SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

AVALIAÇÃO CITOTÓXICA, MUTAGÊNICA, CARCINOGÊNICA E *IN SILICO* DO ÁCIDO BETULÍNICO

Aluno: Victor Constante Oliveira

Orientador: Prof. Dr. Mário Antônio Spanó

UBERLÂNDIA - MG

2022

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Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área Genética).

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VICTOR CONSTANTE OLIVEIRA

COMISSÃO EXAMINADORA

Presidente: Prof. Dr. Mário Antônio Spanó

Examinadores: Prof. Dr. Alexandre Azenha Alves de Rezende
Prof. Dr. Edson José Fragiorge
Profa. Dra. Lee Chen Chen
Profa. Dra. Sandra Morelli

Data da Defesa: 28/01/2022

As sugestões da Comissão Examinadora e as Normas PGGB para o formato da Tese foram contempladas

Prof. Dr. Mário Antônio Spanó

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Coordenação do Programa de Pós-Graduação em Genética e Bioquímica
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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Genética e Bioquímica				
Defesa de:	Doutorado Acadêmico - Nº 02/2022 PPGGB				
Data:	Vinte e oito de janeiro de dois mil e vinte e dois	Hora de início:	08:30h	Hora de encerramento:	11:58h
Matrícula do Discente:	11723GBI012				
Nome do Discente:	Victor Constante Oliveira				
Título do Trabalho:	Avaliação citotóxica, mutagênica, carcinogênica e *in silico* do ácido betulínico.				
Área de concentração:	Genética				
Linha de pesquisa:	Genética, Epigenética, Biologia e Melhoramento de Plantas e Animais.				
Projeto de Pesquisa de vinculação:	Avaliação mutagênica/antimutagênica do ácido betulínico *in vivo* e *in vitro*.				

Aos vinte e oito dias do mês de janeiro de dois mil e vinte e dois, às 08:30 horas, reuniu-se via web conferência pela Plataforma *Zoom*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES e Resolução de nº 06/2020 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Profª. Drª. Lee Chen Chen, Prof. Dr. Edson José Fragiorge, Profª. Drª. Sandra Morelli, Prof. Dr. Alexandre Azenha Alves de Rezende e Prof. Dr. Mário Antônio Spanó, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Prof. Dr. Mário Antônio Spanó, apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

APROVADO (A).

Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do titulo de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pelaBanca Examinadora.

Documento assinado eletronicamente por **Mário Antônio Spano**, **Membro de Comissão**, em 28/01/2022, às 12:00, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, doDecreto nº 8.539, de 8 de outubro de 2015.

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"Aqueles que semeiam com lágrimas, com cantos de alegria colherão".

Salmos 126:5-6

DEDICATÓRIA

Dedico este trabalho à minha amada esposa **Sarah Alves Rodrigues Constante**, pelo amor, carinho e incentivo, e à toda minha família, em especial, meus pais **Nilda Maria Pereira Oliveira** e **Benjamin Constante de Oliveira** e à minha avó **Terezinha Maria Pereira,** que sempre me apoiaram e me ajudaram a chegar até aqui. Vocês são a luz que ilumina minha vida.

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- **Universidade de Franca (UNIFRAN);**

- **Universidade Federal de Uberlândia (UFU).**

LISTA DE ABREVIATURAS

AKT = Protein kinase B

AKR1B10 = Aldo-keto reductase family 1 member B10

ALT = Alanine aminotransferase

AST = Aspartate aminotransferase

AR = aldo-keto reductase

BA = Betulinic acid

BH = Balanced heterozygous

BT-20 = breast cancer cell line

Cav-1 = Caveolin-1

c-Myc = proto-oncogene

COX-2 = Cyclooxygenase-2

CLP = Cecal ligation and puncture

CMN = Congenital melanocytic naevi

CPT = Camptothecin

CRC = Colorectal cancer

CSCs = Colon Cancer stem cells

CYPs = Cytochrome P450 enzymes

DMH = 1,2dimethylhydrazine

DMSO = Dimethyl sulfoxide

DXR = Doxorubicin

ECAR = extracellular acidification

ERK = extracellular signal-regulated kinase

ETT = Epithelial Tumor Test

FAK = focal adhesion kinase

FBS = Fetal Bovine Serum

***flr*^{3}= Flare-3**

GPx = Glutathione peroxidase

GRd = Glutathione reductase

GRP78 = Glucose-regulated protein 78

HB = High bioactivation

HCC = Human hepatocellular carcinoma

HeLa = cervical cancer cell lineage from Henrietta Lacks

HT29 = human colon cancer cell line

JAK2 = Janus kinases 2

JNK = Jun N-terminal kinases

LDH = lactate dehydrogenase

MAPK = Mitogen Activated Protein Kinases

MCF10A = Triple negative breast cells non-tumorigenic

MCF-7 = breast cancer cells

MDA-MB-231 = Triple negative breast cells tumorigenic

MH = Marked trans-heterozygous

MMPs = matrix metalloproteinases

MMS = Methyl methanesulfonate

MNPCEs = Micronucleated polychromatic erythrocytes

MNT = Bone marrow micronucleus test

mTOR = Mammalian target of rapamycin

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

***mwh* =** Multiple wing hairs

NCEs = Normochromatic erythrocytes

NF-κB = F*actor nuclear factor-kappa B*

NO = Nitric oxide

NSCLC = lung cancer cell lines

P-388D1 = Murine pre-B cell lymphoma

p53 = Tumor suppressor gene

PCEs = Polychromatic erythrocytes

PKC = Protein kinase C

POLβ = DNA polymerase beta

PTEN = phosphatase and tensin homologue

Rac1 = Rac Family Small GTPase 1

ROS = Reactive oxygen species

SAE1 = SUMO-activating enzyme subunit 1

SMART = Somatic Mutation and Recombination Test

SOD = Superoxide dismutase

Sp = Specificity protein

Src = Proto-Oncogene, Non-Receptor Tyrosine Kinase

ST = Standard Cross

STAT3 = signal transducers and activators of transcription 3

SUMO = Small Ubiquitin-like Modifier

u937 = *human leukemia cells*

UBA2 = *Ubiquitin-like 1-activating enzyme E1B*

Ubc9 = *SUMO-conjugating enzyme*

URE = Urethane - *Ethyl carbamate*

UV-C = Ultraviolet radiation

VEGF = Vascular endothelial growth factor

VP-16 = Etoposide

wts = Warts

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APRESENTAÇÃO

Tradicionalmente, os produtos naturais extraídos de plantas têm sido alvo de estudo dos grandes centros de pesquisa de novos fármacos. O interesse por esta área de pesquisa aumentou significativamente com o avanço da compreensão da carcinogênese, e da identificação das vias moleculares capazes de interferir neste processo. Dentre as classes de produtos naturais, amplamente encontrados em vegetais, estão os triterpenos pentacíclicos. Os triterpenos formam um grande grupo de compostos que apresentam 30 átomos de carbono distribuídos em cinco anéis, aos quais estão ligados vários átomos de oxigênio. Os efeitos biológicos dos triterpenos incluem atividade bactericida, antioxidante, antinflamatória, antiviral, citotóxica e anticarcinogênica. Diversos produtos naturais com perspectivas farmacêuticas estão incluídos nessa classe, podendo destacar, entre eles, o ácido betulínico (AB).

O AB é um triterpeno pentacíclico de origem vegetal, amplamente distribuído pelo mundo, que apresenta um grupamento ácido carboxílico na posição C-28, e pertence à classe dos lupanos. Este composto é descrito na literatura como potencial agente anti-inflamatório, antioxidante, analgésico, antiviral, antibactericiano, antiproliferativo e antineoplásico. Dentre os benefícios adicionais dessa substância estão a seletividade que ele oferece, reduzindo riscos de efeitos colaterais sistêmicos, e seu amplo mecanismo de ação contra células resistentes a outros agentes terapêuticos, por não ter como alvo o gene *p53*.

O presente trabalho, enquanto pesquisa básica teve como objetivo contribuir com informações sobre os efeitos biológicos do AB por meio de:

1] avaliação *in vivo* dos efeitos genotóxicos e moduladores do AB sobre a genotoxicidade induzida pelo uretano em camundongos Swiss, utilizando o ensaio de micronúcleo da medula óssea;

2] avaliação *in vivo* dos efeitos mutagênicos/recombinogênicos e moduladores do AB sobre a mutagenicidade/recombinogenicidade induzida pelo uretano em células somáticas de asas de *Drosophila melanogaster*, utilizando o teste para detecção de mutação e recombinação somática (SMART — Somatic Mutation and Recombination Test);

3] Avaliação *in vivo* do potencial carcinogênico do AB e protetor contra a carcinogenicidade induzida pela doxorrubicina empregando o teste de tumores epiteliais (ETT) em *D. melanogaster*;

4] Avaliação *in vitro* da atividade antiproliferativa e apoptótica do AB em células mamárias triplo-negativas MCF10A (não-tumorigênicas) e MDA-MB-231

(tumorigênicas) utilizando o ensaio MTT;

5] Avaliação do dano celular causado por AB por meio do ensaio de liberação de LDH; e

6] Avaliação dos alvos moleculares do AB através de análise *in silico* de docking molecular.

Parte do AB foi gentilmente cedido pela Profa. Dra. Denise Crispim Tavares da Universidade de Franca (UNIFRAN). O restante foi adquirido pelo Centro Universitário de Patos de Minas (UNIPAM), sob a responsabilidade da Profa. Dra. Priscila Capelari Orsolin.

As linhagens mutantes de *D. melanogaster* foram fornecidas pelo Dr. Ulrich Graf, do Institute of Toxicology (ETH) and University of Zurich, Schwerzenbach, Suíça. Os testes SMART e ETT foram realizados no Laboratório de Mutagênese da Universidade Federal de Uberlândia e analisados no Laboratório de Mutagênese do Centro Universitário de Patos de Minas.

O teste para avaliação da atividade quimiopreventiva do AB por meio do teste para detecção de micronúcleos em células de medula óssea de camundongos Swiss foi realizado na UNIFRAN, sob a co-orientação da Profª. Dra. Denise Crispim Tavares.

Os testes MTT e LDH para avaliação da atividade antiproliferativa e apoptótica do AB em células tumorais e não tumorais de mama foram realizados na UFU (*Campus* Patos de Minas), sob a co-orientação da Profª. Dra. Thaíse Gonçalves Araújo.

A apresentação deste trabalho foi dividida nos seguintes capítulos:

CAPÍTULO I – FUNDAMENTAÇÃO TEÓRICA, que apresenta sucintamente uma análise literária a que se propõe o trabalho, contemplando informações sobre produtos naturais e triterpenos, com ênfase nos efeitos do AB e, posteriormente, os testes empregados para avaliação da genotoxicidade / antigenotoxicidade do AB em células da medula óssea de camundongos Swiss, mutagenicidade/recombinogenicidade e carcinogenicidade, bem como os efeitos moduladores em *D. melanogaster,* ensaio de viabilidade celular (apoptose), liberação de LDH em células de mama, e análise *in silico* de docking molecular.

CAPÍTULO II: Apresenta o manuscrito intitulado “Betulinic acid modulates urethane-induced genotoxicity and mutagenicity in mice and *Drosophila melanogaster*”, publicado no periódico *“Food and Chemical Toxicology”* (Fator de impacto 6.023), em fevereiro de 2020 (v.138, artigo 111228).

CAPÍTULO III: Apresenta o manuscrito intitulado “Modulating effects of betulinic acid on the carcinogenicity of doxorubicin in *Drosophila melanogaster,* apoptosis in breast cancer cells and *in silico* studies”, que será submetido para publicação no periódico *“Food and Chemical Toxicology”* (Fator de impacto 6.023).

CAPÍTULO I

FUNDAMENTAÇÃO TEÓRICA

1. Triterpenos pentacíclicos

Nos últimos anos, os produtos naturais à base de plantas ganharam a atenção de diversos pesquisadores devido aos inúmeros efeitos colaterais adversos dos medicamentos terapêuticos modernos. Além disso, a aquisição dos fármacos sintéticos apresentam custos elevados em comparação com os produtos à base de plantas (HARUN, et al., 2020). Por isso, remédios seguros com baixos efeitos colaterais e ligantes altamente seletivos que atuam em um único alvo de doença tornaram-se uma missão de centros de pesquisas de desenvolvimento de novos medicamentos (DAVID et al., 2015).

Os produtos naturais derivados de plantas estão disponíveis em abundância pelo mundo inteiro, e ainda proporcionam as melhores possibilidades de descobrir substâncias de interesse terapêutico. De fato, 41% dos fármacos anticarcinogênicos de pequenas moléculas, e 65% dos fármacos antibacterianos, descobertos no período de 1940 a 2010 eram produtos naturais ou derivados semissintéticos de produtos naturais (NEWMAN; CRAGG, 2012). Assim, inúmeros produtos naturais são, constantemente, colocados em fase de testes clínicos, principalmente no tratamento do câncer e de doenças infecciosas (ANDRADE, 2018).

Dentre os grupos mais representativos de fitoquímicos destaca-se o dos triterpenos, que abrange mais de 20.000 compostos conhecidos, e são biossintetizados em plantas através da ciclização do esqualeno (DE JESUS et al., 2021). Os triterpenos são compostos por seis unidades de isopreno do ácido mevalônico, e a maioria dos compostos tem 30 átomos de carbono e são frequentemente encontrados como estruturas tetra ou pentacíclicas. Os triterpenos pentacíclicos se originam da ciclização de um esqualeno epoxidado, que é o precursor de vários triterpenos policíclicos (BORELLA et al., 2019).

Estes são metabólitos secundários de cascas de frutas, folhas, caule e cascas de várias plantas, como casca de vidoeiro, casca plana, folhas de oliveira, bagaço de azeitona, brotos de visco, flor de cravo, bagaço de maçã e folhas de alecrim, principalmente presentes nas camadas externas da cutícula (SHARMA et al., 2018).

Devido à diversidade estrutural associada aos seus efeitos farmacológicos, esses compostos são considerados candidatos promissores para o desenvolvimento de novos fármacos para o tratamento do câncer (DE JESUS et al., 2021), uma vez que eles têm a capacidade de inibir a ativação do fator nuclear κB (NF-κB) e interferir

na transdução de sinal, proliferação celular, apoptose, angiogênese, disfunção mitocondrial e modulação de genes e proteínas MDR (YAN et al., 2014).

Nesse contexto, os triterpenos representam um promissor e expansivo grupo de produtos naturais biologicamente ativos, cujo potencial vem sendo explorado por diversos pesquisadores e companhias farmacêuticas. Eles podem ser divididos em três classes principais: lupano, oleanano e ursano, sendo que cada classe compreende uma série de importantes compostos bioativos com potencial terapêutico (**Figura 1**). Dentre os triterpenos pentacíclicos do tipo lupano consistindo em cinco anéis de seis membros destaca-se o ácido betulínico (AB) (LOBINE et al., 2020).

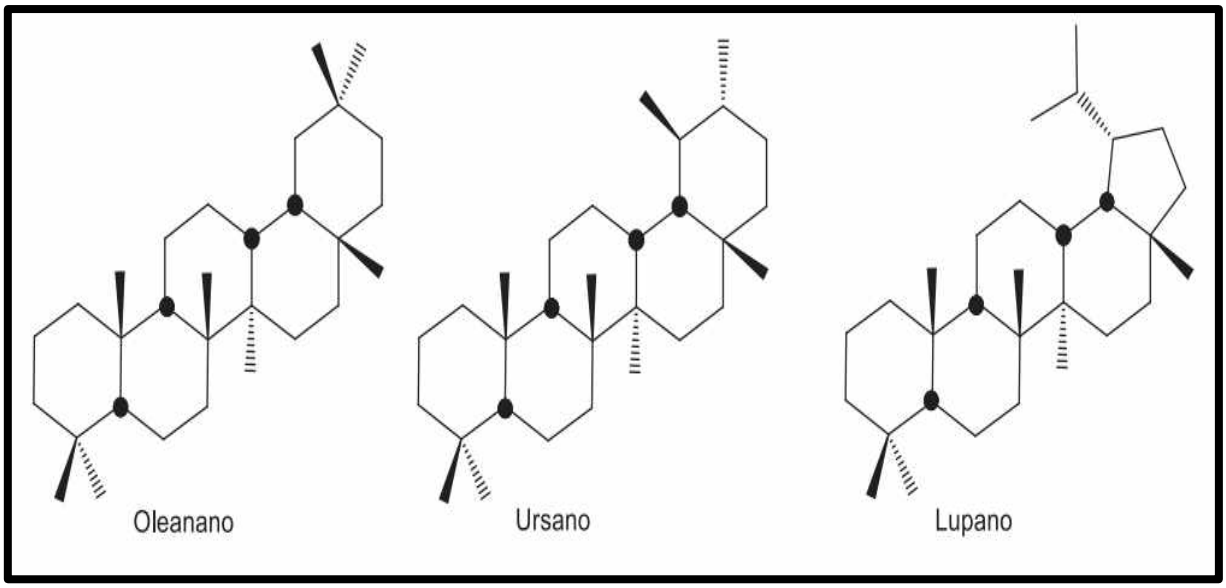

Figura 1. Esqueleto base dos triterpenos pentacíclicos. **Fonte:** Cano-Flores (2013).

2. Ácido betulínico (AB)

O ácido betulínico (AB), ou ácido 3 *β* -hidroxi-lup-20(29)-en-28-óico (**Figura 2**), é um triterpenóide pentacíclico do tipo lupano, de ocorrência natural amplamente distribuído em plantas (ZHONG et al., 2021). Normalmente isolado de plantas medicinais conhecidas, como a bétula (*Betula* sp., Betulaceae), também foi encontrado em várias espécies dos gêneros *Ziziphus* (Rhamnaceae), *Syzygium* (Myrtaceae), *Diospyros* (Ebenaceae) e *Paeonia* (Paeoniaceae). O composto está presente na planta como uma aglicona livre ou como derivados glicosilados (MOGHADDAM et al., 2012), que são facilmente

isolados da fonte com diferentes solventes, mais comumente diclorometano. A purificação do composto pode ser facilmente realizada por meio de uma combinação de diferentes métodos cromatográficos (RÍOS E MÁÑEZ, 2018).

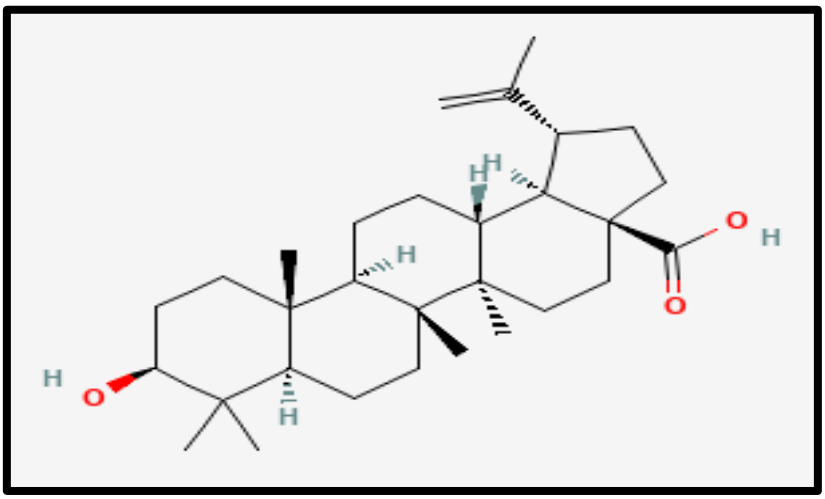

Figura 2. Estrutura química do ácido betulínico.
Fonte: https://pubchem.ncbi.nlm.nih.gov/compound/Betulinic-acid#section=2D-Structure.

Nas décadas anteriores, o AB foi investigado extensivamente devido às suas propriedades benéficas, incluindo efeitos anticarcinogênico, antiinflamatório, antiangiogênico, imunomodulador, anti-helmíntico, e anti-viral (anti-HIV). Seus efeitos antitumorais são maiores em pH reduzido (<6,8), que é uma característica de vários tipos de tumores (DEHELEAN et al., 2012; GHEORGHEOSU et al., 2014; CSUK et al., 2014).

Os efeitos farmacológicos antitumorais do AB consistem no desencadeamento de apoptose por meio da via mitocondrial, regulação do ciclo celular e via angiogênica, incluindo fatores de transcrição de proteínas de especificidade, ciclina D_1 e receptor do fator de crescimento epidérmico, inibindo o transdutor de sinal e ativador da transcrição 3 e vias de sinalização do fator nuclear κB, prevenindo a invasão por metástase de células tumorais, e afetando a expressão de topoisomerase I, proteína p53 e Lamina B1.

Nesse contexto, o AB é capaz de ativar a apoptose, agindo na membrana mitocondrial mediada pela via intrínseca, que gera espécies reativas de oxigênio (ROS), e ativam as quinases pró-apóticas-p38 MAPK e SAP/JNK, que estão envolvidas em vários processos regulatórios, como proliferação celular, expressão

gênica, diferenciação, mitose, sobrevivência celular e apoptose (WANG e FANG, 2009; CSUK et al., 2012).

Estudos recentes demonstraram efeitos terapêuticos antitumorais significativos do AB em células de melanoma humano (CORICOVAC et al., 2021), e vários tipos de tumores sólidos, incluindo glioblastoma (LO et al., 2020), câncer de pulmão (KUTKOWSKA et al., 2021), câncer de mama (QI et al., 2021), câncer colorretal (YURASAKPONG et al., 2021) e câncer de bexiga (KIM et al., 2021). Os mecanismos primários relatados dos efeitos farmacológicos antitumorais do tratamento com AB são apresentados na **Figura 3**.

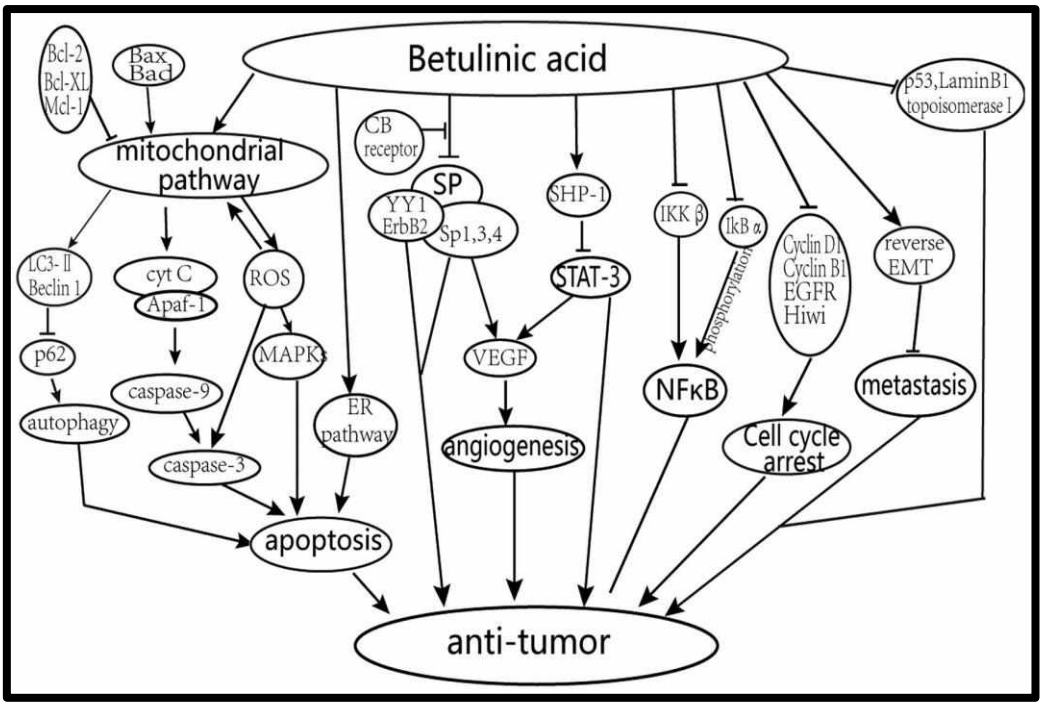

Figura 3. Efeitos farmacológicos antitumorais do ácido betulínico. **Fonte:** Pérez et al., (2009).

Nos últimos anos, o interesse dos pesquisadores em investigar o potencial mutagênico, antimutagênico, recombinogênico, antirecombinogênico, carcinogênico e/ou anticarcinogênico de produtos naturais tem aumentado acentuadamente, pois o conhecimento de tais efeitos pode ser útil na prevenção contra possíveis efeitos adversos à saúde humana, assim como na prevenção contra diferentes neoplasias. A descoberta de novos produtos que reduzem as taxas de mutações certamente diminuiria a incidência do câncer (BHATTACHARYA, 2011).

Devido à sua citotoxicidade específica para diferentes células tumorais humanas e baixa citotoxicidade para células não-tumorigênicas, AB é considerado um agente antitumoral promissor (JIANG et al., 2021). No entanto, ensaios adicionais *in vivo*, *in vitro* e *in silico* ainda são necessários para melhor compreender os mecanismos antitumorais deste composto, uma vez que o conhecimento de tais efeitos pode ser útil na prevenção de possíveis efeitos adversos à saúde humana, e também na prevenção de diferentes tipos de câncer.

3. Ensaio de micronúcleos (MN)

Os seres vivos estão expostos a diferentes agentes xenobióticos presentes no ambiente. Muitos agentes químicos, físicos, ou biológicos podem causar a morte celular. No entanto, existe uma classe de agentes que não necessariamente mata as células, mas apenas danifica seu material genético. Portanto, alguns ensaios são necessários para determinar o nível de exposição e o risco para a saúde.

O Teste de Micronúcleos (MN) é um biomarcador confiável e adequado para avaliação da saúde humana, para o biomonitoramento ambiental, bem como para a avaliação de produtos naturais e sintéticos, tanto *in vitro* como *in vivo* (MANTOVANI et al., 2021).

O ensaio *in vivo* avalia a formação de micronúcleos em eritrócitos amostrados na medula óssea ou em células do sangue periférico de animais. Para tanto são usados, geralmente, roedores ou peixes (NEPOMUCENO et al., 1997; OECD, 2016). O ensaio do MN *in vitro* é realizado com uma variedade de células em cultura como, por exemplo, fibroblastos humanos e células de ovário de hamster chinês (MACHADO et al., 2019; MANTOVANI et al., 2021).

