

UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL
FACULDADE DE CIÊNCIAS FARMACÊUTICAS, ALIMENTOS E NUTRIÇÃO

PROGRAMA DE PÓS-GRADUAÇÃO EM FARMÁCIA

Avaliação dos efeitos do extrato etanólico de *Doliocarpus dentatus* em modelos de genotoxicidade, teratogênese e inflamação

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DEZEMBRO/2017

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Raíssa Borges Ishikawa

Dissertação apresentada ao Programa de Pós-Graduação em Farmácia da Universidade Federal de Mato Grosso do Sul como requisito para a obtenção do título de Mestre.

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Dedicatória

Aos meus pais,

Wilson Mota Ishikawa e Thais Couto Borges

Por cada noite de sono perdida, por escolher deixar uma ou outra conta de lado para que eu não deixasse de estudar, por trabalharem incansavelmente em troca de cada mensalidade, por cada boneco de espuma, cada cenário, cada apresentação realizada papai, por cada brigadeiro, cada pacotinho de biscoito e cada qualquer outra coisa vendida mamãe. Obrigada por não desistirem nunca, obrigada por acreditar em mim, o que mais quero nessa vida é encher vocês de orgulho. Eu amo vocês.

Ao meu filho,

João Pedro Ishikawa Torres de Oliveira

Meu pequeno, você ainda não sabe ler, mas um dia vai fuçar nas coisas da mamãe e achar esse livro bonito. Um dia vai entender o porquê de tantas e tantas vezes que mamãe cheirava a camundongos e passava tanto tempo no computador. Espero que seja inspiração para que sempre persista nas tuas metas e nos teus sonhos, mesmo ouvindo de algumas pessoas que jamais irás alcançá-los. A gente consegue sim filho, não é fácil, mas não tem jeito, o que é destinado a você é seu e ninguém tasca!

Ao meu melhor amigo e esposo,

Edwin José Torres de Oliveira

O que seria tudo isso sem você meu amor? O que me valeria mais um diploma pendurado na parede se não tivesse você e meu pequeno ao lado? Nada! Vocês são as minhas melhores escolhas da vida, minhas maiores conquistas, eu quero viver ao teu lado pra sempre ♥

Avovó Eta

Por todo o amor, carinho e cuidado que me dedicou antes de nos deixar. Eu te amo vó, sei que um dia nossos caminhos irão se cruzar novamente e eu poderei matar toda essa saudade que mora aqui comigo. Sei que você estaria, ou melhor, está cheia de orgulho desse momento.

Agradecimentos

Ao meu orientador, **Rodrigo**, por dar um voto de confiança, me abraçar e me deixar fazer parte do **CeTroGen**. Obrigada pela paciência, por dividir comigo todo o seu conhecimento, por me escutar todas as vezes que eu precisei falar, por me tratar como uma profissional e sempre considerar tudo o que era exposto por mim e por ser/estar presente durante todo o mestrado.

Às professoras, **Andrea Laura, Cândida e Cláudia**, pela parceria estabelecida e pelos auxílios.

À minha irmã **Riane** e minha cunhada **Edylla**, que tanto amo e sempre me ajudaram em todas as situações, me levando, me buscando, me ouvindo, lavando caixas no Biocapri, fazendo piada, socorrendo de todas as maneiras que eram possíveis em todos os momentos necessários.

À minha sogra, **Maria Lucia**, que sempre esteve ao meu lado para resolver qualquer problema, de saúde, emocional, financeiro, não importa o tipo, sempre a postos com sua generosidade e carinho, que incontáveis vezes cuidou com tanto amor do meu filho para que eu pudesse realizar tudo o que já fiz desde o seu nascimento. Obrigada por me receber em sua família e me deixar fazer parte dela.

Aos meus amigos parças que me fazem tão bem e me fazem rir e esquecer um pouco das mazelas da vida acadêmica, **Igor Leal, Ana Paula, João Renato, Lucas Pessatto, Andreza Negreli, Bruno Pelizaro e Giovana Corbucci**, vocês (e o café) foram fundamentais.

Às meninas do CeTroGen, **Joyner, Juliana e Silvia**, que sempre pegaram no pesado e ajudaram nos experimentos, dividindo as eutanásias, os pães com mortadela, a Coca-Cola e tantas outras coisas, muito obrigada meninas.

Às meninas da turma 2016 do mestrado em Farmácia da UFMS, **Cláudia, Camilla e Lanna**, por toda a empatia e carinho durante as partes mais difíceis desses dois anos. Obrigada meninas! Foram-se os dias de luta, aguardamos ansiosamente os dias de glórias.

À **CAPES, CNPQ e FUNDECT**, pelo apoio financeiro.

RESUMO

ISHIKAWA, Raíssa Borges. Avaliação dos efeitos do extrato etanólico de *Doliocarpus dentatus* em modelos de genotoxicidade, teratogênese e inflamação. 2017. Dissertação. Mestrado em Farmácia. Universidade Federal de Mato Grosso do Sul, MS.

O uso de plantas medicinais corrobora com a descoberta de novos compostos bioativos. *Doliocarpus dentatus*, conhecida popularmente como cipó de fogo, é utilizada para o tratamento de cistites e processos inflamatórios. Possui atividade antiedematogênica, antirretroviral, antilipidêmica e antibacteriana. O objetivo do estudo foi avaliar os efeitos anti-inflamatório, antimicobacteriano, genotóxico e teratogênico do extrato etanólico das folhas de *Doliocarpus dentatus* (EEDd). Metodologia: Os ensaios de atividade anti-inflamatória foram induzidos por carragenina e realizados em camundongos machos de linhagem C57BL/6 tratados com 30-300 mg/kg de EEDd. A determinação de atividade anti-*Mycobacterium tuberculosis* foi realizada por meio do ensaio *in vitro* de microtitulação nas doses de 0,98-250 µg/mL de EEDd. Os experimentos de toxicogenética foram realizados com camundongos machos *Swiss* nas doses de 10-1000 mg/Kg, por meio dos ensaios do micronúcleo, cometa e fagocitose esplênica. Para o experimento de teratogênese, fêmeas *Swiss* prenhes foram divididas em 3 grupos, controle, dose 1 (100 mg/kg) e dose 2 (1000 mg/Kg), tratadas do 1º ao 18º dia de gestação. Após retirada dos fetos, foram realizadas análises de malformações externas, viscerais e esqueléticas na prole das fêmeas, parâmetros biométricos, desempenho reprodutivo e genotoxicidade materna. Resultados: O valor da concentração inibitória mínima (MIC) de EEDd para a atividade anti-*Mycobacterium tuberculosis* foi de 62,5 µg/mL. O EEDd induziu uma diminuição de edema, hipersensibilidade mecânica e migração de leucócitos promovidos pela carragenina. A análise estatística nos ensaios de cometa e micronúcleo indicou que o EEDd não tem ação genotóxica e não induz fagocitose esplênica. As doses do extrato testadas nos ensaios de teratogênese não alteraram os parâmetros reprodutivos, desenvolvimento embrionário e genotoxicidade nas fêmeas prenhes. Conclusão: O EEDd possui propriedades anti-inflamatórias, antimicobacterianas e não é genotóxico, o que corrobora para o uso seguro do mesmo. A ausência de teratogênese e genotoxicidade materna confirma indiretamente a segurança no uso do extrato também durante o período gestacional.

Palavras-chave: *Doliocarpus dentatus*; inflamação; genotoxicidade; teratogênese.

ABSTRACT

ISHIKAWA, Raíssa Borges. The ethanolic extract of *Doliocarpus dentatus* leaves has anti-inflammatory properties and is devoid of genotoxic and teratogenic effects. 2017. Dissertation. Master of Science in Pharmacy. Federal University of Mato Grosso do Sul, MS.

The use of medicinal plants corroborates with the discovery of new bioactive compounds. *Doliocarpus dentatus*, popularly known as cipó de fogo, is widely used for the treatment of cystitis and inflammatory processes. Among the biological properties already described there are the antiedematogenic, antiretroviral, antilipidemic and antibacterial. The aim of the present study was to evaluate the anti-inflammatory, antimycobacterial, genotoxic effects, splenic phagocytosis and teratogenic effect of *Doliocarpus dentatus* (EEDd) leaves ethanolic extract. Method: Anti-inflammatory activity assays were induced by carrageenan and performed employing male C57BL/6 mice, treated with 30-300 mg/kg EEDd. The anti-Mycobacterial tuberculosis activity was evaluated using the microtiter assay at the doses of 0.98-250 µg/mL of EEDd. The toxicogenic experiments were also performed with Swiss male mice at doses of 10-1000 mg/kg, using micronucleus, comet and splenic phagocytosis. For the teratogenesis experiment, Swiss pregnant females were divided into 3 groups, control, dose 1 (100 mg/kg) and dose 2 (1000 mg/kg), treated from the 1st to the 18th day of gestation period. After the fetal removal, were performed analyzes of external, visceral and skeletal malformations on the offspring of the females, biometric parameters, reproductive performance and maternal genotoxicity. Results: The minimum inhibitory concentration (MIC) of EEDd for anti-*Mycobacterial tuberculosis* activity was 62.5 µg/mL. EEDd induced a decrease in edema, mechanical hypersensitivity and leukocyte migration induced by carrageenan. Statistical analysis of the comet and micronuclei assays indicated that EEDd has no genotoxic action and does not induce splenic phagocytosis. Both of extract doses tested in the teratogenesis assays did not alter the reproductive parameters, embryofetal development and did not induce micronuclei in the pregnant females. Conclusion: EEDd has anti-inflammatory, antimycobacterial properties and it is not genotoxic, which corroborates for its safe use. The absence of maternal teratogenesis and genotoxicity indirectly confirms the safety in the use of the extract also during the gestational period.

Keywords: *Doliocarpus dentatus*; inflammation; genotoxicity; teratogenesis.

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1. REVISÃO BIBLIOGRÁFICA

1.1. *Dolioscarpus dentatus*

Uma das práticas mais antigas empregadas no tratamento de doenças é o uso de plantas com propriedades medicinais, tal prática corrobora para a descoberta de novos compostos bioativos, uma vez que os produtos farmacêuticos podem ser sintetizados a partir de fontes naturais (ANDRADE et al., 2006).

O Brasil é um país promissor na área de bioprospecção. O Cerrado, seu segundo maior bioma, com mais de 7 mil espécies de vegetais, passa por um processo de degradação, sobretudo devido à ocupação e utilização desordenada de seus recursos naturais. Tal aspecto elucida a necessidade de estudos para a identificação e posterior avaliação do potencial biológico de suas plantas (PEREIRA et al., 2012).

Dentre as plantas medicinais do Cerrado destaca-se a *Dolioscarpus dentatus* (Figura 1), conhecida popularmente como cipó de fogo, murucutua, cipó mata sede, sambaiba e cipó vermelho; é uma planta pertencente à família Dilleniaceae, resistente à secas e temperaturas baixas. Encontrada nas florestas tropicais do México, Peru e Bolívia e nos diferentes ecossistemas brasileiros, como Mata Atlântica, Amazônia e Cerrado (APONTE et al., 2008; BIANKI FILHO et al., 2015). É utilizada empiricamente no tratamento de leishmaniose, inflamações e infecções urinárias (RODRIGUES, 2007; JAGESSAR; PERSID, 2014).

Dentre os metabólitos já isolados da *D. dentatus* estão o aldeído betulínico, o ácido betulínico e a betulina Destaca-se ainda que Sauvain et al. (1996) descreveu que um aldeído betulínico isolado das cascas de *D. dentatus* possui atividade contra formas de *Leishmania amazonensis* em modelo *in vitro*. Dentre as propriedades biológicas da *D. dentatus* já comprovadas estão a atividade antimicrobiana, antiedematogênica e a atividade citotóxica contra células leucêmicas humanas (APONTE et al., 2008; JAGESSAR; PERSID, 2014; BIANKI FILHO et al., 2015; TELES et al., 2015).

Diante do exposto, justifica-se o presente estudo a fim de averiguar a segurança da utilização da *D. dentatus*, por meio de ensaios genotóxicos e

teratogênicos, e comprovar cientificamente sua indicação popular para o tratamento de processos inflamatórios, bem como avaliar o efeito antimicobacteriano e assim contribuir para futuros processos de bioprospecção.



Figura 1 – *D. dentatus* localizada no Campus da UFMS de Campo Grande/MS - Fonte: Arquivo pessoal

1.2. Ensaio de genotoxicidade

Segundo Camargo et al. (1994), a genotoxicidade é decorrente da interação de um agente genotóxico com o material genético, resultando em alterações na estrutura ou função da molécula de DNA (ácido desoxirribonucleico). Essas alterações, quando não corrigidas pelos mecanismos de reparo, podem ser transmitidas às células filhas, e no caso da meiose para os descendentes (GRIFFITHS et al., 1998).

Os danos genotóxicos, que podem ocorrer por razões naturais (transposons), fatores endógenos e fatores externos ao organismo (agentes genotóxicos), são responsáveis pela variabilidade genética das populações. Se as

alterações no DNA forem benéficas e acrescentarem ao indivíduo condições melhores de sobrevivência e reprodução, em um determinado ambiente, elas se estabilizarão na população, garantindo adaptação e a evolução da espécie. Contudo, quando não benéficas, podem, por exemplo, aumentar a incidência de câncer ou até mesmo induzir teratogenicidade e doenças hereditárias (OLIVEIRA et al., 2013).

Alguns vegetais, quando consumidos *in natura* ou usados para a produção de infusões, emplastos, extratos ou outros, podem induzir danos genotóxicos. Nesse contexto, a utilização de plantas para consumo deve ser precedida por ensaios biológicos que garantam o uso seguro das mesmas. Na tentativa de garantir essa segurança toxicogenética podem ser usados ensaios biológicos que avaliem a frequência de aberrações cromossômicas, micronúcleos e outras anomalias nucleares e na molécula de DNA bem como o ensaio do cometa, teste de Ames e bioensaios com *Allium cepa* (OECD, 2014, PRAPHASAWAT; MUNKONG, 2017).

1.2.1. Ensaio do cometa

O ensaio do cometa é um teste genotóxico capaz de detectar lesões no DNA que ainda são passíveis de reparo. Trata-se de uma técnica sensível que utiliza eletroforese em gel e nos permite classificar o dano no DNA de acordo com a fragmentação do material genético, portanto em células não danificadas, com o nucleóide intacto, visualiza-se a cabeça do cometa enquanto que em células que sofreram injúrias, observa-se também a migração dos fragmentos de DNA livres, resultantes de quebras, formando uma cauda de cometa (TICE et al., 2000; OECD, 2014; TRAESEL et al., 2017).

Em 1978, os pesquisadores Rydenberg e Johanson iniciaram a quantificação direta do DNA em células individuais e em 1984, Östling e Johanson melhoraram a sensibilidade do teste empregando a eletroforese em pH semineutro (TICE, 1995). Singh e colaboradores (1988) foram os responsáveis pela adaptação da técnica para o pH alcalino, transformando-a em uma ferramenta de grande importância na avaliação de danos genômicos.

Atualmente, o teste é amplamente utilizado no meio científico por ser relativamente barato e com ótima sensibilidade e reprodutibilidade. Consiste na

deposição de células eucarióticas em gel de agarose que, sobre uma lâmina de vidro, são submetidas a uma solução de lise, com posterior desnaturação do DNA e eletroforese em pH alcalino. A eletroforese promove o deslocamento do DNA fragmentado para longe do nucleóide, formando uma estrutura semelhante a um cometa, sendo a parte fragmentada denominada de cauda. Tanto o nucleóide como os fragmentos (cauda) podem ser corados com reagentes intercalantes fluorescentes, como o brometo de etídeo (ALVAREZ-MOYA et al., 2011).

O cometa pode ser quantificado e classificado comparando o tamanho da cauda com o tamanho do nucleóide da célula (AFANASIEVA; ZAZHYTSKA; SIVOLOB, 2010). De acordo com Speit et al. (1996) os cometas são classificados em: classe 0, quando não são visualizados danos, ou seja, o cometa não apresenta cauda; classe 1, quando o dano resulta em cauda menor que o diâmetro do nucleóide; classe 2, quando o dano resulta em cauda de tamanho entre 1 a 2 vezes o diâmetro do nucleóide; classe 3, presença de dano com cauda 2 ou mais vezes o tamanho do diâmetro do nucleóide.

1.2.2. Ensaio do micronúcleo em sangue periférico

Outra técnica clássica na avaliação de danos genotóxicos é o ensaio do micronúcleo, que difere do ensaio do cometa por avaliar danos cromossômicos já fixados no genoma celular. De acordo com Valente et al. (2017), os micronúcleos são fragmentos de DNA que não são capazes de se incorporar no fuso mitótico durante a fase da anáfase da mitose e que, portanto, não são passíveis de reparo.

Nos ensaios de micronúcleo são utilizadas, principalmente, células provenientes da linhagem hematopoiética, como linfócitos e hemácias. Alguns autores utilizam a medula óssea propriamente dita para análise de micronúcleo. Essa técnica tem desvantagem em relação a que utiliza sangue periférico por permitir somente uma coleta, enquanto que na análise de sangue periférico podem ser realizadas várias coletas, analisando a quantidade de dano cromossomal em diferentes tempos de exposição (SILVA; NEPOMUCENO, 2010).

O ensaio de micronúcleo em sangue periférico utiliza reticulócitos para avaliar a presença de danos cromossômicos. No processo natural de maturação

dessas células, antes de se tornarem eritrócitos, os eritroblastos devem expelir seu núcleo e nesse momento, caso tenha ocorrido dano cromossômico, o dano persistirá no citoplasma da célula, podendo ser visualizado com uso de corantes fluorescentes. A técnica de micronúcleo consiste em depositar sobre uma lâmina coberta previamente com alaranjado de acridina uma gota de sangue periférico. Após o período de maturação das células, utilizando microscopia, é possível visualizar os micronúcleos corados (HAYASHI et al., 1990).

O alaranjado de acridina possui a capacidade de se intercalar ao DNA, e também de se ligar ao RNA. A diferença é que, quando submetido à luz ultravioleta, fluoresce o DNA em amarelo enquanto o RNA é corado em vermelho. Essa diferença de coloração permite distinguir reticulócitos, ricos em RNA citoplasmático, de reticulócitos com micronúcleos, pois o último apresentará em seu citoplasma, além dos fragmentos de RNA corados em vermelho, fragmentos de DNA (micronúcleos) corados em amarelo (HAYASHI et al., 1990).

1.3. Avaliação de fagocitose esplênica

Como descrevem Groom; Schmidt; Macdonald (1991), o baço é um órgão composto de feixes de tecido conjuntivo dividido em três áreas: polpa vermelha, formada pelos seios esplênicos onde se situam os cordões de Billroth, que formam os plexos sanguíneos; polpa branca, formada por nódulos linfáticos onde são encontrados, principalmente, os linfócitos; e zona marginal, situada na periferia da polpa branca e que contém as artérias marginais.

Uma vez que células esplênicas são capazes de capturar e apresentar antígenos aos linfócitos T e B, estimulando os plasmócitos secretores de anticorpos, o baço torna-se um dos principais órgãos do sistema imunológico. É responsável pelo processo de fagocitose de elementos indesejáveis do sangue, removendo 1,2% de todos os glóbulos vermelhos do organismo por meio dos fagócitos e também é fonte de células hematopoiéticas, em especial nos quadros de anemias severas. O baço contém de 30 a 40% das plaquetas do corpo e em casos de aumento do órgão pode ocasionar plaquetopenia uma vez que até 90% da massa total de plaquetas pode estar isolada na polpa vermelha do baço e leucopenia por meio do sequestro de leucócitos (MELBIUS; KRAAL, 2005).

Segundo Guyton e Hall (2000), a fagocitose pode ser desencadeada por três processos; primeiro, a probabilidade de fagocitose tem um aumento diretamente proporcional à rugosidade na superfície dos tecidos; segundo, a presença ou ausência de proteínas que vão repelir os fagócitos; e terceiro, a ativação do sistema imunológico por meio da cascata do complemento.

A fagocitose esplênica também é efetiva no sequestro de células anucleadas e/ou micronucleadas e dessa maneira, pode mascarar a frequência de danos celular, portanto, tal ensaio corrobora para que as análises de frequência de danos no DNA (ensaio de cometa e micronúcleo, por exemplo) sejam mais precisas (CARVALHO et al, 2015).

1.4. Ensaios de teratogênese

Os ensaios *in vivo* na área de teratogênese avaliam os efeitos de compostos durante o período gestacional, servindo para a prevenção de efeitos adversos e malformações congênitas (OLIVEIRA et al., 2009; HOLMES, 2011).

A teratologia experimental demonstrou por meio de estudos com répteis, anfíbios e peixes que, ainda com a proteção exercida pela placenta, os embriões poderiam estar susceptíveis a riscos gerados a partir de fatores externos, não sendo imunes a quaisquer interferências ambientais, como se acreditava até meados do século XIX. Tais estudos corroboraram para a diferenciação de termos até então utilizados como sinônimos: hereditário, transmitidos geneticamente de geração para geração; e congênito, originados durante o processo de embriogênese, podendo ou não ser notados ao nascimento e ter origem genética (RABELLO-GAY et al., 1991).

Uma substância com potencial teratogênico pode determinar alterações no desempenho reprodutivo materno e desenvolvimento embriofetal, ainda que haja diversos mecanismos de proteção oferecidos pelo útero materno (ROSENFELDI, 2015).

1.4.1. Desempenho reprodutivo

Toxicidade materna é definida como alterações na fisiologia materna, de caráter transitório ou permanente, como mudanças na homeostasia, nos níveis

hormonais e até mesmo comportamentais, potencialmente prejudiciais à prole durante o desenvolvimento intra-uterino ou após o nascimento (KHERA, 1987).

Oliveira et al. (2009) descrevem que para avaliação de desempenho reprodutivo de fêmeas prenhes alguns parâmetros são calculados levando em consideração a quantidade de implantações, reabsorções precoces ou tardias, número de fetos vivos e mortos, sexagem, peso do útero, peso do feto e peso placentário. Com esses dados absolutos pode-se calcular a taxa de perdas pós-implantação ($\text{número de implantações} - \text{número de fetos vivos} \times 100 / \text{número de implantações}$), taxa de reabsorção ($\text{número de reabsorções} \times 100 / \text{número de implantações}$), viabilidade fetal ($\text{número de fetos vivos} \times 100 / \text{número de implantações}$), índice placentário ($\text{peso placentário} / \text{peso fetal}$), adequação de peso para a idade de prenhez (média de peso dos fetos do grupo controle mais ou menos o desvio padrão) e razão sexual ($\text{número de fetos machos} / \text{número de fetos fêmeas}$).

