese tumoral mediada por citocinas como o fator de necrose tumoral alfa (TNF-α), a interleucina-6 (IL-6) e a interleucina-1 beta (IL-1β), a qual ativa a enzima cicloxigenase 2 (COX-2), que consequentemente elevará a produção de prostaglandina E2 (PGE2) no tecido tumoral. A IL-1β e a PGE2 regulam os níveis de proteína HIF1α e ativam o fator de crescimento endotelial vascular (VEGF), numa reação que é principalmente mediada via fator nuclear kB (NF-kB). Esta cascata é indispensável para exemplificar o papel da inflamação no desenvolvimento de tumores, inclusive com papel essencial das enzimas N-acetilglucosaminidase (NAG) e mieloperoxidase (MPO) (AL-ZOUGHBI et al., 2014, FEITELSON et al., 2015), que, respectivamente, são indicadoras da presença e atividade de monócitos e neutrófilos no tecido tumoral.

Além da inflamação, o estresse oxidativo deve ser levado em consideração na gênese de neoplasias. O estresse oxidativo é o estado a que células ou tecidos ficam expostos quando as concentrações de espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) e as de antioxidantes estão em desequilíbrio. Podemos afirmar, então, que o estresse oxidativo tem papel fundamental para o desenvolvimento e progressão de tumores (MANTOVANI et al., 2004; RANINGA et al., 2014). Em condições normais os

antioxidantes inibem ou atrasam os processos de oxidação pelos EROs, que podem ser radicais livres como ânion superóxido (O2–•), o hidroxil (OH•), o alcoxil (RO•), o peroxil (ROO•) e o hidroperoxil (ROOH•), bem como não radicais, como o peróxido de hidrogênio (H_2O_2). De outro lado desta balança do estado redox estão os antioxidantes não-enzimáticas, como as vitaminas C e E, e moléculas enzimáticas como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR) e a glicose 6-fosfato desidrogenase (G6PD) (DICHI et al., 2014). Devido à participação na gênese tumoral, e pelo estado redox estar desbalanceado em células cancerosas, é um campo na área farmacológica antitumoral que vem sendo explorado.

Outro fator chave quando se discute o câncer é a apoptose, que é a morte celular programada e fisiologicamente normal. Ela é a responsável por eliminar células anormais e mutadas para, desta forma, suprimir o crescimento tumoral, por exemplo. A apoptose envolve diversos sinalizadores e duas vias são conhecidas, uma intrínseca (via mitocôndria) e uma extrínseca (via receptores). Ambas são responsáveis por ativar as caspases, que levam à desintegração da cromatina e à fragmentação nuclear, seguido da morte da célula. Entretanto, já foi descrito que células neoplásicas podem desenvolver mecanismos que inibem a apoptose, o que se resume numa forma de proteção do tumor contra morte celular (ZHAO et al., 2013; KOFF et al., 2015). A família de genes Bcl-2 (linfoma 2 de células-B) é um exemplo de família genônica que modula a apoptose. Ela é composta de proteínas anti-apoptóticas, como Bcl-2 e Bcl-xL e pró-apoptóticas, como Bax, Bad e Bid (PAROLIN & REASON, 2001; ZIMMER, 2007). Uma vez que o estresse oxidativo induz a ativação das caspases, que ativam a família das proteínas Bcl-2 e modulam as quinases para induzir a morte celular, fica claro a intersecção entre estresse oxidativo, apoptose e câncer (RANINGA et al., 2014).

1.3 Tratamentos antineoplásicos

As modalidades existentes atualmente para tratamento dos mais variados tipos de cânceres envolvem cirurgias, quimioterapia, radioterapia, imunoterapia, transplante de medula óssea e terapia alvo-específica, onde se procura direcionar moléculas de maneira efetiva e, de fato, específicas (DEMBIC, 2020).

O tratamento quimioterápico, no entanto, ainda é o mais comum, mesmo que geralmente esteja associado a outros métodos. Dentre as classes de quimioterápicos utilizadas estão os alquilantes, os antimetabólitos, os antibióticos antitumorais, os inibidores mitóticos, dentre outros. Os agentes alquilantes são efetivos ao combate de inúmeras formas de câncer, por se ligarem ao DNA e impedirem a sua replicação. São exemplos de alquilantes a ciclofosfamida, o bussulfam, as nitrosuréias, a cisplatina e a ifosfamida. Os antimetabólitos impedem a multiplicação e a função das células normais, pois inibem a biossíntese dos componentes essenciais do DNA e RNA. Os principais exemplos são: metotrexato, 5-fluorouracil, 6-mercaptopurina e a pentostatina. Já os antibióticos antitumorais atuam inibindo a produção de ácidos nucleicos ou de proteínas e, consequentemente, aumentam a produção de radicais livres, causando a morte celular. Os principais compostos são: a mitomicina C, actinomicina D, daunorrubicina e doxorrubicina. Ainda, os inibidores mitóticos interrompem a divisão celular, pois paralisam a mitose na metáfase. Neste grupo estão incluídos a vincristina, vimblastina e vindesina, além dos taxanos. Porém, essa gama de drogas antineoplásicas acarreta inúmeros efeitos colaterais, tendo em vista seus vários efeitos citotóxicos (PELLACANI & ELEFTHERIOU, 2020). Além disso, esses agentes quimioterápicos acabam tendo muita resistência à remissão total do tumor, por motivos farmacocinéticos, bioquímicos ou até pela expressão de proteínas de múltipla resistência a drogas (MERCK, 2015). Assim, as terapias utilizando anticorpos monoclonais (mAB) sofreram rápida ascensão no tratamento contra diversos tipos de câncer, como, por exemplo, o mAB anti-CD33 conjugado com toxina (gemtuzumabe-ozogamicina), aprovado para tratar leucemia mielóide aguda CD33 positiva; e a combinação de trastuzumabe (anti-HER2), pertuzumabe (anti-HER2) e um taxano, que é a terapia de primeira linha para pacientes com câncer de mama metastático HER2-positivo (NADER-MARTA, MARTINS-BRANCO, DE AZAMBUJA, 2022). Ainda, a utilização de terapias envolvendo as células CAR-T (células T de receptor de antígeno quimérico) também têm se mostrado boas alternativas, como é o caso do brexucabtagene autoleucel, uma imunoterapia celular que visa o receptor CD19 para pacientes com resistência e recidivas com linfoma (DEMBIC, 2020; RUPPEL et al., 2022; LARSON & MAUS, 2021). Estas novas terapias, entretanto, têm custo elevado, limitando sua utilização como primeira escolha em diversos países. O preço médio dos medicamentos para câncer dobrou na última

década, e a insustentabilidade dos preços dos medicamentos é especialmente preocupante em oncologia e hematologia. Um levantamento mostrou que o preço anual das terapias mAb é cerca de US$ 100.000,00 mais alto em oncologia e hematologia do que em outras doenças (HERNANDEZ et al., 2018). Isto reforça que a busca por novas terapias, eficazes e acessíveis economicamente, é necessária.

1.4 Polissacarídeos

Continuamente a ciência busca novas moléculas com potenciais farmacológicos, dentre elas, as que tenham propriedades antitumorais. Os polissacarídeos vêm aparecendo com frequência na lista de promissoras moléculas com atividades não só antineoplásicas, mas também anti-inflamatórias, imunomoduladoras e antioxidantes (LIU et al., 2015; YU et al., 2018). Estas moléculas são, basicamente, polímeros de resíduos de monossacarídeos unidos por ligações glicosídicas (ZONG et al., 2012). Polissacarídeos com vários e diferentes grupos funcionais em sua estrutura (grupos hidroxila, amino e ácido carboxílico) podem ainda ser modificados para síntese de adjuvantes de vacinas e carreadores de drogas, por exemplo. É certo, também, que a existência destes grupamentos químicos em suas moléculas permite a característica de serem bastante solúveis em água e de não se apresentarem de maneira tão viscosa, apesar da viscosidade ser uma característica importante em outras áreas, como a alimentícia (WEN et al., 2014).

Estudos envolvendo polissacarídeos extraídos de plantas ou outras fontes, como algas ou animais, já são comuns no meio científico, o que é muito interessante. Plantas variadas utilizadas na Medicina Tradicional Chinesa tiveram seus polissacarídeos extraídos e suas habilidades em tratar doenças foram avaliadas. Os resultados foram interessantes e, segundo o grupo de pesquisa responsável pelo estudo, as aplicações dos polissacarídeos na clínica têm grande potencial (CHEN et al., 2020). Outros estudos recentes, desta vez no nosso grupo, mostraram os efeitos antitumorais *in vivo* da fração solúvel de polissacarídeos extraídos de vinho tinto da uva Cabernet Franc em modelo de carcinossarcoma Walker-256 (STIPP et al., 2016), e de polissacarídeos do pimentão verde (ADAMI et al., 2018; ADAMI et al., 2020) e do jambo (TAMIELLO et al., 2018) em modelo de Ehrlich em camundongos, através de diferentes mecanismos celulares.

Nosso enfoque neste trabalho é o estudo dos polissacarídeos extraídos do tucum-do-cerrado (*Bactris setosa* Mart), uma fruta nativa nacional. A caracterização química dos polissacarídeos extraídos desta fruta mostrou que os compostos majoritários são a glucomanana acetilada e a glucuronoarabinoxilana. A glucomanana tem sido destaque em estudos que relatam sua atividade anticancerígena, porém os mais recentes utilizam tal polissacarídeo extraído de uma planta utilizada na Medicina Tradicional Chinesa, o Konjac (*Amorphophallus konjac*). Este polissacarídeo demonstrou ser eficaz no tratamento da diabetes, aterosclerose e câncer (LI et al., 2019), e foi também capaz de reverter a resistência a múltiplas drogas *in vivo* nas células HepG2/5-FU, suprimindo a sinalização da via AKT e aumentando a expressão de p53 (CHEN et al., 2020). O outro polissacarídeo presente no tucum, a glucuronoarabinoxilana, também teve sua atividade antineoplásica descrita, tanto individualmente quanto associada à radioterapia. Neste caso, sua origem foi o farelo de arroz comercial (EL-DIN et al., 2019). LOU et al. (2022) mostraram que estes polissacarídeos extraídos do farelo de arroz eram capazes também de proteger ratos da obesidade frente a uma dieta rica em gordura e de inflamação metabólica através da modulação da microbiota intestinal dos animais. O consumo de arabinoxilanas, inclusive, já foi autorizado pela agência reguladora europeia (*European Food Safety Authority* - EFSA) como um composto que reduz glicemia pós-prandial (KELLOW & WALKER, 2018). Em nosso trabalho, porém, a origem dos polissacarídeos é distinta, a partir do tucum, sobre o qual há poucos estudos de atividades biológicas. O tucum-do-cerrado (*Bactris setosa* Mart) é uma fruta do bioma cerrado brasileiro, bastante importante para alimentação nessa região do país, consumido na maioria das vezes *in natura* ou processado em forma de sucos e geleias. Sua polpa quando ainda verde, tem sabor muito azedo, o qual é substituído para adocicado, quando maduro (SILVA & FONSECA, 2016). Vale destacar que estudos com a combinação de glucomanana acetilada e glucuronoarabinoxilana encontrada na fração de polissacarídeos do tucum, utilizada neste trabalho, são escassos na literatura.