Os MN são expressos em células em divisão que contêm quebras de cromossomos sem centrômeros (fragmentos acêntricos) e/ou cromossomos inteiros que são incapazes de migrar aos polos durante a mitose e ficam para trás na anáfase (**Figura 4**). O mau funcionamento dos centrômeros ou cinetócoro dos cromossomos ou ainda danos na maquinaria mitótica, que inclui o fuso mitótico, necessário para a segregação dos cromossomos depois da replicação do DNA, podem levar à perda de cromossomos inteiros (aneugênese). Na telófase, um envelope nuclear forma-se em torno dos cromossomos e/ou fragmentos, que depois se desenrolam e gradualmente assumem a morfologia de um núcleo em interfase, com a particularidade de serem

menores que o núcleo principal da célula (assim o termo micronúcleo) e fornecer um índice confiável de quebra e perda de cromossomos (MANTOVANI et al., 2021). Assim, o MN é visualisado como um pequeno núcleo, fora do núcleo principal, encontrado no citoplasma.

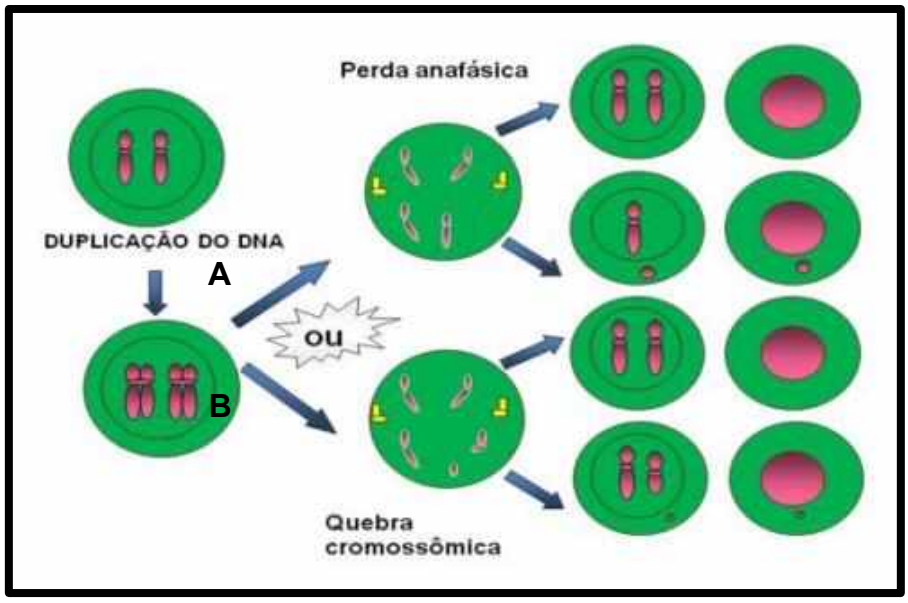

Figura 4. Esquema geral da formação de MN: **A**. Formação de MN após quebras de cromossomos sem centrômeros (fragmentos acêntricos); **B**. Formação de MN após atraso na migração mitótica, levando à aneugênese (perda de cromossomo inteiro). **Fonte:** Castilho et al. (2019).

3.1. Ensaio de micronúcleos (MN) em células da medula óssea *in vivo*

Para a realização dos ensaios de MN em células de medula óssea, geralmente utilizam-se cinco camundongos Swiss machos (7-8 semanas de idade) por grupo, com peso corporal (pc) de aproximadamente 30 g. Os animais são mantidos em microisoladores em uma sala experimental sob condições controladas de temperatura (24 ± 2 ° C) e umidade (50 ± 10%) em um ciclo claro-escuro de 12 horas, com ração padrão para camundongos e água disponíveis *ad libitum*. Para a avaliação da genotoxicidade/antigenotoxicidade, diferentes concentrações de determinado composto são administradas aos animais por gavagem ou intraperitonealmente, isoladamente ou em associação com um agente mutagênico de referência. Controles negativo (água) e solvente também são incluídos. Cinco animais são usados por grupo

de tratamento. Os animais são sacrificados com pentobarbital sódico, 48 horas após o tratamento e amostras de medula óssea são obtidas de acordo com OECD (2016).

Para realização do teste de MN, devem ser utilizadas, de preferência, células que se encontram em altas taxas de divisão, como os eritrócitos, pois além de apresentarem elevada rotatividade, não apresentam núcleo (anucleadas) e diferenciam-se durante o processo de maturação celular em eritrócito policromático (EPC – eritrócito jovem, RNA positivo com presença do ribossomo) e normocromático (ENC – eritrócito maduro, RNA ausente e presença de hemoglobina) (RIBEIRO et al., 2003).

Assim, o MN é facilmente observado em eritrócitos policromáticos (EPCs). O número de micronúcleos no EPC indica o grau de dano genético ao sistema eritropoiético dos seres vivos (MASFRIA et al., 2021). A **Figura 5** apresenta um esquema referente à origem do micronúcleo em células de medula óssea.

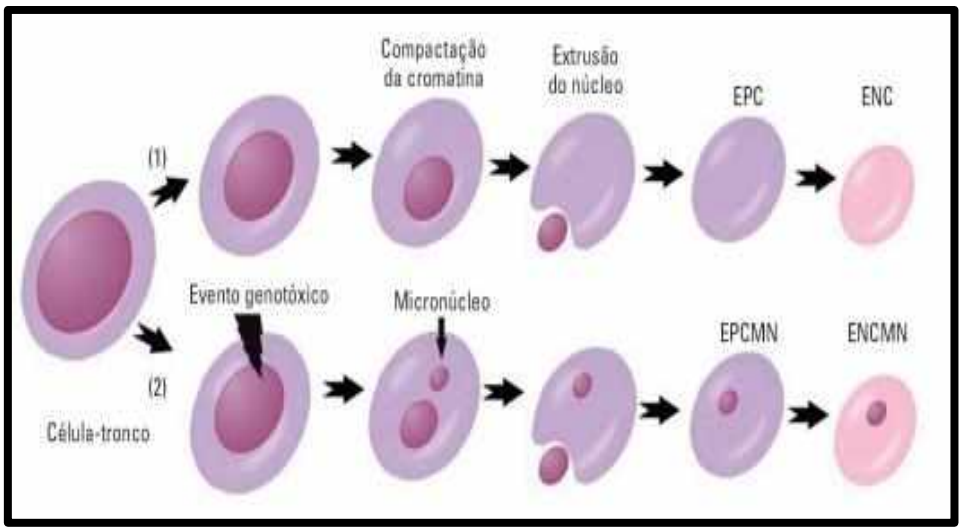

Figura 5. Esquema referente à origem do micronúcleo em células de medula óssea. (1) Durante a maturação do eritrócito, a cromatina compacta e o núcleo picnótico são perdidos, formando o eritrócito policromático (EPC); (2) quando há indução de dano ao DNA o micronúcleo surge do eritrócito nucleado e permanece após a extrusão nuclear formando o eritrócito policromático micronucleado (EPCMN). **Fonte:** Mantovani et al. (2021).

Um total de 4.000 eritrócitos policromáticos (EPCs) são analisados por animal para determinar a frequência de eritrócitos policromáticos micronucleados (EPCMNs).

A relação EPC/ENC (células normocromáticas) é calculada pela análise de 500 eritrócitos para determinar a citotoxicidade dos tratamentos. As lâminas são analisadas em teste cego, usando um microscópio óptico de luz com objetiva de imersão (1000x).

De acordo com Ribeiro et al. (2003), a coloração das lâminas com o corante Giemsa diluído em solução tampão fosfato em pH 6,8, na proporção de 1:10, durante 15 minutos, serve para diferenciar eritrócito policromático (EPC) de eritrócito normocromático (ENC). Eritrócitos policromáticos (EPC) ou jovens se coram de azul claro e eritrócitos normocromáticos se coram de vermelho-telha devido à presença da hemoglobina (**Figura 6**).

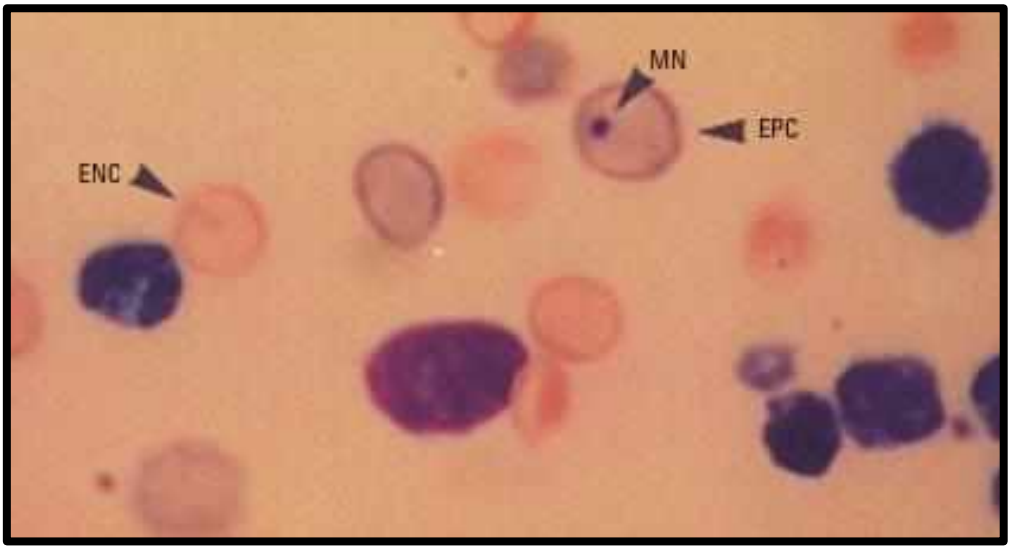

Figura 6. Micronúcleos em medula óssea. Observação em esfregaço de medula óssea de camundongo de eritrócito policromático (EPC) com micronúcleo (MN) e eritrócito normocromático (ENC). Coloração com Giemsa. Aumento: 1.000X. **Fonte:** Mantovani et al. (2021).

Não há consenso em relação ao teste estatístico utilizado na análise. Muitos trabalhos têm testado a normalidade dos dados para verificar se a distribuição de probabilidade associada ao conjunto dos dados pode ser aproximada pela distribuição normal. Caso se assuma uma distribuição normal: ƒ O teste t de student deve ser usado para comparar o tratamento, ao grupo controle negativo; ou ƒ A análise de variância (ANOVA) aplicada para determinar se as médias de três ou mais grupos diferem.

4. Análise bioquímica

Os níveis de creatinina, uréia, aspartato (AST) e alanina (ALT) aminotransferase são mensurados para determinar alterações na função hepato-renal de camundongos Swiss. A análise é realizada em equipamento automatizado (Mindray®) por meio de kits colorimétricos enzimáticos. O método do equipamento Mindray é baseado no princípio de absorção conforme descrito nos estudos de Machado et al. (2018).

Os resultados são avaliados estatisticamente por meio de análise de variância (ANOVA) e teste de Tukey, com $\alpha < 0,05$. O critério experimental é a significância da resposta em relação ao grupo controle. Todas as análises estatísticas são realizadas com o programa GraphPad Prism 5.0 (GraphPad Software) (MACHADO et al., 2018).

5. Somatic Mutation and Recombination Test – SMART

O teste para detecção de mutação e recombinação somática (Somatic Mutation and Recombination Test – SMART) em células de asas de *Drosophila melanogaster*, também conhecido como teste da mancha da asa (wing spot test), é um ensaio *in vivo* de curta duração, baixo custo, simples, versátil, de altas sensibilidade e reprodutibilidade, o qual permite avaliar e quantificar a indução de dano genético (mutação e recombinação) em células somáticas de moscas adultas depois da exposição larval. Ele tem sido empregado com sucesso na avaliação da atividade mutagênica de diferentes agentes, extratos e misturas complexas, assim como em estudos de antimutagenicidade (SPANÓ, 2021).

A *D. melanogaster* (**Figura 7**) é um organismo modelo eucarioto ideal para a pesquisa básica e estudos genéticos contemporâneos por ser um animal pequeno, com baixo número de cromossomos, fácil manutenção, ciclo de vida curto, grande progênie, genoma bem caracterizado, e por possuir um sistema enzimático semelhante ao dos mamíferos, que permite a metabolização de agentes xenobióticos. Muitas propriedades biológicas, fisiológicas e neurológicas básicas são conservadas entre os mamíferos e a *Drosophila melanogaster*. Acredita-se que, aproximadamente, 75% dos genes que causam doenças humanas tenham um homólogo funcional nessas moscas (CONTESTABILE et al., 2020; PANDEY e NICHOLS, 2011; YOUNES et al., 2020).

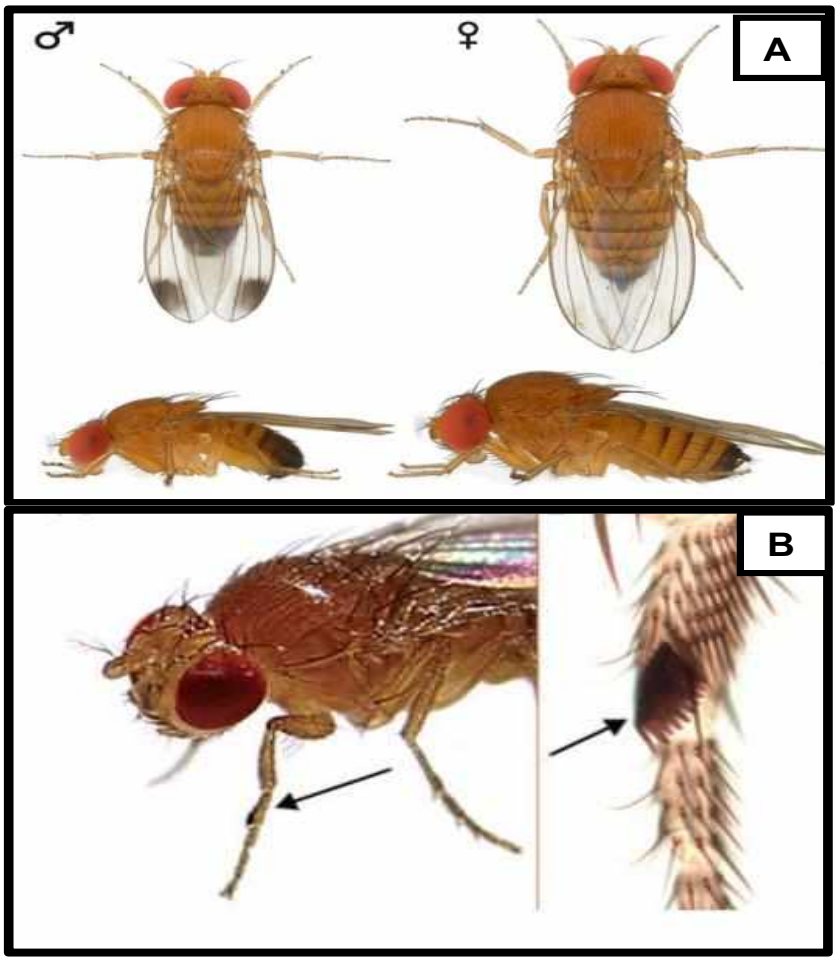

Figura 7. Representação da *D. melanogaster*. **A.** O macho (à esquerda) é menor que a fêmea e possui um pente sexual (tufo de pêlos) no primeiro par de patas. A fêmea (à direita) é maior e não apresenta pente sexual. **B.** Pente sexual (seta). **Fonte:** Oliveira et al. (2017).

O SMART de asas, desenvolvido por Graf et al. (1984) usando *D. melanogaster* como organismo modelo, tem sido amplamente utilizado na avaliação da toxicidade e mutagenicidade/antimutagenicidade de um grande número de agentes xenobióticos (NAVES et al., 2021; OLIVEIRA et al., 2017, 2020; OLIVEIRA et al., 2018; ORSOLIN et al., 2016; REIS et al., 2015; VASCONCELOS et al., 2017).

No SMART são utilizadas moscas portadoras dos genes marcadores *mwh* e *flr*^{3}, sendo que as alterações são detectadas pela perda da heterozigose desses genes. Durante o desenvolvimento embrionário da *D. melanogaster* as células dos discos imaginais proliferam mitoticamente para formar o corpo da mosca adulta.

Alterações genéticas, nas células dos discos imaginais de asas, levam à formação de células mutantes que são detectadas como manchas de pelos mutantes nas asas da mosca adulta. Assim, esse teste é considerado um teste rápido, de baixo custo, e que proporciona resultados confiáveis e altamente reproduzíveis (GRAF et al., 1984; SPANÓ et al., 2001).

5.1. Linhagens mutantes de *D. melanogaster*

Para realização do teste são utilizadas as seguintes linhagens mutantes de *D. melanogaster*: [1] multiple wing hairs (*mwh/mwh*); [2] flare-3 (*flr^3/In(3LR)TM3, ri p^p sep l(3)89Aa bx34^e and Bd^S*); [3] ORR; flare-3 (*ORR/ORR; flr^3/In(3LR)TM3, ri p^p sep l(3)89Aa bx34^e and Bd^S*). A linhagem ORR tem os cromossomos 1 e 2 de uma linhagem DDT-resistente Oregon R(R), responsáveis por altos níveis constitutivos de enzima citocromo P-450, o que torna o SMART mais sensível à ativação de promutágenos via citocromo (GRAF e VAN SCHAIK, 1992).

Essas linhagens são mantidas em frascos de vidro com meio de manutenção (ou seja, banana, sacarose, levedura e metilparabeno) sob ciclos claro/escuro (12 h: 12 h), a 25 ± 1ºC e aproximadamente 60% de umidade em câmara B.O.D.

5.2. Cruzamentos

Dois cruzamentos são realizados para produzir a progênie larval experimental: (1) Cruzamento padrão (ST): machos *mwh/mwh* cruzados com fêmeas virgens *flr^3/In (3LR) TM3, ri p^p sep l (3) 89Aabx^{34e}* e *Bd^s* (GRAF et al., 1984; 1989); (2) Cruzamento de alta bioativação (HB): machos *mwh/mwh* cruzados com fêmeas virgens ORR/*ORR; flr^3/In (3LR) TM3, ri p^p sep l (3) 89Aabx^{34e}* e *Bd^S* (GRAF e VAN SCHAIK, 1992).

Esses cruzamentos produzem dois tipos de descendentes: 1) trans-heterozigoto marcado (MH) (*mwh + / + flr^3*), com asas fenotipicamente do tipo selvagem; e 2) heterozigoto balanceado (BH) (*mwh + / + TM3, Bd^S*) com asas fenotipicamente serrilhadas (**Figura 8**). Esses descendentes são fenotipicamente distintos devido ao marcador TM3, *Bd^S*. Informações detalhadas sobre os símbolos genéticos podem ser encontradas em Lindsley e Zimm (1992).

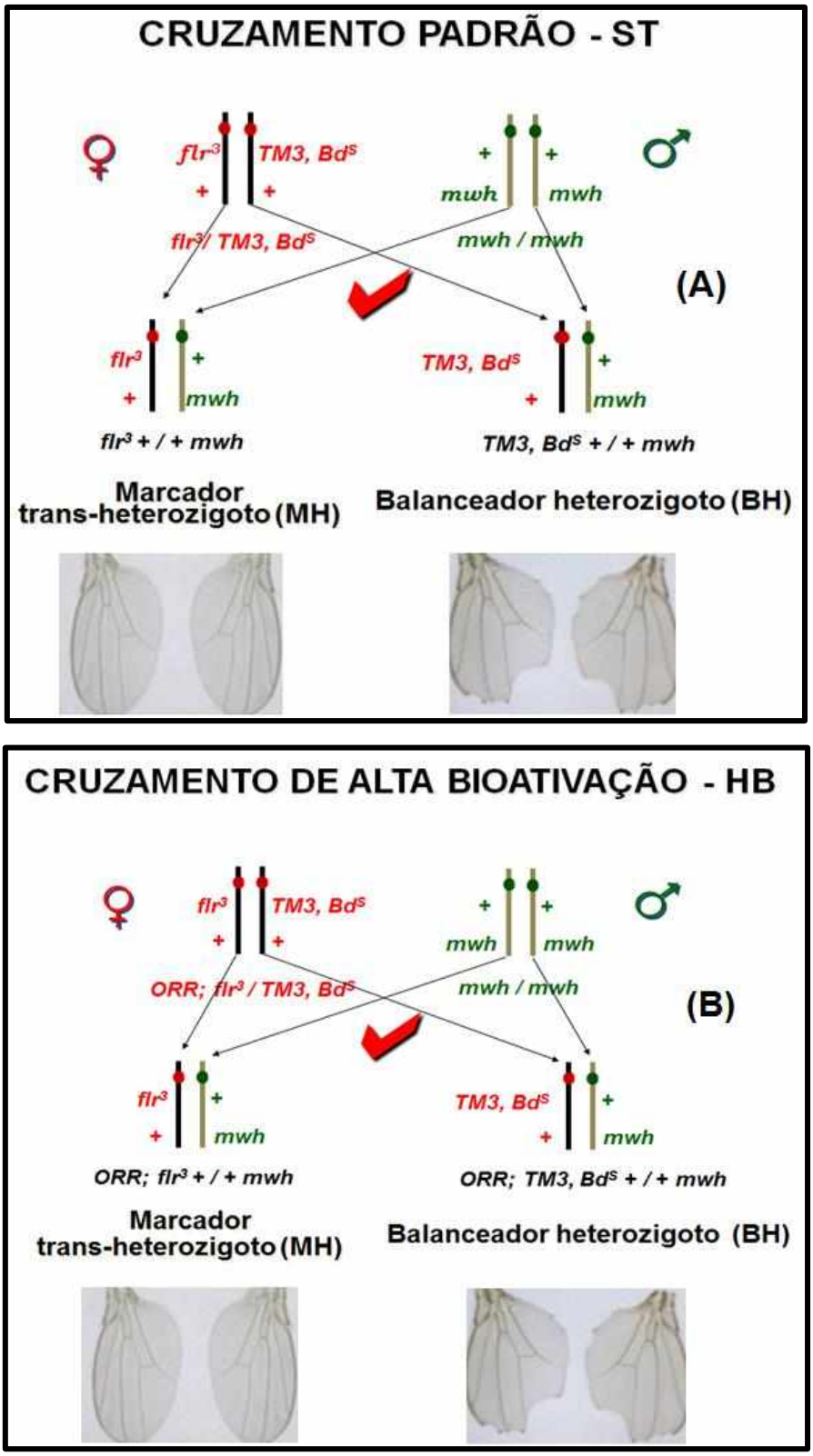

Figura 8. Representação dos cruzamentos realizados no SMART: **(A)** Cruzamento Padrão (ST); **(B)** Cruzamento de Alta Capacidade de Bioativação (HB). **Fonte:** Oliveira et al. (2017).

5.3. Procedimento experimental

Conforme descrito nos estudos de Oliveira et al. (2020), os ovos são coletados durante 8 h em frascos contendo uma base sólida de ágar (ágar 4% em água) e uma camada de levedura (*Saccharomyces cerevisiae*) suplementada com sacarose. Após 72 ± 4 h, as larvas de 3º ínstar são lavadas com água corrente e coletadas com uma peneira de malha fina.

Para realização do tratamento de mutagênese e/ou antimutagênese, grupos de aproximadamente 100 larvas são transferidos para tubos de vidro (2,5 cm de diâmetro e 8,0 cm de altura) contendo 1,5 g de meio de cultura alternativo, preparado com purê de batata instantâneo (segundo Spanó et al., 2001), Yoki® Alimentos SA, e 5,0 mL de diferentes concentrações do composto a ser testado isolado ou em associação com um mutágeno de referência (para co-tratamentos). Os controles também são incluídos: (1) controle negativo (água ultrapura); (2) controle solvente (1% Tween 80) caso necessário; e (3) controle positivo (mutágeno). As concentrações são selecionadas previamente com base em testes de sobrevivência (toxicidade). Para tanto, grupo de 100 larvas são contadas e distribuídas em duas séries de frascos. O número de moscas adultas emergentes é usado para calcular as taxas de sobrevivência após a exposição e o teste qui-quadrado é realizado para comparações estatísticas das taxas de sobrevida para amostras independentes (DE REZENDE et al., 2013).

As moscas adultas emergentes dos diferentes tratamentos são coletadas e colocadas em etanol 70%. As asas são retiradas das moscas, embebidas em solução de Faure (30 g de goma arábica, 20 mL de glicerol, 1,5 g de hidrato de cloral e 5,0 mL de água destilada) e dispostas em lâminas de microscopia. Em seguida, as lâminas são secas por 1h em placa aquecida (40 °C). As lâminas são cobertas com lamínulas e secas à temperatura ambiente.

As asas são examinadas em microscópio óptico de luz (400X) para registrar o número e tipos de manchas, bem como seu tamanho e posição ao longo da asa. São analisadas aproximadamente 24.400 células por asa, ou seja, 48.800 células por indivíduo.

Em asas de indivíduos MH, três categorias diferentes de manchas podem ser observadas: (1) manchas simples pequenas (1–2 células), (2) manchas simples grandes (mais de duas células), expressando pelos múltiplos ou o fenótipo flare; (3)

manchas gêmeas, consistindo em ambos os subclones mwh e flr. Em asas de indivíduos BH, apenas manchas simples mwh podem ser observadas, uma vez que os mesmos não são portadores do gene *flr*^{3} (**Figura 9**).