1.4.2. Desenvolvimento embriofetal

Em estudos de desenvolvimento embriofetal e teratogênese que utilizam camundongos como modelo experimental, recomenda-se a interrupção da gravidez, para obtenção dos fetos e posteriores análises, no décimo oitavo dia de prenhez, diminuindo a probabilidade de canibalismo por parte da genitora em casos de malformação ou baixa viabilidade (TAYLOR, 1986; OLIVEIRA et al., 2009; GONÇALVES et al., 2013; DAVID et al., 2014; PESSATTO et al., 2017).

Após os procedimentos de laparotomia, histerectomia e onfalectomia, os fetos passam por uma análise sistemática para verificação de viabilidade, anomalias estruturais externas e sexagem. Posteriormente, a ninhada é dividida em dois grupos, um destinado às análises de malformações viscerais e outro destinado às análises de malformações esqueléticas. Trabalhos clássicos como os de Taylor (1986), Barrow; Taylor (1969), Wilson (1965) e Manson; Kang (1994) são utilizados para a classificações das malformações e formas de análise.

1.5. Ensaios biológicos de inflamação por carragenina

Os processos inflamatórios são desencadeados de maneira multifatorial e tem por função ativar mecanismos homeostáticos para controle das alterações

biológicas. As atividades inflamatórias são processos fisiológicos gerados pelo sistema imunológico em resposta a injúria tecidual causada por infecções, lesões teciduais, estresse oxidativo, hipertensão, diabetes entre outros. A inflamação apresenta sinais clássicos como rubor, calor, edema e dor, mediados por interleucinas, fator de necrose tumoral (TNF), quimiocinas, prostaglandinas, leucotrienos e fator de ativação plaquetária (CRUVINEL et al, 2010).

Alguns fatores podem desencadear uma exacerbação do quadro inflamatório, como a agressividade de processos infecciosos. Tal cenário ressalta a importância de estudos visando à descoberta de novas substâncias com propriedades anti-inflamatórias.

A carragenina, encontrada principalmente nas algas de espécie *Chondrus crispus*, é um polissacarídeo amplamente utilizado em modelos experimentais de inflamação devido à sua habilidade de induzir uma reação inflamatória do tipo aguda com intensa migração leucocitária, edema e hiperalgesia, por meio da liberação de mediadores de resposta inflamatória, como as interleucinas, eicosanoides, dentre outras. Seu uso foi proposto por Winter, Risley, Nuss (1962) em edema de pata de ratos, posteriormente, sofreu algumas modificações até tornar-se um dos métodos mais utilizados para estudos de terapias anti-inflamatórias (DI ROSA, 1972).

Os leucócitos representam um papel fundamental no processo de inflamação, assim como seu transporte para o local da injúria, uma vez que são capazes de migrar da corrente sanguínea para os tecidos por meio da ativação sequencial das proteínas selectinas, integrinas e imunoglobulinas. Substâncias com propriedades anti-inflamatórias são capazes de diminuir a migração leucocitária por diversos mecanismos de ação, impedindo a liberação de mediadores ou bloqueando uma das fases do processo (VASCONCELOS, 2016).

Com base nas informações supracitadas, a utilização da carragenina como agente indutor de inflamação em modelos experimentais *in vivo*, se faz eficiente para a investigação do potencial anti-inflamatório de uma substância e foi empregado no presente estudo.

1.6. Atividade anti-*Mycobacterium tuberculosis*

Patologias como arteriosclerose, câncer, Alzheimer e tuberculose são associadas a processos inflamatórios acentuados, ocasionando lesões teciduais e disfunções de múltiplos órgãos (VENTURA, 2011).

De acordo com a Organização Mundial de Saúde, mais de 10 milhões de pessoas se infectaram com tuberculose no ano de 2015. Essa doença determinou o registro de 70 mil novos casos no Brasil e cerca de 4500 óbitos, sendo considerado um grave problema de saúde pública, ainda que, com diagnóstico e tratamento realizados gratuitamente pelo Sistema Único de Saúde (BRASIL, 2017).

O agente etiológico responsável pelas infecções por tuberculose em humanos é o *Mycobacterium tuberculosis*, um bacilo álcool-ácido resistente (BAAR), que pode ser transmitido por partículas aerossóis e ocasionalmente assumem um estado de latência, reativando a infecção anos mais tarde (SOUZA, 2013).

Após infecção por *Mycobacterium tuberculosis*, os macrófagos residentes nos alvéolos pulmonares fagocitam parte dos bacilos. Porém, incapazes de conter a infecção, produzem citocinas mediadoras de migração leucocitária. Uma vez estimulados, os leucócitos polimorfonucleares acumulam-se formando uma reação inflamatória inespecífica (LOPES; JANSEN; CAPONE, 2006).

Sabendo-se do número expressivo de casos resistentes aos tratamentos convencionais para tuberculose, o presente trabalho corrobora com a busca por substâncias que além de atuarem contra o *Mycobacterium tuberculosis*, também exerçam atividade frente ao processo inflamatório acentuado observado nessa doença.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar os efeitos toxicogenético, teratogênico, anti-inflamatório e antimicobacteriano do extrato etanólico de *Dolios carpus dentatus* (EEDd).

2.2 Objetivos específicos

Avaliar a atividade anti-inflamatória do EEDd por meio dos ensaios de migração leucocitária, edema e hiperalgesia mecânica induzidos por carragenina;

Avaliar a atividade *in vitro* anti-*Mycobacterium tuberculosis* do EEDd por meio do ensaio de Microtitulação.

Avaliar a genotoxicidade do EEDd por meio dos ensaios de cometa e micronúcleo em sangue periférico;

Avaliar a capacidade do EEDd induzir fagocitose esplênica;

Avaliar a capacidade do EEDd alterar o desempenho reprodutivo de fêmeas prenhes, o desenvolvimento embriofetal e causar teratogênese;

3. REFERÊNCIAS BIBLIOGRÁFICAS

AFANASIEVA, K.; ZAZHYTSKA, M.; SIVOLOB, A. Kinetics of comet formation in single-cell gel electrophoresis: loops and fragments. **Electroph.** v. 31, p. 512-519, 2010.

ALVAREZ-MOYA, C. et al. Evaluation of genetic damage induced by glyphosate isopropylamine salt using *Tradescantia* bioassays. **Gen Mol Biol.** v. 34, n. 1, p. 127-130, 2011.

ANDRADE, S.F. et al. Anti-inflammatory and antinociceptive activities of extract, fractions and populonic acid from bark wood of *Austroplenckia populnea*. **J Ethnopharmacol.** v. 109, p. 464-471, 2006.

APONTE, J.C. et al. Isolation of cytotoxic metabolites from Target Peruvian Amazonian medicinal plants. **J Nat Prod.** v. 71, p. 102-105, 2008.

BARROW, M.V.; TAYLOR, W.J. A rapid method for detecting malformation in rat fetuse. **J Morphol.** v. 127, n. 3, p. 291-305, 1969.

BIANKI FILHO, C.A. et al. Atividade antiedematogênica e inibitória da atividade da mieloperoxidase de extrato de folhas de *Dolioscarpus dentatus* em ratos. **17º Workshop de Plantas Mediciniais do Mato Grosso do Sul. 7º Empório da Agricultura Familiar.** 2015.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância das Doenças Transmissíveis. **Plano nacional pelo fim da tuberculose** / Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância das Doenças Transmissíveis. Brasília, 2017.

CAMARGO, J.L.V. et al. A detecção de substâncias cancerígenas em estudos experimentais. **Rev Bras Cancerol.** v.40, p. 21-30, 1994.

CARVALHO, P.C. et al. Diaryl sulfide analogs of combretastatin A-4: oxigenetic, immunomodulatory and apoptotic valuations and prospects for use as a new chemotherapeutic drug. *Environ. Toxicol Pharmacol.* v. 40, n. 3, p. 715–721, 2015.

CRUVINEL, W.M. et al. Sistema Imunitário – Parte I Fundamentos da imunidade inata com ênfase nos mecanismos moleculares e celulares da resposta inflamatória. **Rev Bras Reumatol.** v. 50, n. 4, p. 434-61. 2010

DAVID, N. de. et al. *Gochnatia polymorpha* ssp. *floccosa*: Bioprospecting of an anti-inflammatory phytotherapy for use during pregnancy. **J Ethnopharmacol.** v. 154, n. 2, p. 370-9, 2014.

DI ROSA, M. Pharmacological properties of carrageenan. **J Pharm Pharmacol.** v. 24, p. 89-102, 1972.

- GONÇALVES, C.A. et al. Evaluation of mutagenic, teratogenic, and immunomodulatory effects of *Annona nutans* hydromethanolic fraction on pregnant mice. **Genet Mol Res.** v. 13, p. 4392-4405, 2013.
- GRIFFITHS, A.J.F. et al. Mutação Cromossômica I: Alterações na Estrutura Cromossômica in: **Introdução à Genética**, Rio de Janeiro, Guanabara Koogan, cap. 8, p. 197-232, ed. 6, 1998.
- GROOM, A.C.; SCHMIDT, E.E.; MacDONALD, I.C. Microcirculatory pathways and blood flow in spleen: new insights from washout kinetics, corrosion casts, and quantitative intravital videomicroscopy. **Scan Microsc.** v. 5, p. 159-174, 1991.
- GUYTON, A.C.; HALL, J.E. **Tratado de Fisiologia Médica**. 10 ed, Rio de Janeiro: Guanabara, 2000.
- HAYASHI M. et al. The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. **Mutation Res.** v. 245, p. 245-249, 1990.
- HOLMES, L.B. Human teratogens: update 2010. **Birth Defects Res A Clin Mol Teratol.** v. 91, n.1, p. 1-7, 2011.
- JAGESSAR, R.C.; PERSID, R. Antimicrobial activity of uncombined and combined extracts of *Doliocarpus dentatus* and *Montricardia arborescens*. **Int J Pharm Sci Res.** v. 5, n. 1, p. 286-293, 2014.
- KHERA, K.S. Maternal toxicity of drugs and metabolic disorders – a possible etiologic factor in the intrauterine death and congenital malformation: a critique on human data. **CRC Crit Rev Toxicol.** v.17, n. 4, p. 345-375, 1987.
- LOPES, J.; JANSEN, J.M.; CAPONE, D. Patogenia e imologia. **Revista HUPE.** v. 5, n. 2, p. 27-34, 2006.
- MANSON, J.M.; KANG, Y.J. Test methods for assessing female reproductive and developmental toxicology. In: Hayes, A.W. (Ed). **Principles and methods of Toxicology**. Raven Press, New York, 1994.
- MELBIUS, R.E.; KRAAL, G. Structure and function of the spleen. **Nat Rev Immunol.** v. 5, p. 606-616, 2005.
- OECD. Test N° 474. Mammalian erythrocyte micronucleus test. **OECD Publishing**. Adopted: 21st July 1997. Paris, 2014.
- OLIVEIRA, R.J. et al. Effects of the polysaccharide Î-glucan on clastogenicity and teratogenicity caused by acute exposure to cyclophosphamide in mice. **Regul Toxicol Pharmacol.** v. 53, n. 3, p. 164-73, 2009.

OLIVEIRA, R. J. et al. In vivo evaluation of the antimutagenic and antigenotoxic effects of β -glucan extracted from *Saccharomyces cerevisiae* in acute treatment with multiple doses. **Genet Mol Biol.**, v. 36, p. 413-424, 2013.

PEREIRA, Z.V. et al. Uso múltiplos de espécies nativas do bioma Cerrado no Assentamento Lagoa Grande, Dourados, Mato Grosso do Sul. **Rev Bras Agroecol.** v. 7, n. 2, p. 126-136, 2012.

PESSATTO, L.R. et al. Effects of dichloromethane and butanol fractions of *Gochnatia polymorpha floccosa* in maternal reproductive outcome, embryo-fetal development and DNA integrity in mice. **J Ethnopharmacol.** 200, p. 205-209. 2017.

PRAPHASAWAT, R.; MUNKONG, N. Anti-genotoxicity evaluation of *Cratoxylum formosum* dyer leaves by comet assay and micronucleus test. **Asian Pac J Cancer Prev.** v. 18 n. 6, p. 1475-1478, 2017.

RABELLO-GAY, M. N. et al. Mutagênese, carcinogênese e teratogênese: métodos e critérios de avaliação. **Soc Bras Gen.** p. 83-90, Ribeirão Preto, 1991.

RODRIGUES, V.E.G. **Etnobotânica e florística de plantas medicinais nativas de remanescentes de floresta estacional semidecidual na região do Alto Rio Grande, MG.** Universidade Federal de Lavras, Minas Gerais, Brasil, 2007.

RODRIGUES, V.E.G.; CARVALHO, D.A. Levantamento etnobotânico de plantas medicinais no domínio do cerrado na região do Alto Rio Grande – Minas Gerais. **Ciênc Agrotec.** v. 25, n. 1, p.102-123, 2001.

ROSENFELD, C.S. Sex-Specific Placental Responses in Fetal Development. **Endocrinology.**v. 156, n. 10, p. 3422-34, 2015.

SAUVAIN, M. et al. Isolation of leishmanicidal triterpenes and ligans from the Amazonian liana *Dolioscarpus dentatus*. (Dilleniaceae). **Phytother Res.** v. 10, p. 1-4, 1996.

SILVA, A.C.; NEPOMUCENO, J.C. Avaliação da frequência de micronúcleos em eritrócitos periféricos de mandi-amarelo (*Pimelodus maculatus*) do rio Paranaíba. **Revista do Núcleo Interdisciplinar de Pesquisa e Extensão do UNIPAM.** Patos de Minas: UNIPAM, n. 7, v. 1, p. 167-179, 2010.

SINGH, N.P. et al. A simple technique for quantitation of low levels of DNA damage in individual cells. **Exper Cell Res.**, v. 175, 184-191, 1988.

SOUZA, P.C. **Atividade anti - Mycobacterium tuberculosis intra e extra celular e citotoxicidade dos complexos de coordenação de metais.** Dissertação (Mestrado) – Universidade Estadual Paulista. “Júlio de Mesquita Filho”. Faculdade de Ciências Farmacêuticas. Programa de Pós Graduação em Biociências e Biotecnologia aplicadas à Farmácia. Araraquara, 2013.

SPEIT, G. et al. Detection of DNA effects in human cells with the comet assay and their relevance for mutagenesis. **Toxicol Let.**, v. 88, p. 91-98, 1996.

TAYLOR, P. Practical Teratology. **Academic Press**, New York, 1986.

TELES, C.B.G. et al. A lupane-triterpene isolated from *Combretum leprosum* Mart. Fruit extracts that interferes with the intracellular development of *Leishmania (L.) amazonensis* *in vitro*. **Off J In Soc Comp Med Res.** v. 15, n. 165, 2015.

TICE, R.R. The single cell gel/ Comet assay: a microgel eletrophoretic technique for the detection of DNA damage and repair in individual cells. In: PHILLIPS, D.H. E VENITT, S. **Environ Mutagen.** Oxford: Bios Scientific Publishers. p. 315-339. 1995.

TICE, R.R. et al. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. **Radiat Res.** 169, p. 110-121, 2000.

TRAESEL, G.K. et al. Safety Assessment of Oil from Pequi (*Caryocar brasiliense* Camb.): Evaluation of the Potential Genotoxic and Clastogenic Effects. **J Med Food.** p. 1-8, 2017.

VALENTE, D. et al. Utilização de biomarcadores de genotoxicidade e expressão gênica na avaliação de trabalhadores de postos de combustíveis expostos a vapores de gasolina. **Rev Bras Saude Ocup.** v. 42, supl. 1, 2017.

VASCONCELOS, P.A. **Avaliação da atividade anti-inflamatória do composto LQFM 147, um candidato a protótipo de fármaco.** Dissertação de mestrado. Instituto de Ciências Biológicas. Programa de Pós Graduação em Biologia. Universidade Federal de Goiás. 2016.

VENTURA, T.L.B. **Atividade anti-inflamatória e antimicobacteriana de espécies vegetais ocorrentes no Brasil com ênfase em *Cecropia pachystachya* e *Vochysia divergens*.** Dissertação. Programa de Pós-Graduação em Biociências e Biotecnologia. Universidade Estadual do Norte Fluminense. Campos dos Goytacazes. 2011.

WILSON, J.G. Methods for administering agents and detecting malformations in experimental animals. In: WILSON, J.G.; WAEKANY, J. (Eds.) **Teratology: Principles and Techniques.** The University of Chicago Press, Chicago, 1965.

WINTER, C.A.; RISLEY, E.A.; NUSS, G.W. Carrageenin-induced edemas in hind paw of the rat as an assay for antiinflammatory drugs. **Proc Soc Exp Biol.** v. 111, p. 544-547. 1962.

*Referências bibliográficas de acordo com as regras da Associação Brasileira de Normas Técnicas (ABNT, 2017).

4. MANUSCRITO I

Artigo publicado na Journal of Ethnopharmacology abril/2017:

Anti-inflammatory, antimycobacterial and genotoxic evaluation of
Doliocarpus dentatus



Anti-inflammatory, antimycobacterial and genotoxic evaluation of *Doliocarpus dentatus*



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ARTICLE INFO

Chemical compounds obtained in this article:

Sitosterol-3-O-β-D-glucopyranoside

(PubChem CID: 5742590)

Kaempferol 3-O-α-L-rhamnopyranoside

(PubChem CID: 5316673)

Betulinic acid (PubChem CID: 64971)

Betulin (PubChem CID: 72326)

Keywords:

Doliocarpus dentatus: genetic toxicity

Mice

Mycobacterium tuberculosis: inflammation

Phagocytosis

ABSTRACT

Ethnopharmacological relevance: *Doliocarpus dentatus* is a medicinal plant widely used in Mato Grosso do Sul State for removing the swelling pain caused by the inflammation process and for treating urine retention. **Aim of the study:** The genotoxic aspects and the anti-inflammatory and antimycobacterial activity of the ethanolic extract obtained from the leaves of *D. dentatus* (EEDd) were investigated.

Materials and methods: The EEDd was evaluated against *Mycobacterium tuberculosis*, and the compound composition was evaluated and identified by nuclear magnetic resonance (NMR). The mice received oral administration of EEDd (30–300 mg/kg) in carrageenan models of inflammation, and EEDd (10–1000 mg/kg) was assayed by the comet, micronucleus, and phagocytosis tests and by the peripheral leukocyte count.

Results: Phenols (204.04 mg/g), flavonoids (89.17 mg/g), and tannins (12.05 mg/g) as well as sitosterol-3-O-β-D-glucopyranoside, kaempferol 3-O-α-L-rhamnopyranoside, betulinic acid and betulin were present in the EEDd. The value of minimal inhibitory concentration (MIC) of EEDd was 62.5 μg/mL. The EEDd induced a significant decrease in the edema, mechanical hypersensitivity and leukocyte migration induced by carrageenan. The comet and micronucleus tests indicated that the EEDd was not genotoxic. The EEDd also did not change the phagocytic activity or the leukocyte peripheral count.

Conclusions: The EEDd does not display genotoxicity, phagocytosis and could act as an antimycobacterial and anti-inflammatory agent. This study should contribute to ensuring the safe use of EEDd.

1. Introduction

Doliocarpus dentatus (Aubl.) Standl. (Dilleniaceae), popularly known in Brazil as cipó-vermelho, can be found in the Amazon and Cerrado regions. The infusions of the leaf and roots of *D. dentatus* have been used for treating cystitis (Rodrigues and Carvalho, 2001), for removing the swelling pain associated with the inflammatory process (especially in the testicles and legs) and for urine retention (Rodrigues, 2007).

Inflammatory processes play a role in various diseases in which

infections (such as *Mycobacterium tuberculosis*) and oxidative stress are present with chronic pathology (hypertension and diabetes). Inflammatory parameters, such as leukocyte migration, edema, protein extravasation, pain (spontaneous and evoked hyperalgesia), and inflammatory mediator levels (cytokines, eicosanoids, and chemokines) could be analyzed in the discovery of new drugs.

Phytochemical studies with different extracts of this plant have shown the presence of butyric acid, steroids, lactones, anthracenoides, betulinic acid, tannins, flavones, and trigonelline (Jagessar et al., 2013). Sauvain et al. (1996) showed the leishmanicidal activity of the

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<http://dx.doi.org/10.1016/j.jep.2017.04.004>

Received 20 February 2017; Received in revised form 3 April 2017; Accepted 4 April 2017

Available online 06 April 2017

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stem extract and the triterpenes and lignins in this plant. The biological properties of compounds isolated from *D. dentatus* against various human tumor cells and against *M. tuberculosis* have also been reported (Aponte et al., 2008).

Thus, the objective of this study was to evaluate the effects of extract obtained from the leaves of *D. dentatus* (EEDd) *in vitro* against *M. tuberculosis*, the oral administration of EEDd in a model of inflammation, and the genotoxic effects of EEDd.

2. Materials and methods

2.1. Animals

For the anti-inflammatory assays, male C57BL/6 mice (30 g) from the Central Biotherium of the Federal University of Grande Dourados (UFGD) were used. For genetic toxicological studies, 25 male Swiss mice (35 g) from the State Agency for Animal and Plant Health Protection (IAGRO) were used. The research was approved by the Ethics Committee in Animal Experimentation of UFMS (776/2016) and of UFGD (32/2015). The animals were kept in polypropylene boxes with sepillos approximately to 22 °C a relative humidity of 55% at air conditioning into the ventilated rack, and they were fed and given filtered water *ad libitum*.

2.1.1. Leukocyte migration induced by carrageenan in the pleurisy test

One hour before being administered with carrageenan, the mice were divided into 6 groups (n=6): one group received a solution of saline with 2% Tween 80 (the vehicle used to dissolve EEDd) by oral route (p.o.) and was called the control group; 3 groups received (p.o.) one single dose of 30, 100 or 300 mg/kg of EEDd (in a solution of saline with 2% Tween 80; 1 group received dexamethasone (1 mg/kg) by subcutaneous injection (s.c.); and the naïve group, which did not receive the intrathoracic injection of carrageenan and which was treated with a solution of saline with 2% Tween 80 (p.o.).