1.5 Tumor de Ehrlich

O tumor de Ehrlich tem origem epitelial e é uma neoplasia maligna. Foi descoberto em 1886 por Paul Ehrlich, através do aparecimento espontâneo no tecido mamário de camundongos fêmeas. No entanto, foi apenas em 1905, que os

pesquisadores Ehrlich e Apolant testaram o tumor experimentalmente, pelo transplante de tecidos tumorais de camundongo para camundongo por via subcutânea (OZASLAN et al., 2011). Em 1932, os pesquisadores Loewenthal e Jahn desenvolveram uma variante especial do carcinoma de Ehrlich. Eles observaram que após a inoculação de uma suspensão de células carcinogênicas de Ehrlich na cavidade peritoneal de camundongos, não só obtiveram tumores na forma sólida, mas também um fluido ascítico que continha um grande número de células neoplásicas, sendo facilmente transmissível a outros animais. Após esta descoberta, esse modelo tumoral passou a ser conhecido como "tumor ascítico de Ehrlich" (KLEIN, 1950). A partir de então, o tumor ascítico de Ehrlich vem sendo amplamente estudado com diferentes propósitos, visto que, é facilmente transplantável in vivo (MIRANDA-VILELA et al., 2011). Quando a inoculação ocorre pela via intraperitoneal de camundongos o tumor de Ehrlich cresce na forma ascítica, aumentando a agressividade tumoral através de passagens repetidas; e quando é inoculado por via subcutânea desenvolve-se na forma de "tumor sólido de Ehrlich". As principais características deste tumor são: origem hiperdiplóide, de rápido crescimento denotando elevado grau de malignidade, elevada capacidade transplantável, não regride e permite avaliação *in vivo* mais curta (KLEIN, 1950; OZASLAN et al., 2011).

Este modelo experimental de neoplasia já está padronizado no Laboratório de Farmacologia & Metabolismo da UFPR, visto que, além das características supracitadas, possui a vantagem da utilização em camundongos, que requerem menor quantidade de fármacos/compostos administrados em relação a ratos. Desta maneira, pretende-se avaliar o efeito antitumoral dos polissacarídeos do tucum-do-cerrado utilizando técnicas farmacológicas e bioquímicas em modelo de tumor *in vivo*, bem como investigar os possíveis mecanismos de ação envolvidos. Ainda, investigar os efeitos hepáticos dos polissacarídeos, considerando que o fígado é o órgão integrador do metabolismo e fundamental na biotransformação de xenobióticos. A hipótese deste trabalho é que os polissacarídeos do tucum possuem efeito antitumoral contra o tumor de Ehrlich e que não alteram a função hepática em camundongos.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a possível atividade antitumoral de polissacarídeos extraídos do tucum-do-cerrado (TUC, glucuronoarabinoxilana e glucomanana) em modelo de carcinoma sólido de Ehrlich em camundongos, bem como os possíveis mecanismos celulares envolvidos.

2.2 Objetivos específicos

a) Avaliar o crescimento tumoral através da medida do volume e peso tumoral em camundongos tratados e não tratados com os polissacarídeos;

b) Investigar alterações hematológicas diante do tumor e dos tratamentos, pela realização de hemograma;

c) Investigar os possíveis mecanismos de ação antitumoral dos polissacarídeos, avaliando algumas vias de desenvolvimento tumoral:

(I) Estresse oxidativo: através da determinação dos níveis ou atividade do GSH, SOD, LPO e proteínas;

(II) Inflamação: avaliando a atividade da mieloperoxidase (MPO) e N-acetilglucosaminidase (NAG), bem como o nível de óxido nítrico (NO) no tecido tumoral;

(III) Necrose e apoptose: através de realização de análise histológica de fragmentos tumorais no intuito de identificar áreas de necrose e de infiltrados inflamatórios, e células em apoptose.

d) Investigar o comprometimento hepático e metabólico diante do tumor e dos tratamentos, através de parâmetros de bioquímica plasmática e de estresse oxidativo hepático, além dos níveis de CYP total e glicogênio;

e) Investigar os efeitos metabólicos diretos do TUC em animais hígidos, avaliando a via de gliconeogênese a partir do lactato em perfusão hepática.

3 ARTIGO CIENTÍFICO

Polysaccharides extracted from tucum-do-cerrado fruits (*Bactris setosa* Mart) have antineoplastic effects in mice without interfering with hepatic metabolism

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ABSTRACT

The purpose of this study was to investigate the antitumor and metabolic effects of polysaccharides that constitute the soluble dietary fibers from tucum (*Bactris setosa*, TUC), a Brazilian native fruit, using the Ehrlich carcinoma as the tumor model in mice. Tumor-bearing female mice were orally treated with 50 and 100 $mg.kg^{-1}$ of TUC or vehicle, once a day, or with 1.5 $mg.kg^{-1}$ methotrexate (MTX) via i.p., every 3 days, along 21 days. Both doses of TUC were able to reduce tumor weight and volume compared to the untreated group. In the tumor tissue of the treated groups, alterations in oxidative stress markers were found, with decreased glutathione (GSH) levels and increased lipoperoxidation (LPO) levels. Also, both biomarkers of inflammation, N-acetylglucosaminidase (NAG) and nitric oxide (NO), were increased in the tumor tissues. The histological analysis showed necrosis and leukocytes infiltration in the tumor microenvironment of TUC groups. The metabolic effects of TUC were investigated by measurement of total cytochrome P (CYP) and glycogen in tumor-bearing mice, and by an *ex vivo* liver perfusion system on non-bearing tumor male mice, using lactate as gluconeogenic precursor. Metabolically, the hepatic glucose and pyruvate productions, oxygen uptake, and the total CYP concentration were not modified by TUC. Thus, tucum-do-cerrado polysaccharides have antitumor effects through the modulation of oxidative stress and inflammation, without induce metabolic changes in liver, the main organ responsible for the metabolism of organic and xenobiotic compounds.

Key words: cancer; complex carbohydrates; gluconeogenesis; tucum palm tree; inflammation; oxidative stress.

1 INTRODUCTION

Cancer is one of the world's major public health problems. The incidence of cancer has been increasing, as well as its mortality, and in 2021 the World Health Organization announced breast cancer as the most prevalent in the world, surpassing the lung cancer. The pathophysiology of different neoplasms can be similar, characterized by differentiated, abnormal and uncontrollable growth of cells, which multiply rapidly (WONGTRAKOONGATE, 2015). The process of carcinogenesis can occur in the most varied ways, that is, through the expression of various specific genes that have mutated, for example, tumor suppressor genes, proto-oncogenes, and DNA repair genes (TSAO et al., 2004). One of the most evident factors arising from environments provided by tumor cells is the oxidative stress, defined as an imbalance between the amount of reactive species and antioxidants, resulting in a toxic environment for the cell and favoring the disease progression (RANINGA et al., 2014; MANTOVANI et al., 2004). Furthermore, the role of reactive oxygen species (ROS) in the tumor microenvironment is linked to mechanisms of cell death, angiogenesis, tumor cell migration, and metastasis (GLASAUER & CHANDEL, 2014), having dual effects depending on the tumor development phase.

The classic treatments for cancer involve surgery, chemotherapy, radiotherapy, immunotherapy, hormonotherapy, and bone marrow transplantation, isolated or in combination. Nowadays also the target therapy has been applied, which seeks to reach specific molecular components. However, in several countries the antineoplastic chemotherapy continues to be the most common cancer treatment, despite their cytotoxic and side effects (PELLACANI & ELEFTHERIOU, 2020; DEMBIC, 2020). From this reason, the search for new molecules that are effective and non-toxic for the treatment of this disease becomes a major challenge. Among molecules that have been studied in this area are polysaccharides. Currently, these molecules have been shown extensive pharmacological actions, such as antitumor, anti-inflammatory, and antimutation (LIU et al., 2015; YU et al., 2018). Arabinoxylans extracted from various sources are effective as antioxidant, immunomodulatory, antitumor, and anti-obesity agents. LOU et al. (2022) showed that these polysaccharides extracted from rice bran were able to protect mice from obesity induced by high fat diet and from metabolic inflammation, by

modulating the gut microbiota. The chemopreventive role in liver cancer, as well as its antitumor activity associated with radiotherapy, also have been described (EL-DIN et al., 2020). Another promising polysaccharide is glucomannan. Extracted from Konjac, coming from Traditional Chinese Medicine, this polysaccharide has been shown to be effective in the treatment of diabetes, atherosclerosis, and cancer (LI et al., 2019) and was also able to reverse multidrug resistance in HepG2/5-FU cells (CHEN et al., 2020). Other recent studies showed the *in vivo* antitumor effects of soluble fraction of polysaccharide extracted from Cabernet Franc red wine in Walker-256 carcinosarcoma model (STIPP et al., 2016), and of polysaccharides from green pepper (ADAMI et al., 2018; ADAMI et al., 2020) and jambo (TAMIELLO et al., 2018) in Ehrlich mouse model, through different cellular mechanisms. Since several polysaccharides possess antineoplastic activity, they emerge as alternative and adjuvant treatment for cancer.

Therefore, the aim of this work was to characterize and test the *in vivo* antineoplastic activity of polysaccharides extracted from the edible fruit of tucum-do-cerrado (*Bactris setosa* Mart, TUC), since studies of polysaccharides extracted from this source had not yet been carried out. This fruit, also known as ticum, a palm tree that belongs to the family of *Aceraceae*, grows in the Brazilian 'Cerrado' biome (NCBI:txid1472304; LORENZI et al., 2006; WORLD CHECKLIST OF MONOCOTYLEDONS, 2004). The possible TUC antitumor mechanisms in mice submitted to the Ehrlich carcinoma model, which mimics the hormone-positive woman breast cancer, was investigated, as well as the TUC influence on hepatic metabolism, since the liver is the main organ involved in the biotransformation of xenobiotics.

2 MATERIAL AND METHODS

2.1 CHEMICALS

Bovine serum albumin, 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), glutathione reductase, NADPH, xylenol orange, K2HPO4, KH2PO4, 1 M Tris, 5 mM ethylenediaminetetraacetic acid (EDTA), TRIS HCl, sodium nitrite, tetramethylbenzidine (TMB), dimethylsulfoxide (DMSO), D_2O, $NaBH_4$, heat-stable *a*-amylase, protease, and amyloglucosidase, standard dextrans (72.2 kDa, 40.2 kDa, 17.2

kDa, 9.4 kDa and 5kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue, 1-chloro-2,4-dinitrobenzene (CDNB), pyrogallol, ethanol, methanol, ferrous ammonium sulfate, hydrogen peroxide, trichloroacetic acid, formaldehyde, acetic acid, ascorbic acid, N,N-dimethylformamide, formaldehyde, citric acid, sodium acetate, sodium chloride, potassium chloride, sodium phosphate, dibasic sodium phosphate, monobasic potassium phosphate, dibasic potassium phosphate, hematoxylin, eosin, sulphanilamide, phosphoric acid, naphthylethylenediamine, Tween 20, sulfuric acid and Triton X-100 were obtained from Vetec (Rio de Janeiro, Brazil). The Bradford Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). ALT (alanine transaminase), AST (aspartate transaminase), creatinine, and total protein assays kits was purchased from Bioclin Quibasa (Belo Horizonte, MG, Brazil).

