Universidade Federal de Juiz de Fora
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Mitracarpus frigidus (Rubiaceae): POTENCIAL FARMACOLÓGICO, AVALIAÇÃO TOXICOLÓGICA E IDENTIFICAÇÃO DE SUBSTÂNCIAS BIOATIVAS

Juiz de Fora
2013

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Mitracarpus frigidus (Rubiaceae): potencial farmacológico, avaliação toxicológica e identificação de substâncias bioativas

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas, Área de concentração Genética e Biotecnologia da Universidade Federal de Juiz de Fora, como requisito à obtenção do Título de Doutor em Ciências Biológicas.

Orientadora: Prof[a]. Dr[a]. Elita Scio Fontes

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Ou se a ciência as fazem fortes.

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RESUMO

Mitracarpus frigidus (Willd. ex Reem Schult.) K. Schum. (Rubiaceae) é uma espécie nativa, amplamente distribuída por todo território brasileiro, com poucos relatos na literatura sobre seu potencial químico-biológico. Estudos preliminares relataram que a planta apresenta atividade antimicrobiana, leishmanicida e antioxidante, e tem como principais constituintes alcaloides, quinonas, flavonoides, terpenos e esteroides. Com o intuito de dar continuidade aos estudos envolvendo esta espécie, este trabalho teve como objetivos avaliar sua toxidade e seu potencial farmacológico, bem como e isolar e caracterizar suas substâncias bioativas. Do extrato metanólico das partes aéreas de *M. frigidus* (MFM) foram isolados os triterpenos ácido ursólico e ursolato de metila, e a naftoquinona psicorubrina. Os flavonoides rutina e campferol também foram identificados. Utilizando essas substâncias como marcadores, MFM foi padronizado e avaliado para as atividades esquistossomicida, anti-inflamatória, laxativa e citotóxica. Além disso, sua toxidade aguda (DL_{50}) e subcrônica foram avaliadas. O potencial biológico e a composição química do extrato em hexano e do óleo essencial da espécie também foram analisados. MFM apresentou atividade esquistossomicida tanto *in vitro* quanto *in vivo* com considerável redução da carga parasitária. Também foi observada atividade anti-inflamatória aguda e crônica para os modelos testados, sendo a resposta aguda mais expressiva. MFM inibiu o processo de migração celular mediado pela inflamação e também diminuiu o processo oxidativo do organismo, evidenciado pela baixa concentração de MDA, catalase e mieloperoxidase. Em relação à expressão de ciclooxigenases, MFM inibiu COX-2. Atividade citotóxica contra células leucêmicas, HL60 e Jurkat, sem indução de apoptose também foi observada. Além disso, MFM induziu aumento do peristaltismo intestinal e da produção de fezes, o que pode estar relacionado com a presença de antraquinonas identificadas em MFM. A partir do estudo toxicológico agudo e subcrônico de MFM foi possível verificar que a planta tem baixa toxidade (DL_{50} > 2000 mg/Kg) e que não provocou alterações bioquímicas e hematológicas durante 42 dias de experimento. As substâncias ácido ursólico, ursolato de metila e psicorubrina, isoladas de MFM, apresentaram atividade citotóxica para células tumorais e para diferentes espécies de *Leishmania*. Da mesma forma, o extrato em hexano apresentou atividade antimicrobiana e leishmanicida, com especificidade e seletividade para formas intracelulares (amastigotas). O óleo essencial apresentou atividade antifúngica e leishmanicida. Linalol e acetato de eugenol foram identificados como as substâncias majoritárias. A partir deste trabalho, foi possível concluir que a espécie *M. frigidus*, que não possui relatos de uso na medicina popular, pode ser uma fonte alternativa de estudos para o tratamento de diversas doenças, como as provocadas por fungos, bactérias e parasitas, além de patologias relacionadas a processos inflamatórios.

Palavras-chave: *Mitracarpus frigidus*; psicorubrina; ácido ursólico; ursolato de metila; óleo essencial; terapia alternativa

ABSTRACT

Mitracarpus frigidus (Willd. ex Schult Reem.) K. Schum. (Rubiaceae) is a native species, widely distributed throughout the Brazilian territory, with few reports in the literature on its chemical-biological potential. Previous studies reported that the plant has antimicrobial, antioxidant and leishmanicidal, and that its main constituents are alkaloids, quinones, flavonoids, terpenes and steroids. In order to continue the studies involving this species, this work aimed to evaluate its toxicity and pharmacological potential, as well as to isolate and characterize its bioactive compounds. From the methanolic extract of the aerial parts of *M. frigidus* (MFM) the triterpenes ursolic acid and methyl ursolate, and the naphthoquinone psycorubrin were isolated. The flavonoids rutin and kaempferol were also identified. Using these compounds as markers, MFM was standardized and evaluated for schistosomicidal, anti-inflammatory, laxative and cytotoxic activity. Moreover, its acute (LD_{50}) and subchronic toxicity were evaluated. The biological potential and chemical composition of the hexane extract and the essential oil of this species were also analyzed. MFM showed both *in vitro* and *in vivo* schistosomicidal activity with considerable reduction in parasite burden. Antiinflammatory activity was also observed for the acute and chronic models tested, with a more proeminent acute response. Those results demonstrated that MFM inhibited the inflammation cell - mediated migration and also decreased the oxidative process of the organism, as evidenced by the observed low concentration of MDA, catalase and myeloperoxidase. Regarding the expression of cyclooxygenase, MFM inhibited COX-2. Cytotoxic activity against leukemia cells, HL60 and Jurkat, without apoptosis induction was also observed. Furthermore, MFM induced increase of intestinal peristalsis and production of faeces, which can be related to the presence of the anthraquinones identified in MFM. From the acute and subchronic toxicity study of MFM it was possible to verify that the plant has low toxicity (LD_{50} > 2000 mg/kg) and caused no hematological and biochemical changes during 42 days of experiment. The compounds ursolic acid, methyl ursolate and psycorubrin, isolated from MFM, showed cytotoxicity against different tumor cells and species of *Leishmania*. Likewise, the hexane extract showed antibacterial and antileishmanial properties, with specificity and selectivity for intracellular forms (amastigotes). The essential oil showed antifungal and leishmanicidal activity. Linalool and eugenol acetate were identified as its major compounds. In conclusion, the species *M. frigidus*, which has no reports on its use in traditional medicine, could be an alternative source of study for the treatment of various diseases such as those caused by fungi, bacteria and parasites, and pathologies related to inflammatory processes.

Keywords: *Mitracarpus frigidus*; psycorubrin; ursolic acid; methyl ursolate; essential oil; alternative therapy

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LISTA DE ABREVIATURAS E SIGLAS

AcOEt	Acetato de etila
CH_2Cl_2	Diclorometano
$CHCl_3$	Clorofórmio
Da	Dalton
DMSO	Dimetilsulfóxido
ED_{50}	*Median Effective Dose*/ Dose Efetiva Mediana
EPP	*Ethyl Phenylpropiolate*/ Fenilpropiolato de Etila
GC/MS	*Gas Chromatography - Mass Spectometry*/ Cromatografia Gasosa - Espectrômetro de Massas
HH-COSY	*Homonuclear Correlated Sprectroscopy*/ Espectroscopia de Correlação
HMBC	*Heteronuclear Multipe Bond Correlation*/ Correlação Heteronuclear de Múltiplas Ligações
HMQC	*Heteronuclear Multiple Quantum Coherence*/ Coerência Heteronuclear Múltiplo-Quântica
HPLC	*High Pressure Liquid Chromatography*/ Cromatografia Líquida de Alta Eficiência
IC_{50}	*Median Inhibitory Concentration*/ Concentração Mediana de Inibição
IV	Infravermelho
LC_{50}	*Median Lethal Concentration*/ Concentração Letal Mediana
LD_{50}/ DL_{50}	*Median Lethal Dose* / Dose Letal Mediana
MDA	*Malondialdehyde*/ Malonaldeído
MeOH	Metanol

MFH	Extrato Hexânico das Partes Aéreas de *Mitracarpus frigidus*
MFM	Extrato Metanólico das Partes Aéreas de *Mitracarpus frigidus*
MIC	*Minimum Inhibitory Concentration*/ Concentração Inibitória Mínima
MSI	*Microbial Susceptible Index*/ Índice de Susceptibilidade Microbiana
NP/PEG	*Natural Products Reagent*/ Reagente de Produtos Naturais
OD	*Optical Density/* Densidade Óptica
RMN de ^{13}C	Ressonância Magnética Nuclear de Carbono
RMN de ^{1}H	Ressonância Magnética Nuclear de Hidrogênio
ROS	*Reactive Oxigen Species*/ Espécies Reativas de Oxigênio
TLC	*Thin Layer Chromatography*/ Cromatografia de Camada Delgada
UV	Ultravioleta
WHO/OMS	*World Health Organization*/ Organização Mundial da Saúde

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos, os quais se encontram no item ARTIGOS CIENTÍFICOS. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências encontram-se nos próprios artigos e representam a íntegra deste estudo.

O item CONSIDERAÇÕES FINAIS encontrado no final desta tese, apresenta comentários gerais sobre todos os artigos científicos contidos neste trabalho. E no item ANEXO, estão todas as informações das substâncias identificadas e isoladas.

O item REFERÊNCIAS refere-se somente as citações que aparecem nos itens INTRODUÇÃO e REVISÃO BIBLIOGRÁFICA.

1 INTRODUÇÃO

Nos últimos anos, a frequência de resistência microbiana e a associação deste evento com doenças infecciosas graves têm aumentado de forma progressiva (N'GUESSAN et al., 2007). Muitos micro-organismos têm desenvolvido resistência tanto contra os já bem estabelecidos antibióticos de uso convencional, quanto contra aqueles de última geração, causando graves problemas de saúde pública e prejuízos econômicos (SPELLBERG, BARTLETT e GILBERT, 2013).

Além dos problemas enfrentados devido a resistência microbiana, destacam-se aqueles específicos de cada doença, como os relacionados a leishmaniose, a esquistossomose e a neoplasias. As duas primeiras são consideradas doenças negligenciadas e que causam sérios problemas de saúde pública na maioria dos países tropicais e subtropicais (STEINMANN et al., 2006) e o câncer, por sua vez, ocorre em todas as regiões do planeta e acomete um número cada vez maior de pessoas, sendo uma das principais causas de morte em todo o mundo (WHO, 2008).

Se forem traçados paralelos entre estas doenças, um elo entre as mesmas seria, sem dúvida, as limitações em seu tratamento. Isto ocorre devido a vários problemas relacionados à toxidez, resistência ou variações de sensibilidade, além daqueles específicos a cada uma delas (BOTROS et al., 2003; HAGAN et al., 2004). Também o aparecimento de muitos micro-organismos resistentes aos já bem estabelecidos antibióticos de uso convencional justifica a busca constante de drogas mais eficazes, seletivas e com menos efeitos colaterais.

Durante séculos as plantas representaram a única fonte de agentes terapêuticos para o homem (COUTINHO et al., 2004; MELO et al., 2009), sendo utilizadas no tratamento de doenças e disfunções do organismo humano devido às suas propriedades terapêuticas e tóxicas (MARTINS et al., 2000). No início do século XIX, com o desenvolvimento da química, as plantas passaram a ser a primeira fonte de substâncias para o desenvolvimento de medicamentos (MELO et al., 2009). Nas últimas décadas tem-se verificado um grande avanço científico envolvendo estudos químicos e farmacológicos de plantas medicinais que visam obter novas moléculas com propriedades terapêuticas (LU et al., 2007; MBWAMBO et al., 2009). As plantas produzem uma grande variedade de metabólitos que

ganham importância devido a suas aplicações terapêuticas, biotecnológicase nutracêuticos. A produção dos mesmos é influenciada por fatores ambientais e genéticos e está relacionada com sua defesa contra patógenos (bactérias, fungos ou vírus) e herbívoros ou com a competição com espécies vizinhas, inibindo seu crescimento (SINGH e BHAT, 2003).

Tais metabólitos possuem grandes potencialidades como princípios ativos, podendo ser utilizados diretamente na terapia, como precursores na semi-síntese farmacêutica ou em modelos para síntese de novos princípios ativos (VIEGAS, BOLZANI e BARREIRO, 2006). Assim, apesar dos avanços da química tradicional e da farmacologia, o reino vegetal pode prover uma rica fonte de novos fármacos e medicamentos (SINGH e BHAT, 2003).

A biodiversidade brasileira é considerada fonte de substâncias biologicamente ativas tornando sua preservação essencial, não apenas pelo valor intrínseco dessa imensa riqueza biológica, mas também pelo seu enorme potencial como fonte de novos fármacos. Diante disso, várias pesquisas de bioprospecção dos nossos biomas vêm sendo incrementadas objetivando a busca racional de bioprodutos de valor agregado (BARREIRO, 2009).

Na última década, a maneira de se pesquisar novas substâncias biologicamente ativas sofreu grandes mudanças, principalmente devido aos avanços tecnológicos. A indústria farmacêutica tem um papel importante no desenvolvimento de novos métodos que podem propiciar, de forma mais rápida, o surgimento de novos medicamentos no mercado (PONTIN et al., 2008).

Um dos mais importantes fatores para o sucesso no descobrimento de um fármaco novo é a diversidade química das substâncias a serem selecionadas, cujas fontes podem ser moléculas sintéticas, produtos naturais ou química combinatória (via biotecnologia). Entre estas possibilidades, os produtos naturais são considerados como uma das maiores fontes de diversidade química (DA SILVA FILHO et al., 2004). Dessa forma, uma das estratégias para a descoberta de novas drogas consiste na triagem de extratos naturais (plantas, fungos, bactérias, animais, organismos marinhos) em modelos biológicos adequados para a identificação de substâncias que sirvam de modelo para o desenvolvimento de novos agentes quimioterápicos. Esta estratégia é especialmente importante para os países

tropicais, que detêm a maior parte da biodiversidade do planeta (BRANDON et al., 2005).

Os extratos de muitos organismos vivos são ricos em substâncias químicas produzidas pelo metabolismo especial para a comunicação intra- e inter- específica, para defesa contra infecções e ataques de predadores, proteção contra formas radicalares de oxigênio e em resposta ao estresse ambiental. Estas substâncias são denominadas produtos naturais (PNs) e apresentam grande diversidade estrutural e uma gama enorme de atividades farmacológicas (HARVEY, 2008). A maioria dos PNs bioativos são de baixo peso molecular (<1000 Da), o que facilita sua absorção e metabolização, tornando-os úteis como drogas para o tratamento de diversas doenças (COLEGATE e MOLYNEUX, 2008). Segundo estes mesmos autores, a busca de PNs bioativos se constitui em uma estratégia de sucesso na descoberta de novos medicamentos.

Enquanto alguns extratos são analisados do ponto de vista da sua composição química, outros têm suas atividades biológicas testadas em vários sistemas de bioensaios. Estas abordagens, quando conduzidas em separado, se constituem num fator limitante na busca de PNs bioativos, pois não correlacionam informações químicas e biológicas (HARVEY, 2008). Por considerar importante a correlação entre estas informações, buscou-se por meio desta, identificar PNs bioativos de uma das espécies do programa de triagem biológica realizado no Laboratório de Produtos Naturais Bioativos do Departamento de Bioquímica – ICB/UFJF, no qual foram testadas dezenas de extratos vegetais em vários modelos biológicos, muitos deles mostraram-se suficientemente ativos para justificar seu fracionamento químico.

A espécie selecionada para esta pesquisa foi *Mitracarpus frigidus* (Willd. ex Reem Schult.) K. Schum, nativa do Brasil, que apresenta poucos relatos na literatura sobre as suas propriedades biológicas. Estudos preliminares demonstraram que a espécie possui atividade leishmanicida, antimicrobiana e antioxidante, o que a torna bastante interessante do ponto de vista farmacológico (FABRI, 2008; FABRI et al., 2009).

Com o intuito de dar continuidade a estudos envolvendo esta espécie, propõe-se isolar e caracterizar as substâncias bioativas, bem como avaliar sua toxidade e seu potencial farmacológico.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Realizar estudos farmacológicos/biológicos e toxicológicos de *Mitracarpus frigidus* e identificar as substâncias potencialmente bioativas.

2.2 OBJETIVOS ESPECÍFICOS

2.2.1 Avaliar o potencial antimicrobiano e leishmanicida da partição hexânica das partes aéreas de *M. frigidus;*

2.2.2 Isolar e identificar as substâncias potencialmente ativas de *M. frigidus*;

2.2.3 Verificar o potencial biológico das substâncias isoladas no item 2.2.2;

2.2.4 Padronizar o extrato metanólico das partes aéreas de *M. frigidus*;

2.2.5 Avaliar o potencial esquistossomicida *in vitro* e *in vivo* do extrato metanólico de *M. frigidus;*

2.2.6 Avaliar o potencial anti-inflamatório *in vivo* do extrato metanólico de *M. frigidus;*

2.2.7 Avaliar o potencial laxativo *in vivo* do extrato metanólico de *M. frigidus;*

2.2.8 Avaliar o potencial tóxico para células tumorais *in vitro* do extrato metanólico de *M. frigidus;*

2.2.9 Avaliar o potencial toxicológico agudo (DL_{50}) e subcrônico do extrato metanólico de *M. frigidus;*

2.2.10 Analisar a composição química e o potencial antimicrobiano, antioxidante e leishmanicida do óleo essencial *de M. frigidus*.

3 REVISÃO BIBLIOGRÁFICA

3.1 DOENÇAS TROPICAIS E NEGLIGENCIADAS

3.1.1 ESQUISTOSSOMOSE MANSÔNICA

3.1.1.1 ASPECTOS GERAIS DA ESQUISTOSSOMOSE MASÔNICA

A esquistossomose está presente na população humana desde a antiguidade. Há registros de que foram encontradas lesões típicas da doença e antígenos do parasito detectados por anticorpos monoclonais em eluato de tecido de múmias egípcias da XX dinastia (aproximadamente 3000 anos de idade). O principal hospedeiro vertebrado do *Schistosoma mansoni* é o homem, que possui vital importância para a manutenção da sua sobrevivência, o que torna bastante razoável supor que a relação entre o homem e o *S. mansoni* se situe em tempos ainda mais remotos (NEVES et al., 2005).

A esquistossomose é uma das doenças parasitárias mais importantes em todo o mundo, sendo prevalente em regiões tropicais e subáreas tropicais. Mais de 230 milhões de pessoas estão infectadas, com uma estimativa de 700 milhões de pessoas em situação de risco em 77 países endêmicos (WHO, 2012).

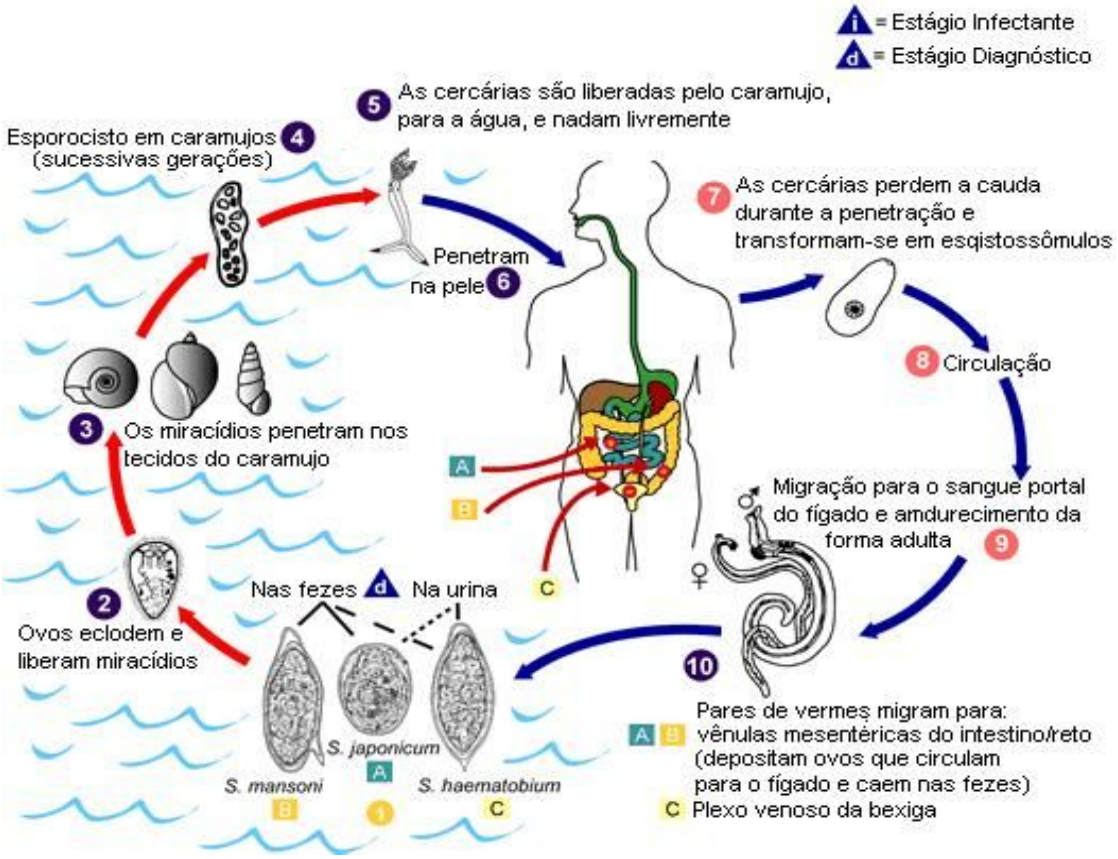
Essa infecção é endêmica na América do Sul (Brasil, Suriname e Venezuela), Caribe (incluindo Porto Rico, Santa Lúcia, República Dominicana), maioria dos países do oeste equatorial e centro sul da África e da península Arábica (FERRARI, MOREIRA e CUNHA, 2008; WHO, 2012).