After that, the animals received an intrapleural injection of 100 µL of either 1% carrageenan suspension or sterile saline (naïve group only) (Velo et al., 1973; Vinegar et al., 1973). After four hours, the mice were killed with an overdose of isoflurane (1.5%), and the pleural exudates were obtained after rinsing with 1 mL of phosphate buffered saline. The pleural exudate (50 µL) was diluted with 1000 µL of Turk's solution to count the total leukocytes in a Neubauer chamber.

2.1.2. Edema and mechanical hyperalgesia induced by carrageenan

Four different groups of mice (n =6) were treated (p.o.) with the EEDd (100 and 300 mg/kg), vehicle (control) or dexamethasone (s.c., 1 mg/kg). After 1 h, the animals received an injection (s.c.) into right paw of a 50-µL solution of carrageenan (300 µg/paw) and the same volume of saline in the left paw. The thickness of the paw edema was measured using a plethysmometer before any treatment and at 1, 2, and 4 h after the injection of carrageenan. The results were expressed in mL, and the difference between basal and post-injection values were quantified as edema. Mechanical hyperalgesia was measured with an analgesimeter at 3 and 4 h after the carrageenan injection.

2.1.3. Genotoxic evaluation

Mice were divided into 5 groups (n=5): the negative control (CN), in which the animals were treated intraperitoneally (i.p.) with a physiological solution in the proportion of 10 mL/Kg of body weight (b.w.) and the EEDd vehicle in the same proportion (p.o.); the positive control (CP), in which animals were treated with cyclophosphamide (100 mg/kg, b.w., i.p.) and the EEDd vehicle (10 mL/Kg, b.w., p.o.); the EEDd groups (EEDd1, EEDd2 and EEDd3), in which animals were treated with EEDd at 3 different doses (10, 100 and 1000 mg/kg, p.o., respectively) and a physiological solution (10 mL/Kg, b.w., i.p.).

Blood samples were taken at 24, 48 and 72 h after administration of

the tested substances. After the last blood collection, the animals were euthanized by cervical dislocation to collect their organs.

2.1.4. Micronucleus assay in peripheral blood

In a slide previously prepared with a layer of 20 µL of acridine orange (1.0 mg/mL), 20 µL of peripheral blood were deposited and then covered by a coverslip. This material remained in freezer for a minimum of 7 days, and 2000 cells/animal were analyzed under a fluorescence microscope with a 420–490-nm excitation filter and 520-nm barrier filter (Hayashi et al., 1990; Oliveira et al., 2009).

2.1.5. Comet assay in peripheral blood

To a slide with normal agarose (5%) was added 20 µL of homogenized peripheral blood in 120 µL of LMP agarose (1.5%) at 37 °C. It was then covered by a glass cover, cooled to 4 °C for 20 min, immersed in lysis solution (89 mL of lysis stock (2.5 M NaCl, 100.0 mM EDTA, 10.0 mM Tris, pH 10, corrected with solid NaOH, 890 mL of distilled water and 1% sodium lauryl sarcosinate), 1.0 mL of Triton X-100 and 10 mL of DMSO) for 1 h at 4 °C, and protected from light. The slides were brought into an electrophoresis vessel with pH buffer > 13.0 (300 mM NaOH and 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA, pH 10.0) for a period of 20 min at 4 °C for DNA denaturation. Electrophoresis was performed at 25 V and 300 mA (1.25 V/cm). The samples were neutralized with pH 7.5 buffer (0.4 M Tris-HCl) for 3 cycles of five minutes each, dried in the open air and fixed in absolute ethanol for 10 min. Subsequently, they were stained with 100 µL of ethidium bromide (20 µg/mL), and 100 cells/animal were analyzed by fluorescence microscopy with a 40× magnification, 420–490-nm excitation filter and 520-nm barrier filter. One hundred cells/animal were analyzed using the following classification system: class 0 - undamaged, tail-free cells; class 1 - cells with a tail smaller than the nucleoid diameter; class 2 - cells with tail sizes between 1 and 2 times the nucleoid diameter; and class 3 - cells with a tail greater than 2 times the nucleoid diameter. Apoptotic cells (those with a completely fragmented nucleoid) were not counted. The total score was calculated by multiplication of the total cells observed in each lesion class (Kobayashi et al., 1995).

2.1.6. Phagocytosis assay and differential cell count

The spleen was macerated in a physiological solution to obtain a homogeneous cell suspension by successive aspirations with a Pasteur pipette. One hundred microliters of the cell suspension was placed on a lamina previously stained with acridine orange (1 mg/mL) and covered by a cover slip. The slide were then stored in a freezer until the time of analysis, which was done using a fluorescence microscope with a 400× magnification, 420–490-nm filter and 520-nm barrier filter at 200 cells per animal. The absence or presence of phagocytosis was based on the description of Carvalho et al. (2015).

Twenty microliters of peripheral blood were used for histological slide smears, which were air dried and stained with Panótico® kit, developed by Laborclin® laboratory. Slide analysis was performed using brightfield microscopy at 1000 magnification. A total of 100 cells/animal were analyzed and differentiated into lymphocytes, neutrophils, monocytes, eosinophils, and basophils (Ishii et al., 2011).

2.2. Anti-*Mycobacterium tuberculosis* activity

Standard strain of *Mycobacterium tuberculosis* virulent type (H₃₇Rv) American Type Culture Collection (ATCC27294) was grown in Ogawa–Kudoh (OK) medium for 10 days at 37 °C. A sample was cultured in Middlebrook 7H9 broth (Difco) supplemented with oleic acid, bovine serum albumin, dextrose, catalase (Baltimore Biological Laboratory Becton, Dickinson and Company (BBL/Becton-Dickinson)), 0.5% glycerol (carbon source), and 0.5% Tween 80 for 15 days at 37 °C. The bacterial suspensions were prepared and adjusted to the McFarland scale. Stock solutions of the tested extract were solubilized

in dimethyl sulfoxide (Sigma-Aldrich) and diluted in Middlebrook 7H9 broth (Difco) supplemented with oleic acid, bovine serum albumin, dextrose and catalase (BBL/Becton Dickinson). Rifampicin and isoniazid were solubilized according to the manufacturer's recommendations (Sigma, USA) and used as positive control drugs. The determination of antimycobacterial activity was performed using the resazurin microtiter assay (REMA) (Palomino et al., 2002). Briefly, 100 μ L of supplemented Middlebrook 7H9 broth was dispensed into each well of a sterile, flat-bottom 96-well plate, and then serial dilutions were made of the solutions to obtain varying concentrations of the test extract (0.98–250 μ g/mL) and reference drugs (0.004–1 μ g/mL). After these dilutions, 100 μ L of bacterial suspension was added to each well (5×10^5 UFC/mL). Plates were incubated for 7 days at 37 °C, and, after this period, 30 μ L of resazurin solution (Sigma-Aldrich) diluted to 0.01% in sterile water was added to each well. The samples were incubated for 24 h at 37 °C. The reading was performed based on the color change and the absorbance on a microplate reader (Thermo Plate, TP-Reader) at 492 nm. The EEDd was analyzed in triplicate on alternate days. The minimal inhibitory concentration (MIC) was defined as the lowest concentration resulting in 90% growth inhibition of *M. tuberculosis*.

2.2.1. Plant material, ethanolic extract preparation and compound analysis

The leaves of *Dolioscarpus dentatus* (Aubl.) Standl. were collected from young and mature samples in the pre-flowering stage at the Federal University of the State of Mato Grosso do Sul - (UFMS, Campo Grande). The botanical identification was carried out by Prof. Dr. Arnildo Pott, and a voucher was deposited in the Campo Grande Mato Grosso do Sul (CGMS) herbarium (49860) of the UFMS.

The collected leaves were dried at 40 °C. Afterwards, the leaves were crushed to a particle size of ≤ 1 mm by granulometry. The material was extracted with ethanol (95%) for seven consecutive days by the maceration process. The obtained extracts were pooled and concentrated to dryness in an evaporator, and then they were lyophilized to produce the crude extract.

A solution of the extract was dissolved in water at a concentration of 10 μ g/mL and was evaluated in relation to the flavonoid content by employing the method described by Lin and Tang (2007). The result was expressed as mg of quercetin equivalents/g of extract.

Phenols were assayed with the same samples used in the flavonoid quantification, and we employed the method described by Djeridane et al. (2006). The result was expressed as mg of gallic acid equivalents/g of extract.

Tannins were quantified using the vanillin reaction according to the method proposed by Broadhurst and Jones (1978) and adapted by Agostini-Costa et al. (1999). The results were expressed as mg of catechin equivalents/g of extract. All assays were performed in triplicate.

The extract was fractionated by liquid chromatography employing XAD-2 (Supelco, Bellefonte, PA, USA) resin column chromatography (30 cm x 3 cm). The extract (2.45 g) was eluted with 0.5 L of water, followed by elution with 0.5 L of methanol and with 0.4 L of ethyl acetate. An aliquot of the methanolic fraction (1.16 g) was dissolved in 30 mL of methanol and fractionated by liquid chromatography employing Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) column chromatography (70cmx3 cm) at a rate of 0.3 mL/min. In total, 33 fractions were collected. The fractions were combined based on their chemical profile, as determined by thin layer chromatography. Samples were applied onto silica gel plates using ethyl acetate–n-propanol–water (123:7:70, v/v/v) as the eluent (upper phase). Fractions 20–23 and 26–28 were purified using polyvinylpyrrolidone (Sigma, USA) column chromatography (10x2 cm) with elution by methanol, which led to the identification of the compounds sitosterol-3-O- β -D-glucopyranoside (C₃₅H₆₀O₆) (3 mg) and kaempferol 3-O- α -L-rhamnopyranoside (C₂₁H₂₀O₁₀) (4 mg), respectively. An aliquot of

0.67 g of the ethyl acetate fraction was dissolved in 20 mL of methanol, fractionated by liquid chromatography on a Sephadex LH-20 column (80x2 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), and eluted with methanol at a rate of 0.3 mL 28 fractions were collected. The fractions were combined according to their chemical profile, as determined by thin layer chromatography. Samples were applied onto silica gel plates using ethyl acetate–methanol (75:25, v/v) as the eluent. Fractions 9–11 and 18–19 gave the isolated compounds betulinic acid (C₃₀H₄₈O₃) (3 mg) and betulin (C₃₀H₅₀O₂) (2 mg), respectively.

Chemical characterization was performed using nuclear magnetic resonance (Bruker DPX-300, 300 MHz for ¹H and 75 MHz for ¹³C) and the chemical structures were confirmed by comparison with literature data (Mahato and Kundu, 1994; Kojima, 1990; Agrawal, 1989; Harborne, 1996).

3. Results

In the pleurisy test, the carrageenan was able to significantly increase (by approximately eight times) the leukocyte migration to the pleura, as determined by comparing the control group to the naive group. The treatment with a dose of 300 mg/kg, but not with doses of 30 and 100 mg/kg, of EEDd significantly reduced the leukocyte migration to the pleura in relation to control group with inhibition of approximately 31%. Dexamethasone also inhibited approximately 78% of the leukocyte migration (Fig. 1).

In edema test, the EEDd displayed significant inhibitory activity at a dose of 100 mg/kg at 1 (69%) and 2 h (40%) after the carrageenan injection, while the dose of 300 mg/kg exhibited inhibition at 1 (56%), 2 (75%) and 4 h (58%) after the injection. Dexamethasone also showed significant inhibition at all time points (Fig. 2a).

In the mechanical hyperalgesia test, the EEDd blocked the induction of mechanical hypersensitivity at a dose of 300 mg/kg (after 3 and 4 h) and of 100 mg/kg (after 3 h). Dexamethasone also displayed significant inhibition at all time points (Fig. 2b).

The weight values of the body and organs treated with EEDd did not show significant differences when compared to control group, which suggested the absence of toxicity. In addition, the cyclophosphamide treatment caused a reduction in the absolute and relative spleen weight (Table 1).

The frequency of lesioned cells in the comet assay was 1.00 ± 0.32 for the control group, 78.40 ± 21.60 for the positive control and 0.40 ± 0.24 , 0.80 ± 0.37 and 0.60 ± 0.24 for the groups treated with 10, 100

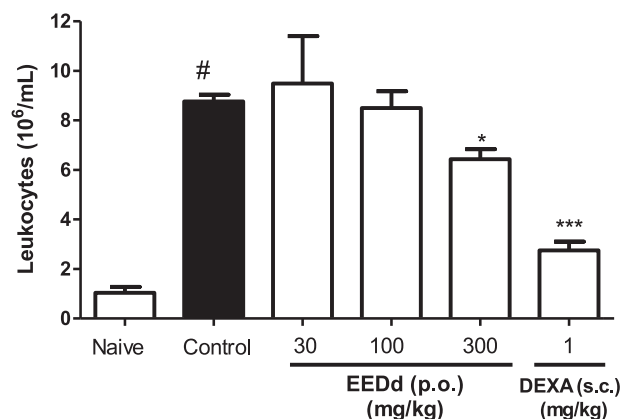


Fig. 1. Effects of oral administration of *D. dentatus* (EEDd) on the inhibition of leukocyte migration in the pleurisy test. The animals received EEDd (30, 100 or 300 mg/kg, p.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.), and 1 h later, an intrathoracic injection of carrageenan was administered. The naive group (# indicates a statistically significant difference from the vehicle group) received an intrapleural injection of sterile saline instead of carrageenan and was also treated with saline solution. Each bar represents the mean \pm SEM of 6 animals. * $p < 0.05$, *** $p < 0.001$, # $p < 0.001$ compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

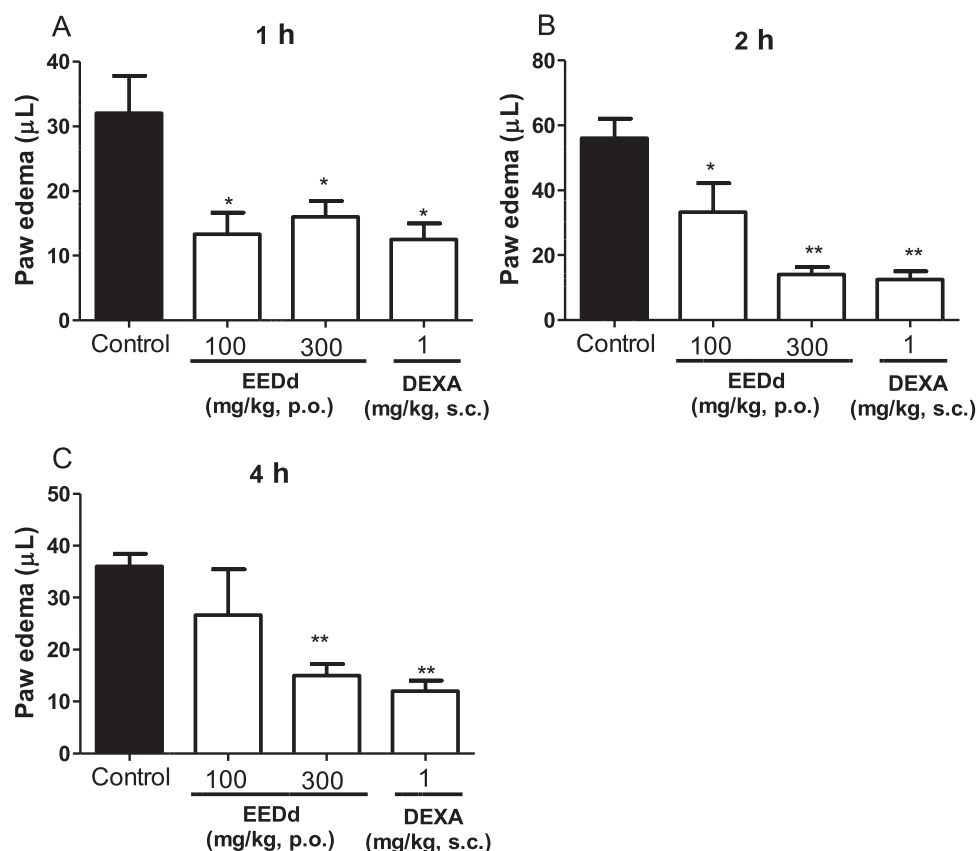


Fig. 2. Effect of oral administration of EEDd on the carrageenan-induced paw edema in mice. The animals received EEDd (100 and 300 mg/kg, p.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.), and 1 h later, an intraplantar injection of carrageenan (300 µg/paw) was administered. Graphs (A), (B), and (C) represent the evaluation of the paw edema at 1, 2, and 4 h, respectively, after carrageenan injection. Each bar represents the mean \pm SEM of 6 animals. * $p < 0.05$, ** $p < 0.01$ compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

Table 1

Mean values of Biometric parameters (body and organs weights) of evaluation of ethanolic extract of *D. dentatus* (EEDd).

Biometric parameters						
Experimental groups		Initial weight (g)		Final weight (g)		
CN		36.40 \pm 0.68 ^a		36.00 \pm 1.41 ^a		
CP		36.40 \pm 1.12 ^a		35.60 \pm 1.21 ^a		
EEDd1		36.20 \pm 0.86 ^a		35.40 \pm 1.12 ^a		
EEDd2		34.80 \pm 0.58 ^a		35.20 \pm 0.49 ^a		
EEDd3		34.60 \pm 1.20 ^a		34.80 \pm 1.62 ^a		
Absolute weight organs (g)						
Heart		Lung	Spleen	Liver	Kidneys	
CN		0.22 \pm 0.01 ^{a,b}	0.25 \pm 0.00 ^a	0.23 \pm 0.01 ^b	1.91 \pm 0.09 ^a	0.50 \pm 0.02 ^a
CP		0.25 \pm 0.01 ^b	0.26 \pm 0.01 ^a	0.08 \pm 0.00 ^a	1.93 \pm 0.08 ^a	0.53 \pm 0.02 ^a
EEDd1		0.20 \pm 0.00 ^{a,b}	0.25 \pm 0.13 ^a	0.23 \pm 0.02 ^b	2.04 \pm 0.10 ^a	0.50 \pm 0.05 ^a
EEDd2		0.20 \pm 0.00 ^{a,b}	0.26 \pm 0.02 ^a	0.25 \pm 0.00 ^b	2.03 \pm 0.10 ^a	0.48 \pm 0.01 ^a
EEDd3		0.19 \pm 0.02 ^a	0.24 \pm 0.01 ^a	0.22 \pm 0.01 ^b	1.85 \pm 0.11 ^a	0.49 \pm 0.03 ^a
Relative weight organs (g)						
Heart		Lung	Spleen	Liver	Kidneys	
CN		0.006 \pm 0.0005 ^{a,b}	0.007 \pm 0.0003 ^a	0.006 \pm 0.0005 ^b	0.053 \pm 0.0060 ^a	0.014 \pm 0.0016 ^a
CP		0.007 \pm 0.0003 ^b	0.007 \pm 0.0002 ^a	0.002 \pm 7.0711 ^a	0.054 \pm 0.0020 ^a	0.015 \pm 0.0020 ^a
EEDd1		0.006 \pm 0.0004 ^{a,b}	0.007 \pm 0.0004 ^a	0.006 \pm 0.0005 ^b	0.058 \pm 0.0061 ^a	0.014 \pm 0.0020 ^a
EEDd2		0.006 \pm 0.0002 ^{a,b}	0.007 \pm 0.0005 ^a	0.007 \pm 0.0001 ^b	0.058 \pm 0.0080 ^a	0.014 \pm 0.0005 ^a
EEDd3		0.005 \pm 0.0003 ^a	0.007 \pm 0.0001 ^a	0.006 \pm 0.0002 ^b	0.053 \pm 0.0037 ^a	0.014 \pm 0.0007 ^a

Legend: Negative Control (CN) – the mice were treated with physiological solution by intraperitoneal (i.p.) route (10 ml/Kg body weight (b.w.) and the vehicle used to dissolve EEDd was used in the same way by oral route (p.o.); Positive control (CP) – the animals were treated with cyclophosphamide with 100 mg/Kg (b.w., i.p.) and the vehicle used to dissolve EEDd (10 ml/Kg b.w., p.o.); groups EEDd (EEDd1, EEDd2 and EEDd3) – the animals were treated by oral route with EEDd in 3 different doses (10, 100 and, 1000 mg/kg, p.o., respectively) and with physiological solution (10 ml/Kg, b.w., i.p.). The letters “a” and “b” indicate statistical significant differences. Statistical tests used: ANOVA/Tukey-Kramer ($P < 0,05$).

Table 2Mean values of frequency of cells with DNA damage, distribution between classes of damage and score in comet assay in mice treated with Ethanolic Extract of *D. dentatus* (EEDd).

Experimental groups	Cells with DNA damage	Classification of DNA damage				Score
		0	1	2	3	
CN	1.00 ± 0.32 ^a	99.00 ± 0.32	1.00 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.32 ^a
CP	78.40 ± 0.93 ^b	21.60 ± 0.93	34.60 ± 2.38	23.20 ± 1.12	20.60 ± 0.81	142.80 ± 2.40 ^b
EEDd1	0.40 ± 0.24 ^a	99.60 ± 0.24	0.40 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.24 ^a
EEDd2	0.80 ± 0.37 ^a	99.20 ± 0.37	0.80 ± 0.37	0.00 ± 0.00	0.00 ± 0.00	0.80 ± 0.37 ^a
EEDd3	0.60 ± 0.24 ^a	99.40 ± 0.24	0.60 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.60 ± 0.24 ^a

Legend: Negative Control (CN) – the mice were treated with physiological solution by intraperitoneal (i.p) route (10 ml/Kg body weight (b.w.) and the vehicle used to dissolve EEDd was used in the same way by oral route (p.o.); Positive control (CP) – the animals were treated with cyclophosphamide with 100 mg/Kg (b.w., i.p.) and the vehicle used to dissolve EEDd (10 ml/Kg b.w., p.o.); groups EEDd (EEDd1, EEDd2 and EEDd3) – the animals were treated by oral route with EEDd in 3 different doses (10, 100 and, 1000 mg/kg, p.o., respectively) and with physiological solution (10 ml/Kg, b.w., i.p.). The letters “a” and “b” indicate statistical significant differences. Statistical tests used: ANOVA/Tukey-Kramer ($P < 0,05$).

and 1000 mg/kg doses of EEDd, respectively (Table 2).

