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DISSERTAÇÃO DE MESTRADO

AVALIAÇÃO TOXICOLÓGICA E ATIVIDADES ANTIMICROBIANA E ANTI-INFLAMATÓRIA DE EXTRATO ETANÓLICO DE FOLHAS DE *Morus alba* L. (Moraceae)

ALISSON MACÁRIO DE OLIVEIRA

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COORIENTADORA: PROFA. DRA. IVONE ANTÔNIA DE SOUZA

RECIFE
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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

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e amigos por todo incentivo e colaboração
durante toda minha vida, servindo de inspiração
e exemplo.

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“Não seja o de hoje. Não suspires por ontem. Não queiras ser o de amanhã. Faze-te sem limites no tempo”.

(Cecília Meireles)

RESUMO

Morus alba L. (amoreira branca) é uma planta utilizada em todo o mundo no tratamento de diversas enfermidades, incluindo distúrbios inflamatórios. Essa planta tem origem asiática, mas demonstrou boa adaptação ao solo brasileiro. Diante da expansão do uso medicinal de *M. alba*, inclusive comercialmente, o presente trabalho teve por objetivos construir um painel de toxicidade para extrato etanólico das folhas dessa planta e determinar seu potencial como agente antimicrobiano e anti-inflamatório. O extrato foi analisado quanto à composição química por cromatografia em camada delgada. As análises toxicológicas realizadas foram: ensaio de letalidade para o microcrustáceo *Artemia salina* (concentrações: 100–1000 µg/mL); ensaio de toxicidade aguda por via oral para camundongos (doses: 300 e 2000 mg/kg); determinação da genotoxicidade *in vivo* para camundongos através do teste do micronúcleo (doses: 75, 150 e 300 mg/kg v.o.); e ensaios de citotoxicidade para as células tumorais humanas NCI-H292, HEp-2, HT-29, MCF-7 e HL-60 (concentração: 50 µg/mL). Na avaliação do potencial anti-inflamatório, foi utilizado o modelo de inflamação aguda induzida por carragenina em bolsão de ar (doses: 75, 150 e 300 mg/kg v.o.). A ação anti-inflamatória foi avaliada através da quantificação do número de leucócitos nos exsudatos coletados dos bolsões. Atividade antimicrobiana foi avaliada contra bactérias e fungos de importância médica. A análise fitoquímica revelou a presença de cumarinas, flavonoides, taninos e triterpenos no extrato, o qual não promoveu mortalidade dos náuplios de *A. salina.* No teste de toxicidade aguda, não houve mortalidade nem alterações comportamentais nos camundongos tratados com o extrato durante 14 dias. Contudo, a análise de parâmetros hematológicos e bioquímicos revelou que os animais que receberam a dose mais alta apresentaram valores significativamente ($p < 0,05$) menores de volume corpuscular médio eritrocitário (VCM) e concentração média de hemoglobina corpuscular (CMHC), bem como atividade aumentada de fosfatase alcalina no plasma. Nos tratamentos com o extrato a 300 e 2000 mg/kg, houve redução no número de leucócitos, com diminuição da percentagem de linfócitos e aumento na proporção de células segmentadas. Análise histopatológica dos órgãos dos animais tratados com o extrato a 2000 mg/kg revelou turgidez dos túbulos contorcidos renais, presença de infiltrado leucocitário ao redor da veia centrilobular hepática e elevada dispersão da polpa branca esplênica. Avaliação do potencial genotóxico *in vivo* revelou que o extrato não causou aumento na frequência de micronúcleos, em comparação com o controle negativo. O extrato também não demonstrou citotoxicidade para as linhagens celulares testadas. Com base nos resultados obtidos nos testes de toxicidade, o potencial anti-inflamatório do extrato foi avaliado utilizando doses menores e igual a 300 mg/kg. Foi verificada redução do número de leucócitos nos grupos tratados com 75 (56,8%), 150 (62,9%) e 300 (65,6%) mg/kg, em comparação com o grupo controle. A dose de 300 mg/kg mostrou efeito de inibição similar ao observado no grupo padrão, que recebeu indometacina a 10 mg/kg, i.p (64,7%). Por fim, o extrato apresentou atividade antimicrobiana contra *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida krusei*, *Candida tropicalis* e *Aspergillus flavus*. Em conclusão, o extrato etanólico de folhas de *M. alba* apresentou potencial anti-inflamatório e antimicrobiano, não sendo letal nem apresentando efeito genotóxico para camundongos quando ingerido na dose de 300 mg/kg. Contudo, a utilização do extrato na dose de 2000 mg/kg deve ser evitada, devido às alterações bioquímicas, hematológicas e histopatológicas detectadas no ensaio de toxicidade aguda.

Palavras-chave: amoreira branca; análise toxicológica; genotoxicidade; citotoxicidade; atividade antimicrobiana; inflamação aguda.

ABSTRACT

Morus alba L. (white mulberry) is a plant used around the world for treatment of several infirmities, including inflammatory disorders. This plant is originated from Asia but showed good adaptation to Brazilian soil. In view of the expansion of the medicinal use of *M. alba*, including commercially, the present work aimed to build a toxicity panel for an ethanolic extract from leaves of this plant and to determine its potential as antimicrobial and anti-inflammatory agents. The extract was analyzed for chemical composition through thin layer chromatography. The toxicological analyses performed were: lethality assay with the microcrustacean *Artemia salina* (concentrations: 100–1000 µg/mL); acute oral toxicity assay to mice (doses: 300 and 2000 mg/kg); determination of *in vivo* genotoxicity to mice by micronucleus test (doses: 75, 150 and 300 mg/kg *per os*); and cytotoxicity assays to the human tumor cells NCI-H292, HEp-2, HT-29, MCF-7 and HL-60 (concentration: 50 µg/mL). In the evaluation of anti-inflammatory potential, it was used the model of acute inflammation induced by carrageenan in air pouch (doses: 75, 150 and 300 mg/kg *per os*). The anti-inflammatory action was evaluated by the quantification of the number of leukocytes in exudates collected from the pouches. Antimicrobial activity was evaluated against bacteria and fungi of medical importance. The phytochemical analysis revealed the presence of coumarins, flavonoids, tannins and triterpenes in the extract, which did not promote mortality of *A. salina* nauplii. In the acute oral toxicity assay, there was no mortality or behavioral alterations in mice treated with the extract during 14 days. However, the analysis of hematologic and biochemical parameters revealed that animals that received the highest dose showed significantly ($p < 0.05$) lower values of mean erythrocyte corpuscular volume (MCV) and mean concentration of corpuscular hemoglobin (MCHC) as well as increased activity of alkaline phosphatase in plasma. In the treatments with the extract at 300 and 2000 mg/kg, there was reduction in the number of leukocytes, with decrease of lymphocyte percentage and increase in the proportion of segmented cells. Histopathological analysis of the organs from animals treated with the extract at 2000 mg/kg revealed turgidity of renal contorted tubules, presence of leukocyte infiltration around the hepatic centrilobular vein and high dispersion of the spleen white pulp. Evaluation of the *in vivo* genotoxic potential revealed that the extract did not cause increase in the frequency of micronuclei, in comparison with negative control. The extract also did not show cytotoxicity to the cell lines tested. Based on the results obtained in the toxicity assays, the anti-inflammatory potential of the extract was evaluated using doses lower and equal to 300 mg/kg. It was verified a reduction in the number of leukocytes in the groups treated with the extract at 75 (56.8%), 150 (62.9%) and 300 (65.6%) mg/kg, in comparison with control group. The dose of 300 mg/kg showed inhibitory effect similar to that observed in the standard group, which received indometacin 10 mg/kg, i.p (64.7%). Finally, the extract showed antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida krusei*, *Candida tropicalis* and *Aspergillus flavus*. In conclusion, the ethanolic extract from *M. alba* leaves showed anti-inflammatory and antimicrobial potential, being not lethal and showing no genotoxic effect when ingested by mice at the dose of 300 mg/kg. However, the use of the extract at 2000 mg/kg should be avoided, due to the biochemical, hematological and histopathological alterations detected in the acute toxicity assay.

Keywords: white mulberry; toxicological analysis; genotoxicity; cytotoxicity; antimicrobial activity; acute inflammation.

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ALT – Alanina aminotransferase

ANVISA – Agência Nacional de Vigilância Sanitária

AST – Aspartato aminotransferase

CMI – Concentração mínima inibitória (em inglês **MIC –** *minimal inhibitory concentration*)

CMHC – Concentração média de hemoglobina corpuscular (em inglês **MCHC –** *mean corpuscular hemoglobin concentration*)

DMEM – *Dulbecco's Modified Eagle's Medium*

DMSO – Dimetilsulfóxido

GGT – Gama glutamil transferase

HCM – Hemoglobina corpuscular média (em inglês **MCH** – *mean corpuscular hemoglobin*)

IL-1β **–** Interleucina 1β

INCA – Instituto Nacional do Câncer

MTT – Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazólio

OECD – *The Organization for Economic Co-operation and Development*

OMS – Organização Mundial de Saúde

SBMCTA – Sociedade Brasileira de Mutagênese Carcinogênese e Teratogênese Ambiental

TLC – *Thin layer chromatography*

TMN – Teste de Micronúcleo

TNF-α – Fator de Necrose Tumoral

VCM – Volume corpuscular médio (em inglês **MCV –** *mean corpuscular volume*)

SUMÁRIO

1. INTRODUÇÃO

As plantas têm tido papel fundamental para o desenvolvimento humano, tanto como componentes na alimentação quanto na busca de estratégias para a cura de enfermidades. Além da eficácia atribuída a diversas espécies, conhecidas como plantas medicinais, o contínuo uso de vegetais pela população para fins terapêuticos está também relacionado com o custo elevado dos medicamentos alopáticos convencionais e a falta de acesso aos mesmos VEIGA-JUNIOR et al., 2005; SAAD et al., 2006; SHAW, 2010).

A fitoterapia, também conhecida como medicina alternativa ou medicina popular, é mundialmente responsável pela forma mais comum de tratamento de doenças e, segundo a Organização Mundial de Saúde (OMS), 80% das populações dos países em desenvolvimento utilizam esse tipo de terapia (OMS, 2011). Contudo, as plantas medicinais têm sido empregadas de modo predominantemente empírico pelas populações, seja pela ausência de estudos científicos e toxicológicos ou pelo desconhecimento dos resultados dessas pesquisas. O crescente aumento na utilização e comercialização de plantas medicinais tem impulsionado a busca de informações comprovadas cientificamente sobre sua segurança e eficácia terapêutica (TUROLLA e NASCIMENTO, 2005; SAAD et al., 2006; RIBEIRO et al., 2012).

Os vegetais são utilizados de diversas maneiras para propósitos terapêuticos: *in natura*, com partes inteiras ou rasuradas para a preparação de chás e infusões, por exemplo; ou sob a forma de drogas pulverizadas, extratos brutos ou frações, tinturas, comprimidos e cápsulas. Finalmente, as preparações vegetais podem ser submetidas a sucessivos processos de extração e purificação, para isolamento de substâncias de interesse (RATES, 2001).

Segundo a Agência de Vigilância Sanitária (ANVISA), um medicamento fitoterápico é um produto obtido por processos tecnologicamente adequados, empregando exclusivamente matérias-primas vegetais, com finalidade profilática, curativa, paliativa ou para fins de

diagnóstico. É caracterizado pelo conhecimento da eficácia e dos riscos de seu uso, bem como pela reprodutibilidade e constância de sua qualidade. Não se considera medicamento fitoterápico aquele que, na sua composição, inclua substâncias ativas isoladas de qualquer outra origem (RDC nº 17/2000).

No Brasil, o uso de plantas medicinais como alternativa terapêutica é uma prática realizada por milhares de pessoas, sendo influenciada por fatores sociais, econômicos e culturais. Frente a esta realidade, faz-se necessária a adoção de medidas que visem a ampliação das pesquisas sobre o uso seguro dessas plantas (VENSON et al., 2011).

Morus alba é uma planta nativa da Ásia, conhecida como "amoreira branca", com grande importância na medicina popular chinesa. Essa planta foi introduzida no Brasil e tem sido bastante explorada do ponto de vista medicinal, inclusive comercialmente. Diversos estudos relatam propriedades farmacológicas dessa planta visando o tratamento de enfermidades em animais e humanos. Como exemplos, podem-se citar atividades antitumoral, antioxidante, antimicrobiana, neuroprotetora e hipoglicêmica, além de potencial aliviador dos sintomas associados à osteoartrite (KIM et al., 1993; FANG et al., 2005; WANG et al., 2012; QIN et al., 2015; SEO et al., 2015; YIMAN et al., 2015).