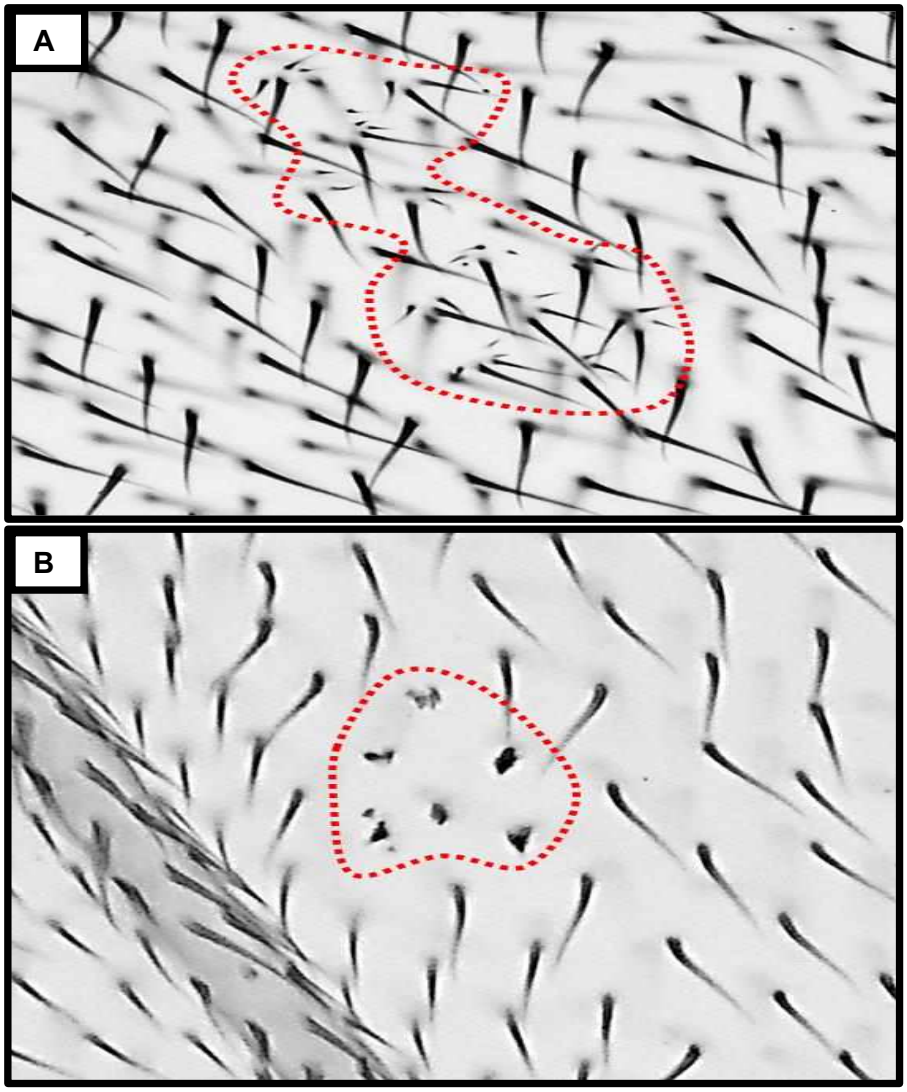

Figura 9. Fotomicrografia de asas de *D. melanogaster* ao microscópio óptico de luz (aumento de 400x) mostrando pelos normais e: **A.** Pelos múltiplos e **B.** Pelos flare (contornados em vermelho).

5.4. Análise estatística

A análise estatística para possíveis efeitos mutagênicos / recombinogênicos e antimutagênicos / antirecombinogênicos é realizada usando o teste binomial condicional de Kastenbaum e Bowman (1970). Frei e Würgler (1988) descreveram

esse procedimento e os resultados são classificados como positivos, fracamente positivos, negativos ou inconclusivos, com $\alpha \leq 0,05$. A porcentagem de mutação (M) é calculada por meio da frequência de clones em moscas BH / frequência de clones em moscas MH x 100. A porcentagem de recombinação (R) = 100 - M. A frequência total de manchas (FT) é calculada dividindo-se o total de manchas observadas nas moscas MH (considerando manchas mwh e flr) pelo número de moscas (SANTOS et al., 1999; SINIGAGLIA et al., 2004, 2006). Com base nas frequências de manchas corrigidas pelo controle por 10^5 células, o percentual de inibição de determinado composto é calculada como: {[mutágeno sozinho - (mutágeno + composto) / mutágeno sozinho]} x 100 (ABRAHAM, 1994).

6. Teste de tumores epiteliais (Epithelial Tumor Test – ETT)

O teste de tumores epiteliais **(**Epithelial Tumor Test – ETT**),** também realizado em *D. melanogaster*, é utilizado para avaliação do efeito carcinogênico/anticarcinogênico de diferentes agentes químicos naturais ou sintéticos. Neste teste são empregadas moscas portadoras de genes marcadores *mwh* e *wts*, sendo que a formação de tumores também é detectada pela perda da heterozigose desses genes (NEPOMUCENO, 2015).

Durante o desenvolvimento embrionário inicial da *D. melanogaster*, grupos de células (discos imaginais) são reproduzidos e se ocorrer alteração genética em uma dessas células, essa alteração estará presente em todas as células descendentes e formará um clone de células mutantes (tumor) (JUSTICE et al., 1995; NEPOMUCENO, 2015; SIDOROV et al., 2001).

6.1. Linhagens mutantes de *D. melanogaster*

Duas linhagens mutantes de *D. melanogaster* são usadas para investigar a carcinogenicidade de determinado composto quando administrado sozinho, ou sua anticarcinogenicidade quando administrado simultaneamente com um carcinógeno de referência: [1] *multiple wing hairs* (constituição genética: *mwh/mwh; y; mwh jv,* 3 [3–0.3]); e [2] “warts” (constituição genética: *st* [1] *in*[1] *kni*[*ri*-1] *p*[*p*] *wts* [3–17]/TM3, *Sb*[1]). As linhagens são mantidas nas condições citadas no item 5.1.

6.2. Cruzamentos e tratamentos

Fêmeas virgens *wts*/TM3, *Sb1* são cruzadas com machos *mwh/mwh* (NEPOMUCENO, 2015). Deste cruzamento são obtidos dois tipos de descendentes: [1] Descendentes trans-heterozigotos marcados (*wts* + / + *mwh*); [2] Descendentes heterozigotos balanceados (*TM3*, sb^1 + / + *mwh*) (**Figura 10**).

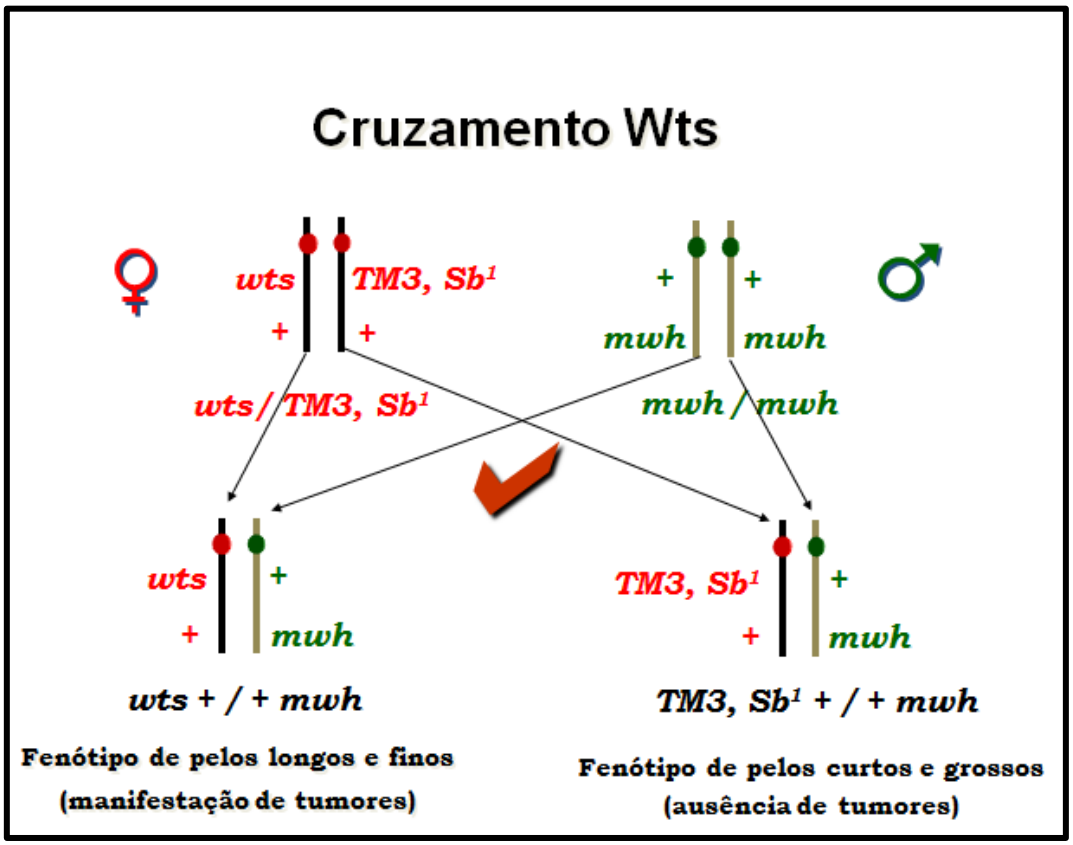

Figura 10. Representação do cruzamento realizado no ETT: Fêmeas virgens *wts/TM3,* Sb^1 cruzadas com machos *mwh/mwh*; sendo os descendentes *wts/mwh* caracterizados pelo fenótipo de pelos longos e finos, e os descendentes *TM3,*Sb^1*/mwh* caracterizados pelo fenótipo de pelos curtos e grossos. **Fonte:** Oliveira et al. (2017).

6.3. Análise das moscas

Moscas adultas emergentes são coletadas e armazenadas em frascos contendo etanol 70%. A análise dos indivíduos é realizada em lâminas côncavas contendo glicerol, sob microscópio estereoscópico, para visualização e contagem dos

tumores. Os tumores epiteliais podem aparecer em diferentes partes da mosca, incluindo olhos, cabeça, asas, corpo, pernas e halteres (**Figura 11**). A presença de tumores é avaliada e registrada em uma planilha padrão.

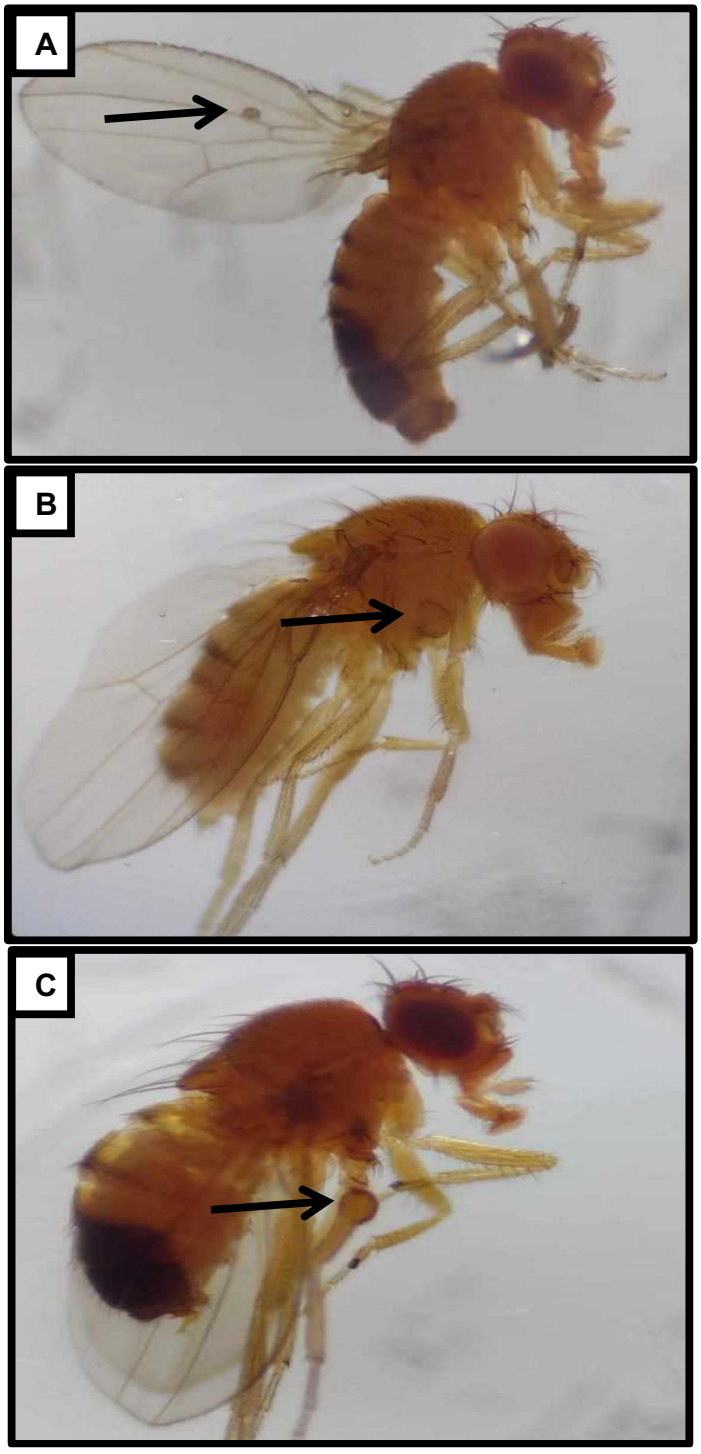

Figura 11. Expressão de tumors nos diferentes segmentos da *D. melanogaster* (indicado pelas setas). **A.** asa. **B.** torax. **C.** perna. **Fonte:** Oliveira et al. (2017).

6.4. Análise estatística

O potencial carcinogênico / anticarcinogênico do agente testado é validado pelo teste U não paramétrico de Mann, Whitney e Wilcoxon, com nível de significância $\alpha = 0,05$.

7. Ensaios de citotoxicidade *in vitro*

Os ensaios *in vitro* com células tumorigênicas e não-tumorigênicas humanas são uma excelente alternativa para avaliar a atividade antineoplásica de diferentes compostos (KLEINPOPPEN et al., 2019). Diferentes linhagens celulares humanas podem ser usadas para este propósito, em especial células de câncer de mama.

De acordo com estatísticas mundiais, o câncer de mama feminino ultrapassou o câncer de pulmão e se tornou o tumor mais comum, com aproximadamente 2,3 milhões de novos casos (11,7%) (SIEGEL et al. 2020; SUNG et al. 2021). O câncer de mama costuma ser acompanhado de alta taxa de recorrência, de modo que a busca por medicamentos específicos para tratá-lo continua indispensável (QI et al., 2021).

Linhagens celulares epiteliais não-tumorigênicas de mama humana (MCF-10A) e células tumorais de mama triplo negativas, da linhagem MDA-MB-231, foram empregadas com sucesso em estudos de citotoxicidade usando o ensaio de MTT [3-(4,5-dimetiltiazol- -2yl)-2,5-difenil brometo de tetrazolina] (CHEN et al., 2021; YURASAKPONG et al., 2021). O MTT é um ensaio colorimétrico utilizado para determinar o potencial citotóxico de diferentes substâncias e é um indicador de ativação antiproliferativa, ativação celular e citotoxicidade (SHARIF et al., 2017).

A avaliação do dano celular induzido por um agente qualquer ocorre pela análise da atividade de enzimas desidrogenases mitocondriais. A viabilidade mitocondrial e, consequentemente, a viabilidade celular, é então mensurada pela redução do MTT (sal amarelo solúvel em água) a formazan (cristais insolúveis de coloração púrpura), em células metabolicamente ativas (**Figura 12**). Dessa forma, a redução do MTT a formazan é diretamente proporcional à atividade mitocondrial e à viabilidade celular (MAGALHÃES et al., 2018).

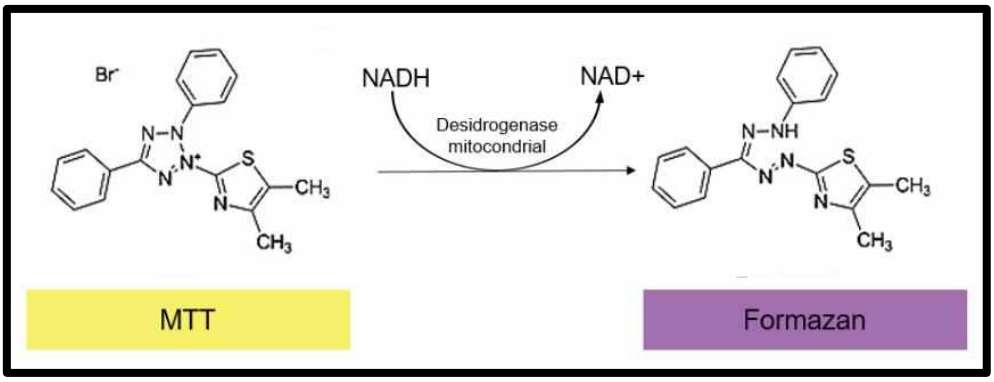

Figura 12. Reação de redução do MTT (um sal de coloração amarela e solúvel em água) a formazan (sal de coloração arroxeada e insolúvel em água). **Fonte:** Magalhães et al., (2018).

Por sua vez, a viabilidade também pode ser observada por meio da liberação de lactato desidrogenase (LDH) (WANG et al., 2017; BRAGA et al., 2018; COSTA et al., 2020). Parte do sobrenadante da cultura de células pode ser usada para medir LDH, uma enzima que catalisa a conversão de glicose em ácido láctico e que tem atividade irregular em células cancerosas (FORKASIEWICZ et al., 2020). Assim, a liberação de LDH do citoplasma celular para o meio de cultura é o resultado do dano à membrana celular e da lise celular, sendo proporcional ao número de células mortas por necrose (PAWLAK et al., 2021).

7.1. Linhagens celulares e condições de cultura

Células MCF-10A (não-tumorigênicas) e TNBC MDA-MB-231 (tumorigênica) são cultivadas em meio DMEM / F-12 suplementado com 10 µg / mL de EGF, 0,25 µg / mL de hidrocortisona, 10 µg / mL de insulina e meio L15. Todas as cepas são suplementadas com 10% de soro fetal bovino (FBS), 50 µg / mL de gentamicina e mantidas a 37 °C. A linhagem MCF-10A também é mantida em uma atmosfera de 5% de CO_2 (Thermo Scientific ™ Forma Series 3 Water Jacketed CO_2 Incubator). Para a linhagem celular MDA-MB-231, os frascos são fechados, livres de CO_2. O meio deve ser trocado em dias alternados, até que as células atinjam 80-90% de confluência, quando são utilizadas nos ensaios subsequentes. As linhagens celulares são obtidas na American Type Culture Collection e confirmadas como isentas de contaminação por micoplasma.

7.2. Ensaio citotóxico – MTT

O ensaio do brometo de 3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio (MTT) é realizado de acordo com Gerlier e Thomasset (1986) com modificações conforme o esquema resumido apresentado na **Figura 13**. As células são semeadas em placas de 96 poços a uma concentração de 1,5 x 10^4 células por poço e incubadas durante 24 h para aderência. Após esse período, ambas as linhagens são tratadas com diferentes concentrações do composto a ser testado por 24 e 48 h. Solução de MTT preparada em meio transparente (5 mg / mL) é adicionada a cada poço e incubada a 37 ˚C por mais 4 h.

O sobrenadante é cuidadosamente descartado. Os cristais de formazan insolúveis produzidos pela desidrogenase intracelular são solubilizados com DMSO e a absorbância (Ab) de cada poço é lida a 570 nm usando um espectrofotômetro. Cada amostra é medida em triplicata e cada experimento deve ser repetido três vezes (n = 3).

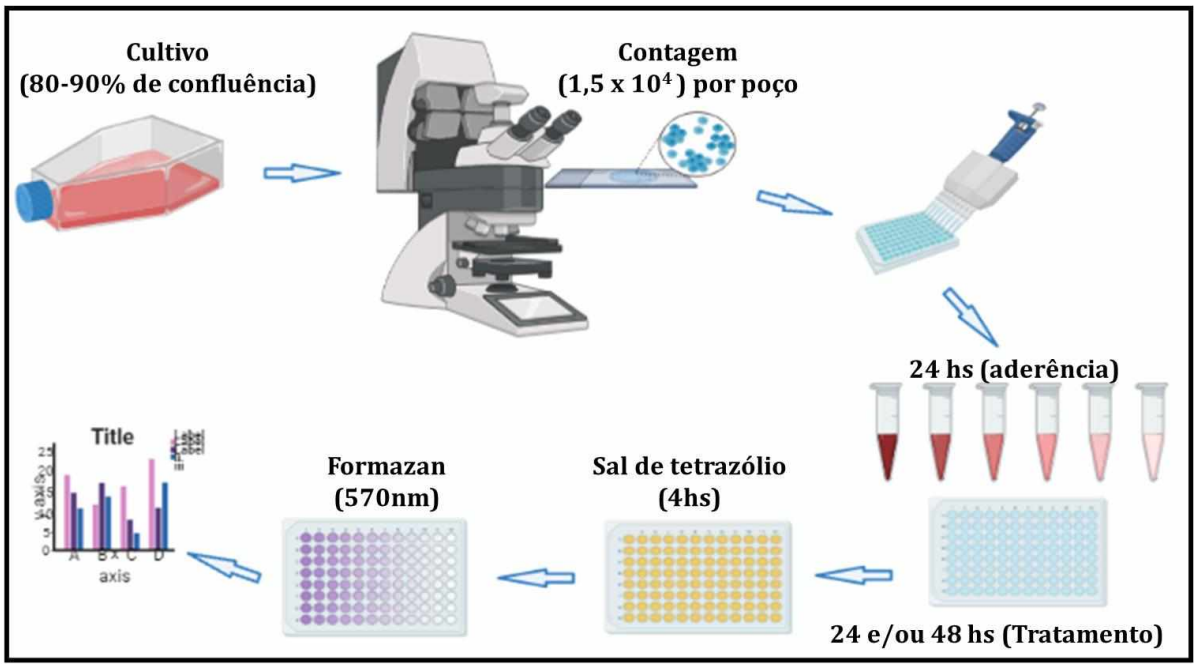

Figura 13. Esquema resumido dos procedimentos realizados no ensaio de MTT.

7.3. Ensaio de liberação de LDH

O ensaio de LDH é utilizado para complementar os resultados do MTT e baseia-se na liberação da enzima LDH para o meio de cultura após dano à membrana celular. Normalmente esta enzima é encontrada no citoplasma das células, no entanto, quando a célula sofre um dano que cause o ruptura de sua membrana, sua viabilidade é afetada e ocorre liberação dessa enzima para o meio extracelular (ASLANTÜRK, 2018).

Assim, a liberação de LDH é um indicador de dano celular (KOROTH et al., 2019). Para medir a liberação de LDH no sobrenadante celular, o ensaio é realizado utilizando o kit CyQUANTTM LDH Cytotoxicity Assay (Invitrogen), de acordo com as recomendações do fabricante. As células tumorigênicas são semeadas em placas de 96 poços, 1,5 x 10^4 células por poço e em triplicata. As células são tratadas com as diferentes concentrações do composto a ser testado, que são definidas com base no valor IC50. As absorvâncias são lidas em um leitor de placas a 492 nm. A lise celular é quantificada pela razão de aumento de LDH no sobrenadante das células tratadas em comparação com as células de controle lisadas.

7. 4. Análise estatística

Os dados são apresentados como média ± desvio padrão (DP) dos três experimentos independentes. As diferenças entre as linhagens de células tratadas com o composto em questão são determinadas usando ANOVA de uma via e testes post hoc de Tukey. $\alpha < 0,05$ é considerado significativo.

8. Análises *In silico*

Ensaios *in silico* são empregados na obtenção e/ou análise de dados utilizando simulações computacionais. Além disso, otimizam e economizam tempo e recursos concentrando a atenção na estrutura química de alvos de interesse. Esses ensaios têm sido amplamente utilizados por pesquisadores envolvidos com a produção de novos fármacos a fim de fornecer previsões de interações moleculares mais significativas e correlacionar com resultados experimentais *in vivo* e *in vitro*. Nesse contexto, o docking molecular está entre os ensaios *in silico* mais populares e

bem-sucedidos, que ajudam a prever as interações que ocorrem entre moléculas e alvos biológicos (WALKER, 2009; PINZI; RASTELLI, 2019).

O Docking molecular (DM) é um método de design de fármacos que baseia-se na simulação da interação molecular e prevê o modo de ligação e a afinidade entre receptores e ligantes. O objetivo do acoplamento ligante-proteína é prever o(s) modo(s) de ligação predominante(s) de um ligante com uma proteína de estrutura tridimensional conhecida (MORRIS et al., 2008).

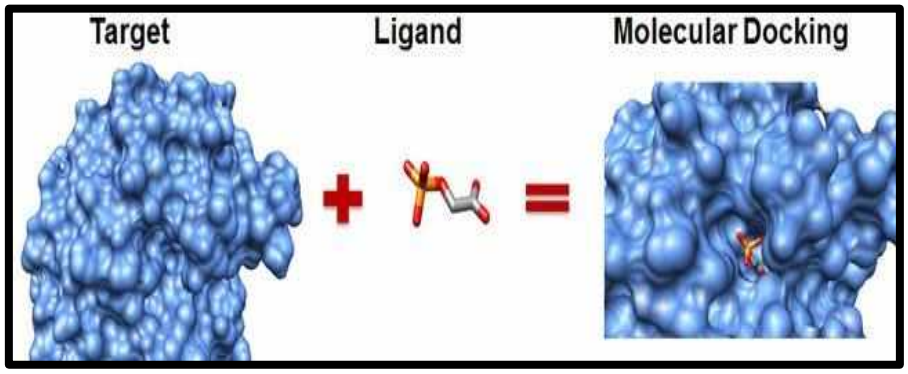

Figura 14. Elementos em docking molecular. **Fonte:** Hernández-Santoyo et al. (2013).

Assim, estudos complementares de DM são úteis para uma previsão mais confiável de atividades biológicas (KAUR DHANJAL et al., 2014). Muitas funções de pontuação e várias metodologias de docking podem estimar a interação da proteína de ligação e, de acordo com essas informações estruturais, pode-se prever informações de alvos específicos com maior afinidade de ligação, com mais rapidez e menor custo (HAMMOUDI et al., 2020).

8.1. Pesquisa de potenciais alvos

A busca por potenciais alvos para determinado composto é realizada usando diferentes ferramentas, como a Swiss Target Prediction (DAINA et al., 2019). Esta ferramenta estima a similaridade 1D e 2D do composto submetido com uma biblioteca de 370.000 compostos ativos em mais de 3.000 proteínas de diferentes espécies e, finalmente, sugere seus alvos mais prováveis. Para esta busca, a estrutura 2D do composto é obtida do banco de dados PUBCHEM (CID 64971).