The micronucleus test showed a frequency of 5.00 ± 0.32 , 26.00 ± 2.81 , 5.80 ± 0.73 , 4.60 ± 0.51 and 5.20 ± 0.37 at 24 h; 5.20 ± 0.58 , 28.40 ± 1.83 , 5.60 ± 0.93 , 6.40 ± 0.75 and 7.00 ± 1.05 at 48 h; and 6.40 ± 0.87 , 28.00 ± 1.00 , 7.80 ± 0.73 , 8.20 ± 0.58 and 7.80 ± 0.58 at 72 h for the negative control, positive control and the three doses of EEDd, respectively (Table 3). These results demonstrated that the EEDd had no genotoxic activity at the concentrations tested. (Fig. 3).

The frequency of phagocytosis was not significantly altered between the different groups. The value of the control group was 5.20 ± 0.66 , that of the cyclophosphamide group was 12.00 ± 1.14 and that of EEDd1, EEDd2 and EEDd3 were 7.20 ± 0.71 , 5.00 ± 0.63 and 6.00 ± 1.18 , respectively (Table 4). The experimental groups did not exhibit significantly different differential counts (Table 5).

The value of MIC ($\mu\text{g/mL}$) of EEDd was $62.5 \mu\text{g/mL}$, while the value of MIC of isoniazid and rifampicin were 0.05 and $0.01 \mu\text{g/mL}$, respectively.

The content of phenols was $204.04 \pm 3.2 \text{ mg/g}$, flavonoids was $89.17 \pm 1.7 \text{ mg/g}$ and tannins $12.05 \pm 0.2 \text{ mg/g}$. We also identified the four compounds. Sitosterol-3-O- β -D-glucopyranoside: [¹H NMR (300 MHz, pyridine-*d*₅, J (Hz)): 5.36 (1 H, m, H-6), 5.07 (1 H, d, J = 7.7 Hz, H-1'), 4.58 (1 H, dd, J = 11.7, 2.4 Hz, H-6'), 4.43 (1 H, dd, J = 11.7, 5.2 Hz, H-6'), 4.31 (2 H, m, H-3' and H-4'), 4.08 (1 H, brt, J = 8.1 Hz, H-2'), 3.99 (2 H, m, H-3 and H-5'), 1.00 (3 H, d, J = 6.5 Hz, H-21), 0.95 (3 H, s, H-19), 0.92 (3 H, d, J = 7.3 Hz, H-26), 0.89 (3 H, t, J = 7.4 Hz, H-29), 0.88 (3 H, d, J = 7.0 Hz, H-27), 0.67 (3 H, s, H-18). ¹³C NMR (75 MHz, pyridine-*d*₅) d: 37.52 (C-1), 30.30 (C-2), 78.16 (C-3), 39.39 (C-4), 140.96 (C-5), 121.96 (C-6), 32.22 (C-7), 32.10 (C-8), 50.39 (C-9), 36.97 (C-10), 21.33 (C-11), 39.99 (C-12), 42.53 (C-13), 56.87 (C-14), 24.55 (C-15), 28.58 (C-16), 56.29 (C-17), 12.02 (C-18),

Table 3Mean values of frequency of Micronucleus in blood peripheral cells in mice treated with Ethanolic Extract of *D. dentatus* (EEDd).

Experimental Groups	Mean ± EPM		
	24 h	48 h	72 h
CN	5.00 ± 0.32 ^a	5.20 ± 0.58 ^a	6.40 ± 0.87 ^a
CP	26.00 ± 2.81 ^b	28.40 ± 1.83 ^b	28.00 ± 1.00 ^b
EEDd1	5.80 ± 0.73 ^a	5.60 ± 0.93 ^a	7.80 ± 0.73 ^a
EEDd2	4.60 ± 0.51 ^a	6.40 ± 0.75 ^a	8.20 ± 0.58 ^a
EEDd3	5.20 ± 0.37 ^a	7.00 ± 1.05 ^a	7.80 ± 0.58 ^a

Legend: Negative Control (CN) – the mice were treated with physiological solution by intraperitoneal (i.p) route (10 ml/Kg body weight (b.w.) and the vehicle used to dissolve EEDd was used in the same way by oral route (p.o.); Positive control (CP) – the animals were treated with cyclophosphamide with 100 mg/Kg (b.w., i.p.) and the vehicle used to dissolve EEDd (10 ml/Kg b.w., p.o.); groups EEDd (EEDd1, EEDd2 and EEDd3) – the animals were treated by oral route with EEDd in 3 different doses (10, 100 and, 1000 mg/kg, p.o., respectively) and with physiological solution (10 ml/Kg, b.w., i.p.). The letters “a” and “b” indicate statistical significant differences. Statistical tests used: ANOVA/Tukey-Kramer ($P < 0,05$).

19.26 (C-19), 36.43 (C-20), 19.05 (C-21), 34.26 (C-22), 26.44 (C-23), 46.09 (C-24), 29.51 (C-25), 19.46 (C-26), 20.02 (C-27), 23.44 (C-28), 12.20 (C-29), 102.62 (C-1'), 75.38 (C-2'), 78.65 (C-3'), 71.75 (C-4'), 78.55 (C-5'), 62.88 (C-6'). Kaempferol 3-O- α -L-rhamnopyranoside: [¹H NMR, 300 MHz, pyridine-*d*₅, J (Hz): d 6.19 (s, H-6), 6.39 (s, H-8), 7.76 (d, J = 8.4 Hz, H-2', H-6'), 6.91 (d, J = 8.4 Hz, H-3', 5'), 5.36 (d, J = 1.8 Hz, H-1''), 0.90 (d, J = 5.4 Hz, H-6''), 3.2–4.3 (m, sugar). ¹³C NMR (75 MHz, pyridine-*d*₅): d 157.8 (C-2), 134.0 (C-3), 177.6 (C-4), 161.9 (C-5), 98.5 (C-6), 164.1 (C-7), 93.5 (C-8), 156.8 (C-9), 103.9 (C-10), 121.8 (C-1'), 130.3 (C-2', C-6'), 115.3 (C-3', C-5'), 161.3 (C-4'), Rha": 101.9 (C-1''), 70.4 (C-2''), 70.6 (C-3''), 70.8 (C-4''), 69.8 (C-5''), 16.6 (C-6'').] Betulinic acid [¹H NMR, 300 MHz, CDCl₃, J (Hz): 1.66 (3 H, s, H-29), 4.60 (2 H, d, 24 Hz, H-30). ¹³C NMR (75 MHz, CDCl₃): 38.7 (C-1), 27.4 (C-2), 78.9 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 34.3 (C-7), 40.7 (C-8), 50.5 (C-9), 37.2 (C-10), 20.8 (C-11), 25.5 (C-12), 38.4 (C-13), 42.4 (C-14), 30.5 (C-15), 32.1 (C-16), 56.3 (C-17), 46.8 (C-18), 49.2 (C-19), 150.3 (C-20), 29.7 (C-21), 37.0 (C-22), 27.9 (C-23), 15.3 (C-24), 16.0 (C-25), 16.1 (C-26), 14.7 (C-27), 180.5 (C-28), 109.6 (C-29), 19.4 (C-30). Betulin [¹H NMR, 300 MHz, CDCl₃, J (Hz): 1.69 (3 H, s, H-29), 4.70 (2 H, d, H-30), 9.68 (1H, s, H-28). ¹³C NMR (75 MHz, CDCl₃): 38.8 (C-1), 27.4 (C-2), 79.0 (C-3), 38.3 (C-4), 55.4 (C-5), 18.3 (C-6), 34.3 (C-7), 41.0 (C-8), 50.6 (C-9), 37.4 (C-10), 20.9 (C-11), 25.6 (C-12), 37.0 (C-13), 42.8 (C-14), 27.1 (C-15), 29.3 (C-16), 47.8 (C-17, C-18), 48.8 (C-19), 150.3 (C-20), 29.8 (C-21), 34.0 (C-22), 28.0 (C-23), 15.3 (C-24), 16.1 (C-25), 16.1 (C-26), 14.7 (C-27), 60.8 (C-28), 109.6 (C-29), 19.4 (C-30).

4. Discussion

Our studies showed the antimycobacterial and anti-inflammatory activities of EEDd. The treatment of Multidrug-resistant tuberculosis is very expensive, takes a long time to complete, disrupts lives, and has potentially life-threatening side effects, there is an urgent need to develop new natural or synthetic antitubercular drugs (Caminero et al., 2010; Copp and Pearce, 2007; Velayati et al., 2016). The folk use of *D. dentatus* has indicated its anti-malarial and anti-inflammatory properties, although only its antileishmanial activity had previously been shown in its isolated compounds (Aponte et al., 2008). In previous studies, betulin and betulinic acid displayed a MIC ranging from 12.5 to 32.0 $\mu\text{g/ml}$ and 15–100 $\mu\text{g/ml}$, respectively (Fomogne-Fodjo et al., 2017; Gu et al., 2004; Hasan et al., 2012; Li et al., 2015; Morrison et al., 2016; Suksamrarn et al., 2006; Wächter et al., 1999). Betulinic acid exhibited antiplasmodial and antileishmanial activity. *M. tuberculosis*, *P. falciparum*, *Leishmania infantum* and *L. danovani* are intracellular organisms, which may indicate that this component has important action in the cytoplasm of host cells (Lenta et al., 2009; Sousa et al., 2014; Wert et al., 2011). In the present study, the antimycobacterial activity with EEDd were carried out because the literature indicated that this plant has isolated compounds with this efficacy, and no signs of genetic toxicity were detected in the genotoxic

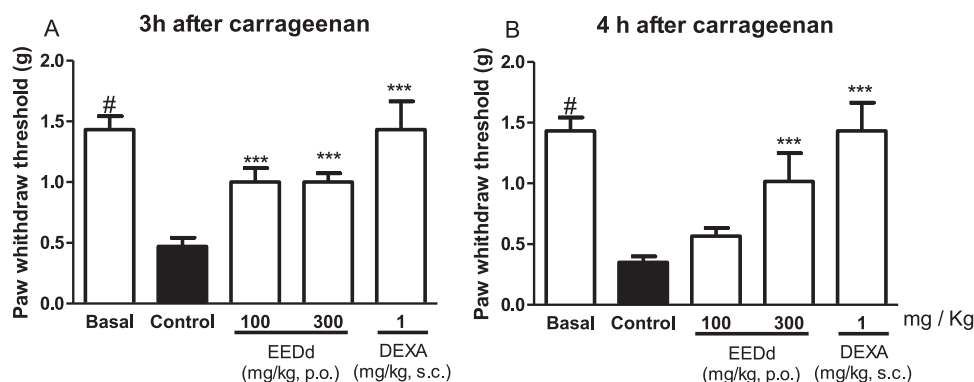


Fig. 3. Effect of oral administration of EEDd on the mechanical hyperalgesia in mice. The animals received EEDd (100 and 300 mg/kg, p.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.). The mechanical hyperalgesia was measured with a digital analgesymeter 3 and 4 h after carrageenan administration. Each bar represents the mean \pm SEM of 6 animals. * $p < 0.05$, *** $p < 0.001$, # $p < 0.001$ when compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

Table 4

Mean values of frequency of splenic phagocytosis of Ethanolic Extract of *D. dentatus* (EEDd).

Cells with evidence of splenic phagocytosis		
Experimental groups	Mean \pm EPM	Percentage (%)
CN	5.20 \pm 0.66 ^a	2.60
CP	12.00 \pm 1.14 ^b	6.00
EEDd1	7.20 \pm 0.71 ^a	3.60
EEDd2	5.00 \pm 0.63 ^a	2.50
EEDd3	6.00 \pm 1.18 ^a	3.00

Legend: Negative Control (CN) – the mice were treated with physiological solution by intraperitoneal (i.p) route (10 ml/Kg body weight (b.w.) and the vehicle used to dissolve EEDd was used in the same way by oral route (p.o.); Positive control (CP) – the animals were treated with cyclophosphamide with 100 mg/Kg (b.w., i.p.) and the vehicle used to dissolve EEDd (10 ml/Kg b.w., p.o.); groups EEDd (EEDd1, EEDd2 and EEDd3) – the animals were treated by oral route with EEDd in 3 different doses (10, 100 and, 1000 mg/kg, p.o., respectively) and with physiological solution (10 ml/Kg, b.w., i.p.). The letters “a” and “b” indicate statistical significant differences. Statistical tests used: ANOVA/Tukey-Kramer ($P < 0.05$).

models, even with the high dose of 1000 mg/kg.

Using inflammatory markers, such as leukocyte migration, edema and mechanical hyperalgesia induced by carrageenan, the present work showed the anti-inflammatory effect of the 100 and 300 mg/kg doses of EEDd. *D. dentatus* has many widespread uses (Sano et al., 2008). For swelling and inflammation, fresh leaves and root are used, while for urine retention, young branches are applied (Rodrigues, 2007). The anti-inflammatory activity of EEDd was verified by an important parameter of inflammation: leukocyte migration. Leukocyte migration is an inflammatory phenomenon observed in several inflammatory models, such as the pleurisy test. Several factors are involved in leukocyte migration, such as chemotactic mediators and adhesion molecules, and the EEDd could modulate one or more inflammatory

Table 5

Mean values of leukocyte parameters in mice treated with Ethanolic Extract of *D. dentatus* (EEDd).

Leukocyte Type	Differential leukocyte analysis					
	Reference value	CN	CP	EEDd1	EEDd2	EEDd3
Neutrophil	10–40%	59.80 \pm 3.55 ^a	55.80 \pm 2.83 ^a	57.00 \pm 3.11 ^a	57.20 \pm 1.43 ^a	59.60 \pm 2.23 ^a
Lymphocyte	55–95%	37.40 \pm 3.47 ^a	41.00 \pm 3.27 ^a	41.20 \pm 3.01 ^a	40.40 \pm 1.17 ^a	38.20 \pm 1.74 ^a
Monocyte	0.1–3.5%	3.00 \pm 0.01 ^a	3.20 \pm 0.58 ^a	2.20 \pm 0.20 ^a	2.40 \pm 0.40 ^a	2.20 \pm 0.49 ^a
Eosinophil	0–0.4%	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a
Basophil	0–0.3%	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a

Legend: Negative Control (CN) – the mice were treated with physiological solution by intraperitoneal (i.p) route (10 ml/Kg body weight (b.w.) and the vehicle used to dissolve EEDd was used in the same way by oral route (p.o.); Positive control (CP) – the animals were treated with cyclophosphamide with 100 mg/Kg (b.w., i.p.) and the vehicle used to dissolve EEDd (10 ml/Kg b.w., p.o.); groups EEDd (EEDd1, EEDd2 and EEDd3) – the animals were treated by oral route with EEDd in 3 different doses (10, 100 and, 1000 mg/kg, p.o., respectively) and with physiological solution (10 ml/Kg, b.w., i.p.). The letters “a” and “b” indicate statistical significant differences. Statistical tests used: ANOVA/Tukey-Kramer ($P < 0.05$).

factors. Is difficult to indicate the exact mechanism of action and the only evidence that we could affirm is that EEDd is an anti-inflammatory agent. Since several works have indicated betulinic acid as an important active anti-inflammatory compound found in medicinal plants (Costa et al., 2014; Jingbo et al., 2015; Lingaraju et al., 2015a, 2015b, 2015c), and because two compounds that have originated from betulinic acid are found in *D. dentatus*, we suggest that this molecule could be involved in the anti-inflammatory activity of EEDd. Betulinic acid inhibits NF- κ B activation by stabilizing the NF- κ B inhibitory protein I κ B α (Wang et al., 2016) and significantly inhibits IL-1 β , NO, PGE2, and MMP production in chondrocytes (Jingbo et al., 2015). The treatment only with higher doses of 100 or 300 mg/kg of EEDd induced inhibitory effects in edema and mechanical hyperalgesia. Oyebanji et al. (2013) showed that betulinic acid presented anti-inflammatory, analgesic and antipyretic activity with higher doses for pure compound with dose of 10, 20 or 40 mg/kg by intraperitoneal route. But it is difficult to do a direct relation of this anti-inflammatory compound and EEDd. Therefore, until the betulinic acid or other compound is tested under the same conditions of EEDd, it is not possible to confirm what compound is really responsible for EEDd activity, and further work is needed to clarify this point. Because of their popular use, this work focused on the use of leaves to produce the extract, and the resulting EEDd showed anti-inflammatory activity in carrageenan-induced leukocyte migration, edema formation and mechanical hyperalgesia. Hence, this work showed for the first time that *D. dentatus* really is an important source of this anti-inflammatory agent. This work did not contribute directly to validate the use of the extract (or other product) formulated as *D. dentatus* popular uses as natural anti-inflammatory in humans (only in rodents) but contributed indirectly.

Regulatory agencies require genotoxicity tests, such as the micronucleus test (ANVISA, 2013), to characterize the toxicological potential of phytotherapeutic products. The evaluation of the safety of use

beyond the effectiveness is crucial for proving the safe use of products derived from medicinal plants. The tests with herbal products in models of genetic toxicity can be performed *in vivo* and are used to detect the induction of genetic damage to deoxyribonucleic acid (DNA) or genotoxic damage. A genetic change is indicative of early changes that can lead to cancer and other diseases, and the detection of such changes contribute to ensure the safe use of the product (Cardoso et al., 2016; Loeb et al., 2003).

In genetic toxicological assays, toxicogenic damages in mice treated with EEDd could be detected. The genetic tests revealed that there were no statistically significant differences in relation to the cell damage in blood cells. The micronucleus test has been used to measure genotoxicity both *in vivo* and *in vitro*, although *in vivo* tests are especially relevant because they provide more information about the experimental metabolism, pharmacokinetics and DNA processes (OECD, 2014). The comet assay can also be performed *in vivo* and *in vitro*. However, *in vivo* tests are used because they evaluate the metabolism, similar to the micronucleus assay (Morita et al., 2016). The biometric parameter also indicated that EEDd was not toxic to mice because it did not alter the temporal body weight or the organ weight compared to the control group (David et al., 2014; Gonçalves et al., 2013; Pessatto et al., 2017). Similar to Kanno et al. (2009), our results with cyclophosphamide in Swiss mice, with doses of 100–250 mg/kg, showed reduced body and testicle weights and produced hepatotoxic and nephrotoxic effects. The EEDd is not a pure compound but, even with a dose of 1000 mg/kg, it did not alter the observed parameters. The EEDd did not induce increases in phagocytic activity or even change the leukocyte peripheral count. Hence, no significant differences were detected between control and EEDd-treated groups, suggesting that EEDd does not have genotoxic potential, does not alter splenic phagocytosis and/or leukometry at doses of 10, 100 and 1000 mg/kg. These results suggest that its use in therapies not exceeding the tested doses would be safe.

In relation to anti-TB activity, no compound isolated from *D. dentatus* had presented this property (Aponte et al., 2008), although our study showed that EEDd displayed efficacy against *M. tuberculosis* with MIC of 62.5 µg/ml. The EEDd showed moderate efficacy against *M. tuberculosis*, and perhaps the compounds responsible for this activity could possess even more efficacy than EEDd. Therefore, we are planning their isolation to test the pure compounds because the work of Aponte et al. (2008) showed that betulinic acid did not have anti-TB activity.

Sauvain et al. (1996) and Jagessar et al. (2013) have prepared extracts from *D. dentatus*, and the most commonly occurring compound they found was betulinic acid. Research with betulinic acid in *D. schottianus* confirmed the presence of betulinic acid in the leaves and wood; this metabolite was also found in the leaves, bark, and wood of the plant (de Oliveira et al., 2002). In the present study, we also found betulinic acid and betulin, as well as sitosterol-3-O-β-D-glucopyranoside, kaempferol 3-O-α-L-rhamnopyranoside. Betulin is a pentacyclic triterpene that can be converted into betulinic acid (Alakurtti et al., 2010). This compound, in turn, has shown anti-malarial activity, anti-inflammatory, antioxidant, immunomodulatory, anthelmintic, anti-HIV, and anticancer properties (Alakurtti et al., 2010; Dantas, 2012). Other important works contributed to show that pure betulinic acid or isolated betulinic acid from other plants really presented anti-inflammatory, cancer, and immune activity (Csuk et al., 2011; Fulda et al., 2008; Huguet et al., 2000; Moghaddam et al., 2012; Oyebanji et al., 2013). EEDd displayed anti-inflammatory properties, and it is possible that betulinic acid and betulin were the compounds found in *D. dentatus* that were responsible for the inhibition of the inflammatory parameters.

5. Conclusion

The present scientific studies described for the first time the anti-inflammatory and antimycobacterial activity of *D. dentatus* leaves. The

genetic toxicity tests showed that the EEDd did not induce any signs of toxicity until treatment with a dose 10 times higher than the effective dose.