No Brasil, a introdução do *S. mansoni* ocorreu por meio do tráfico de escravos vindos da África. Em terras brasileiras o *S. mansoni* encontrou o seu hospedeiro intermediário *(Biomphalaria* sp.), o que permitiu a instalação e proliferação desta espécie. O *S. haematobium* foi também introduzido pelos escravos africanos, e posteriormente o *S. japonicum* com os imigrantes oriundos do continente asiático, mas a instalação dessas duas últimas espécies não ocorreu devido à ausência dos hospedeiros intermediários suscetíveis (*S. japonicum*, cujo hospedeiro suscetível é o caramujo do gênero *Oncomelania* e o *S. haematobium* é o caramujo do gênero

Bulinus). Como os escravos africanos foram trazidos inicialmente para trabalharem nos engenhos de cana-de-açúcar, os primeiros focos da doença se instalaram na região canavieira do Nordeste, posteriormente com os movimentos migratórios à doença foi se expandindo para outras regiões do país. A existência de clima apropriado para a transmissão e as condições sócio-econômicas precárias (saneamento básico, educação sanitária, entre outras) permitem a manutenção da endemia nas áreas onde foram implantadas (NEVES et al., 2005).

3.1.1.2 CARACTERÍSTICAS MORFOLÓGICAS E CICLO EVOLUTIVO DO *Schistosoma mansoni*

A morfologia do *S. mansoni* deve ser estudada nas várias fases do seu complexo ciclo biológico, que é composto por duas fases parasitárias: uma no hospedeiro definitivo (vertebrado/homem) e outra no hospedeiro intermediário (invertebrado/caramujo). Há ainda duas passagens de larvas de vida livre no meio aquático, que se alternam com as fases parasitárias. As fases evolutivas consistem no verme adulto (macho e fêmea), ovo, miracídio, esporocisto e cercária (CIMERMAN e CIMERMAN, 2002). O ciclo evolutivo do parasito leva por volta de 80 dias para ser completado, isso considerando condições favoráveis. O ciclo é sexuado no homem e o período decorrido entre a penetração das cercárias e o encontro de ovos nas fezes é cerca de 40 dias. No molusco, o ciclo é feito de modo assexuado e tem uma duração aproximada de 40 dias (Figura 1) (KATZ e ALMEIDA, 2003).



Fonte: http://www.cve.saude.sp.gov.br/gif/hidrica/ciclo_esquisto.jpg

Figura 1 - Ciclo biológico do *Schistosoma mansoni.*

O *S. mansoni* macho mede aproximadamente 1 cm, possui cor esbranquiçada com tegumento recoberto de minúsculas projeções denominadas de tubérculos e apresenta o corpo dividido em duas porções: anterior, na qual é encontrada a ventosa oral e a ventosa ventral chamada acetábulo, e a posterior onde é encontrado o canal ginecóforo que consiste em dobras laterais do corpo no sentido longitudinal, para albergar a fêmea e fecundá-la. O verme não possui órgão copulador, então os espermatozoides passam pelos canais deferentes, que se abrem no poro genital dentro do canal ginecóforo, alcançando à fêmea e fecundando-a (NEVES et al., 2005).

A fêmea possui corpo cilíndrico, mais longo e fino comparado com o macho (REY, 2002). Ela tem um comprimento de aproximadamente 1,5 cm, com tegumento liso e apresenta uma coloração escura devido ao ceco conter sangue semi digerido. Na metade anterior é encontrada a ventosa oral e o acetábulo, seguidos destes temos a vulva, depois útero com um ou dois ovos, e ovário. A metade posterior é

preenchida pelas glândulas vitelogênicas e o ceco (NEVES et al., 2005). O revestimento externo dos vermes adultos é formado por uma espessa citomembrana que recobre o tegumento e que apresenta em sua superfície grande quantidade de pequenos tubérculos, mais abundantes na superfície dorsal (REY, 2002).

O ovo apresenta formato oval e a parte mais larga um espículo voltado para trás. O que caracteriza o ovo maduro é a presença de uma miracídio formado e visível pela transparência da casca (KATZ e ALMEIDA, 2003). Na ocasião da postura, os embriões (miracídios) dos ovos estão em formação, e apenas depois de alguns dias que os mesmos amadurecem e se tornam aptos a eclodir. Logo após abandonarem a casca, as larvas nadam ativamente, percorrendo grandes círculos. O miracídio é o primeiro estágio de vida livre do *Schistosoma* (REY, 2002).

Os miracídios de *S. mansoni* apresentam fototropismo, buscando sempre áreas mais claras, embora não tenham olhos ou órgão fotossensíveis conhecidos. Nadam preferencialmente próximo a superfície da água, sendo capazes de buscar o molusco hospedeiro em profundidades de um ou dois metros (CIMERMAN e CIMERMAN, 2002).

O miracídio, após penetrar no interior do molusco, se transforma em esporocisto (CIMERMAN e CIMERMAN, 2002). Na sua fase de desenvolvimento se apresenta como esporocisto primário (forma de tubo enovelado imóvel e contendo várias células germinativas em multiplicação) e como esporocisto secundário que possui a mesma arquitetura, mas depois se rompe liberando 20 a 40 esporocistos filhos (FERREIRA, FORONDA e SCHUMAKER, 2003).

A formação do esporocisto secundário no interior do esporocisto primário inicia-se a partir do 14^{o} dia após a penetração do miracídio no molusco, no interior do esporocisto secundário encontram-se células germinativas aglomeradas em grandes vacúolos (KATZ e ALMEIDA, 2003). Os esporocistos secundários migram para o hepatopâncreas e o ovostestis do molusco, o qual continuam a crescer até alcançar a maturidade. Apresenta na extremidade anterior uma protuberância móvel e um poro para a eliminação de cercarias, levando em média 3 a 4 semanas o tempo para a maturação dos esporocistos filhos e formação das primeiras cercárias (REY, 2002).

As cercárias quando saem dos esporocistos, entram nos espaços sanguíneos que envolvem o hepatopâncreas e ovotéstis, migrando pela corrente

circulatória do molusco chegam a algumas áreas muito vascularizadas do tegumento. Provocam a formação de pequenas vesículas na superfície externa, e ao romper a vesícula saem para o meio exterior (FERREIRA, FORONDA e SCHUMAKER, 2003). As cercárias possuem ventosas que auxiliam na sua fixação na pele do hospedeiro facilitando o processo de penetração (NEVES et al., 2005).

Após penetração no hospedeiro, as cercarias se transformas em esquistossômulos que consistem em larvas alongadas, cujos tegumentos modificaram e adquiriram propriedades novas, inclusive resistência a ação lítica do soro. Os esquistossômulos logo se adaptam ao meio interno isotônico do hospedeiro definitivo facilitando a sua entrada nos vasos sanguíneos ou linfáticos do mesmo (KATZ e ALMEIDA, 2003).

O esquistossômulo fica no tecido por dois a três dias, e no fim desse período, se o parasita não foi destruído pelo sistema de defesa do hospedeiro, penetra em um vaso cutâneo e segue via circulação sanguínea em direção ao coração e aos pulmões. Após uma semana, os esquistossômulos se fixam no sistema porta hepático, aí crescem e amadurecem até o fim da quarta semana (REY, 2002).

O desenvolvimento dos helmintos se completa no sistema porta intra-hepático depois de quatro semanas após a chegada do esquistossômulo nesse local, depois migram para as vênulas mais finas, que são tributárias da veia mesentérica inferior e para o plexo hemorroidário superior (REY, 2002). Quando atinge a vida adulta do seu ciclo biológico no sistema vascular do homem e de outros mamíferos, o *S. mansoni* alcança as veias mesentéricas (principalmente a veia mesentérica inferior), migrando contra a corrente circulatória (NEVES et al., 2005).

O acasalamento entre os parasitas ocorre geralmente depois da terceira semana após a chegada do esquistossômulo ao fígado, ocorrendo nesse período o início da atração entre os sexos. O aparelho reprodutor feminino se desenvolve muito lentamente, se não houver acasalamento com os machos e fecundação (REY, 2002). As fêmeas fazem postura no nível da submucosa, sendo que cada fêmea faz postura de aproximadamente 400 ovos por dia, na parede de capilares e vênulas, e cerca de 50% destes chegam ao meio externo (NEVES et al., 2005).

Os ovos colocados nos tecidos levam em média cerca de uma semana para amadurecerem, ou seja, formarem miracídio e da submucosa chegam à luz intestinal. Essa migração demora dias, decorre um período mínimo de seis dias, tal

tempo é necessário para que haja a maturação dos ovos. Os ovos podem ficar presos na mucosa intestinal ou serem arrastados para o fígado. Quando chegam à luz intestinal, são arrastados para o exterior juntamente com o bolo fecal, alcançando a água, os ovos liberam o miracídio, estimulado por fatores como temperatura alta, luz intensa e oxigenação da água (NEVES et al., 2005).

Os ovos maduros do *S. mansoni* têm uma expectativa de vida de aproximadamente 20 dias, e caso não ocorra a expulsão do ovo dentro de três a quatro semanas após a oviposição o miracídio morre. No meio exterior, os ovos sobrevivem de 2 a 5 dias na massa fecal sólida e na líquida apenas 1 dia, devido aos processos de fermentação ou de putrefação das fezes (KATZ e ALMEIDA, 2003).

3.1.1.3 CARACTERÍSTICAS CLÍNICAS E PATOLÓGICAS DA ESQUISTOSSOMOSE

No hospedeiro há o desenvolvimento de três fases evolutivas importante para a patogenia da doença que são o esquistossômulo, adulto e ovos, relacionados com alterações morfológicas, bioquímicas e antigênicas do verme (HUGGINS, BATISTA e MEDEIROS, 1998). A patogenia da esquistossomose mansônica é dependente de diversos fatores importantes como a carga parasitária, a linhagem do parasito e as características do hospedeiro e seu meio, do qual são relevantes aspectos relacionados à idade, estado nutricional e estado imunológico que é importante para regular a carga parasitária, determinação da sintomatologia e controle de reinfecções; sendo difícil a distinção entre ação direta do parasita e seus elementos ou se é resposta do hospedeiro (CIMERMAN e CIMERMAN, 2002).

Um mesmo individuo em determinado decurso de tempo apresenta uma variação na carga parasitária. Nas áreas endêmicas a carga parasitaria pode aumentar de acordo com a idade, alcançando pico máximo entre as idades de 15, 20 e 25 anos. A carga parasitária é um importante fator na determinação da gravidade da doença (REY, 2002).

Nos diferentes estágios que o verme apresenta, são desenvolvidos mecanismos de escape. O esquistossômulo até chegar à fase adulta desenvolve algumas mudanças que o torna menos vulnerável a ação do sistema de defesa do

hospedeiro. O verme adulto apresenta uma maior resistência às defesas do hospedeiro. O ovo é o principal fator patogênico na esquistossomose, sendo que o ovo maduro e o antígeno solúvel eliminado pelos poros dos ovos são responsáveis pela reação inflamatória granulomatosa nos tecidos. As cercarias de *S. mansoni* produzem dermatite cercariana quando penetram na pele do homem, ocorrendo no local da penetração eritema, edema, pápula e dor (CIMERMAN e CIMERMAN, 2002).

As manifestações clínicas da doença esquistossomose ocorrem em três fases: a primeira, ou dermatite cercarial, que é uma reação aguda à penetração da pele por cercarias. A segunda é uma reação da doença do soro, febre de Katayama, que ocorre 6 a 8 semanas depois da exposição e apresenta como manifestação febre, urticária, dor abdominal, diarréia e dor muscular. O terceiro estágio consiste numa infecção crônica da maioria dos órgãos internos como fígado, baço, bexiga, coração e pulmões (ROWLAND, 2002).

A febre de Katayama se inicia com a produção de ovos e ao aumento da estimulação antigênica do hospedeiro. As manifestações ocorrem aproximadamente de 3 a 7 semanas após a infecção, podendo haver sintomas como febre, anorexia, mal-estar, dor abdominal, diarréia, cefaléia, hepatoesplenomegalia e eosinofilia. Depois de 6 meses, o quadro clínico da infecção pode evoluir para a esquistossomose crônica (SILVA, 2008a).

Na fase aguda, as manifestações mais graves acontecem após reinfecções e em indivíduos mais hipersensíveis, podendo manter-se por dois ou três dias. Além disso, pode ocorrer um moderado processo inflamatório nos pulmões e fígado, devido à migração dos esquistossômulos, e a partir dos vermes adultos pode acontecer obstrução embólica de vasos, necrose em volta dos tecidos por morte dos vermes, sendo posteriormente ser substituído por tecido cicatricial (CIMERMAN e CIMERMAN, 2002).

O estágio crônico é caracterizado pela reação granulomatosa, desenvolvida a partir da liberação de antígenos e enzimas dos ovos do *S.mansoni.* Isso demonstra a importância do ovo como agente patogênico, que produz efeito mais nocivo que o próprio verme adulto. Apenas metade dos ovos produzidos consegue alcançar a luz intestinal para serem eliminados, mas muitos dos ovos que não conseguem deixar o organismo do hospedeiro continuam a evoluir até produzirem miracídio, mesmo

estando na parede do intestino, fígado ou em outros órgãos, tais ovos desencadeiam uma reação inflamatória e posterior formação de granuloma (CIMERMAN e CIMERMAN, 2002).

As formas clínicas crônica da esquistossomose são: I) Formas assintomáticas e intestinais, os sintomas são geralmente vagos, com predominância de diarréia, sangue e muco de permeio com as fezes, dores abdominais, anorexia, estado nauseoso e sintomas nervosos como fadiga e cefaléia (PESSÔA e MARTINS, 1988); II) Forma hepatointestinal, com diarréia, epigastralgia e hepatomegalia, havendo presença de lesões hepáticas discretas; III) Forma hepatoesplênica compensada é caracterizada pelo envolvimento do fígado e do baço, havendo hepatoesplenomegalia, hipertensão portal, presença de varizes esofagogástricas cuja ruptura é responsável por hemorragias geralmente graves (SILVA, 2008a). Na forma hepatoesplênica descompensada há maior tendência a hemorragia, ascite e edemas, com aparecimento de circulação colateral superficial e insuficiência hepática severa; IV) Forma cardiopulmonar, decorrente das condições hemodinâmicas da esquistossomose hepatoesplênica, havendo tosse com laivos de sangue, febre, sinais de bronquite ou broncopneumonia e crises asmáticas (BRASIL, 2005).

A sintomatologia da infecção pode variar muito, pois depende da localização e intensidade das lesões, fator que leva a diferentes formas clínicas da doença que tem início na fase aguda, passando pela fase crônica, chegando até a forma compensada que pode se apresentar com ou sem hipertensão, podendo evoluir para a forma descompensada com formação de ascite e também complicações vasculopulmonares, icterícia e encefalopatias (NEVES et al., 2005).

Há várias formas ectópicas de esquistossomose mansônica, principalmente com envolvimento do sistema nervoso central, podendo encontrar os ovos do *S. mansoni* em praticamente todos os órgãos do hospedeiro como os pulmões, pâncreas, rins, sistema genito-urinário, linfonodos, tireóide e coração (SILVA, 2008a). Ovos e granulomas podem ser encontrados no esôfago, estômago, apêndice, vesícula biliar (causando colecistite catarral ou ulcerosa) e peritônio, atingindo excepcionalmente o ureter, bexiga, pênis, escroto, vesícula seminal e testículo. No sistema genital feminino é de incidência mais rara, acometendo tubas,

ovários, endométrio (produzindo hemorragias), abscesso perianal, fístula retovaginal e anal (BOGLIOLO e BRASILEIRO FILHO, 2006).

3.1.1.4 DIAGNÓSTICO E TRATAMENTO DA ESQUISTOSSOMOSE MANSÔNICA

O diagnóstico da esquistossomose crônica geralmente baseia-se no encontro de ovos de *S. mansoni* nas fezes. Recomenda-se submeter pelo menos três amostras fecais a técnicas rotineiras de concentração ou de Kato-Katz para o diagnóstico de infecções leves. A escolha entre técnicas quali ou quantitativas depende do contexto clínico e epidemiológico em que os resultados serão interpretados (FERREIRA, FORONDA e SCHIMAKER, 2003).

O teste de eclosão de miracídeos é empregado em situações em que se deseja avaliar a viabilidade dos ovos eliminados nas fezes, principalmente no acompanhamento de pacientes em tratamento (NEVES et al., 2005).

Nos casos de infecção crônica, a eliminação dos ovos pelas fezes pode ficar reduzida em função de sua retenção na mucosa do intestino grosso e reto. Portanto, torna-se necessária a realização de uma biópsia retal para a identificação dos ovos retidos em casos suspeitos e que o exame de fezes são repetidamente negativo (FERREIRA, FORONDA e SCHIMAKER, 2003).

O objetivo básico da farmacoterapia no controle da esquistossomose pressupõe a redução da prevalência e prevenção de formas mais graves da doença. Três fármacos são utilizados atualmente: o metrifonato, ativo contra *S. haematobium*; a oxamniquina, ativa contra *S. mansoni* e o praziquantel, ativo contra todas as espécies de *Schistosoma* de interesse humano (BRASIL, 2002). Tem sido observada em experiências controladas, a existência de helmintos com baixa susceptibilidade aos fármacos empregados (DRESCHER et al., 1993; COURA, 1995; FALLON, SMITH e DUNNE, 1995; PENIDO, COELHO e NELSON, 1999), desencadeando a emergente necessidade de se desenvolverem novos esquistossomicidas, bem como moduladores dos processos inflamatórios presentes nessa parasitose (PYRRHO et al., 2002; RIBEIRO-DOS-SANTOS, VERJOVSKI-ALMEIDA e LEITE, 2006).

Diversas plantas já possuem sua atividade esquistossomicida relatada, sendo uma alternativa para o tratamento dessa doença. Ruiz et al. (2005) identificaram que

o uso do extrato hidroetanólico de *Eleocharis sellowiana* Kunth, em experimento *in vitro*, mostrou-se ser ativo no processo de desova do caramujo *Biomphalaria glabrata*, uma vez que a maioria dos embriões morria entre o terceiro e o sétimo dia após a exposição ao extrato. Já no estudo realizado por Hamed e Hetta (2005), *Citrus reticulata* L., foi utilizada para o tratamento da esquistossomose em modelos experimentais *in vivo* e foi verificado que além da atividade esquistossomicida, os extratos foram capazes de proteger o fígado (hepatoproteção).

Outros pesquisadores também observaram a relação direta da atividade esquistossomicida com a capacidade antioxidante dos extratos, os quais protegem o tecido hepático do dano oxidativo da infecção por *S. mansoni* (EL-SHENAWY, SOLIMAN e ABDEL-NABI, 2006; EL-SHENAWY, SOLIMAN e REYAD, 2008).

Os extratos de *Zingiber officinalis* Roscoe (SANDERSON, BARTLETT e WHITFIELD, 2002) e dos frutos de *Balanites aegyptiaca* (L.) Del. (Hegleig) (KOKO et al., 2005), apresentaram excelentes resultados quando administrados para o tratamento da doença em modelos experimentais *in vitro* e *in vivo*. Ambos foram capazes de reduzir o número de parasitas recuperados dos animais tratados, por perda de motilidade ou completa morte dos parasitas, redução da carga parasitária e contagem de ovos encontrados nos tecidos e nas fezes.

Além de extratos de plantas medicinais, compostos isolados de vegetais também já foram relatados. A maioria dessas substâncias apresentou atividade esquistossomicida *in vitro,* por meio de destruição do tegumento do verme, alterando sua motilidade, ou por provocando a sua morte (MAGALHÃES et al., 2009; MORAES et al., 2010; HOLTFRETER et al., 2011; MAGALHÃES et al., 2011).

Curcumina, um derivado polifenólico extraído de pimentas turméricas, mostrou-se significativo modulador da resposta imune celular e humoral em camundongos infectados, reduzindo o número de parasitas presentes nesses animais e patologias hepáticas decorrentes da infecção aguda por essa parasitose (ALLAM, 2009).