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CAPÍTULO II

BETULINIC ACID MODULATES URETHANE-INDUCED GENOTOXICITY AND MUTAGENICITY IN MICE AND *Drosophila melanogaster*

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Betulinic acid modulates urethane-induced genotoxicity and mutagenicity in mice and *Drosophila melanogaster*

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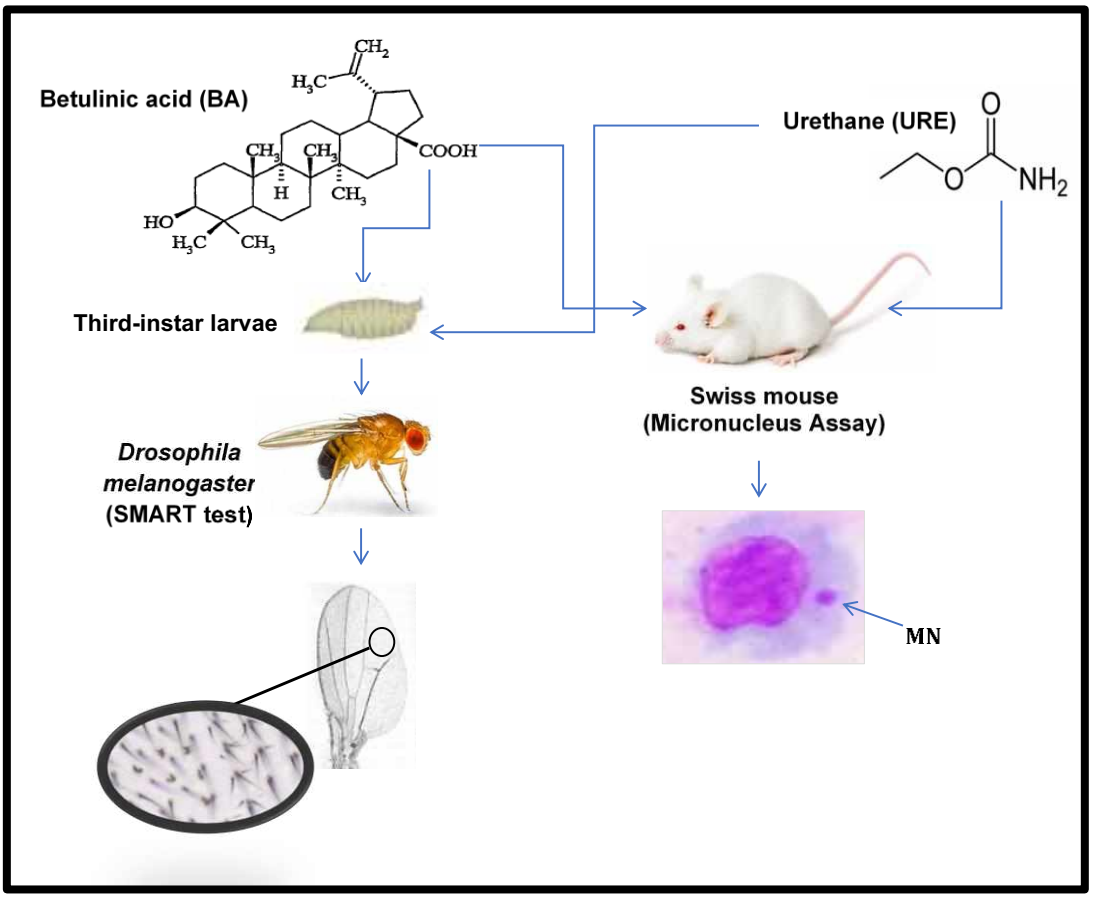
Graphical abstract



Highlights

- BA was not toxic neither genotoxic to mice.

- BA has modulatory effects on the genotoxicity induced by URE in mice.

- BA was not toxic neither mutagenic to *D. melanogaster*.

- BA has modulatory effects on the mutagenicity induced by URE in somatic cells of *Drosophila*.

- Modulatory effects of BA may be due to its antioxidant and apoptotic properties.

ABSTRACT

Betulinic acid (BA) is a pentacyclic triterpenoid found in several plant species. Urethane (URE) is a known promutagen. Here, we examine the genotoxicity and mutagenicity of BA alone or in combination with URE using the bone marrow micronucleus assay in mice bone marrow cells and the Somatic Mutation and Recombination Test in *Drosophila melanogaster*. Findings revealed that BA alone was not genotoxic but reduced the frequency of micronucleus when compared to the positive control. No significant differences were observed in the cytotoxicity. Biochemical analyzes showed no significant differences for liver (AST and ALT) or renal (creatinine and urea) function parameters, indicating the absence of hepatotoxic and nephrotoxic effects. BA alone did not increase the frequency of mutant spots but reduced the total frequency of mutant spots when co-administered with URE in both ST and HB crosses. In addition, BA reduced the recombinogenic effect of URE at the highest concentrations of both crosses. In conclusion, under experimental conditions, BA has modulatory effects on the genotoxicity induced by URE in mice, as well as in somatic cells of *D. melanogaster*. We suggest that the modulatory effects of BA may be mainly due to its antioxidant and apoptotic properties.

Keywords: Antigenotoxicity. Antimutagenicity. Cytochrome P450. Micronucleus test. Somatic Mutation and Recombination Test. SMART.

1. Introduction

Betulinic acid (BA) is a naturally occurring pentacyclic triterpene belonging to the class of lupan, being found in food, plants and medicinal herbs, especially birch bark, widely distributed throughout the world, which has a carboxylic acid group at the C-28 position (Flekhter et al., 2002; Martin et al., 2016). Triterpenes form a large group of compounds that have 30 carbon atoms distributed in five rings, to which are attached various oxygen atoms (Alakurtti et al., 2006).

Several natural products with pharmaceutical perspectives are included in this group, highlighting BA, due to its known anti-inflammatory (Araújo et al., 2019; Costa et al., 2014; Khan et al., 2018; Wang et al., 2019), analgesic (Oyebanji et al., 2014), antiviral (Dang et al., 2013; Visalli et al., 2015; Karagöz et al., 2019), antibacterial (Mullauer et al., 2010; Bildziukevich et al., 2019), antioxidant (Lingaraju et al., 2015, Yi et al., 2016; Lv et al., 2018; Nile et al., 2019; Wu et al., 2019) and apoptotic activities (Potze et al., 2016a; Khan et al., 2016; Shankar et al., 2017), in addition to its potential as anticancer agent by inhibition of topoisomerase (Zhang et al., 2015, 2016; Król et al., 2015).

A review of the physicochemical, pharmacokinetic, and toxicological properties of BA, including mutagenicity and carcinogenicity is presented by Khan et al. (2018). The potential molecular mechanisms of action and medical applications of BA, including previous studies of its anticancer activity, with listed cancer cell types susceptible to therapy is presented by Hordyjewska et al. (2019). However, the genotoxicity and modulatory effects of BA on the genotoxicity induced by different mutagens are still controversial (Acésio et al., 2016). Previous studies have shown that BA was unable to induce genotoxic/mutagenic effects after analysis by the Ames test and SOS chromotest (Frolova et al., 2015); somatic mutation and recombination test (SMART) in *Drosophila melanogaster* (Freitas et al., 2015); cytokinesis-block micronucleus assay using V79 cells (Acésio et al., 2016); and *Salmonella*/microsome test (Yoshida et al., 2016). Other investigations demonstrated that BA induces DNA damage and apoptosis (Goswami et al., 2018).

On the other hand, antigenotoxic/antimutagenic activities of BA were demonstrated when it was used in co-treatment trials with methyl methanesulfonate (MMS) through the cytokinesis-block micronucleus assay with V79 cells (Acésio et al., 2016); human lymphocytes incubated with BA followed by an exposure to either MMS

or H_2O_2 (Goswami et al., 2019); SMART with *D. melanogaster* (Freitas et al., 2015); and SOS response in *Staphylococcus aureus* (Carvalho Júnior et al., 2019).

In recent years, the interest of researchers in investigating the genotoxic/mutagenic and antigenotoxic/antimutagenic potentials of natural products has increased markedly, as knowledge of such effects may be useful in preventing possible adverse effects on human health, as well as in preventing different types of cancer. The discovery of new products that induce or reduce mutation rates would certainly decrease the incidence of diseases (Bhattacharya, 2011). The evaluation of these effects on the genetic material encompasses several *in vivo* tests, including the bone marrow micronucleus test (MNT) and the Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster*.

The mammalian *in vivo* MNT developed by Schmid (1975) is widely used as a screening test for detection of structural or numerical chromosomal anomalies induced by clastogenic and/or aneugenic substances, which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes (OECD, 2016). The mice and rat bone marrow MNT has been successfully used to detect the genotoxic/antigenotoxic potential of chemical compounds (Alves et al., 2018; Belgacem et al., 2019; Carnizello et al., 2019; Díez-Quijada et al., 2019; Fernandes et al., 2019).

Non-mammalian animals, such as the common fruit fly *Drosophila melanogaster*, have shown to be excellent *in vivo* models for genotoxicity tests due to their quick reproductive cycles, greater ethical acceptance and lower need for infrastructure (Lombardot et al., 2015). Besides that, *Drosophila* has been used as a model organism to study human disease because many biological, physiological and neurological properties are conserved between mammals and fly (Huo et al., 2014). Nearly 75% of human disease-causing genes are believed to have a functional homolog in the fly (Pandey and Nichols, 2011).

SMART has been successfully used to detect mutagenic/recombinogenic as well as the modulatory effects of many hundred chemical substances on the DNA damage (Orsolin et al., 2016; Oliveira et al., 2017; Cardozo et al., 2019; Naves et al., 2019). In addition to presenting highly reliable and reproducible results, it allows to quantify the recombinogenic activity against the mutagenic activity of different compounds and mixtures (Spanó et al., 2001). Thus, although BA has been previously evaluated through SMART (Freitas et al., 2015), we used the short-term *in vivo* SMART in *D.*

melanogaster (Graf et al., 1984; Graf and van Schaik, 1992) as a model for the study of the mutagenic/recombinogenic or antimutagenic/anti-recombinogenic activities associated with BA exposure, since in the present work we considered different concentrations and a high number of individuals in order to estimate precisely the contribution of mutation and recombination to the total observed spots.

Ethyl carbamate (also called urethane - URE), considered as positive control in this study, is an ester of carbamic acid that induces the production of reactive oxygen species (ROS), DNA depurination and mitochondrial dysfunction, hence causing DNA damage and promoting the formation of tumors (Chun et al., 2013; Liu et al., 2017). Previous studies have confirmed the effect of this compound on the formation of micronuclei in mice (Premkumar et al., 2004; Stankowski et al., 2015; Koul and Abraham, 2018) and induction of mutations in *Drosophila* wings, therefore being commonly used as positive control in the SMART assay (De Rezende et al., 2013; Machado et al., 2016; Morais et al., 2016; Naves et al., 2018, 2019; Reis et al., 2016). As previous studies have shown that URE induce oxidative stress (Liu et al., 2017) and BA is an antioxidant agent (Lingaraju et al., 2015; Yi et al., 2016; Lv et al., 2018), in the present study URE was associated with BA for antigenotoxicity tests.

Thus, the aim of the present study was to investigate the antigenotoxic potency of BA against URE-induced genotoxicity using the mice bone marrow MNT, as well as the mutagenicity and recombinogenicity of BA when administered alone or simultaneously with URE using SMART.

2. Material and methods

2.1. Chemical agents

Betulinic acid (BA) (3β-hydroxy-lup-20(29)-en-28-oic acid), CAS: 472-15-1 (# STBF5901V); dimethylsulfoxide (DMSO), CAS: 67-68-5; Tween 80, CAS: 67-68-5; and ethyl carbamate, CAS: 51-79-6 (#WXBC6663V) were obtained from Sigma-Aldrich. The structural formulas of BA and URE are shown in **Fig. 1**.

2.2. Mice bone marrow MNT

2.2.1. Animals and experimental design

Male Swiss mice (Mus musculus) (7–8 weeks old) weighing 25–30g were obtained from the animal house of the University of São Paulo, Ribeirão Preto, São Paulo, Brazil. Animals were kept in micro isolators in an experimental room under controlled conditions of temperature (24 ± 2 °C) and humidity (50 ± 10%) on a 12 h light-dark cycle, with standard mouse chow and water being available ad libitum. This study was approved by the Animal Use Ethics Committee of the University of Franca, Franca, SP, Brazil, under the protocol number 8768010319. Five animals were used per treatment group. Different BA doses (0.25; 0.5 and 1 mg/kg b.w.) were administered to animals by gavage (0.3 mL/animal).

The BA concentrations were chosen based on literature (Wu et al., 2019). The mutagen urethane (URE, 400 mg/kg b.w.) was administered intraperitoneally (i.p.; 0.3 mL/animal). Negative (water) and solvent (DMSO – dimethylsulfoxide, 1%) groups were also included. Therefore, two treatments were performed, administered at 24-h intervals. The bone marrow samples were obtained 24 h following the final treatment (OECD 474, 2016). Animals were euthanized with sodium pentobarbital (840 mg/kg b.w., i.p.) at the end of the treatment periods. The three independent experiments were carried out, with about two animals per treatment group.

2.2.2. MNT

The bone marrow micronucleus assay was performed according to OECD 474 (2016). A total of 4,000 polychromatic erythrocytes (PCEs) were analyzed per animal to determine the frequency of micronucleated polychromatic erythrocytes (MNPCEs). The PCEs: total erythrocytes (PCEs + NCEs [normochromatic erythrocytes]) ratio was calculated by the analysis of 500 erythrocytes in order to determine the cytotoxicity of the treatments. Slides were scored blindly using a light microscope with a 1,000X immersion objective.

2.2.3. Biochemical analysis

Blood samples were collected by cardiac puncture under anesthesia (sodium pentobarbital, 45 mg/kg b.w., i.p., 0.3 mL). Serum alanine aminotransferase (ALT, Modified Glutamate Pyruvate Transaminase Kit, IFCC - REF: D98624; DIALAB) and aspartate aminotransferase (AST, Modified Glutamate Oxaloacetate Transaminase Kit, IFCC - REF: D98616; DIALAB) activities were measured to assess hepatotoxicity. Renal function was evaluated by the measurement of creatinine (Enzymatic Creatinine Assay Kit, PAP - REF: D95704; DIALAB) and urea (Urea UV Auto Urease Kit, GLDH - REF: D95595; DIALAB). For this purpose, approximately 400 µL blood was collected from the heart of euthanized animals and the samples were centrifuged for 15 min at 3,000 rpm for the separation of plasma. Serum levels of urea and creatinine were assessed 48 h after the last treatment, whose experimental design is in accordance with OECD 474. The samples were analyzed in an automated Mindray BS 200® spectrophotometer based on the principle of absorption (wavelength of 340 nm to urea, ALT and ASP; and 540 nm to creatinine).

2.2.4. Statistical analysis

The results were evaluated by analyses of variance (ANOVA) and Tukey test at $\alpha < 0.05$. The experimental criteria were the significance of the response in relation to the control group. All statistical analyses were performed using the GraphPad Prism 5.0 program (GraphPad Software).

2.3. Somatic mutation and recombination test – SMART

2.3.1. Strains and stock

The following strains of *D. melanogaster* were used: [1] multiple wing hairs (*mwh/mwh*); [2] flare-3 (*flr*^{3}/*In*(3LR) TM3, *ri p*^{p} *sep l (3) 89Aa bx34*^{e} *and Bd*^{S}); [3] ORR; flare-3 (ORR/ORR; *flr*^{3}/*In*(3LR) TM3, *ri p*^{p} *sep l (3)89Aa bx34*^{e} *and Bd*^{S}). These strains were maintained in glass vials filled with a maintenance medium (i.e., banana, agar-agar, yeast, methylparaben and penicillin/streptomycin) under light/dark cycles (12 h:12 h), at 25 ± 1 °C and approximately 60% humidity in a BioOxygen Demand-type

chamber (Model: SL224, SOLAB – Equipamentos para Laboratórios Ltda., São Paulo, SP, Brazil).

2.3.2. Crosses and treatments

Two crosses were carried out to produce the experimental larval progeny: (1) Standard (ST) cross: *mwh/mwh* males crossed with *flr*^{3}*/In* (3LR)TM3, *ri p*^{p} *sep l(3)89Aa bx34*^{e} *and Bd*^{S} virgin females (Graf et al., 1984; 1989); (2) High bioactivation (HB) cross: mwh/mwh males crossed with ORR/ORR; *flr*^{3}*/In*(3LR)TM3, *ri p*^{p} *sep l(3)89Aa bx34*^{e} *and Bd*^{S} virgin females (Graf and van Schaik, 1992). These crosses yielded two types of offspring: marked trans-heterozygous (MH) (*mwh +/+ flr*^{3}) flies with phenotypically wild-type wings and balanced heterozygous (BH) (*mwh+/+TM3, Bd*^{S}) flies with phenotypically serrated wings. These offspring are phenotypically distinct due to the marker TM3, *Bd*^{S}.

2.3.3. Experimental procedure

Eggs were collected over 8 h in vials containing a solid agar base (4% agar in water) and a layer of yeast (*Saccharomyces cerevisiae*) supplemented with sugar. After 72 ± 4 h, the third instar larvae were washed with running water and collected using a fine mesh sieve. We performed a pilot study to test the BA toxicity in the SMART. The BA concentrations were based on previous studies of Freitas et al. (2015), who treated *Drosophila* larvae with high concentrations of BA (1.64; 3.28 and 6.57 mM which correspond to 0.75; 1.5 and 3.0 mg/mL) and survival assays in *D. melanogaster*.

To calculate the survival rates upon exposure, larvae were counted before the distribution into glass tubes (2.5 cm in diameter and 8.0 cm in height) containing 1.5 g of alternative culture medium, prepared with instant potato puree Yoki® Alimentos SA (according to Spanó et al., 2001) and different low concentrations of BA (0.0312; 0.0625; 0.125; 0.25 or 0.50 mg/mL) solubilized in water. The hatched flies were counted and stored in 70% ethanol. Chi-squared test was performed for statistical comparisons of the survival rate ratios for independent samples (De Rezende et al., 2013). Treatments were done in two independent experiments, with two replicates, with five different concentrations of BA (0.0312; 0.0625; 0.125; 0.25 or 0.50 mg/mL)

alone or in association with URE (10 mM) (for co-treatments). Two controls were included: (1) negative control (ultrapure water); and (2) positive control (urethane, 10 mM URE).

2.3.4. Analysis of flies

Emerging adult flies from the different treatments were collected and fixed in 70% ethanol, and the wings were subsequently mounted on slides. The wings were removed from the flies, soaked in Faure's solution (30 g of gum arabic, 20 mL of glycerol, 1.5 g of chloral hydrate and 5.0 mL of distilled water) and arranged on a dry slide. Then, the slides were dried for 1 h on a hot plate (40 °C). Slides were cover slipped and dried at room temperature.

Wings were examined on a microscope (Nikon Eclipse E200, 400X) to record the number and types of spots as well as their size and position along the wing. For each treatment, we analyzed the wings of 60 flies (approximately 24,400 cells per wing), achieving a total of approximately 48,800 cells analyzed per fly.

On marker-heterozygous (MH) wings (*mwh/flr*^{3}), three different categories of spots can be observed: (1) small single spots (1–2 cells in size) and (2) large single spots (more than two cells), expressing either the multiple wing hairs (*mwh*) or the flare (*flr*^{3}) phenotype, as well as (3) twin spots, consisting of both *mwh* and *flr*^{3} sub clones. On balancer heterozygous (BH) wings (*mwh*/TM3), only *mwh* single spots can be observed, as they do not carry *flr*^{3} or any other suitable marker mutation.

2.3.5. Statistical analysis

The wings of 60 flies from each treated series were scored, including controls. The data were evaluated according to the multiple-decision procedure of Frei and Würgler (1988, 1995), resulting in three different diagnoses: negative, positive or inconclusive. The frequency of each type of spot (small single, large single or twin) and the total frequency of spots per fly for each treatment were compared pair-wise, i.e., positive control (URE) alone vs. URE plus BA, following recommendations of Kastenbaum and Bowman (1970) with $\alpha = 0.05$. All inconclusive and weak results were analyzed with the non-parametric U test of Mann, Whitney and Wilcoxon ($a = b = 0.05$, one sided) to exclude false positives (Frei and Würgler, 1995). The U test takes into

account the rank values in controls and treatments and considers over-dispersion in a non-normal distribution (Kastenbaum and Bowman, 1970).

The modulatory effects of BA on the recombinogenic and mutagenic activities of URE were quantified by comparing the two genotypes (*mwh/flr*^{3} and *mwh*/TM3) and by applying the formulas: Recombination (R) = 1 - [(control corrected n/negative control in *mwh*/TM3 flies)/ (control corrected n/negative control in *mwh/flr*^{3} flies)] x 100; Mutation (M) = 100 – R (Frei and Würgler, 1996). Based on the control-corrected spot frequencies per 105 cells, the percentage of BA inhibition was calculated as: [URE alone – (URE + BA)/URE alone] x 100 (Abraham, 1994).

3. Results

3.1. MNT

Considering the need to reduce the use of animals in experiments, in the present study the genotoxic potential of BA was conducted only for the highest dose employed (1.0 mg/kg b.w.). The micronucleus frequencies obtained in male Swiss mice treated with BA alone (1 mg/kg b.w.) or BA (0.25; 0.5 and 1.0 mg/kg b.w.) in combination with URE (400 mg/kg b.w.) are shown in **Table 1**.

Table 1. Mean frequencies and standard deviation of PCEMNs and PCE/NCE + PCE ratio obtained in bone marrow cells from Swiss mice treated with betulinic acid (AB) and/or urethane (URE) and their respective controls.

The animals treated with BA plus URE showed significantly lower chromosomal damage frequencies when compared to those in the URE group. A gradual increase in the BA dose did not produce a proportional reduction in URE-induced micronucleus, demonstrating the absence of a dose-response relationship. No significant differences were observed in the PCE/total erythrocytes ratios between the different treatment groups, revealing absence of cytotoxicity (**Table 1**).

Fig. 2 shows photomicrographs of polychromatic erythrocytes, normochromatic erythrocytes and micronucleated polychromatic erythrocytes in bone marrow cells from Swiss mice treated with BA and URE.

3.2. Biochemical analysis

No significant differences were found for liver (AST and ALT) or renal (creatinine and urea) function parameters when the treatment was compared with the negative control group. These findings indicate the absence of hepatotoxic and nephrotoxic effects of the different treatments (**Fig. 3**).

3.3. Somatic mutation and recombination test – SMART

The Somatic Mutation and Recombination Test (SMART) in wing somatic cells of *D. melanogaster* was performed to assess the mutagenic and recombinogenic potential of BA and its possible effects on modulating DNA damage induced by urethane (URE). The different concentrations of BA used alone or in combination with URE were selected based on previous studies of Freitas et al. (2015) and on survival assays with *Drosophila*. The survival rates are depicted in **Fig. 4**.

According to the results, it was possible to observe a statistically significant decrease in survival rate on those treated with BA alone at concentrations of 0.0625 and 0.50 in the ST cross. However, these concentrations were used in the experiments performed, since at the same concentrations in the HB cross and associated with urethane in both ST and HB crosses, there was no reduction in the rate of survivors, as well as the fact that are lower than those used previously by Freitas et al. (2015). Thus, the authors concluded that the observed decreases must have been due to the manipulation of larvae during counting and not to BA toxicity. In this context, these survival data validated the use of five concentrations (0.0312, 0.0625, 0.125, 0.25 and 0.50 mg/mL) of BA alone or in combination with URE, which were tested in two independent experiments. The data were pooled after verifying that there were no significant differences between repetitions.

The results for the marker trans-heterozygous (MH) and balancer heterozygous (BH) descendants, derived from the Standard Cross (ST), treated with different concentrations of BA alone or in combination with URE are shown in **Table 2**. In the MH individuals, BA alone did not show any mutagenicity at the concentrations used. URE treatment, as expected, induced positive results for single, large and total spots when compared to the negative control ($\alpha < 0.05$). The simultaneous administration of

lower concentrations of BA (0.0312 and 0.0625 mM) with URE (10 mM) was not able to significantly reduce URE-induced total spots ($\alpha > 0.05$).

However, the simultaneous administration of URE with BA (0.125; 0.25 or 050 mM) reduced significantly ($\alpha < 0.05$) the number of URE-induced wing total spots (56.67%, 49.45 and 60.28%, respectively) when compared to URE alone. Due to the significant reduction observed in flies simultaneously treated with BA (0.125, 0.25 or 0.50 mg/mL) with URE (10 mM), the wings of the BH flies resulting from these treatments were also scored. Based on the clone induction frequency per 105 cells, we compared the number of observed spots in the MH and BH flies and quantified the contribution (%) of mutation and recombination to the total number of observed spots (Frei and Würgler, 1996).

The results of the HB cross of the SMART assays with *D. melanogaster* are summarized in **Table 3**. The findings obtained with the MH individuals treated with BA alone were negative at all tested concentrations. URE statistically increased ($\alpha < 0.05$), as expected, all categories of spots when compared to the negative control. The mutagenic activity was the major response to URE-induced DNA damage (72.87%). When administered with URE, all concentrations of BA (0.0312, 0.0625, 0.125, 0.25 or 0.50 mg/mL) were found to significantly decrease the number of spots ($\alpha < 0.05$) induced by URE. The inhibition rate was, respectively, 21.09, 20.63, 19.65, 29.36 and 39.82%. By comparing the number of observed spots in the MH flies and BH flies, we found that the induced spots were mainly due to mutation. **Fig. 5** shows some different categories of spots that can be observed on marker-heterozygous wings.

4. Discussion

Our results demonstrated that BA reduced the number of micronuclei generated by the URE in Swiss mouse using the bone marrow micronucleus assay. Also, we revealed that BA is not mutagenic/recombinogenic and displays antimutagenic/anti-recombinogenic effects when co-administered with URE on *Drosophila* using the SMART. The concentrations used and the observed results are valid only for these tested organisms, under these experimental conditions.

Prior studies found in the literature regarding genotoxicity/mutagenicity or antigenotoxicity/antimutagenicity of BA are controversial. However, some of them describe BA as a non-genotoxic/mutagenic substance, and report that the molecule is

able to modulate the effect of different mutagens in different experimental models, which reinforces the results of the present study. The genotoxicity and mutagenicity of BA analyzed by the Ames test and SOS chromotest demonstrated that the substance did not show mutagenic and genotoxic activities (Frolova et al., 2015; Yoshida et al., 2016). Salti et al. (2001) reported the protective effect of BA against DNA damage induced by UV-C (254 nm) on cultured congenital melanocytic naevi (CMN) cells, indicating that BA may have an application as a chemopreventive agent in patients with CMN.

Other studies conducted by Acésio et al. (2016) evaluated the genotoxic potential of different BA concentrations (2.7, 5.5, 11 or 22 μM) and its effects on the genotoxicity induced by different mutagens in V79 cells using the cytokinesis-block micronucleus assay. The frequencies of micronuclei in cultures treated with different BA concentrations alone did not differ from those of the negative control. When associated with camptothecin (CPT), BA had no effect on CPT-induced genotoxicity. Treatment with BA and MMS resulted in lower micronucleus frequencies than those observed for cultures treated with MMS alone. On the other hand, a significant increase in MN frequencies was observed in cultures treated with BA combined with doxorubicin (DXR) or etoposide (VP-16) in comparison to these mutagens alone. Thus, the authors concluded that the modulatory effect of BA depends on the type of mutagen and concentrations used. Evaluation of serum biochemical parameters is critical to identify the possible occurrence of toxicity induced by one or more substances (Ramaiah, 2007).