Authors' contributions

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; RMK, ML and LFM conducted the anti-inflammatory assays; RI conducted the genetic toxicological analysis; FM did the antimycobacterial assay; AP performed the botanical identification; CALC, RGC and GG were involved in the preparation and isolation of extract; CALK, JC and RJO performed data analyses and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors would like to thank FUNDECT - Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul, FNDCT - Fundo Nacional de Desenvolvimento Científico e Tecnológico, CNPq- Conselho Nacional de Desenvolvimento Científico e Tecnológico, CAPES- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, MCT - Ministério da Ciência e Tecnologia and UFGD - Universidade Federal da Grande Dourados. We are grateful to CNPq for providing financial support (407791/2013-2)

References

- Agostini-Costa, T.S., Garruti, D., Lima, L., Freire, S., Abreu, F.A.P., Feitosa, T., 1999. Avaliação de metodologias para determinação de taninos no suco de caju. Bol. CEPPA 17, 167–176.
- Agrawal, P.K., 1989. Carbon-13 NMR of Flavonoids. Elsevier, Amsterdam.
- Alakurtti, S., Heiska, T., Kiriazis, A., Sacerdoti-Sierra, N., Jaffe, C.L., Yli-Kauhaluoma, J., 2010. Synthesis and anti-leishmanial activity of heterocyclic betulin derivatives. Bioorg. Med. Chem. 18, 1573–1582.
- ANVISA, Agência Nacional de Vigilância Sanitária, 2013. Guia para a condução de estudos não clínicos de toxicologia e segurança farmacológica necessários ao desenvolvimento de medicamentos. Gerênc. De. Aval. De. Segur. e Eficácia – Gesef. Bras.
- Aponte, J.C., Vaisberg, A.J., Rojas, R., Caviedes, L., Lewis, W.H., Lamas, G., Sarasara, C., Gilman, R.H., Hammond, G.B., 2008. Isolation of cytotoxic metabolites from targeted peruvian amazonian medicinal plants. J. Nat. Prod. 71, 102–105.
- Broadhurst, R.B., Jones, W.T., 1978. Analysis of condensed tannins using acidified vanillin. J. Sci. Food Agric. 29, 788–794.
- Caminero, J.A., Sotgiu, G., Zumla, A., Migliori, G.B., 2010. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. Lancet Infect. Dis. 10 (9), 621–629.
- Cardoso, V.S., Vermelho, A.B., de Lima, C.A.R., Oliveira, J.M., de Lima, M.E.F., da Silva, L.H.P., Direito, G.M., Danelli, M.G.M., 2016. Antigenotoxic effect of piperine in broiler chickens intoxicated with aflatoxin B1. Toxins (Basel) 8 (11), 316.
- Carvalho, P.C., Santos, E.A., Schneider, B.U.C., Matuo, R., Pesarini, J.R., Cunha-Laura, A.L., Monreal, A.C.D., Lima, D.P., Antonioli, A.C.M.B., Oliveira, R.J., 2015. Diaryl sulfide analogs of combretastatin A-4: toxicogenetic, immunomodulatory and apoptotic evaluations and prospects for use as a new chemotherapeutic drug. Environ. Toxicol. Pharmacol. 40 (3), 715–721.
- Copp, B.R., Pearce, A.N., 2007. Natural product growth inhibitors of *Mycobacterium tuberculosis*. Nat. Prod. Rep. 24, 278–297.
- Costa, J.F., Barbosa-Filho, J.M., Maia, G.L., Guimarães, E.T., Meira, C.S., Ribeiro-dos-Santos, R., de Carvalho, L.C., Soares, M.B., 2014. Potent anti-inflammatory activity of betulinic acid treatment in a model of lethal endotoxemia. Int Immunopharmacol. 23, 469–474.
- Csuk, R., Barthel, A., Sczepak, R., Siewert, B., Schwarz, S., 2011. Synthesis, encapsulation and antitumor activity of new betulin derivatives. Arch. Pharm. (Weinh.). 344 (1), 37–49.
- Dantas, M.B., 2012. Efeito do ácido betulínico no tratamento de dislipidemia e diabetes em camundongos. Universidade Federal do Ceará, Ceará, Brasil.
- David, N., Mauro, M.O., Gonçalves, C.A., Pesarini, J.R., Strapasson, R.L.B., Kassuya, C.A.L., Stefanello, M.E.A., Cunha-Laura, A.L., Monreal, A.C.D., Oliveira, R.J., 2014. *Gochmatia polymorpha* ssp. *Floccosa*: bioprospecting of a anti-inflammatory phytotherapy for use during pregnancy. J. Ethnopharmacol. 154, 370–379.
- Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P., Vidal, N., 2006. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chem. 97, 654–660.
- Fomogie-Fodjo, M.C., Ndinteh, D.T., Olivier, D.K., Kempgens, P., Van Vuuren, S., Krause, R.W., 2017. Secondary metabolites from *Tetracera* potatoria stem bark with anti-mycobacterial activity. J. Ethnopharmacol. 4 (195), 238–245.

- Fulda, S., 2008. Betulinic acid for cancer treatment and prevention. *Int. J. Mol. Sci.* 9 (6), 1096–1107.
- Gonçalves, C.A., Siqueira, J.M., Carollo, C.A., Mauro, M.O., David, N., Cunha-Laura, A.L., Monreal, A.C.D., Castro, A.H., Fernandes, L., Chagas, R.R., Auharek, S.A., Oliveira, R.J., 2013. Gestational exposure to *Byrsonima verbascifolia*: teratogenicity, mutagenicity and immunomodulation evaluation in female Swiss mice. *J. Ethnopharmacol.* 150, 843–850.
- Gu, J.Q., Wang, Y., Franzblau, S.G., Montenegro, G., Yang, D., Timmermann, B.N., 2004. Antitubercular constituents of *Valeriana laxiflora*. *Planta Med.* 70 (6), 509–514.
- Harborne, J.B., 1996. *The Flavonoids Advances in Research since 1986*. Chapman and Hall, London, 675.
- Hasan, N., Osman, H., Mohamad, S., Chong, W.K., Awang, K., Zahariluddin, A.S., 2012. The chemical components of *Sesbania grandiflora* root and their Antituberculosis activity. *Pharm. (Basel)* 23 (8), 882–889.
- Hayashi, M., Morita, T., Kodama, Y., Sofuni, T., Ishidate-Junior, M., 1990. The micronucleus assay with mouse peripheral blood reticulocytes using acridina orange-coated slides. *Mut. Res.* 245, 245–249.
- Huguet, A., del Carmen Recio, M., Máñez, S., Giner, R., Ríos, J., 2000. Effect of triterpenoids on the inflammation induced by protein kinase C activators, neuronally acting irritants and other agents. *Eur. J. Pharmacol.* 410 (1), 69–81.
- Ishii, P.L., Prado, C.K., Mauro, M.O., Carreira, C.M., Mantovani, M.S., Ribeiro, L.R., Dichi, J.B., Oliveira, R.J., 2011. Evaluation of *Agaricus blazei* in vivo for antigenotoxic, anticarcinogenic, phagocytic and immunomodulatory activities. *Regul. Toxicol. Pharmacol.* 59, 412–422.
- Jagessar, R.C., Hoolas, G., Maxwell, A.R., 2013. Phytochemical screening, isolation of betulinic acid, trigonelline and evaluation of heavy metals ion content of *Doliocarpus dentatus*. *J. Nat. Prod.* 6, 5–16.
- Jingbo, W., Aimin, C., Qi, W., Xin, L., Huaining, L., 2015. Betulinic acid inhibits IL-1 β -induced inflammation by activating PPAR- γ in human osteoarthritis chondrocytes. *Int. Immunopharmacol.* 29 (2), 687–692.
- Kanno, T.Y.K., Sensiate, L.A., de Paula, N.A., Salle, M.J.S., 2009. Toxic effects of different doses of cyclophosphamide on the reproductive parameters of male mice. *Braz. J. Pharm. Sci.* 45, 313–319.
- Kobayashi, H., Sugiyama, C., Morikawa, Y., Hayashi, m., Sofuni, T., 1995. A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis assay. *MMS Commun.* 3, 103–115.
- Kojima, H., Sato, N., Hatano, A., Ogura, H., 1990. Sterol glucosides from *Prunella vulgaris*. *Phytochem* 29, 2351–2355.
- Lenta, B.N., Tantangmo, F., Devkota, K.P., Wansi, J.D., Chouna, J.R., Soh, R.C., Neumann, B., Stammer, H.G., Tsamo, E., Sewald, N., 2009. Bioactive constituents of the stem bark of *Beilschmiedia zenkeri*. *J. Nat. Prod.* 72 (12), 2130–2134.
- Li, H., Webster, D., Johnson, J.A., Gray, C.A., 2015. Anti-mycobacterial triterpenes from the Canadian medicinal plant *Alnus incana*. *J. Ethnopharmacol.* 13 (165), 148–151.
- Lin, J.Y., Tang, C.Y., 2007. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. *Food Chem.* 101, 140–147.
- Lingaraju, M.C., Pathak, N.N., Begum, J., Balaganur, V., Bhat, R.A., Ram, M., Kumar, D., Kumar, D., Tandan, S.K., 2015a. Betulinic acid negates oxidative lung injury in surgical sepsis model. *J. Surg. Res.* 193, 856–867.
- Lingaraju, M.C., Pathak, N.N., Begum, J., Balaganur, V., Bhat, R.A., Ramachandra, H.D., Ayanur, A., Ram, M., Singh, V., Kumar, D., Kumar, D., Tandan, S.K., 2015b. Betulinic acid attenuates lung injury by modulation of inflammatory cytokine response in experimentally-induced polymicrobial sepsis in mice. *Cytokine* 71, 101–108.
- Lingaraju, M.C., Pathak, N.N., Begum, J., Balaganur, V., Ramachandra, H.D., Bhat, R.A., Ram, M., Singh, V., Kandasamy, K., Kumar, D., Kumar, D., Tandan, S.K., 2015c. Betulinic acid attenuates renal oxidative stress and inflammation in experimental model of murine polymicrobial sepsis. *Eur. J. Pharm. Sci.* 70, 12–21.
- Loeb, L.A., Loeb, K.R., Anderson, J.P., 2003. Multiple mutations and cancer. *Proc. Natl. Acad. Sci. Usa.* 100 (3), 776–781.
- Mahato, S.B., Kundu, A.P., 1994. Triterpene. *Phytochem.* 37, 1517–1575.
- Moghaddam, M.G., Ahmad, F.B.H., Samzadeh-Kermani, A., 2012. Biological activity of betulinic acid: a review. *Pharmacol. Pharm.* 3, 119–123.
- Morita, T., Hamada, S., Masumura, K., Wakata, A., Maniwa, J., Takasawa, H., Yasunaga, K., Hashizume, T., Homma, M., 2016. Evaluation of the sensitivity and specificity of *in vivo* erythrocyte micronucleus and transgenic rodent gene mutation tests to detect rodent carcinogens. *Mutat. Res.* 802, 1–29.
- Morrison, S.A., Li, H., Webster, D., Johnson, J.A., Gray, C.A., 2016. Antimycobacterial triterpenes from the Canadian medicinal plant *Sarracenia purpurea*. *J. Ethnopharmacol.* 21 (188), 200–203.
- OECD, *Guidelines for the Testing of Chemicals*, 2014. Test No. 474: Mammalian Erythrocyte Micronucleus Test. OECD Publishing, Paris. <http://dx.doi.org/10.1787/9789264224292-en>, (Accessed 16.02.2017).
- de Oliveira, B.H., Santos, C.A.M., Espindola, A.P.D.M., 2002. Determination of the triterpenoid, Betulinic acid. *Doliocarpus schottianus* Hplc. *Phytochem. Anal.* 13, 95–98.
- Oliveira, R.J., Kanno, T.Y.N., Salles, M.J.S., Lourenã, O.A.C.S., Ribeiro, L.R., Freira, G.A., Matiaz, H.J.O., Mantovani, M.S., Silva, A.F., 2009. Effects of the polysaccharide beta-glucan on clastogenicity and teratogenicity caused by acute exposure to cyclophosphamide in mice. *Regul. Toxicol. Pharmacol.* 53 (3), 164–173.
- Oyebanji, B.O., Saba, A.B., Oridupa, O.A., 2013. Studies on the anti-inflammatory, analgesic and antipyretic activities of betulinic acid derived from *Tetracera potatoria*. *Afr. J. Tradit. Complement Altern Med.* 11 (1), 30–33.
- Palomino, J.C., Martin, A., Camacho, M., Guerra, H., Swings, J., Portaels, F., 2002. Resazurin microtiter assay for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 46, 2720–2722.
- Pessatto, L.R., Auharek, S.A., Gonçalves, C.A., David, N., Monreal, A.C.D., Kassuya, C.A.L., Antonioli-Silva, A.C.M.B., Stefanello, M.E.A., Oliveira, R.J., 2017. Effects of dichloromethane and butanol fractions of *Gochnatia polymorpha floccosa* in maternal reproductive outcome, embryo-fetal development and DNA integrity in mice. *J. Ethnopharmacol.*, 205–209.
- Rodrigues, V.E.G., 2007. *Etnobotânica e florística de plantas medicinais nativas de remanescentes de floresta estacional semidecidual na região do Alto Rio Grande*, MG. Universidade Federal de Lavras, Minas Gerais, Brasil.
- Rodrigues, V.E.G., Carvalho, A.D., 2001. Levantamento etnobotânico de plantas medicinais no domínio do cerrado na região do alto Rio Grande - Minas Gerais. *Ciênc. Agrotec.* 25, 102–123.
- Sano, S.M., de Almeida, S.P., Ribeiro, J.F., 2008. Cerrado: Ecologia e flora. *Embrap. Cerrad.* 2, 688.
- Sauvain, M., Kunesch, N., Poisson, J., Gantier, J.C., Gayral, P., Dedet, J.P., 1996. Isolation of leishmanicidal triterpenes and lignans from the Amazonian Liana *Doliocarpus dentatus* (Dilleniaceae). *Phytother. Res.* 10, 1–4.
- Sousa, M.C., Varandas, R., Santos, R.C., Santos-Rosa, M., Alves, V., Salvador, J.A., 2014. Antileishmanial activity of semisynthetic lupane triterpenoids betulin and betulinic acid derivatives: synergistic effects with miltefosine. *PLoS One* 9 (3), e89939.
- Suksamran, S., Panseeta, P., Kunchanawatta, S., Distaporn, T., Ruktasing, S., Suksamran, A., 2006. Ceanothane- and lupane-type triterpenes with antiplasmodial and antimycobacterial activities from *Ziziphus cambodiana*. *Chem. Pharm. Bull.* 54 (4), 535–537.
- Velayati, A.A., Farnia, P., Farahbod, A.M., 2016. Overview of drug-resistant tuberculosis worldwide. *Int. J. Mycobacteriol.* 5 (Suppl 1), S161.
- Velo, G.P., Dunn, C.J., Giroud, J.P., Timsit, J., Willoughby, D.A., 1973. Distribution of prostaglandins in inflammatory exudate. *J. Pathol.* 111 (3), 149–158.
- Vinegar, R., Truax, J.F., Selph, J.L., 1973. Some quantitative temporal characteristics of carrageenin-induced pleurisy in the rat. *Proc. Soc. Exp. Biol. Med.* 143 (3), 711–714.
- Wächter, G.A., Valcic, S., Flagg, M.L., Franzblau, S.G., Montenegro, G., Suarez, E., Timmermann, B.N., 1999. Antitubercular activity of pentacyclic triterpenoids from plants of Argentina and Chile. *Phytomedicine* 6 (5), 341–345.
- Wang, S., Yang, Z., Xiong, F., Chen, C., Chao, X., Huang, J., Huang, H., 2016. Betulinic acid ameliorates experimental diabetic-induced renal inflammation and fibrosis via inhibiting the activation of NF- κ B signaling pathway. *Mol. Cell Endocrinol.* 434, 135–143.
- Wert, L., Alakurti, S., Corral, M.J., Sánchez-Fortún, S., Yli-Kauhaluoma, J., Alunda, J.M., 2011. Toxicity of botulin derivatives and *in vitro* effect on promastigotes and amastigotes of *Leishmania infantum* and *L. donovani*. *J. Antibiot. (Tokyo)*. 64 (7), 475–481.

5. MANUSCRITO II

Artigo submetido à Journal of Ethnopharmacology nov/2017:

Uso seguro de *Doliocarpus dentatus* no período gestacional: ausência de alterações no desempenho reprodutivo materno, desenvolvimento embriofetal e integridade do DNA

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Resumo

Relevância etnofarmacológica: *Doliocarpus dentatus* (Dilleniaceae) é comumente utilizada no Brasil para o tratamento da dor do processo inflamatório e retenção de urina. Estudos anteriores de nosso grupo comprovaram a ação anti-inflamatória e antimicobacteriana do extrato etanólico de *Doliocarpus dentatus* (EEDd) bem como a segurança de uso.

Objetivo do estudo: Investigou-se os efeitos do EEDd sobre o desempenho reprodutivo, desenvolvimento fetal e integridade do DNA em camundongos *Swiss* fêmeas prenhes.

Material e métodos: Foram utilizadas 30 fêmeas prenhes de camundongos *Swiss* divididas em três grupos experimentais (n = 10): grupo Controle tratadas com Tween 80 a 1% e solução fisiológica e grupos EEDd1 e EEDd2 tratadas com EEDd nas doses de 100 e 1000 mg/Kg, respectivamente. O tratamento ocorreu por gavagem durante todo o período gestacional. Ao final do período gestacional foram avaliados os parâmetros relativos ao desempenho reprodutivo, desenvolvimento embriofetal e integridade do DNA.

Resultados: As duas doses do extrato testadas não alteraram os parâmetros reprodutivos, não apresentaram diferenças significativas no desenvolvimento embriofetal quando comparadas ao grupo controle e também não induziram a formação de micronúcleos.

Conclusão: O EEDd não altera desempenho reprodutivo e desenvolvimento embriofetal e não induz genotoxicidade. Esses fatos sugerem segurança de uso mesmo no período gestacional.

1. Introdução

Doliocarpus dentatus, conhecida popularmente como cipó de fogo, cipó mata sede e cipó vermelho, é uma planta da família Dilleniaceae com crescimento moderado, resistente à seca e às baixas temperaturas. Essa planta é encontrada nas florestas tropicais do México, do Peru e da Bolívia e em ecossistemas brasileiros como a Mata Atlântica, a Amazônia e o Cerrado (Aponte et al., 2008; Bianki Filho et al., 2015). Sua seiva aquosa é utilizada popularmente para aliviar a sede, suas cascas são empregadas em infusões para tratamento de leishmaniose e suas folhas e raízes são utilizadas para

tratamento de cistites, dor induzida por processos inflamatórios e retenção urinária (Rodrigues, 2007; Jagessar; Persid, 2014).

Estudo anterior de nosso grupo determinou a presença de fenóis, flavonóides e taninos, bem como do sitosterol-3-O- β -D-glucopiranosídeo, kaempferol 3-O- α -L-rhamnopiranosídeo, ácido betulínico e betulina no extrato etanólico de *D. dentatus* (EEDd) (Ishikawa et al., 2017). Outro estudo fitoquímico também demonstrou que esta planta apresenta ácido butírico, esteróides, lactonas, antracenosídeos, ácido betulínico, taninos, flavonas e trigonelina (Jagessar et al., 2013).

Estudos sobre a atividade biológica de *D. dentatus* comprovaram ação anti-*Leishmania in vitro* contra formas amastigotas de *Leishmania (L.) amazonensis* (Sauvain et al., 1996), citotóxica em células leucêmicas da linhagem K562 (Aponte et al., 2008), antiedematogênica (Bianki Filho et al., 2015), antimicrobiana contra cepas de *Escherichia coli*, *Klebsiella pneumoniae* e *Staphylococcus aureus* (Jagessar; Persid, 2014) e atividades antimicobacteriana e anti-inflamatória (Ishikawa et al., 2017). Além disso, nosso grupo de pesquisa demonstrou recentemente que o EEDd não causa danos genômicos e cromossômicos o que sugere segurança de uso (Ishikawa et al., 2017). Uma vez que não são encontrados dados adicionais sobre os efeitos do EEDd no período gestacional, o presente estudo fez-se pioneiro e teve como objetivo avaliar os efeitos do EEDd no desempenho reprodutivo, no desenvolvimento embrionário e na integridade do DNA de camundongos fêmeas *Swiss* prenhes.

2. Metodologia

2.1. Extrato etanólico de *Dolioscarpus dentatus*

O EEDd utilizado no estudo já foi previamente descrito por Ishikawa et al. (2017). Em síntese as folhas jovens e maduras de *Dolioscarpus dentatus* foram coletadas na Universidade Federal de Mato Grosso do Sul (UFMS), na cidade de Campo Grande - MS. A identificação botânica foi realizada pelo professor Dr. Arnildo Pott e uma amostra foi depositada no herbário da UFMS sob o número #49860.

As folhas coletadas foram secas a 40°C, trituradas e extraídas com etanol (95%) pelo processo de maceração por um período de 7 dias consecutivos. Os extratos obtidos foram reunidos, secos em evaporador e, posteriormente, liofilizados para a obtenção do extrato bruto seco. Para administração nos animais o extrato foi diluído em Tween 80 a 1% e solução fisiológica e as doses utilizadas foram de 100 e 1000 mg/Kg peso corpóreo (p.c.) administradas por gavagem (v.o.).

A dose de 100 mg/Kg (p.c., v.o.) foi escolhida devido a sua eficácia anti-inflamatória comprovada por Ishikawa et al. (2017). Optou-se também por uma dose 10x maior, como preconizam os guidelines na área de toxicologia reprodutiva (OECD, 2009) e toxicologia genética (OECD, 1997; OECD, 2012), bem como a Agência Nacional de Vigilância Sanitária (ANVISA, 2010), que afirmam que os ensaios pré-clínicos devem ser conduzidos com as doses que se pretende usar em humanos e outra 10x maior, somente se essa segunda estiver livre de efeitos adversos, a dose menor poderá ser considerada segura.

2.2. Delineamento experimental

Foram utilizados 15 camundongos machos e 30 camundongos fêmeas, *Mus musculus* da variedade *Swiss*, com peso médio de 35g, em idade reprodutiva, provenientes da Agência Estadual de Defesa Sanitária Animal e Vegetal (IAGRO). O presente estudo foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal de Mato Grosso do Sul sob parecer #776/2016.

Os animais foram mantidos em caixas de propileno recobertas por sepilho, sob condições padronizadas de climatização (com temperatura mantendo-se em torno de $22 \pm 2^\circ\text{C}$ e umidade relativa de $55 \pm 10\%$) e alimentados com ração comercial (Nuvital®) e água filtrada *ad libitum*.

O cruzamento *overnight* foi realizado na proporção de 1 macho: 2 fêmeas, e a detecção da prenhez constatada por observação do plug vaginal, sendo este dia considerado o dia zero de gestação (Oliveira et al., 2015). As fêmeas foram divididas em 3 grupos experimentais (n=10): Grupo Controle – os animais receberam solução fisiológica e Tween 80 a 1% (veículo do EEDd) na

proporção de 0,1mL/10g peso corpóreo (p.c.) por gavage (v.o.) durante todos os dias da gestação (1º ao 18º dia gestacional). Grupo Gestacional – os animais receberam o EEDd (v.o.) nas doses de 100 mg/kg (EEDd1) e 1000 mg/kg (EEDd2) durante todos os dias da gestação.