Frente aos diversos relatos de propriedades medicinais das folhas de *M. alba*, e a ampliação do seu uso no Brasil, esse trabalho avaliou a toxicidade *in vitro* (usando *Artemia salina*), toxicidade aguda para camundongos (incluindo análise de sobrevivência, comportamento, alterações histológicas e parâmetros hematológicos e bioquímicos) e o potencial genotóxico e citotóxico de extrato etanólico obtido a partir desse tecido. De posse dos resultados, foi então determinado o potencial anti-inflamatório *in vivo* do extrato de folhas

em dose considerada segura. Adicionalmente, foi avaliada a atividade antimicrobiana frente a bactérias e fungos de importância médica.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Toxicidade e produtos naturais

As células vegetais, utilizando substâncias químicas simples, conseguem sintetizar diversas substâncias complexas e que possuem grande importância para o homem, sendo denominadas de "produtos naturais" (CASTELLANO, 1981). O número total de produtos naturais produzidos por plantas foi estimado em torno de 500.000 (DEMAIN, 2000). Ao se estudar uma planta com relação às suas características fitoquímicas, deve-se considerar a existência de dois grupos distintos de metabólitos, que são importantes para seu desenvolvimento: metabólitos primários e metabólitos secundários (WANG et al., 2012)

Os metabólitos secundários são substâncias produzidas em pequenas quantidades, e, em contraste com os primários, nem sempre estão envolvidos em funções vitais do vegetal ou mesmo presente em todos eles. Além disto, são conhecidos por serem sintetizados em tipos celulares especializados e em distintos estágios de desenvolvimento. Estes constituintes químicos são extremamente diversos e cada família, gênero e espécie produz uma categoria química característica ou uma mistura delas, o que pode ser utilizado na classificação taxonômica das plantas (BELL et al. 1980; WAKSMUNDZKA-HAJNOS; SHERMA; KOWALSKA, 2008; SIMÕES et al., 2010). Alguns metabólitos secundários possuem reconhecida importância comercial, pois podem ser aplicados industrialmente e possuem propriedades terapêuticas, sendo considerados princípios ativos para obtenção de medicamentos (OLIVEIRA, et al., 2011). Contudo, diferentemente do que muitos acreditam, a origem natural desses produtos não os torna isentos de toxicidade para seres humanos.

O conceito de que "todas as substâncias são tóxicas, sendo a dose o que diferencia um medicamento de um agente tóxico" foi introduzido por Paracelsos (1493

-1541). Um agente tóxico é todo aquele que, em determinada dose, provoca desestabilização do organismo, enfermidades e até a morte (VEIGA JR.; PINTO; MACIEL, 2005). Em outras palavras, a toxicidade de uma substância para um organismo vivo corresponde à capacidade de causar dano grave ou morte (OECD, 2001).

Todos os organismos apresentam a capacidade de responder a variações externas, de modo a manter sua homeostasia. Dessa forma, através de modificações bioquímicas e morfológicas e de mecanismos fisiológicos, os organismos podem se adaptar a condições adversas de exposição a substâncias estranhas, sem manifestações de toxicidade. Entretanto, quando o organismo não é capaz de se adaptar à presença dessas substâncias, manifesta-se o efeito tóxico (BARROS, DAVINO, 2003).

A maioria dos consumidores de produtos de origem natural são motivados, além dos custos menores, pela prerrogativa de que tais produtos são isentos de efeitos adversos, desconhecendo que a toxicidade, efeitos colaterais, interações com outras drogas ou alimentos, e os produtos de sua biotransformação são causas frequentes de problemas de saúde pública e internações em hospitais (VEIGA JR.; PINTO; MACIEL, 2005; LANGMAN; KAPUR, 2006).

Em relação às plantas medicinais, a toxicidade de seus produtos é ainda mais subestimada devido ao fato de que a ocorrência de efeitos indesejáveis, geralmente, é menor quando comparada a drogas sintéticas (FERREIRA-MACHADO et al., 2004). Entretanto, tanto o uso como também estudos de plantas medicinais comprovaram que várias espécies vegetais possuem substâncias potencialmente perigosas e, por esta razão, devem ser utilizadas respeitando-se os riscos toxicológicos (VEIGA JR; PINTO; MACIEL, 2005).

O uso de uma espécie vegetal pela população não é suficiente para validar sua segurança e eficácia, já que frequentemente a indicação é feita por pessoas leigas, sendo a intoxicação um resultado frequente (RATES, 2001). Os estudos toxicológicos de xenobióticos

visam garantir a segurança do homem frente a possíveis efeitos negativos, estabelecendo limites seguros para a aplicabilidade de uma nova substância e contribuindo, assim, para o desenvolvimento de novos fármacos (HODGSON, 2003). Os testes toxicológicos permitem avaliações de parâmetros, como alterações de massa corporal do animal, consumo de água e ração (JAHN; GÜNZEL, 1997), condições anatomopatológicas dos órgãos, parâmetros bioquímicos e hematológicos e influência na reprodução (REBOREDO et al., 2007).

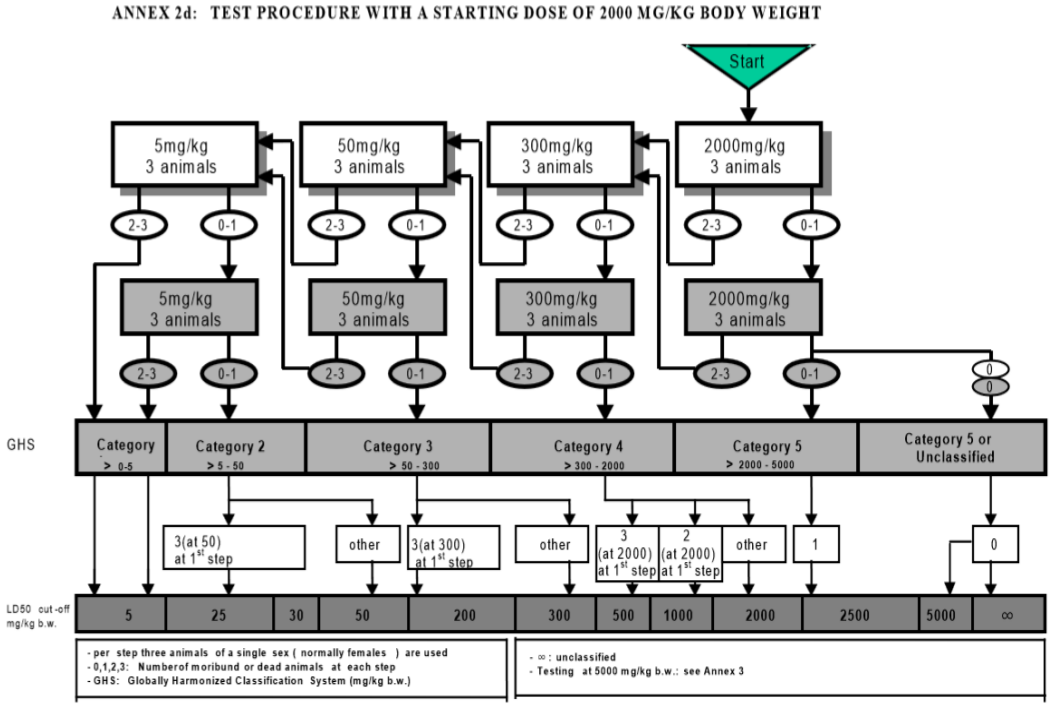
A toxicidade e a segurança de uma determinada substância química estão relacionadas com sua concentração e tempo de permanência e/ou exposição, de forma que os testes de toxicidade devem ser realizados de forma padronizada e em condições replicáveis (SILVERIA, 2007). Além das interações com medicamentos e/ou alimentos, possíveis reações adversas inerentes de preparações vegetais podem depender de características do paciente como gênero, idade e estado de nutricional, entre outros (BALBINO; DIAS, 2010).

Dentre os bioensaios de toxicidade, encontra-se o ensaio de letalidade frente a *Artemia salina* Leach, um microcrustáceo de água salgada comumente utilizdo como alimento para peixes. A simplicidade com que pode ser manuseado, a rapidez dos ensaios e o baixo custo favorece a sua utilização rotineira em análises preliminares de toxicidade geral (LUNA et al., 2005; NASCIMENTO et al., 2008). A literatura relata que existe uma correlação entre a toxicidade geral frente *A. salina* e atividades como antifúngica, antiviral, antimicrobiana (MacRAE; HUDSON; TOWERS, 1988), antiparasitária (SAHPAZ et al., 1994) e tripanocida (ZANI et al., 1995), entre outras. Há, também, correlação com a citotoxicidade para linhagens celulares tumorais humanas (MacLAUGHLIN, 1991).

Dentre vários métodos ou testes empregados para avaliar efeitos toxicológicos *in vivo*, o teste de toxicidade aguda permite definir o grau de toxicidade intrínseca do composto, identificar órgãos que podem ser alvos de efeitos indesejados e selecionar doses para estudos de longa duração (ALMEIDA, 2011). A Figura 1 apresenta o protocolo estabelecido pela Organização para Cooperação e Desenvolvimento Econômico (www.oecd.org) com

orientações para avaliação da toxicidade aguda de drogas. O teste é iniciado a partir da administração dos produtos na dose de 2000 mg/kg, e estabelece classificações do nível de toxicidade de acordo com a mortalidade em diferentes doses.

Figura 1- Avaliação de toxicidade aguda de acordo com o guia da Organização para Cooperação e Desenvolvimento Econômico (OECD).



Fonte: OECD (2001).

Além da avaliação da mortalidade, a análise toxicológica pode ser complementada pela avaliação de alterações em órgãos e em parâmetros bioquímicos e fisiológicos. Adicionalmente, a triagem comportamental permite avaliar se uma determinada droga modifica a atividade cerebral, através do registro de alguns sinais ou alterações de condutas apresentados pelos animais (ALMEIDA, 2011). Dessa forma, esse modelo permite avaliar se determinadas drogas apresentam toxicidade, possibilitando o estudo farmacológico de plantas e/ou fitoconstituintes com uma determinada margem de segurança (LIMA, 2011).

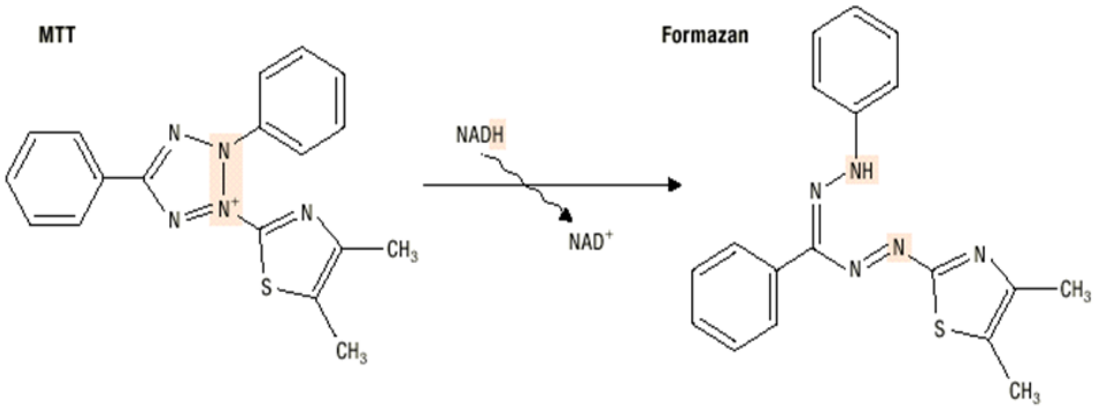
A citotoxicidade é definida como os efeitos resultantes da interferência de drogas nos processos essenciais para a sobrevivência, proliferação e funções comuns a todas as células do organismo (EKWALL, 1999). Os estudos de citotoxicidade fornecem informações a respeito do potencial tóxico de moléculas estudadas de forma mais rápida e com custos menores que os estudos *in vivo*. Neste tipo de ensaio, pode ser avaliada a viabilidade, os aspectos morfológicos, a integridade da membrana celular, bem como crescimento/proliferação das células (CASTAÑO; LECHÓN, 2005).

Um dos métodos mais utilizados é a avaliação da citotoxicidade basal utilizando o brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazólio (MTT) (CASTAÑO; LECHÓN, 2005). O MTT é um corante amarelo que é reduzido por células que mantêm a integridade mitocondrial para um composto azul (formazan), insolúvel em solução aquosa (Figura 2). Uma vez solubilizado, a quantidade de formazan pode ser determinada espectroscopicamente. A redução do MTT para formazan é realizada principalmente pela enzima succinato desidrogenase, sendo muito utilizada em ensaios de avaliação de sobrevivência e proliferação celular. Somente as células viáveis reduzem o MTT, portanto a quantidade de formazan produzido é proporcional ao número de células viáveis presentes (MOSMANN, 1983; DENIZOT; LANG, 1986).

Algumas substâncias vegetais são capazes de interagir com o DNA, induzindo alterações no material genético, tais como quebras cromossômicas e mutações, o que justifica o interesse no estudo potencial genotóxico de extratos de plantas medicinais (CHACON et al., 2002). A avaliação dos efeitos genotóxicos é importante para referendar o emprego de plantas medicinais, acrescentando segurança ao usuário de fitoterápicos (RIBEIRO et al., 2003). A avaliação da genotoxicidade *in vivo* oferece como vantagens a participação doo metabolismo do animal, que nunca pode ser totalmente reproduzido num sistema *in vitro*, bem como é possível utilizar as condições de exposição a que o homem está sujeito, seja

quanto a via de administração, duração do tratamento e condições nutricionais (RABELLO-GAY et al., 1991).