2.2 BOTANIC MATERIAL AND POLYSACCHARIDE EXTRACTION AND CHARACTERIZATION

2.2.1 Preparation of the fruit pulp

Ripe *Bactris setosa* fruits (tucum-do-cerrado) were collected in Corumbá city (February/2017, state of Mato Grosso do Sul, at 18 34 35.6 S/57 1 5.6 W) and deposited at Federal University of Mato Grosso do Sul Herbarium under number CGMS 48441. Besides, the research project was registered at the SisGen under n° A23EE4B and A3F60F0.

Fruits were cleaned, frozen and manually pulped. Pulp (together with the peel) was freeze-dried (Liobras® - Lp1010 equipment) for 72 h, homogenized in a blender and sieved in through a granulometric sieve (24 mesh). The tucum flour was stored in sterile packages protected from light for further analysis.

2.2.2 Extraction of soluble dietary fibers

Tucum flour was defatted with hexane (1:7, w/v, 2x), air dried and submitted to the standard enzymatic-gravimetric method AOAC official (Method 991.43) to obtain soluble and insoluble dietary fibers. Briefly, samples undergo sequential enzymatic digestion by heat-stable *a*-amylase, protease, and amyloglucosidase to remove starch and protein. The enzymatic treatments were performed in a heated water bath with

temperature adjusted for each enzyme, under constant stirring. Samples were suspended in water (1:24, w:v), and heated until 90°C. Thermostable *a*-amylase was added (5 units/g of sample) and samples were treated for 3 hours. The suspension was cooled to 60 °C, amyloglucosidase (1.3 units/g of sample) was added and remained for 1 hour. At the end of this treatment, the Lugol's iodine test was performed to confirm the absence of starch. Then, pH was adjusted to 7.0 with 10% NaOH solution and protease ($\leqslant$1 unit/g of sample) was added to the suspension and incubated for 1 hour.

The enzymatically-treated suspensions were centrifuged (5000 rpm/25 min) and supernatant (containing soluble dietary fiber- TUC fraction) was separated from the precipitated (containing insoluble dietary fiber). Samples were then submitted to dialysis against tap water at room temperature, for 48 h, in 12-14 kDa size exclusion membranes (Spectra Por®). Each material was freeze-dried, and yields were determined and expressed as a percentage based on the weight of dry tucum flour (20 g).

2.2.3 Monosaccharide composition

Neutral monosaccharide components of the TUC fraction were determined after hydrolysis with 2 M TFA (8 h/100°C), followed by conversion to alditol acetates with successive $NaBH_4$ reduction and acetylation with Ac_2O-pyridine (1:1, v/v, 1mL) at 100°C for 30 min. These were analyzed trough GC–MS using a Varian gas chromatograph and mass spectrometer, with He as carrier gas. A capillary column of DB-1, held at 100°C during injection for 3 min, then programmed at 10°C/min to 250°C and held at this constant temperature for 24 min was used for the quantitative analysis.

Uronic acid contents were determined spectrophotometrically using the modified *m*-hydroxybiphenyl method (FILISETTI-COZZI & CARPITA, 1991).

2.2.4 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR spectrum of TUC fraction was acquired at 70°C on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{1}H at 400.13 MHz and ^{13}C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. The samples were acquired in D_2O with chemical shifts expressed as δ PPM, using the $^{13}C/^{1}H$

resonances of CH_3 groups of acetone (δ_C 30.2/δ_H 2.22) as internal reference. All pulse programs were supplied by Bruker.

2.2.5 Size exclusion chromatography

High performance size exclusion chromatography (HPSEC) was applied to access the homogeneity and relative molecular weight (M_w) of soluble polysaccharides of tucum (TUC). The samples were dissolved in the mobile phase at a concentration of 1 mg/mL, filtered in 0.22 μm membrane (Millipore) and analysed on a Waters chromatograph equipped with four Ultrahydrogel columns connected in series (2000, 500, 250, 120; with size exclusion of 7×10^6 Da, 4×10^5 Da, 8×10^4 Da and 5×10^3 Da; Milford, MA, USA), coupled to a Waters 2410 (Milford, MA, USA) differential refractometer (RI) detector. The mobile phase used was 0.1 mol/L sodium nitrite containing 0.2 $g.L^{-1}$ sodium azide at a flow rate of 0.6 $mL.min^{-1}$ and at 25°C. To calculate the molecular weight, standard dextrans (72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa and 5kDa, from Sigma) were employed to obtain the calibration curve.

2.3 ANIMAL MODEL AND EHRLICH TUMOR CELLS INOCULATION

All experiments were approved by the Ethics Committee of Animal Experimentation. Female (CEUA/UFPR nº 1374) and male (CEUA/UEM nº 8309170117) Swiss mice (*Mus musculus*) weighing from 25 to 30 g were used. The animals were allocated in groups of 8 in cages with environmental enrichment to maintain animal welfare, using igloos to form nests and rolls of paper to chew. Along the experiments the animals had free access to water and feed (Nuvilab®, Colombo, Brazil), the room temperature was controlled at 22±2°C, and the light/dark cycle of 12/12h was applied.

To make the frozen Ehrlich cells viable, it was used the method of weekly passages by intraperitoneal injections of 1 x 10^6 cells/mouse. The cells were collected aseptically in 1 mL of phosphate buffered saline (PBS; 16.5 mM phosphate, 137 mM NaCl, and 2.7 mM KCl) at pH 7.4 and solution of ethylenediaminetetraacetic acid (EDTA) (0.5 M, pH 8.0). The ascitic form of the tumor occurs 4 to 7 days after intraperitoneal injections, and after 4 or 5 passages the cell viability was calculated using the trypan

blue exclusion method in a Neubauer chamber (MARTINS et al., 2015; VICENTINO, CONSTANTIN, BRACHT, & YAMAMOTO, 2002). Achieving cell viability > 95%, the amount of 1 x 10^6 cells/animals was inoculated subcutaneously into the right pelvic limb of the mice, to induce the Ehrlich solid tumor.

2.4 EXPERIMENTAL DESIGN

Oral treatment by gavage started on the first day after inoculation of Ehrlich cells in the subcutaneous tissue and lasted until day 21, daily. The doses of 50 mg.kg^{-1} and 100 mg.kg^{-1} were based on previous studies with polysaccharides (ADAMI et al., 2018; 2020). The groups (n=8) were divided as follows: G1 (Naive, animals without tumor and treated orally with vehicle); G2 (Vehicle, animals with tumor and treated with p.o. vehicle); G3 (TUC50, animals with tumor and treated p.o, with the lowest dose of Tucum polysaccharides, 50 mg.kg^{-1}); G4 (TUC100, animals with tumor and treated p.o. with the highest dose of Tucum polysaccharides, 100 mg.kg^{-1}); G5 (Positive control, animals with tumor and treated intraperitoneally every 3 days with the antineoplastic drug Methotrexate, at a dose of 1.5 mg.kg^{-1}) (Fig 1). Tucum polysaccharides were dissolved in distilled water every day before administration. Methotrexate was dissolved in 0.9% saline solution every day of administration.

From the 7th day of treatment, the tumor volume was measured using a digital pachymeter, obtaining the smallest and largest diameter of the tumor. The volume (*V*) was calculated by the following formula (MISHRA et al., 2018):
$V(cm^3) = L \text{ x } (W^2) \text{ x } 0.52$, where *W*: Smallest tumor diameter (cm), *L*: Largest tumor diameter (cm).

The tumor weight was also recorded at the end of treatment. At the end of the 21st day of treatment, the animals were fasted for 12 hours with free access to water and then anesthetized with ketamine hydrochloride (100 mg.kg^{-1}) and xylazine hydrochloride (10 mg.kg^{-1}) for collection of biological material. Blood was collected from the inferior cava vein for hematological and biochemical analysis. Fragments of tumors and livers were separated and introduced in formalin solution for histological analysis, and the rest were immediately frozen in liquid nitrogen and stored in a -80°C freezer for

further assays. Euthanasia was performed under anesthesia by a puncture of the diaphragm to induce respiratory arrest.

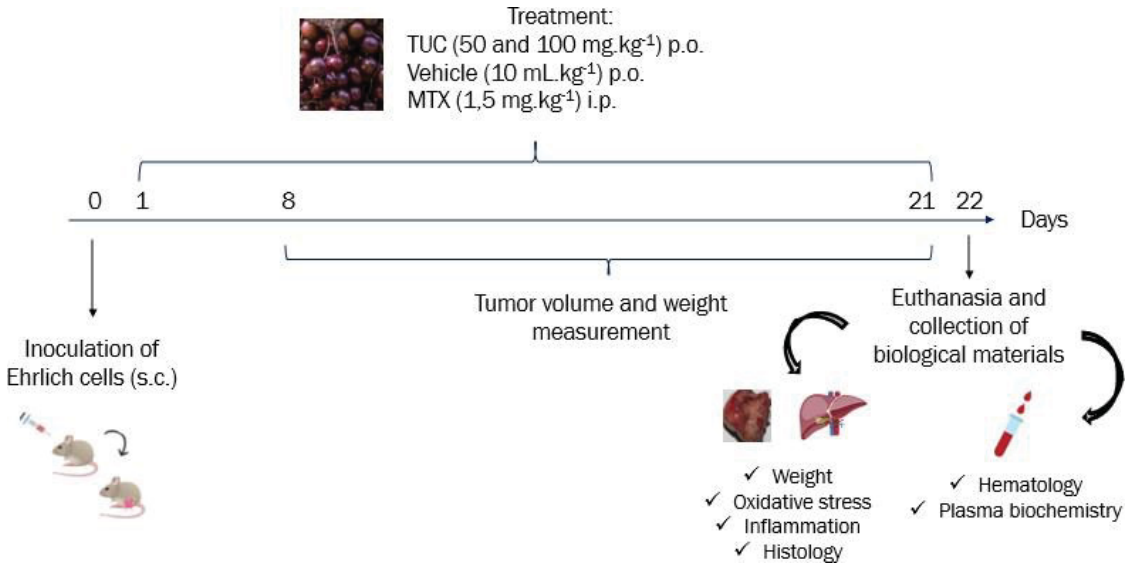


Figure 1. Experimental time-course of the Ehrlich tumor model in female Swiss mice. Legend: TUC, tucum-do-cerrado polysaccharides; MTX, methotrexate; p.o. *per os*; i.p., intraperitoneal; s.c., subcutaneous.