2.3. Desempenho reprodutivo e ensaio de teratogenicidade

Com 18 dias de gestação, os camundongos foram submetidos à eutanásia, por deslocamento cervical, seguida de laparotomia, histerectomia e onfalectomia. Os órgãos, fetos e placentas foram coletados e pesados. Os fetos foram submetidos à avaliação externa para a detecção de possíveis malformações e, posteriormente, foram sexados. O número de implantações, reabsorções, fetos vivos e fetos mortos foram registrados e com base nos dados obteve-se a viabilidade fetal (número de fetos vivos x 100 / número de implantações), taxa de perdas pós-implantação [(número de implantações - número de fetos vivos) x 100 / número de implantações], taxa de reabsorção (número de reabsorções x 100 / número de implantações), índice placentário (peso placentário / peso fetal) e razão sexual (número de fetos machos / número de fetos fêmeas). Após análise externa, os fetos foram distribuídos aleatoriamente em dois subgrupos, o primeiro foi destinado à análise visceral e, portanto, foram fixados em solução de Bodian's e submetidos à microdissecação com cortes estratégicos (Barrow & Taylor, 1969; Wilson, 1965; Oliveira et al., 2009). As alterações viscerais foram classificadas com base nos estudos de Taylor (1986), Manson e Kang (1994), Damasceno (2008) Oliveira et al. (2009) e Oliveira et al. (2015). O segundo grupo, destinado à análise esquelética, foram submetidos à fixação em acetona e, posteriormente, à diafanização em KOH (solução trocada a cada 24h ao longo de sete dias) e corados com Alizarina Vermelha durante o processo de diafanização como proposto por Straples & Schnell (1964) e modificações de Oliveira et al. (2015).

2.4. Ensaio do micronúcleo em sangue periférico

Para o ensaio do micronúcleo, 20 µL de sangue periférico foram depositados em uma lâmina previamente preparada com 20 µL de alaranjado

de acridina (1,0 mg/mL) e então recoberta por lamínula. O material permaneceu em *freezer* (-20°C) por um período mínimo de 7 dias e 2.000 células/animal foram analisadas em microscópio de fluorescência com filtro de excitação 420-490 nm e filtro de barreira 520 nm, como proposto por Hayashi et al. (1990) e modificado por Oliveira et al. (2009).

2.5. Análises estatísticas

Os dados foram apresentados em média \pm erro padrão da média (EPM) e avaliados de acordo com a natureza de sua distribuição (teste paramétrico - ANOVA/Tukey e não paramétricos - Kruskal-Wallis/Dunn). Para as comparações de frequências entre o grupo controle e os grupos experimentais, utilizou-se o teste de Qui-quadrado. O nível de significância foi estabelecido em $p < 0,05$.

3. Resultados

3.1. Avaliação dos parâmetros biométricos de fêmeas prenhes tratadas com extrato etanólico de *Doliocarpus dentatus*

Não houve diferenças significativas entre os pesos iniciais, finais e ganho de peso, tampouco nos pesos absolutos e relativos dos órgãos das fêmeas dos diferentes grupos experimentais (Tabela 1).

3.2. Avaliação dos parâmetros reprodutivos de fêmeas prenhes tratadas com extrato etanólico de *Doliocarpus dentatus*

Em relação à média de implantes, fetos vivos, fetos mortos, viabilidade fetal, taxa de perda pós-implantacional (TPPI), reabsorção, taxa de reabsorção, índice placentário e razão sexual não foram observadas diferenças significativas entre os grupos. Já o peso placentário e o peso fetal aumentaram ($p < 0,05$) nos grupos tratados com a dose de 100 mg/Kg. Quando realizada a adequação do peso à idade gestacional observou-se que os fetos das mães tratadas com a dose de 100 mg/Kg apresentavam peso elevado para idade gestacional e os fetos das mães tratadas com a dose de 1000 mg/kg apresentavam peso adequado para a idade gestacional (Tabela 2).

3.3. Avaliação do desenvolvimento embriofetal: malformações externas, viscerais e esqueléticas

As taxas de malformações externas, viscerais e esqueléticas não apresentaram diferenças estatisticamente significativas entre os grupos experimentais (Tabela 3, 4 e 5).

As malformações externas observadas foram retroversão de membros anteriores (unilateral) e posteriores (uni e bilateral), cauda em vírgula e escoliose (Tabela 3).

As malformações viscerais encontradas foram hidrocefalia e hidronefrose (Tabela 4). Já as malformações esqueléticas observadas foram ossificação reduzida, ossificação irregular ou agenesia de falanges e de esternóbrios (Tabela 5).

3.4. Avaliação da genotoxicidade em fêmeas prenhes tratadas com extrato etanólico de *Doliocarpus dentatus*

A avaliação da integridade genética, pelo ensaio de micronúcleo, indicou que o EEDd não é genotóxico para as fêmeas prenhes ($p > 0,05$). A média da frequência de eritrócitos micronucleados foi de $7,1 \pm 1,03$ para o grupo controle, $8,5 \pm 0,82$ para o grupo EEDd1 e $7,2 \pm 0,51$ para o grupo EEDd2 (Figura 1).

4. Discussão

Os estudos científicos na área de bioprospecção tem como finalidade a comprovação científica de propriedades biológicas já descritas empiricamente, a determinação do uso correto das substâncias e o desenvolvimento de novos produtos farmacêuticos a partir de plantas medicinais, uma vez que os fármacos podem originar-se a partir de moléculas bioativas isoladas de produtos naturais. Além disso, os extratos de plantas medicinais podem também originar fitofármacos e/ou fitoterápicos (Saccaro-Junior, 2011).

De acordo com a medicina popular, as folhas de *Doliocarpus dentatus* são usadas no tratamento da dor induzida por processo inflamatório e de infecção e retenção urinária (Rodrigues; Carvalho, 2001; Rodrigues, 2007). No entanto, a literatura não possui estudos sobre a segurança do consumo dessa planta, exceto o trabalho de Ishikawa et al. (2017) que relatou pela primeira vez que o EEDd não é genotóxico. Mas, no que se refere ao uso durante a gestação nenhum estudo foi encontrado. Assim, de forma pioneira o presente estudo demonstrou que esse extrato pode ser consumido durante a gestação sem causar alterações na integridade do DNA e efeitos adversos no desempenho reprodutivo de fêmeas e no desenvolvimento embriofetal.

São indicativos dessa segurança a ausência de variações nos parâmetros biométricos (peso final, ganho de peso, peso líquido e peso absoluto e relativo dos órgãos) associadas à ausência de manifestações clínicas (alterações de pele e das mucosas, ocorrência de diarreia e vômito, redução da ingestão alimentar e hídrica, e modificações no comportamento) como relatados na literatura (Gonçalves et al, 2013).

Ainda corroboram com a segurança de uso a ausência de danos genotóxicos comprovados pelo ensaio do micronúcleo. Esse mesmo resultado de ausência de danos no DNA já havia sido relatado em camundongos adultos machos (Ishikawa et al., 2017). A presente pesquisa, pioneiramente, comprova esse mesmo fato em fêmeas prenhes.

Os dados relativos ao desempenho reprodutivo, de modo geral, também corroboram com a segurança de uso do extrato e permitem inferir na ausência de danos tóxicos, no entanto, foi observado aumento do peso placentário e do peso fetal nas fêmeas tratadas com a menor dose do extrato. Um fato que pode explicar essas variações é que o número de fetos nesse grupo foi menor do que nos outros dois (grupo controle e EEDd2). Essa redução do número de filhotes pode ser compreendida como um achado biológico de ocorrência aleatória visto que nenhum dos parâmetros relativos ao número de implantes, viabilidade fetal, reabsorção e perdas pós-implantacionais apresentaram diferenças estatisticamente significativas. Outro evento que corrobora ainda a ocorrência aleatória é a ausência de variação do índice placentário que se trata da relação entre o peso da placenta e o peso do

feto, ou seja, os dois cresceram proporcionalmente uma vez que o índice placentário é estatisticamente semelhante entre os grupos.

De acordo com a literatura o aumento da placenta é correlacionado ao aumento do suporte nutricional que é destinado ao feto (El Behery, 2011). Essa pode ser uma adaptação do organismo materno diante de doenças e/ou exposição a agentes tóxicos (Roberts, 2014). Tal aspecto geralmente está associado com alterações no índice placentário, fato que não foi observado nesse estudo.

Reforçam ainda a segurança de uso do extrato no período gestacional a ausência de alterações no desenvolvimento embriofetal visto que a ocorrência de malformações externas, viscerais e esqueléticas foram semelhantes em todos os grupos. Esse fato permite inferir que o EEDd, nas condições experimentais do presente estudo, não altera o desenvolvimento embriofetal e não é teratogênico.

Esses resultados abrem perspectivas de uso seguro para o EEDd visto que há diferentes atividades biológicas já descritas para essa planta tais como ação anti-Leishmania *in vitro* (Sauvain et al., 1996), citotóxica (Aponte et al., 2008), antimicrobiana (Jagessar; Persid, 2014), antiedematogênica (Bianki Filho et al., 2015), antimicobacteriana e anti-inflamatória (Ishikawa et al., 2017). Além disso, o EEDd mostra-se promissor para a prospecção de anti-inflamatórios para uso seguro durante a gestação já que os medicamentos comerciais apresentam riscos potenciais (David et al., 2014; Pessatto et al., 2017).

5. Conclusão

O presente estudo permite inferir que o extrato etanólico de *Doliocarpus dentatus* não altera o desempenho reprodutivo de fêmeas prenhes, não altera o desenvolvimento embriofetal, não é teratogênico e também não induz danos no DNA. Essas características indicam boas condições de uso desse extrato em processos de bioprospecção inclusive para o desenvolvimento de anti-inflamatórios seguros para o uso no período gestacional.

6. Referências Bibliográficas

ANVISA - Agência Nacional de Vigilância Sanitária. **Guia para a condução de estudos não clínicos de segurança necessários ao desenvolvimento de medicamentos.** Brasília, 2010.

APONTE, J.C. et al. Isolation of cytotoxic metabolites from Target Peruvian Amazonian medicinal plants. **J Nat Prod.** v. 71, p. 102-105, 2008.

BARROW, M.V.; TAYLOR, W.J. A rapid method for detecting malformation in rat fetuse. **J Morphol.** v. 127, n. 3, p. 291-305, 1969.

BIANKI FILHO, C.A. et al. Atividade antiedematogênica e inibitória da atividade da mieloperoxidase de extrato de folhas de *Dolioscarpus dentatus* em ratos. **17º Workshop de Plantas Medicinais do Mato Grosso do Sul.** 7º Empório da Agricultura Familiar. 2015.

DAMASCENO, D.C. et al. **Anomalias congênitas: estudos experimentais.** Editora Média, Belo Horizonte, 2008.

DAVID, N. de. et al. *Gochnatia polymorpha ssp. floccosa*: Bioprospecting of an anti-inflammatory phytotherapy for use during pregnancy. **J Ethnopharmacol.** v. 154, n. 2, p. 370-9, 2014.

EL BEHERY, M.M. et al. Effect of umbilical vein blood flow on perinatal outcome of fetuses with lean and/or hypo-coiled umbilical cord. **Arch Gynecol Obstet.** v. 283. p. 53. 2011.

GONÇALVES, C.A. et al. Evaluation of mutagenic, teratogenic, and immunomodulatory effects of *Annona nutans* hydromethanolic fraction on pregnant mice. **Genet Mol Res.** v. 13, p. 4392-4405, 2013.

HAYASHI, M. et al. The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. **Mut Res.** v. 245, p. 245-249, 1990.

ISHIKAWA, R.B. et al. Anti-inflammatory, antimycobacterial and genotoxic evaluation of *Dolioscarpus dentatus*. **J Ethnopharmacol.** 204 (2017) 18–25.

JAGESSAR, R.C.; PERSID, R. Antimicrobial activity of uncombined and combined extracts of *Dolioscarpus dentatus* and *Montrcardia arborescens*. **Int J Pharm Sci Res.** v. 5, n. 1, p. 286-293, 2014.

MANSON, J.M.; KANG, Y.J. Test methods for assessing female reproductive and developmental toxicology. In: Hayes, A.W. (Ed). **Principles and methods of Toxicology.** Raven Press, New York, 1994.

OECD- Guidelines for Testing of Chemicals. Mammalian Erythrocyte Micronucleus Test. Test. No. 474,1997.

OECD- Guidelines for Testing of Chemicals. Draft Proposal for an Extended One-Generation Reproductive Toxicity Study. No.28, 2009.

OECD- Guidelines for Testing of Chemicals. *In vitro* mammalian Cell Micronucleus Test. Test. No. 487, 2012.

OLIVEIRA, R.J. et al. Effects of the polysaccharide β -glucan on clastogenicity and teratogenicity caused by acute exposure to cyclophosphamide in mice. **Regul Toxicol Pharmacol.** v. 53, n. 3, p. 164-73, 2009.

OLIVEIRA, R.J. et al. 6-Dimethylaminopurine and cyclohexamide are mutagenic and alter reproductive performance and intrauterine development in vivo. **Genet Mol Res.** v. 14, p. 834-849, 2015.

PESSATTO, L.R. et al. Effects of dichloromethane and butanol fractions of *Gochnatia polymorpha floccosa* in maternal reproductive outcome, embryo-fetal development and DNA integrity in mice. **J Ethnopharmacol.** 200, p. 205-209. 2017.

RODRIGUES, V.E.G. **Etnobotânica e florística de plantas medicinais nativas de remanescentes de floresta estacional semidecidual na região do Alto Rio Grande, MG.** Universidade Federal de Lavras, Minas Gerais, Brasil, 2007.

RODRIGUES, V.E.G.; CARVALHO, D.A. Levantamento etnobotânico de plantas medicinais no domínio do cerrado na região do Alto Rio Grande – Minas Gerais. **Ciênc Agrotec.** v. 25, n. 1, p.102-123, 2001.

ROBERTS, M.D. Pathophysiology of ischemic placental disease. **Semin Perinatol.** v. 38, n. 3, p. 139-145. 2014.

SACCARO-JUNIOR, N.L. Desafios da bioprospecção no Brasil. Instituto de Pesquisa Econômica Aplicada. Brasília, 2011.

SAUVAIN, M. et al. Isolation of leishmanicidal triterpenes and ligans from the Amazonian liana *Dolioscarpus dentatus*. (Dilleniaceae). **Phytother Res.** v. 10, p. 1-4, 1996.

STRAPLES, R.E.; SCHENELL, V.L. Refinements in rapid clearing technic in the KOH-alizarin red method for fetal bone. **Stain Technol.** v. 39, p. 61-63, 1964.

TAYLOR, P. Pratical Teratology. **Academic Press**, New York, 1986.

WILSON, J.G. Methods for administering agents and detecting malformations in experimental animals. In: WILSON, J.G.; WAEKANY, J. (Eds.) **Teratology: Principles and Techniques.** The University of Chicago Press, Chicago, 1965.

*Referências bibliográficas de acordo com as regras da Journal of Ethnopharmacology (2017).

Tabela 1 – Parâmetros biométricos de fêmeas prenhes tratadas com Extrato Etanólico de *D. dentatus* ou veículo de diluição do extrato.

Parâmetros Biométricos					
Grupos Experimentais	Peso Inicial	Peso Final	Ganho de Peso	Peso do Útero	Ganho de Peso Líquido
Controle	34,20 ± 1,05 ^a	52,83 ± 1,60 ^a	18,63 ± 1,25 ^a	19,64 ± 1,50 ^a	-1,00 ± 0,85 ^a
EEDd1	35,20 ± 1,15 ^a	53,71 ± 1,88 ^a	18,51 ± 1,99 ^a	17,75 ± 2,28 ^a	0,76 ± 0,71 ^a
EEDd2	36,60 ± 1,23 ^a	54,14 ± 1,43 ^a	17,54 ± 1,36 ^a	18,32 ± 1,49 ^a	-0,78 ± 1,02 ^a
Peso Absoluto (g)					
	Coração	Pulmão	Baço	Fígado	Rins
Controle	0,18 ± 0,01 ^a	0,28 ± 0,02 ^a	0,14 ± 0,01 ^a	2,23 ± 0,07 ^a	0,41 ± 0,01 ^a
EEDd1	0,19 ± 0,01 ^a	0,30 ± 0,02 ^a	0,17 ± 0,01 ^a	2,34 ± 0,10 ^a	0,43 ± 0,01 ^a
EEDd2	0,16 ± 0,01 ^a	0,28 ± 0,01 ^a	0,16 ± 0,01 ^a	2,39 ± 0,07 ^a	0,40 ± 0,01 ^a
Peso Relativo (g)					
	Coração	Pulmão	Baço	Fígado	Rins
Controle	0,003 ± 0,000 ^a	0,006 ± 0,000 ^a	0,003 ± 0,000 ^a	0,042 ± 0,001 ^a	0,008 ± 0,000 ^a
EEDd1	0,003 ± 0,000 ^a	0,006 ± 0,000 ^a	0,003 ± 0,000 ^a	0,044 ± 0,001 ^a	0,008 ± 0,000 ^a
EEDd2	0,003 ± 0,000 ^a	0,005 ± 0,000 ^a	0,003 ± 0,000 ^a	0,044 ± 0,001 ^a	0,007 ± 0,000 ^a

Legenda: Controle – animais tratados por gavagem, via oral, com veículo de diluição de extrato (Tween 80 1%) na proporção de 0,1mL/10g peso corpóreo (p.c.); EEDd1 – animais tratados por gavagem, via oral (v.o.) com extrato etanólico de *Doliocarpus dentatus* na dose de 100 mg/kg (p.c.); EEDd2 – animais tratados com extrato etanólico de *Doliocarpus dentatus* na dose 1000 mg/kg (p.c., v.o.). Os resultados estão apresentados em média ± erro padrão da média. Letras iguais na coluna indicam ausência de diferenças estatisticamente significativas entre os grupos experimentais (Teste Estatístico: ANOVA de uma via de medidas repetitivas; p>0,05).

Tabela 2 – Parâmetros reprodutivos de fêmeas prenhes tratadas com Extrato Etanólico de *D. dentatus* ou veículo de diluição do extrato.

Parâmetros	Grupos Experimentais		
	Controle	EEDd1	EEDd2
Implantes ¹	15,00 ± 1,00 ^a	13,00 ± 1,74 ^a	15,10 ± 0,72 ^a
Fetos Vivos ¹	13,70 ± 1,15 ^a	11,40 ± 1,54 ^a	13,00 ± 1,12 ^a
Fetos Mortos ¹	0,00 ± 0,00 ^a	0,01 ± 0,10 ^a	0,00 ± 0,00 ^a
Viabilidade Fetal ¹	90,34 ± 2,54 ^a	90,56 ± 5,11 ^a	85,47 ± 5,10 ^a
TPPI ¹	9,66 ± 2,54 ^a	9,44 ± 5,11 ^a	14,53 ± 5,10 ^a
Reabsorção ¹	1,30 ± 0,26 ^a	1,50 ± 0,83 ^a	2,10 ± 0,71 ^a
Taxa de Reabsorção ¹	9,66 ± 2,54 ^a	8,91 ± 4,69 ^a	14,53 ± 5,10 ^a
Peso Placentário (g) ²	0,09 ± 0,001 ^a	0,14 ± 0,051 ^b	0,09 ± 0,001 ^a
Índice Placentário ²	0,08 ± 0,01 ^a	0,12 ± 0,048 ^a	0,08 ± 0,002 ^a
Peso Fetal (g) ¹	1,13 ± 0,01 ^a	1,23 ± 0,01 ^b	1,11 ± 0,01 ^a
APIP	-	PEIP	PAIP
Razão Sexual ¹	1,57 ± 0,45 ^a	1,93 ± 0,40 ^a	1,13 ± 0,25 ^a

Legenda: TPPI – Taxa de perdas pós implantacionais; APIP – Adequação de peso para a idade de prenhez; PEIP – Peso elevado para a idade de prenhez; PAIP – Peso adequado para a idade de prenhez. Controle – animais tratados por gavagem, via oral, com veículo de diluição de extrato (Tween 80 1%) na proporção de 0,1mL/10g peso corpóreo (p.c.); EEDd1 – animais tratados por gavagem, via oral (v.o.) com extrato etanólico de *Doliocarpus dentatus* na dose de 100 mg/kg (p.c.); EEDd2 – animais tratados com extrato etanólico de *Doliocarpus dentatus* na dose 1000 mg/kg (p.c., v.o.). Os resultados estão apresentados em média ± erro padrão da média. . Letras iguais na coluna indicam ausência de diferenças estatisticamente significativas entre os grupos experimentais. (Teste Estatístico: ¹ANOVA de uma via de medidas repetitivas; p>0,05; ²Kruskal-Wallis; p>0,05).

Tabela 3 – Frequência de malformações externas na prole de fêmeas prenhes tratadas com Extrato Etanólico de *D. dentatus* ou veículo de diluição do extrato.

Parâmetros	Grupos Experimentais		
	Controle	EEDd1	EEDd2
Malformações Externas de Membros Anteriores e Posteriores			
Fetos Analisados	137	113	130
Fetos Normais	119	94	112
Retroversão Anterior Unilateral	3	2	7
Retroversão Posterior Unilateral	14	14	10
Retroversão Posterior Bilateral	1	3	1
Frequência de Malformações Externas	18	19	18
% Malformações Externas	13,14%	16,81%	13,85%
Malformações Externas de Cauda			
Fetos Analisados	137	113	130
Fetos Normais	129	105	125
Cauda em Vírgula	8	8	5
Frequência de Malformações Externas	8	8	5
% Malformações Externas	5,84%	7,07%	3,85%
Malformações Externas de Coluna			
Fetos Analisados	137	113	130
Fetos Normais	135	112	130
Escoliose	2	1	0
Frequência de Malformações Externas	2	1	0
% Malformações Externas	1,46%	0,88%	0%

Legenda: Controle – animais tratados por gavagem, via oral, com veículo de diluição de extrato (Tween 80 1%) na proporção de 0,1mL/10g peso corpóreo (p.c.); EEDd1 – animais tratados por gavagem, via oral (v.o.) com extrato etanólico de *Doliocarpus dentatus* na dose de 100 mg/kg (p.c.); EEDd2 – animais tratados com extrato etanólico de *Doliocarpus dentatus* na dose 1000 mg/kg (p.c., v.o.). (Teste Estatístico: Qui-quadrado; $p > 0,05$).

Tabela 4 – Frequência de malformações viscerais na prole de fêmeas prenhes tratadas com Extrato Etanólico de *D. dentatus* ou veículo de diluição do extrato.