3.1.2 LEISHMANIOSE

3.1.2.1 ASPECTOS GERAIS DA LEISHMANIOSE

As leishmanioses são doenças infecto-parasitárias de caráter antropozoonótico, não contagiosas, de transmissão vetorial, causadas por diferentes espécies de protozoários flagelados pertencentes ao gênero *Leishmania* (ASHFORD, 2000; CRUZ et al., 2009). Este gênero compreende várias espécies pertencentes à ordem *Kinetoplastida*. São parasitos unicelulares, com ciclo de vida heteroxênico, apresentando-se sob a forma promastigota flagelada extracelular no trato digestivo dos hospedeiros invertebrados (flebótomos) e sob a forma amastigota intracelular obrigatória de células do sistema mononuclear fagocitário (SMF) dos hospedeiros vertebrados (DINIZ, 2004; RODRIGUES, 2005).

As leishmanioses possuem ampla distribuição geográfica incluindo regiões tropicais, subtropicais e certas zonas temperadas, sendo considerada endêmica em parte da Ásia, Índia, América Central e do Sul, Baixo Mediterrâneo e alguns países da Europa (WHO, 2010).

De acordo com a Organização Mundial de Saúde (OMS), as leishmanioses acometem 15 milhões de pessoas no mundo, com 2 milhões de novos casos a cada ano (WHO, 2010) e, por esta razão, tem sido considerada como uma das seis principais doenças tropicais infecciosas. No Brasil, de acordo com os dados do Ministério da Saúde/ Fundação Nacional de Saúde (BRASIL, 2002) as leishmanioses encontram em franca expansão geográfica, constituindo um importante problema de saúde pública pela sua magnitude, transcendência e pouca vulnerabilidade às medidas de controle.

Das 14 espécies de Leishmanias encontradas no Brasil, 10 conhecidamente infectam o homem (CRUZ et al., 2009). Dependendo de diversos fatores, como a espécie envolvida, a interação com a resposta imune do hospedeiro vertebrado, a patogenicidade do parasito e sua capacidade de invasão e tropismo, as infecções por *Leishmania* podem assumir diferentes formas clínicas (RODRIGUES, 2005; MONZOTE et al., 2007). Estas podem ser agrupadas em dois grupos: leishmaniose visceral ou calazar, que corresponde à forma clínica mais grave da doença, potencialmente fatal quando não instituído o tratamento adequado e leishmaniose

cutânea ou tegumentar que inclui um espectro da doença geralmente referida pelas suas características clínicas e patológicas: leishmaniose cutânea, mucocutânea e difusa (GRIMALDI e TESH, 1993; ASHFORD e BATES, 1998; BASANO e CAMARGO, 2004).

3.1.2.2 O PARASITA E SEU CICLO BIOLÓGICO

Parasitos de todas as espécies de *Leishmania* são morfologicamente similares e, enquanto a identificação presuntiva pode ser realizada por meios substanciais. Uma análise bioquímica é requerida para a identificação dos níveis da espécie (ASHFORD, 2000).

Existem dois estágios de vida das leishmanias: amastigosta e promastigota. As amastigotas são formas intracelulares obrigatórias, ovais ou fusiformes, com diâmetro de no máximo 5 μm, contendo um único núcleo, um cinetoplasto e uma invaginação na região anterior do corpo formando a bolsa flagelar, onde se localiza o flagelo. Dependendo da preparação, quando fixadas e coradas pelos métodos derivados de Romanovsky como Giemsa e Leishman, aparecem, à microscopia ótica, como citoplasma corado de azul-claro, onde são encontrados núcleos grandes e arredondados, ocupando às vezes um terço do corpo do parasito, e cinetoplasto em forma de pequeno bastonete, ambos corados em vermelho-púrpura, além de vacúolos que podem ser ou não visualizados (NEVES et al., 2005).

As formas amastigotas são adaptadas a se desenvolver no interior de fagolisossomo das células hospedeiras. O vacúolo parasitóforo apresenta um forte sistema ácido hidrolítico, o que não constitui um problema, visto que os parasitos são acidófilos e a presença de enzimas e lipofosfoglicanas (LPGs) na sua superfície celular os protegem das enzimas lisossomais (BURCHMORE e BARRET, 2001). As formas amastigotas se dividem repetidamente por divisão binária simples, longitudinal, sendo deslocadas para os vacúolos até que a célula hospedeira se rompa, liberando-as (ASHFORD, 2000). O isolamento das formas amastigotas pode ser feito por meio de animais infectados ou de culturas de células de macrófagos infectados *in vitro* (ASHFORD e BATES, 1998). Apesar de apresentarem maior dificuldade de manipulação e cuidados específicos, algumas espécies já possuem

seu cultivo axênico descrito, sendo denominada amastigotas-*like*, muito úteis para *screening* primário de drogas (SILVA, 2008b; CRUZ et al., 2009).

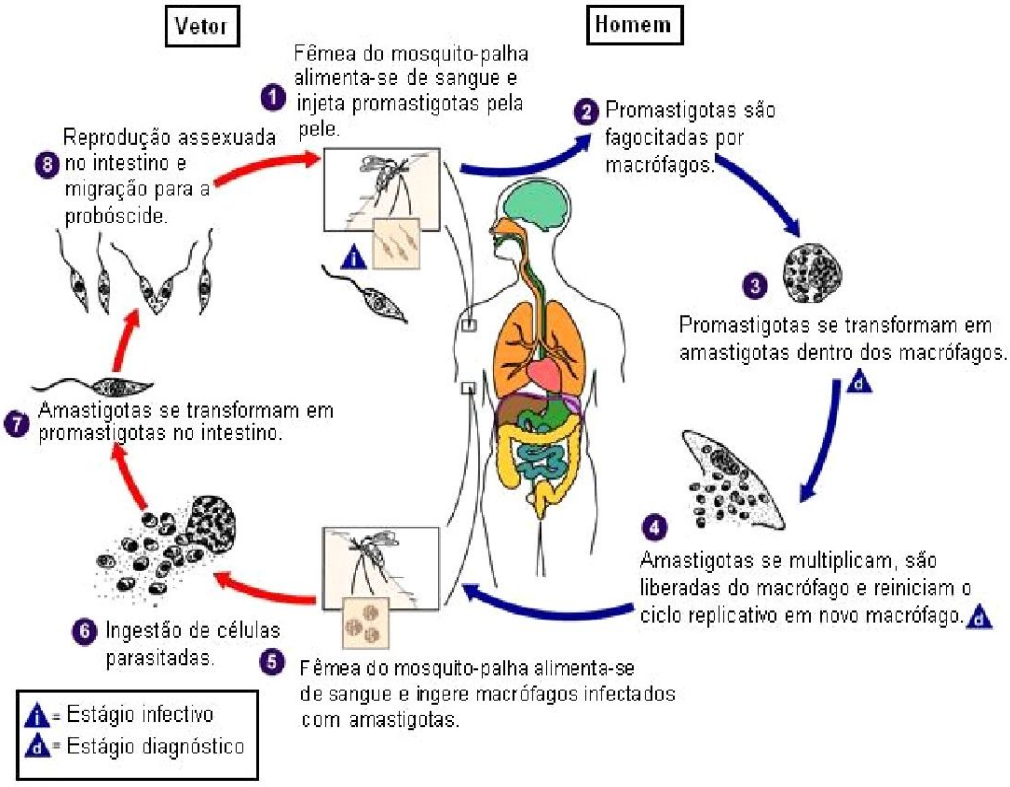
As promastigotas são formas flageladas encontradas no trato digestório do hospedeiro invertebrado. São alongadas, com flagelo livre e longo, emergindo do corpo do parasito na porção anterior. O núcleo é arredondado ou oval, e está situado na região mediana ou ligeiramente na porção anterior do corpo. O cinetoplasto em forma de bastão localiza-se na posição mediana entre a extremidade anterior e o núcleo. As formas promastigotas possuem de 10 a 15 μm de comprimento por 2 a 3,5 μm de largura e seu flagelo apresenta sempre medidas iguais ou superiores ao maior diâmetro do corpo (NEVES et al., 2005). Durante o desenvolvimento no intestino do inseto vetor, as diferentes espécies de Leishmania sofrem transformações morfológicas e alguns autores referem-se a estas formas por diferentes nomes. Bates (2007) classificou o desenvolvimento das promastigotas em cinco formas: I) promastigotas procíclicas, que é o primeiro estágio do vetor, sendo uma forma com pouco movimento e que se multiplica no sangue ingerido; II) promastigotas nectomonadas, nesse estágio a replicação do parasito torna-se mais lenta e diferencia-se em uma forma alongada e com uma forte motilidade, pois nesse estágio possui caráter migratório; III) promastigotas leptomonades, são formas curtas resultantes da diferenciação das promastigotas nectomonadas que se destinam a replicação na fase de alimentação com açúcares do inseto vetor; IV) promastigotas haptomonadas, são formas que possuem expansão da extremidade flagelar e ficam aderidas na superfície cuticular da válvula estomacal e V) promastigotas metacíclicos, são formas resultantes da diferenciação de alguns promastigotas leptomonades, sendo o estágio infectivo para os mamíferos.

Todas as formas de leishmaniose são transmitidas pelo vetor flebotomídeo. No momento do repasto sanguíneo em um indivíduo ou animal infectado, as formas amastigotas são ingeridas juntamente com o sangue e/ou linfa intersticial pelo inseto vetor. O alimento, juntamente com as formas amastigotas, é envolvido pela matriz peritrófica, uma membrana quitinosa secretada pelas células epiteliais do intestino. Após 4-5 dias do repasto infectante para o vetor, as formas amastigotas se transformam em promastigotas (CHANG, CHAUDHURI e FONG, 1990; ASHFORD e BATES, 1998; SACKS e KAMHAWI, 2001). Essas formas promastigotas multiplicam-se por divisão binária, sendo que a sua diferenciação depende do tempo de infecção

e do lugar onde se encontra. As formas infectantes para os mamíferos são as formas metacíclicas que são as formas livres, migram rapidamente e possuem corpo pequeno e flagelos longos. Esta corresponde ao último estágio evolutivo dos promastigotas (BATES, 2007).

Os hospedeiros vertebrados são infectados quando as formas promastigotas metacíclicas são inoculadas pelas fêmeas dos insetos vetores, durante o repasto sanguíneo. Estes insetos possuem o aparelho bucal muito curto e adaptado para dilacerar o tecido do hospedeiro, formando condições para obter sangue durante a alimentação. A saliva do inseto é inoculada neste ambiente e exerce papel importante como anticoagulante, vasodilatador e na antiagregação de plaquetas, favorecendo o fluxo de sangue e linfa para o alimento. Além destes efeitos, sabe-se que fatores presentes na saliva de flebotomídeos têm ação quimiotática para monócitos e imunorreguladores, com a capacidade de interagir com macrófagos, aumentando sua proliferação e impedindo a ação efetora destas células na destruição dos parasitos. As formas promastigotas metacíclicas são resistentes à lise pelo sistema complemento, devido, em parte, a modificações estruturais nas lipofosfoglicanas. Elas podem ainda interagir com proteínas do soro para ativar o sistema complemento, facilitando à adesão à membrana do macrófago (NEVES et al., 2005; BATES, 2007).

A internalização do parasito se faz por meio de endocitose mediada por receptores na superfície do macrófago. Sabe-se que durante esse processo, devido a causas fisiológicas, a célula hospedeira aumenta intensamente a atividade respiratória e ocorre a liberação de óxido nítrico, radicais livres, que são lesivos às membranas celulares (BURCHMORE e BARRET, 2001). Porém, os parasitos possuem mecanismos de escape que inutilizam este ataque. Uma vez no interior do macrófago, o parasito reside dentro do vacúolo parasitóforo. Neste, a forma promastigota diferencia-se em amastigota, que multiplica-se por divisão binária e, após sucessivas divisões, o macrófago rompe e as amastigotas serão fagocitadas por novas células hospedeiras, propagando a infecção no hospedeiro vertebrado (Figura 4) (BURCHMORE e BARRET, 2001; NEVES et al., 2005).



Fonte: http://i9projetos.com.br/infectologiaemfoco_blog/?p=163

Figura 4 – Ciclo biológico da *Leishmania* sp.

3.1.2.3 TRATAMENTO DA LEISHMANIOSE

A escolha do tratamento depende da espécie do parasito, da sensibilidade aos fármacos, do tipo e estágio da doença e do estado imunológico do paciente (SANTOS et al., 2008). Os antimoniais pentavalentes são os medicamentos indicados para o tratamento de todas as leishmanioses, sendo que nos casos de falha dos tratamentos com antimoniais, os fármacos utilizados como segunda escolha são anfotericina B e pentamidina. No entanto, o uso dessas drogas deve ser feito sob vigilância, em serviços especializados e com o paciente hospitalizado (BRASIL, 2005). No Brasil, o tratamento preconizado pelo Ministério da Saúde (BRASIL, 2007) baseia-se, principalmente, no antimonial pentavente (Glucantime®),

entretanto, todos os medicamentos utilizados na terapia possuem muitos efeitos adversos devido à sua toxidade (ALMEIDA e SANTOS, 2011). Por este motivo, é de fundamental importância a busca por novas substâncias leishmanicidas desprovidas de efeitos tóxicos e capazes de atuarem contra as espécies mais resistentes (SANTOS et al., 2008).

Estudos em diversos países como Brasil, Argentina, México e Paraguai identificaram o potencial de muitas plantas contra espécies de Leishmanias (FERREIRA et al., 2002; CHAN-BACAB et al., 2003; BRAGA et al., 2007; FABRI et al., 2009; SULSEN et al., 2007). Essa atividade se deve a diversos metabólitos destacando naftoquinonas, ligninas, alcaloides, chalconas e triterpenos (TANAKA et al., 2007), apesar de, até o momento, nenhuma dessas substâncias possuírem sua eficácia e segurança comprovadas. Mesmo assim, o estudo de produtos naturais constitui uma estratégia importante para a obtenção de novas formas de tratamento para as leishmanioses (GIL et al., 2008).

3.2 A COMPLEXA PROBLEMÁTICA DO EVENTO DE RESISTÊNCIA E SUAS CONSEQUÊNCIAS

O surgimento da resistência a antimicrobianos é um exemplo clássico de evolução em resposta a uma forte pressão seletiva. Hospitais e outros estabelecimentos constituem um ambiente ecológica particular, na qual diversos tipos de micro-organismos circulam e interagem entre si diretamente, por meio da reprodução e da troca de plasmídeos e/ou indiretamente, por meio de interações entre pacientes e funcionários (PRATES e BLOCH-JÚNIOR, 2001; RODRÍGUES-VERDUGO, GAUT e TENAILLON, 2013).

Estudos realizados no período de 1973 a 1994, pela Organização Europeia para Pesquisa e Tratamento do Câncer, mostraram que durante o período de 1973 a 1985, bactérias Gram-negativas eram as principais causadoras de infecções em pacientes oncológicos, enquanto nos últimos anos desta análise, a bactérias Gram-positivas eram as predominantes. Esta troca na dominância de patógenos foi coincidente com a introdução, em 1985, da terceira geração de cefalosporinas, ceftazidima e ceftriaxona, e foi possivelmente causada pelo uso generalizado destas drogas (PETERSON, 2009). Entre as bactérias mais comumente envolvidas com

resistência às drogas e infecção hospitalar estão *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Klebsiella* sp, *Streptococcus pneumoniae*, *Acinetobacter sp*, *Proteus mirabilis* e *Escherichia coli*, e em muitas delas esta resistência ocorre em função da produção de amplo espectro de β-lactamases (PETERSON, 2009).

A incidência de infecções fúngicas, tanto por fungos filamentosos como por leveduras, e a resistência dos mesmos às drogas também têm aumentado drasticamente nos últimos 15 anos (GUPTE, KULKARNI e GANGULI, 2002).

Infecções causadas por micro-organismos resistentes não atingem somente a saúde humana. Na pecuária, grandes são os problemas enfrentados pelos criadores de gado leiteiro susceptível à mastite, uma condição inflamatória da glândula mamária causada por bactérias, principalmente *S. aureus*, *E. coli* e *P. aeruginosa*, que acarreta mudanças nas características físicas do úbere e do leite de vaca (PRATES e BLOCH-JÚNIOR, 2001). O mesmo quadro pode ser verificado no que diz respeito aos fitopatógenos. O surgimento da resistência em fungos fitopatogênicos de importância agronômica é considerado um fator limitante da eficácia e da vida útil das mais variadas estratégias de controle de doenças (HEANEY et al., 2000). Muitos gêneros de fungos, tais como *Aspergillus*, *Botrytis*, *Venturia*, *Ustilago*, *Marnaporte*, *Colletotrichum*, *Alternaria*, *Cercospora*, *Cladosporium* e *Penicillium*, têm desenvolvido estratégias de resistência contra os fungicidas mais amplamente empregados, como os das classes dos benzimidazóis, das estrobilurinas, das fenoxiquinolinas e das anilinopirimidinas, sendo o uso indiscriminado destas drogas o principal fator de indução de resistência (STEFFENS, PELL e TIEN, 1996).

Espécies vegetais com atividade antimicrobiana estão amplamente distribuídas em diversas famílias vegetais como Piperaceae (LENTZ et al., 1998), Hypericaceae, Asteraceae, Caryophyllaceae, Euphorbiaceae, Fumariaceae, Gesneriaceae, Lamiaceae, Rosaceae (TAYLOR, MANANDHAR e TOWERS, 1995), Lyrthaceae, Myrtaceae, Apocynaceae, Casuarinaceae (AHMAD e BEG, 2001) entre outras.

As substâncias antimicrobianas isoladas de plantas apresentam, com raras exceções, grandes diferenças estruturais em relação aos antibióticos derivados de micro-organismos. Podem agir como reguladores do metabolismo intermediário,

ativando ou bloqueando reações enzimáticas, afetando diretamente uma síntese enzimática seja em nível nuclear ou ribossomal, ou mesmo alterando estruturas de membranas (SINGH e SHUKLA, 1984).

Os principais grupos de compostos com propriedades antimicrobianas, extraídos de plantas incluem: terpenoides e óleos essenciais (NASCIMENTO et al., 2008); alcalóides (MOREL et al., 2005); lectinas e polipeptídeos (ZHANG e LEWIS, 1997) e substâncias fenólicas e polifenóis, que são: fenóis simples, ácidos fenólicos, quinonas (STERN et al., 1996), flavonas, flavonóis e flavonoides (KUSTER, ARNOLD e WESSGOHANN, 2009), taninos (SCALBERT, 1991) e cumarinas (O'KENNEDY e THORNES, 1997).

Mecanismos de ação antimicrobiana para algumas dessas classes já foram descritos. Para os alcaloides, essa propriedade pode ser atribuída a sua habilidade de intercalarem com o DNA microbiano (PHILLIPSON e O'NEIL, 1989). Para os terpenoides, o mecanismo de ação ainda não está bem esclarecido, mas especula-se que estejam envolvidos na ruptura de compostos lipofílicos das membranas microbianas (BAGANBOULA, UYTTENDAELE e DEBEVERE, 2004).

Os compostos fenólicos possuem uma relativa toxicidez a micro-organismos devido à localização e ao número de grupos hidroxilas presentes nos grupamentos fenólicos, sendo que quanto maior o número de hidroxilações, maior a toxidade (GEISSMAN, 1963). Além disso, já foi descrito que quanto maior a oxidação dos fenóis, maior seu efeito inibitório (SCALBERT, 1991). O mecanismo responsável pela toxidade de compostos fenólicos para micro-organismos inclui a inibição enzimática por compostos oxidados, possivelmente por meio de reações com grupos sulfidrilas ou por interações não específicas com as proteínas (MASON e WASSERMAN, 1987). Os taninos possuem a habilidade de inativar adesinas microbianas, enzimas, proteínas transportadoras de membrana, etc. Os taninos condensados podem se ligar às paredes celulares de bactérias de ruminantes, impedindo seu crescimento (JONES et al., 1994). As antraquinonas possuem anéis aromáticos com duas cetonas substituídas. Elas são ubiquitinadas *in natura* e são altamente reativas. Conhecidas como estabilizadores de radicais livres e também por complexarem irreversivelmente com aminoácidos nucleofílicos em proteínas, frequentemente conduzindo a sua inativação e perda de função. Os alvos das antraquinonas nas células microbianas são adesinas expostas à superfície,

polipeptídeos da parede celular e enzimas ligadas á membrana (STERN et al., 1996).

Já os flavonoides, de maneira geral, são conhecidos por serem sintetizados por plantas em resposta a infecção microbiana (DIXON, DEY e LAMB, 1983). Sua atividade é provavelmente devido à habilidade de se complexarem com as proteínas extracelulares e solúveis e com a parede celular bacteriana (CUSHNIE e LAMB, 2005). Flavonoides lipofílicos podem romper a membrana microbiana (TSUCHIYA et al., 1994). Segundo CHABOT et al. (1992), flavonoides desprovidos de grupamentos hidroxila em seus anéis B são mais ativos contra micro-organismos do que aqueles hidroxilados, o que corrobora com a proposta de que o alvo antimicrobiano seja a membrana. No entanto, alguns autores se opõem a esta teoria, argumentando que quanto maior o número de hidroxilações, mais ativo serão os compostos (SATO et al., 1996).