Figura 2 - Reação de redução do MTT ([brometo de (3-(4,5-dimetiltiazol-2-yl)-2,5-difenil tetrazólio]) a formazan**.**



Fonte: MOSMANN (1983)

Dentre os testes de avaliação toxicológica recomendados por agências internacionais e instituições governamentais, o teste de micronúcleo (TMN) em células de sangue periférico de roedores é amplamente utilizado como um estudo toxicológico para avaliação e registro de novos produtos químicos e farmacêuticos que entram no mercado mundial, visto que os resultados obtidos são considerados fortemente relevantes no contexto humano (MORITA et al., 1997; RIBEIRO et al., 2003; TAGLIATI et al., 2008). O TMN é reconhecido por sua robustez, sensibilidade e poder estatístico para avaliar alterações nucleares resultantes da perda de informação genética no núcleo principal, em consequência de dano cromossômico estrutural ou no aparelho mitótico, bem como quebras de DNA em consequência de mutagenicidade (FENECH, 2000; ARALDI et al., 2015).

Os micronúcleos consistem em núcleos pequenos, separados e adicionais ao núcleo principal da célula que aparecem nas células filhas (como, por exemplo, eritrócitos

policromáticos) em decorrência de danos induzidos nas células precursoras, de forma que ocorre a perda de fragmentos cromossômicos ou cromossomos inteiros durante a telófase da mitose. Os eritrócitos policromáticos resultam da diferenciação dos eritroblastos, após sofrerem replicação cromossômica, sendo ainda eritrócitos imaturos, em um estágio intermediário de desenvolvimento. Eles ainda contêm ribossomos e, portanto, podem ser distinguidos dos eritrócitos normocromáticos por coloração seletiva para ribossomos (SBMCTA, 2007). Os fragmentos cromossômicos que resultam de quebras após a mitose podem ser envoltos por uma membrana nuclear, sendo visíveis como um pequeno núcleo (Figura 3) adjacente ao núcleo principal dos eritrócitos policromáticos (RIBEIRO, SALVADORI & MARQUES, 2003; SBMCTA, 2007).

Figura 3 – Visualização de micronúcleo em lâmina corada com laranja de acridina em microscópio de fluorescência (seta).

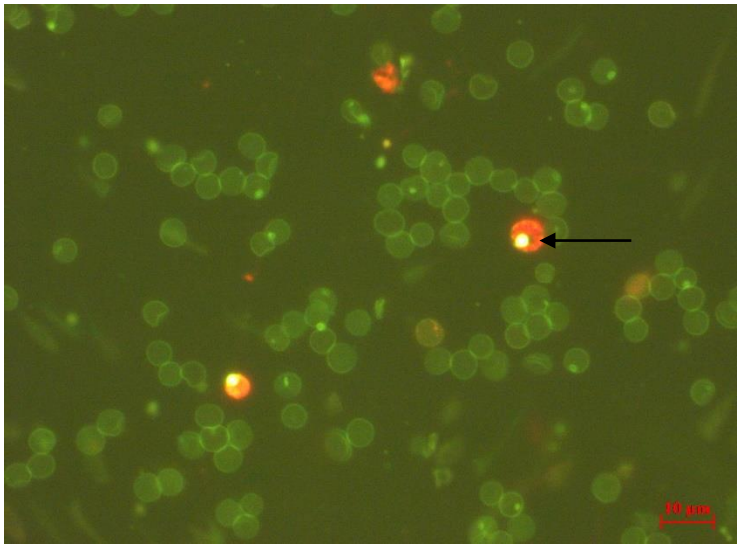


Foto: Rafael Albuquerque.

As características básicas do teste são: (1) o efeito do agente químico é observado em eritrócitos policromáticos; (2) o eritrócito policromático tem um tempo de vida relativamente

curto, de modo que qualquer micronúcleo que ele contenha deve ter sido gerado como resultado de danos cromossômicos induzidos recentemente; (3) os micronúcleos são facilmente identificáveis e a sua distribuição é bem definida; e (4) a frequência de micronúcleos é dependente do tempo de amostragem (RIBEIRO, SALVADORI & MARQUES, 2003). Ainda, deve-se ter o cuidado de coletar essas células, no mínimo, 36 horas após a administração da substância, que é quando os eritrócitos micronucleados começam a aparecer na corrente circulatória (HAYASHI, 1999). O teste de micronúcleo permite a classificação de substâncias em agentes aneugênicos (que induzem a aneuploidia ou segregação cromossômica anormal) (JOSEPH, 2004) e agentes clastogênicos (que quebram cromossomos) (WAHNSCHAFFE et al., 2005).

A associação entre citotoxicidade e genotoxicidade está bem estabelecida, pois danos ao DNA são considerados como um importante mecanismo indutor de apoptose celular. Quando ocorre uma lesão no DNA, o ciclo celular é interrompido e processos de reparo são ativados. Porém, caso a lesão seja muito complexa, inicia-se o processo de apoptose (BARTEK & LUKAS, 2007; HOUTGRAAF; VAN DER GIESSEN, 2006).

2.2 *Morus alba* L.

Morus L. (Moraceae) é um gênero composto por 10–16 espécies (dependendo das sinonímias consideradas em cada sistema de classificação) de árvores conhecidas como "amoreiras", caducifólias e nativas de regiões temperadas e subtropicais, sendo a maioria das espécies nativas da Ásia (PIAO et al., 2011). As amoreiras são amplamente cultivadas e consumidas na China, Coréia e Japão como alimento nutracêutico e hipoglicemiante, sendo essa última propriedade atribuída à presença do composto 1-deoxinojirimicina, um dos mais potentes inibidores de α-glicosidase (KIM et al., 2003).

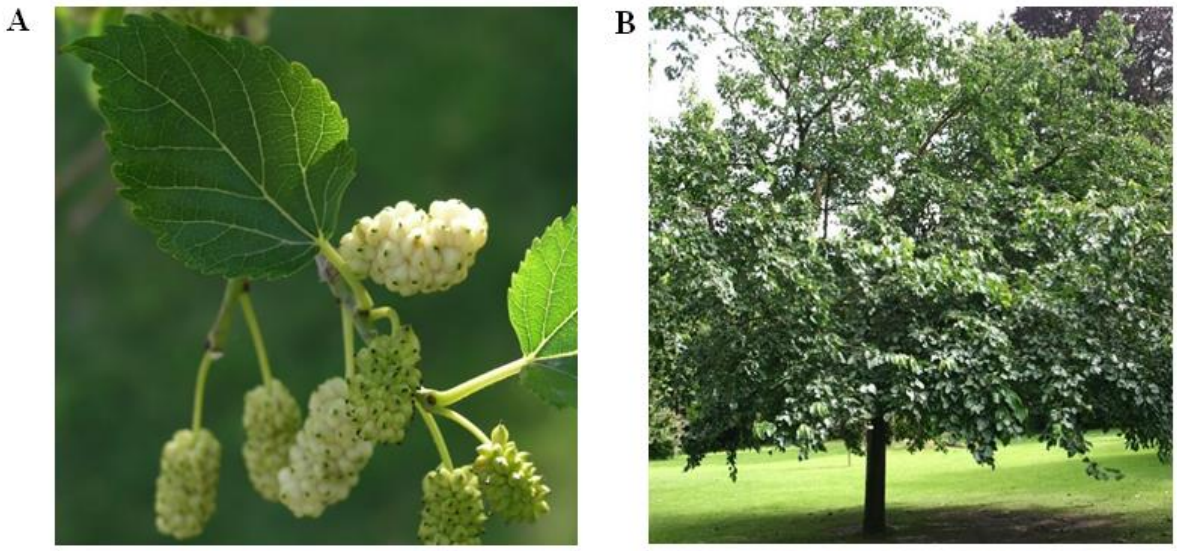
As folhas de amoreiras têm sido muito utilizadas na medicina tradicional chinesa para tratar a febre, melhorar a visão, fortalecer as articulações, reduzir a pressão arterial e níveis elevados de colesterol, evitar a formação de trombos, tratar a constipação, combater o diabetes, e como agente diurético (DOI et al., 2001; HARAUMA et al., 2003; DUGO et al., 2009; PIAO et al., 2011). Toshio et al. (2005) isolaram de espécies de *Morus* uma substância denominada chalcomoracina, a qual apresentou considerável atividade antimicrobiana contra *Staphylococcus aureus* resistente à meticilina, com atividade similar à vancomicina.

A *Morus alba*, conhecida como amoreira branca (Figura 4), é uma planta perene, rústica, de fácil cultivo e de excelente desenvolvimento, mesmo em períodos de estiagens prolongadas (MIRANDA et al., 2002). É explorada economicamente para nutrição das lagartas do bicho-da-seda (*Bombyx mori* L.), que se alimentam exclusivamente de suas folhas (OKAMOTO; RODELLA, 2006). Em função de suas características produtivas e da composição bromatológica, a amoreira branca tem sido mundialmente avaliada como alimento volumoso (de baixo teor energético, mas com altos teores em fibra ou em água) para ruminantes (MIRANDA et al., 2002). De acordo com Sanchez (2002), o valor nutricional das folhas da amoreira pode ser considerado como equivalente ao de concentrados baseados em grãos. Na medicina tradicional chinesa, suas folhas, frutos e caules são usados no tratamento do diabetes mellitus e da hipercolesterolemia, além de como sedativo, expectorante, analgésico, diurético e antiepilético (ZENI; DALL'MOLIN, 2010).

Estudos vêm sendo realizados visando confirmar o potencial farmacológico dessa planta. Uma mistura de extratos padronizados de folhas de *Uncaria gambir* e casca da raiz de *M. alba* foi relatada como terapia alternativa para aliviar a osteoartrite e seus sintomas associados (YIMAM et al, 2015). Kim et al. (2003) relataram que extratos de folhas e frutos de *M. alba* foram capazes de reduzir os déficits cognitivos em ratos. Outros relatos mencionam atividades antioxidante, antibacteriana (WANG et al., 2012), antiviral (LEE et al.,

2014) e neuroprotetora (SEE et al, 2015) de extratos e compostos isolados a partir de frutos, folhas, caule e raiz de *M. alba*.

Figura 4 – Amoreira branca (*Morus alba*). (A) Detalhe do fruto (amora) e da folha. (B) Árvore de porte médio.



Fonte: www.esacademic.com

2.3. Infecções bacterianas e fúngicas

Antibióticos são definidos como moléculas de origem natural, sintética ou semissintética, capazes de matar ou inibir o crescimento de micro-organismos. O uso de antibióticos pode ser considerado como um dos principais avanços terapêuticos do século passado (SILVA, 2010).

Os micro-organismos considerados patogênicos ao homem são responsáveis por desde infecções leves e de fácil cura até infecções graves e de difícil tratamento. Por exemplo, bactérias do gênero *Klebsiella* podem atingir diversas estruturas do corpo humano, em especial, os alvéolos pulmonares ocasionando pneumonia lobar, com formação de cavidades e

escarro sanguinolento (MURRAY et al., 2006). O gênero *Salmonella* representa um grupo de bactérias que habita o trato intestinal humano, mas que podem causar diarreia constante associada a dor e febre (CVE/CCD-SES, 2005). A salmonela, juntamente com *Escherichia coli* e espécies de *Shigella* estão entre as bactérias enteropatogênicas mais isoladas nas infecções ocorridas em enfermarias e pediatrias de unidades hospitalares (HERKENHOFF et al, 2006; NOGUEIRA et al., 2008; VERONESI et al., 2010).

Staphylococcus aureus é uma bactéria Gram-positiva, que cresce melhor em ambientes anaeróbios, mas poder ser aeróbia facultativa. Esta espécie é documentada como um patógeno oportunista em humanos, responsável por diversos casos de morbidade e mortalidade; suas infecções podem ocorrer em diversos tecidos do corpo humano e incluem: pneumonia, osteomielite, miocardite, pericardite, meningites, infecções do trato urogenital, sistema nervoso central e órgãos intra-abdominais, entre outras, além de também estar associada a intoxicações alimentares (MURRAY, 1995).

Relata-se que a prevalência de *Staphylococcus aureus* é influenciada pela idade, doença subjacente, raça, comportamento e o ambiente no qual a pessoa está inserida; cepas de *S. aureus* oportunistas vêm tornando-se cada vez mais predominantes não só em hospitais como também em comunidades, o que configura um desafio para a pesquisadores (SCHIJFFELEN et al., 2010; KHATTAK et al., 2015).

Pseudomonas aeruginosa é uma bactéria Gram-negativa e anaeróbia facultativa, responsável por infecções principalmente em ambiente hospitalar, afetando inclusive indivíduos imunocompetentes. São exemplos mais comuns peritonites, bacteremias, infecções do trato urinário e de cirurgias (MURRAY, 1995; SAVIOLLI, 2010).

Os fungos são seres dispersos no meio ambiente, sendo encontrados no ar atmosférico, solo e água, bem como habitando estruturas de outros organismos. Os fungos de interesse médico, agentes de micoses, são de dois tipos morfológicos: leveduras, que são unicelulares, e

os fungos filamentosos, também conhecidos como bolores, que são multicelulares (CAMBUIM, 2007).