Parâmetros	Grupos Experimentais		
	Controle	EEDd1	EEDd2
Malformações Viscerais no Cérebro			
Fetos Analisados	69	57	65
Fetos Normais	66	55	59
Hidrocefalia Severa	3	2	6
Frequência de Malformações Viscerais	3	2	6
% Malformações Viscerais	4,35%	3,51%	9,23%
Malformações Viscerais na Região Urogenital			
Fetos Analisados	69	57	65
Fetos Normais	66	51	59
Hidronefroze Leve	3	6	6
Frequência de Malformações Viscerais	3	6	6
% Malformações Viscerais	4,35%	10,53%	9,23%

Legenda: Controle – animais tratados por gavage, via oral, com veículo de diluição de extrato (Tween 80 1%) na proporção de 0,1mL/10g peso corpóreo (p.c.); EEDd1 – animais tratados por gavage, via oral (v.o.) com extrato etanólico de *Doliocarpus dentatus* na dose de 100 mg/kg (p.c.); EEDd2 – animais tratados com extrato etanólico de *Doliocarpus dentatus* na dose 1000 mg/kg (p.c., v.o.) (Teste Estatístico: Qui-quadrado; $p > 0,05$).

Tabela 5 – Frequência de malformações esqueléticas na prole de fêmeas prenhes tratadas com Extrato Etanólico de *D. dentatus* ou veículo de diluição do extrato.

Parâmetros	Grupos Experimentais		
	Controle	EEDd1	EEDd2
Malformações Esqueléticas de Membros Anteriores e Posteriores			
Fetos Analisados	68	56	65
Fetos Normais	61	48	60
Ossificação Reduzida/Ausência de Falanges	7	8	5
Frequência de Malformações Esqueléticas	7	8	5
% Malformações Esqueléticas	10,29%	14,28%	7,69%
Malformações Esqueléticas de Esternébrio			
Fetos Analisados	68	56	65
Fetos Normais	57	44	51
Esternébrio Anormal/Ossificação Incompleta	11	12	14
Frequência de Malformações Esqueléticas	11	12	14
% Malformações Esqueléticas	16,18%	21,43%	21,54%

Legenda: Controle – animais tratados por gavagem, via oral, com veículo de diluição de extrato (Tween 80 1%) na proporção de 0,1mL/10g peso corpóreo (p.c.); EEDd1 – animais tratados por gavagem, via oral (v.o.) com extrato etanólico de *Doliocarpus dentatus* na dose de 100 mg/kg (p.c.); EEDd2 – animais tratados com extrato etanólico de *Doliocarpus dentatus* na dose 1000 mg/kg (p.c., v.o.). (Teste Estatístico: Qui-quadrado; $p > 0,05$).

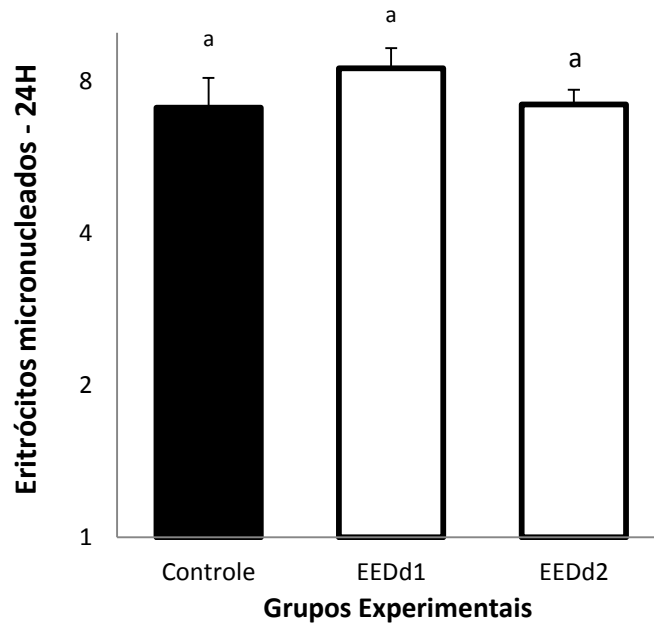


Figura 1 – Frequência de micronúcleos analisados em 2.000 hemácias de camundongos fêmeas prenhes tratadas com Extrato Etanólico de *Doliocarpus dentatus* ou veículo de diluição do extrato. Controle – animais tratados por gavagem, via oral, com veículo de diluição de extrato (Tween 80 1%) na proporção de 0,1mL/10g peso corpóreo (p.c.); EEDd1 – animais tratados por gavagem, via oral (v.o.) com extrato etanólico de *Doliocarpus dentatus* na dose de 100 mg/kg (p.c.); EEDd2 – animais tratados com extrato etanólico de *Doliocarpus dentatus* na dose 1000 mg/kg (p.c., v..o.). Os resultados estão apresentados em média \pm erro padrão da média. Letras iguais indicam que não há diferenças estatisticamente significativas; $p > 0,05$).

The safe use of *Doliocarpus dentatus* in the gestational period: absence of changes in maternal reproductive performance, embryo-fetal development and DNA integrity

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Running Title: Effects of ethanolic extract of *Doliocarpus dentatus* on maternal reproductive performance, embryofetal development and genotoxicity in Swiss mice

Authors' contributions

Ishikawa, RB; Vani, JM; and Neves, SC conducted the reproductive performance, teratogenicity and micronucleus assays; Rabacow, APM conducted the translation and helped to draft the manuscript; Cardoso, CAL was involved in the preparation and isolation of the extract; Monreal, ACDF; Antonioli, CMB and Laura, ALC; performed statistical analysis; Kassuya, CAL and Croda, J; performed data analyses and helped to write the manuscript. Oliveira, RJ conceived of the study and served as principal investigator throughout its execution. All authors read and approved the final manuscript. conducted and analyzed the experimental assays, participated in the design of the study and performed the statistical analysis.

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Abstract

Ethnopharmacological relevance: *Dolioscarpus dentatus* (Dilleniaceae) is commonly used in Brazil for the treatment of inflammatory process pain and urinary retention. Previous studies of our group have demonstrated the anti-inflammatory and antimycobacterial action of the ethanolic extract of *Dolioscarpus dentatus* (EEDd) as well as the safety of its use.

Objective: we investigated the effects of EEDd on reproductive performance, fetal development and DNA integrity in pregnant female Swiss mice.

Methods: thirty female Swiss mice were divided into three experimental groups (n=10): control group treated with 1% tween-80 and EEDd1 and EEDd2 groups treated with EEDd at doses of 100 and 1000 mg/kg, respectively. The treatment occurred by oral gavage throughout the gestational period. At the end of pregnancy, parameters related to reproductive performance, embryofoetal development and DNA integrity was evaluated.

Results: both doses of the extract tested did not alter the reproductive parameters, did not present significant differences in the embryofetal development when compared to the control group and also did not induce the formation of micronuclei.

Conclusion: the EEDd do not alter the reproductive parameters, embryofetal development and DNA integrity, ensuring its safe use during pregnancy.

Keywords: *Dolioscarpus dentatus*; teratogenesis; reproductive toxicology.

1. Introduction

Dolioscarpus dentatus, popularly known as “cipó de fogo”, “cipó mata sede” or “cipó vermelho” is a plant of the Dilleniaceae family with moderate growth, resistant to the drought and low temperatures. It is found in the tropical forests of Mexico, Peru and Bolivia and in Brazilian ecosystems such as the Atlantic Forest, the Amazon and the Cerrado (Aponte et al., 2008; Bianki Filho et al., 2015). Its aqueous sap is popularly used to relieve thirst, its barks are used in infusions to treat leishmaniosis, and its leaves and roots to treat cystitis, pain induced by inflammation and urinary retention (Rodrigues, 2007; Jagessar, Persid, 2014).

A previous research from our group determined the presence of phenols (204.04 mg/g), flavonoids (89.17 mg/g) and tannins (12.05 mg/g) as well as sitosterol-3-O-D-glucopyranoside, kaempferol 3-O-L-aminopyranoside, betulinic acid and betulin in the ethanolic extract of *D. dentatus* (EEDd) (Ishikawa et al., 2017). Another phytochemical study has also shown that this plant contains butyric acid, steroids, lactones, anthracensides, betulinic acid, tannins, flavones and trigonellin (Jagessar et al., 2013).

In vitro studies on the biologic activity of *D. dentatus* demonstrated anti-Leishmania action against amastigotes of *Leishmania (L.) amazonensis* (Sauvain et al., 1996), antimicrobial effect against strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Jagessar; Persid, 2014), anti-*Mycobacterium tuberculosis*, anti-inflammatory (Ishikawa et al., 2017) and cytotoxic in leukemic cells of the K562 lineage (Aponte et al. , 2008). In addition, our research group has recently demonstrated that EEDd does not cause genomic and chromosomal damage which suggests safety of use (Ishikawa et al., 2017). However, no additional data on the effects of EEDd in the gestational period were found in the literature. Hence, this study is a pioneer and aimed to evaluate the effects of EEDd on the reproductive performance, embryofetal development and DNA integrity of pregnant female Swiss mice.

2. Methods and Materials

2.1 Ethanolic Extract of *Doliocarpus dentatus*

The EEDd employed in this research has previously been studied and described by Ishikawa et al. (2017). Briefly, the young and mature leaves of *Doliocarpus dentatus* were collected at the Universidade Federal de Mato Grosso do Sul (UFMS), in the city of Campo Grande - MS. The botanical identification was performed by Professor Dr. Arnildo Pott and a sample was deposited in the UFMS herbarium under the number # 49860.

The collected leaves were dried at 40°C, ground to powder and extracted with ethanol (95%) by the maceration for 7 consecutive days. The extracts were collected, dried in an evaporator and then lyophilized to obtain the crude dried extract. For administration to the animals the extract was diluted in 1% tween-80 and saline and the doses used were 100 and 1000 mg/kg body weight (b.w.) via oral gavage.

The dose of 100 mg/kg was chosen due its anti-inflammatory efficacy as proven by Ishikawa et al. (2017). It was also resolved to test a 10x higher dose owing to the fact that according to the guidelines in the area of reproductive toxicology (OECD, 2012) and genetic toxicology (OECD, 2010, OECD, 2014), as well as the National Agency of Sanitary Surveillance (ANVISA, 2013), preclinical trials should be conducted with the doses intended for use in humans

and another test with 10x larger dose. Only if the highest dose is free of side effects, the lowest dose may be considered safe.

2.2 Chemical composition

The extract was fractionated by liquid chromatography employing XAD-2 (Supelco, Bellefonte, PA, USA) resin column chromatography (30 cm x 3 cm). The extract (2.45 g) was eluted with 0.5 L of water, followed by elution with 0.5 L of methanol and with 0.4 L of ethyl acetate. An aliquot of 0.67 g of the ethyl acetate fraction was dissolved in 20 mL of methanol, fractionated by liquid chromatography on a Sephadex LH-20 column (80x2 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), and eluted with methanol at a rate of 0.3 mL, 28 fractions were collected. The fractions were combined according to their chemical profile, as determined by thin layer chromatography. Samples were applied onto silica gel plates using ethyl acetate-methanol (75:25, v/v) as the eluent. Fraction 21-24 gave the isolated compound quercetin (2 mg) and fraction 26-27 Kampferol (1 mg), respectively. Chemical characterization was performed using nuclear magnetic resonance (Bruker DPX-300, 300 MHz for ¹H and 75 MHz for ¹³C) and the chemical structures were confirmed by comparison with literature data (Agrawal, 1989; Harborne, 1996).

2.3 Experimental design

15 male and 30 female mice, *Mus musculus* (Swiss), with 35 g average weight, at reproductive age, were obtained from the State Agency of Animal and Plant Health Protection (IAGRO). The present study was approved by the Ethics Committee on the Use of Animals of the UFMS under authorization #776/2016.

The animals were kept in polypropylene boxes, covered by wood shaving at a controlled temperature of 22 ± 2°C and relative humidity of (55 ± 10%) Water and food were provided *ad libitum*. The fed was with commercial ration (Nuvital®). The mice were mated overnight in the proportion of 1 male: 2 females. It was checked females for vaginal plugs to determine if pregnancy has occurred. If the plugged female is pregnant, the first day of gestation is considered to be the day after the plug is found (Oliveira et al., 2015). Females were divided into 3 experimental groups (n = 10): control group

- animals received 0.1 mL/10g b.w. of EEDd vehicle per oral gavage throughout gestation (1st to 18th). Gestational Group - animals received EEDd per oral gavage at doses of 100 mg/kg (EEDd1) and 1000 mg/kg (EEDd2) throughout gestation.

2.4 Reproductive performance and teratogenicity testing

On the eighteenth day of gestation, the mice were submitted to euthanasia, by cervical dislocation, followed by laparotomy, hysterectomy and omphalectomy. Organs, fetuses and placentas were collected and weighed. The fetuses underwent external evaluation to detect possible malformations and were subsequently sexed. The number of implantations, resorptions, live fetuses and dead fetuses were recorded and based on the data, it was obtained the: fetal viability (number of live fetuses x 100/number of implantations), post-implantation loss rate [(number of implantations - (number of resorptions x 100/number of implantations)], placental index (placental weight/fetal weight) and sex ratio (number of male fetuses/number of female fetuses). After external analysis, the fetuses were randomly distributed into two subgroups. The first one was aimed at the visceral analysis and, therefore, were fixed in a solution of Bodian's and submitted to microdissection with strategic cuts (Barrow & Taylor, 1969, Wilson, 1965 and Oliveira et al., 2009). The visceral changes were classified based on the studies of Taylor (1986), Manson & Kang (1994), Damasceno et al. (2008) Oliveira et al. (2009) and Oliveira et al. (2015). For skeletal analysis, the second group was subjected to fixation in acetone and subsequently to KOH and stained with Alizarin Red during the diaphanization process as proposed by Straples & Schnell (1964) and modified by Oliveira et al. (2015).

2.5 Micronucleus assay in peripheral blood

For the micronucleus assay, 20 µL of peripheral blood was stained by dropping blood on an Acridine Orange-coated slide (1.0 mg/mL) and covering the sample with a coverslip. The material remained in freezer (-20°C) for a minimum of 7 days and 2.000 cells/animal were analyzed under epifluorescence microscope at 400x magnification with a 420-490 nm excitation

and a 520 nm barrier filter, as proposed by Hayashi et al. (1990) and modified by Oliveira et al. 2009.

2.6 Statistical analysis

The results were shown as mean \pm standard error of the mean (SEM) and the comparison of quantitative results was carried out using parametric and non-parametric tests (ANOVA/Tukey, Kruskal-Wallis/Dunn and Chi-square depending on the nature of the data distribution. In all cases, differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1 Evaluation of the biometric parameters of pregnant females treated with ethanolic extract of *Doliocarpus dentatus*

There were no significant differences between the initial and final weights and weight gain, or in the absolute and relative weights of the female organs of the different experimental groups (Table 1).

3.2 Evaluation of the reproductive parameters of pregnant females treated with ethanolic extract of *Doliocarpus dentatus*

In relation to the mean number of implants, live fetuses, dead fetuses, fetal viability, post-implantation loss rate (PILR), resorption, resorption rate, placental index and sex ratio, no significant differences were observed between groups. Placental weight and fetal weight increased ($p < 0.05$) in the groups treated with the dose of 100mg/kg. When the weight was adjusted to gestational age, it was observed that the fetuses of the females treated with the dose of 100mg/kg had a high weight for gestational age and the fetuses of the females treated with the dose of 1000mg/kg had adequate weight for gestational age (Table 2).

3.3 Evaluation of embryofetal development: external, visceral and skeletal malformations

The rates of external, visceral and skeletal malformations did not present statistically significant differences between the experimental groups (Table 3, 4 and 5).

The external malformations observed were forelimb (unilateral) and hindlimb (uni and bilateral) retroversion, rolled up tail and scoliosis (Table 3).

The visceral malformations detected were hydrocephalus and hydronephrosis (Table 4). The skeletal malformations observed were reduced ossification, irregular ossification or agenesis of phalanges and sternum (Table 5).

3.4 Evaluation of DNA integrity of pregnant females treated with ethanolic extract of *Doliocarpus dentatus*

Genetic integrity evaluation by micronucleus assay, indicated that EEDd is not genotoxic ($p > 0.05$) for pregnant females. The mean frequency of micronucleated erythrocytes was 7.1 ± 1.03 for the control group, 8.5 ± 0.82 for the EEDd1 group and 7.2 ± 0.51 for the EEDd2 group (Figure 1).

4. Discussion

The scientific researches in the area of bioprospecting have as purpose the scientific verification of biological properties already described empirically, the determination of the correct use of the substances and the development of new pharmaceuticals products from medicinal plants, since the drugs can be originated by bioactive molecules isolated from natural products. In addition, medicinal plant extracts can also originate phytopharmaceuticals and/or phytoterapics (Saccaro-Junior, 2011).

The compounds present in the ethanolic extract of *Doliocarpus dentatus* were firstly identified as betulinic acid, betulin, sitosterol 3-O- β -D-glucopyranoside, kaempferol 3-O- α -Lhamnopyranoside (Ishikawa et al., 2017). The continuity of the fractionation and purification of this extract led to the identification of two other compounds, quercetin and kampferol, which are present in the human diet. Its biological potentials are already described, specialist antioxidant, anticarcinogenic and protective activities of the renal and cardiovascular systems attributed mainly to the capacity of free radical sequestration (Behling et al., 2004).

Matsubara and Rodriguez-Amaya (2006) describe in extracts of dried leaves of *Camellia sinensis* marketed as different types of tea (dry leaf) with 2.5 mg/g of quercetin and 1.0 mg/g of kaempferol. However, Toyoda (et al., 1997)

quantified dry leaf with 1.6 mg/g of quercetin and 1.5 mg/g of kaempferol. The values of the previously mentioned works are close to those obtained in the present work with the EEDd, serving as source for the consumption of flavonoids.

According to folk medicine, the leaves of *Doliocarpus dentatus* are employed in the treatment of pain induced by inflammation and infection and urinary retention (Rodrigues; Carvalho, 2001; Rodrigues, 2007). However, the literature does not have studies on the safety of the consumption of this plant, except the work of Ishikawa et al. (2017) who reported for the first time that EEDd does not cause genomic damage. But with regard to use during gestation no study was found yet. Therefore, the present study demonstrated that this extract can be consumed during pregnancy without causing changes in DNA integrity neither side effects on the reproductive performance of females and embryofetal development.

Are indicative of this safety: The absence of variations in the biometric parameters (final weight, weight gain, net weight and absolute and relative body weight) associated to the absence of clinical manifestations (skin and mucosal alterations, diarrhea and vomiting, reduction of food and water intake, and changes in behavior) as reported in the literature (Gonçalves et al., 2014).

The absence of genomic damage verified by the micronucleus test also corroborates for the safe use. The DNA-damaging absence results had previously been reported in male adult mice (Ishikawa et al., 2017). The present research, demonstrated, for the first time, this same fact in pregnant females mice.

Data on reproductive performance, in general, also corroborate for the safety use of the extract and allow us to infer that there is no maternal toxic and/or toxic reproductive harm. However, placental weight gain and fetal weight gain were observed in the females treated with lower dose of the extract. A fact that may explain these variations is that the number of fetuses in this group was lower than in the other two (control group and group treated with the highest dose of the extract). This reduction in the number of offspring born can be understood as a biological finding of random occurrence once none of the parameters related to the number of implants, fetal viability, resorption and post-implantational losses presented statistically significant differences. Another fact

that still corroborates the random occurrence is the absence of variation of the placental index which is the relationship between the weight of the placenta and the weight of the fetus, that is, the placenta and the fetus of this group grew proportionally since the placental index is statistically similar between groups.

According to the literature, placental enlargement is correlated with increased nutritional support for the fetus (El Behery, 2011). This may occur due an adaptation of the maternal organism to diseases and/or exposure to toxic agents (Roberts, 2014). However, this aspect is usually associated with alterations in the placental index, which was not observed in this study.

We also reinforce the safety of the extract use in the gestational period, the absence of alterations in embryofetal development once the occurrence of external, visceral and skeletal malformations were similar in all groups. This fact allows implying that the EEDd, in the experimental conditions of the present search, does not alter the embryofetal development and it is not teratogenic.

These findings open perspectives for the safe use of EEDd since there are different biological activities already described for this plant, such as anti-*Leishmania in vitro* (Sauvain et al., 1996), antimicrobial (Jagessar, Persid, 2014), antimycobacterial, anti-inflammatory (Ishikawa et al., 2017) and cytotoxic (Aponte et al., 2008). In addition, EEDd is promising for the prospection of anti-inflammatory drugs for safe use during pregnancy considering that commercial drugs present potential risks (David et al., 2014; Pessatto et al., 2017).

5. Conclusion

The present study allows us to infer that the ethanolic extract of *Doliocarpus dentatus* does not alter the reproductive performance of pregnant females, does not alter embryofetal development, is not teratogenic and does not induce DNA damage. These features indicate good conditions for the use of this extract in bioprospecting processes, including for the development of safe anti-inflammatory drugs for use in the gestational period.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors would like to thank FUNDECT - Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul, CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico and CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for the support.

References

Agrawal, P.K., 1989. Carbon 13 NMR of flavonoids. Elsevier: Amsterdam, 564.

Andrade, S.F., Cardoso, L.G., Carvalho, J.C., Bastos, J.K., 2006. Anti-inflammatory and antinociceptive activities of extract, fractions and populonic acid from bark wood of *Austroplenckia populnea*. J Ethnopharmacol, 109, 464-471.

ANVISA - Agência Nacional de Vigilância Sanitária, 2013. Guia para a condução de estudos não clínicos de segurança necessários ao desenvolvimento de medicamentos, Brasília.

Aponte, J.C., Vaisberg, A.J., Rojas, R., Caviedes, L., Lewis, W.H., Lamas, G., Sarasara, C., Gilman, R.H., Hammond, G.B., 2008. Isolation of cytotoxic metabolites from Target Peruvian Amazonian medicinal plants. J. Nat. Prod, 71, 102-105.

Barrow, M.V., Taylor, W.J., 1969. A rapid method for detecting malformation in rat fetuse. J Morphol, 127, 3, 291-305.

Behling, E.B., Sendão, M.C., Francescato, H.D.C., Antunes, L.M.G., Bianchi, M.L.P., 2004. Flavonóide quercetina: Aspectos gerais e ações biológicas. Alim. Nutr., 15, 3, 285-292.

Bianki Filho, C.A., Cardoso, C.A.L., Coelho, R.G., Lanza-Junior, U., Balen, E., Kassuya, C.A.L., 2015. Atividade anti-edematogênica e inibitória da atividade da mieloperoxidase de extrato de folhas de *Dolioscarpus dentatus* em ratos. 17^o Workshop de Plantas Mediciniais do Mato Grosso do Sul. 7^o Empório da Agricultura Familiar.