3.3 PROCESSO INFLAMATÓRIO

3.3.1 ASPECTOS GERAIS DA INFLAMAÇÃO

Nos últimos anos, um grande interesse tem sido demonstrado na tentativa de buscar respostas referentes à etiologia fisiopatológica e controle farmacológico do processo inflamatório. Este interesse, dentro da medicina experimental, deve-se à descoberta de que os danos teciduais que ocorrem em diversas doenças existentes resultam de uma resposta inflamatória (MONTHANA, 2011).

A palavra inflamação, do grego *phlogosis* e do latim *flamma*, significa fogo, área em chamas, foi descrita pela primeira vez por Celsus, um escritor romano que viveu entre os anos 30 aC e 38 dC, onde observou os quatro sinais cardinais da inflamação: calor (aumento da temperatura local), rubor (hiperemia), tumor (edema) e dor, as quais Virchow acrescentou o quinto sinal clínico, a perda da função (*functio laesa*) (ROCHA E SILVA e GARCIA LEME, 2006). Em 1793, John Hunter, um cirurgião escocês, observou que a inflamação não é uma doença, e sim uma resposta inespecífica que tem um efeito salutar sobre seu hospedeiro (ROCHA E SILVA e GARCIA LEME, 2006).

Assim, a resposta inflamatória representa um dos mecanismos de defesa do organismo. Supondo que não existisse o processo inflamatório, teríamos consequências graves: haveria um descontrole das infecções, não haveria cicatrização das feridas e, além disso, o processo destrutivo nos órgãos atacados seria permanente. As reações inflamatórias, por exemplo, são o pilar de doenças crônicas, tais como a artrite reumatoide, a aterosclerose e a fibrose pulmonar, assim como de reações de hipersensibilidade potencialmente fatais a picadas de insetos, drogas e toxinas (BHANDARE et al., 2010; MUELLER, HOBIGER e JUNGBAUER, 2010).

Existe uma série de fenômenos biológicos envolvidos no processo inflamatório, que se associam e se complementam e, com isso, formam reações em cascata, envolvendo a interação complexa entre as células inflamatórias, tais como macrófagos, neutrófilos e linfócitos (TEDGUI e MALLAT, 2001). Esses fenômenos podem ser desencadeados por vários fatores, chamados agentes flogogênicos. Dentre os tipos flogogênicos, destacam-se: I) agentes físicos, em que os fatores físicos, como o calor excessivo (queimaduras), o frio exagerado (congelamento), traumatismos, fraturas, incisões, raios ultravioletas e radiações ionizantes são as causas importantes da inflamação; II) agentes químicos, neste caso, a inflamação é determinada pela ação de substâncias químicas de natureza muito variadas, como substâncias cáusticas, ácidos, álcalis, óleo de cróton, terebentina, formaldeído, carragenina; III) agentes infecciosos, em que o fator desencadeante da inflamação é de origem biológica, como bactérias, fungos, vírus, protozoários (CARVALHO, 2004)

Além do combate ao agente agressor, de forma a evitar a sua disseminação às outras regiões do corpo, a resposta inflamatória tem a finalidade de eliminar produtos resultantes da destruição celular, assim, promovendo condições ideais para a reparação do tecido lesado. Todo esse processo de restituição da normalidade tecidual é concluído pela reparação, fenômeno inseparável da inflamação (TEDGUI e MALLAT, 2001).

A inflamação é uma resposta celular e humoral de intensidade variável com repercussões locais, loco-regionais ou sistêmicas. A cascata de eventos desencadeada após uma agressão ou estímulo tem como resultado o aumento do calibre vascular, aumento da permeabilidade vascular, recrutamento de leucócitos como resultado de uma interação complexa entre diferentes tipos celulares

residentes no tecido e vários mediadores pró-inflamatórios (NICKOLOFF e NESTLE, 2004; SHERWOOD e TOLIVER-KINSKY, 2004).

O tempo de duração e a intensidade do agente inflamatório determinam diferentes graus ou fases de transformação nos tecidos. Categoricamente, a resposta inflamatória é dividida em três distintas fases, sendo que cada fase é mediada aparentemente por diferentes mecanismos. Inicialmente, é evidenciada uma fase aguda, caracterizada por ser de duração variável e alterações vasculares (fluxo e calibre vascular, aumento da permeabilidade vascular), seguida de uma fase subaguda, em que nota-se, predominantemente, a infiltração leucocitária e de outras células fagocíticas (quimiotaxia, adesão, transmigração e fagocitose) e posteriormente, ocorre à fase crônica, onde a proliferação é fato de destaque, em que ocorre a regeneração tecidual e fibrose (SANTOS JUNIOR, 2003).

Primeiramente, os fenômenos vasculares predominam no foco da inflamação, onde ocorre um aumento da dilatação dos vasos e ainda, um aumento da permeabilidade capilar (CIRINO et al., 2003) gerados principalmente pelos seguintes mediadores: prostaglandinas, histamina, serotonina, leucotrienos, C5a, e substâncias liberadas localmente nas terminações nervosas (taquicininas e o peptídeo relacionado ao gene da calcitonina – CGRP) (HEDQVIST, GAUTAN e LINBOM, 2000; LECCI et al., 2000). Este aumento da permeabilidade vascular culmina com a saída de exsudato, ou seja, um fluído rico em proteínas para o meio extravascular resultando em acúmulo deste, no local da lesão, o que caracteriza o edema (PATTERSON e LUM, 2001; LEY, 2002).

Posteriormente, evidencia-se uma fase subaguda em que ocorre o fenômeno da quimiotaxia, caracterizada por migração e infiltração leucocitária e de células fagocíticas, no sítio da lesão (ALI et al., 1997; FRANGOGIANNIS, SMITH e ENTMAN, 2002). No local da lesão, ocorre o reconhecimento do agente agressor por meio de neutrófilos e macrófagos, após este reconhecimento, essas células englobam e o destroem (fagocitose) e liberam enzimas lisossomais a fim de destruir o agente agressor (RODRIGUES et al., 2002). Ainda no foco inflamatório, ocorre um importante evento: a ativação dos mastócitos e agregação plaquetária, que também resultam na liberação de mais mediadores quimiotáticos. Dentre os mediadores quimiotáticos citam-se: os prostanoides (PGI_2, PGD_2, PGE_2 e PGF2α), derivados da

via da lipoxigenase (5 HETE, LTB_4), cininas, substâncias liberadas de neutrófilos ativados, entre outros (TAN, LUSCINKAS e HONNER-VANNIASNKIM, 1999).

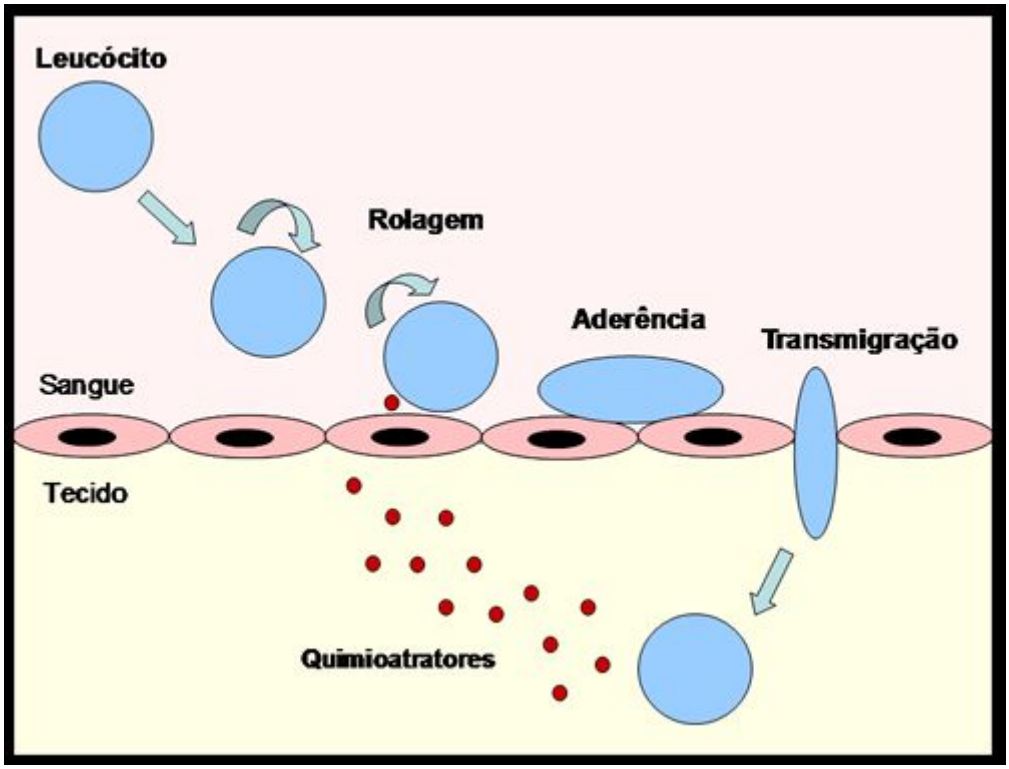
Finalmente, após a fase subaguda, ocorre uma complexa série de eventos na tentativa de reconstituir o tecido danificado por meio da ativação de células mesenquimatosas (resposta primária), incluindo hiperplasia, hipertrofia, e produção de matriz extracelular. A injúria inflamatória (injúria secundária) é mantida e induz o processo de cicatrização (resposta secundária) que freqüentemente reconstitui o sítio lesado com tecidos fibroblásticos menos especializados (SUZUKI et al., 2003).

3.3.2 MARGINAÇÃO, ROLAMENTO, ADESÃO E MIGRAÇÃO LEUCOCITÁRIA

O recrutamento leucocitário promove uma série de mudanças não só nas células leucocitárias como nas células endoteliais vasculares ativadas por várias citosinas, como IL-1 e TNF. As células endoteliais secretam substâncias quimioatrativas que conduzem os neutrófilos para extremidade dos vasos sanguíneos, este processo é de extrema importância para o seu recrutamento ao sítio inflamatório (ABBAS, LICHTMAN e POBER, 2003).

Inicia-se o rolamento por intermédio das seletinas, que são moléculas de adesão presentes na superfície das células do endotélio e do leucócito, essas moléculas são compostas por oligossacarídeos chamados de Syalil-Lewis X que tem a finalidade de promover um maior número de pontes de ligações entre a superfície do leucócito e a superfície do endotélio. Entretanto, para que o processo ocorra os leucócitos precisam aderir-se de maneira eficaz as células do endotélio, permitindo que as integrinas possam ser estimuladas e ativadas, por ação de mediadores inflamatórios, tornando-se ávidas por se ligarem principalmente a receptores ICAM-1 (molécula de adesão intercelular-1), se tornando um pré-requisito para ativação do leucócito (YANG et al., 2005; HEIT, COLARUSSO e KUBES, 2005). Esse mecanismo é fundamental para gerar respostas rápidas como na reorganização do citoesqueleto (WEI et al., 2004; HORDIJK et al., 2003). Quando ligadas aos receptores ICAM-1 gera-se um sinal para dentro da célula via proteína ERM, responsáveis pela formação da actina e miosina o que resultará na contração endotelial (HORDIJK, 2003). Algumas ligações resultam na formação da ROS no qual se gera um estresse da fibra citoesquelética, alterando a estrutura da actina e

miosina. A formação da ROS também resulta na degradação da junção protéica do endotélio o que leva a sua abertura, assim finalmente os leucócitos conseguem atravessar a membrana endotelial e chegar ao local em que ocorreu o dano tecidual (Figura 2) (DEKKER e SEGAL, 2000; JASON et al., 2001).



Fonte: COUTINHO, MUZITANO e COSTA, 2009.

Figura 2 – Esquema ilustrativo da migração de leucócitos através do endotélio vascular.

3.3.3 ANTI-INFLAMATÓRIOS E SUAS LIMITAÇÕES

Os anti-inflamatórios estão entre os agentes terapêuticos mais utilizados, porém apresentam algumas limitações com relação à sua potência, eficácia e efeitos adversos (PARENTE, 2001; FIORUCCI et al., 2001). Os AINES (anti-inflamatórios não esteroidais) apresentam efeitos gastrointestinais adversos e os AINEs seletivos para COX-2 foram associados a pequenas alterações cardiovasculares, porém proeminentes em alguns pacientes (PARENTE, 2001; BOERS, 2001). Os anti-inflamatórios esteroidais apresentam acentuados efeitos adversos devido à ação

comum aos esteroides. Já os recentes moduladores ou anti-citocinas (anti-IL-1*β* e anti-TNF-*α*) são mais caros e a via de administração é subcutânea, pelo menos duas vezes por semana, o que diminui a adesão ao tratamento (CALIXTO et al., 2004). Devido a esses inconvenientes, torna-se necessária a busca por substâncias naturais com potencial anti-inflamatório que possam dar origem a novos fármacos de baixo custo e com efeitos adversos reduzidos, ou que ao menos possam auxiliar no entendimento de mecanismos de ação.

Dados da literatura mostram que moléculas de origem vegetal apresentam importantes atividades antiinflamatórias e que muitas de suas ações são relacionadas à habilidade de inibir a síntese ou ação de citocinas, quimiocinas e moléculas de adesão, vias do ácido araquidônico e óxido nítrico (CALIXTO et al., 2004; WERZ, 2007).

As preparações de plantas, por sua vez, frequentemente inibem mais de uma via de ação, maximizando os efeitos anti-inflamatórios e minimizando efeitos adversos (SCHMITZ e BACHER, 2005). Isso se deve ao fato de haver uma mistura de substâncias que podem agir sinergicamente ou antagonicamente, por meio de diferentes mecanismos de ação. Além disso, sugere-se que os compostos derivados de plantas possam ser utilizados na forma de monopreparados ou em associações aos medicamentos atuais com o objetivo de diminuir custos e aumentar a eficácia (CALIXTO et al., 2004).

3.4 CONSTIPAÇÃO INTESTINAL E AGENTES LAXATIVOS

A constipação intestinal consiste em dificuldade para evacuar, fezes endurecidas e secas e dores abdominais. A presença de constipação está associada à falta de resíduos dentro do cólon, perda de sensibilidade dos órgãos que desencadeiam os mecanismos da defecação, perda das contrações dos músculos envolvidos com a defecação e obstrução mecânica, daí os mais idosos apresentarem maior frequência de constipação do que os mais jovens (DANTAS, 2004). Também ocorre mais frequentemente em mulheres do que nos homens, principalmente naquelas com inatividade física diária, baixa renda, baixo nível educacional, baixo consumo de líquidos e alimentos ricos em fibras (COTA e MIRANDA, 2006).

A maioria dos pacientes que apresenta o quadro de constipação não procura ajuda médica para realizar o tratamento. Quando os sintomas permanecem, é necessário buscar as causas primárias e determinar qual é o tratamento mais adequado (WANNMACHER, 2005).

A modificação dos hábitos alimentares, proveniente da ocidentalização e industrialização, tem introduzido um elevado consumo de alimentos refinados desprovidos de fibras alimentares, contidas em maiores quantidades em frutas, vegetais e cereais integrais. Por esse motivo, atualmente, existe uma elevada incidência de doenças que eram pouco frequentes no passado, as chamadas “doenças de civilização”, fazendo parte deste elenco à constipação intestinal (ANDRE et al., 2000; COTA e MIRANDA, 2006).

Dessa forma, a mudança no estilo de vida, que inclui modificações na dieta, maior atividade física, ingestão de maior quantidade de líquidos, reeducação intestinal e auxílio de preparados de fibras vegetais podem ser os componentes de terapêutica de sucesso para a maioria dos casos de constipação intestinal (COTA e MIRANDA, 2006).

A constipação pode ter causa motora, por distúrbios da motilidade enterocólica, consequente a condições metabólicas, miopatias e neuropatias, ou causa mecânica, por suboclusão intestinal. Entre as causas motoras, estão à síndrome do cólon irritável, uso crônico de laxativos, megacólon, alimentação inadequada, lesões neurológicas, sedentarismo, desequilíbrio hidroeletrolítico, fatores psicogênicos, hipotireoidismo e efeito colateral de medicamentos, como antiácidos (com cálcio e alumínio), antiespasmódicos, diuréticos, analgésicos, anticonvulsivantes e antidepressivos. Entre as causas mecânicas estão os tumores benignos e malignos, diverticulite, colite isquêmica e compressão extrínseca. Devem ser consideradas, também, as afecções proctológicas, como fissuras, hemorroidas e proctite, que dificultam a evacuação (DANTAS, 2004).

Constipação é um sintoma e não uma doença. Pode estar associada, sem que possamos estabelecer a relação causa-efeito, com infecção urinária, enurese noturna e incapacidade de contrair a bexiga. A incidência é três vezes maior nos homens obesos, e duas vezes maior nas mulheres obesas, do que na população com peso normal. Não podemos esquecer que a causa mais frequente é a funcional,

mas, para que possamos ter certeza desse diagnóstico, as causas orgânicas devem ser excluídas (DANTAS, 2004).

No contexto da constipação crônica, medidas não-medicamentosas constituem a primeira escolha para manejo inicial, com ênfase em abordagem dietética e de hábitos de vida. Dependendo do mecanismo gerador do problema, outras abordagens não-medicamentosas são propostas. Os laxativos estão indicados quando as primeiras alternativas falham, não demonstrando diferença significativa de eficácia entre eles, sobretudo por longo prazo. Quando usados em esquemas recomendados, sua segurança se equivale (WANNMACHER, 2005).

Diversos tipos de laxantes são prescritos, e um número ainda maior é obtido sem prescrição, indicando uma preocupação cultural pela regularidade intestinal. Em geral, esses fármacos são classificados, com base no seu mecanismo de ação, em agentes expansores da massa, agentes osmóticos e agentes irritantes ou estimulantes (BURROWS e MERRITT, 1983).

Laxantes expansores de massa são polímeros orgânicos que interagem com a água e têm a capacidade de mantê-la retida nas fezes. Dentre tais polímeros podemos incluir as fibras dietéticas como farelo, medicamentos como *Psyllium*, polímeros sintéticos como metilcelulose. Sua principal função é evitar a absorção de água pelo intestino delgado e conservá-la retida nas fezes. O aumento do volume e peso das fezes estimula a motilidade e acelera o trânsito intestinal do bolo fecal. Um dos principais efeitos colaterais que ocorrem são provocados pela fermentação dessas fibras, causando a formação de gases e flatulências, inchaços e retenção de líquidos (BURROWS e MERRITT, 1983).

Os agentes osmóticos são íons ou moléculas volumosas adicionadas ao conteúdo intestinal, que retêm água dentro do lúmen por efeito de força osmótica, ou que estimulam secreções intestinais ou motilidade, assim causando o aumento da frequência e facilidade de defecação (SCHILLER, 2001). Íons como o magnésio, fosfato ou sulfato retêm água no intestino por força osmótica e provocam distensão, estimulando suas contrações. As soluções eletrolíticas isosmóticas que contêm polietilenoglicol são utilizadas como soluções para lavagem do cólon, principalmente, na preparação de procedimentos radiológicos ou endoscópicos, bem como para remoção de toxinas ingeridas (KATSUNG, 2003). Outro exemplo é a lactulose, um dissacarídeo sintético (galactose-frutose), que não é absorvido pelo

intestino, mas pode ser metabolizado por bactérias intestinais e se transformar em um ácido graxo de cadeia curta, que é facilmente absorvido, ou pode permanecer no lúmen, exercendo suas atividades osmóticas (HAMMER et al., 1989). Sorbitol e manitol são alcoóis de açúcares, assim como a lactulose; também não são absorvidos pelo intestino. Em um estudo realizado por Lederle et al. (1990), observou-se que o tratamento com sorbitol era tão eficaz quanto à lactulose. Sua maior vantagem é o baixo custo.

Laxantes amolecedores do bolo fecal têm a capacidade de se emulsificarem com as fezes e amolecê-las, facilitando seu trânsito (KATSUNG, 2003). Destaca-se o óleo mineral, que administrado via oral, não é quimicamente ativo no corpo, porém altera as características físicas das fezes agindo como um lubrificante do bolo fecal. Sua utilização em longo prazo pode causar má absorção de vitaminas lipossolúveis, e sua absorção pelo intestino pode causar reações de corpo estranho na mucosa intestinal e linfonodos regionais. Além disso, pode causar problemas no tônus do esfíncter anal (SCHILLER, 2001).

Laxantes estimulantes e irritantes do trato intestinal são chamados assim porque têm a finalidade de estimular a motilidade (SCHILLER, 1997). Incluem o óleo de rícino, o bisacodil e também as antraquinonas, que são metabólicos especiais encontrados em diversas plantas medicinais tais como as dos gêneros *Cascara* e *Senna* (KATSUNG, 2003). Os derivados de difenilmetano, como por exemplo, o bisacodil - um éster diacético - utilizado como catártico e hidrolisado no intestino delgado e cólon por enterases endógenas, depois convertido para sua forma livre que apresenta os grupos difenólicos. Tal medicamento tem seu efeito obtido entre seis e oito horas (FLIG, HERMANN e ZABEL, 2000).