Biochemical parameters are widely used as indicators of the physiological behavior of the animal in response to endogenous alterations and also as diagnostic biomarkers. In fact, the occurrence of changes may suggest organ or tissue injury (Shahsavani et al., 2010).

Therefore, the evaluation of these parameters in the mice used in the present study is of great importance for a correct interpretation of the experimental data. Studies conducted by Yi et al. (2016) revealed a potent hepatoprotective effect of BA and reduced liver damage caused by alcohol in mice. This effect was attributed to its antioxidant capacity, which was able to improve the tissue redox system and to decrease lipid peroxidation in the liver. In the present study, the influence of BA on URE-induced mutant spots in *Drosophila* was also evaluated. BA alone or in association with URE was tested in two independent experiments with two replicates.

The data were pooled after verifying that there were no significant differences between repetitions.

We considered URE as a reference mutagen based on its genotoxic potential in *Drosophila*, which exhibits a clear dependence on metabolic activation (Frölich and Würgler, 1990; Morais et al., 2016; Naves et al., 2019). BA alone did not significantly increase the frequency of mutant spots in both ST and HB crosses, but it was able to reduce the total frequency of mutant spots induced by URE, in the three highest concentrations in the ST cross (0.125; 0.25 and 0.50 mg/mL) and in all concentrations in the HB cross. In addition, BA was able to reduce the recombinogenic effect of URE at the highest concentrations of both crosses (ST and HB).

Ours findings reinforce the protective effects of BA on the damage generated by URE directly and also after its metabolization. BA is metabolized via CYP enzyme in humans, and the free carboxylic acid group (C-28) of this triterpene can act as a substrate for CYP2C9, being C-23 and C-6a carbons the most likely sites of oxidative metabolism. In addition to CYP2C9, a homologous model for human CYP3A4 predicted similar metabolic sites: The C-6 and C-23 or C-24 positions (Ríos and Manez, 2018). The differences in the results observed with URE in both crosses are related to the basal and high levels of metabolic enzymes of cytochrome P450 complex present in the ST and HB crosses, respectively. Cytochrome P450 enzymes (CYPs) constitute one of the enzyme families involved in the metabolism of xenobiotics, including natural compounds. CYPs comprise many isoforms which catalyze a wide variety of reactions leading to different metabolites (Olsen et al., 2015). The first cytochrome P450 cloned from *Drosophila* was the phase I enzyme cytochrome P450 6A2, which exhibits a peak of expression in the third larval and pupal stage. CYP6A2 mRNA was found to be present in the insecticide-resistant strain [OR(R)] at higher levels than in the insecticide-sensitive (*flr*^{3}) strain (Saner et al., 1996), which may explain the different frequencies of mutations observed with URE in the ST and HB crosses.

Studies conducted by Freitas et al. (2015) using the SMART test in *Drosophila melanogaster* demonstrated that BA (1.64, 3.28 or 6.57 mM) alone was not capable of causing DNA damage. However, after combination with URE, the frequency of spots was significantly reduced in the lowest concentration of the ST cross and in the three concentrations of the HB cross. In this study, the main effect of BA also occurred at the HB crossing, which contains high levels of metabolic enzymes of the cytochrome

P450 complex. Based on the literature, it is believed that the protective effect of BA in Swiss mouse and in *Drosophila* is related mainly to the antioxidative and apoptotic potential of this compound. It is possible that BA has attenuated the production of ROS generated by URE modulating its genotoxicity. The antioxidant potential of BA has been reported in the literature. The effect of BA on oxidative lung injury was demonstrated in mice, using cecal ligation and puncture (CLP) as model. BA pretreatment decreased the levels of oxidants and increased the levels of antioxidants in lungs and plasma, thereby reducing the oxidative lung injury in CLP mice.

Furthermore, BA was found to scavenge the superoxide and nitric oxide radical *in vitro* (Lingaraju et al., 2015); had promising free radical scavenging and appreciable tyrosinase, xanthine oxidase and urease inhibitory effects (Nile et al., 2019) and reduced the oxidative damage induced by T-2 toxin in mice (Wu et al., 2019). Besides that, BA exhibited anti-inflammatory activity against λ-carrageenan-induced paw edema model in mice through the inhibition of COX-2 and NO levels and increased activity of the antioxidant enzymes SOD, GPx and GRd (Tsai et al., 2011). BA can effectively attenuate hyperalgesia induced by remifentanil by inhibiting oxidative stress and subsequently downregulating matrix metalloproteinase-9 related pro-inflammatory cytokines in spinal dorsal horn (Lv et al., 2018). In the literature, there are also several reports regarding the apoptotic effect of BA. It is possible that part of the BA protection against genotoxicity induced by URE is due to apoptosis of damaged cells.

A previous study performed by Wang et al. (2017) showed that BA exerted its anticancer effect by triggering cell proliferation inhibition and apoptosis in colorectal cancer (CRC) cells. In addition, BA induced a protective autophagy by inhibiting the AKT/MTOR signaling pathway. Shankar et al. (2017) demonstrated that BA may induce apoptosis by stabilizing p53 and downregulating NF-κB pathway in human prostate cancer cells, irrespective of the androgen association, and therefore can potentially be developed as a molecule of interest in cancer chemoprevention. Goswami et al. (2018) also indicated that BA effectively induced DNA damage and apoptosis in SiHa cells preferentially in a concentration-dependent manner with a 50% inhibition of the cells at 39.83 µg/mL. The mechanism of apoptosis was caspase independent and through mitochondrial pathways.

Finally, BA has also been highlighted in the literature as a potent antitumor agent. The compound can induce apoptosis of tumor cells through different ways, including activation of the mitochondrial pathway; regulation of Bcl-2 family proteins; modulation

of NF-κB activity; specificity protein (Sp) transcription factors; and by vascular endothelial growth factor (VEGF) signaling (Fulda, 2008; Luo et al., 2016). Chintharlapalli et al. (2007), after using LNCaP prostate cancer cells as model, showed that BA reduces the proliferation of these cells through the targeted degradation of SP proteins that are highly expressed in tumors.

Studies *in vitro* performed by Potze et al. (2016b) stablished that BA induces rapid apoptosis in all colon cancer stem cells (CSCs) tested and is able to affect the CSCs directly via the loss of clonogenic capacity. Cai et al. (2018) also demonstrated that BA chemosensitized breast cancer through directly targeting glucose-regulated protein 78 (GRP78) to trigger apoptotic pathway. The results observed in our study allow us to conclude that, under the experimental conditions, BA has antigenotoxic effect against the genotoxicity induced by URE in Swiss mice. Also, the compound was not mutagenic in *Drosophila*, but modulated DNA damage directly and, mainly, after metabolization. Based on literature data, we can suggest that the modulatory effects of BA can be explained by its antioxidant and apoptotic effects.

Taken together, our findings reveal that BA can interact with the enzymatic system that catalyzes the metabolic detoxification of URE, by inhibiting the activity of the mitochondrial complex I, thereby capturing free radicals. In addition, BA can also modulate the metabolic activation of URE, inhibiting the formation of DNA binding metabolites and inducing damaged cells to apoptosis. Further studies should be conducted using other reference mutagens and different organisms in order to confirm the use of this compound in the production of new drugs.

CRediT authorship contribution statement

Victor Constante Oliveira: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Writing - original draft. **Maria Paula Carvalho Naves:** Data curation, Formal analysis, Methodology, Writing - review & editing. **Cássio Resende de Morais:** Data curation, Formal analysis. **Sarah Alves Rodrigues Constante:** Data curation, Formal analysis. **Priscila Capelari Orsolin:** Data curation, Formal analysis. **Bianca Silva Alves:** Methodology, Data curation, Formal analysis. **Francisco Rinaldi Neto:** Methodology, Data curation, Formal analysis. **Lucas Henrique Domingos da Silva:** Methodology, Data curation, Formal analysis. **Lucas Teixeira Souza de Oliveira**: Methodology, Data curation, Formal analysis. **Natália**

Helen Ferreira: Methodology, Data curation, Formal analysis. **Tábata Rodrigues Esperandim**: Methodology, Data curation, Formal analysis. **Wilson Roberto Cunha**: Resources. **Denise Crispim Tavares**: Conceptualization, Project administration, Resources, Supervision, Writing - review & editing. **Mário Antônio Spanó**: Conceptualization, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of competing interest

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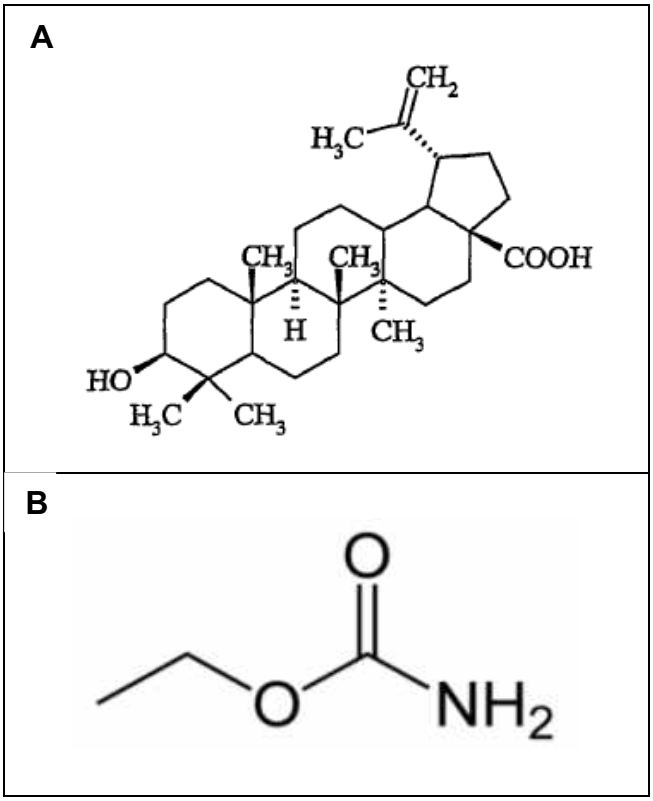

Fig. 1. Structural formula of (**A)** betulinic acid, and (**B)** urethane.

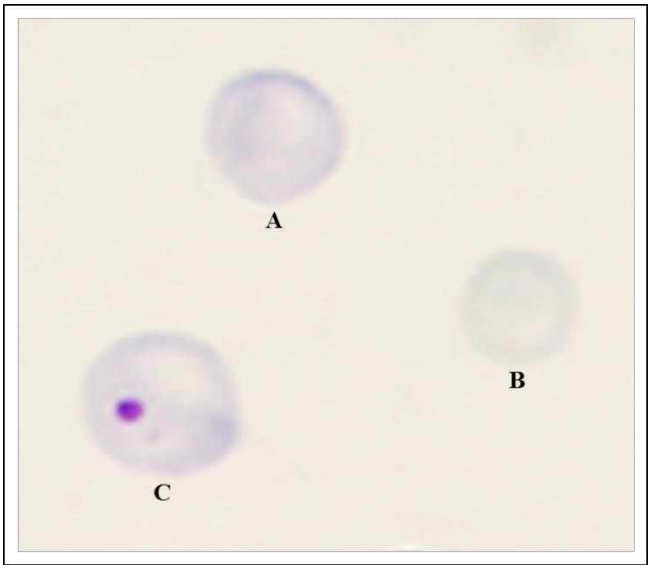

Fig. 2. Photomicrographs of **(A)** polychromatic erythrocyte, **(B)** normochromatic erythrocyte and **(C)** micronucleated polychromatic erythrocyte in bone marrow cells from Swiss mice treated with betulinic acid and urethane (1,000X magnification, Giemsa).

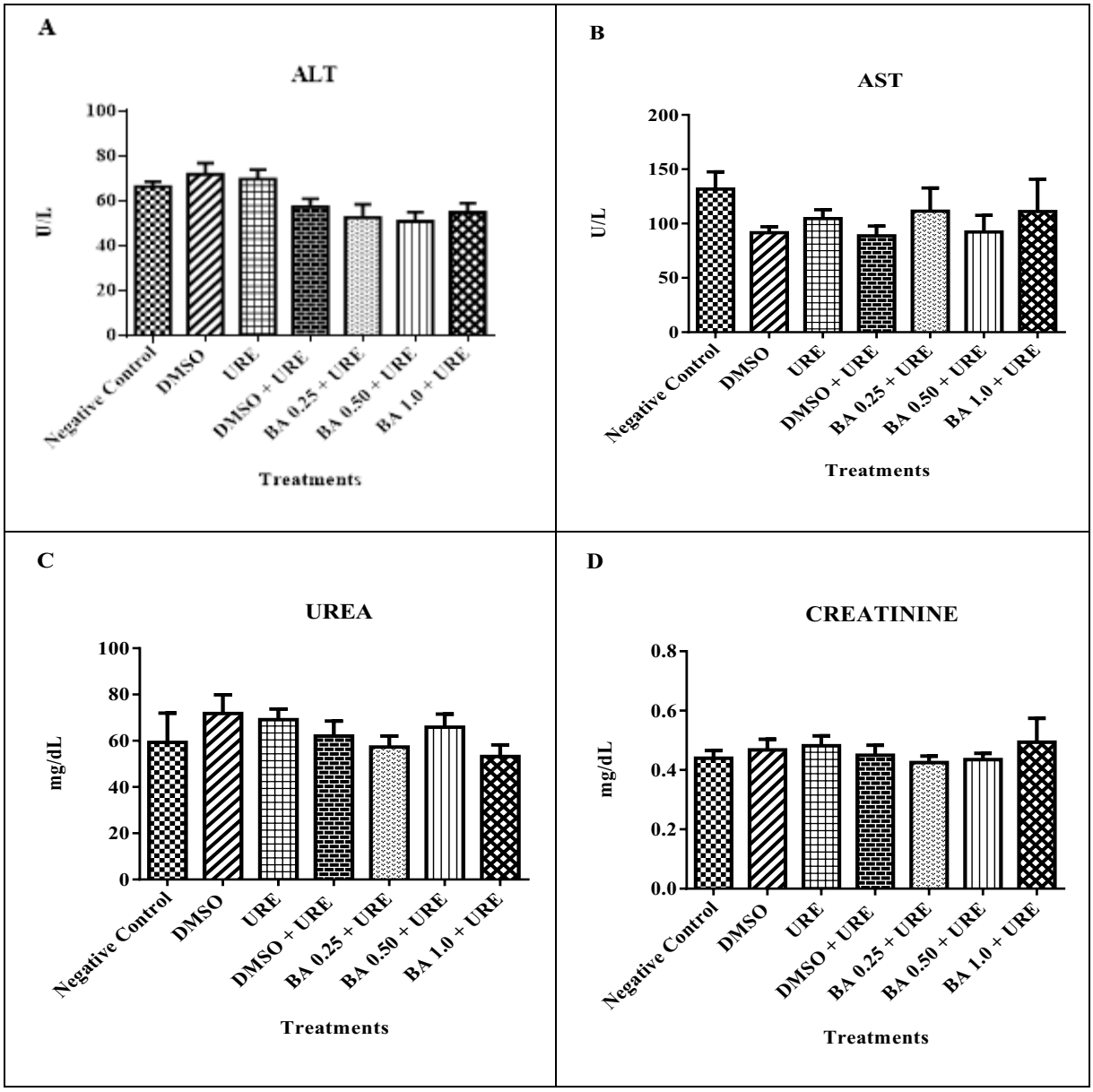

Fig. 3. Serum levels of **(A)** ALT, **(B)** AST, **(C)** urea, and **(D)** creatinine obtained from Swiss mice treated with different doses of betulinic acid and urethane, and their respective controls. **URE:** Urethane (400 mg/kg b.w.); **DMSO:** dimethylsulfoxide (1%); **BA:** betulinic acid (0.25, 0.50 and 1.0 mg/kg b.w.); **ALT:** alanine aminotransferase; **AST:** aspartate aminotransferase. Data are representative of the three independent experiments, with about two animals per treatment group. Values are mean ± standard deviation.

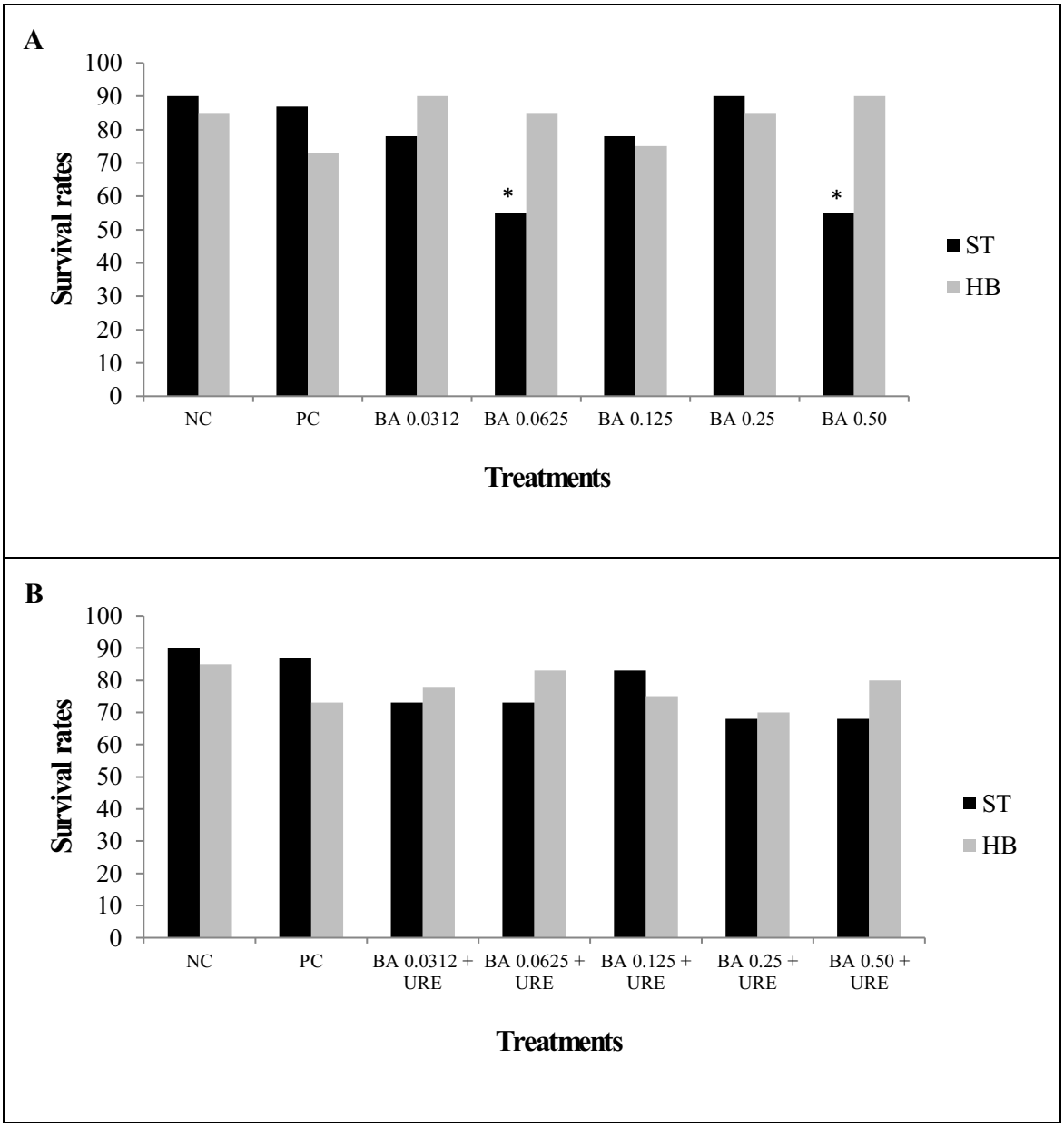

Fig. 4. Survival rates (%) of individuals from ST and HB crosses upon exposure to different concentrations of **(A)** BA (Betulinic acid - mg/mL) alone and **(B)** BA in combination with URE (Urethane 10 mM). **NC:** Negative control; **PC:** Positive control; *$\alpha < 0.05$. Data are representative of survival tests performed only once, without replica.

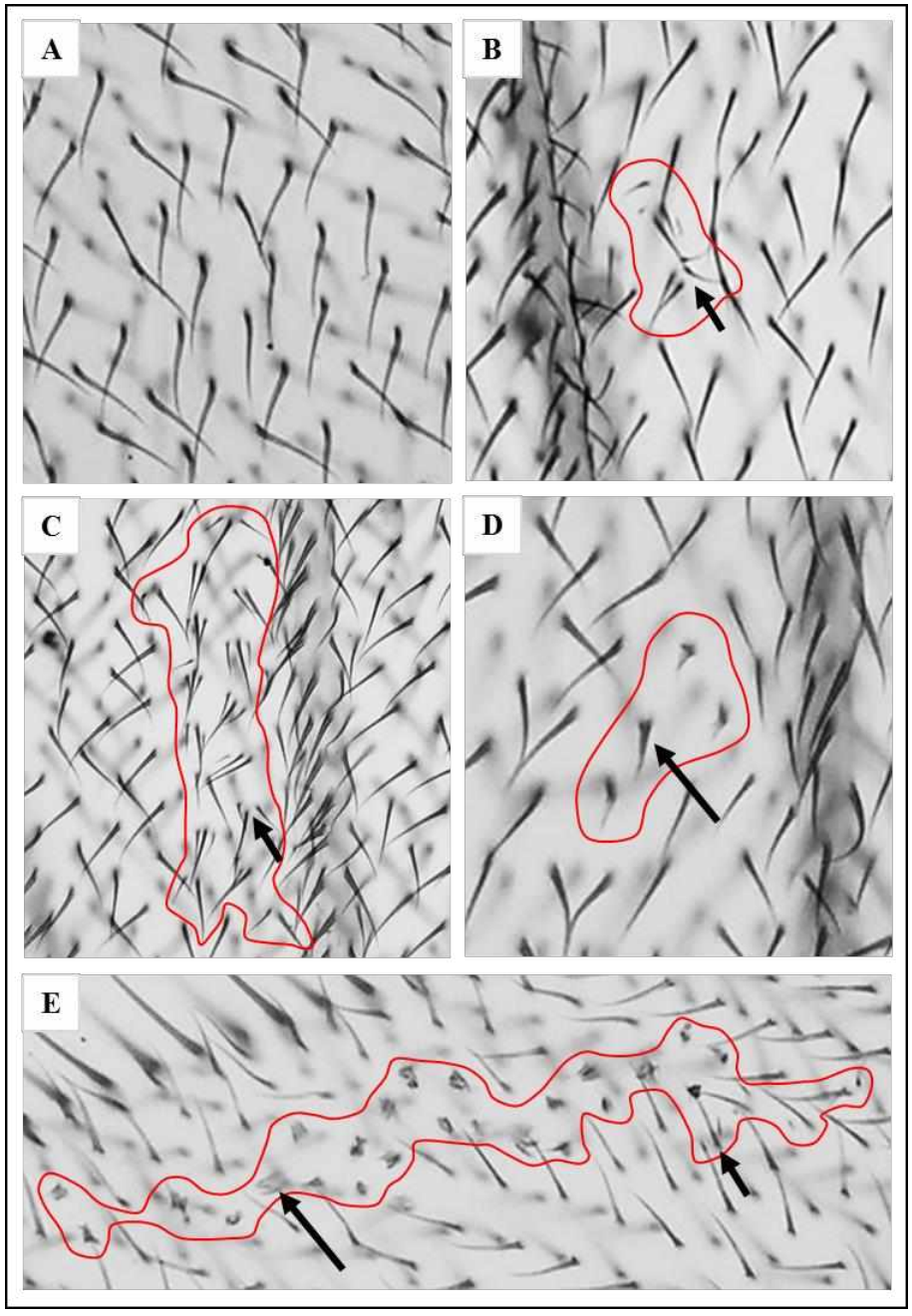

Fig. 5. Photomicrographs (400X magnification) of *D. melanogaster* wings surface obtained by light microscopy. **(A)** Normal hairs or trichomes; **(B)** Small single mwh spot; **(C)** Large single mwh spot; **(D)** Large single flare spot; **(E)** Twin spot. Small arrows showing mwh phenotype; large arrows showing flare phenotype.

Table 1. Mean frequencies and standard deviation of PCEMNs and PCE/NCE + PCE ratio obtained in bone marrow cells from Swiss mice treated with betulinic acid (AB) and/or urethane (URE) and their respective controls.

Treatments	**PCEMNs[a]**	**PCE/NCE+PCE[b]**
(mg/Kg b.w.)	**Mean ± SD**	**Mean ± SD**
Negative control	9.33 ± 2.52	0.57 ± 0.03
DMSO	12.50 ± 5.32	0.64 ± 0.11
URE	86.00 ± 20.35[c]	0.64 ± 0.06
DMSO + URE	112 ± 62.36[c]	0.55 ± 0.08
BA 0.25 + URE	20.00 ± 7.46[d]	0.68 ± 0.09
BA 0.50 + URE	28.67 ± 23.36[d]	0.70 ± 0.13
BA 1.0 + URE	43.17 ± 35.97	0.67 ± 0.08

URE: Urethane (800 mg/kg b.w.); **DMSO:** Dimethylsulfoxide (1%); **BA:** Betulinic acid; **PCE:** Polychromatic Erythrocytes; **NCE:** Normochromatic erythrocytes; **MN:** Micronucleus.

[a] Analysis of 4,000 polychromatic erythrocytes per animal.

[b] Analysis of 500 total erythrocytes per animal.

[c] Significantly different from the negative control group ($\alpha < 0.05$).

[d] Significantly different from the Urethane group ($\alpha < 0.05$).

Table 2. Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the standard (ST) cross after chronic treatment of larvae with betulinic acid (BA) and urethane URE).