2.5 BIOCHEMICAL AND HEMATOLOGICAL ASSAYS

After the complete blood cells count in an automatic system (BC2800-Vet, Mindray, Shenzhen, China), the blood samples were centrifuged at 4000 revolutions per minute (rpm) for 5 minutes to obtaining the plasma. Plasmatic levels of ALT (alanine transaminase), AST (aspartate transaminase), creatinine, and total protein were measured in an automated system (Cobas Mira, Roche Diagnostics, Germany) using commercial kits (Bioclin Quibasa, Belo Horizonte, MG, Brazil).

2.6 OXIDATIVE STRESS PARAMETERS

For oxidative stress analyses, 0.3 g of liver and tumor tissue samples were homogenized in 1.7 mL of phosphate buffer solution (pH 6.5) at a dilution of 1:10 volume: volume and centrifuged at a speed of 6911 rpm at 4°C for 10 min and 4887 rpm at 4°C for 5 min, respectively. These spectrophotometric assays used 96-wells

microplates, which readings were performed in a microplate reader (Synergy HT, Biotek, VT, USA).

2.6.1 Determination of Superoxide Dismutase (SOD) activity

It was performed through the ability of SOD to inhibit the auto-oxidation of the pyrogallol reagent, according to GAO et al (1998). The SOD unit is defined by the amount of enzyme that inhibits the reaction in 50% (IC_{50}), and the enzyme activity is expressed in units of SOD per milligram of tissue protein (U SOD.mg protein^{-1}).

2.6.2 Determination of Lipid Peroxidation (LPO) levels

The determination of lipid peroxidation in liver and tumor samples was performed by the FOX or orange xylenol method, as described by JIANG et al (1991). The absorbance was read at 560 nm, and the concentration expressed in nmol hydroperoxides.mg protein^{-1}.

2.6.3 Determination of Reduced Glutathione (GSH) levels

For the measurement of tumor and liver GSH levels, the samples were prepared according to the method described by Sedlak and Lindsay (1968), using thehomogenates from the conjugation of DTNB with reduced glutathione. The readings were performed in 415 nm. The results were expressed in μg of GSH.g^{-1} of tissue.

2.6.4 Determination of Tissue Protein levels

The determination of tissue proteins in the liver and tumor was estimated at 595 nm, according to the Bradford method (1976), using the Bradford reagent based on Brilliant Blue G with phosphoric acid and methanol, and the concentration curve of bovine serum albumin (125-1000 μg.μL^{-1}) as standard. The protein amount was also used to normalize SOD activity and LPO level.

2.7 INFLAMMATORY PARAMETERS

2.7.1 Determination of Myeloperoxidase (MPO) activity

To evaluate the tissue neutrophil infiltration, tumor samples were weighed, homogenized with 2 mL of X-100 Triton saline, and centrifuged at 6911 rpm at 4°C for 10 min. The activity of MPO was determined in 620 nm after reaction of the supernatants with TMB in aqueous dimethylformamide
(BRADLEY et al, 1982).

2.7.2 Determination of N-acetylglucosaminidase (NAG) activity

NAG activity in tissue would indicate the presence of macrophages (mononuclear cells) and was analyzed by the method described by Bailey (1988). After reaction of the supernatants with NAG reagent in Citrate/Phosphate buffer (39 mM, pH 4.5), the samples were analyzed by spectrophotometry at 405 nm and the results expressed in μmol.g tissue^{-1}.

2.7.3 Determination of Nitrite levels

As the half-life of nitric oxide (NO) is short due to its rapid oxidation to nitrite and nitrate (FILHO & ZILBERSTEIN, 2000), nitrite levels in the tumor tissue were measured as an indirect indicator of NO, according to the method described by Green and collaborators (1982). The tissue homogenate was prepared using Griess solution (0.1% N-1-naphthyl-tilediamine and 1% sulfanilamide in 5% H_3PO_4) and measured at 540 nm.

2.8 HISTOLOGY

Fragments of the tumors were removed, fixed and stained with hematoxylin & eosin. All slides containing histological sections of the tumors, obtained from 3 mice/group, were evaluated blindly and in random order by a veterinarian pathologist. The tumor features analyzed were the infiltration of leukocytes and the degree of necrosis and apoptosis (MIRANDA-VILELA et al, 2011). The necrosis was classified according to its intensity, from I (lower) to V (higher). The inflammatory infiltrate was classified as negative (-), mild (+), moderate (++), or intense (+++) cell infiltration.

2.9 HEPATIC METABOLISM EVALUATION

2.9.1 Total Cytochrome P (CYP) levels measurement

The liver homogenates of mice (groups G1–G5 described in section 2.4) were prepared with 10 mg wet tissue·mL^{-1} in a 50 mM Tris-HCl buffer (pH 7.4), containing 150 mM KCl and 10 mM $MgCl_2$. CO was bubbled through the suspension for about 1 min. The samples were divided in microplates in duplicate and stand for 5 minutes. The baseline was recorded, then 6 µl of sodium dithionite was added to the contents in the sample and the spectrum was obtained during 2 min. The concentration of CYP was determined from the spectrum using the molar extinction (104 mM^{-1}·cm^{-1}) for the absorption difference between the peaks in 450 nm and 490 nm, according to Matsubara et al (1976). The results were expressed in nmol CYP·mg hepatic $tissue^{-1}$.

2.9.2 Determination of Hepatic Glycogen levels

The determination of liver glycogen was performed by the method of Kepler & Decker (1974). The amount of 0.3 g of liver was macerated with 0.6 N perchloric acid. The glucose content measured in the homogenate was designated basal glucose. With the addition of the solution adjusted to pH 6.0 made from 1 M potassium bicarbonate, 0.2 M amyloglucosidase, and acetate buffer (pH 4.8) to the homogenate, hydrolysis of the glycogen occurred. After incubating the samples for 2 h at 40°C, the reaction was stopped with 0.6 N perchloric acid, followed by centrifugation at 6000 rpm for 5 min at 4°C. The total glucose in the supernatant (final glucose) was determined with a commercial kit and a spectrophotometer at 505 nm. The difference between basal and final glucose was considered as the glycogen content.

2.9.3 Liver Perfusion and Gluconeogenesis evaluation

Healthy Swiss male mice (non-tumor bearing) were used in experiments after 3 days of acclimatization and 15 h of fasting. Hemoglobin-free non-recirculating liver perfusion was performed as previously described (BATAGLINI et al, 2021). For this, animals were deeply anesthetized and submitted to midline laparotomy to liver exposure. After cannulation of the portal and cava veins, the liver was perfused *in situ*

with Krebs/Henseleit-bicarbonate buffer (KH buffer; pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and it was adjusted to approximately 4 $mL.min^{-1}$. After an initial period of 10 min of perfusion to stabilize the preparation, samples of the effluent perfusion fluid were collected at two-minutes intervals and analyzed for their glucose and pyruvate content (BERGMEYER, 1974). Oxygen concentration in the venous perfusate was monitored by a teflon-shielded platinum electrode (COMAR et al., 2003). During this period, liver perfusion was performed as follows: 10 min with KH buffer, 30 min with KH buffer plus the gluconeogenic precursor L-lactate (2.0 mM) and another 30 min with KH buffer plus L-lactate plus tucum (50 $\mu g.mL^{-1}$). L-lactate and TUC were directly added to the perfusion fluid to achieve the desired final concentration.

2.10 STATISTICAL ANALYSIS

The data were represented as the mean ± standard error of the mean. All data were submitted to the Kolmogorov-Smirnov normality test. The differences between the groups were evaluated by the analysis of variance (ANOVA) of one- or two-ways followed by Dunnet's or Bonferroni post-test, when applicable. The analysis and construction of the graphics were performed using the software Graphpad Prism (Graphpad Software, San Diego, USA, v. 8.0). A value of $p < 0.05$ was considered significant.

3 RESULTS

3.1 CHARACTERIZATION OF POLYSACCHARIDES EXTRACTED FROM TUCUM

The tucum flour (20 g) was defatted and submitted to the enzymatic-gravimetric method, centrifuged, and dialyzed against tap water, yielding approximately 63.5% of total dietary fiber (TDF, g/100 g of dried weight) in which, 22% (4.4 g) were soluble and 41.5% (8.3 g) were insoluble dietary fibers.

On monosaccharide composition, the soluble dietary fiber fraction (TUC) presented a complex monosaccharide composition, with 27.5% of uronic acids and mannose (26.5%), xylose (12.0%), glucose (11.3%), arabinose (10.6%), galactose (9.8%), rhamnose (1.9%) and fucose (0.5%) as neutral sugars. On size exclusion chromatography (Fig 2A) it presented a main peak with the relative molecular weight of 70 kDa. The ^{13}C NMR spectrum (Fig 2B) was in agreement with monosaccharide composition and presented at least seven anomeric signals. Those at δ 102.4 and δ 100.1 are probably from anomeric signals of β-D-Glc*p* and β-D-Man*p*. Others at δ 101.7 and δ 101.2 are from β-D-Xyl*p* units, at δ 104.3 from β-D-Gal*p* units and that at δ 97.7 and δ 107.6 can be assigned to α-D-Glc*p*A and α-L-Ara*f* units, respectively (Fig. 2B). Signals of acetyl groups at δ 20.0 and δ 20.2 were also observed. All these assignments are in agreement with published literature data (BENDAHOU et al., 2007; ISHURD et al., 2001; KATSURAYA et al., 2003; ODONMAZIG et al., 1990; SHATALOV, EVTUGUIN and NETO, 1999; SIMAS-TOSIN et al., 2014; THUDE and CLASSEN, 2005) and suggest the presence of a mixture of acetylated glucomannan and glucuronoarabinoxylan in TUC fraction (as confirmed by analysis of the purified polysaccharides – unpublished data).