As leveduras do gênero *Candida* vivem em diversos sítios anatômicos no homem, como a pele, mucosas, trato geniturinário e trato intestinal (ALONSO-VALLE, et al., 2003; EMIRA, et al., 2011). Entre 80 e 90% da microbiota fúngica é constituída por *Candida albicans*, sendo o restante atribuído a outras espécies, tais como *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida lusitaniae* e *Candida guilliermondi* (PIATO, 1997; FREITAS et al., 2003; EGGIMANN et al., 2003; SHOHAM et al, 2005; HAZEN et al., 2006)

Determinadas espécies de *Candida* podem se tornar patogênicas frente a alguns fatores, como comprometimento de barreira epitelial e imunodeficiência do hospedeiro, favorecendo a infecção. Atualmente, as espécies de *Candida* são a quarta causa de infecções nosocomiais sistêmicas, ocasionando alta mortalidade e aumento do tempo de hospitalização (VIDIGAL, et al., 2009).

Nas últimas três décadas, a incidência de infecções fúngicas apresentou aumento significativo devido a diversos fatores, tais como: desenvolvimento de técnicas terapêuticas invasivas, utilização em larga escala de antibióticos de amplo espectro, número crescente de transplantes hematológicos e de órgãos sólidos, expansão da Síndrome da Imunodeficiência Adquirida e surgimento de novos e potentes imunossupressores (PAPAS et al.; NUCCI, et al., 2010; MICELI, 2011).

Apesar do desenvolvimento de novos antibióticos nas últimas décadas, o aparecimento de cepas de micro-organismos resistentes aumentou, em parte devido ao uso indiscriminado pela população e nos serviços de saúde (GULCIN; TEL; KIRECCI, 2008; SILVA et al., 2010). A resistência microbiana está relacionada à seleção de células microbianas não sensíveis aos antimicrobianos, levando ao estabelecimento de cepas capazes de se multiplicar, necessitando o aumento da dose terapêutica convencional para conter a infecção, ou até

mesmo não sendo possível mais o uso de determinado antibiótico. Apesar desse fenômeno ser algumas vezes decorrente da evolução natural dos micro-organismos, ele vem sendo acelerado pelo uso irracional das substâncias antimicrobianas nas práticas médicas, agrárias e veterinárias. Esse problema de saúde pública mundial torna-se um obstáculo para o sucesso dos tratamentos e diminui o número de antimicrobianos válidos disponíveis (DA SILVA et al., 2010).

Tendo em vista que bactérias e fungos resistentes a múltiplos antimicrobianos representam um desafio no tratamento de infecções, tem crescido a procura por novas substâncias com propriedades antimicrobianas (PEREIRA et al., 2004; FIRMO et al., 2014). É nessa perspectiva que vem crescendo o estudo de produtos de origem vegetal com atividade antimicrobiana (CECHINEL FILHO, 2001; CARMO et al. 2008b; OLIVEIRA et al., 2011; MIYASAKI, 2013).

Guiados pelo uso popular das espécies nativas, alguns grupos de pesquisadores estudam a atividade antimicrobiana de plantas medicinais originárias de diversas regiões do mundo. Tem-se observado o aumento no uso de antibióticos de ocorrência natural devido ao fato de os micro-organismos estarem se mostrando resistentes à maioria dos antimicrobianos conhecidos (DUARTE, 2009). O uso de extratos de plantas como agentes antimicrobianos apresenta um baixo risco de aumento da resistência microbiana porque são misturas complexas, geralmente atuando por vários mecanismos de ação, fazendo com que haja maiores dificuldades para adaptabilidade microbiana (DAFERERA, 2003; OLIVEIRA et al., 2011; MIYASAKI, 2013). Espera-se que os compostos naturais atinjam, nas células microbianas, alvos diferentes daqueles utilizados polos antibióticos conhecidos e sejam ativos contra patógenos resistentes (FERRONATO, 2007).

A técnica de microdiluição é considerada como padrão ouro na investigação da concentração inibitória mínima (CIM) em ensaios antimicrobianos. Esse método é vantajoso

por sua fácil execução e por requerer pouca quantidade de amostra e meio de cultura, podendo ser usado para grande número de produtos. Além disso, possui boa reprodutibilidade, sendo trinta vezes mais sensível que outros métodos usados na literatura (SCORZONI et al., 2007; OSTROSKY et al, 2008). Esse método tem a vantagem de poder ser utilizado tanto para produtos solúveis em água, como aqueles lipossolúveis (SCORZONI et al., 2007).

2.4. Câncer

O câncer ou neoplasia maligna é o nome dado a um conjunto de mais de 100 doenças que têm em comum o crescimento desordenado de células, que invadem tecidos e órgãos. Dividindo-se rapidamente, estas células tendem a ser muito agressivas e incontroláveis, determinando a formação de tumores malignos, que podem espalhar-se para outras regiões do corpo (metástase). As causas do câncer são variadas, podendo ser externas ou internas ao organismo, estando inter-relacionadas. As causas externas referem-se ao meio ambiente e aos hábitos ou costumes próprios de uma sociedade. As causas internas são, na maioria das vezes, geneticamente pré-determinadas, e estão ligadas à capacidade do organismo de se defender das agressões externas (INCA, 2013).

A estimativa para essa doença é que, em 2030, haverá 21,4 milhões de novos casos e 13,2 milhões de novas mortes globais (IYER et al., 2013). No Brasil, a estimativa para o ano de 2014, que será válida também para o ano de 2015, aponta para a ocorrência de aproximadamente 576 mil casos novos de câncer, incluindo os casos de pele não melanoma, reforçando a magnitude do problema do câncer no país.

A quimioterapia convencional utiliza, em geral, moléculas sintéticas e produtos naturais que esbarram, além da resistência, em outras dificuldades, tais como pouca

seletividade para o local desejado e ações adversas em células normais, causando efeitos colaterais (PAI et al., 2006; AAGAARD e ROSSI, 2007).

Dessa forma, é de fundamental importância a contínua realização de pesquisas que visem o desenvolvimento de novos fármacos com atividade antitumoral e que apresentem baixa ou nenhuma toxicidade para células normais (ALMEIDA et al., 2004).

2.5. Inflamação

O processo inflamatório é, fundamentalmente, um mecanismo de proteção com a função de livrar o organismo de agentes que causam danos em células e tecidos, remover restos celulares que foram danificados em consequência de uma injúria e promover a reparação tecidual. No entanto, os processos de inflamação podem ser potencialmente danosos ao organismo quando sua intensidade ou duração ultrapassam o nível necessário para conter o agente agressor (RANKIN, 2004). Por esta razão muitas drogas com a capacidade de interferir no processo inflamatório, atenuando sua intensidade ou reduzindo seu tempo de duração, são objeto de pesquisas (SOLEDADE, 2008).

A inflamação é predominantemente caracterizada pela presença de dor, vermelhidão (hiperemia ou rubor), calor, edema e perda de função, chamados sinais cardinais. Esses sinais são consequência de uma série de eventos que promovem aumento do fluxo e permeabilidade vascular, exsudação de fluidos, migração de leucócitos e outros efeitos induzidos pelos mediadores químicos no foco inflamatório (JANEWAY et al., 2002; KUMAR, ABBAS, FAUSTO, 2005).

Inicialmente, ocorrem os fenômenos de vasodilatação e exsudação, iniciados muitas vezes pela ação da histamina. Isso resulta em aumento localizado e imediato da irrigação sanguínea, que se traduz em um halo avermelhado em torno da lesão (rubor). Em seguida, a

ação de mediadores inflamatórios no local – tais como citocinas (pequenas proteínas liberadas por várias células do organismo e envolvidas na comunicação celular durante a resposta imune) e fragmentos de componentes do sistema complemento – promove aumento da permeabilidade, quimiotaxia (atração de células polimorfonucleares, neutrófilos e macrófagos para o foco da lesão) e efeitos sobre a adesão dos leucócitos às células endoteliais. A migração celular para os tecidos e suas ações locais determinam a dor (JANEWAY et al., 2002).

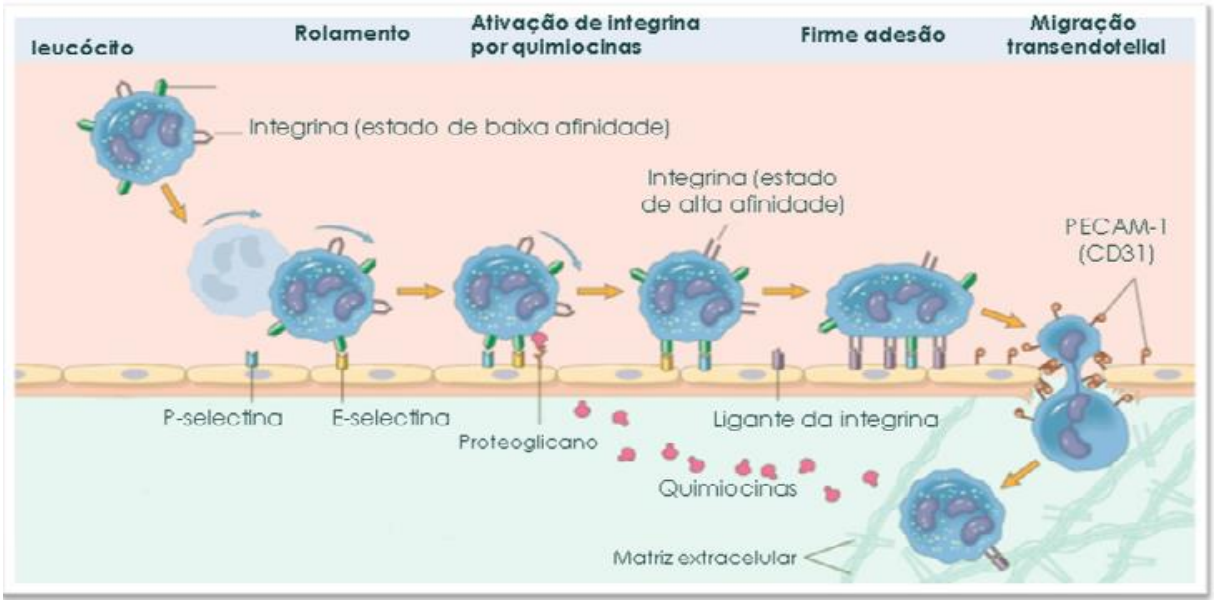
A inflamação é caracterizada como aguda e crônica. A forma aguda se caracteriza por ter uma curta duração (minutos, horas ou até duas semanas), predomínio da ação de neutrófilos e exibir fenômenos vasculares e exsudativos intensos. A inflamação crônica, sempre precedida pela forma aguda, possui longa duração (além de duas semanas), é mais específica, tem como principais células envolvidas os linfócitos, plasmócitos e macrófagos, e exibe fenômenos mais destrutivos e regenerativos do que o processo agudo. Na inflamação crônica não são observados os sinais cardinais, mas as alterações vasculares e exsudativas permanecem e há um prolongamento da fase de reparação, seja por permanência do agente agressor ou pela complexidade do processo de reparação (BRUNETTI et al., 2007).

A inflamação aguda é caracterizada pelos sinais cardinais descritos acima (KUMAR, ABBAS, FAUSTO, 2005). Além do acúmulo de líquido na região inflamada, há acúmulo de fibrina, leucócitos (especialmente neutrófilos, que são as primeiras células a chegarem nos sítios inflamatórios) e hemácias (ALBERTINE et al., 2004). Os leucócitos são atraídos pela ação das quimiocinas, as quais constituem uma classe de citocinas com propriedades quimioatratentes, induzindo células com receptores apropriados a migrarem através da fonte de quimiocinas. Essas moléculas são também comumente chamadas de interleucinas. Durante a fase tardia da inflamação aguda, há predominância dos eventos celulares, que se caracterizam pela marginação, aderência endotelial, diapedese e migração dos leucócitos para

o foco da lesão, em decorrência dos estímulos quimiotáticos (KUMAR, ABBAS, FAUSTO, 2005).

As moléculas de adesão, cuja expressão está aumentada no processo inflamatório, estão presentes tanto na superfície dos leucócitos como na parede dos vasos e permitem o rolamento e adesão firme dos leucócitos ao endotélio, o que representa fator crucial para que essas células cheguem à área lesada. Estas moléculas são as imunoglobulinas, integrinas, caderinas e selectinas (PHILLIPSON; KUBES, 2011). As selectinas estão envolvidas com a interação de pequena intensidade e rolamento inicial dos leucócitos pelo endotélio. Após o rolamento, ocorre a ativação (adesão firme ao endotélio) culminando com a migração transendotelial. Estas etapas, por sua vez, são governadas pelas integrinas e imunoglobulinas (Figura 5).

Figura 5 – Fases da migração leucocitária por controle quimiotático e moléculas de adesão.



Fonte: ROBBINS; COTRAN; KUMAR (2009).

Inúmeras citocinas participam do processo inflamatório, porém as principais são a interleucina-1B (IL-1β) e o Fator de Necrose Tumoral (TNF-α) (ZHANG, 2008). Estas duas citocinas são reconhecidas como iniciadoras do processo inflamatório, pois são responsáveis por ativar uma complexa cascata que envolve mais outras 20 citocinas (SANTOS et al., 2004).