Carvalho, P.C., Santos, E.A., Schneider, B.U.C., Matuo, R., Pesarini, J.R., Cunha-Laura, A.L., Monreal, A.C.D., Lima, D.P., Antonioli, A.C.M.B., Oliveira, R.J., 2015. Diaryl sulfide analogs of combretastatin A-4: toxicogenetic, immunomodulatory and apoptotic valuations and prospects for use as a new chemotherapeutic drug. Environ. Toxicol. Pharmacol. v. 40, n. 3, p. 715–721.

Damasceno, D.C., Empinas, W.G., 2008. Anomalias congênitas: estudos experimentais. Editora Média, Belo Horizonte.

David, N., Mauro, M.O., Gonçalves, C.A., Pesarini, J.R., Strapasson, R.L.B., Kassuya, C.A.L., Stefanello, M.E.A., Cunha-Laura, A.L., Monreal, A.C.D., Oliveira, R.J., 2014. *Gochnatia polymorpha* ssp. *Floccosa*: bioprospecting of na anti-inflammatory phytotherapy for use during pregnancy. J Ethnopharmacol. 154, 370-379.

El Behery, M.M., Nouh, A.A., Alanwar, A.M., Diab, A.E., 2011. Effect of umbilical vein blood flow on perinatal outcome of fetuses with lean and/or hypo-coiled umbilical cord. Arch Gynecol Obstet., 283, 53.

Gonçalves, C.A., Silva, N.L., Mauro, M.O., David, N., Cunha-Laura, A.L., Auharek, S.A., Monreal, A.C.D., Vieira, M.C., Silva, D.B., Santos, F.J.L., Siqueira, J.M., Oliveira, R.J., 2014. Evaluation of mutagenic, teratogenic, and immunomodulatory effects of *Annona nutans* hydromethanolic fraction on pregnant mice. Genet Mol Res., 13, 4392-4405.

Harborne, J.B., 1996. The Flavonoids advances in research since 1986. Chapman and Hall, London, 675.

Hayashi, M., Morita, T., Kodama, Y., Sofuni, T., Ishidate-Junior, M., 1990. The micronucleus assay with mouse peripheral blood reticulocytes using acridina orange-coated slides. Mut Res. 245, 245-249.

Ishikawa, R.B., Leitão, M.M., Kassuya, R. M., Marcorini, F.M.F.M., Cardoso, C.A.L., Coelho, R.G., Pott, A., Gelfuso, G.M., Croda, J., Oliveira, R.J., Kassuya, C.A.L., 2017. Anti-inflammatory, antimycobacterial and genotoxic evaluation of *Doliocarpus dentatus*. J Ethnopharmacol., 204, 18-25.

Jagessar, R.C., Persid, R., 2014. Antimicrobial activity of uncombined and combined extracts of *Doliocarpus dentatus* and *Montrichardia arborescens*. Int. J. Pharm. Sci. Res., 5, 1, 286-293.

Manson, J.M., Kang, Y.J., 1994. Test methods for assessing female reproductive and developmental toxicology. In: Hayes, A.W. (Ed). Principles and methods of Toxicology. Raven Press, New York.

Matsubara, S., Rodriguez-Amaya, D.B., 2006. Conteúdo de miricetina, quercetina e kaempferol em chás comercializados no Brasil. Ciênc. Tecnol. Aliment., 26, 2, 380-385.

OECD - Guidelines for Testing of Chemicals, 2010. *In vitro* mammalian Cell Micronucleus Test. No. 487.

OECD - Guidelines for Testing of Chemicals, 2012. Draft Proposal for an Extended One-Generation Reproductive Toxicity Study. No. 28.

OECD - Guidelines for Testing of Chemicals, 2014. Mammalian Erythrocyte Micronucleus Test. No. 474.

Oliveira, R.J., Kanno, T.Y.N., Salles, M.J.S., Lourenã, O.A.C.S., Ribeiro, L.R., Freira, G.A., Matiazi, .H.J.O., Mantovani, M.S., Silva, A.F., 2009. Effects of the polysaccharide I - glucan on clastogenicity and teratogenicity caused by acute exposure to cyclophosphamide in mice. *Regul. Toxicol. Pharmacol.*, 53, 3, 164-173.

Oliveira, R.J., Mantovani, M.S., Pesarini, J.R., Mauro, M.O., Silva, A.F., Souza, T.R., Ribeiro, L.R., 2015. 6-Dimethylaminopurine and cyclohexamide are mutagenic and alter reproductive performance and intrauterine development *in vivo*. *Genet Mol Res.*, 14, 834-849.

Pessatto, L.R., Auharek, S.A., Gonçalves, C.A., David, N., Monreal, A.C.D., Kassuya, C.A.L., Antonioli-Silva, A.C.M.B., Stefanello, M.E.A., Oliveira, R.J., 2017. Effects of dichloromethane and butanol fractions of *Gochnatia polymorpha floccosa* in maternal reproductive outcome, embryo-fetal development and DNA integrity in mice. *J Ethnopharmacol.*, 200, 205-209.

Rodrigues, V.E.G., 2007. Etnobotânica e florística de plantas medicinais nativas de remanescentes de floresta estacional semidecidual na região do Alto Rio Grande, MG. Universidade Federal de Lavras, Minas Gerais, Brasil.

Rodrigues, V.E.G.; Carvalho, D.A., 2001. Levantamento etnobotânico de plantas medicinais no domínio do cerrado na região do Alto Rio Grande – Minas Gerais. *Ciênc. Agrotec.*, 25, 1, 102-123.

Roberts, M.D., 2014. Pathophysiology of ischemic placental disease. *Semin Perinatol.*, 38, 3, 139-145.

Saccaro-Junior, N.L., 2011. Desafios da bioprospecção no Brasil. Instituto de Pesquisa Econômica Aplicada. Brasília.

Sauvain, M., Kunesch, N., Poisson, J., Gantier, J.C., Gayral, P., Dedet, J.P., 1996. Isolation of Leishmanicidal Triterpenes and Lignans from the Amazonian Liana *Doliosarpus dentatus* (Dilleniaceae). *Phytotherap Res.* 10, 1-4.

Straples, R.E.; Schenell, V.L., 1964. Refinements in rapid clearing technic in the KOH-alizarin red method for fetal bone. *Stain Technol.*, 39, 61-63.

Taylor, P., 1986. *Practical Teratology*. Academic Press, New York.

Wilson, J.G., 1965. Methods for administering agents and detecting malformations in experimental animals. In: WILSON, J.G.; WAEKANY, J. (Eds.) *Teratology: Principles and Techniques*. The University of Chicago Press, Chicago.

Table 1 - Biometric parameters of pregnant females treated with ethanol extract of *Doliocarpus dentatus* or vehicle of dilution of the extract.

Biometric parameters					
Experimental Groups	Inicial Weight	Final Weight	Weight Gain	Uterine Weith	Net Weight Gain
Control	34.20 ± 1.05 ^a	52.83 ± 1.60 ^a	18.63 ± 1.25 ^a	19.64 ± 1.50 ^a	-1.00 ± 0.85 ^a
EEDd1	35.20 ± 1.15 ^a	53.71 ± 1.88 ^a	18.51 ± 1.99 ^a	17.75 ± 2.28 ^a	0.76 ± 0.71 ^a
EEDd2	36.60 ± 1.23 ^a	54.14 ± 1.43 ^a	17.54 ± 1.36 ^a	18.32 ± 1.49 ^a	-0.78 ± 1.02 ^a
Absolute Weight (g)					
	Hart	Lung	Spleen	Liver	Kidneys
Control	0.18 ± 0.01 ^a	0.28 ± 0.02 ^a	0.14 ± 0.01 ^a	2.23 ± 0.07 ^a	0.41 ± 0.01 ^a
EEDd1	0.19 ± 0.01 ^a	0.30 ± 0.02 ^a	0.17 ± 0.01 ^a	2.34 ± 0.10 ^a	0.43 ± 0.01 ^a
EEDd2	0.16 ± 0.01 ^a	0.28 ± 0.01 ^a	0.16 ± 0.01 ^a	2.39 ± 0.07 ^a	0.40 ± 0.01 ^a
Relative Weight (g)					
	Hart	Lung	Spleen	Liver	Kidneys
Control	0.003 ± 0.000 ^a	0.006 ± 0.000 ^a	0.003 ± 0.000 ^a	0.042 ± 0.001 ^a	0.008 ± 0.000 ^a
EEDd1	0.003 ± 0.000 ^a	0.006 ± 0.000 ^a	0.003 ± 0.000 ^a	0.044 ± 0.001 ^a	0.008 ± 0.000 ^a
EEDd2	0.003 ± 0.000 ^a	0.005 ± 0.000 ^a	0.003 ± 0.000 ^a	0.044 ± 0.001 ^a	0.007 ± 0.000 ^a

Animals orally treated with: control - extract dilution vehicle (1% tween-80) in the proportion of 0.1mL/10g body weight (b.w.); EEDd1 - ethanolic extract of *Doliocarpus dentatus* at dose of 100 mg/kg (b.w.); EEDd2 - ethanolic extract of *Doliocarpus dentatus* at dose 1000 mg/kg (b.w.). Results are presented as mean ± standard error of the mean. Equal letters in the column indicate absence of statistically significant differences between the experimental groups (Statistical Test: one-way ANOVA with repetitive measures, p>0.05).

Table 2 – Reproductive parameters of pregnant females treated with *Doliocarpus dentatus* ethanol extract or extract dilution vehicle.

Parameters	Experimental Groups		
	Control	EEDd1	EEDd2
Implants ¹	15.00 ± 1.00 ^a	13.00 ± 1.74 ^a	15,10 ± 0,72 ^a
Live fetus ¹	13.70 ± 1.15 ^a	11.40 ± 1.54 ^a	13,00 ± 1,12 ^a
Dead fetus ¹	0.00 ± 0.00 ^a	0.01 ± 0.10 ^a	0,00 ± 0,00 ^a
Fetal viability ¹	90.34 ± 2.54 ^a	90.56 ± 5.11 ^a	85.47 ± 5.10 ^a
PILR ¹	9.66 ± 2.54 ^a	9.44 ± 5.11 ^a	14.53 ± 5.10 ^a
Resorption ¹	1.30 ± 0.26 ^a	1.50 ± 0.83 ^a	2.10 ± 0.71 ^a
Resorption rates ¹	9.66 ± 2.54 ^a	8.91 ± 4.69 ^a	14.53 ± 5.10 ^a
Placental weight (g) ²	0.09 ± 0.001 ^a	0.14 ± 0.051 ^b	0.09 ± 0.001 ^a
Placental index ²	0.08 ± 0.01 ^a	0.12 ± 0.048 ^a	0.08 ± 0.002 ^a
Fetal weight (g) ¹	1.13 ± 0.01 ^a	1.23 ± 0.01 ^b	1.11 ± 0.01 ^a
AWGA	-	HWGA	AWGA ₂
Sexual rate ¹	1.57 ± 0.45 ^a	1.93 ± 0.40 ^a	1.13 ± 0.25 ^a

PILR – Post-implantation loss rate; AWGA – Adequacy of weight to gestational age; HWGA – High weight for gestational age; AWGA₂ – Appropriate weight for the gestational age. Animals orally treated with: control: extract dilution vehicle (1% tween-80) in the proportion of 0.1mL/10g body weight (b.w.); EEDd1 - ethanolic extract of *Doliocarpus dentatus* at dose of 100 mg/kg (b.w.); EEDd2 - ethanolic extract of *Doliocarpus dentatus* at dose 1000 mg/kg (b.w.). Results are presented as mean ± standard error of the mean. Equal letters in the line indicate absence of statistically significant differences between the experimental groups (Statistical Test: one-way ¹ANOVA with repetitive measures, p>0.05; ²Kruskal-Wallis, p>0.05).

Table 3 - External malformations frequency in the offspring born of pregnant females treated with *Doliocarpus dentatus* Ethanol Extract or extract dilution vehicle.

Parameters	Experimental Groups		
	Control	EEDd1	EEDd2
External malformations in the forelimbs and hindlimbs			
Analyzed fetuses	137	113	130
Normal fetuses	119	94	112
Unilateral forelimb	3	2	7
Unilateral hindlimb	14	14	10
Bilateral hindlimb	1	3	1
External malformation frequency	18	19	18
% External malformation	13.14%	16.81%	13.85%
External malformations of tail			
Analyzed fetuses	137	113	130
Normal fetuses	129	105	125
Rolled up tail	8	8	5
External malformation frequency	8	8	5
% External malformation	5.84%	7.07%	3.85%
External malformations of column			
Analyzed fetuses	137	113	130
Normal fetuses	135	112	130
Scoliosis	2	1	0
External malformation frequency	2	1	0
% External malformation	1.46%	0.88%	0%

Animals orally treated with: control: extract dilution vehicle (1% tween-80) in the proportion of 0.1mL/10g body weight (b.w.); EEDd1 - ethanolic extract of *Doliocarpus dentatus* at dose of 100 mg/kg (b.w.); EEDd2 - ethanolic extract of *Doliocarpus dentatus* at dose 1000 mg/kg (b.w.). (Statistical Test: Chi-square, p>0.05).

Table 4 - Visceral malformations frequency in the offspring born of pregnant females treated with *Doliocarpus dentatus* Ethanol Extract or extract dilution vehicle.

Parameters	Experimental Groups		
	Control	EEDd1	EEDd2
Brain visceral malformation			
Analyzed fetuses	69	57	65
Normal fetuses	66	55	59
Severe Hydrocephalus	3	2	6
Visceral malformation frequency	3	2	6
% Visceral malformation	4.35%	3.51%	9.23%
Urogenital visceral malformation			
Analyzed fetuses	69	57	65
Normal fetuses	66	51	59
Light hydronephrosis	3	6	6
Visceral malformation frequency	3	6	6
% Visceral malformation	4.35%	10.53%	9.23%

Animals orally treated with: control: extract dilution vehicle (1% tween-80) in the proportion of 0.1mL/10g body weight (b.w.); EEDd1 - ethanolic extract of *Doliocarpus dentatus* at dose of 100 mg/kg (b.w.); EEDd2 - ethanolic extract of *Doliocarpus dentatus* at dose 1000 mg/kg (b.w.). (Statistical Test: Chi-square, $p > 0.05$).

Table 5 – Skeletal malformations frequency in the offspring born of pregnant females treated with *Doliocarpus dentatus* Ethanol Extract or extract dilution vehicle.

Parameters	Experimental Groups		
	Control	EEDd1	EEDd2
Skeletal malformations in the forelimbs and hindlimbs			
Analyzed fetuses	68	56	65
Normal fetuses	61	48	60
Reduced Ossification/ Absence of phalanges	7	8	5
Skeletal malformation frequency	7	8	5
% Skeletal malformation	10.29%	14.28%	7.69%
Skeletal malformations in the sternum bone			
Analyzed fetuses	68	56	65
Normal fetuses	57	44	51
Abnormal sternum Abnormal/Incomplet ossification	11	12	14
Skeletal malformation frequency	11	12	14
% Skeletal malformation	16.18%	21.43%	21.54%

Animals orally treated with: control: extract dilution vehicle (1% tween-80) in the proportion of 0.1mL/10g body weight (b.w.); EEDd1 - ethanolic extract of *Doliocarpus dentatus* at dose of 100 mg/kg (b.w.); EEDd2 - ethanolic extract of *Doliocarpus dentatus* at dose 1000 mg/kg (b.w.). (Statistical Test: Chi-square, $p > 0.05$).

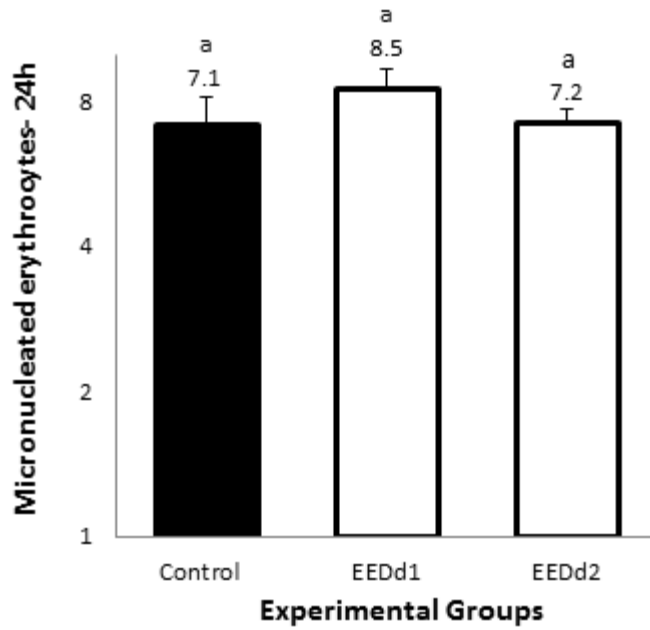


Figure 1 – Frequency of micronuclei analyzed in 2.000 erythrocytes of pregnant female mice treated with - ethanolic extract of *Doliocarpus dentatus* or extract dilution vehicle. Treated animals by gavage, orally with: control: extract dilution vehicle (1% tween-80) in the proportion of 0.1mL/10g body weight (b.w.); EEDd1 - ethanolic extract of *Doliocarpus dentatus* at dose of 100 mg/kg (b.w.); EEDd2 - ethanolic extract of *Doliocarpus dentatus* at dose 1000 mg/kg (b.w.). Results are presented as mean \pm standard error of the mean. Same letters indicate that there is no statistically significant differences between the experimental groups (Statistical Test: one-way ¹ANOVA with repetitive measures, $p > 0.05$).

6. CONSIDERAÇÕES FINAIS

Com base nos modelos experimentais e doses utilizadas nos ensaios executados, conclui-se que o extrato etanólico das folhas de *Doliocarpus dentatus*:

- Possui Atividade anti-inflamatória;
- Possui atividade contra *Mycobacterium tuberculosis*;
- Não é genotóxico;
- Não altera o desempenho reprodutivo;
- Não altera desenvolvimento embriofetal;
- Não é teratogênico.

Frente ao exposto, o EEDd indica boas condições para processos de bioprospecção.

ANEXOS



COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 20 de julho de 2016.

CERTIFICADO

Certificamos que a proposta intitulada **"Avaliação do efeito antihiperalgésico, antidepressivo e tóxico do extrato de *Blutaparon portulacoides* (raízes) e *Doliocarpus dentatus* em modelos experimentais"**, registrada sob o protocolo de nº 32/2015, sob a responsabilidade de *Cândida Aparecida Leite Kassuya* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 11/12/2015.

<i>Finalidade</i>	() Ensino (X) Pesquisa Científica
<i>Vigência da autorização</i>	01/08/2016 a 01/06/2019
<i>Espécie/linhagem/raça</i>	<i>Rattus norvegicus</i> /Wistar <i>Mus musculus</i> /Swiss e C57BL/6
<i>Nº de animais</i>	205
<i>Peso/idade</i>	200-250 g Wistar / 20-30 g <i>M. musculus</i>
<i>Sexo</i>	50 machos e 40 fêmeas Wistar / 95 machos Swiss e 20 machos C57BL/6
<i>Origem</i>	Biotério Central Da Universidade Fereal da Grande Dourados

Melissa Negrão Sepulveda
Coordenadora CEUA



C E R T I F I C A D O

Certificamos que a proposta intitulada "Avaliação dos efeitos antigenotóxico, antimutagênico, anticarcinogênico, apoptótico, imunoestimulatório e teratogênico do extrato etanólico de *Dolichocarpus dentatus* em sistemas testes *in vivo*", registrada com o nº 776/2016, sob a responsabilidade de **Rodrigo Juliano Oliveira** - que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA DA UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL/UFMS, na 2ª reunião extraordinária do dia 26/07/2016.

FINALIDADE	() Ensino (x) Pesquisa Científica
Vigência da autorização	01/06/2016 a 10/03/2018
Espécie/Linhagem/Raça	<i>Mus musculus</i> / Swiss <i>Rattus norvegicus</i> / Wistar
Nº de animais	100 / 40 80 / -
Peso/Idade	60 dias / 30 g 60 dias / 250 g
Sexo	Macho e Fêmea
Origem	Biotério Central/CCBS/UFMS

Maria Araújo Teixeira
Maria Araújo Teixeira
Coordenadora da CEUA/UFMS
Campo Grande, 26 de julho de 2016.



JOURNAL OF ETHNOPHARMACOLOGY

An Interdisciplinary Journal Devoted to Indigenous Drugs

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ISSN: 0378-8741

DESCRIPTION

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their **biological** and **pharmacological effects** based on the principles established through international conventions. Early people confronted with illness and disease, discovered a wealth of useful **therapeutic agents** in the plant and animal kingdoms. The empirical knowledge of these **medicinal substances** and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of **indigenous remedies**. Chemists continue to use **plant-derived drugs** (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

In recent years the preservation of local knowledge, the promotion of indigenous medical systems in primary health care, and the conservation of biodiversity have become even more of a concern to all scientists working at the interface of social and natural sciences but especially to ethnopharmacologists. Recognizing the sovereign rights of States over their natural resources, ethnopharmacologists are particularly concerned with local people's rights to further use and develop their autochthonous resources.

Accordingly, today's ethnopharmacological research embraces the multidisciplinary effort in the:

- documentation of **indigenous medical knowledge**,
- scientific study of **indigenous medicines** in order to contribute in the long-run to improved health care in the regions of study, as well as
- search for pharmacologically unique principles from existing indigenous remedies.

The *Journal of Ethnopharmacology* publishes original articles concerned with the observation and experimental investigation of the biological activities of plant and animal substances used in the traditional medicine of past and present cultures. The journal will particularly welcome interdisciplinary papers with an **ethnopharmacological**, an **ethnobotanical** or an **ethnochemical** approach to the study of indigenous drugs. Reports of **anthropological** and **ethnobotanical** field studies fall within the journal's scope. Studies involving **pharmacological** and **toxicological** mechanisms of action are especially welcome. Clinical studies on efficacy will be considered if contributing to the understanding of specific ethnopharmacological problems. The journal welcomes review articles in the above mentioned fields especially those highlighting the multi-disciplinary nature of ethnopharmacology. Commentaries are by invitation only.

AUDIENCE

Ethnopharmacologists, Medicinal Chemists, Pharmacologists, Toxicologists, Anthropologists, Pharmacognosists, Ethnobotanists, Economic Botanists, Ethnobiologists

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Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith, R.Z. (Eds.), *Introduction to the Electronic Age*. E-Publishing Inc., New York, pp. 281–304.

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