Genotypes and Treatments		Number of flies	Spots per fly (number of spots); statistical diagnoses[a]				Spots with *mwh* clone[c]	Frequency of clone formation/10^5 cells per division[d]		Recombination (%)	Inhibition[e] (%)
URE (mM)	BA (mM)		Small single spots (1-2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control Corrected		
***mwh*/*flr*³**											
0	0	60	0.45 (27)	0.02 (01)	0.03 (02)-	0.50 (30)	30	1.02			
0	0.0312	60	0.33 (20)-	0.05 (03)-	0.00 (00)-	0.38 (23)-	23	0.79	- 0.24		
0	0.0625	60	0.27 (16)-	0.02 (01)-	0.00 (00)-	0.28 (17)-	17	0.58	- 0.44		
0	0.125	60	0.30 (18)-	0.05 (03)-	0.00 (00)-	0.35 (21)-	21	0.72	- 0.31		
0	0.25	60	0.23 (14)-	0.02 (01)-	0.02 (01)-	0.27 (16)-	16	0.55	- 0.48		
0	0.50	60	0.13 (08)-	0.07 (04) -	0.02 (01)-	0.22 (13)-	13	0.44	- 0.58		
10	0	60	1.87 (112)+	0.18 (11)+	0.00 (00)-	2.05 (123)+	118	4.03	3.01	41.44	
10	0.0312	60	1.58 (95)	0.22 (13)	0.05 (03)	1.85 (111)	110	3.76	2.73		
10	0.0625	60	1.40 (84)*	0.15 (09)	0.05 (03)	1.60 (96)	93	3.18	2.15		
10	0.125	60	1.08 (65)*	0.12 (07)	0.00 (00)	1.20 (72)*	72	2.46	1.43	50.00	52.49
10	0.25	60	1.18 (71)*	0.12 (07)	0.00 (00)	1.30 (78)*	78	2.66	1.64	50.00	45.51
10	0.50	60	1.02 (61)*	0.13 (08)	0.00 (00)	1.15 (69)*	69	2.36	1.33	29.24	55.81
***mwh*/TM3**											
10	0	60	1.12 (67)	0.03 (02)	[f]	1.15 (69)	69	2.36			
10	0.125	60	0.58 (35)*	0.02 (01)		0.60 (36)*	36	1.23			
10	0.25	60	0.65 (39)*	0.00 (00)		0.65 (39)*	39	1.33			
10	0.50	60	0.82 (49)*	0.00 (00)		0.82 (49)*	49	1.67			

Marker-trans-heterozygous flies (*mwh*/*flr*³) and balancer-heterozygous flies (*mwh*/TM3) were evaluated.

[a] Statistical diagnoses according to Frei and Würgler (1988, 1995). U-test, two sided; probability levels: -, negative; +, positive; i, inconclusive; $\alpha < 0.05$ vs. negative control; *, positive; $\alpha \leq 0.05$ vs. URE (10 mM) only.

[b] Including rare *flr*^{3} single spots.

[c] Considering mwh clones from mwh single and twin spots.

[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction).

[e] Calculated as{[URE alone – URE +BA] /URE} X 100, according to Abraham (1994).

[f] Balancer chromosome TM3 does not carry the *flr*^{3} mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

Table 3. Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the high bioactivation (HB) cross after chronic treatment of larvae with betulinic acid (BA) and urethane URE)

Genotypes and Treatments		Number of flies	Spots per fly (number of spots); statistical diagnoses[a]				Spots with *mwh* clone[c]	Frequency of clone formation/10^5 cells per division[d]		Recombination (%)	Inhibition[e] (%)
URE (mM)	BA (mM)		Small single spots (1-2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control Corrected		
***mwh*/*flr*³**											
0	0	60	0.50 (30)	0.05 (03)	0.03 (02)	0.58 (35)	34	1.16			
0	0.0312	60	0.57 (34)-	0.02 (01)-	0.00 (00)-	0.58 (35)-	35	1.20	0.04		
0	0.0625	60	0.48 (29)-	0.05 (03)-	0.00 (00)-	0.53 (32)-	31	1.06	- 0.10		
0	0.125	60	0.43 (26)-	0.05 (03)-	0.00 (00)-	0.48 (29)-	29	0.99	- 0.17		
0	0.25	60	0.38 (23)-	0.08 (05)-	0.00 (00)-	0.47 (28)-	28	0.96	- 0.20		
0	0.50	60	0.43 (26)-	0.07 (04)-	0.00 (00)-	0.50 (30)-	30	1.02	- 0.14		
						12.08				25.64	
10	0	60	9.73 (584)+	1.22 (73)+	1.13 (68)+	(725)+	675	23.05	21.89		
10	0.0312	60	7.62 (457)*	1.08 (65)	0.47 (28)*	9.17 (550)*	542	18.51	17.35	20.10	20.74
10	0.0625	60	7.72 (463)*	1.12 (67)	0.30 (18)*	9.13 (548)*	545	18.61	17.45	22.19	20.28
10	0.125	60	7.45 (447)*	1.38 (83)	0.45 (27)*	9.28 (557)*	551	18.82	17.66	17.06	19.32
10	0.25	60	7.18 (431)*	0.68 (41)*	0.32 (19)*	8.18 (491)*	490	16.73	15.57	15.66	28.87
10	0.50	60	6.20 (372)*	0.75 (45)*	0.25 (15)*	7.20 (432)*	424	14.48	13.32	3.52	39.15
***mwh*/TM3**											
10	0	60	8.12 (487)	0.25 (15)	[f]	8.37 (502)		17.14			
10	0.0312	60	7.03 (422)*	0.18 (11)		7.22 (433)*		14.79			
10	0.0625	60	6.92 (415)*	0.15 (09)		7.04 (424)*		14.48			
10	0.125	60	7.52 (451)	0.10 (06)*		7.62 (457)		15.61			
10	0.25	60	6.82 (409)*	0.07 (04)*		6.88 (413)*		14.11			
10	0.50	60	6.68 (401)*	0.13 (08)		6.82 (409)*		13.97			

Marker-trans-heterozygous flies (*mwh*/*flr*³) and balancer-heterozygous flies (*mwh*/TM3) were evaluated.

[a] Statistical diagnoses according to Frei and Würgler (1988, 1995). U-test, two sided; probability levels: -, negative; +, positive; i, inconclusive; $\alpha < 0.05$ vs. negative control; *, positive; $\alpha \leq 0.05$ vs. URE (10 mM) only.

[b] Including rare *flr*^{3} single spots.

[c] Considering mwh clones from mwh single and twin spots.

[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction).

[e] Calculated as{[URE alone – URE +BA] /URE} X 100, according to Abraham (1994).

[f] Balancer chromosome TM3 does not carry the *flr*^{3} mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

CAPÍTULO III

MODULATING EFFECTS OF BETULINIC ACID ON THE CARCINOGENICITY OF DOXORUBICIN IN *Drosophila melanogaster,* APOPTOSIS IN BREAST CANCER CELLS AND *IN SILICO STUDIES*

Modulating effects of betulinic acid on the carcinogenicity of doxorubicin in *Drosophila melanogaster, apoptosis in breast cancer cells* and *in silico* studies

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Highlights:

- BA was not toxic neither carcinogenic to *D. melanogaster*.
- BA has modulatory effects on the carcinogenicity induced by DXR in *Drosophila.*
- Modulatory effects of BA may be due to its antioxidant and apoptotic properties.
- BA reduces MCF10A and MDA-MB-231 cells viability after 48 h.
- BA showed selective apoptotic effect against tumorigenic strains MDA-MB-231.
- BA does not induce necrosis by LDH release.
- BA showed a stable interaction with SUMO proteins (SAE1 and UBA2), DNA POLβ and AKR1B10 in the *in silico* analysis.

Abstract

Betulinic acid (BA) is a pentacyclic triterpenoid found in several plant species. BA has anti-inflammatory, analgesic, antiviral, antibacterial, antioxidant, apoptotic and antineoplastic properties. Doxorubicin (DXR) is a known genotoxic with carcinogenic potential in *Drosophila melanogaster*. BA (0.0312, 0.0625, 0.125, 0.25 or 0.50 mg/mL) alone was examined for carcinogenicity and combined with DXR (0.4 mM) for anticarcinogenicity, using the Epithelial Tumor Test (ETT) in *Drosophila melanogaster*. BA alone did not significantly increase the frequency of tumors but was able to reduce the total frequency of tumors when co-administered with DXR, in all concentrations. Triple-negative breast cells MCF10A (non-tumorigenic) and MDA-MB-231 (tumorigenic) were treated with BA at different concentrations ranging from 1.5 to 200 μM for 24 and 48 h and cell viability was evaluated by the MTT tetrazolium salt colorimetric assay. BA reduced the cell viability of MCF10A and MDA-MB-231 cells in a concentration-dependent manner compared to the control (untreated cells). However, the cytotoxic effect of BA was more significant after 48 hours, where the reduction of growth viability was more efficient in MDA-MB-231 cells. The LDH release assay was used to measure cell damage caused by BA. It was observed that there was no significant difference in LDH release when comparing untreated and BA-treated cells. To elucidate some of the ways in which BA works, we performed an *in silico* analysis of molecular docking. The result of the search for the most likely targets of BA resulted that POLB, AKR1B10 and SUMO (SAE1/UBA2) had a 97.16, 97.16 and 81.26% chance of being targets of the BA ligand, respectively.. Taken together, our data suggest that BA can modulate the carcinogenic effect of DXR, inducing damaged cells to apoptosis through different pathways involving SUMO proteins (SAE1 and UBA2), DNA POLβ and AKR1B10. Further studies should be conducted using other reference carcinogens and different organisms to confirm the use of this compound in the production of new drugs.

Keywords: Natural products, Pentacyclic triterpene, Anticarcinogenicity, Epithelial tumor test, ETT, Apoptotic effect, Molecular docking.

1. Introduction

Traditionally, phytochemicals and derivatives present in plants are promising options to improve the efficiency of treatment in cancer patients and reduce adverse reactions (Choudhari et al., 2020). Among the naturally occurring biologically active phytochemicals that have significant antitumor potential is betulinic acid (BA). BA is a lupane-type pentacyclic triterpenoid widely distributed throughout the plant kingdom, which can be isolated from various sources, but the most reported is birch bark (*Betula* spp.) (Li et al., 2021; Sousa et al., 2021).

In recent years, this compound has drawn considerable scientific interest due to its diverse biological and pharmacological properties, such as antibacterial (Bildziukevich et al., 2019), antiviral (Loe et al., 2021), antidiabetic (Song et al., 2021), anti-inflammatory (Lou et al., 2021; Zhou et al., 2021), antioxidant (Kong et al., 2021; Zhu et al., 2020), antiproliferative (Kim et al., 2021) and apoptotic activities (Chen et al., 2020; Liao et al., 2020; Zhang et al. 2021), as well as potential as an antineoplastic agent by inhibiting topoisomerase (Król et al., 2015; Zhang et al., 2015; 2016).

Owing to its specific cytotoxicity to different human tumor cells and low cytotoxicity to normal cells, BA is considered a promising antitumor agent (Jiang et al., 2021). Nevertheless, further *in vivo*, *in vitro* and *in silico* assays are still required to better understand the antitumor mechanisms of this compound, as knowledge of such effects can be useful in preventing possible adverse effects on human health, and also in preventing different types of cancer.

In vivo carcinogenesis assays are valuable tools to elucidate possible modulatory effects of chemical and natural compounds. There are several model organisms available for this purpose, however, due to the need to minimize the use of mammals in laboratory tests, efforts have been devoted to developing alternative models, such as *Drosophila melanogaster* (Doke and Dhawale, 2015; Hifzur et al. 2005).

D. melanogaster has about 60% of genes identical to human genes and almost 75% of cancer-related genes have a functional homolog in this fly. Furthermore, it has a fast life cycle, small size and low maintenance cost compared to other organisms (Baenas and Wagner, 2019; Mirzoyan et al., 2019; Demir, 2021). Remarkably, *D.*

melanogaster has been a reference organism in scientific research to assess the toxicity, mutagenicity and carcinogenicity of different compounds (Machado et al., 2016; Mendonça et al., 2019; Naves et al., 2021; Oliveira et al., 2018; 2020; Orsolin et al., 2016; Spanó, 2021; Vasconcelos et al., 2020). The evaluation of these effects includes numerous *in vivo* tests, such as the Epithelial Tumor Test (ETT). During the initial embryonic development of *D. melanogaster*, groups of cells (imaginal discs) are separated and if a genetic alteration occurs in one of these cells, this alteration will be present in all descendant cells and will form a clone of mutant cells (tumor) (Justice et al., 1995; Nepomuceno, 2015; Sidorov et al., 2001).

In ETT, doxorubicin (DXR) has been commonly used as a tumor inducer (positive control) (Oliveira et al., 2017; Vasconcelos et al., 2020). DXR is an anthracycline antibiotic, also known as adriamycin, used in cancer chemotherapy. It is an intercalating agent that inhibits topoisomerase II activity and blocks DNA replication and transcription. DXR is also able to induce the production of reactive oxygen species (ROS) and lipid peroxidation (Bellance et al., 2020; Fant et al., 2021).

In vitro assays with human tumor and non-tumor cells have also been an excellent alternative to test different compounds against cancer (Kleinpoppen et al., 2019). Several human cell lines can be used for this purpose, especially breast cancer cells. According to world statistics, female breast cancer surpassed lung cancer and became the most common tumor, with approximately 2.3 million new cases (11.7%) (Siegel et al. 2020; Sung et al. 2021). Breast cancer is often accompanied by a high recurrence rate, so the search for specific drugs to treat it remains mandatory (Qi et al., 2021).

Triple-negative breast cancer (TNBC) is a genetically heterogeneous subtype of breast cancer characterized by the lack of expression of estrogen receptor (ER) and progesterone receptor (PR) and the amplification of human epidermal growth factor receptor 2 (HER2). It comprises 15-20% of all breast cancers, being a more aggressive subtype of cancer, with a worse prognosis, with restricted targeted therapies (Ozawa et al., 2018; Ünal et al., 2021; Zhou et al., 2021).

Human non-tumorigenic breast epithelial (MCF-10A) and metastatic TNBC (MDA-MB-231) cell lines have been successfully employed in cytotoxicity studies using the MTT assay (Du et al., 2021; Grbović et al., 2013; Maleki et al., 2020; Vale et al., 2020). MTT is a colorimetric assay used to determine the cytotoxic potential of different

substances, and is an indicator of antiproliferative activation, cell activation and cytotoxicity (Sharif et al., 2017). In turn, viability has been extensively observed through lactate dehydrogenase (LDH) release (Wang et al., 2017; Braga et al., 2018; Costa et al., 2020). Part of the cell culture supernatant can be used to measure LDH, an enzyme that catalyzes the conversion of glucose to lactic acid and that has irregular activity in cancer cells (Forkasiewicz et al., 2020). Thus, the release of LDH from the cell cytoplasm into the culture medium is the result of damage to the cell membrane and cell lysis, being proportional to the number of cells killed by necrosis (Pawlak et al., 2021).

Complementary molecular docking studies are useful for more reliable prediction of biological activities (Kaur Dhanjal et al., 2014). Many scoring functions and various docking methodologies can estimate the binding protein interaction and, according to this structural information, the design of potent new compounds for a specific target with higher binding affinity becomes faster and cheaper (Hammoudi et al., 2020).

Therefore, the aim of the present study was to investigate the modulatory potential of BA against DXR-induced carcinogenicity using the ETT in *D. melanogaster;* the BA cytotoxicity and viability of MCF-10A and MDA-MB-231 TNBC lineages, using the MTT and LDH release assays. Finally, *in silico* analyses were performed to predict BA targets.

2. Material and Methods

2.1. Chemical agents

Betulinic acid (BA) (3β-Hydroxy-20(29)-lupaene-28-oic acid), CAS: 472-15-1, was purchased from Sigma Aldrich - Brazil. Tween 80 P.S. (Polysorbate 80), CAS: 9005-65-6, was from Vetec Química Fina Ltda. - Brazil. Doxorubicin (DXR), CAS: 25316-40-9, commercially known as Adriblastina® RD, Batch 5PL5111, obtained from Pfizer - Brazil, was used as a positive control. Ultrapure water (18.2 MΩ), obtained from the MilliQ System (Millipore S.p.A.; Vimodrone, Milano, Italy), was used as a negative control. The flake-mashed potatoes, used as an alternative culture medium, were obtained from Yoki Hikari® Alimentos S.A. - Brazil. The structural formulas of BA

and DXR are shown in **Fig. 1**.

2.2. *In vivo* Epithelial Tumor Test - ETT

2.2.1. Strains and stock

Two *D. melanogaster* strains were used to investigate the carcinogenicity of BA when administered alone, or its anticarcinogenicity when administered simultaneously with DXR: [1] multiple wing hairs (genetic constitution: *mwh/mwh; y; mwh jv,* 3 [3–0.3]) and [2] “warts” (genetic constitution: *st* [1] *in*[1] *kni*[*ri*-1] *p*[*p*] *wts* [3–17]/TM3, *Sb*[1]). These strains were maintained in glass vials filled with a maintenance medium [i.e., water, agar-agar, banana, yeast (*Saccharomyces cerevisiae)*, methylparaben and penicillin/streptomycin] under light/dark cycles (12 h: 12 h), at 25 ± 1 ºC and approximately 60% humidity in a Bio-Oxygen Demand-type chamber (Model: SL224, SOLAB - Equipamentos para Laboratórios Ltda., São Paulo, SP, Brazil).

2.2.2. Experimental procedure

The ETT was performed by crossing multiple wing hairs males (mwh) with virgin females warts (wts). This cross produced two types of progeny: marked trans-heterozygous flies (MH - *wts* +/+ *mwh*), phenotypically with long and fine hairs on the chest, and balanced heterozygous flies (BH - TM3, sb1 +/+ mwh) with short and thick hairs on the chest. Eggs were collected over 8 h in vials containing a solid agar base (4% agar in water) and a layer of yeast (*Saccharomyces cerevisiae*) supplemented with sucrose. After 72 ± 4 h, third-instar larvae were washed with running water and collected using a fine mesh sieve.

BA concentrations (0.0312; 0.0625; 0.125; 0.25 and 0.50 mg/mL) were selected after performing survival assays with *D. melanogaster*. To calculate the survival rates upon exposure, 100 larvae of 72 ± 4 h were counted before the distribution into glass vials (2.5 cm in diameter and 8.0 cm in height) containing 1.5 g of alternative culture medium, prepared with instant mashed potato (according to Spanó et al., 2001) hydrated with 5 mL of different concentrations of BA. BA concentrations from 0.0312 to 0.50 mg/mL did not alter survival rates compared to the negative control. Therefore, these concentrations were used in the ETT. The survival tests were carried out only

once, without replicates. The chi-square test was applied for statistical comparisons of the survival rate ratios for independent samples (De Rezende et al., 2013).

ETT treatments were done in two independent experiments, with replicates, and five different concentrations of BA (0.0312 - 0.50 mg/mL) were used alone or in association (co-treatments) with DXR (0.4 mM). Three controls were included: (1) negative control (ultrapure water); (2) solvent control (1% Tween + 3% ethanol) and (3) positive control (DXR 0.4 mM).

2.2.3. Analysis of flies

Emerging adult flies were collected and stored in vials containing 70% ethanol. The analysis of individuals was carried out on concave slides containing glycerol, under a stereoscopic microscope (Bel® Photonics), for visualization and tumor counting. The epithelial tumors can appear in different parts of the fly, including eyes, head, wings, body, legs and halters.

The presence of tumors was evaluated and recorded in a standard spreadsheet.

2.2.4. Statistical analysis

The frequency of each tumor type and the total frequency of tumors for each treatment were compared pair-wise (i.e. solvent control *vs.* negative control; DXR *vs*. negative control; BA *vs*. negative control; BA + DXR *vs*. DXR) and the possible carcinogenic/anticarcinogenic effects were validated by the non-parametric *U*-test of Mann, Whitney and Wilcoxon, using $\alpha \leq 0.05$ as the significance level.

2.3. *In vitro* cytotoxicity assays

2.3.1. Cell lines and culture conditions

MCF-10A (non-tumorigenic) and TNBC MDA-MB-231 (tumorigenic) cells were cultured in DMEM/F-12 medium supplemented with 10 µg/mL EGF, 0.25 µg/mL Hydrocortisone, 10 µg/mL insulin and L15 medium. All strains were supplemented with 10% fetal bovine serum (FBS), 50 µg/mL gentamycin, and maintained at 37 °C. MCF-

10A was also kept in an atmosphere of 5% CO_2 (Thermo Scientific™ Forma Series 3 Water Jacketed CO_2 Incubator). For the MDA-MB-231 cell line, the flasks were closed, free of CO_2. The medium was changed on alternate days, until the cells reached 80-90% confluence, when they were used in subsequent assays. The cell lines were obtained from the American Type Culture Collection and confirmed to be free of mycoplasma contamination.

2.3.2. MTT Cytotoxic assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed according to Gerlier and Thomasset (1986) with modifications. Cells were seeded into 96-well plates at a concentration of 1.5 x 10^4 cells per well and incubated for 24 h for adherence. After this period, both lineages were treated with 1.5, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM of BA for 24 and 48 h. Freshly MTT solution prepared in clear medium (5 mg/mL) was added to each well and incubated at 37 ˚C for a further 4 h.

The supernatant was carefully discarded. The insoluble formazan crystals produced by intracellular dehydrogenase were solubilized with DMSO and the absorbance (Ab) of each well was read at 570 nm using a spectrophotometer (IndiaMART, DD Bioinfotech / Nathupura, New Delhi). Each sample was measured in triplicate and each experiment was repeated three times (n = 3). Cell viability was calculated according to formula:

$$\text{Cell viability} = \left[\frac{Ab\ Sample - Ab\ Blank}{Ab\ DMSO - Ab\ Blank}\right] \times 100$$

Ab Sample: treated cells

Ab Blank: untreated cells

Ab DMSO: cells treated with vehicle only - DMSO

Half-maximal inhibitory concentration (IC_{50}) was calculated by non-linear regression from a dose-response curve between compound concentration and percent growth inhibition (Vichai and Kirtikara, 2006), using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA).

2.3.3. LDH release assay

LDH release is an indicator of cell damage (Koroth et al., 2019). To measure LDH release into the cell supernatant, the assay was performed using CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen), according to the manufacturer's recommendations. Tumorigenic cells (MDA-MB-231) were seeded in 96-well plates, 1.5 x 10^4 cells per well and in triplicate. Cells were treated with BA at different concentrations, which were defined based on the IC_{50} value for MDA-MB-231 cells (0.12; 0.25; 0.50; 1.0; 2.0, 4.0; 8.0 and 16.0 μM).

Absorbances were read on an IndiaMART plate reader (DD Bioinfotech / Nathupura, New Delhi) at 492 nm. Cell lysis was quantified by the ratio of LDH increase in the supernatant of treated cells compared to that of lysed control cells. LDH release was calculated by formula:

$$\text{Cell lysis} = \left[\frac{Ab\ Sample - Ab\ Spontaneous\ LDH\ activity}{Ab\ Maximum\ LDH\ activity - Ab\ Spontaneous\ LDH\ activity}\right] \times 100$$

Ab Sample: treated cells

Ab Spontaneous LDH activity: untreated cells

Ab Maximum LDH activity: untreated cells + Lysis Buffer

2.3.4. Statistical analysis

Data were presented as mean ± standard deviation (SD) of the three independent experiments. Differences between BA-treated cell lines were determined using one-way ANOVA and Tukey's post hoc tests. $\alpha < 0.05$ was considered significant.

2.4. *In silico* analysis

2.4.1. Search for potential targets for betulinic acid

The search for potential targets for BA was performed using the Swiss Target Prediction tool (Daina et al., 2019). This tool estimates the 1D and 2D similarity of the

submitted compound with a library of 370,000 active compounds in more than 3,000 proteins from different species and finally suggests its most likely targets. For this search, the 2D structure of BA was obtained from PUBCHEM database (CID 64971).

2.4.2. Protein-binder docking of the main targets found

Prior to docking, potential binding sites for putative targets were identified. For this purpose, the DeepSite program (Jiménez et al., 2017) was used for the SAE1 and UBA2 complex proteins and for the POLB protein. Experimental information was used for the AKR1B10 protein. DeepSite is a protein-binding site predictor based on deep neural networks and was used to identify the central x,y,z coordinates of potential protein binding sites from the SAE1 and UBA2 complex and from the POLB protein. With respect to the AKR1B10 protein, information on the coordinates of the flufenamic acid inhibitor present in the complex was obtained from the PDB (4I5X) and referenced in the work by Zhang et al. (2013).After defining the targets and obtaining their three-dimensional structures, the binding sites for each one was defined using the DeepSite program.

After defining the binding sites, docking was carried out using GOLD program (Jones et al., 1997). GOLD performs a search for the best position of the chosen molecule at the receptor binding site using genetic algorithm (GA) and ChemPL score. The AG's flexible running search parameters were set to 200% and 10 docking solutions were generated for each analysis.The positions generated post-docking were ranked and the solutions with the best scores were selected. The best 3D complexes were analyzed using the DS Visualizer program (BIOVIA, Dassault Systèmes, Discovery Studio Visualizer, v 20.1, San Diego: Dassault Systèmes, 2020). 2D diagrams were generated to assess the molecular interactions between ligands and proteins using the LigPLot+ program (Laskowski and Swindells, 2011).

3. Results

3.1. *In vivo* Epithelial Tumor Test – ETT

Different concentrations of BA alone or in combination with DXR were selected

based on the survival assay with *D. melanogaster*. The survival rates are illustrated in **Fig. 2**. None of the concentrations tested, including controls and solvent, showed toxic effects ($\alpha > 0.05$) in *D. melanogaster*. These survival data validated the use of five concentrations of BA (0.0312, 0.0625, 0.125, 0.25 and 0.50 mg/mL) alone or in combination with DXR (0.4 mM) (for co-treatments), which were tested in two independent experiments, with replicates. Data from two independent experiments and replicates were pooled after verifying that there were no significant differences between repetitions.

Only MH adult flies were analyzed because they have the gene of interest. The epithelial tumors were observed in the eyes, head, wings, body, legs and halters (**Fig. 3**).

Table 1 depicts the frequency of tumors found in the different body segments of *D. melanogaster* treated with different concentrations of BA alone. The total frequency of tumors observed in the MH descendants was not statistically significant ($\alpha > 0.05$) when compared to the frequencies observed in the negative control. As expected, the frequencies of tumors observed in *D. melanogaster* treated with DXR (0.4 mM) were statistically significant ($\alpha < 0.05$) when compared to the frequencies in the negative control.

Table 2 shows the frequency of tumors found in the different body segments of *D. melanogaster* treated with different concentrations of BA in combination with DXR. The total frequencies of tumors observed in the MH descendants were statistically significant ($\alpha < 0.05$) in those treated with BA in combination with DXR, when compared to the frequency observed in the positive control. The reduction in tumor frequency was concentration-related and ranged from 34.96 to 71.32%.