Modelos *in vivo* de inflamação são definitivamente as melhores ferramentas para estudo, porque nenhuma outra abordagem permite a investigação simultânea de células e mediadores que interagem durante o processo inflamatório (KNAPP, 2009). O uso da carragenina como agente inflamatório apresenta inúmeras vantagens, como eficácia em níveis não tóxicos, baixa variabilidade e produção de uma curva dose-resposta com fármacos de conhecida eficácia terapêutica, sendo considerado um modelo padrão para ensaios de determinação da atividade de fármacos anti-inflamatórios não esteroidais (AINEs) (VAJJA et al., 2004).

A carragenina é um polissacarídeo derivado de algumas espécies de algas, sendo amplamente utilizado em diversos estudos experimentais acerca da migração de neutrófilos por ser uma substância com intensa ação quimiotática (SZABÓ; BECHARA, CUNHA, 2005). Ela desencadeia uma inflamação aguda envolvendo a liberação de vários mediadores inflamatórios, sobretudo histamina, serotonina, cininas, prostaglandinas e tromboxanos (DAMAS et al., 1990; PANTHONG, et al., 2004).

Com base nos acontecimentos moleculares que levam à inflamação, alguns trabalhos sugerem que o processo inflamatório que acompanha doenças crônicas pode ser melhorado e possivelmente até mesmo impedido pelo uso de fitoterápicos. As propriedades anti-inflamatórias de medicamentos de origem vegetal têm sido atribuídas à presença de substâncias como flavonoides e cumarinas, que agem como inibidores dos alvos moleculares e de mediadores pró-inflamatórios (IWALEWA et al., 2007). Triterpenoides de diferentes

grupos estruturais foram estudados e descritos como agentes anti-inflamatórios e imunomoduladores através da sua capacidade de inibir tanto os fatores de transcrição implicados nestes processos, como os mediadores produzidos pela sua ativação (RIOS, 2010).

O protocolo experimental do bolsão de ar tem sido empregado por sua relevante semelhança, do ponto de vista histológico, com a membrana da cavidade sinovial e, quando induzida a inflamação por injeção de carragenina, a reação é semelhante à observada em uma inflamação sinovial crônica, como a artrite reumatoide (SEDGWICK, LEES, 1986). Sendo assim, fármacos com promissores resultados em teste de bolsão de ar podem ser considerados como potenciais candidatos a fármacos antiartríticos.

3. OBJETIVOS

3.1. Geral

Obter um painel referente à toxicidade do extrato etanólico das folhas de *Morus alba* L. (Moraceae) e avaliar sua atividade antimicrobiana e anti-inflamatória.

3.2. Específicos

- Identificar as classes de metabólitos secundários presentes em extrato etanólico de folhas de *M. alba*.
- Avaliar a toxicidade do extrato frente ao microcrustáceo *Artemia salina*.
- Avaliar o extrato quanto à toxicidade aguda por via oral para camundongos, determinando os efeitos sobre sobrevivência, parâmetros hematológicos e bioquímicos e histologia de fígado, baço e rins.
- Avaliar a genotoxicidade *in vivo* do extrato quando ingerido por camundongos.
- Avaliar a citotoxicidade do extrato utilizando células tumorais humanas.
- Avaliar o potencial anti-inflamatório *in vivo* do extrato em modelo de inflamação aguda.
- Avaliar o potencial antimicrobiano do extrato sobre as bactérias *Staphylococcus aureus* (ATCC – 13150, M-177 e LM-197) e *Pseudomonas aeruginosa* (ATCC -9027 e LM-6) e fungos *Candida albicans* (ATCC-76645 e LM-106), *Candida tropicalis* (ATCC-13803 e LM-6), *Candida krusei* (LM-656 e LM-978), *Aspergillus flavus* (LM-118) and *Aspergillus niger* (LM-108).

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5. ARTIGO 1

Evaluation of toxicity and antimicrobial activity of an ethanolic extract from leaves of *Morus alba* L. (Moraceae)

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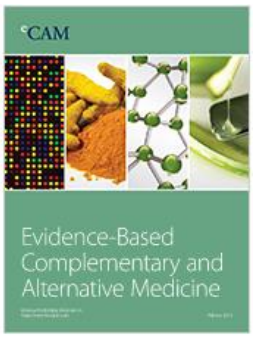

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Research Article

Evaluation of Toxicity and Antimicrobial Activity of an Ethanolic Extract from Leaves of *Morus alba* L. (Moraceae)

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This work evaluated an ethanolic extract from *Morus alba* leaves for toxicity to *Artemia salina*, oral toxicity to mice, and antimicrobial activity. Phytochemical analysis revealed the presence of coumarins, flavonoids, tannins, and triterpenes in the extract, which did not show toxicity to *A. salina* nauplii. No mortality and behavioral alterations were detected for mice treated with the extract (300 and 2000 mg/kg b.w.) for 14 days. However, animals that received the highest dose showed reduced MCV and MCHC as well as increased serum alkaline phosphatase activity. In treatments with the extract at both 300 and 2000 mg/kg, there was a reduction in number of leukocytes, with decrease in percentage of lymphocytes and increase in proportion of segmented cells. Histopathological analysis of organs from mice treated with the extract at 2000 mg/kg revealed turgidity of contorted tubules in kidneys, presence of leukocyte infiltration around the liver centrilobular vein, and high dispersion of the spleen white pulp. The extract showed antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, Candida krusei, Candida tropicalis*, and *Aspergillus flavus*. In conclusion, the extract contains antimicrobial agents and was not lethal for mice when ingested; however, its use requires caution because it promoted biochemical, hematological, and histopathological alterations.

1. Introduction

The use of plants as therapeutic tools is based on the popular culture and plant preparations are continually used by people, despite the large number of synthetic drugs developed. This has been mainly associated with the higher costs of allopathic medicines and a limited access of population to them, especially in developing countries where most of the people depend essentially on natural resources to address problems related to diseases of primary care [1]. The relevance of medicinal plants over the centuries has stimulated the search for scientifically proven information on their efficacy and safety for humans [2–4].

Morus genus (Moraceae) comprises a group of trees native from Asia, popularly known as mulberries, and with a great importance in folk medicine. In China, the leaves, roots, and branches of *Morus* species are used for treatment of fevers, hepatic protection, vision improvement, strengthening of joints, and reduction of blood pressure [5–7]. The leaves of different mulberries are consumed in Korea and Japan as a nutraceutical food and used for controlling blood glucose levels; this last property is attributed to the

presence of the compound 1-deoxynojirimycin, a potent α-glucosidase inhibitor [8]. Fukai et al. [9] isolated from *Morus* species a substance deemed chalcomoracin, which showed antimicrobial activity against methicillin-resistant *Staphylococcus aureus*. Dai et al. [10] isolated three compounds from *Morus macroura* bark, called guangsangon H, guangsangon I, and guangsangon J, which showed anti-inflammatory and antioxidant activities.

The pharmacological potential of the species *Morus alba* L. (white mulberry), for benefit of animals and humans, has been investigated. Flavonoids from root showed antiparasitic activity against *Ichthyophthirius multifiliis*, a parasite of gills and skin of freshwater fishes [11] and it was reported that a methanolic extract from the plant foliage might be used as a dietary supplement in order to alleviate *Aeromonas hydrophila* infection in catfish *Clarias gariepinus* [12]. Diels-Alder adducts and prenylated flavanones isolated from the root bark of *M. alba* showed cytotoxic activity against human tumor cells [13]. In another study, a composition containing a blend of standardized extracts from *Uncaria gambir* leaves and *M. alba* root bark was reported to be useful as an alternative therapy for alleviating osteoarthritis and its associated symptoms [14]. Kim et al. [15] reported that extracts from *M. alba* leaves and fruits were able to reduce cognitive deficits in mice induced by the high-fat diet. Other reports mention antioxidant, antibacterial, antiviral, and neuroprotective activities from extracts and isolated compounds from fruits, leaves, stem, and root of *M. alba* [16–20].

In face of the several reports on the medicinal properties of *M. alba* leaves, we investigated in this work the toxicity of an ethanolic extract from this tissue by using two models: *Artemia salina* lethality assay and assessment of *in vivo* oral toxicity to mice. In the last, biochemical, hematological, and histopathological analyses were also performed. In addition, it reports the phytochemical composition of the extract and the evaluation of its antimicrobial activity against pathogens with medical relevance.

2. Materials and Methods

2.1. Plant Material. Leaves of *M. alba* were collected in Petrolina city, Pernambuco, northeast Brazil. Taxonomic identification was performed and a voucher specimen (number 88372) is deposited in the herbarium of the *Instituto Agronômico de Pernambuco*, Recife, Brazil.

2.2. Extract Preparation. The ethanolic extract was chosen for this study because this solvent is able to dissolve both polar and nonpolar substances, including a variety of plant-derived compounds. The leaves were washed with distilled water and dried at 28°C during 24 h and next in an oven at 45°C for 15 days; subsequently, the leaves were powdered. The leaf powder (180 g) was mixed with 70% (v/v) ethanol in distilled water and the mixture was allowed to rest for 48 h, followed by mechanical agitation for 48 h using an orbital shaker. The extract was filtered and the solvent was removed using a rotary evaporator.

Table 1: Phytochemical screening of ethanolic extract from *Morus alba* leaves through thin layer chromatography.

Compound classes	Revealer	Result
Alkaloids	Dragendorff's reagent	Negative
Anthraquinones	KOH	Negative
Coumarins	KOH	Positive
Steroids	Liebermann-Burchard	Negative
Flavonoids	Aluminum chloride	Positive
Tannins	Iron (III) chloride	Positive for condensed tannins
Triterpenes	Liebermann-Burchard	Positive

2.3. Phytochemical Screening. The extract was evaluated for the presence of coumarins, flavonoids, tannins, steroids, alkaloids, anthraquinones, and triterpenes by thin layer chromatography (TLC). The assays were performed using the revealers listed in Table 1 and following the instructions described by Markhan [21], Wagner and Bladt [22], and Abreu [23].

2.4. Toxicity to Artemia salina. The assay was performed according to Meyer et al. [24] using *A. salina* cysts purchased from San Francisco Bay Brand, Inc. (USA). First, an artificial saline solution (3.8%, w/v) was prepared using sea salt (Marinex, Brazil) and distilled water. In each assay, 10 nauplii were added to extract solutions at different concentrations (100–1,000 μg/mL) prepared by diluting the extract in 10 mL of the artificial saline solution. In controls, the nauplii were incubated with the saline solution. Each concentration was tested in triplicate and three independent experiments were performed. The assays were maintained under artificial lighting for 24 h at 27 ± 2°C. After this period, the mortality rates were determined.

2.5. Oral Toxicity Assay. The experiments were performed using female Swiss mice (*Mus musculus*) weighing 38–50 g and reared in the vivarium of the *Laboratório de Farmacologia e Cancerologia Experimental* of the *Universidade Federal de Pernambuco*. The animals are maintained at temperature of 21 ± 1°C, 12L : 12D photoperiod and it is given *ad libitum* access to food (Purina) and water. The experimental procedures were approved by Animal Ethics Committee of the *Universidade Federal de Pernambuco* (process number 23076.061132/2014-33).

The dried extract was dissolved in 0.9% (w/v) NaCl [25] and acute toxicity (mortality and behavioral alterations) was evaluated by oral administration. The mice were separated in three groups ($n = 3$ for each group), according to the instructions of the Organization for Economic Cooperation and Development [26]: control group, which received saline solution (vehicle), and two test groups, which received the extract at 300 mg/kg and 2000 mg/kg b.w. The mice were observed during 14 days.

2.6. Biochemical and Hematological Analyses. At the end of oral toxicity assays, the blood of animals was collected and the following biochemical parameters were evaluated: total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphate, gamma-glutamyl transferase (GGT), urea, and creatinine, using specific kits (Labtest Diagnóstica, Lagoa Santa, Brazil) and a COBAS Mira Plus analyzer (Roche Diagnostics Systems, Basel, Switzerland). Hematologic analysis was performed using an automatic analyzer (Animal Blood Counter: ABC Vet, Montpellier, France) and optical microscopy; the parameters evaluated were as follows: erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and total and differentiated analysis of leukocytes.

2.7. Histopathological Analysis. Histological analyses of liver, kidney, and spleen of animals from control and extract treatments were performed by optical microscopy. Fragments of the organs were fixed in buffered formalin (10%, v/v) and then dehydrated through a graded ethanol series (70–100%), diaphonized in xylol, and embedded in paraffin. Histological sections (5 μm) were stained with hematoxylin-eosin and mounted using cover slips with Entellan resin (Merck, Germany) [27]. The materials were observed under a Motic BA200 microscopy coupled to a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd., Causeway Bay, Hong Kong).

2.8. Antimicrobial Activity. The extract was evaluated for antibacterial activity against three strains of *Staphylococcus aureus* (ATCC-13150, M-177, and LM-197) and two strains of *Pseudomonas aeruginosa* (ATCC-9027 and P-03). Antifungal activity was evaluated against strains of *Candida albicans* (ATCC-76645 and LM-106), *Candida tropicalis* (ATCC-13803 and LM-6), *Candida krusei* (LM-656 and LM-978), *Aspergillus flavus* (LM-118), and *Aspergillus niger* (LM-108). Bacterial suspensions were prepared in Nutrient Broth (Difco Laboratories, France) and fungal suspensions in RPMI 1640 medium (Acumedia, India). The suspensions were standardized for 10^6 colony-forming units (CFU) per mL using a Leitz-Photometer 340–800 spectrophotometer.