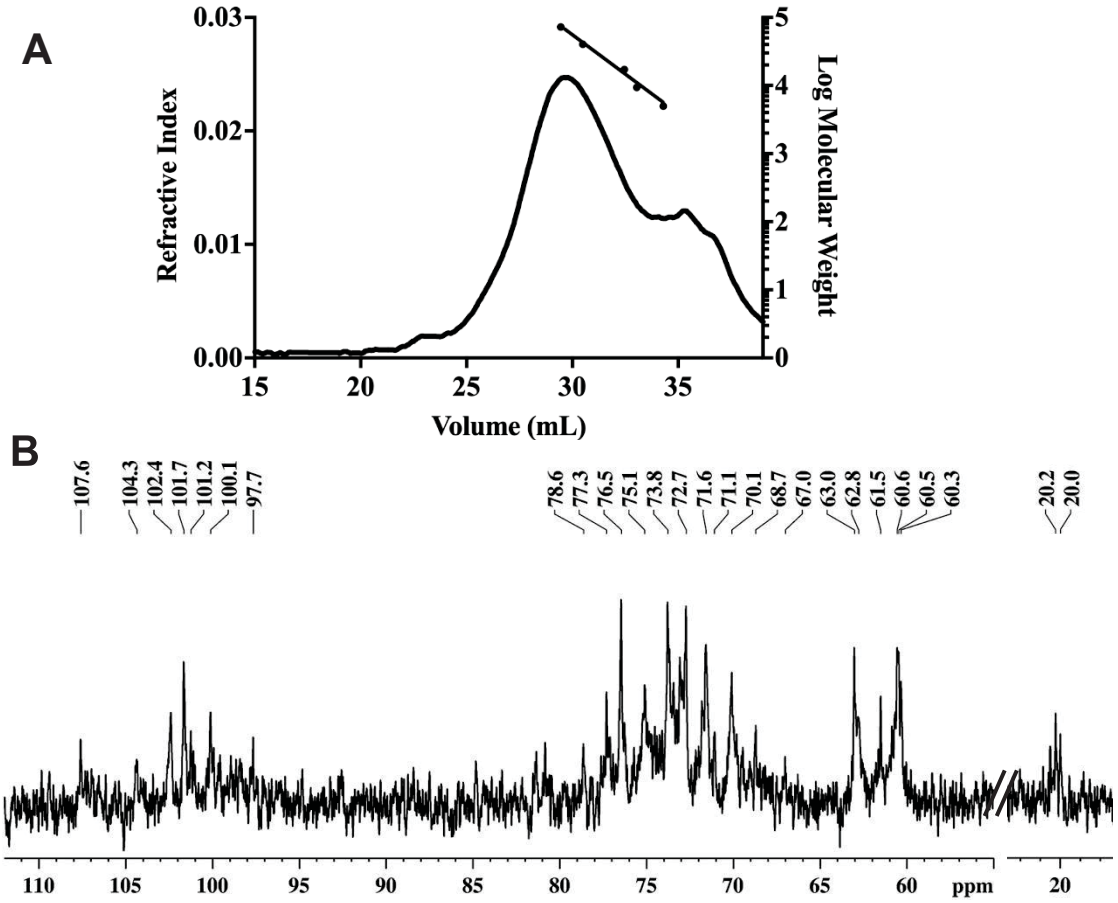


Figure 2. HPSEC elution profile of TUC fraction **(A)**; and ^{13}C NMR spectrum of TUC fraction, in D_2O at 50°C **(B)**.

3.2 TUC TREATMENT DECREASED THE TUMOR DEVELOPEMENT

From day 7 of inoculation of Ehrlich cells, the tumors were already visible. The group treated with the highest dose of TUC had tumor volumes reduced by 71.4% and the group treated with the lowest dose by 57.1%, compared to the control (vehicle) group. (Fig 3A, 3B). In addition, at the end of the treatment the tumors were collected and weighed and, in accordance, the treatment with TUC was favorable to reduction of the tumor weights in comparison to the control group (Fig 3C). The development of tumors from both TUC groups was similar to that of the group treated with the chemotherapeutic MTX (Fig 3A-C).

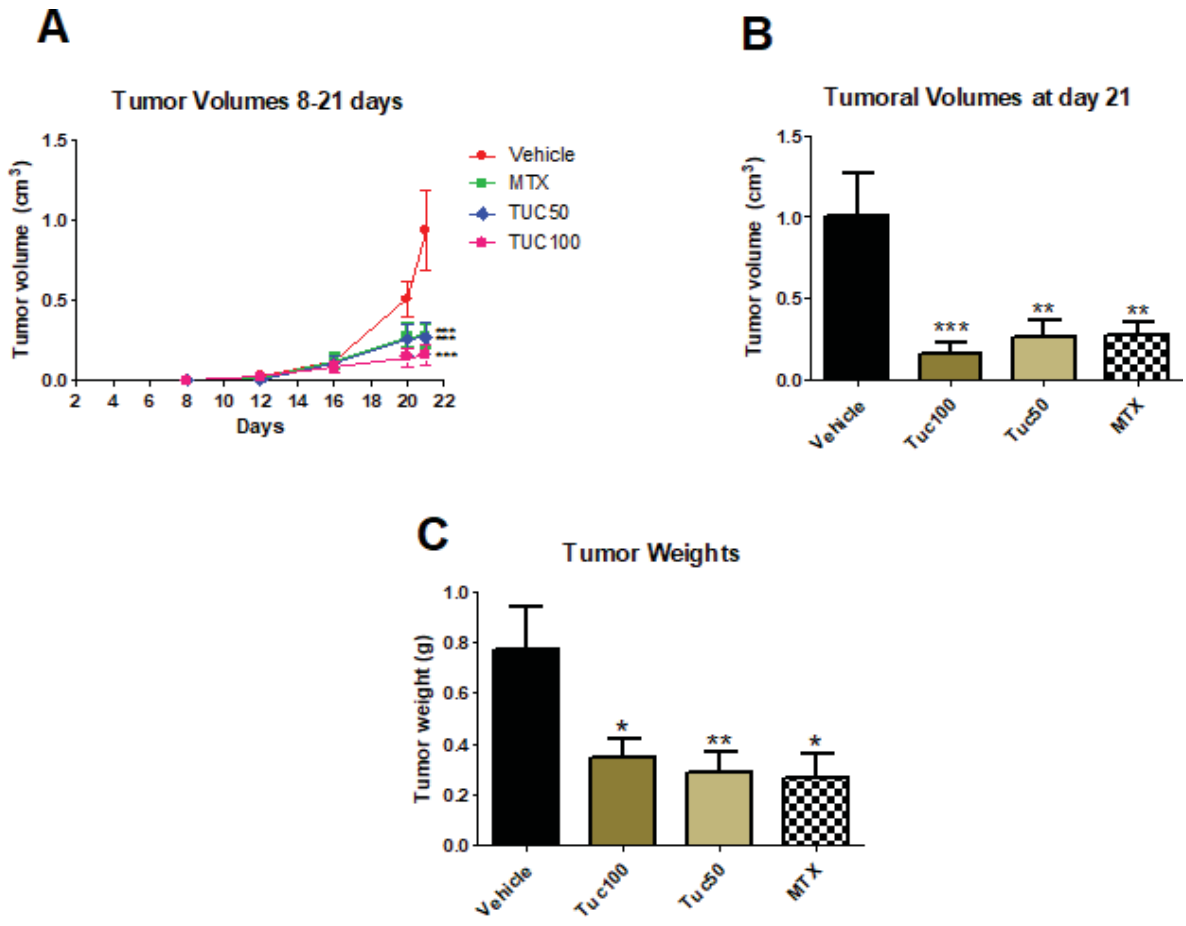


Figure 3. Ehrlich tumor volume **(A** and **B)** and weight **(C)**. Tumor-bearing mice were treated for 21 days with TUC (50 mg.kg^{-1} and 100 mg.kg^{-1}, p.o.), MTX (1.5 mg.kg^{-1}, i.p.) or Vehicle (p.o.), and tumor was withdrawal after euthanasia at the 22nd day. Data represent mean ± SEM (n=8), analyzed by two-way (A) or one-way (B, C) ANOVA followed of post-hoc test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared with Vehicle.

3.3 TUC DID NOT INDUCE CHANGES IN HEMATOLOGICAL ANALYSIS

In the hematological analysis the white blood cells, red blood cells, platelets, hemoglobin, and hematocrit were measured, and no changes were found when the groups were compared. Also, no changes were observed in the creatinine and total protein levels among the groups. The ALT enzyme was reduced in all groups in relation to the Naive, while AST levels were increase. These effects, however, were related with the tumor presence and not specifically with the treatments (Table 1).

Table 1. Hematological parameters of tumor bearing-mice treated with both doses of TUC or vehicle, and non-tumor bearing mice (naive).

	Experimental group				
Parameter	Naive	Vehicle	TUC100	TUC50	MTX
HTC	41.71 ± 2.23	41.14 ± 6.43	39.22 ± 2.17	38.66 ± 1.93	39.37 ± 4.72
HBG	13.74 ± 0.94	13.91 ± 2.28	12.86 ± 0.55	12.49 ± 0.57	12.89 ± 1.99
Platelets	376.70 ± 131.30	307.82 ± 114.38	399.06 ± 104.30	376.54 ± 123.80	380.09 ± 110.28
RBC	7.19 ± 1.63	6.60 ± 2.00	7.61 ± 1.35	7.19 ± 1.81	5.75 ± 1.99
WBC	5.86 ± 1.24	4.95 ± 1.01	6.53 ± 1.75	5.35 ± 0.86	5.59 ± 2.03
Total proteins	5.55 ± 0.26	5.28 ± 0.59	5.3 ± 0.46	5.01 ± 0.74	5.25 ± 0.49
Creatinine	0.39 ± 0.04	0.35 ± 0.06	0.38 ± 0.04	0.36 ± 0.05	0.35 ± 0.06
AST	128.25 ± 44.94	217.37 ± 50.61##	222.48 ± 55.23 ##	221.56 ± 49.40 ##	258.24 ± 54.62 ###
ALT	70.88 ± 12.31*	55.7 ± 4.8	61.65 ± 9.42	57.07 ± 9.20	66.34 ± 11.71

Animals without tumor (Naive) or with tumor were treated for 21 days with vehicle, 50 and 100 mg.kg^{-1} polysaccharides from *Bactris setosa* Mart (TUC; v.o.), or 1.5 mg.kg^{-1} methotrexate (MTX; i.p.). The results are expressed as mean ± SEM (n=8). The statistical analyses were performed using one-way ANOVA followed by Dunnett's post hoc. Legend: HTC: hematocrit (%); HBC: hemoglobin (g.dL^{-1}); RBC: red blood cells (x10^{6}.uL^{-1}); WBC: white blood cells (x10^{3}.uL^{-1}); platelets (x10^{3}.uL^{-1}); AST: aspartate aminotransferase (U.L^{-1}); ALT: alanine amino transferase (U.L^{-1}); creatinine (mg.dL^{-1}); total proteins (mg.dL^{-1}); * $p < 0.05$ compared with Vehicle; $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$ comparedwith Naive.

3.4 TUC INDUCED ALTERATION IN OXIDATIVE STRESS PARAMETERS IN TUMOR TISSUES

In tumor tissue the GSH level was reduced in the groups treated with TUC in comparison to the vehicle group (Fig 4A). On the other hand, LPO increased in the tumor tissues of the TUC-treated groups, denoting a redox disequilibrium (Fig 4B). The SOD, in turn, increased only in the group treated with the highest dose of TUC (Fig 4C). Additionally, the proteins amount in tumor tissues decreased in all treated groups compared to the vehicle group (Fig 4D).