Antraquinonas apresentam diferentes formas de ação laxativa dependendo da sua estrutura base. Os glicosídeos constituem as formas de transporte e de maior potência farmacológica, porém, pela reduzida lipossolubilidade, têm menores índices de absorção (menor biodisponibilidade) que as correspondentes antraquinonas livres (SIMÕES et al., 2007). As antronas e diantronas são mais ativas que as formas oxidadas e constituem as formas realmente ativas dos compostos antracênicos (KOBASHI et al., 1980). Outro ponto importante é a presença de hidroxilas nesses compostos, sendo as posições C-1 e C-8 essenciais para a ação laxativa (WAGNER, 1988).

Vários mecanismos de ação têm sido propostos para explicar o efeito laxativo de antraquinonas, os quais incluem a inibição da Na/K-ATPase (SIMÕES et al., 2007), a estimulação direta de contração da musculatura lisa do intestino pelo aumento da motilidade, relacionada com a liberação de prostaglandina (BEUBLER e KOLLAR, 1988), histamina (CAPASSO et al., 1986) ou serotonina (BEUBLER e SCHIRGI-DEGEN, 1993) e inibição dos canais de cloreto (SIMÕES et al., 2007).

3.5 CÂNCER

O termo câncer é a tradução latina do grego carcinoma (de *karkinos* = crustáceo, caranguejo). Essa terminologia foi utilizada pela primeira vez por Galeno a fim de descrever um termo maligno na mama, com veias superficiais túrgidas e ramificadas, semelhando-se ás patas de caranguejo (FLECK, 1992). Atualmente, esse termo é utilizado como sinonímia de neoplasia maligna.

Neoplasia pode ser entendida como proliferação celular anormal, descontrolada e autônoma, ou seja, fora do controle dos mecanismos que regulam a multiplicação celular. As células reduzem ou perdem a capacidade de se diferenciar em consequência de alterações nos genes que regulam o crescimento e a diferenciação celular (BOGLIOLO e BRASILEIRO FILHO, 2006).

O câncer, segundo a Agência Internacional de Pesquisa sobre Câncer, é a principal causa de óbito no mundo, correspondendo a 7,6 milhões de óbitos (cerca de 13% do total). Em 2008, o câncer de pulmão, fígado, estômago, cólon e mama foram responsáveis pela maioria dos óbitos por essa doença. Cerca de 30% das mortes por câncer se devem a cinco fatores comportamentais e dietéticos: índice de massa corpórea elevada, baixo consumo de frutas e legumes, falta de atividade física, tabagismo e consumo de álcool (WHO/GLOBOCAN, 2010). Em 2009, ocorreram no Brasil aproximadamente 172 mil óbitos por neoplasias, correspondendo a 16% do total de óbitos no país, perdendo somente para doenças do aparelho circulatório (BRASIL, 2012).

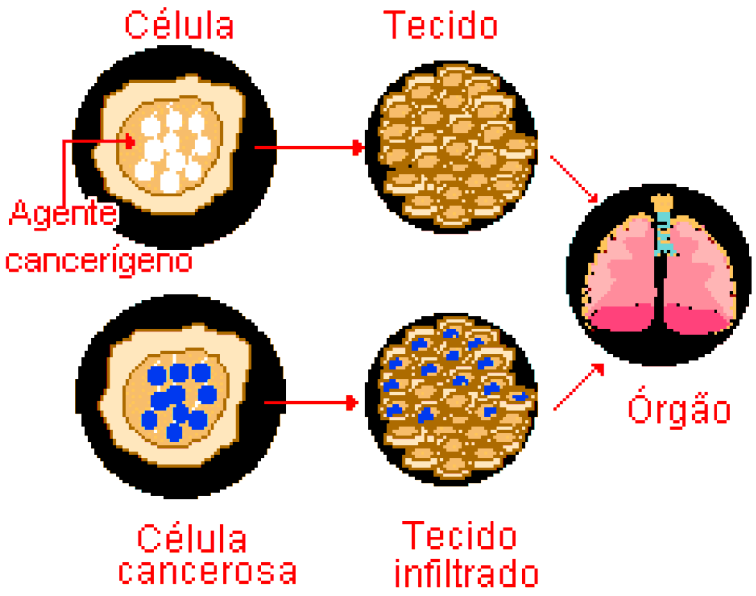
3.5.1 A CÉLULA CANCEROSA

As principais características das células cancerosas são a perda da inibição por contato, independência com relação aos fatores de crescimento, imortalidade, tumorogênese, desvio no controle da apoptose e potencial replicativo limitado. Porém, essa célula preserva certo grau de diferenciação característico do tecido original (HANAHAN e WEINGERG, 2000).

As características morfológicas mais marcantes desse tipo de célula são anormalidades no núcleo: volumoso, múltiplos, nucléolos visíveis, hiperploidia; anormalidades no tamanho das células: heterogêneas; anormalidades citoplasmáticas: aumento da relação citoplasma-núcleo; anormalidades da membrana citoplasmática: perda da inibição por contato, modificação da adesividade e modificação dos antígenos de superfície (APTSIAURI, 2007).

3.5.2 A PROGRESSÃO TUMORAL

A formação e o desenvolvimento das neoplasias, denominado de carcinogênese, é o processo complexo que ocorre em três etapas consecutivas: iniciação, promoção e progressão (Figura 3) (BOGLIOLO e BRASILEIRO FILHO, 2006).



Fonte: http://bioquimicacancer.blogspot.com/2011/06/carcinogenese-o-comeco-da-patologia.html

Figura 3 – Etapas do desenvolvimento das neoplasias.

A iniciação acontece com uma desregulação genômica, devido a um agente carcinogênico ou oncoiniciador, que provoca modificações permanentes em alguns de seus genes e transforma a célula. Uma célula iniciada torna-se menos responsiva a fatores que inibem o crescimento celular, ou aos indutores de diferenciação celular, ou a apoptose. Os agentes oncoiniciadores são substâncias eletrofílicas commuita afinidade por compostos nucleofílicos, como proteínas, RNA e DNA. Exemplos de agentes carcinógenos: sulfato de dimetila, metilnitrosureia, cloreto de vinila, aflatoxinas, dimetilnitrosamina e benzopireno (KUMAR, 2005).

A promoção acontece por meio de uma série de interações entre citocinas, fatores de crescimento e seus receptores, consistindo na proliferação ou expansão das células iniciadas. Há uma perda da homeostasia tissular e o surgimento de clones celulares transformados pelo efeito dos agentes cancerígenos classificados como oncopromotores. A célula iniciada é transformada em célula maligna de forma lenta e gradual. Para que ocorra essa transformação é necessário um longo e continuado contato com o agente cancerígeno promotor. A suspensão do contato muitas vezes interrompe o processo nesse estágio, pois a ação do promotor é reversível. Os promotores são substâncias que têm em comum a propriedade de irritar os tecidos e de provocar reações inflamatórias e proliferativas. O promotor

mais conhecido é o acetato de 12-*o*-tetradecanoilforbol (TPA) (BOGLIOLO e BRASILEIRO FILHO, 2006), além de outros ésteres de forbol e fenóis (SILVA e HARTMANN, 2006).

Após seu surgimento, o câncer sofre, com frequência, modificações biológicas que o tornam, em geral, mais agressivo e maligno. Esse fenômeno é denominado de progressão tumoral. Essa etapa dá origem ao fenômeno de disseminação metastática, caracterizando-se pela multiplicação descontrolada, sendo um processo irreversível. A doença já está instalada, evoluindo até o surgimento das primeiras manifestações clínicas. Além da aquisição de novas características intrínsecas das células tumorais, a progressão dos tumores depende também de fatores do paciente como o estado hormonal e sua resposta imunitária (SILVA e HARTMANN, 2006).

3.5.3 TIPOS DE CÂNCER (CLASSIFICAÇÃO)

Os tumores podem ser classificados de acordo com: I) o comportamento clínico (benignos ou malignos); II) o aspecto microscópico (critério histomorfológico) e III) a origem da neoplasia (critério histogenético). O critério mais frequentemente adotado é o histomorfológico, ou seja, aquele que identifica a neoplasia pelo tecido ou célula que está proliferando. Nesse sentido, algumas regras são importantes: I) o sufixo “oma” é empregado na denominação de qualquer neoplasia benigna ou maligna; II) a palavra carcinoma indica tumor maligno que reproduz epitélio de revestimento e indica malignidade quando usada como sufixo, como no caso das adenocarcinomas; III) o termo sarcoma refere-se a uma neoplasia maligna mesenquimal e usado como sufixo, indica tumor maligno de determinado tecido, como os fibrossarcomas; IV) a palavra bastoma pode ser usada como sinônimo de neoplasias e quando empregada como sufixo, indica que o tumor reproduz estruturas com características embrionárias como as nefrobastomas (BOGLIOLO e BRASILEIRO FILHO, 2006).

Em resumo, para se denominar um tumor, toma-se o nome da célula, tecido ou órgão afetado e acrescentam-se os sufixos – oma ou – sarcoma. Além disso, o nome do tumor pode conter outros termos para identificar certas propriedades da lesão ou sua diferenciação: carcinoma epidermoide (epitélio neoplásico com diferenciação semelhante à da epiderme). Ainda existem os casos onde epônimos

são utilizados na denominação da lesão, como nos casos dos tumores de Wilms, linfomas de Hodgkin e tumores de Burkitt (TEIXEIRA e FONSECA, 2007).

3.5.4 TIPOS DE TRATAMENTO

Com o objetivo de tratar o câncer com maior eficiência, esquemas terapêuticos utilizando cirurgias, radioterapia e quimioterapia têm sido cada vez mais prevalentes (GUIDA et al., 2008; REINER et al., 2009).

A cirurgia oncológica com intuito curativo realiza a remoção do tumor, de sua drenagem linfática, dos linfonodos locais e do tecido normal, em quantidade suficiente, a fim de manter uma adequada margem de segurança (TEICH e FRANKS, 1990).

A radioterapia é um método capaz de destruir células tumorais, empregando feixe de radiações ionizantes. Uma dose pré-calculada de radiação é aplicada, em um determinado tempo, a um volume de tecido que engloba o tumor, buscando erradicar todas as células tumorais, com menor dano possível às células normais circunvizinhas, à custa das quais se fará a regeneração da área irradiada (BRASIL, 2008).

A quimioterapia consiste na utilização de medicamentos a fim de destruir as células cancerosas bloqueando o seu desenvolvimento. A maior parte destes medicamentos é derivada de plantas, organismos marinhos e micro-organismos. Esses agentes incluem uma grande variedade de compostos que atuam por diversos mecanismos. As substâncias naturais ou derivadas têm-se mostrado importantes no tratamento do câncer, destacando os antitumorais vincristina e vinblastina, extraídos de *Catharanthus roseus* (L.) G. Don, que contribuíram para remissão e cura de leucemias em crianças, teratomas testiculares, linfomas de Hogkin e muitos outros tipos de câncer; o etoposídeo, encontrado no *Podophyllum pellatum* Linn., é amplamente utilizado em casos de câncer de pulmão e o taxol, produto isolado de *Taxus brevifolia* Nutt., utilizado para o tratamento de câncer de mama e de ovário, sendo líder em vendas entre as drogas antitumorais (MANN, 2002).

A cirurgia e a radioterapia são efetivas no tratamento de tumores sólidos, mas no tratamento de tumores disseminados atuam somente como paliativos. Nesses

casos, a quimioterapia permanece sendo o tratamento de escolha, mas seu efeito tóxico sobre os tecidos normais geralmente limitam seu uso (BAXEVANIS, PEREZ e PAPAMICHAIL, 2009).

As células do câncer frequentemente expressam antígenos associados que podem ser reconhecidos pelas células T de defesa. Esta condição proporciona o emprego de imunoterapias para o tratamento de diversos tipos de câncer (BRUSERUD et al., 2008), com o intuito de aumentar as respostas imunes antitumorais (BAXEVANIS, PEREZ e PAPAMICHAIL, 2009)

As estratégias possíveis de imunoterapia para o tratamento do câncer incluem várias formas de vacinas, como as de peptídeo ou células dendríticas pulsadas com lisados de células ou transfectadas com RNA das células do câncer (YU e FINN, 2006).

Essas vacinas têm sido testadas em diversos ensaios clínicos, principalmente fase I e II (DALGLEISH e WHELAN, 2006) como tratamento único do câncer, no entanto no futuro elas devem ser utilizadas como parte integrante dos esquemas terapêuticos, incluindo quimioterapia inicial, podendo ser combinada com cirurgia e radioterapia, a fim de reduzir o tumor e facilitar a apresentação do antígeno (BRUSERUD et al., 2008).

3.6 TOXIDADE DE PLANTAS MEDICINAIS

Apesar do amplo uso de medicamentos sintéticos no mercado mundial, a utilização de produtos naturais vem aumentando de forma significativa, não somente pela aceitabilidade cultural, como também pela comprovação científica de eficácia de algumas espécies vegetais. Contudo, várias plantas da medicina tradicional ainda não foram submetidas a algum tipo de avaliação toxicológica, podendo, dessa forma, representar risco tóxico ao usuário (PAL e SHUKLA, 2003; DA SILVEIRA, BANDEIRA e ARRAIS, 2008).

A partir do final do século XX, as plantas medicinais passaram a ser veiculadas nos meios de comunicação como produtos associados a um modo alternativo de vida. Com o objetivo de atingir o público-alvo dos adeptos do estilo de “vida natural”, a propaganda camuflou os riscos da utilização das plantas medicinais, atribuindo-lhes uma fisionomia “não agressora” e passando para o paciente toda

responsabilidade e autonomia de seu consumo (TAGLIATI e FÉRES, 2009). Atualmente, a crença na “naturalidade inócua” das plantas medicinais continua a ser um mito que deve ser enfrentado. Trata-se de um conceito popular equivocado de que os produtos naturais seriam isentos de efeitos indesejáveis ou desprovidos de toxidade (DA SILVEIRA, BANDEIRA e ARRAIS, 2008).

Como forma de contrastar essa afirmação, podem-se citar várias espécies vegetais como a *Datura suaveolens* L. (trombeteira) e a *Conium maculatum* L. (cicuta), que são bem conhecidas por sua capacidade de produzir risco à saúde humana. Outras, ainda que de amplo uso na medicina popular, tiveram seu potencial tóxico também reconhecido após serem submetidas aos testes de segurança, como foi o caso do efeito hepatotóxico verificado para a *Symphytum officinale* L. (confrei), hoje restrita ao uso tópico (TAGLIATI e FÉRES, 2009). Por outro lado, a utilização inadequada de um produto, mesmo que de baixa toxidade, pode induzir a problemas graves desde que existam outros fatores de risco, tais como contraindicações, uso prolongado ou concomitante de outros medicamentos, sintéticos ou naturais (DOS REIS e DE SOUZA, 2006).

Por isso, a determinação da segurança de um medicamento, independentemente de sua origem, é fundamental para a promoção da saúde (KLEIN, LONGHINI e MELLO, 2009). É com essa preocupação que as agências regulamentadoras de vários países têm preconizado a realização de estudos que comprovem a eficácia e segurança das drogas vegetais e fitoterápicos (SAHOO; MANCHIKANTI e DEY, 2010). Essas diretrizes compreendem os estudos toxicológicos pré-clínicos e ensaios clínicos, bem como ensaios de controle de qualidade que comprovem a inexistência de contaminantes nocivos à saúde (metais pesados, agrotóxicos, micro-organismos, aflotoxinas e produtos de degradação) ou sua existência dentro dos limites considerados seguros (KLEIN, LONGHINI e MELLO, 2009).

No Brasil, os testes de toxidade pré-clínica exigidos para registro e renovação de registro de fitoterápicos são estipulados pelo “Guia para a realização de estudos de toxidade pré-clínica de fitoterápicos” da Agência Nacional de Vigilância Sanitária (ANVISA), publicado pela Resolução RE 90/04. Nesse guia, constam os ensaios de toxidade aguda e o de doses repetidas (longa duração); genotoxidade, que deve ser efetuada quando há indicação de uso contínuo ou prolongado em humanos;

sensibilização dérmica, irritação cutânea e ocular, no caso de medicamentos fitoterápicos de uso tópico. Nos estudos agudos, o guia não especifica o método a ser utilizado, mas sugerem a utilização de doses suficientes para se observarem efeitos tóxicos e/ou adversos. No caso da não observação destes, recomenda-se a utilização da maior dose possível (BRASIL, 2004).

Um dos primeiros testes realizados para a avaliação do potencial tóxico de quaisquer substâncias é o teste de toxidade aguda. A dose letal mediana (DL_{50}) e outros efeitos tóxicos podem ser determinados após administração aguda por uma ou mais vias (rota de exposição) em uma ou mais espécies (ratos e camundongos são os mais frequentemente usados). Os estudos são realizados com animais adultos de ambos os sexos e verifica-se o número de animais que morrem durante o período de observação de 14 dias. A avaliação dos resultados permite conhecer a espécie ou sexo mais sensível. Além de dados de mortalidade e peso corporal, podem ser avaliados outros parâmetros como sinais de intoxicação, letargia, mudanças comportamentais, morbidade e alteração no consumo de alimento. Os testes de toxidade aguda fornecem uma estimativa quantitativa de toxidade aguda em comparação com outras substâncias; a identificação dos órgãos-alvo ou outras manifestações clínicas agudas; o estabelecimento do caráter reversível das respostas tóxicas e um guia de doses para outros estudos (KLAASSEN e EATON, 1994).

O ensaio de toxidade aguda oral avalia a exposição a uma dose única ou fracionada no período de 24 horas em roedores. A análise estatística de resultados provenientes de diversos testes convencionais de toxidade aguda (determinação da DL_{50}), com animais de ambos os sexos, demonstrou que, embora a diferença de sensibilidade entre os sexos seja pequena, nos casos em que está presente, observa-se que as fêmeas são ligeiramente mais sensíveis (OECD, 2001). Com este teste é possível evidenciar risco de intoxicações agudas, inadvertidas ou não, e a forma de preveni-las (KLEIN, LONGHINI e MELLO, 2009). No Brasil, 1728 casos de intoxicações foram registrados pelo Sistema Nacional de Informações Tóxico-Farmacológicas (SINTOX) devido ao uso indevido de plantas medicinais (ano-base 2002) (DA SILVEIRA, BANDEIRA e ARRAIS, 2008). Os resultados obtidos no ensaio agudo também dão suporte à escolha das doses para os demais testes de toxidade (KLEIN, LONGHINI e MELLO, 2009). Portanto, as informações obtidas por

meio do estudo de toxidade aguda oral são de crucial importância na avaliação da segurança de substâncias químicas para uso humano e animal, entre as quais também se encontram as drogas vegetais e fitoterápicos.

Além desse teste, os testes de toxidade subaguda são amplamente utilizados e fornecem informações sobre a toxidade das substâncias químicas após administrações repetidas, além de estabelecer doses para estudos subcrônicos posteriores. Um protocolo típico para estes testes é a administração de três diferentes doses e a avaliação das alterações macroscópicas é realizada após 14 dias de exposição ao xenobiótico (KLAASSEN e EATON, 1994).

3.7 GÊNERO *Mitracarpus* ZUCC. EX SHULT. F.

Classificação botânica

Reino: Plantae
Divisão: Magnoliophyta
Classe: Magnoliopsida
Ordem Gentianales
Família: Rubiaceae
Gênero: *Mitracarpus*

O gênero *Mitracarpus* é nativo do Brasil, sendo encontrado principalmente nos países tropicais e subtropicais. As espécies de *Mitracarpus* se diferenciam principalmente pela ornamentação do tegumento da semente e indumento do caule. Exemplos incluem *M. longicalyx* E.B. Souza & M.F. Sales (tegumento da face ventral da semente com depressão em forma de “x” que se alonga na fase dorsal em duas depressões semi-circulares; indumento do caule viloso a grabrescente) e *M. frigidus* (Willd. ex Reem Schult.) K. Schum (depressão em forma de “y” na vista ventral e a face dorsal lisa, indumento híspido) (PEREIRA, CARVALHO-OKANO e GARCIA, 2006).

Algumas espécies de *Mitracarpus* possuem o uso etnofarmacológico relatado, destacando *M. scaber* que é empregada extensamente na medicina tradicional da África ocidental para cefaleia, dor de dente, amenorreia, dispepsia, doenças

hepáticas, doenças venéreas e doenças ulcerativas. O suco da planta é aplicado topicamente para o tratamento dermatológico (DALZIEL, 1936; KERHARO e ADAM, 1974).

Moulis et al. (1992) relataram o isolamento do harounosídeo, uma hidroquinona pentalongina diglicosilada das partes aéreas frescas de *M. scaber* que demonstrou uma potente atividade antifúngica contra *Candida albicans* e *Trichophytum soudanense*. Recentemente patentes do extrato etanólico das partes aéreas dessa espécie foram adquiridas em produtos dermocosméticos para o clareamento de pele (GREFF, 1998; SPINDLER e URBANEC, 2009; TSUJI e NAKANISHI, 2010) e esta propriedade pode estar relacionada com a presença deste composto no extrato (MADHOGARIA e AHMED, 2009).