3.2. Cytotoxicity assay

Non-tumorigenic (MCF-10A) and metastatic TNBC (MDA-MB-231) cells were treated with BA at different concentrations ranging from 1.5 to 200 μM for 24 and 48 h, and cytotoxicity was evaluated by the MTT tetrazolium salt colorimetric assay. The findings revealed that BA reduced cell viability of MCF-10A and MDA-MB-231 cells in a concentration-dependent manner in comparison to the control (non-treated cells) (**Fig. 4**).

The half-maximal inhibitory concentrations (IC_{50}) for the MCF-10A and MDA-MB-231 cell lines after 24 h were 20.98 and 10.14 µg/mL, respectively, whereas after 48 h the values were 12.13 and 2.67, respectively (**Fig. 5**). However, the cytotoxic effect of BA was more significant after 48 hours, where the reduction of growth viability was more efficient in MDA-MB-231 cells. Thus, under the present experimental conditions, BA had a selective effect against tumorigenic cells after 48 h.

3.3. LDH release assay

The LDH release assay was conducted to measure cell damage caused by BA using IC_{50} values for MDA-MB-231 cells (IC_{50} = 2.67 µM) (**Fig. 6**). Cell lysis was quantified by the increased ratio of LDH in the supernatant of treated cells compared to lysed control cells. No significant difference was observed in LDH release when comparing untreated and BA-treated cells. Although the presence of LDH released in the medium can confirm the result of MTT assay, its absence suggests that the mechanisms involved do not promote cell lysis. Thus, under the present experimental conditions, BA did not induce necrosis in tumor cells, but was able to induce apoptosis without lysis cell membranes.

3.4. *In silico* analysis

The most likely targets found for BA were obtained from the Protein Database and are: [1] SUMO-activating enzyme, SAE1 and UBA2 complex (PDBid: 6CWZ); [2] DNA polymerase beta, POLB (PDBid: 1BPX); [3] Aldo-keto reductase family 1 member B10, AKR1B10 (PDBid: 4I5X). The result of the search for the most likely targets of BA resulted that POLB, AKR1B10 and SUMO (SAE1/UBA2) had a 97.16, 97.16 and 81.26% chance of being targets of the BA ligand, respectively.

For the SAE1 and UBA2 protein complex, two sites were identified with a high probability of being binding sites (score 0.99), which we call pocket 1 and 2 with the coordinates x,y,z (103.2, -12.8, 357.0) and (107.2, -2.8, 399.0). For the POLB protein, two sites with a high probability of being binding sites were also identified (score 0.97), which we call pocket 1 and 2 with the coordinates x,y,z (17.8, -3.2, 4.2) and (1.8, 16.8, 6.2). Regarding the AKR1B10 protein, the information on the coordinates of the flufenamic acid inhibitor was used to reference pocket 1.

After defining the sites, dockings were generated and the highest values of the ChemPLP scores were used to select the best complexes (**Table 3**). The best docking solutions were analyzed for their atomic protein-ligand interactions and the 3D and 2D images are listed below (**Fig. 7** and **Fig. 8**).

4. Discussion

Our study used the Epithelial Tumor Test (ETT) in *D. melanogaster* to assess the toxicity and carcinogenicity of BA isolated or co-treated with the chemotherapeutic agent DXR. The results of the survival test demonstrated that BA was not toxic to *D. melanogaster* at any concentration in both treatments performed, which is in agreement with previously published studies using the same model organism (Freitas et al., 2015; Oliveira et al., 2020).

BA alone did not increase the total frequency of tumors, but significantly reduced the total frequency of tumors when co-administered with DXR. There was tumor reduction in all five concentrations tested (34.96, 56.64, 55.24, 69.23 and 71.32%, respectively). Our findings agree with *in vivo* studies with different model organisms that reported the antimutagenic/antigenotoxic and anticarcinogenic potential of BA (Acésio et al., 2016; Freitas et al., 2015; Carvalho Júnior et al., 2019).

Recently, our research group demonstrated that BA, besides being non-toxic and non-genotoxic, was able to modulate urethane-induced genotoxicity (URE) in the micronucleus test in mouse bone marrow. In the same study, BA was neither toxic nor mutagenic and showed modulatory effects on URE-induced mutagenicity in the Somatic Mutation and Recombination Test (SMART) in *D. melanogaster* somatic cells (Oliveira et al., 2020).

Ferreira et al. (2021) also revealed that BA exerted an antigenotoxic effect against doxorubicin (DXR) in the micronucleus test in mice, in addition to presenting an anticarcinogenic effect against 1,2-dimethylhydrazine (DMH)-induced colorectal lesions in rats through aberrant crypt foci. The study also found that BA modulated the activation of the NF-kB complex, inhibiting COX-2 and consequently blocking cell proliferation.

Based on the literature, it is believed that the protective effect of BA in *Drosophila* is mainly related to the antioxidant and apoptotic potential of this

compound. It is possible that BA attenuated the production of ROS generated by DXR modulating its carcinogenicity. Indeed, the antioxidant potential of BA has been reported in the literature (Kong et al., 2021; Zhu et al., 2020).

Studies by Zhu et al. (2018) reported BA protection against dexamethasone-induced oxidative stress through the JNK-P38 MAPK signaling pathway. Wu et al. (2019) demonstrated that BA attenuates T-2 toxin-induced oxidative damage of the testis by regulating the JAK2/STAT3 signaling pathway in mice. Additionally, BA can effectively attenuate remifentanil-induced hyperalgesia by inhibiting oxidative stress and subsequently downregulating matrix metalloproteinase-9 related pro-inflammatory cytokines in spinal dorsal horn (Lv et al., 2018).

In the literature, there are also several reports of the apoptotic effect of BA. It is possible that part of the BA protection against DXR-induced carcinogenicity in *D. melanogaster* is due to apoptosis of damaged cells. To reinforce this hypothesis, our study investigated *in vitro* the effect of BA on MCF-10A (non-tumorigenic) and MDA-MB-231 (tumorigenic) TNBC for 24h and 48h.

It was observed that BA was cytotoxic to MCF-10A and MDA-MB-231 cells in a concentration-dependent manner compared to the control (untreated cells). However, the cytotoxic effect of BA was more significant after 48 hours, where the reduction of growth viability was more efficient in MDA-MB-231 cells. Therefore, under the present experimental conditions, BA had a selective effect against tumorigenic cells after 48 h. To measure the cell damage caused by BA in the same cell lines, the LDH release assay was used. The results showed no significant difference in LDH release when comparing untreated and BA-treated cells, thereby revealing that BA did not induce necrosis in tumor cells but can induce apoptosis without breaking cell membranes.

Our findings agree with studies that demonstrated that BA induces apoptosis and ultrastructural changes in MDA-MB-231 TNBC (Gao et al., 2018). BA also decreased the viability of three breast cancer cell lines and markedly impaired cell migration and invasion. Furthermore, BA can inhibit the activation of STAT3 and FAK, which resulted in the reduction of matrix metalloproteinases (MMPs) and increased expression of the inhibitor of MMPs (TIMP-2) (Zeng et al., 2017). Cai et al. (2018) also evidenced that BA chemosensitized breast cancer through directly targeting glucose-regulated protein 78 (GRP78) to trigger the apoptotic pathway.

Other studies have shown the effects of BA on other types of tumor cells. Recently, Kutkowska et al. (2021) reported that BA induced apoptosis and reduced clonogenic activity in lung cancer cell lines (NSCLC) under hypoxic conditions. Chen et al. (2020) revealed that BA triggered apoptosis and inhibited the migration and invasion of gastric cancer cells (SNU-16 and NCI-N87) by preventing the progress of migration, invasion, and epithelial-mesenchymal transition. Park et al. (2021) also described that the anticancer effect of BA on u937 human leukemia cells is mediated by ROS-dependent cell cycle arrest and apoptosis.

We believe that the absence of necrosis is because BA does not disrupt cell membranes and/or induce apoptosis by other mechanisms, such as inhibiting aerobic glycolysis and blocking lactate production. BA can inhibit aerobic glycolysis activity in MCF-7 and MDA-MB-231 breast cancer cell lines via Cav-1 / NF-κB / c-Myc pathway, preventing lactate production, glucose uptake and rate of extracellular acidification (ECAR), as well as suppressing proteins related to aerobic glycolysis, including c-Myc, lactate dehydrogenase A (LDH-A) and p-PDK1/PDK1 (pyruvate dehydrogenase kinase 1) (Jiao et al., 2019). Moreover, BA can suppress aerobic glycolysis of breast cancer cells, presenting itself as a reduction in lactate production, quiescent energy phenotype transition and downregulation of proteins related to aerobic glycolysis (Zeng et al., 2019).

Thus, to elucidate how BA works, we performed an *in silico* analysis through molecular docking. The most likely BA targets were POLB, AKR1B10 and SUMO (SAE1/UBA2) had a 97.16, 97.16 and 81.26% chance of being targets of the BA ligand, respectively.

DNA polymerase beta (pol β) has a significant effect on resistance to chemotherapeutic agents, as its overexpression minimizes the effectiveness of anticancer drug therapies. As a result of contouring errors, the enzyme can help cancer cells tolerate DNA damage (Barakat et al., 2012). According to the literature, BA has DNA polymerase inhibitory activity. Studies by Ma et al. (1999) demonstrated that BA potentiated the activity of bleomycin, a DNA strand break mediator, through inhibition of poly β DNA in P-388D1 cells. Gao et al. (2008) found that the inhibition of poly β by BA results in its cytotoxic activity through the inhibition of DNA repair synthesis.

BA also targets the Aldo-keto reductase family 1 member B10 protein (AKR1B10). AKR1B10 is a protein that has been identified in human hepatocellular

carcinoma (HCC) (Cao et al., 1998). AKR1B10 is a member of the aldo-keto reductase (AR) superfamily, a group of proteins involved in intracellular detoxification, cell carcinogenesis and drug resistance. In normal tissues, AKR1B10 is mainly expressed in the intestinal system and protects cells against carbonyl damage (Liu et al., 2019). However, AKR1B10 is overexpressed in breast cancer, hepatocellular carcinoma, non-small cell lung carcinoma, cervical and endometrial cancer. In breast cancer cells, overexpression of AKR1B10 promotes migration and invasion of MCF-7 and BT-20 cells by activating the ERK signaling pathway (Li et al., 2017). Previous study conducted by Huang et al. (2016) showed that AKR1B10 promotes breast cancer metastasis through activation of FAK/Src/Rac1 signaling pathway mediated by α5 integrin and δ-catenin. Huang et al. (2018) with breast cancer cells also exhibited that AKR1B10 promoted clonogenic growth and proliferation of breast cancer cells in two-dimensional (2D) and three-dimensional (3D) cultures and tumor growth in immunodeficient female nude mice through activation of the PKC/ERK pathway. Takemura et al. (2011), using HeLa and HT29 cells, revealed that several pentacyclic triterpenes showed selective and potent inhibition of AKR1B10, including BA, which may be related to a possible cancer inhibitory role.

Finally, most SUMO substrates that have been identified so far are involved in transcription, RNA processing, DNA repair and chromatin remodeling (Welchman et al., 2005). Importantly, preventing enhanced SUMO conjugation of cellular substrates in breast cancer cells reduces tumorigenesis (Bawa-Khalfe and Yeh, 2010). Studies indicate that SAE1/UBA2, a heterodimer that catalyzes the first step of the SUMOylation cascade, is involved in regulating tumor growth and metastasis. For example, elevated levels of SAE1 and UBA2 (SAE2) are correlated with decreased survival and increased metastatic capacity in breast cancer patients (Kessler et al., 2012). Mo et al. (2005) showed that augmentation of SUMO conjugation in breast cancer cells increases tumor formation and stable overexpression of Ubc9 in the breast cancer cell line MCF-7 increases SUMOylation and tumor volume when xenografted into mice. Therefore, it is possible that the link between BA and the SUMO enzyme influences the inhibition of proliferation of breast cancer cells tested in the present study. BA decreases the level of specificity of protein 1 (Sp1) by increasing the SUMOylation of Sp1 to inhibit lung cancer growth (Hsu et al., 2012). Furthermore, BA increased radiosensitization of oral squamous cell carcinoma by inducing Sp1

SUMOylation and PTEN expression (Jiang et al., 2021).

In conclusion, the results of our study indicated that, under the experimental conditions, BA was not carcinogenic in *Drosophila*, but significantly reduced the total frequency of tumors when co-administered with DXR. BA showed an antiproliferative effect on breast cells, the most significant effect after 48 h. Furthermore, the compound had a selective effect against tumor cells compared to non-tumor cells. We also observed that BA did not disrupt the membranes of treated cells, hence indicating an apoptotic rather than necrotic effect. The results of molecular docking demonstrated the interaction of BA with SUMO proteins (SAE1 and UBA2), DNA POLB and AKR1B10. Based on literature data, we can suggest that the modulatory effects of BA can be explained by its antioxidant and apoptotic effects. Taken together, our data suggest that BA can modulate the carcinogenic effect of DXR, inducing damaged cells to apoptosis through different pathways involving SUMO proteins (SAE1 and UBA2), DNA POLB and AKR1B10. Further studies should be conducted using other reference carcinogens and different organisms to confirm the use of this compound in the production of new drugs.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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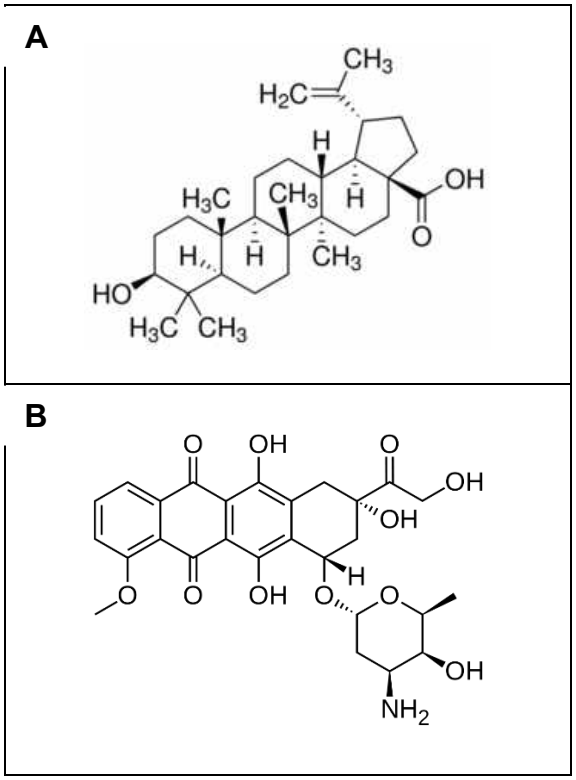


Fig. 1. Structural formula of (**A**) Betulinic acid and (**B**) Doxorubicin.

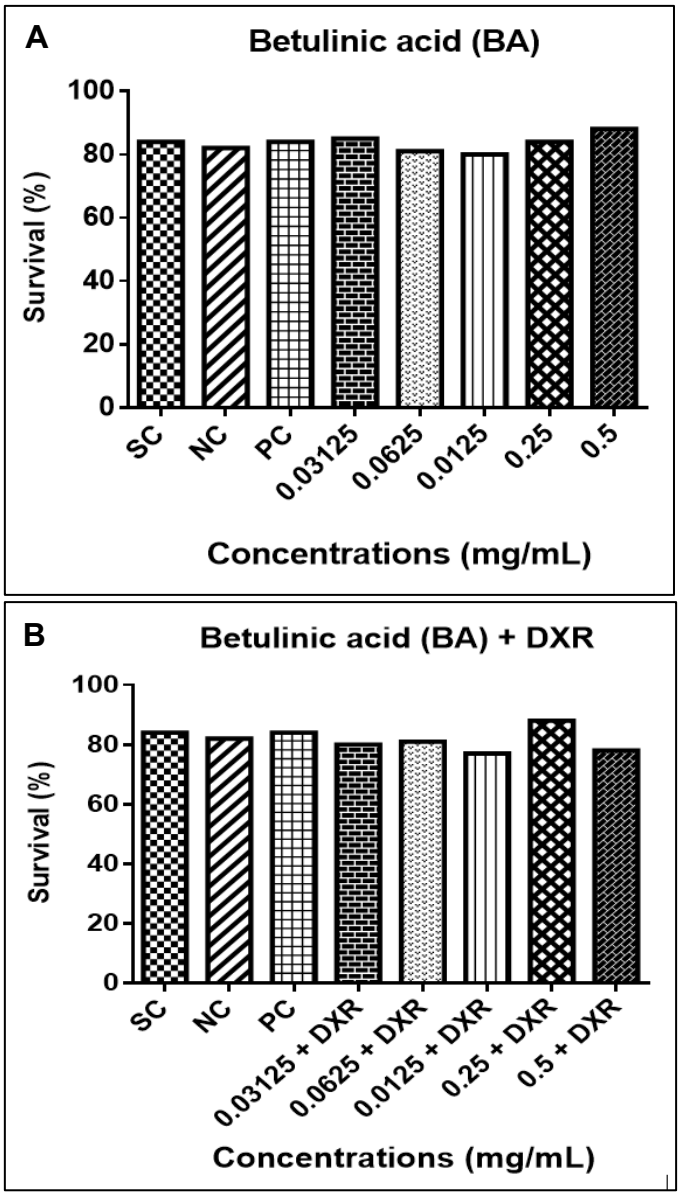


Fig. 2. Survival rates (%) of individuals from ETT (**A**) upon exposure to different concentrations of betulinic acid (BA 0.03125 - 0.5 mg/mL) alone; (**B**) upon exposure to BA (0.03125 - 0.5 mg/mL) in combination with doxorubicin (DXR 0.4 mM). **SC:** solvent control (1% tween 80 and 3% ethanol); **NC:** negative control (ultrapure water) and **PC:** positive control (DXR 0.4 mM). Data are representative of survival assays performed only once, without replication. Statistical comparisons were made using the Chi-square test for ratios for independent samples ($\alpha < 0.05$).

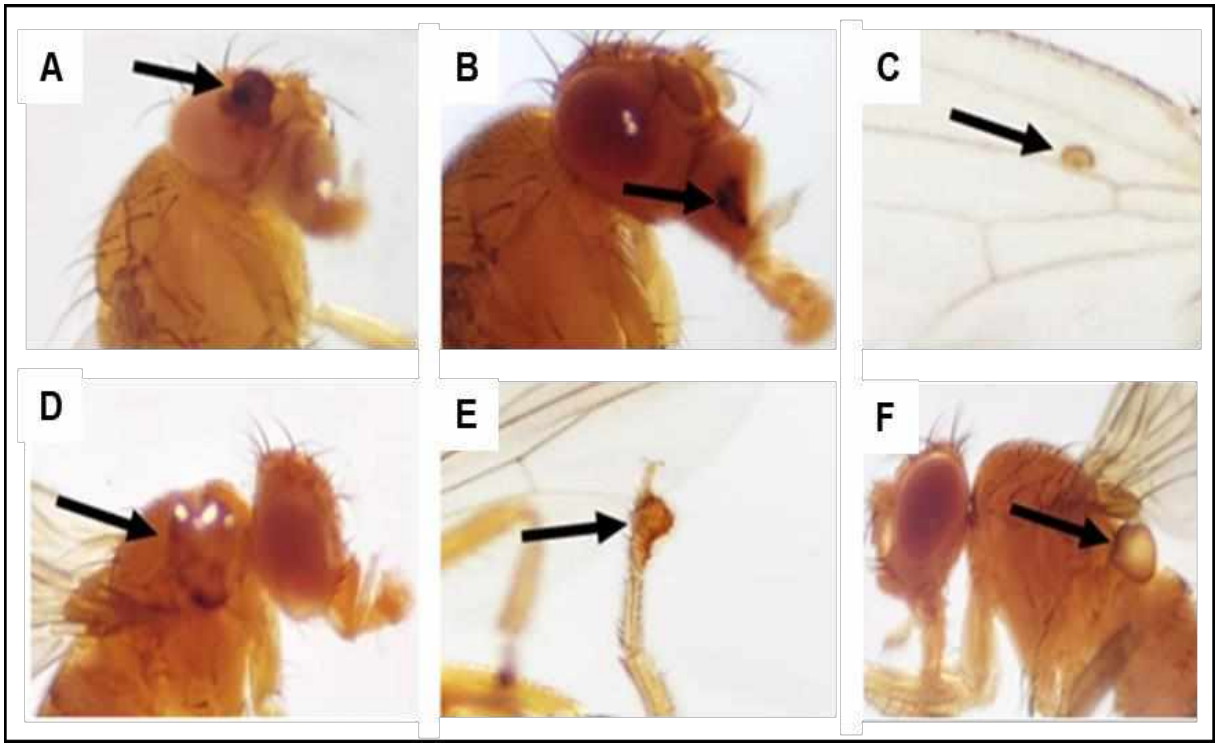


Fig. 3. Photomicrographs of *D. melanogaster*, obtained by light stereomicroscope at 25x magnification, showing epithelial tumors (arrows) in different parts of the fly: (**A**) eyes; (**B**) head; (**C**) wings; (**D**) body; (**E**) legs; (**F**) halters.

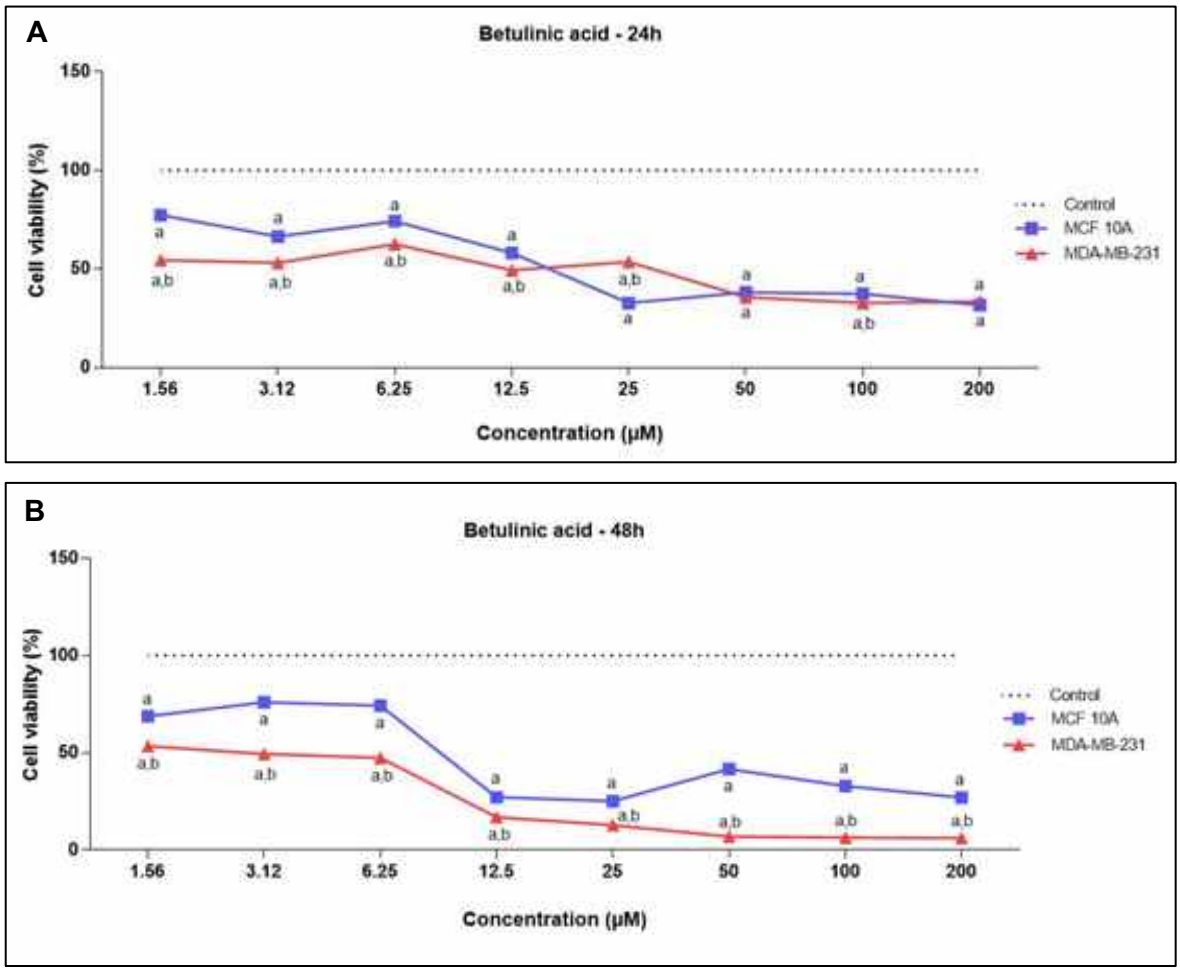


Fig. 4. Cell viability (%) by MTT assay. The cytotoxicity of betulinic acid (1.56 - 200 μM) was evaluated in triple-negative human breast cancer strain (MDA-MB-231) and in the non-tumorigenic strain (MCF-10A). The trial was conducted in 24h (**A**) and 48h (**B**) of treatment. Three independent experiments were performed (n = 3).

[a] Values considered different from the control (untreated cells), $\alpha < 0.05$.

[b] Values considered different from the non-tumorigenic cell line (MCF 10A) ($\alpha < 0.05$).