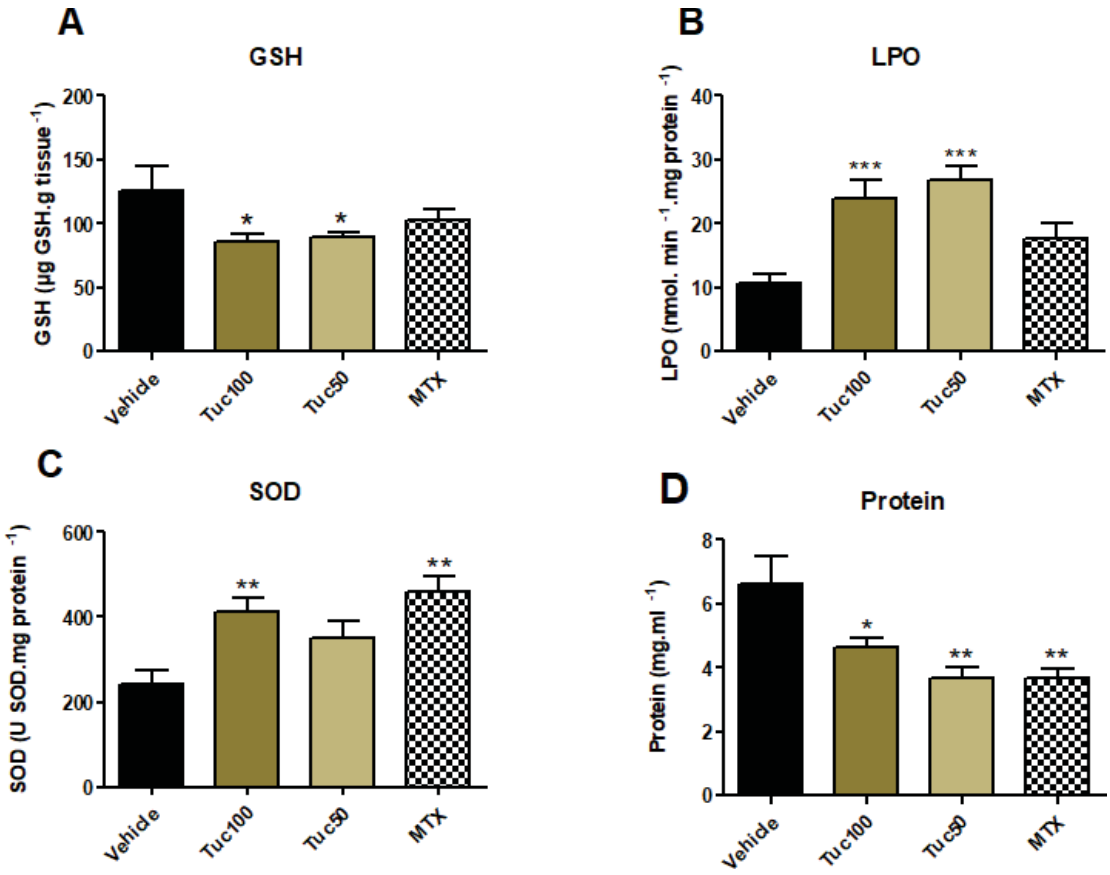


Figure 4. Oxidative stress parameters in tumor tissue. **(A)** GSH levels, **(B)** LPO levels, **(C)** SOD activity and **(D)** Protein levels. Mice were treated for 21 days with vehicle, 50 (TUC50) and 100 mg.kg^{-1} (TUC100) of tucum polysaccharides, or methotrexate (MTX). The data are presented as mean ± SEM (n=8), and analyzed by the one-way ANOVA followed by the Dunnett post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the vehicle group.

3.5 TUC INDUCED ALTERATION IN INFLAMMATORY PARAMETERS IN TUMOR TISSUES

The inflammatory parameters also presented alterations in the groups treated with TUC. NAG activity increased in the tumor tissues of the groups treated with both doses of TUC compared to the control (Fig 5A). However, NO level increased only in the group treated with the highest dose of TUC (Fig 5B), while MPO activity increased significantly with the MTX treatment (Fig 5C).

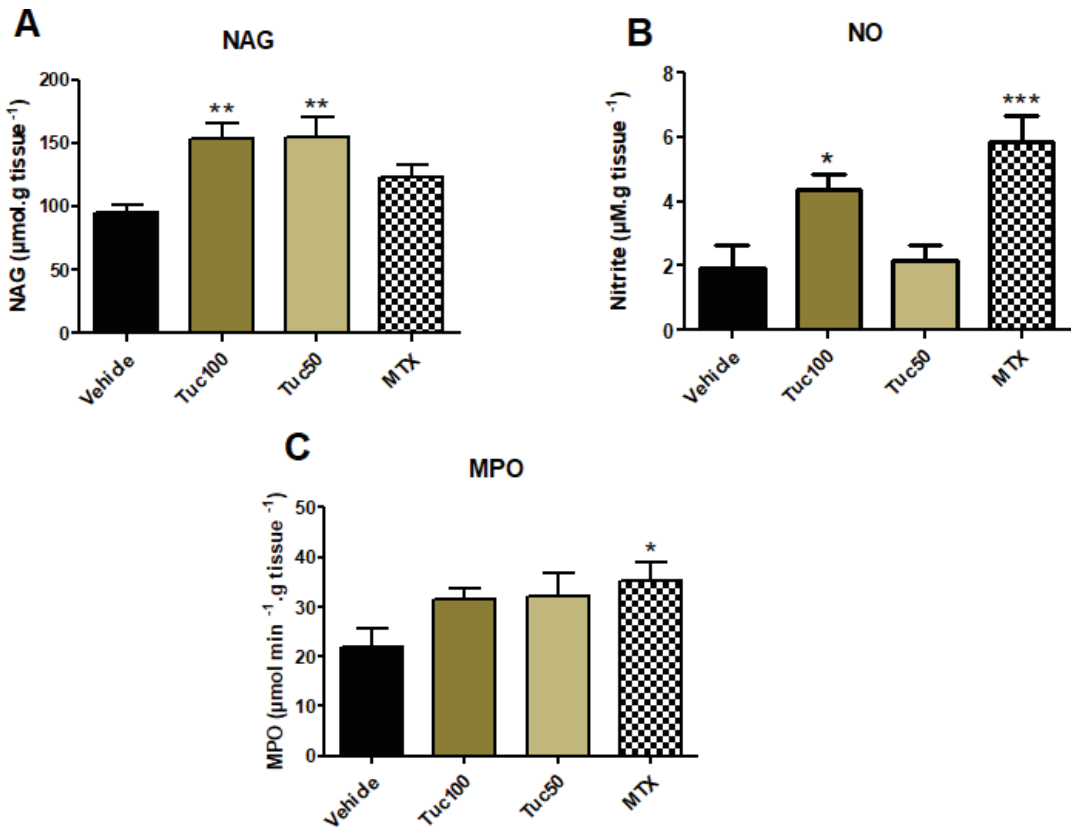
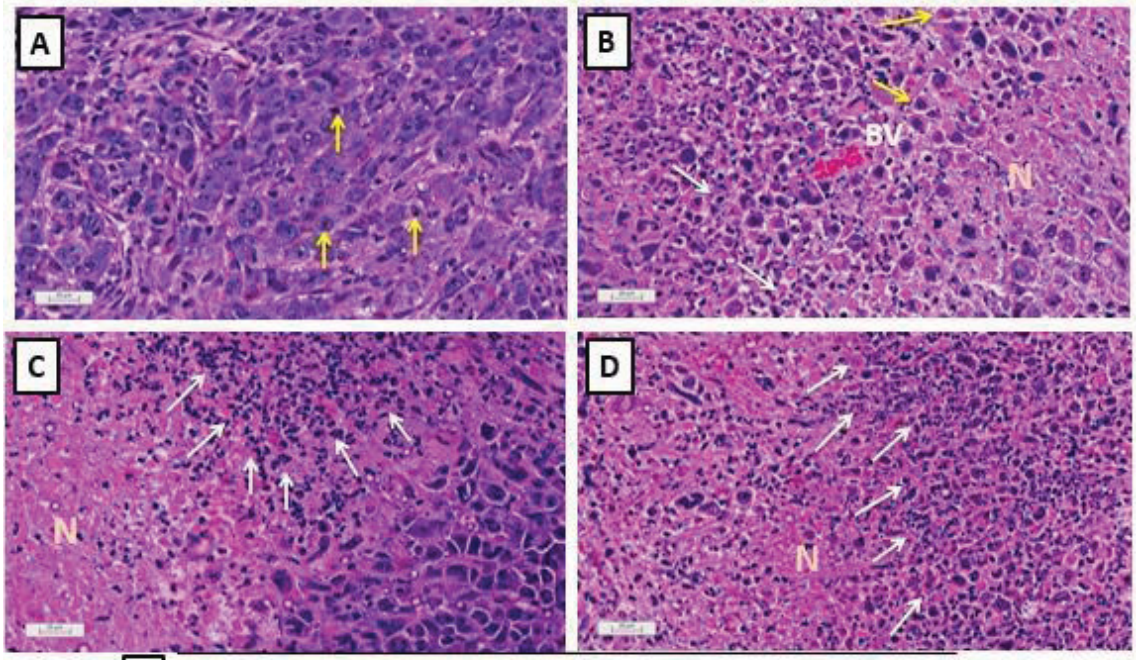


Figure 5. Inflammatory parameters in tumor tissue. **(A)** NAG activity, **(B)** NO levels, and **(C)** MPO activity. Mice were treated for 21 days with vehicle, 50 (TUC50) and 100 mg.kg^{-1} (TUC100) of tucum polysaccharides, or methotrexate (MTX). The data are presented as mean ± SEM (n=8) and analyzed by the one-way ANOVA followed by the Dunnett post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the vehicle group.

3.6 TUC INDUCED NECROSIS AND INFLAMMATORY CELLS INFILTRATION IN TUMOR

The histological analysis of tumor slides indicated different degrees of necrosis. The vehicle group presented grade II necrosis. The group treated with the lowest dose of TUC exhibited grade III necrosis, while the group treated with the highest dose the grade IV. Both groups treated with TUC presented intense (+++) degree of inflammatory infiltrate, compared to the mild (+) degree of the vehicle group (Fig 6).



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Treatment	Necrosis	Inflammatory infiltration
(A) Vehicle	II	+
(B) MTX	IV	++
(C) TUC50	III	+++
(D) TUC100	IV	+++

Figure 6. Representative histology of Ehrlich tumors from mice submitted to treatment with vehicle **(A)**, methotrexate **(B)** or polysaccharides of tucum (TUC) at 50 $mg.kg^{-1}$ **(C)** and 100 $mg.kg^{-1}$ **(D)**, and the degrees of necrosis and inflammation **(E)**. Legend: N: necrosis; BV: blood vessel; yellow arrow: viable tumor cells; white arrow: inflammatory cells. Magnification 20x.

3.7 TUC SLIGHTLY MODIFIED THE OXIDATIVE STRESS PARAMETERS IN LIVER TISSUE

The analysis of antioxidant parameters in liver from tumor-bearing mice did not present relevant alterations, since SOD activity and GSH levels showed no differences among the groups (Fig 7C, 7B). However, an increase in hepatic LPO levels of the groups treated with both doses of TUC was observed (Fig 7A). In addition, a reduction around 19% of total protein level was observed in liver of all animals with tumor, independently of the treatment, relatively to the naive group (Fig 7D).