Outro estudo também mostrou que o extrato em éter dietílico das partes aéreas de *M. scaber* exibiu um amplo espectro antibacteriano e antifúngico contra cepas padrões e isolados clínicos de *Staphylococcus aureus* e de *Candida albicans* responsáveis por infecções comuns da pele (SANOGO et al., 1996). Germano et al. (1999) relataram o efeito hepatoprotetor do decoto dessa espécie na hepatotoxidade induzida por CCl_4 *in vivo* assim como *in vitro* usando hepatócitos isolados. Além disso, o extrato etanólico dessa espécie apresentou resultados significativos contra dermatofitoses em bovinos (ALI-EMMANUEL et al., 2003).

Já se tem conhecimento de alguns compostos presentes em *M. scaber*, como psolareno, rutina, campferol, campferol-3-*O*-rutinosídeo, ácido gálico, ácido 3,4,5-trimetoxi benzoico, 4-metoxiacetofenona e 3,4,5-trimetoxiacetofenona. Destes, o ácido gálico e o 3,4,5-trimetoxiacetofenona apresentaram expressiva atividade antimicrobiana contra as cepas de *S. aureus* (BISIGNANO et al., 2000). Dois ácidos triterpênicos isoméricos foram identificados nesta espécie, o ácido oleanólico e o ursólico e foi observada a atividade antimicrobiana para *Dermatophilus congolensis* (GBAGUIDI et al., 2005).

As espécies de *Mitracarpus* são caracterizadas por serem ricas em alcaloides. Okunade et al. (1999) isolou um alcaloide com atividade antimicrobiana a partir do extrato etanólico das partes aéreas de *M. scaber*, o azaantraquinona (benzilisoquinolina-5,10-diona). Outros trabalhos (NOK, 2002; NOK e NOK, 2002; GBAGUIDI et al., 2005) confirmaram a potente atividade antimicrobiana deste alcaloide e também seu potencial como agente tripanomicida.

Outra espécie *de Mitracarpus, M. villosus* (Sw) DC., foi estudada quanto às suas propriedades antimicrobianas. De acordo com Irob e Daramola (1993; 1994), o extrato etanólico de suas partes aéreas apresentou uma atividade significativa contra *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Candida albicans, Aspergillus niger* entre outros*.* Epkendu, Adesomoju e Okogun (2001) identificaram a presença de estigmasterol, 24-metilcolesta-5-em-3*β*-ol e ácido ursólico na espécie.

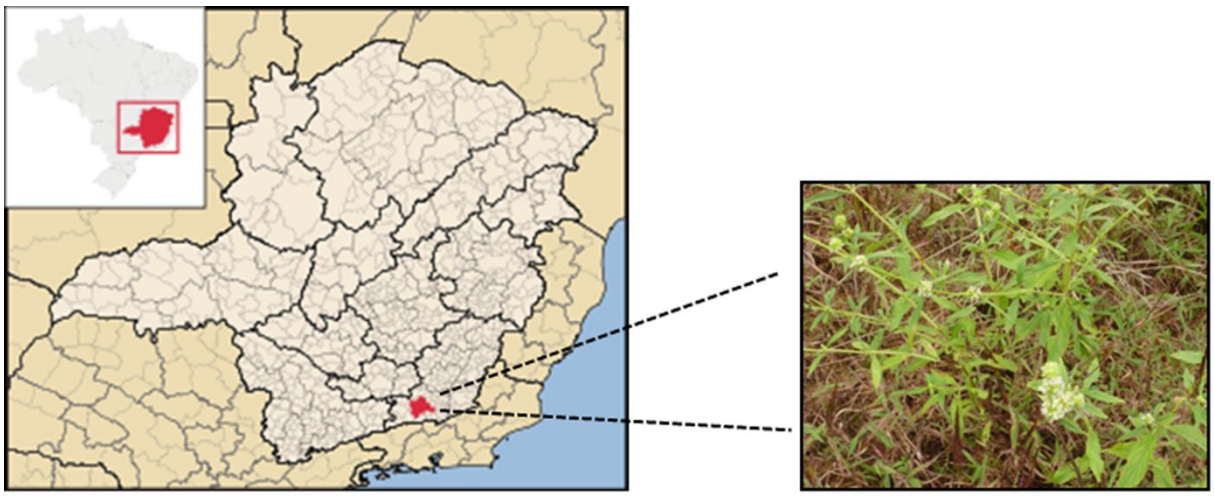
3.8 A ESPÉCIE *Mitracarpus frigidus* (WILLD. EX REEM SCHULT.) K. SCHUM.

Segundo Pereira, Carvalho-Okano e Garcia (2006), a espécie *Mitracarpus frigidus* distribui-se na América Tropical e Antilhas, sendo encontrada no Brasil desde o Amazonas até o Rio Grande do Sul. É uma espécie vegetativamente muito semelhante às espécies do gênero *Borreria,* sendo possível distinguí-la pelos ramos pilosos somente nos ângulos, estípulas cerdosas, deiscência circuncisa do fruto e semente com depressão em forma de Y na superfície ventral.

Consiste em ervas perenes, eretas de 70 cm, com ramos tetragonais, pilosos nos ângulos, folhas subsésseis, verde amareladas, lâmina elíptico-lanceolada (3-8,5× 0,5-1,5 cm), hirsuta, nervuras primárias e secundárias proeminentes na superfície dorsal, ápice agudo, base atenuada, estípulas persistentes, (6-9 cerdosas). Possui inflorescências em glomérulos terminais e axilares, multifloras, 4 brácteas, lanceoladas, flores sésseis, 4-meras, cálice com 4 lobos triangulares, iguais dois a dois, corola tubulosa, branca (2-2,5 mm), glabra, internamente com um anel de tricomas na metade do tubo, lobos oval-triangulares (0,6-1,1 mm), estames exsertos, filetes (0,3-0,5 mm), anteras subelipsoides, estilete exserto (5 mm), estigma bífido. Seu fruto possui cápsula, subglobosa, deiscência circuncisa (1×0,6 mm), pericarpo coriáceo, com pilosidade translúcida na metade superior, sementes subelipsoides, castanhas, planoconvexas, superfície ventral com depressão em forma de Y de coloração esbranquiçada (PEREIRA, CARVALHO-OKANO e GARCIA, 2006).

Segundo Fabri et al. (2009), esta espécie apresenta potencial leishmanicida, antimicrobiano e antioxidante. Tais atividades foram identificadas por meio da avaliação de seis extratos das partes aéreas de *M. frigidus* coletadas na cidade de Juiz de Fora, Minas Gerais (Figura 5). Além disso, a análise fitoquímica e a

citotoxidade também foram testadas. Os resultados identificaram a presença de alcaloides, triterpenos, esteróis, saponinas, cumarinas, fenóis, taninos, flavonoides, antraquinonas, leucocianidinas, catequinas e flavonas como principais grupos de fitoconstituintes presentes nessa espécie e uma correlação positiva dos conteúdos de compostos fenólicos e flavonoides com a atividade antioxidante dessas amostras. Em relação à atividade leishmanicida, o extrato em diclorometano apresentou-se o mais ativo contra a espécie *Leishmania chagasi* e o hidrometanólico para *L. amazonensis*. Os micro-organismos mais susceptíveis foram *Shigella sonnei, Bacillus cereus e Cryptococcus neoformans*, sendo o extrato em diclorometano com maior espectro de ação. A citotoxidade contra células de mamíferos foi moderada para todas as amostras testadas.



Fonte: Adaptado de http://pt.wikipedia.org/wiki/Mesorregi%C3%A3o_da_Zona_da_Mata

Figura 5 – Local da coleta da espécie de estudo. *Mitracarpus frigidus* foi coletada na região de Juiz de Fora, Zona da Mata Mineira.

4 REFERÊNCIAS

ABBAS, A.K.; LICHTMAN, A.H.; POBER, J.S. 2003. **Imunologia celular e molecular**. 4º ed. Rio de Janeiro: Ed. Revinter, p. 274-283.

ALI, H.; HARIBABU, B.; RICHARDSON, R.M.; SNYDERMAN, R.C. 1997.Mechanisms of inflammation and leukocyte activation. **Medical Clinics of North America, 81:** 1-28.

ALI-EMMANUEL, N.; MOUDACHIROU, M.; AKAKPO, J.A.; QUERTIN-LECLERCQ, J. 2003. Treatment of bovine dermatophilosis with *Senna alata*, *Lantana camara* and *Mitracarpus scaber* leaf extracts. **Journal of Ethnopharmacology, 86 (2-3):** 167-171.

ALLAM, G. Immunomodulatory effects of curcumin treatment on murine *Schistosomiasis mansoni*. 2009. **Immunobiology, 214:** 712-727.

ALMEIDA, O.L.; SANTOS, J.B. 2011. Avanços no tratamento de leishmaniose tegumentar do novo mundo nos últimos dez anos: uma revisão sistemática da literatura. **Anais Brasileiros de Dermatologia, 86 (3):** 497-506.

AHMAD, I.; BEG, A.Z. 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. **Journal of Ethnopharmacology, 74:** 113-123.

ANDRE, S.B.; RODRIGUEZ, T. N.; MORAES-FILHO, J. P. P. 2000. Constipação intestinal funcional. **Revista Brasileira de Medicina, 57 (11):** 1240-1252.

APTSIAURI, N.; CABRERA, T. MENDEZ, R.; GARCIA-LORA, A.; RUIZ-CABELLO, F.; GARRIDO, F. 2007. Role of altered expression of HLA class I molecules in cancer progression. **Advances in Experimental Medicine in Biology, 601:**123-31.

ASHFORD, R.W.; BATES, P.A. 1998. **Leishmaniasis on the world.** In: COX, F.E.G.; KREIER, J.P.; WAKELIN, D. (eds). Microbiology and Microbial infection. New York: Oxford University Press. 215 p.

ASHFORD, R.W. The leishmaniasis as emerging and reemerging zoonoses. 2000. **International Journal for Parasitology, 30:** 1269-1281.

BAGAMBOULA, C. F.; UYTTENDAELE, M.; DEBEVERE, J. 2004. Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptanha* (Montbret et Aucher ex Benth.) and *Salvia multicaulis* (Vahl.). **Food Chemistry, 84 (4):** 519-525.

BARREIRO, E.J. 2009. Biodiversidade: fonte potencial para a descoberta de fármacos. **Química Nova, 32 (3):** 679-688.

BASANO S.A.; CAMARGO, L.M. 2004. Leishmaniose tegumentar americana: histórico, epidemiologia e perspectivas de controle. **Revista Brasileira de Epidemiologia, 7 (3):** 328-337.

BATES, P.A. Transmission of *Leishmania* metacyclic promastigotas by phlebotomines and flies. 2007. **International Journal for Parasitology, 37:** 1097-1106.

BAXEVANIS, C.N.; PEREZ, S.A.; PAPAMICHAIL, M. 2009. Combinatorial treatments including vaccines, chemotherapy and monoclonal antibodies for cancer therapy. **Cancer Immunology, Immunotherapy, 58:** 317-324.

BEUBLER, E.; KOLLAR, G. 1988. Prostaglandin-mediated action of sennosides. **Pharmacology, 36 (Supl. 1):** 85-91.

BEUBLER, E.; SCHIRGI-DEGEN, A. 1993, Serotonin antagonists inhibit sennoside-induced fluid secretion and diarrhea. **Pharmacology, 47 (Supl. 1):** 64-69.

BHANDARE, A.M.; KSHIRSAGAR, A.D.; VYAWAHARE, N.S.; HADAMBAR, A.A.; THORVE, V.S. 2010. Potential analgesic, anti-inflammatory and antioxidant activities of hydroalcoholic extract of *Areca catecu* L. **Food and Chemical Toxicolology, 48:** 3412-3417.

BISIGNANO, G.; SANOGO, R.; MARINO, A.; AQUINO, R.; D`ANGELO, V.; GERMANO, M.P.; De PASQUALE, R.; PIZZA, C. 2000. Antimicrobial activity of *Mitracarpus scaber* extract and isolated constituents. **Letters In Applied Microbiology, 30 (2):** 105-108.

BOERS, M. 2001. NSAIDS and selective COX-2 inhibitors: competition between gastroprotection and cardioprotection. **The Lancet, 357:** 1222-1223.

BOGLIOLO, L.; BRASILEIRO-FILHO, G**.** 2006. **Bogliolo: patologia.** 7 ed. Rio de Janeiro: Guanabara Koogan.

BOTROS, S.; WILLIAM S.; HAMMAM O.; ZIDEK Z.; HOLY, A. 2003. Activity of 9-(S)-[3-hydroxy-2 (phophonomethoxy)propyl]adenine against *Schistossoma mansoni* in mice. **Antimicrobial Agents and Chemotherapy, 47(12):** 3853-3858.

BRAGA, F.G.; BOUZADA, M.L.M.; FABRI, R.L.; MATOS, M.O.; MOREIRA, F.O.; SCIO, E. COIMBRA, E.S. 2007. Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. **Journal of Ethnopharmacology, 111:** 396-402.

BRANDON, K.; FONSECA, G.A.B.; RYLANDS, A.B.; DA SILVA, J.M.C. 2005. Conservação brasileira: desafios e oportunidades. **Megadiversidade, 1(1):** 7-13.

BRASIL. Ministério da Saúde, FUNASA. 2002. **Vigilância epidemiológica – Situação da prevenção e controle das doenças transmissíveis no Brasil**.

BRASIL. Resolução RE nº 90, de 16 de março de 2004 (DOU 18.03.04). 2004. **Guia para realização de estudos de toxicidade pré-clínica de fitoterápicos.** Brasília: Agência Nacional de Vigilância Sanitária.

BRASIL. Ministério da Saúde. 2005. **Guia de vigilância epidemiológica**. Série A. Normas e Manuais Técnicos. 6ª Ed. Brasília-DF.

BRASIL. Ministério da Saúde. Secretária de Vigilância da Saúde. 2007. **Manual de vigilância da leishmaniose tegumentar americana.** 2 ed. Brasília: Editora do Ministério da Saúde.

BRASIL. Ministério da Saúde. Instituto Nacional do Câncer no Brasil. 2008. **Estimativas 2008: Incidência de câncer no Brasil.** Rio de Janeiro: INCA.

BRASIL. Ministério da Saúde. 2012. **Datasus: informações de saúde.** Disponível em: <www.datasus.gov.br/tabnet/tabnet.htm>. Acesso em: 14 jan. 2012.

BRUSERUD, O.; ERSVAER, E.; OLSNES, A.; GJERTSEN, B.T. 2008. Anticancer immunotherapy in combination with proapoptotic therapy. **Current Cancer Drug Targets, 8:** 666-675.

BURCHMORE, R.J.S.; BARRET, M.P. 2001.bLife in vacuoles – nutrient acquisions by *Leishmania* amastigotes. **International Journal for Parasitology, 31:** 1131-1320,

BURROWS, C.F.; MERRITT, A.M. 1983. Influence of α-cellulose on my electric activity of proximal canine colon. **American Journal of Physiology, 245:** 301-306.

CALIXTO, J.B.; CAMPOS, M.M.; OTUKI, M.F.; SANTOS, A.R. 2004. Anti-inflammatory compounds of plant origin. Part II. Modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. **Planta Medica, 70:** 93-103.

CAPASSO, F.; MASCOLO, N.; AUTORE, G.; ROMANO, V. 1986. Laxatives and the production of autacoids by rat colon. **Journal of Pharmacy and Pharmacology, 38:** 627-62.

CARVALHO, J.C.T. 2004. **Fitoterápicos anti-inflamatórios: aspectos químicos, farmacológicos e aplicações terapêuticas.** Ribeirão Preto: Tecmedd.

CIMERMAN, B.; CIMERMAN, S. 2002. **Parasitologia humana e seus fundamentos gerais.** São Paulo: Atheneu, 390 p.

CIRINO, C.; FIORUCCI, S.; SESSA, W. C. 2003. Endothelial nitric oxide synthase: the cinderella of inflammation? **Trends in Pharmacological Sciences, 24:** p. 91-95.

CHABOT, S.; BEL-RHILD, R.; CHENEVERT, R.; PICHE, Y. 1992. Hyphal growth promotion in vitro of the VA mycorrhizal fungus. *Gigaspora margarita* Becker and Hall, by the activity of structurally specific flavonóide compounds under CO_2-enriched conditions. **New Phytologist, 122:** 461-467.

CHAN-BACAB, M.J.; BALANZA, E.; DEHARO, E.; MUÑOZ, V.; GARCIA, R.D.; PEÑA-RODRIGUEZ, L.M. 2003. Variation of leishmanicidal activity in four populations of *Urechites andrieuxii.* **Journal of Ethnopharmacology, 86:** 243-247.

CHANG, K.P.; CHAUDHURI, G.; FONG, D. 1990. Molecular determinants of *Leishmania* virulence. **Annual Review of Microbiology, 44:** 499-529.

COLEGATE, S.M.; MOLYNEUX, R.J. 2008. **Bioactive Natural Products - Detection, Isolation, and Structural Determination.** 2 ed. Londres: Taylor & Francis Group.

COTA, R.P.; MIRANDA, L.S. 2006. Associação entre constipação intestinal e estilo de vida em estudantes universitários. **Revista Brasileira de Nutrição Cliníca, 21 (4):** 296-301.

COURA, J.R. 1995. Control of schistosomiasis in Brazil: perpectives and proposals. **Memórias do Instituto Oswaldo Cruz, 90 (2):** 101-106.

COUTINHO, M.R.; QUADRI, M.B.; MOREIRA, R.F.P.M.; QUADRI, M.G.N. 2004. Partial purification of anthocyanins from *Brassica oleracea* (red cabbage). **Separation Science and Technology, 39(16):** 3769-3782.

COUTINHO, M.A.S.; MUZITANO, M.F.; COSTA, S.S. 2009. Flavonoides: Potenciais agentes terapêuticos para o processo inflamatório. **Revista Virtual de Química, 1 (3):** 241-256.

CRUZ, A.K.; DE TOLEDO, J.S.; FALADE, M.; TERRÃO, M.C.; KAMCHONWONGPAISAN, S.; UTHAIPIBULL, C. 2009. Current treatment and drug discovery against *Leismania* ssp. and *Plasmodium* ssp.: a review. **Current Drug Targets, 10:** 178-192.

CUSHNIE, T.P.T.; LAMB, A.J. 2005. Antimicrobial activity of flavonoids. **International Journal of Antimicrobial Agents, 26 (5):** 343-356.

DA SILVA FILHO, A.A.; BUENO, P.C.P.; GREGÓRIO, L.E.; SILVA, M.L.A.; ALBUQUERQUE, S.; BASTOS, J.K. 2004. In vitro trypanocidal activity evaluation of crude extract and isolated compounds from *Baccharis dracunculifolia* D.C. (Asteraceae). **Journal of Pharmacy and Pharmacology, 56:** 1195-1199.

DA SILVEIRA, P.F.; BANDEIRAS, M.A.M.; ARRAIS, P.S.D. 2008. Farmacovigilância e reações adversas às plantas medicinais e fitoterápicos: uma realidade. **Revista Brasileira de Farmacognosia, 18 (4):** 618-626.

DALGLEISH, A.G.; WHELAN, M.A. 2006. Cancer vaccines as a therapeutic modality: the long trek. **Cancer Immunology, Immunotherapy, 55:** 1025-1032.

DANTAS, R.O. 2004. Diarréia e constipação intestinal. **Medicina, 37:** 262-266.

DALZIER, J.M. 1936. **Useful Plants of West tropical Africa.** London: The Crown Agents for Overareas Colonica.

DEKKER, L. V.; SEGAL, A W. 2000. Perspectives: signal transduction. Signals to move cells. **Science, 287 (5455):** 982-985.

DINIZ, A.G. 2004. **Resistência de Leishmania braziliensis e L. amazonensis ao óxido nítrico e correlação com a doença.** Dissertação (Mestrado em imunologia), Universidade Federal da Bahia, Salvador, Brasil, 95 p.

DIXON, R.A.; DEY, P.M.; LAMB, C.J. 1983. Phytoalexins: anzimology and molecular biology. **Advances in Enzimology and Related Areas of Molecular Biology, 55**: 1-69.

DOS REIS, M.S.T.; DE SOUZA, E.N. 2006. Informações toxicológicas de alguns fitoterápicos utilizados no Brasil. **Revista Brasileira de Ciências Farmacêuticas, 42 (2):** 289-306.

DRESCHER, K.M.; ROGERS, E.J.; BRUCE, J.I.; KATZ, N.; DIAS, L.C.; COLES, G.C. 1999. Response of drug resistant isolates of *Schistosoma mansoni* to antishistosomal agents. **Memórias do Instituto Oswaldo Cruz, 88 (1):** 89-95.

EL-SHENAWY, N.S.; SOLIMAN, M.F.M.; ABDEL-NABI, M. 2006. Does *Cleome droserifolia* have anti-schistosomiasis mansoni activity? **Revista do Instituto de Medicina Tropical de São Paulo, 48 (4):** 223-228.

EL-SHENAWY, N.S.; SOLIMAN, M.F.M.; REYAD, S.I. 2008. The effect of antioxidant properties of aqueous garlic extract and *Nigella sativa* as anti-schistosomiasis agents in mice. **Revista do Instituto de Medicina Tropical de São Paulo, 50 (1):** 29-36.