The assays for determination of the minimal inhibitory concentrations (MIC) were performed in U-bottom 96-well microplates. In an assay, each plate well received 100 μL of Nutrient Broth (for bacteria) or RPMI medium (for yeasts and filamentous fungi). Next, 100 μL of the extract, dissolved in distilled water, was added in the wells of the first column of the plate and then a two-fold serial dilution was performed in each row to reach extract concentrations ranging from 32 to 1,024 μg/mL. Finally, 10 μL of microorganism suspension was added to each well. A 100% growth control was done in absence of the extract and positive controls were performed using chloramphenicol (100 μg/mL) for bacteria as well as nystatin (100 IU) and fluconazole (100 μg/mL) for fungi. The plates were incubated for 24 h at 35°C in assays with bacteria and yeasts or for 7–10 days at 28°C in assays with filamentous fungi. After 24 h, bacterial growth was revealed by addition of 20 μL of 0.01% (w/v) resazurin (Inlab Confiança, São Paulo, Brazil); a change in color from blue to red was indicative of microbial growth and thus the MIC was recorded as the lowest extract concentration at which there was no color change [28]. For the fungi, the MIC was defined as the lowest concentration able to inhibit the visual growth in comparison with the 100% growth control. Each antimicrobial assay was performed in duplicate and repeated three times. The antimicrobial activity of the extract was classified according to MIC values as follows: 50–500 μg/mL = strong activity; 600–1500 μg/mL = moderate activity; and >1500 μg/mL = weak activity or inactive [29].

2.9. Statistical Analysis. The data were expressed as mean ± SD and submitted to one-way ANOVA followed by Bonferroni's test, with significance level at $p < 0.05$.

3. Results and Discussion

Health treatments using medicinal plants are the less expensive and more accessible to the population, mainly in developing countries. However, the indiscriminate use of plant preparations or products with doubtful constitution and without rigorous standardization may pose a risk due to the toxicity of some plant compounds to human organism. For this reason, studies that evaluated the side effects and toxicity degree of preparations and compounds from medicinal plants are imperative. Within this context, this work evaluated the toxicity of an ethanolic extract from leaves of *M. alba*, a plant whose tissues have been extensively used with medicinal purposes, including as commercial formulations.

The phytochemical screening of the extract revealed the presence of coumarins, flavonoids, tannins, and triterpenes (Table 1). Toxicity of the extract was firstly evaluated using the microcrustacean *A. salina*, which is often used as a preliminary indicator of general toxicity of plant compounds; also, it is reported that toxicity to *A. salina* has a correlation with possible antitumor activity [30, 31]. The results showed that the extract had no toxicity at all the tested concentrations, suggesting that it does not have a general toxicity.

In the oral toxicity assays, the groups of mice from control and extract treatments at both 300 and 2000 mg/kg b.w. did not show alterations in behavioral signals during the 14 days of experiments. However, the hematological analysis (Table 2) demonstrated significant ($p < 0.05$) reductions in MCV and MCHC in animals treated with the extract at 2000 mg/kg, in comparison with control; MCHC was also lower than control in treatment at 300 mg/kg. This result indicates that components of the *M. alba* extract may be acting on the erythrocytes causing a reduction of hemoglobin content and may be interfering with the hematopoiesis, causing the production of cells with lower volume. It has been reported that plant secondary metabolites may act directly on erythrocytes interfering with the integrity of plasma membrane and then causing shrinkage, reduction in hemoglobin content, and even destruction of the cells [32].

Table 2: Hematological parameters of animals from control group and treated with ethanolic extract of *Morus alba* leaves for 14 days.

Parameter	Control	Extract	
		300 mg/kg b.w.	2000 mg/kg b.w.
Erythrocytes (10^6/mm^3)	7.05 ± 0.39	7.84 ± 0.58	6.82 ± 0.51
Hematocrit (%)	39.95 ± 0.21	41.47 ± 2.41	36.50 ± 4.95
Hemoglobin (g/dL)	17.35 ± 1.20	15.62 ± 1.05	16.00 ± 1.41
MCV (fL)	64.00 ± 5.00	64.33 ± 5.03$^{\Delta}$	50.00 ± 1.00$^{*\Delta}$
MCH (pg)	26.97 ± 1.91	24.80 ± 0.85	21.20 ± 4.53
MCHC (%)	38.20 ± 1.01	32.67 ± 0.85*	32.20 ± 2.01*
Leukocytes (10^3/mm^3)	8.26 ± 0.43	9.57 ± 0.66$^{\Delta}$	6.81 ± 0.27$^{*\Delta}$
Segmented (%)	22.68 ± 1.16	43.90 ± 3.41$^{*\Delta}$	55.71 ± 3.12$^{*\Delta}$
Lymphocytes (%)	58.88 ± 1.70	42.39 ± 2.57$^{*\Delta}$	29.65 ± 0.92$^{*\Delta}$
Monocytes (%)	18.44 ± 3.55	13.71 ± 1.35	14.61 ± 0.64

*Significantly different ($p < 0.05$) from control treatment. $^{\Delta}$Significantly different ($p < 0.05$) from the other dose of *M. alba* leaf extract. MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

Table 3: Biochemical parameters of blood of animals from control and treatments with the ethanolic extract from *Morus alba* leaves for 14 days.

Parameters	Control	Extract	
		300 mg/kg b.w.	2000 mg/kg b.w.
Albumin (g/dL)	2.14 ± 0.17	1.92 ± 0.13	2.25 ± 0.25
ALT (U/L)	78.50 ± 2.12	80.3 ± 8.02$^{\Delta}$	66.33 ± 4.73$^{*\Delta}$
AST (U/L)	147.50 ± 4.95	149.67 ± 6.81	149.33 ± 5.03
Total protein (g/dL)	6.16 ± 0.23	6.09 ± 0.35	6.13 ± 0.17
Alkaline phosphatase (IU/L)	14.00 ± 1.0	12.33 ± 2.52$^{\Delta}$	37.00 ± 2.65$^{*\Delta}$
GGT (U/L)	11.00 ± 1.41	12.67 ± 1.15	14.00 ± 1.50
Urea (mg/dL)	54.33 ± 4.93	50.33 ± 1.53	53.33 ± 2.08
Creatinine (mg/dL)	0.28 ± 0.02	0.28 ± 0.02	0.30 ± 0.04

*Significantly different ($p < 0.05$) from control treatment. $^{\Delta}$Significantly different ($p < 0.05$) from the other dose of *M. alba* leaf extract.

Table 2 also shows that the number of leukocytes in animals treated with the extract at 2000 mg/kg was significantly lower ($p < 0.05$) than in control animals, having observed a reduction in the proportion of lymphocytes but an increase of segmented leukocytes. At the dose of 300 mg/kg, the extract of *M. alba* also decreased the proportion of lymphocytes and increased the percentage of segmented leukocytes, although the number of total leukocytes was not altered. A possible explanation is that compounds of the extract resulted in a drastic decrease of blood lymphocytes, which stimulated the production of new leukocytes, leading to the increase in proportion of segmented cells. In summary, these results constitute an indication that this extract has an important effect on the immune cells.

Biochemical analysis (Table 3) revealed significant ($p < 0.05$) alterations in the serum levels of the enzymes ALT and alkaline phosphatase in animals that received the extract at 2000 mg/kg, in comparison with control. The group treated with the dose 300 mg/kg did not show significant ($p > 0.05$) variations. Since damage of liver cells usually results in a release of several types of enzymes, we can infer that the *M. alba* extract did not cause strong impairment of hepatocytes membrane. Indeed, no alterations or a decrease only of ALT levels, which is a more specific marker for damage to liver cells, has been interpreted as a signal of stability of the membranes of these cells [33]. An increase only in alkaline phosphatase level is usually indicative of obstruction of bile flow [4] and it is possible that the *M. alba* extract is acting at this level and not by disturbing the membrane of hepatocytes. The levels of GGT, which is a more specific marker for hepatic damage [34], were similar in all groups, corroborating that the *M. alba* extract did not exert hepatotoxic effect.

Table 4: Evaluation of food and water consumption and weight gain of animals from control and treated with the ethanolic extract from leaves of *Morus alba*.

Parameter	Control	Extract	
		300 mg/kg b.w.	2000 mg/kg b.w.
Water consumed (mL)	35.00 ± 1.05	34.82 ± 1.68	34.29 ± 1.27
Food consumed (g)	28.68 ± 1.23	28.13 ± 1.00	27.09 ± 1.28
Weight gain (g)	12.04 ± 0.79	11.98 ± 0.49	12.00 ± 0.38

No differences in food and water consumption were detected between the groups as well as regarding the biomass gain (Table 4). The results from oral toxicity evaluation

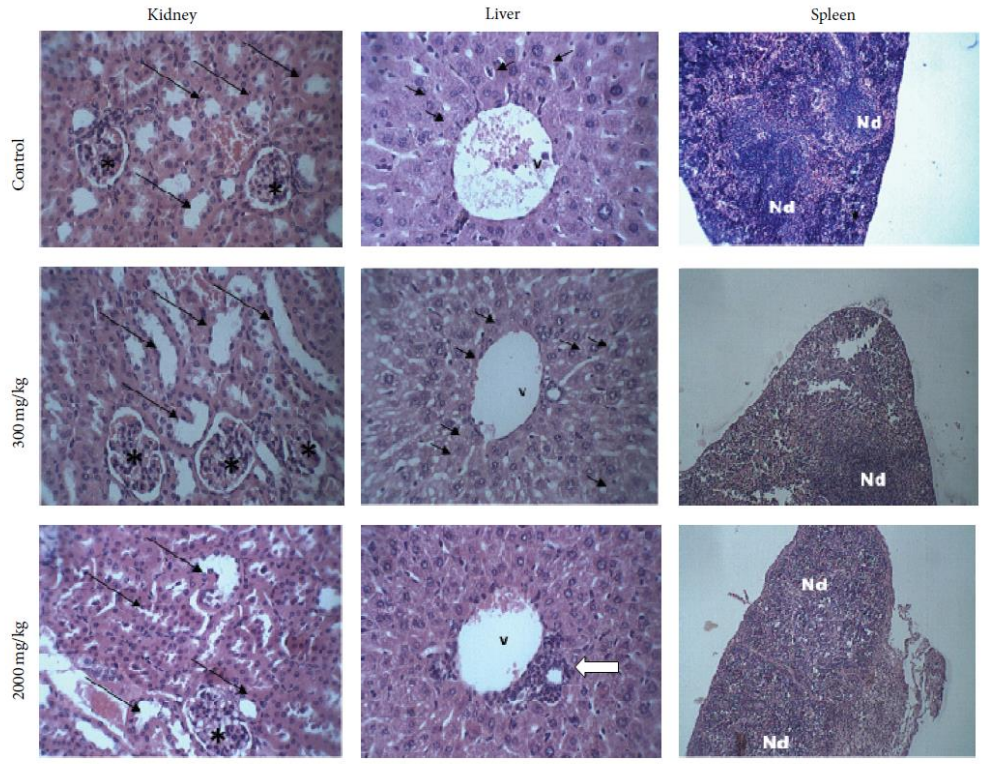

FIGURE 1: Histopathological analysis of kidneys, livers, and spleens of mice from control and treatments with the ethanolic extract from *Morus alba* leaves at 300 and 2000 mg/kg b.w. Kidneys: in control and treatment with 300 mg/kg, the cortical region with the renal glomeruli (∗) and contorted tubules (arrows) without alterations can be seen. In treatment with 2000 mg/kg, a reduction of the subcapsular space and turgid contorted tubules (arrows) is observed. Livers: photomicrography from control treatment shows the centrilobular vein (v) and a preserved organization of the hepatocytes bundles. In the image from treatment with 300 mg/kg, reversible-type cytoplasm vacuolizations (arrows) can be seen while the photomicrograph of an animal that received 2000 mg/kg of extract shows a lymphocyte infiltration around the centrilobular vein (white arrow). Spleens: in control, the well-delimited lymphatic nodes (Nd) can be noticed, while, in images for treatments with 300 and 2000 mg/kg, there is an expansion of lymphatic node (Nd) in the white pulp. All sections were stained with hematoxylin/eosin. Kidney and liver images: 400x. Spleen images: 100x.

indicated that the ethanolic extract from leaves of *M. alba* has low toxicity, since no death was observed even in assays at 2000 mg/kg dose [26]. However, the alterations found in hematological and biochemical analyses stimulated us to perform a histopathological study of the livers, kidneys, and spleens of the animals, since damage to these organs may be linked to the changes detected [35].

Photomicrographs obtained in the histopathological analysis are showed in Figure 1. The structure of the organs was preserved in control animals while a slight presence of reversible-type vacuolization was observed in hepatocytes of animals treated with the extract at 300 mg/kg. On the other hand, all the organs from animals that received the extract at 2000 mg/kg showed alterations. The kidneys showed a decreasing of the subcapsular space and turgidity of the contorted tubules. In the livers, the presence of leukocyte infiltration around the centrilobular vein was detected, which can be associated with intracellular lesions. A high dispersion of the spleen white pulp was also observed, which is a region of lymphatic tissue containing B and T lymphocytes; this can explain the reduction in the number of lymphocytes recorded in hematological analysis.