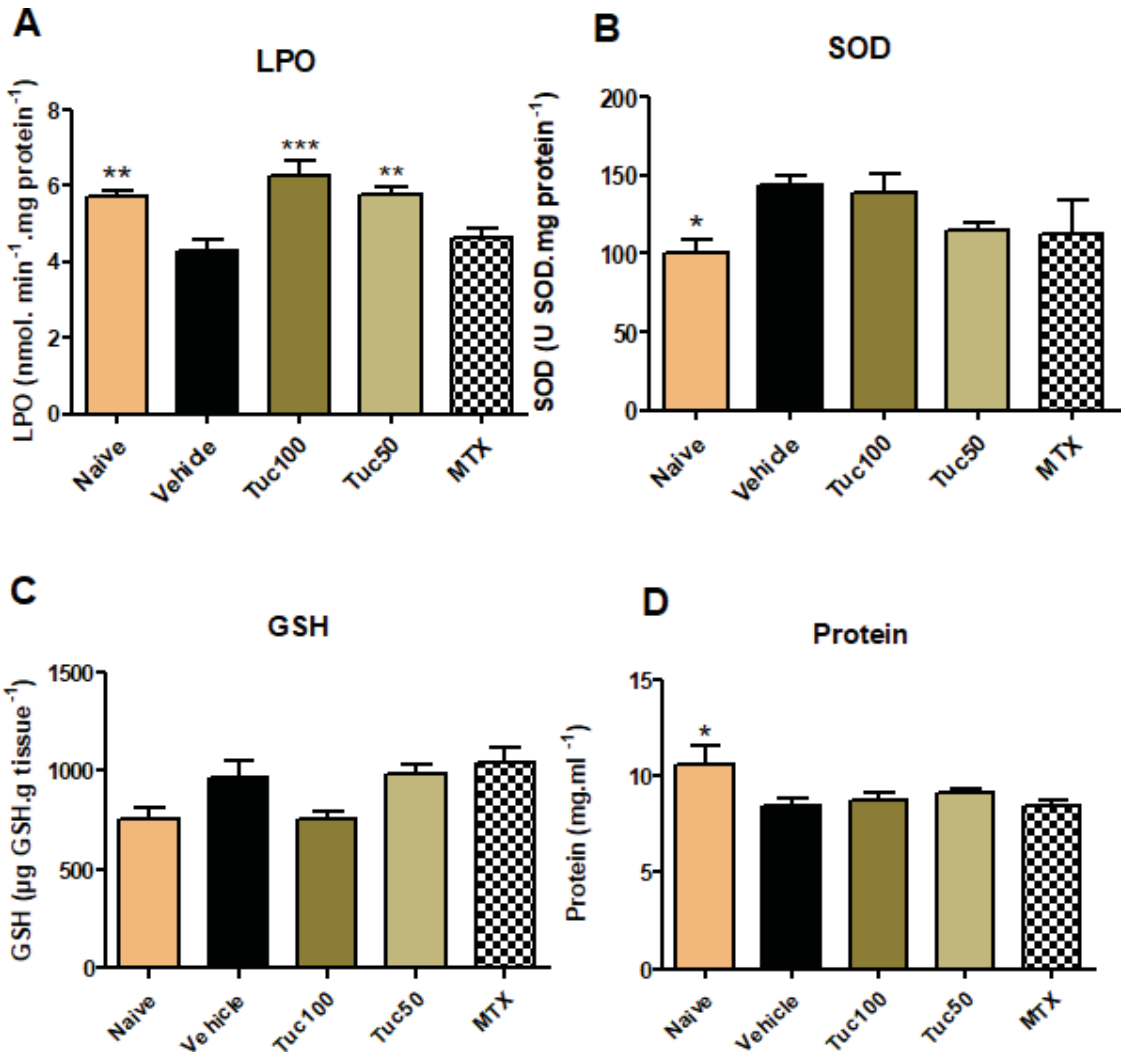

Figure 7. Oxidative stress parameters in liver tissue. **(A)** LPO levels, **(B)** SOD activity, **(C)** GSH levels, and **(D)** Protein levels. Mice were treated for 21 days with vehicle, 50 (TUC50) and 100 mg.kg^{-1} (TUC100) of tucum polysaccharides, or methotrexate (MTX). The data are presented as mean ± SEM (n=8) and analyzed by the one-way ANOVA followed by the Dunnett post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the vehicle group.

3.8 HEPATIC TOTAL CYP AND GLYCOGEN LEVELS ARE ALTERED DUE TO THE TUMOR PRESENCE

In hepatic tissue, total CYP levels were reduced in all tumor-bearing mice, with statistical significance in vehicle, MTX and the group treated with the highest dose of TUC, compared to the naive group (Fig 8A). It is inferred from these data that the

presence of Ehrlich tumor or the reduction of liver protein levels (Fig 7D) influenced more the total CYP concentration than the treatments.

Following the same rationality, liver glycogen levels of all animals in the tumor groups were decreased compared to the naive group, with the highest reduction in the vehicle group (80%). However, treatment with the highest dose of TUC was able to partially recover the glycogen, almost matching the naive group (Fig 8B).

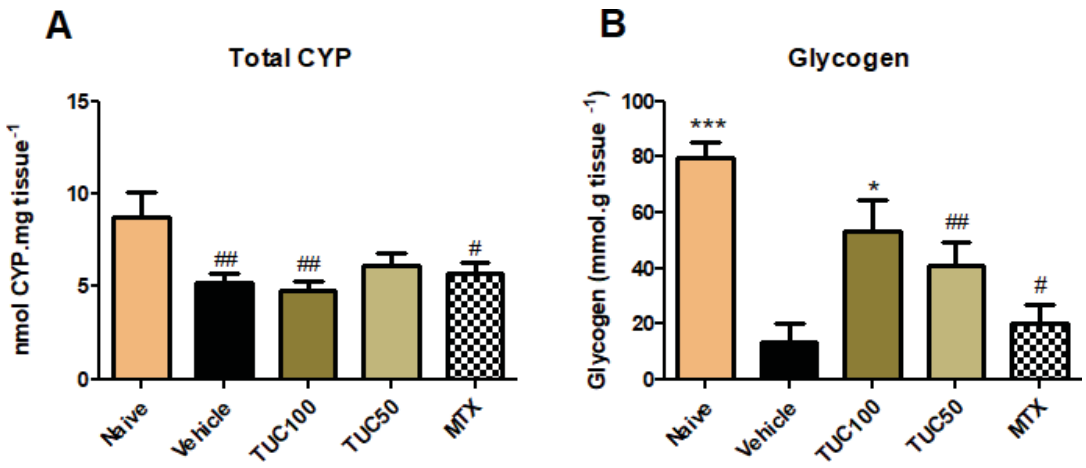

Figure 8. Total CYP **(A)** and glycogen **(B)** levels in liver tissue. Mice were treated for 21 days with vehicle, 50 (TUC50) and 100 mg.kg^{-1} (TUC100) of tucum polysaccharides, methotrexate (MTX), and in the case of animals without tumor, with vehicle. The data are presented as mean ± SEM (n=8) and analyzed by the one-way ANOVA followed by the Dunnett post-hoc test. * $p < 0.05$, *** $p < 0.0001$ compared with the vehicle group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ compared with the naive group.

3.9 TUC DID NOT CHANGE THE HEPATIC METABOLISM

The effects of TUC on gluconeogenesis were investigated in perfused livers of non-bearing tumor mice using lactate as precursor, which allows to evaluate the complete gluconeogenic machinery going through all gluconeogenic steps from pyruvate up to glucose. Figure 9A shows the time courses of the modifications in the glucose and pyruvate production and oxygen consumption caused by TUC at a concentration of 50 µg.ml^{-1}. This concentration was based in previous work of our group with in vitro cell viability tests using different polysaccharides (ADAMI et al, 2018, 2020). Livers from 15-

hours fasted mice were perfused to ensure low glycogen levels. Under such conditions the rate of glucose output reflects mainly the rate of gluconeogenesis (COMAR et al., 2016). The basal rates of glucose and pyruvate production were minimal. Lactate infusion produced progressive increases in glucose and pyruvate productions and oxygen uptake. These increases tended to stabilize at 30-40 minutes perfusion time. In general terms, the lactate-induced stimulus of all parameters was not modified by TUC introduction from the 40th to 70th min of perfusion (Fig 9B-D).

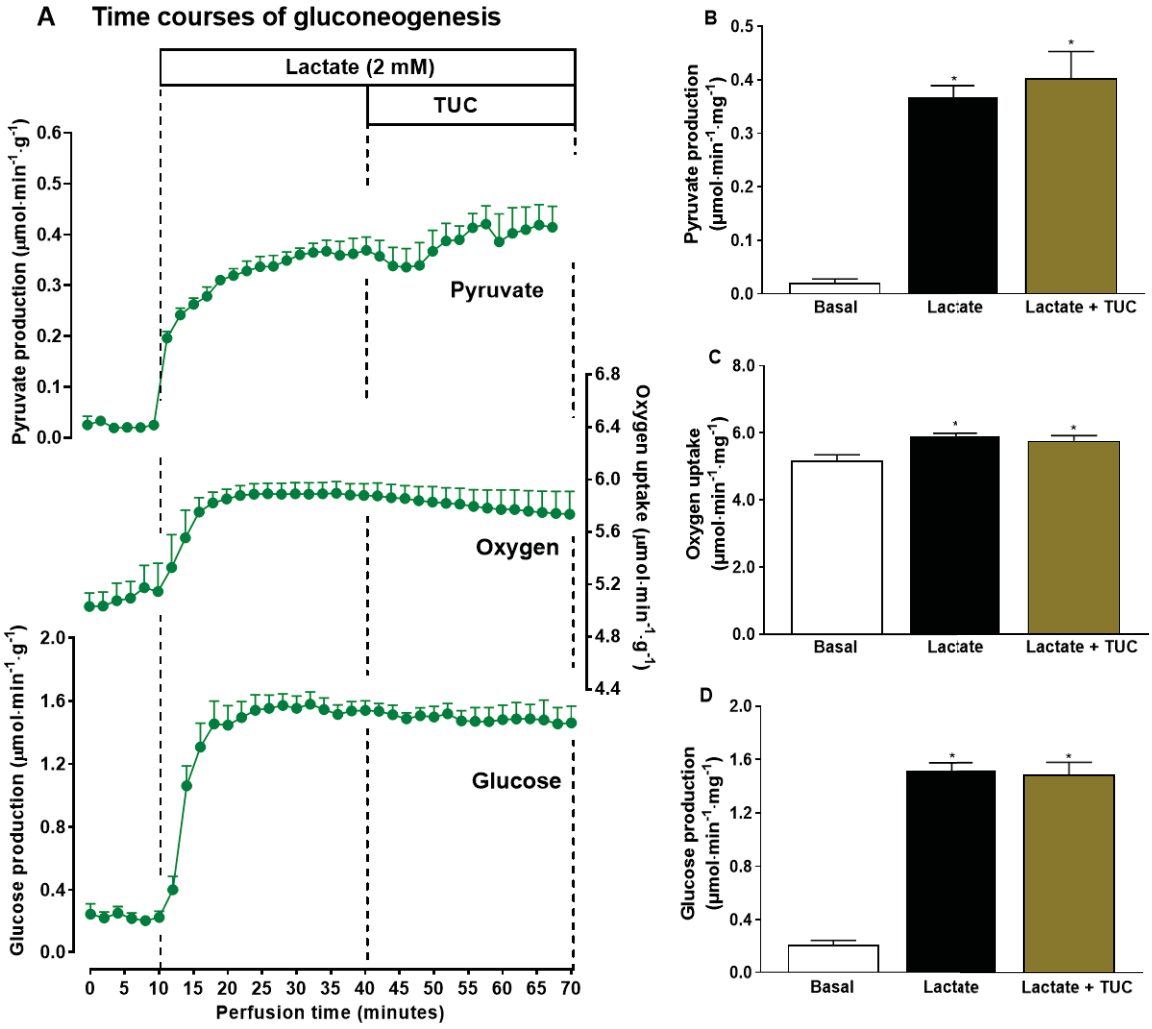

Figure 9. Effects of TUC on gluconeogenesis in perfused mouse livers. (**A)** Time courses of glucose and pyruvate production and oxygen consumption due to infusion of lactate (2 mM) in the absence and presence of TUC (50 µg.mL^{-1}). Livers from 15-hour fasted mice were perfused with Krebs/Henseleit bicarbonate buffer (basal). Lactate (2 mM) and

tucum were introduced as indicated by the horizontal bars. The outflowing perfusate was sampled at regular intervals and analyzed for its glucose and pyruvate contents. Oxygen uptake was monitored by polarography. The values in panels **(B), (C)** and **(D)** are the steady state rates in the basal period (time 8 min in panel A) and after the introduction of lactate in the absence (time 36 min in panel A) and presence of TUC (time 64 min in panel A) for pyruvate production **(B)**, oxygen production **(C)** and glucose production **(D)**. Data are the mean ± SEM obtained from 3-4 animals for each condition, analyzed by one-way ANOVA and Dunnett post hoc. $^{\#}$ $p < 0.05$ compared with Basal condition.