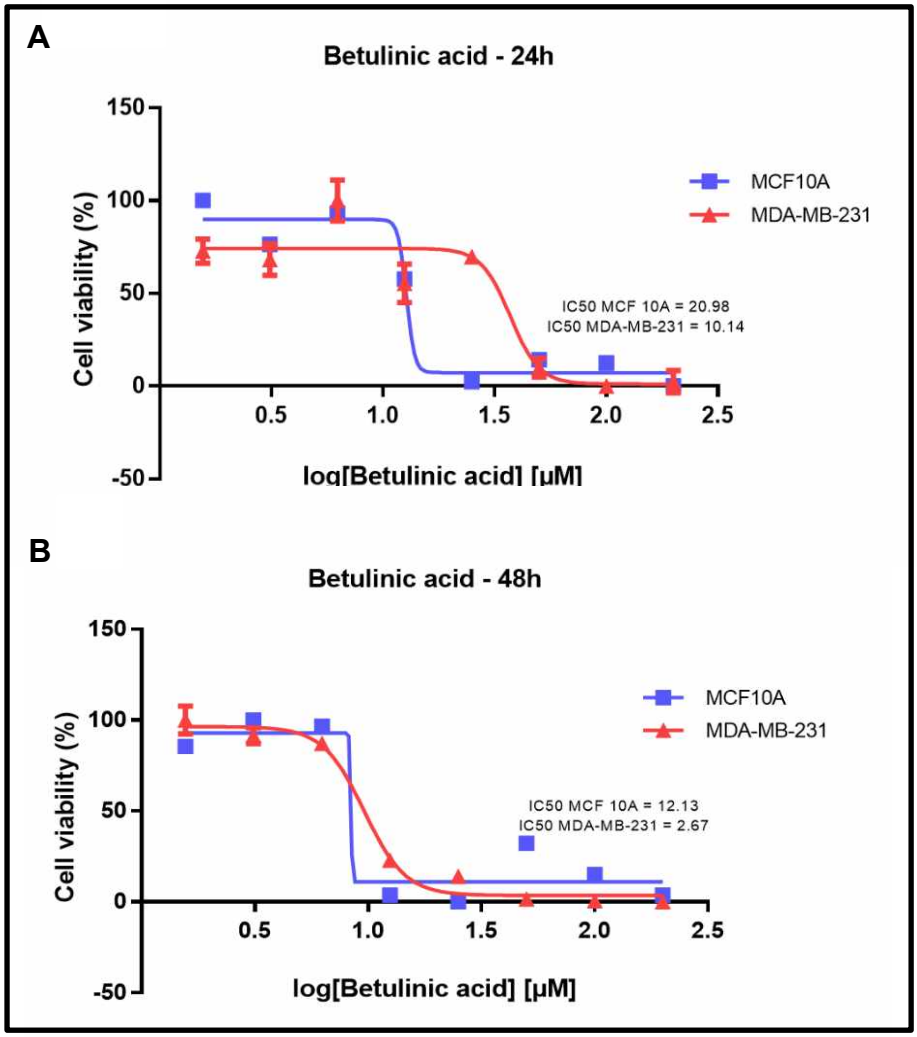


Fig. 5. IC_{50} values of betulinic acid based on cytotoxicity assay - MTT with triple-negative breast cancer cell line (MDA-MB-231) and non-tumorigenic breast line (MCF-10A). Values were calculated for 24h (**A**) and 48h (**B**) of treatment.

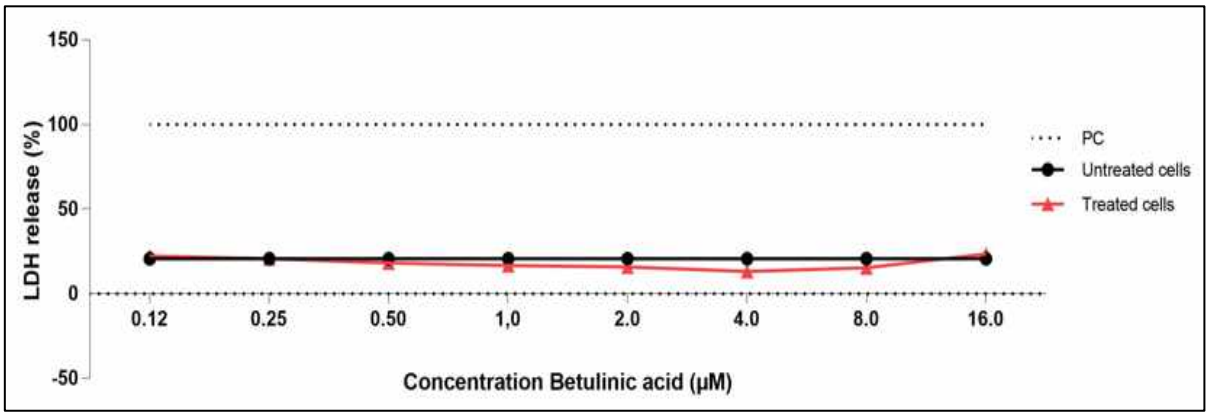


Fig. 6. Lactate dehydrogenase (LDH) assay, in the release of LDH in the cell culture medium after treatment of triple-negative breast cancer cell (MDA-MB-231) with different concentrations of betulinic acid (0.12 - 16.0 µM) for 48 hours.

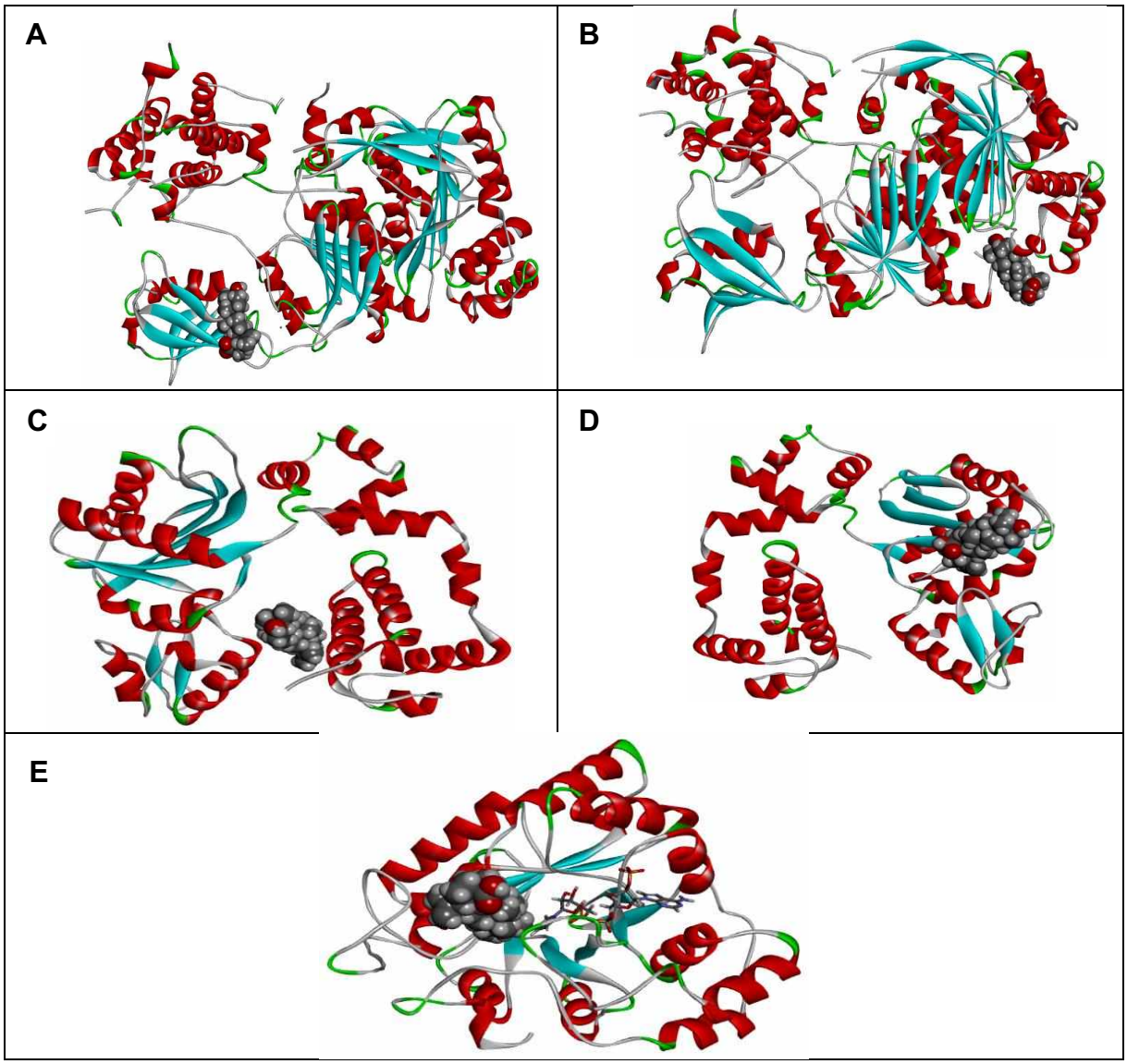
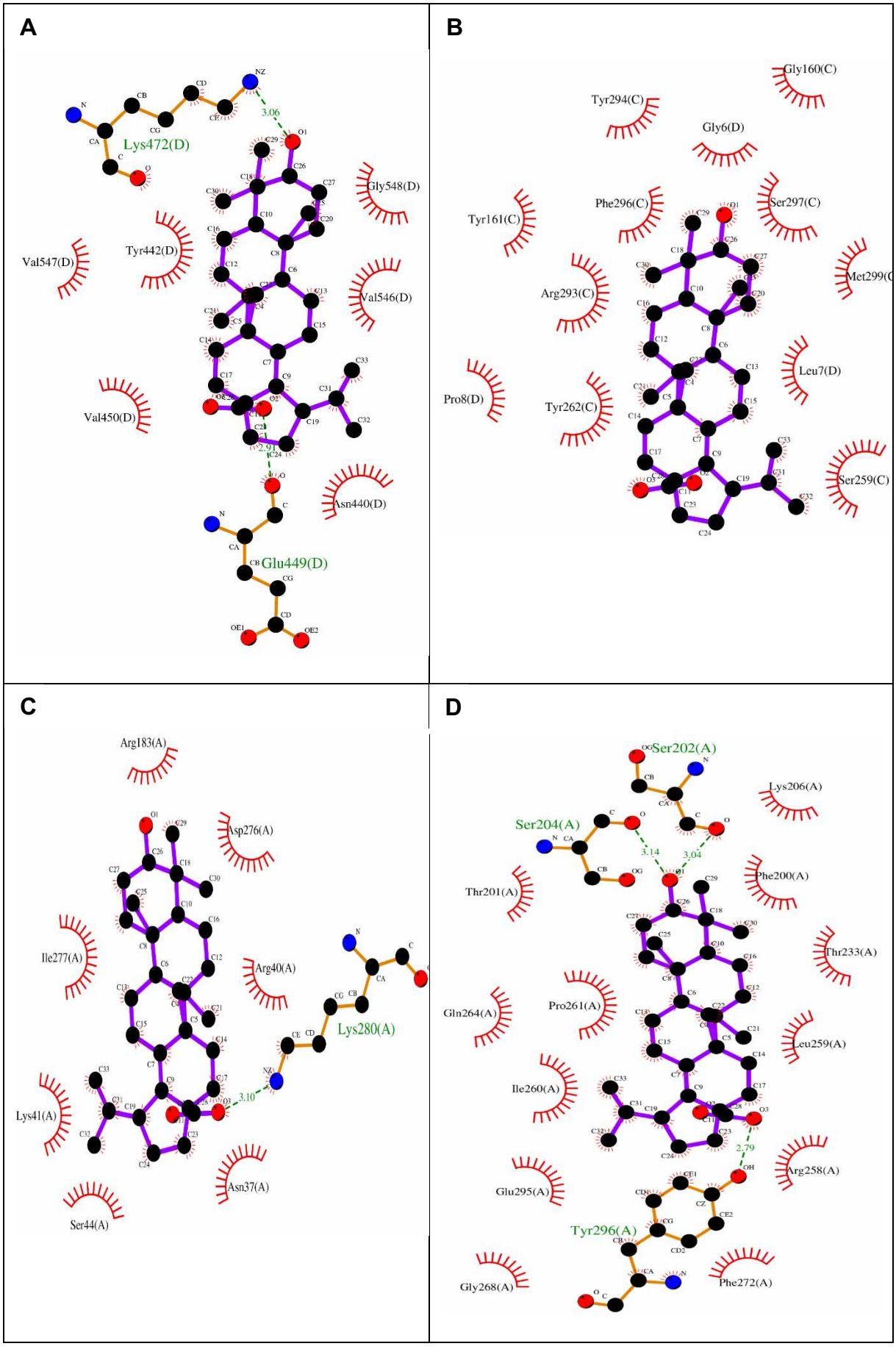


Fig. 7. 3D images of the best docking solutions that were analyzed for their atomic-protein ligand *vs.* betulinic acid (BA) interactions. **A.** SAE (pocket1) *vs.* BA. **B.** SAE (pocket2) *vs.* BA. **C.** POLB (pocket1) *vs*. BA. **D.** POLB (pocket2) *vs*. BA. **E.** AKR1B1 (pocket1) *vs.* BA.


A
Lys472(D)
Gly548(D)
Tyr442(D)
Val547(D)
Val546(D)
Val450(D)
Asn440(D)
Glu449(D)
B
Gly160(C)
Tyr294(C)
Gly6(D)
Phe296(C)
Ser297(C)
Tyr161(C)
Met299(C
Arg293(C)
Leu7(D)
Pro8(D)
Tyr262(C)
Ser259(C)
C
Arg183(A)
Asp276(A)
Ile277(A)
Arg40(A)
Lys280(A)
Lys41(A)
Asn37(A)
Ser44(A)
D
Ser202(A)
Lys206(A)
Ser204(A)
Phe200(A)
Thr201(A)
Thr233(A)
Gln264(A)
Pro261(A)
Leu259(A)
Ile260(A)
Arg258(A)
Glu295(A)
Tyr296(A)
Gly268(A)
Phe272(A)

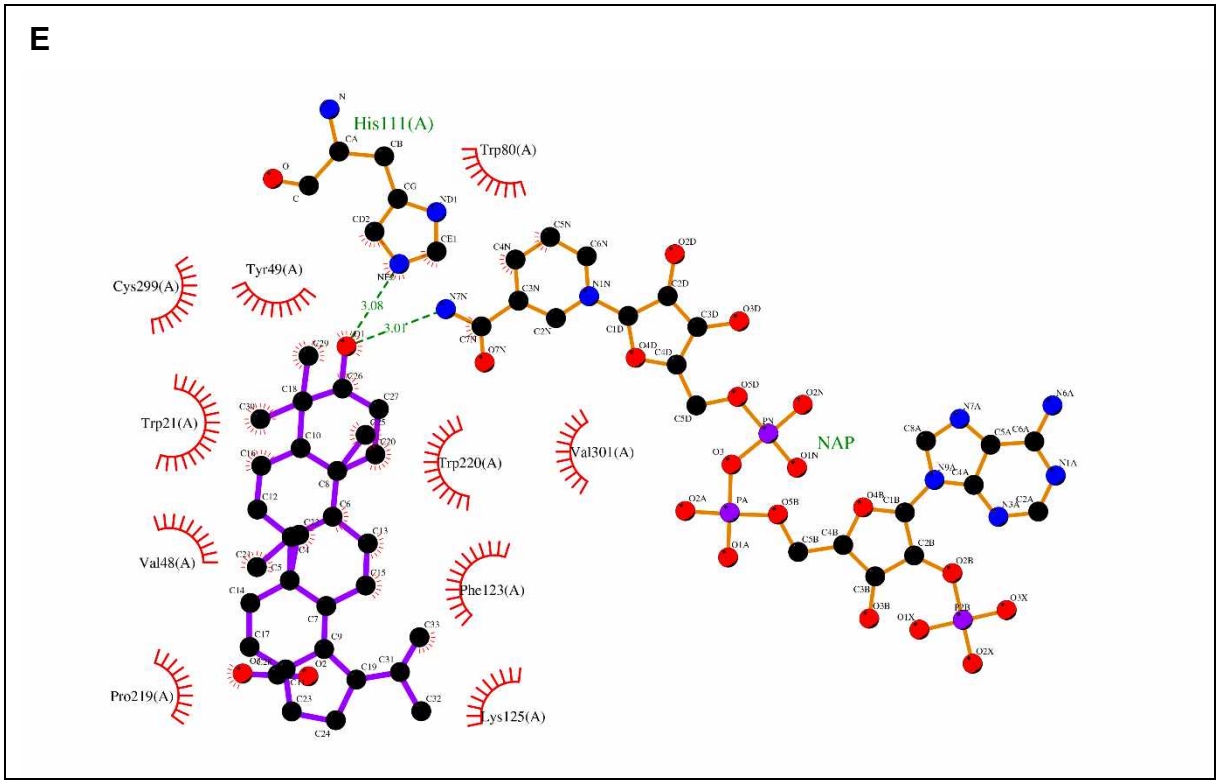


Fig. 8. 2D plotting of ligand-protein interactions between: **A.** SAE (pocket1) *vs.* BA. **B.** SAE (pocket2) *vs.* BA. **C.** POLB (pocket1) *vs.* BA. **D.** POLB (pocket2) *vs.* BA. **E.** AKR1B1 (pocket1) *vs.* BA. In lilac the ligand atoms, and in beige the protein atoms. Green dashes represent hydrogen bond-type interaction and red dashed arcs indicate hydrophobic interactions between the ligand and the protein.

Table 1. Summary of results obtained with the *D. melanogaster* Epithelial Tumor Test (ETT) after chronic treatment of larvae with 1% tween 80 and 3% ethanol as solvent control (SC); ultrapure water as negative control (NC); doxorubicin (DXR 0.4 mM) as a positive control and different concentrations of betulinic acid (BA 0.03125 - 0.5 mg/mL)

Treatments	**N° of flies**	**Frequency of tumors**						
		Eyes	**Head**	**Wings**	**Body**	**Legs**	**Halters**	**Total tumors**
Tween 80 + ethanol	200	0.040 (08)	0.035 (07)	0.050 (10)	0.065 (13)	0.070 (14)	0.010 (02)	0.270 (54)
Ultrapure water	200	0.030 (06)	0.030 (06)	0.045 (09)	0.060 (12)	0.055 (11)	0.010 (02)	0.230 (46)
DXR	200	0.080 (16)*	0.150 (30)*	0.500 (100)*	0.300 (60)*	0.310 (62)*	0.090 (18)*	1.430 (286)*
BA								
0.03125	200	0.005 (01)	0.040 (08)	0.055 (11)	0.040 (08)	0.040 (08)	0.000 (00)	0.180 (36)
0.0625	200	0.030 (06)	0.040 (08)	0.030 (06)	0.040 (08)	0.035 (07)	0.010 (02)	0.185 (37)
0.0125	200	0.045 (09)	0.035 (07)	0.035 (07)	0.055 (11)	0.025 (05)	0.010 (01)	0.200 (40)
0.25	200	0.045 (09)	0.035 (07)	0.020 (04)	0.045 (09)	0.040 (08)	0.005 (01)	0.190 (38)
0.50	200	0.005 (01)	0.030 (06)	0.015 (03)	0.070 (14)	0.065 (13)	0.005 (01)	0.190 (38)

Statistical diagnoses according to the Mann-Whitney test. Significance level $\alpha \leq 0.05$.

* Values considered different from the negative control (ultrapure water).

Table 2. Summary of results obtained with the *D. melanogaster* Epithelial Tumor Test (ETT) after chronic treatment of larvae with 1% tween 80 and 3% ethanol as solvent control (SC); ultrapure water as negative control (NC); doxorubicin (DXR 0.4 mM) as a positive control and different concentrations of betulinic acid (BA 0.03125 - 0.5 mg/mL) co-treated with DXR 0.4 mM

Treatments	N° of flies	Frequency of tumors							Reduction (%)
		Eyes	Head	Wings	Body	Legs	Halters	Total tumors	
Tween 80 + ethanol	200	0.040 (08)	0.035 (07)	0.050 (10)	0.065 (13)	0.070 (14)	0.010 (02)	0.270 (54)	
Ultrapure water	200	0.030 (06)	0.030 (06)	0.045 (09)	0.060 (12)	0.055 (11)	0.010 (02)	0.230 (46)	
DXR	200	0.080 (16)*	0.150 (30)*	0.500 (100)*	0.300 (60)*	0.310 (62)*	0.090 (18)*	1.430 (286)*	
BA + DXR									
0.03125 + 0.4	200	0.060 (12)	0.090 (18)	0.310 (62)	0.220 (44)	0.250 (50)	0.000 (00)**	0.930 (186)**	34.96
0.0625 + 0.4	200	0.090 (18)	0.050 (10)**	0.190 (38)**	0.170 (34)**	0.090 (18)**	0.030 (06)**	0.620 (124)**	56.64
0.125 + 0.4	200	0.030 (06)**	0.080 (16)	0.220 (44)**	0.200 (40)	0.090 (18)**	0.020 (04)**	0.640 (128)**	55.24
0.25 + 0.4	200	0.030 (06)**	0.090 (18)	0.200 (40)**	0.070 (14)**	0.060 (12)**	0.000 (00)**	0.440 (88)**	69.23
0.5 + 0.4	200	0.060 (12)	0.050 (10)**	0.100 (20)**	0.050 (10)**	0.150 (30)**	0.000 (00)**	0.410 (82)**	71.32

Statistical diagnoses according to the Mann-Whitney test. Significance level $\alpha \leq 0.05$.

* Values considered different from DXR (PC) *vs.* ultrapure water (NC).

** Values considered different from BA + DXR *vs.* DXR (PC).

Table 3. Best scores for each docking solution based on defined binding sites.

Target		CHEMPLP score
SAE1	Pocket 1	50.21
	Pocket 2	54.61
POLβ	Pocket 1	41.18
	Pocket 2	59.72
AKR1B1	Pocket 1	62.33

CONCLUSÕES FINAIS

Os resultados observados em nosso estudo permitem concluir que, nas condições experimentais, o AB apresentou efeito antigenotóxico contra a genotoxicidade induzida pelo URE em camundongos Swiss. Não foi mutagênico em *Drosophila*, mas modulou o dano ao DNA principalmente após metabolização. Com base em dados da literatura, podemos sugerir que os efeitos modulatórios do AB podem ser explicados por seus efeitos antioxidantes e apoptóticos. Em conjunto, nossos dados sugerem que o AB pode interagir com o sistema enzimático que catalisa a desintoxicação metabólica do URE, inibindo a atividade do complexo mitocondrial I, capturando assim os radicais livres. Além disso, AB também pode modular a ativação metabólica de URE, inibindo a formação de metabólitos de ligação ao DNA e induzindo células danificadas à apoptose.

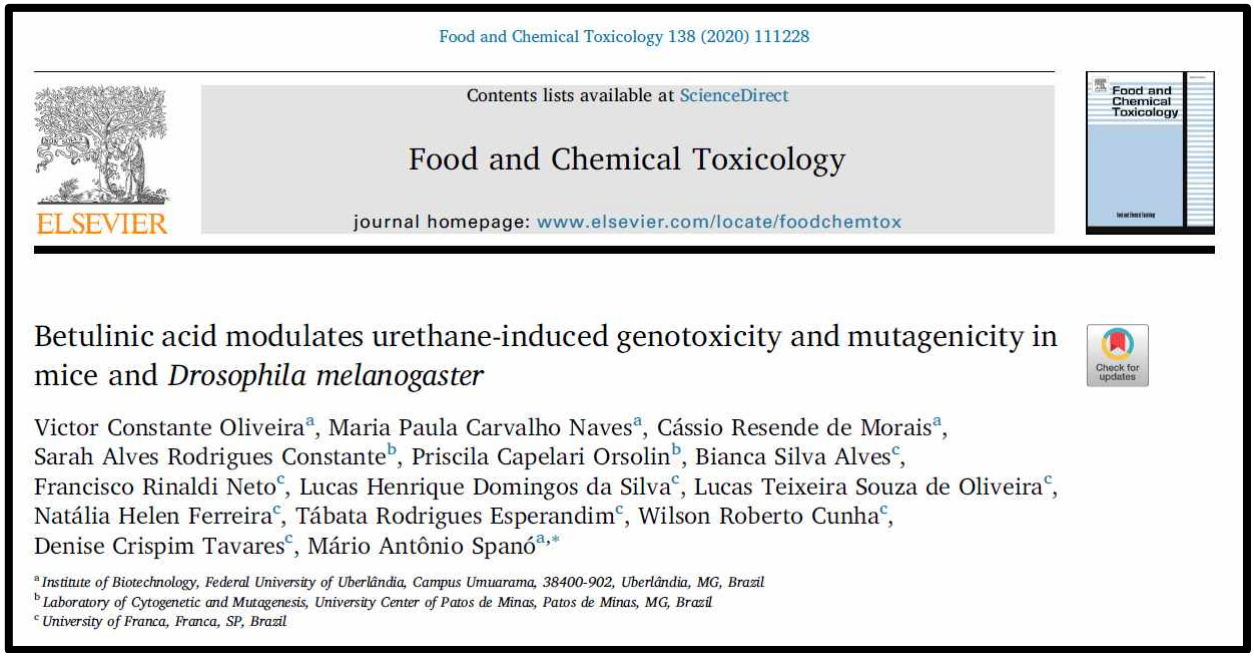
Além disso, nossos resultados também indicaram que, nas condições experimentais, AB não foi cancerígeno em *Drosophila*, mas reduziu significativamente a frequência total de tumores quando coadministrado com DXR. AB mostrou um efeito antiproliferativo nas células da mama, sendo o efeito mais significativo após 48 h. Além disso, o composto teve um efeito seletivo contra células tumorais em comparação com células não tumorais. Também observamos que o AB não rompeu as membranas das células tratadas, indicando um efeito apoptótico em vez de necrótico. Os resultados do docking molecular demonstraram a interação de BA com proteínas SUMO (SAE1 e UBA2), DNA POLB e AKR1B10. Com base em dados da literatura, podemos sugerir que os efeitos modulatórios do AB podem ser explicados por seus efeitos antioxidantes e apoptóticos. Em conjunto, nossos dados sugerem que AB pode modular o efeito carcinogênico de DXR, induzindo células danificadas à apoptose por meio de diferentes vias envolvendo as proteínas SUMO (SAE1 e UBA2), DNA POLB e AKR1B10. Novos estudos devem ser realizados utilizando outros mutágenos e/ou carcinógenos de referência e diferentes organismos para confirmar o uso desse composto na produção de novos medicamentos.

Anexo I

Published scientific article

Journal: Food and Chemical Toxicology.

Impact factor: 6.023.



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journal homepage: www.elsevier.com/locate/foodchemtox

Betulinic acid modulates urethane-induced genotoxicity and mutagenicity in mice and *Drosophila melanogaster*

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Anexo II

Premiação de melhor trabalho

Evento: XIV Congresso da Associação Brasileira de Mutagênese e Genômica Ambiental - MutaGen-Brasil.

Ano/Local: 2019, Bento Gonçalves - RS.

Título do trabalho: Modulatory effects of betulinic acid on the mutagenicity induced by urethane in wing somatic cells of *Drosophila melanogaster*.

Anexo III

Premiação de melhor trabalho

Evento: XV Congresso da Associação Brasileira de Mutagênese e Genômica Ambiental - MutaGen-Brasil.

Ano/Local: 2021, virtual.

Título do trabalho: Modulatory effects of betulinic acid on epithelial tumor incidence in doxorubicin-treated *Drosophila melanogaster*.