The ethanolic extract of *M. alba* leaves can be considered more toxic than an ethanolic extract from *Ocimum sanctum* leaves, which did not promote mortality at 200, 600, and 2000 mg/kg in 14 days and did not affect water consumption, body weight, and hematological and biochemical parameters. In addition, there were no alterations in the structure of liver, kidney, and spleen of mice treated with the *O. sanctum* extract at a dose of 800 mg/kg [36]. On the other hand, a leaf extract from *Lawsonia inermis* showed higher toxicity than the *M. alba* extract since it promoted pathological changes in liver

Table 5: Minimal inhibitory concentrations (MIC) of ethanolic extract from *Morus alba* leaves against bacteria and fungi.

Microbial strain	MIC (μg/mL)
Staphylococcus aureus ATCC-13150	512
Staphylococcus aureus M-177	1,024
Pseudomonas aeruginosa ATCC-9027	1,024
Pseudomonas aeruginosa ATCC-P-03	1,024
Candida albicans ATCC-76645	1,024
Candida albicans LM-106	256
Candida tropicalis ATCC-13083	512
Candida tropicalis LM-6	1,024
Candida krusei LM-656	512
Candida krusei LM-978	512
Aspergillus flavus KM-714	512
Aspergillus niger LM-108	NI

NI: no inhibition.

and kidney of rats at a dose of 1000 mg/kg, with signals of degenerative/apoptotic changes [37].

Coumarins are known for their hepatotoxicity and have been reported to be toxic to mice and rats, including being able to increase the incidence of adenomas and carcinomas in lungs of rats [38, 39]. Thus, this class of compounds may be involved in the alterations promoted by *M. alba* extract in oral toxicity assays. On the other hand, flavonoids are known to exert hepatoprotective and antioxidant effects [40] and thus may counterbalance the effects of potentially toxic compounds in the extract.

Among the several applications described for *Morus* plants, there is the use for combating infections caused by bacteria and fungi. The increase in the number of microbial strains resistant to the mostly used antibiotics has stimulated the search for new antimicrobial agents, especially those with natural origin [41]. In this way, we evaluated the antimicrobial activity of the ethanolic extract from *M. alba* leaves against bacteria and fungi with medical relevance. The results are summarized in Table 5 and show that the extract presented a strong activity (MIC of 256 μg/mL) against *C. albicans* LM-106 and moderate antimicrobial activity on the other strains, except *A. niger*, which was not sensitive to the extract. The bacterium most sensitive to the extract was *S. aureus* ATCC-13150. The strains *C. albicans* ATCC-76645, *C. albicans* LM-106, and *C. krusei* LM-656 were resistant to the positive controls used.

4. Conclusions

Ethanolic extract of *M. alba* leaves showed low oral toxicity to mice since no animal death was detected at a dose of 2000 mg/kg. However, the extract promoted biochemical, hematological, and histopathological alterations at this dose, which indicates that caution is required regarding its use. At 300 mg/kg, the extract did not show toxicity or cause irreversible cellular damages but altered the proportion of leukocyte types. The extract showed mainly moderate activity against the microbial pathogens evaluated.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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6. ARTIGO 2

Evaluation of ethanolic extract from *Morus alba* L. (Moraceae) leaves for genotoxicity *in vivo*, cytotoxicity and inhibitory effect in acute inflammation model

ARTIGO A SER SUBMETIDO AO PERIÓDICO

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Short communication

Evaluation of ethanolic extract from *Morus alba* L. (Moraceae) leaves for genotoxicity *in vivo*, cytotoxicity and inhibitory effect in acute inflammation model

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Abstract

Ethnopharmacological relevance: The use of *Morus alba* L. (white mulberry) in traditional medicine has expanded around the world, including in Brazil. The Leaves of this plant are used to treat inflammatory disorders. This scenario stimulate studies on the toxicological safety and scientific substantiation of *M. alba* medicinal properties.

Aim of the study: This work evaluated an ethanolic extract from *M. alba* leaves for *in vivo* genotoxicity, cytotoxicity to human cancer cells, and potential to reduce acute inflammation.

Materials and Methods: Genotoxicity was evaluated by micronucleus test and administration of the extract to Swiss mice at 75, 150 and 300 mg/kg b.w. *per os*. After 48 h, the number of micronucleated polychromatic erythrocytes was determined. Cytotoxicity of the extract (50 µg/mL) to NCI-H292, HEp-2, HT-29, MCF-7, and HL-60 cells was evaluated by MTT assay. The anti-inflammatory effect was investigated using the carrageenan-induced inflammation in the air pouch model The same doses of the genotoxicity test were administered *per os* to Swiss mice.

Results: The extract, containing coumarins, flavonoids, tannins, and triterpenes, did not show genotoxic effect and was also not cytotoxic to the cell lines evaluated. The oral administration of *M. alba* extract considerably reduced (58.6–65.6% inhibition) the leukocyte migration in all doses evaluated, in comparison with negative control.

Conclusions: The ethanolic extract from *M. alba* leaves, which did not show genotoxicity and general cytotoxicity to human cells, exerted a high inhibitory effect of acute inflammation. The work contributes in the determination of the safety of the medicinal use of *M. alba* leaves, which has expanded all over the world.

Keywords: white mulberry; micronucleus test; anti-inflammatory effect; toxicological safety.

1. Introduction

Several populations traditionally use medicinal plants to alleviate, prevent or cure physical and mental infirmities, mainly based on the beliefs of people resulting from empiric practices (Gaikwad et al., 2014). *Morus alba* L. is one of these plants, being used for centuries in Chinese medicine and now all over the world, including in Brazil (Zeni and Dall'Molin, 2010; Singh et al., 2013). Ethnopharmacological surveys have listed its use to treat diabetes, atherosclerosis, chancre, and throat irritation, for example (Butt et al., 2008; Amjad, 2015). Leaves and root bark of this plant are also used to treat inflammatory disorders (Chen et al., 2013; Zafar et al., 2013; Eo et al., 2014)

The compounds mulberroside A and oxyresveratrol obtained from *M. alba* cortex showed anti-inflammatory effect by reducing paw edema induced by carrageenan in mice; the oxyresveratrol was also extracted from *M. alba* branches and suppressed the migration of leukemic Jurkat T cells in response to stromal cell-derived factor 1 *in vitro* (Chung et al., 2003; Chen et al., 2013). Regarding the leaves of this plant, Kim et al. (2011) showed that ethanolic extract from leaves fermented with *Hericium erinaceum* mycelium was able to inhibit *in vitro* the production of the pro-inflammatory mediators nitric oxide, tumor necrosis factor-α, and interleukins IL-1β and IL-6 in LPS-stimulated RAW 264.7 cells. Pereira et al. (2013) reported that a hydroethanolic extract from *M. alba* leaves showed was able to inhibit the chronic inflammation in an *in vivo* granuloma tissue model.

Although plant medicines is often accepted by people as safer than allopathic drugs, the natural origin of plant products did not become them exempt of toxicity to humans. Among the damaging effects to health that can result from the indiscriminate use of natural medicines, there is the possibility that some plant substances interact with DNA promoting alterations in structure of this molecule as well as inducing mutations (Neto et al., 2005). In

addition, the absence of lethal effect of a plant preparation is not guarantee of safety from a genotoxicological standpoint. For example, the water extract from *Arisolochiae manshuriensis* stem was genotoxic to bone marrow erythrocytes when orally administered to mice, although it did not cause death and clinical alterations (Hwang et al., 2012).

A previous study on the oral toxicity of ethanolic extract from *M. alba* leaves revealed that it was not lethal and did not cause histopathological changes in liver, spleen and kidney of mice at a dose 300 mg/kg, being only observed a reduction of leukocytes number (Oliveira et al., 2015). In order to additionally contribute with the toxicological survey of *M. alba* leaves, in the present work we evaluated the ethanolic leaf extract for *in vivo* genotoxicity to mice polychromatic erythrocytes and for cytotoxicity to human cancer cells. After these evaluations, we determined its anti-inflammatory potential using an acute inflammation model.

2. Materials and methods

2.1. Plant material

Leaves of *Morus alba* L. (checked with www.theplantlist.org), known as white mulberry, were collected in Petrolina city (9°23′39″ S, 40°30′35″W), State of Pernambuco, northeast of Brazil. A voucher specimen (number 88372) is deposited in the herbarium of the *Instituto Agronômico de Pernambuco*, Recife, Brazil.

2.2. Preparation of extract and phytochemical analysis

The ethanolic extract was obtained as described by Oliveira et al. (2015). After collection, the leaves were washed with distilled water, maintained at 28°C during 24 h and next in an oven at 45°C for 15 days. The powder of dried leaves (180 g) was suspended in 70% (v/v) ethanol in distilled water and allowed to rest for 48 h. Next, the suspension was mechanically stirred for 48 h using an orbital shaker. The extract was obtained after filtration and removal of solvent by rotary evaporation. The extract was evaluated for the presence of coumarins, flavonoids, tannins, steroids, alkaloids, anthraquinones and triterpenes by thin layer chromatography (TLC) as described by Markhan (1982), Wagner and Bladt (2001) and Abreu (2000).

2.3. Animals

The *in vivo* assays were performed using male and female Swiss mice (*Mus musculus*) weighing 38–50 g and reared in the vivarium of the *Laboratório de Farmacologia e Cancerologia Experimental* from the *Universidade Federal de Pernambuco*. The animals were maintained at temperature of 21±1°C, 12L:12D photoperiod with *ad libitum* access to food (Purina®) and water. The Animal Ethics Committee of the *Universidade Federal de Pernambuco* approved all experimental procedures (process number 23076.061132/2014-33).

2.4. In vivo genotoxicity assay

The genotoxicity of *M. alba* leaf extract was evaluated by the micronucleus test. Groups of male mice (n=5) were treated with 0.9% saline *per os* (negative control), cyclophosphamide (25 mg/kg b.w. i.p.; positive control) or leaf extract (75, 150 or 300 mg/kg b.w. *per os*). After 48 h of treatment administration, the animals were anesthetized with

ketamine/xylazine solution and their blood was sampled from the orbital plexus. For each animal, two slides previously stained with acridine orange received 10 µL of blood and the presence of micronuclei was evaluated using a fluorescence microscope (OECD, 1997). For each sample, a number of 2000 polychromatic erythrocytes was counted, being the analyses performed in a blinded manner by a single person.

2.5. Cytotoxicity assays

Cytotoxicity of leaf extract was evaluated using the human tumor cell lines NCI-H292 (lung mucoepidermoid carcinoma), HEp-2 (larynx epidermoid carcinoma), HT-29 (human colon adenocarcinoma), MCF-7 (human breast adenocarcinoma), and HL-60 (acute promyelocytic leukemia). The NCI-H292, HEp-2 and Ht-29 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) while the MCF-7 and HL-60 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium. Both culture media were supplemented with 10% (w/v) fetal bovine serum and 1% (v/v) of antibiotic solution (100 U/mL penicillin, and 100 µg/mL streptomycin). The cultures were kept at 37°C in an atmosphere with 5% CO_2.

The NCI-H292, HEp-2, MCF-7 (10^5 cells/mL) and HL-60 (3×10^5 cells/mL) were separately plated in 96-well microplates and incubated for 24 h. Next, the extract was dissolved in 0.5% (v/v) dimethylsulfoxide (DMSO) and added in the plate wells to a final concentration of 50 µg/mL. Doxorubicin (5 µg/mL) was used as standard drug. After 72 h of incubation, it was added to each well 25 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5.0 mg/mL) and after additional 3 h, the culture medium containing MTT was removed and 100 µL of DMSO was added to each well. The absorbance at 560 nm was recorded using a microplate reader (Alley et al., 1988; Mosmann,

1983). The cytotoxic effect was classified as follows: strong (91–100% of inhibition), moderate (71–90% inhibition), weak (51–70% inhibition) and no effect (inhibition lower than or equal to 50 %).

2.6. In vivo anti-inflammatory assay

Anti-inflammatory potential of *M. alba* leaf extract in the air pouch model was investigated. Female mice were separated in the following groups (n=6): negative control (0.9% saline *per os*), positive control (indometacin 10 mg/kg b.w. i.p) and experimental groups (leaf extract at 75, 150 or 300 mg/kg b.w. *per os*). Each group received a subcutaneous injection of sterile air (2.5 mL) two times, with an interval of 3 days. At the 7^{th} day, 1% (w/v) carrageenan was injected in the air pouch and after 1 h, the animals received the respective treatments. A control group (n=6) that did not receive carrageenan injection and any treatment was also performed. After 6 h of treatment, the exudates in air pouches were collected and the number of leukocytes was counted using an automated hematology analyzer.

2.7. Statistical analysis

The results were expressed as mean of replicates ± SD. In the analysis of results from genotoxicity assay, the variance homogeneity was verified by the Levene's test and the comparisons were performed using the Kruskal-Wallis's test. For evaluation of the anti-inflammatory potential, analysis of variance (ANOVA) was performed followed by Bonferroni's test for multiple comparisons. A p value < 0.05 was adopted as significance level.