4 DISCUSSION

The present results demonstrate the reduction of Ehrlich tumor development in the groups treated with 50 $mg.kg^{-1}$ and 100 $mg.kg^{-1}$ of polysaccharides extracted from tucum-do-cerrado fruits. The antitumor activity of polysaccharides extracted from different sources has already been confirmed in other pre-clinical studies. Both tumors of mammary origin, the Walker-256 carcinoma in rats and the Ehrlich carcinoma in mice, had the tumor development reduced by the treatment with polysaccharides extracted from the soluble fraction of cabernet franc red wine (STIPP et al, 2016) and from the sweet green pepper (ADAMI et al, 2018; 2020), respectively. However, the mechanisms of action referring the antineoplastic activity are different according to the polysaccharide composition and structure.

In this study we demonstrate for the first time the structure of polysaccharides extracted from tucum, a native fruit of the Brazilian Cerrado biome, composed basically of the glucuronoarabinoxylans and glucomannan molecules. Glucuronoarabinoxylan is one of the most abundant polysaccharides of cereal grain cell walls, found in rye, wheat, barley, oat, rice, sorghum, maize and millet, as well as in other plants such as psyllium (IZYDORCZYK, 2009). Arabinoxylans extracted from rice bran have already shown biological effects, isolated or as an adjuvant to radiotherapy in an animal carcinoma model (EL-DIN et al, 2019). The mechanism involved in this antineoplastic effect was by activation of apoptosis and necrosis pathways. Glucomannan, extracted from Konjac, has also been shown to be effective as a tumor-reducing agent and was also able to reverse multidrug resistance in vivo in HepG2/5-FU cells by suppressing AKT pathway

signaling and increasing p53 expression (CHEN et al., 2018). The results obtained in our experiments with TUC also revealed induction of tumor necrosis. However, the antitumor effect of TUC polysaccharides may be related to more than one mechanism, involving oxidative stress and inflammation pathways.

First, we evaluated oxidative stress markers in tumor tissue. There was an increase in LPO levels along with a decrease in GSH levels in the tumor tissues of the groups treated with TUC, accompanied by an increase in SOD, but only in the group treated with the highest dose. The redox system in the tumor works different of normal cells, since neoplastic cells generate higher amount of reactive oxygen species, often leading to deregulation of the antioxidant defense system (GLASAUER & CHANDEL, 2014). This feature can be explored therapeutically. Herein, the increase of lipid peroxidation products (LPO) combined with the decrease in the amount of antioxidant molecules (such as GSH) can indicate that TUC induced oxidative stress in the tumor microenvironment, leading to tumor cells death. This hypothesis corroborates previous data that showed decreased viability of Caco-2 cells (colorectal adenocarcinoma) upon tucum extract exposure, trough down-regulation of genes with antioxidant functions, and elevation of mitochondrial ROS levels (DA SILVA et al, 2022). However, we observed an increase of lipid peroxidation also in the hepatic tissues of the TUC treated groups, which denotes a mechanism not exclusive of the tumor tissue.

Previous observations suggest that oxidative stress, chronic inflammation, and cancer are closely related (REUTER et al, 2010; MURATA, 2018), thus inflammatory biomarkers were also evaluated. It was observed an increase of NO levels and NAG activity in tumors of groups treated with TUC, despite no alterations in MPO activity. Previous studies have shown the increase of inflammatory parameters promoted by polysaccharides in tumor tissues (ZHANG et al, 2017a; MA et al, 2017). The inflammatory process in tumor microenvironment induced by polysaccharides can trigger tumor growth suppression. For example, a TNF-α increase induced by polysaccharides is related to tumor cell death through the necroptosis pathway (STIPP et al, 2018). Specifically in the present work, the augmented NAG activity indicates the recruitment of macrophages to increase the phagocytosis in tumor microenvironment. The modulation of inflammatory cells by the glucomannan from the fungus *Heterodermia obscurata* was already demonstrated (PEREIRA et al, 2010).

The tumor histological finds corroborate the TUC mechanisms through both oxidative and inflammatory involvement. The presence of inflammatory infiltrates in the tumor observed in histological slides corroborates the increase of NAG activity measured in the tumor tissue. Furthermore, the relatively high degrees of necrosis in the groups treated with TUC compared to the control group reinforce the hypothesis that the antineoplastic action of the TUC follows the oxidative stress pathway, as well as could indicate the engagement of necroptotic cell death pathway.

Besides the antineoplastic effects of TUC polysaccharides, their hepatic effects were also investigated since the liver is the main organ of metabolism and drugs biotransformation. Although there was an increase of lipid peroxidation induced by TUC in livers, the other alterations related with hepatic function, such as increased plasmatic AST, and reduced amount of liver proteins, glycogen and hepatic CYP concentration, are related to the tumor presence more than to the treatments. This fact reinforces the hypothesis that TUC polysaccharides are not associated to hepatotoxicity. Additionally, the experiment of hepatic perfusion shows that TUC did not modify the gluconeogenesis and the oxygen consumption when introduced at the concentrations of 50 $\mu g.mL^{-1}$ in the liver of healthy mice. In this experimental protocol, the compounds could modify the gluconeogenesis by acting directly on the mitochondrial respiration or other intracellular mechanism, such as the modification of enzymes activity, but only if they enter the cells. However, as a polysaccharide TUC should not enter the cells in a manner that their actions should occur via interaction with receptors on the plasma membrane of hepatic cells. In this regard, gluconeogenesis is extremely sensitive to alterations in the cellular integrity because the pathway occurs in different intracellular compartments and requires energy from an aerobic system associated to membranes (CASTRO-GUIZONI et al, 2017; AMES-SIBIN et al, 2018). Measurements of hepatic gluconeogenesis can then provide information about the toxicity of substances used against tumors. If similar concentrations (50 $\mu g.mL^{-1}$) are achieved in the plasma after oral ingestion of TUC still require investigation. Despite the absence of gluconeogenesis alterations by TUC, a previous work demonstrated that the consumption of pulp tucum fruit may inhibit the glucose-6-phosphatase (G6Pase), a gluconeogenic rate limiting enzyme, suppressing the gluconeogenesis, and upregulates GLUT2 hepatic glucose

uptake, without changes in glycogen contents (HEIBEL et al, 2018). These data corroborate in part with our results, since herein expressive reduction on glycogen levels was induced by the tumor presence instead of the TUC treatment. Additionally, the data of Heibel et al (2018) were obtained experimentally with rats in a different condition, represented by a long consumption (12 weeks) of whole tucum pulp fruit together with the chow. Possibly, the responsible for the effect on gluconeogenesis are other compounds rather than polysaccharides, since phenolic compounds and carotenoids have also been identified in the tucum pulp extract (BOEING et al, 2017), including catechins, cyanidin, peonidin, and quercetin (DANTAS et al, 2022). The results of our protocol allow concluding that the tucum polysaccharides do not impair the liver metabolic function.

In conclusion, the polysaccharides extracted from the tucum, composed primarily of the glucuronoarabinoxylan and glucomannan, have antineoplastic effects against Ehrlich carcinoma in mice, modulating the oxidative stress and the inflammation in the tumor microenvironment, without induce neither changes in gluconeogenesis nor hepatotoxicity. Thus, these polysaccharides have therapeutic potential against solid tumors, mainly the mammary tumors.

ACKNOWLEDGMENTS

The authors thank the Brazilian funding agencies CAPES (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* – Financial code 001) and CNPq (*Conselho Nacional de Desenvolvimento Científico e Tecnológico* – process 305787/2018-7, 310290/2021-0 to A.A.; and 404717/2016-0, 310332/2015-0, 307314/2018-9 to L.M.C.C) for financial support. The authors thank to Melina Pazzin, Olair Carlos Beltrame, Rosângela Locatelli Dittrich, and Mayara Amorim for their help with the experiments, and to CTAF (*Centro de Tecnologias Avançadas em Fluorescência*) of UFPR for the technical support.

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4 CONSIDERAÇÕES FINAIS

O presente estudo se propôs a avaliar o efeito antitumoral dos polissacarídeos extraídos do tucum-do-cerrado frente ao modelo de carcinoma de Ehrlich. Esses carboidratos foram caracterizados e concluiu-se que são compostos majoritariamente de glucuronoarabinoxilana e glucomanana. O tratamento com esses polissacarídeos reduziu significativamente os volumes e pesos tumorais, comparado com o grupo sem tratamento. Essa redução está relacionada com a via de estresse oxidativo, bem como vias inflamatórias, uma vez que os parâmetros redox e inflamatórios estavam alterados nos grupos tratados. Na histologia dos tumores observou-se a presença de necrose e infiltrados inflamatórios nos tratamentos com TUC, o que está praticamente ausente no grupo sem tratamento. Podemos inferir que as vias que levaram à redução dos volumes tumorais nos grupos tratados com TUC têm relação com estresse oxidativo e inflamação.

Além disso, ensaios envolvendo o funcionamento do fígado em diferentes condições experimentais (animais hígidos e com tumor), como perfusão hepática e dosagem de CYP total e glicogênio, mostraram que os polissacarídeos não alteram o metabolismo hepático. Inclusive, o TUC foi capaz de recuperar, pelo menos em parte, o glicogênio hepático reduzido como consequência metabólica da presença do tumor subcutâneo. Desta forma, TUC parece promissor como terapia antitumoral, sem induzir toxicidade hepática ou sistêmica. Novos estudos devem ser encorajados para avaliar seu efeito em longo prazo, em outros modelos de tumor, e sua eficácia quando associado a outros agentes anticancerígenos.

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