EKPENDU, T.O.E.; ADESOMOJU, A.A.; OKOGUN, J.I. 2001. Chemical studies of *Mitracarpus villosus* (Sw.) DC - a medicinal rubiaceous weed. **Journal of Chemical Society of Nigeria, 26:** 69-74.

FABRI, R.L. 2008. **Estudo fitoquímico de *Mitracarpus frigidus* (Willd. ex Beem Schult) K. Schum biomonitorado pela atividade antimicrobiana e avaliação das atividades citotóxica, antioxidante e leishmanicida.** Dissertação (Mestrado em Ciências Biológicas) – Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Juiz de Fora, Brasil, 140 p.

FABRI, R.L.; NOGUEIRA, M.S.; BRAGA, F.G.; COIMBRA, E.S.; SCIO, E. 2009 *Mitracarpus frigidus* aerial parts exhibited potent antimicrobial, antileishmanial and antioxidants effects. **Bioresource Technology, 100:** 428-433.

FALLON, P.G.; SMITH, P. DUNNE, D.W. 1998. Type 1 and type 2 cytokine producing mouse CD4+ and CD8+ T cells in acute *Schistosoma mansoni* infection. **European Journal of Immunology, 28:** 1408-1416.

FERREIRA, M.E.; ROJAS De ARIAS, A.; TORRES DE ORTIZ, S.; INCHAUSTI, A.; NAKAYAMA, H.; THOUVENEL, C.; HOCQUEMILLER, R.; FOURNET, A. 2002. Leishmanicidal activity of two canthin-6-one alkaloids, two major constituents of

Zanthoxylum chiloperone var. *angustifolium*. **Journal of Ethnopharmacology, 80:** 199-202.

FERREIRA, M.V.; FORONDA, A.S.; SCHUMAKER, T.T. 2003. **Fundamentos biológicos da parasitologia humana**. Rio de Janeiro: Ed. Manole.

FERRARI, T.C.A.; MOREIRA, P.R.R.; CUNHA, A.S. 2008. Clinical characterization of neuroschistosomiasis due to Schistosoma mansoni and its treatment. **Acta Tropica, 108 (2-3):** 87-97.

FIORUCCI, S.; MELI, R.; BUCCI, M. CIRINO, G. 2001. Dual inhibitors of cyclooxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy? **Biochemical Pharmacology, 62:** 1433-1438.

FLECK, J. 1992. **Câncer – Integração Clínico-biológico**. Rio de Janeiro: Ed. Médica e Científica Ltda.

FLIG, E.; HERMANN, T.W.; ZABEL, M. 2000. Is bisacodyl absorbed at all from suppositories in man? **International Journal of Pharmacy, 196:** 11-20.

FRANGOGIANNIS, N. G.; SMITH, C. W.; ENTMAN, M. L. 2002. The inflammatory response in myocardial infarction. **Cardiovascular Research, 53:** 31-47.

GBAGUIDI, F.; ACCROMBESSI, G.; MOUDACHIROU, M.; QUETIN-LECLERCQ, J. 2005. HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis*. **Journal of Pharmaceutical and Biomedical Analysis, 39:** 990-995.

GEISSMAN, C.S. 1963. **Flavonoid compounds, tannins, lignins and related compounds**, In: FLORKIN, M.; STOTZ; E.H. (ed.). Pyrrole pigments, isoprenoid compounds and phenolic plant constituents. vol. 9. New York: Elsevier, p. 265.

GERMANO, M.P.; SANOGO, R.; COSTA, C.; FULCO, R.; D'ANGELO, V.; TORRE, E.A.; VISCOMI, M.G.; De PASQUALE, R. 1999. Hepatoprotective properties of *Mitracarpus scaber* (Rubiaceae). **Journal of Pharmacy and Pharmacology, 51:** 729-734.

GIL, E.S.; PAULA, J.R.; NASCIMENTO, F.R.F.; BEZERRA, J.C.B. 2008. Produtos naturais com potencial leishmanicida. **Revista de Ciências Farmacêuticas Básica e Aplicada, 29 (3):** 223-230.

GREFF, D. 1998. Cosmetic and pharmaceutical compositions for beautifying and lightening skin containing *Mitracarpus* extracts. **PCT Int. Appl. WO 9805299**.

GRIMALDI, G.Jr.; TESH, R.B. 1993. Leishmaniasis of new world: current concenpts and implication for future research. **Clinical Microbiology Reviews, 6:** 230-250.

GUIDA, C.; MAIONE, P.; ROSSI, A.; BARESCHINO, M.; SCHETTINO, C.; BARZAGHI, D.; ELMO, M.; GRIDELL, C. 2008. Combined chemo-radiotherapy for

locally advanced non-small cell lung câncer: Current status and future development. **Critical Reviews in Oncology/Hematology, 68:** 222-232.

GUPTE, M., KULKARNI, P., GANGULI, B.N. 2002 Antifungal antibiotics. **Applied Microbiology and Biotechnology, 58:** 46-57.

HAGAN, P.; APPLETON, C.C.; COLES, G.C.; KUSEL, J.R. 2004. Human schistossomiasis. **Lancet, 368:** 1106-1118.

HAMED, M.A.; HETTA, M.H. 2005. Efficacy of *Citrus reticulata* and Mirazid in treatment of *Schistosoma mansoni*. **Memórias do Instituto Oswaldo Cruz,100 (7):** 771-778.

HAMMER, H.F.; SANTA ANA, C.A.; SCHILLER, L.R.; FORDTRAN, J.S. 1989. Studies of osmotic diarrhea induced in normal subjects by ingestion of polyethylene glycol and lactulose. **Journal Clinical Investiment, 83:** 66-73.

HANAHAN, D.; WEINGERG, R.A. 2000. The hall marks of cancer. **Cell, 100:** 57-70.

HARVEY, A.L. 2008. Natural products in drug discovery. **Drug Discovery Today, 13(19/20):** 894-901.

HEANEY, S.P.; HALL, A.A.; DAVIS, S.A.; OLAYA, G. 2000. **Resistance to fungicides in the QoISTAR cross resistance group: current perspectives.** *In:* BRIGHTON CROP PROTECTION CONFERENCE. Pests and Diseases, *Brighton Proceedings*, Brighton-UK, pp 755-762.

HEDQVIST, P.; GAUTAN, N.; LINBOM, L. 2000. Interactions between leukotrienes and other inflammatory mediators/modulators in the microvasculature. **Americam Journal of Respiratory and Critical Care Medicine, 161**: 117-119.

HEIT, B.; COLARUSSO, P.; KUBES, P. 2005. Fundamentally different roles for LFA-1, MAC-1 and α 4-integrin in neutrophil chemotaxis. **Journal of Cell Science, 118:** 5205-5220.

HOLTFRETER, M.C.; LOEBERMANN, M.; KLAMMT, S.; SOMBETZKI, M.; BODAMMER, D.; KINZELBACH, R.; REISINGER, E.C. 2011. *Schsitosoma mansoni*: schistomicidal effect of mefloquine and primaquine *in vitro*. **Experimental Parasitology, 127 (1):** 270-276.

HORDIJK, P. 2003. Endothelial signaling in leukocyte transmigration. **Cell Biochemistry and Biophysics, 38 (3):** 305-322.

HUGGINS, D.W.; BATISTA, R.S.; MEDEIROS, L.B. 1998. Esquistossomose mansoni. São Paulo: Grupo editorial Moreira JR, 108p.

IROB, O.N.; DARAMOLA, S.O. 1993. Antifungal activities of crude extracts of *Mitracarpus-villosus* (Rubiaceae). **Journal of Ethnopharmacology, 40:** 137-140.

IROB, O.N.; DARAMOLA, S.O. 1994. Bactericidal properties of crude extracts of *Mitracarpus-villosus*. **Journal of Ethnopharmacology, *42* :** 39-43.

JASON, J.; ARCHIBALD, L.K.; NWANYAANWU, O.C.; BYRD, M.G.; KAZEMBE, P.N.; DOBBIE, H.; JARVIS, W.R. 2001. Comparasion of serum and cell-specific cytokines in humans. **Clinical and Diagnostic Laboratory Immunology, 8 (6):** 1097-1103.

JONES, G.A.; MCALLISTER, T.A.; MUIR, A.D.; CHENG, K.J. 1994. Effects of sainfoin (*Onobrychis vivifolia* scop.) condensed tannins on grotwn and proteolysis by four strains of ruminal bacteria. **Applied and Environmental Microbiology, 60:** 1374-1378.

JONES, R.N. 2001. Resistance patterns among nosocomial pathogens - Trends over the past few years. **CHEST, 119*:*** 397-404.

KATSUNG, B.G. 2003. **Fármacos utilizados nas doenças gastrointestinais**. In: DAVID, F.; ALTMAN, M.D. Farmacologia básica & clinica. 8ª ed. Rio de Janeiro: Guanabara Koogan, 925p.

KATZ, N.; ALMEIDA, K. Esquistossomose, xistosa, barriga d'água. 2003. **Ciência e Cultura,** 55 (1): 38-43.

KERHARO, J.; ADAM, J.G. 1974. In : **La Pharmacopée Sénegalaise Traditionnelle: Plantas Médicinales Et Toxiques.** Paris: Editions Vigot Frères, p. 692-693.

KLAASSEN, C.D.; EATON, D.L. 1994. **Principles of Toxicology**. In: AMDUR, M. O.; DOULL, J.; KLAASSEN, C.D. Casarett & Doull's Toxicology. The Basic Science of Poisons. 4 ed. New York/ St. Louis/San Francisco: McGraw-Hill Inc., p. 12-49.

KLEIN, T.; LONGHINI, R.; MELLO, J.C.P. 2009. Fitoterápicos: um mercado promissor. **Revista de Ciências Farmacêuticas Básica e Aplicada, 30 (3):** 241-248.

KOBASHI, K.; NISHIMURA, T.; KUSAKA, M.; HATTORI, M.; NAMBA, T. 1980. Metabolism of sennosides by human intestinal bacteria. **Planta Medica, 40 (3):** 225-236.

KOKO, W.S.; ABDALLA, H.S.; GALAL, M.; KHALID, H.S. 2005. Evaluation of oral therapy on Mansonial Schistosomiasis using single dose of *Balanites aegyptiaca* fruits and praziquantel. **Fitoterapia, 76:** 30-34.

KUMMAR, V.; ABBAS, A.; FAUSTO, N.; ROBBINS, S.L.; COTRAN, R.S. 2005. **Pathology Basis of Disease.** 7 ed. Rio de Janeiro: Elsevier.

KUSTER, R.M.; ARNOLD, N.; WESSJOHANN, L. 2009. Anti-fungal flavonoids from *Tibouchina grandifolia*. **Biochemical Systematics and Ecology, 37 (1):** 63-65.

LECCI, A.; GIULIANI, S.; TRAMONTANA, M.; CARINI, F.; MAGGI, C. A. 2000. Peripheral actions of thachykininis. **Neuropepitides, 34:** 303-313.

LEDERLE, F.A.; BUSCH D.L.; MATTOX, K.M.; WEST, M.J.; ASKE; D.M. 1990. Costeffective treatment of constipation in the elderly: a randomized double-blind comparison of sorbitol and lactulose. **American Journal Medicine, 89:** 597-601.

LENTZ, D.L.; CLARK, A.M.; HUFFORD, C.D.; MEURER-GRIMES, B.; PASSREITER, C.M.; CORDERO, J.; IBRAHIMI, O.; OKUNADE, A.L. 1998. Antimicrobial properties of Honduran medicinal plants. **Journal of Ethnopharmacology, 63 (3):** 253-263

LEY, K. 2002. Integration of inflammatory signals by rooling neutrophils. **Immunology Reviews, 186:** 8-18.

LU, Y.; ZHAO, Y.P.; WANG, Z.C.; CHEN, S.Y.; FU, C.X. 2007. Composition and antimicrobial activity of the essential oil of *Actinidia Macrosperma* from China. **Natural Product Research, 21 (3):** 227-233.

MADHOGARIA, S.; AHMED, I. 2009. Leucoderma after use of a skin-lightening cream containing kojic dipalmitate, liquorice root extract and *Mitracarpus scaber* extract. **Clinical and Experimental Dermatology, 35:** 103-105.

MAGALHÃES , L.C.; MACHADO, C.B.; MORAIS, E.R.; MOREIRA, E.B.C.; SOARES, C.S.; SILVA, S.H.; FILHO, A.A.S; RODRIGUES, V. 2009. *In vitro* schistosomicidal activity of curcumin against *Schistosoma mansoni* adult worms. **Parasitology Research, 104:** 1197-1201.

MAGALHÃES, L.C.; KAPADIA, G.J.; TONUCI, L.R.S.; CAIXETA, S.C.; PARREIRA, N.A.; RODRIGUES, V. FILHO, A.A.S. 2011. *In vitro* schistosomicidal effects of some phloroglucinol derivates from *Dryopteris* species against *Schistosoma mansoni* adult worms. **Parasitology Research, 106:** 395-401.

MANN, J. 2002. Natural products in cancer chemotherapy: past, present and future. **Nature Reviews: Cancer, 2 (2):** 143-148.

MARTINS, E.R.; CASTRO, D.M.; CASTELLANE, D.C.; DIAS, J.E. 2000. **Plantas Medicinais**. Viçosa: UFV. 220p.

MASON, T.L.; WASSERMAN, B.P. 1987. Inactivation of red beet betaglucan synthase by native and oxidized phenolic compounds. **Phytochemistry, 26**: 2197-2202.

MBWAMBO, Z.H.; MOSHI, M.J.; MASIMBA, P.J.; KAPINGU, M.C.; NONDO R.S. 2007. Antimicrobial activity and brine shrimp toxicity of extracts of *Terminalia brownii* roots and stem. **BMC Complementary and Alternative Medicine, 7 (9):** 1-5.

MELO, R.R.; ARAÚJO, E.R.S.; SILVA, A.A.L.; RANDAU, K.P.; XIMENES, E.C.P.A. 2009. Características farmacobotânicas, químicas e biológicas de *Syzygium*

malaccense (l.) Merr. & l. M. Perry. **Revista Brasileira de Farmácia**, 90 (4): 298-302.

MONTHANA, R.A.A. 2011. Anti-inflammatory, antinociceptive and antioxidant activities of the endemic Soqotraen *Boswellia elongata* Balf f. and *Jatropha unicostata* Balf f. different experimental models. **Food and Chemical Toxicology, 49 (10):** 2594-24999.

MONZOTE, L.; GARCIA, M.; MONTALVO, A.M.; SCULL, R.; MIRANDA, M.; ABREU, J. 2007. *In vitro* activity of an essential oil against *Leishmania donovani*. **Phytotherapy Research, 21 (11):** 1055-1058.

MORAES, J.; NASCIMENTO, C.; LOPES, P.O.M.V.; NAKANO, E.; YAMAGUCHI, L.F. KATO, M.J.; KAWANO, T. 2010. *Schistosoma mansoni*: *in vitro* schistosomicidal activity of piplartine. **Experimental Parasitology, 127 (2):** 357-364.

MOREL, A.F.; MALDANER, G.; ILHA, V.; MISSAU, F.; SILVA, U.F.; DALCOL, I.I. 2005. Cyclopeptide alkaloids from *Scutia buxifolia* Reiss and their antimicrobial activity. **Phytochemistry, 66:** 2571–2576.

MOULIS, C.; PELISIER, J.; BAMBA, D.; FOURASTÉ, L. 1992. **Pentalongin, antifungal naphthoquinoid pigment from *Mitracarpus scaber*.** Second International Congress on Ethnopharmacology, Uppsala, Sweden. Stockholm: The Swedish Academy of Pharmaceutical Sciences.

MUELLER, M.; HOBIGER, S.; JUNGBAUER, A. 2010. Anti-inflammatory activity of extracts from fruits, herbs and spices. **Food Chemistry, 122:** 987-996.

N'GUESSAN, J.D; DINZEDI, M.R.; GUESSENND, N.; COULIBALY, A.; DOSSO, M.; DJAMAN, A.J.; GUEDE-GUINA, F. 2007. Antibacterial activity of the aqueous extract of *Thonningia sanguinea* against extended-spectrum-β-lactamases (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* strains. **Tropical Journal of Pharmaceutical Research, 6 (3):** 779-783.

NASCIMENTO, A.; MORENO, P.R.H; SOUZA. A.; YOUNG, M.C.M. 2008. Chemical composition and antimicrobial activity of the essential oil from *Bidens segetum* Mart. Ex Colla leaves, flowers and fruits. **Planta Medica, 74 (9):** 1199-1210.

NEVES, D.P.; MELO, A.L; LINARDI, P.M.; VITOR, R.W. 2005. **Parasitologia humana.** 11 ed. São Paulo: Atheneu. 494 p.

NICKOLOFF, B. J.; NESTLÉ, F. O. 2004. Recent insights into the immunopathogenesis of psoriasis provide new therapeutic opportunities. **Journal Clinical Investigation, 13:** 1664-1675.

NOK, A.J. 2002. Azaanthraquinone inhibits respiration and in vitro growth of long slender bloodstream forms of *Trypanosoma congolense*. **Cell Biochemistry and Function, 20:** 205-212.

NOK, A.J.; NOK, I.H. 2002. Transferrin coupled azanthraquinone enhances the killing effect on trypanosomes. The role of lysosomal mannosidase. **Parasite, 9 (4):** 375-379.

O'KENNEDY, R.; THORNES, R.D. 1997. **Coumarins: biology, applications and mode of action.** New York: John Willey.

OKUNADE, A.L.; CLARK, A.M.; HUFFORD, C.D.; OGUNTIMEIN, B.O. 1999. Azaanthraquinone: An antimicrobial alkaloid from *Mitracarpus scaber.* **Planta Medica, 65:** 447-448.

ORGANIZATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (OECD). 2001. **Guideline 423: Acute Oral Toxicity – Acute Toxic Class Method.** Paris: Head of Publications Service.

PAL, S. K.; SHUKLA, Y. 2003. Herbal medicine: current status and the future. **Asian Pacific Journal of Cancer Prevention, 4 (4):** 281-288.

PARENTE, L. 2001. Pros and cons of selective inhibition of cyclooxygenase-2 versus lipoxygenase/cyclooxygenase inhibition: is two better than one? **The Journal of Rheumatology, 28:** 2375-2382.

PATTERSON, C. E.; LUM, H. 2001. Update on pulmonary edema: the role and regulation of endothelial barrier function. **Endothelium, 8:** 75-105.

PESSÔA, S. B.; MARTINS, A. V. 1988. **Parasitologia Médica**. Rio de Janeiro: Guanabara Koogan.

PENIDO, M.L.; COELHO, P.M.; NELSON, D.L. 1999. Efficacy of a new schistosomicidal agent 2-[(methylpropyl)amino]-1-octanethiosulfuric acid against an oxamniquine resistant *Schistosoma mansoni* isolte. **Memórias do Instituto Oswaldo Cruz, 94 (6):** 811-813.

PEREIRA, Z.V.; CARVALHO-OKANO, R.M.; GARCIA, F.C.P. 2006. Rubiaceae Juss. da Reserva Florestal Mata do Paraíso, Viçosa, MG, Brasil. **Acta Botanica Brasilica, 20 (1):** 207-224.

PHILLIPSON, J.D.; O'NEIL, M.J. 1989. New leads to the treatment of protozoal infections based on natural product molecules. **Acta Pharmaceutica Nordica, 1:** 131-144.

PONTIN, K.; DA SILVA FILHO, A.A.; SANTOS, F.F.; SILVA, M.L.A.; CUNHA, W.R.; NANAYAKKARA, N.P.D.; BASTOS, J.K. 2008. *In vitro* and *in vivo* antileishmanicidal activities of Brazilian Green própolis extract. **Parasitology Research, 103:** 487-492.

PRATES, M.V.; BLOCH JÚNIOR, C. 2001. Peptídeos antimicrobianos. **Biotecnology, 23:** 30-36.

PYRRHO, S.A.; RAMOS, J.A.; NETO, R.M.; SILVA, C.S.; LENZI, H.L.; TAKIYA, C.M.; GATTASS, C.R. 2002. Dexamethasone, a drug for attenuation of *Schistosoma*

mansoni infection morbidity. **Antimicrobial Agents Chemotherapy, 46 (1):** 3490-3498.

REINER, T.; POZAS, A.D.L.; GOMEZ, L.A.; PEREZ-STABLE, C. 2009. Low dose combinations 2-methoxyestradiol and docetaxel block prostate cancer in mitosis and increase apoptosis. **Cancer Letters, 276 (1):** 21-31.

REY; L. 2002. **Bases da parasitologia médica.** 2. ed. Rio de Janeiro, Guanabara Koogan. 579 p.

RIBEIRO-DOS-SANTOS, G.; VERJOVSKI-ALMEIDA, S.; LEITE, S.C. 2006. Schsitosomiasis – a century searching of chemotherapic drugs. **Parasitology Research, 99 (5):** 505-521.

ROCHA E SILVA, M.; GARCIA LEME, J. 2006. Chemical mediators of the acute inflammatory reaction. In: International series of monographs in pure and applied biology. **Modern Trends in Physiological Sciences**, 37: 1-47.