3. Results and discussion

Phytochemical analysis of the ethanolic extract from *M. alba* leaves indicated the presence of coumarins, flavonoids, tannins, and triterpenes, similarly to found by Oliveira et al. (2015). Figure 1 shows the numbers of micronuclei counted in samples from animals of all treatments. It can be noticed that the samples from mice of negative control and treatments with the extract contained similar numbers (< 10) of micronucleated polychromatic erythrocytes. Statistical analysis indicated that there are no significant differences ($p > 0.05$) between these groups. On the other hand, a high number of micronuclei was determined in samples from animals that received the positive control drug.

The micronucleus assay is considered an important biomarker of cytogenetic damages caused by genotoxic agents (Araldi et al., 2015). A drug is defined as genotoxic when the frequency of micronuclei is at least 20% higher than that in negative control (OECD, 1997). In this sense, the *M. alba* leaf extract can be considered as safe. Similarly, the plant flavonoid myricitrin, used in food production, did not show genotoxicity by *in vivo* micronucleus test, supporting its safety for use in food and beverage industry (Hobbs et al., 2015).

The cytotoxicity of *M. alba* leaf extract was evaluated using human tumor cell lines. These assays allowed us to determine if the extracts would have a general toxicity to human cell as well as to evaluate a possible antitumor potential against one or some of the cancer types tested. The results (Table 1) show that *M. alba* extract did not show cytotoxic effect to the cell lines evaluated, promoting inhibition no higher than 37.7%.

Together with the previous results published, the ethanolic extract from *M. alba* leaves prepared as described here can be considered safe at doses lower or equal to 300 mg/kg. At the conditions evaluated, the extract was non-toxic when ingested by mice (Oliveira et al.,

2015), was not damaging for genetic material when also orally administered and did not exert unselective toxic effect on human cells.

As mentioned above, *M. alba* is broadly used in traditional medicine to treat inflammatory disorders. Based on the results of toxicological assays, we evaluated the potential of the ethanolic extract (at doses ≤ 300 mg/kg) in reduce the leukocyte migration in an acute inflammation model. The air pouch model is considered as a first choice assay for the verification *in vivo* of the anti-inflammatory potential of natural products (Sedgwick and Lees, 1986). The oral administration of *M. alba* extract considerably reduced the leukocyte migration in all doses evaluated, in comparison with negative control (Table 2). In addition, the effects of the doses 150 and 300 mg/kg were not significantly different ($p > 0.05$) from that of the standard drug indomethacin.

Pereira et al. (2013) studied the anti-inflammatory effect of an ethanolic extract from *M. alba* using a model of chronic inflammation (granuloma tissue). They showed that the extract reduced the inflammation in 20.3% at a dose of 300 mg/kg and suggested that flavonoids are involved in the anti-inflammatory effect. Although the extraction methods were distinct, the comparison of this study with our results suggests that ethanolic extract from *M. alba* leaves has a higher potential in combating acute phase of inflammation.

Among the compounds detected in *M. alba* leaf extract, the flavonoids and coumarins have been reported as natural anti-inflammatory agents. Prenylated flavonoids isolated from *M. alba* root bark showed *in vitro* anti-inflammatory effect on LPS-treated cells (Kollar et al., 2013) and the anti-inflammatory activities of extracts from leaves of *Phyllantus acidus* and *Bidens pilosa* were also attributed to the presence of this class of compounds (Chakraborty et al., 2012; Fotso et al., 2014). Coumarins have been shown to exert anti-inflammatory effect *in vitro* on HT-29 human colon carcinoma cells (Kang et al., 2009) and *in vivo* effect on guinea pigs submitted to the carrageenan-induced paw edema model (Backhouse et al., 2001).

In conclusion, ethanolic extract from *M. alba* leaves did not show genotoxicity and cytotoxicity and exerted a high inhibitory effect of acute inflammation in the model evaluated here. The work contributes in the determination of the safety of the medicinal use of *M. alba* leaves, which has expanded all over the world.

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Table 1. Evaluation of cytotoxicity of ethanolic extract from *M. alba* leaves to human cancer cell lines.

Cell line	Inhibitory effect (%)	
	M. alba extract	Doxorubicin
HEp-2	37.7 ± 2.0	79.4 ± 2.6
MCF-7	20.1 ± 1.5	74.8 ± 2.1
HL-60	35.9 ± 3.3	92.9 ± 0.6
NCI-H292	23.2 ± 2.7	94.1 ± 2.0
HT-29	20.6 ± 0.0	64.1 ± 1.1

Table 2. Effect of ethanolic extract from *M. alba* leaves on leukocyte migration in the acute inflammation model of air pouch.

Groups and treatments	**Cell migration (10^3 cells/mm^3)**	**Inhibition (%)**
Negative control		
Saline *per os*	11.63±0.55 a	-
Positive control		
Indometacine 10 mg/kg i.p.	4.11±0.09 b	64.7
Treated		
Extract 75 mg/kg *per os*	5.02±0.04 c	56.8
Extract 150 mg/kg *per os*	4.31±0.01 b	62.9
Extract 300 mg/kg *per os*	4.00±0.06 b	65.6

The number of leukocytes in exsudate from control group that did not receive carrageenan injection and any treatment was 0.22×10^3 ($\pm0.04\times10^3$) cells/mm^3. Different letters indicate significant differences between groups at $p < 0.05$.

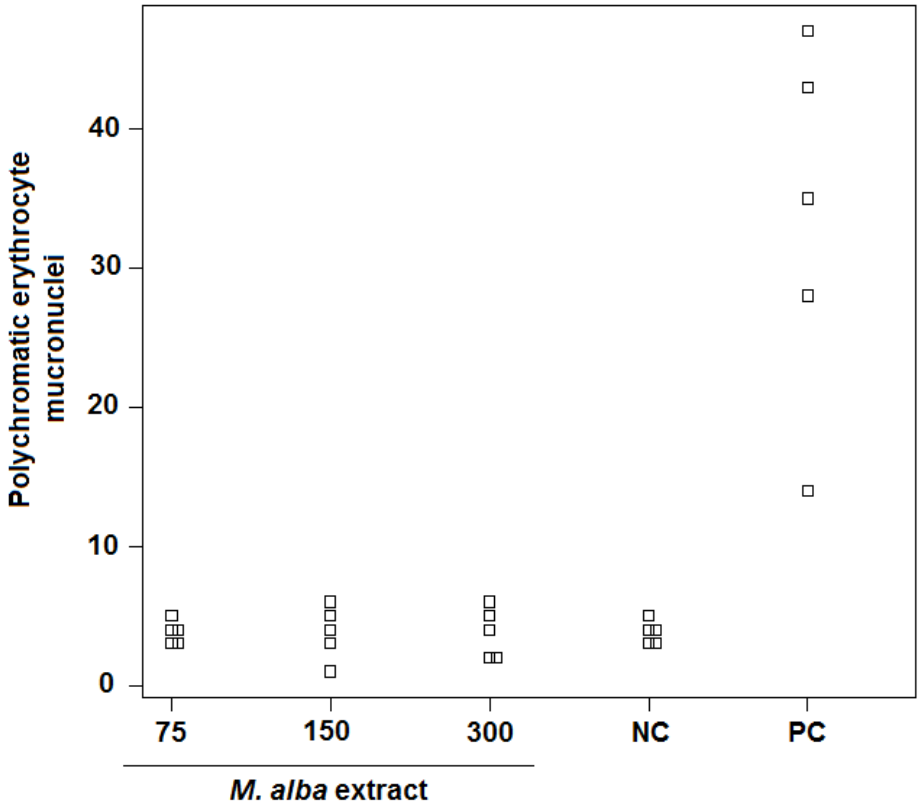


Figure 1. Evaluation of *in vivo* genotoxicity of ethanolic extract from *M. alba* leaves (75, 150 and 300 mg/kg b.w. *per os*) by determination of the micronuclei frequencies in murine polychromatic erythrocytes. Each dot represents an animal (n=5) of the group. NC: negative control. PC: positive control (cyclophosphamide 25 mg/kg b.w. i.p.).

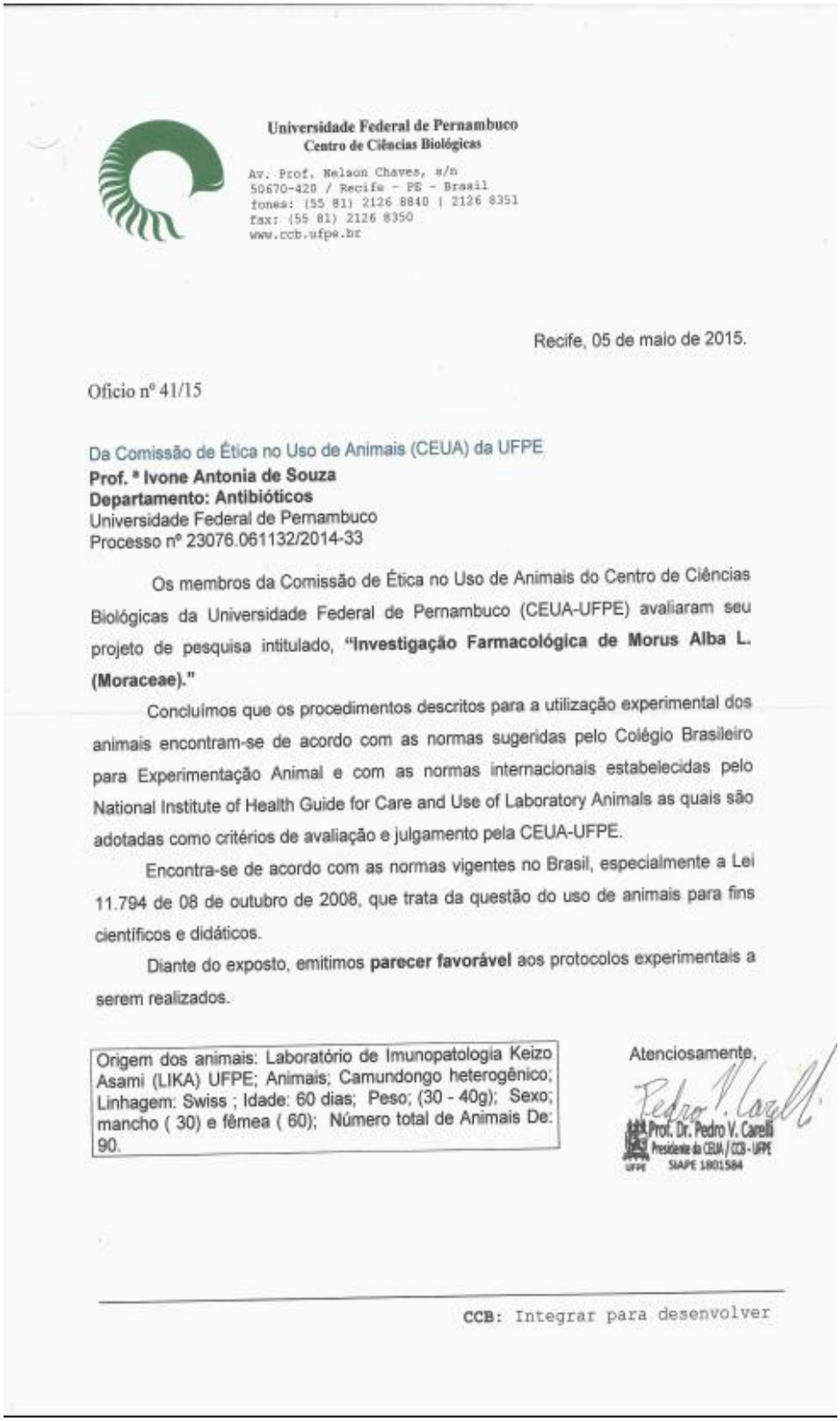
7. CONCLUSÃO

Avaliação da toxicidade de etanólico de folhas de *M. alba* revelou que o mesmo não foi letal nem apresentou efeito genotóxico para camundongos quando administrado por via oral na dose de 300 mg/kg. Contudo, a utilização do extrato em dose de 2000 mg/kg deve ser evitada, devido às alterações bioquímicas, hematológicas e histopatológicas detectadas no ensaio de toxicidade aguda. O extrato também não apresentou citoxicidade *in vitro* para linhagens celulares humanas.

Em dose segura, de acordo com os ensaios toxicológicos, o extrato apresentou potencial inibitório em modelo de inflamação aguda, reduzindo a migração de leucócitos. Ainda, o extrato apresentou atividade antimicrobiana contra bactérias e fungos de importância médica.

8. ANEXOS

Anexo 1: Parecer do Comitê de Ética em Experimentação Animal (UFPE)



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Recife, 05 de maio de 2015.

Oficio nº 41/15

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Prof.ª Ivone Antonia de Souza
Departamento: Antibióticos
Universidade Federal de Pernambuco
Processo nº 23076.061132/2014-33

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Investigação Farmacológica de Morus Alba L. (Moraceae)."**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Laboratório de Imunopatologia Keizo Asami (LIKA) UFPE; Animais; Camundongo heterogênico; Linhagem: Swiss ; Idade: 60 dias; Peso; (30 - 40g); Sexo; mancho (30) e fêmea (60); Número total de Animais De: 90.

Atenciosamente,

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
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