RODRIGUES, M.R.; RODRIGUEZ, D.; RUSSO, M.; CAMPA, A. 2002. Macrophage activation includes high intracellular myeloperoxidase activity. **Biochemical and Biophysical Research Communications, 292:** 869-873

RODRIGUES, F.H. 2005. Efeito do imidocarb e do levamisol sobre a infecção experimental de camundongos BALB/C por *Leishmania amazonensis*. Dissertação (Mestrado em Imunologia e Parasitologia Aplicadas), Universidade Federal de Uberlândia, Uberlândia, Brasil, 81 p.

ROWLAND, L.P. 2002. (Ed.). **Merritt tratado de neurologia**. Tradução de: ARAÚJO, C.L.C.; MUNDIM, F.D. Rio de Janeiro: Guanabara Koogan. 887 p.

RUIZ, A.L.T.G.; MAGALHÃES, E.G.; MAGALHÃES, A.F.; FARIA, A.D.; AMARAL, M.C.E.; SERRANO, D.R.; ZANOTTI-MAGALHÃES, E.M.; MAGALHÃES, L.A. 2005. Avaliação da atividade tóxica em *Artemia salina* e *Biomphalaria glabrata* de extratos de quatro espécies do gênero *Eleocharis* (Cyperaceae). **Revista Brasileira de Farmacognosia, 15 (2):** 98-102.

SACKS, D.; KAMHAWI, S. 2001.Molecular aspects of parasite-vetor and vetor-host interactions in leishmaniasis. **Annual Review of Microbiology, 55:** 453-483.

SAHOO, N.; MANCHIKANTI, P.; DEY, S. 2010. Herbal drugs: Standards and regulation. **Fitoterapia, 81 (6):** 462-471.

SANOGO, R.; GERMANÓ, M.P.; De PASQUALE, R.; KEITA, A.; BISIGNANO, G. 1996. Selective antimicrobial activities of *Mitracarpus scaber* Zucc against *Candida* and *Staphylococcus* sp. **Phytomedicine, 2:** 265-268.

SANDERSON, L.; BARTLETT, A.; WHITFIELD, P.J. 2002. *In vitro* and *in vivo* studies on the bioactivity of a ginger (*Zingiber officinale*) extract towards adult schistosomes and their egg production. **Journal of Helminthology, 76 (3):** 241-247.

SANTOS, D.O.; COUTINHO, C.E.R.; MADEIRA, M.F.; BOTTINO, C.G.; VIEIRA, R.T.; NASCIMENTO, S.B.; BERNARDINO, A.; BOURGUIGNON, S.C.; CORTE-REAL, S.; PINHO, R.T.; RODRIGUES, C.R.; CASTRO, H.C. 2008. Leishmaniasis treatment – a challenge that remains: a review. **Parasitology Research, 103 (1):** 1-10.

SANTOS JUNIOR, J.C.M. 2003. Rubor, calor, tumor e dor e o paciente grave. **Revista Brasileira de Coloproctologia, 23 (3):** 206-210.

SATO, M.; FUJIWARA, S.; TSUCHIYA, H.; FUJII, T.; IINUMA, M.; TOSA, H.; OHKAWA, Y. 1996. Flavones with antibacterial activity against cariogenic bacteria. **Journal of Ethnopharmacology, 54:** 171-176.

SCALBERT, A. 1991. Antimicrobial properties of tannins. **Phytochemistry, 30:** 3875-3883.

SCHILLER, L.R. 1997. **Cathartics, laxatives, and lavage solutions.** In: FRIEDMAN, G.; JACOBSON, E.D.; MCCALLUM R.W. (eds.) Gastrointestinal Pharmacology and Therapeutics. Philadelphia: Lippincott-Raven, 159-174.

SCHILLER, L. R. 2001. Review article: the therapy of constipation. **Alimentary and Pharmacology Therapeutic, 15:** 749-763.

SCHIMITZ, L.M.; BACHER, S. 2005. Novel molecular targets in the search for anti-inflammatory agents. **Phytochemistry Reviews, 4:** 19-25.

SHERWOOD, E.R.; TOLIVER-KINSKY, T. 2004. Mechanisms of the inflammatory response. **Best Practice & Research Clinical Anaesthesiology, 18:** 385-405.

SILVA, H.T.H.; HARTMANN, A.A. 2006. A relação do uso crônico de fenobarbital com áreas potencialmente pré-neoplásicas em fígados de ratos. **Arquivos de Gastroenterologia, 43:** 121-124.

SILVA, C.M.R. 2008a. **Perfil clínico e epidemiológico dos portadores de mielorradiculopatia esquistossomótica atendidos em uma unidade de saúde de Pernambuco**. Dissertação (Mestrado em Saúde Publica). Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Brasil. 154 p.

SILVA, D.G. 2008b. **Padronização do cultivo de amastigotas axênicos e intracelulares de *Leishmania* spp. e análise da atividade leishmanicida de chalconas.** Dissertação (Mestrado em Biotecnologia), Universidade Federal de Santa Catarina, Florianópolis, Brasil. 120 p.

SIMÕES, C.O.M.; MENTZ, L.A.; SCHENKELL, E.P.; GOSMANN, G.; DE MELLO, J.C.P.E.; PETROVICK P.L. 2007. **Farmacognosia da planta ao medicamento.** Porto Alegre: Editora da Universidade/UFRGS.

SINGH, K.V.; SHUKLA, N.P. 1984 Activity on multiple resistant bacteria of garlic (*Allium sativum*) extract. **Fitoterapia, 55:** 313-315.

SINGH, B.; BHAT, T.K. 2003. Potential therapeutic applications of some antinutritional plant secondary metabolites. **Journal of Agricultural and Food Chemistry**, 51: 5579-5597.

SPINDLER, R.; URBANEC, S.J. 2009. Improved skin brightening compositions. **PCT Int. Appl. WO 2009/099911**

STEFFENS, J.J.; PELL, E.J.; TIEN, M. 1996. Mechanisms of fungicide resistance in phytopathogenic fungi. **Current Opinion in Biotechnology, 7(3):** 348-355.

STEINMANN, P.; KEISER, J.; BOS, R.; TANNER, M. UTZINGER, J. 2006. Schistossomiasis and water resources development; systematic review, meta-analysis, and estimates of people at risk. **The lancet Infectious Diseases, 6:** 411-425.

STERN, J.L.; HAGERMAN, A.E.; STEINBERG, P.D.; MASON, P.K. 1996 Phlorotannin-protein interactions. **Journal of Chemical Ecology, 22:** 1887-1899

SÜLSEN, V.P.; CAZORLA, S.I.; FRANK, F.M.; REDKO, F.C.; ANESINI, C.A.; COUSSIO, J.D.; MALCHIODI, E.L.; MARTINO, V.S.; MUSCHIETTI, L.V. 2007. Trypanocidal and leishmanicidal activities of flavonoids from argentine medicinal plants. **The American Journal of Tropical Medicine and Hygiene, 77 (4):** 654-659.

SUZUKI, Y.; RUIZ-ORTEGA, M.; LORENZO, O.: RUPEREZ, M.; ESTEBAN, V.; EGIDO, J. 2003. Inflammation and angiotensin II. **International Journal of Biochemistry and Cell Biology, 35:** 881-900.

TAGLIATI, C.A.; FÉRES, C.A.O. 2009. **Pesquisas toxicológicas e farmacológicas.** In: LEITE, J.P.V. (Ed.). Fitoterapia: Bases científicas e tecnológicas. São Paulo: Atheneu. p. 119-140.

TAN, P.; LUSCINKAS, F. W.; HONNER-VANNIASINKAM, S. 1999. Cellular and molecular mechanisms of inflammation and thrombosis. **European Journal of Vascular and Endovascular Surgery, 17:** 373-389.

TANAKA, J.C.; DA SILVA, C.C.; FERREIRA, I.C.; MACHADO, G.M.; LEON, L.L. DE OLIVEIRA A.J. 2007. Antileishmanial activity of indole alkaloids from *Aspidosperma ramiflorum*. **Phytomedine, 4 (6):** 377-380.

TAYLOR, R.S.; MANANDHAR, N.P.; TOWERS, G.H.N. 1994. Screening of selected medicinal plants of Nepal for antimicrobial activities. **Journal of Ethnopharmacology, 46 (3):** 153-159.

TEDGUI, A.; MALLAT, Z. 2001. Anti-inflammatory mechanisms in the vascular wall. **Circulation Research, 88:** 877-887.

TEICH, N.M.; FRANKS, L.M. 1990. **Introdução a biologia celular e molecular do câncer.** 1 ed. São Paulo: Ed. Roca.

TEIXEIRA, L.A.; FONSECA, C.M.O. 2007. **De doença desconhecida a problema de saúde pública: o INCA e o controle do Câncer no Brasil .** Rio de Janeiro: Ministério da Saúde. 172p.

TSUCHIYA, H.; SATO, M.; IINUMA, M.; YOKOYAMA, J.; OHYAMA, M.; TANAKA, T.; TAKASE, I.; NAMIKAWA, I. 1994. Inhibition of the growth of cariogenic bactéria *in vitro* by plant flavanones. **Experientia, 50:** 846-849

TSUJI, D.; NAKANISHI, H. 2010. Skin-conditioning compositions containing fucoxanthin and hydroquinone derivative and cosmetics, skin-lightening compositions, and cosmetic creams containing them. **JP 2010/209023.**

VIEGAS, C J.; BOLZANI, V. S.; BARREIRO, E.J. 2006. Os produtos naturais e a química medicinal moderna. **Química Nova, 29 (2):** 326-337.

WAGNER, H. 1988. **Pharmazeutische Biologie 2: Drogen und ihre Inhaltsstoffe.** 4 ed. Stuttgart: Gustav Fisher.

WANNMACHER, L. 2005. Constipação intestinal crônica no adulto e na criança: quando não se precisa de medicamentos. **Uso Racional de Medicamentos: Temas Selecionados, 3 (1).**

WEI, H.; SONG, J.; FANG, L.; LI, G.; CHATTERJEE S. 2004. Identification of a novel transcript of human PECAM-1 and its role in the transendothelial migration of monocytes and CA^{+2} mobilization. **Biochemical and Biophysical Research Communications, 320 (4):** 1228-1235.

WERZ, O. 2007. Inhibition of 5-lipoxygenase product synthesis by natural compounds of plant origin. **Planta Medica, 73:** 1331-1357.

WORLD HEALTH ORGANIZATION. 2008**. 2008-2013 Action plan for the global strategy for the prevention and control of noncommunicable diseases.** WHO Press. Geneva.

WORLD HEALTH ORGANIZATION. 2010. **Leishmaniasis.** Disponível em < http://www.who.int/en/> Acesso em: 20 de outubro, 2010.

WORLD HEALTH ORGANIZATION/ GLOBOCAN. 2010. **Cancer incidence, mortality and prevalence worldwide in 2008.** IARC, v.12.

WORLD HEALTH ORGANIZATION. 2012. **Schistosomiasis.** Fact sheet N°115. Disponível em <http://www.who.int/mediacentre/factsheets/fs115/en/index.html> Acesso em: 01 de fevereiro, 2012.

YANG, L.; FROIO, R.M.; SCIUTO, T.E.; DUORAK, A.M.; ALON, R.; LUSCIN SKAS, F.W. 2005. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. **Blood, 106 (2):** 584-592.

YU, M.; FINN, O.J. 2006. DNA vaccines for câncer too. **Cancer Immunology, Immunotherapy, 55:** 119-130.

ZHANG, Y.; LEWIS, K. 1997. Fabatins: new antimicrobial plant peptides. **FEMS Microbiological Letters, 149:** 59-64.

5 ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos. Os itens Materiais e Métodos, Resultados e Discussão e Referências Bibliográficas encontram-se nos próprios artigos. Os artigos estão dispostos da mesma forma que foram publicados nas revistas científica (**Artigos 1** a **3)** e que foram submetidos **(Artigos 4 a 6)**. Além disso, será apresentado o **Artigo 7**, que está em fase final de preparação.

5.1 O artigo a seguir relata a toxidade *in vitro* em células tumorais e o potencial toxicológico agudo (DL_{50}) e subcrônico e a atividade laxativa *in vivo* do extrato metanólico das partes aéreas de *Mitracarpus frigidus.*

Artigo 1

IN VIVO LAXATIVE AND TOXICOLOGICAL EVALUATION AND *IN VITRO* ANTITUMOR EFFECTS OF *Mitracarpus frigidus* AERIAL PARTS

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JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

In-vivo laxative and toxicological evaluation and in-vitro antitumour effects of *Mitracarpus frigidus* aerial parts

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Keywords
antitumour; intestinal motility; laxative; *Mitracarpus frigidus*; toxicity

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Abstract

Objectives To evaluate the in-vitro antitumour properties, and the in-vivo laxative and toxicological effects of the methanolic extract of the aerial parts of *Mitracarpus frigidus* (MFM).

Methods The in-vitro antitumour activity of MFM was evaluated against three human tumour cell lines: Jurkat, HL60 and MCF-7. The laxative activity and the effect of MFM on intestinal motility were evaluated in rats at the doses of 100, 300 and 1000 mg/kg. Acute oral toxicity was performed at 10, 100, 1000 and 2000 mg/kg and subchronic toxicity was evaluated at 100, 300 and 1000 mg/kg of MFM during a 42-day period. After subchronic administration of MFM the biochemical, haematological and histopathological parameters were analysed. Also, the total content of anthraquinones was determined.

Key findings MFM was cytotoxic only against HL60 and Jurkat cells with 89 and 83% growth inhibition, respectively. The laxative activity of MFM was similar to bisacodyl. Regarding the effect on intestinal motility, MFM showed a significant increase in the pathway of charcoal compared with the group treated with saline. Furthermore, MFM showed no in-vivo toxicity at the doses tested. Free and anthraquinone *C*- and *O*-glycosides were detected in MFM.

Conclusions MFM showed significant antitumour activity for leukaemic cells. Moreover, it presented laxative potential and no in-vivo toxicity.

Introduction

The medicinal plants used to treat many diseases are associated with folk medicine from different parts of the world.[1] Different cultures know and use the therapeutic potential of plants in the treatment of disease, and it is a practice that has evolved over centuries.[2]

Some species of the genus *Mitracarpus* (Rubiaceae) have many ethnopharmacological uses. For example, *Mitracarpus scaber* Zucc. ex Schult. & Schult. F. is used in traditional medicine in West Africa for headaches, toothache, amenorrhoea, dyspepsia, hepatic and venereal diseases, and leprosy. The juice of the plant is applied topically for the traditional treatment of diseases of the skin.[3] Germano *et al.*[4] reported the hepatoprotective effects of a decoction of *M. scaber* on CCl_4-induced hepatotoxicity *in vivo*, as well as *in vitro*, using isolated hepatocytes.

There are no reports however on the traditional uses of *M. frigidus* (Willd. ex Roem. & Schult.) K. Shum, an annual shrub commonly found in Brazil. However, the methanolic extract of the aerial parts of *M. frigidus* has shown significant leishmanicidal activity against *Leishmania chagasi* and *L. amazonensis* promastigote forms. Also, it has shown significant growth inhibition of pathogenic bacteria and yeasts strains.[5] Phytochemical screening of this extract revealed the presence of alkaloids, terpenoids, steroids, saponins, anthraquinones and phenolic compounds.[6]

This study has evaluated the in-vivo laxative and toxicological effects, and the in-vitro antitumour properties of the methanolic extract of the aerial parts of *M. frigidus*. The total content of anthraquinones was determined also.

Materials and Methods

Plant material

M. frigidus aerial parts were collected in Juiz de Fora, Minas Gerais, Brazil, in May, 2009. The plant was identified by Dr Tatiana Konno. A voucher specimen (CESJ 46076) was deposited at the Herbarium Leopoldo Krieger of Federal University of Juiz de Fora.

Preparation of the extract

The aerial parts (1 kg) of *M. frigidus* were powdered and macerated with methanol (5 × 2000 ml) for five days at room temperature. After evaporation of the solvent under reduced pressure at 45°C, the methanolic extract was obtained (MFM), and kept in tightly stoppered bottles under refrigeration until used for the biological testing and phytochemical assays.

Detection of free and combined anthraquinones

A portion of the MFM that was subjected to biological screening was used for the identification of free and conjugated anthraquinones, employing the protocols described by Matos.[7]

Quantitative analysis of total anthraquinones and total anthraquinone glycosides

The amounts of total anthraquinones and anthraquinone glycosides were determined as previously described by Sakulpanich and Gritsanapan[8] with slight modifications. The calibration curve was made using emodin as reference at five concentrations, from 1.56 to 25.00 μg/ml. The contents of total anthraquinones and total anthraquinone glycosides in MFM were calculated using the linear regression equation of emodin. The contents were expressed as mean ± standard deviation (SD) (% w/w).

In-vitro antitumour assay

Cell lines

Three human tumour cell lines were used, Jurkat (human immortalized line of T lymphocyte), HL60 (human promyelocytic leukaemia) and MCF-7 (breast cancer). HL60 and Jurkat cells were kindly furnished by Dr Gustavo Amarante-Mendes (São Paulo University, Brazil). MCF-7 was generously provided by Alfredo Goes (Federal University of Minas Gerais, Brazil). All lineages were maintained in the logarithmic phase of growth in RPMI 1640 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY, USA) enriched with 2 mM L-glutamine and 10% fetal bovine serum. All cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air. The media were changed twice weekly and they were regularly examined. All cell lines were used until 20 passages.

Evaluation of cytotoxic effect against human tumour cell lines

Tumour cell lines (Jurkat, HL60 and MCF-7) were inoculated at 50 000 cells per well. The plates were pre-incubated for 24 h at 37°C to allow adaptation of cells before addition of the samples. A freshly prepared solution of sample was tested for 48 h in an atmosphere of 5% CO_2 and 100% relative humidity. The samples included 20 μg/ml MFM, 0.05% dimethyl sulfoxide (DMSO; negative control) and 14 μM etoposide (positive control). Cell viability was estimated by measuring the rate of mitochondrial reduction of tetrazolium dye (MTT). The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only.[9] All samples were tested in triplicate, in three independent experiments.[10] The optical densities (OD) were measured with a spectrophotometer at 590 nm. Results were expressed as percentage of cell proliferation, comparing with 0.05% DMSO control and were calculated as follows: viability (%) = (mean OD treated – mean OD background)/mean OD untreated cultured, i.e. 0.05% DMSO – mean OD blank wells) × 100. Interactions of MFM and media were estimated on the basis of the variations between extract-containing medium and extract-free medium to avoid false-positive or false-negative results.[11]

DNA fragmentation assay

Cell cycle status and quantification of DNA fragmentation (hypodiploid DNA content) were performed by propidium iodide (PI) staining according to Nicolleti *et al*.[12] Cells were treated with MFM at 20 μg/ml in a 5% CO_2/95% air-humidified atmosphere at 37°C for 24 h. After incubation, the cells were centrifuged and re-suspended in hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated at 4°C for 4 h, and immediately analysed by flow cytometry. The PI fluorescence of 20 000 individual nuclei was measured using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Data were analysed by FlowJo software (TreeStar Inc, CA, USA).

Animals

Male Wistar rats (60-days-old, 160–200 g) were obtained from the Reproduction Biology Center of the Federal University of Juiz de Fora. They were housed in a room kept under controlled conditions with temperature maintained at

22 ± 2°C, on a 12-h light/dark cycle, with free access to water and complete commercial chow (Nuvital, Colombo, PR, Brazil). Throughout the experiments animals were processed according to the suggested ethical guidelines for the care of laboratory animals. The study was approved by the Brazilian College of Animal Experimentation (COBEA – Protocol no. 009/2009).

Laxative activity

After acclimatization, rats previously fasted for 8 h were divided into five groups ($n = 6$) as follows: group A, negative control received normal saline + 3% DMSO; group B, reference group received bisacodyl (Dulcolax) in saline (0.25 mg/kg); groups C–E, received MFM at 100, 300 or 1000 mg/kg body weight, respectively. All extracts were re-suspended in saline + 3% DMSO and administered orally, by gavage (1 ml). The test was performed according to Capasso *et al*.[13] Immediately after dosing, the animals were separately placed in cages suitable for collection of the faeces. After 8 h of drug administration, the faeces were collected and weighed. Thereafter, food and water were given to all rats and faecal outputs were weighed after 16 h.

Effect on intestinal motility

The method described by Jansen and Jageneau[14] and Wong and Wai[15] was used to test the effect of MFM on intestinal motility. The animals were divided into five groups ($n = 6$) as follows: group A, negative control received normal saline orally; group B, reference group received bisacodyl in saline + 3% DMSO (0.25 mg/kg); groups C–E received MFM at 100, 300 or 1000 mg/kg body weight, respectively. The animals were previously fasted for 12 h, but were allowed free access to water. All extracts were resuspended in saline + 3% DMSO and administered orally, by gavage (1 ml). After 40 min, 1 ml 10% charcoal suspension in 5% acacia solution was administered to each animal orally. The animals were killed after 20 min and the abdomens were opened. The small intestines were dissected out and placed on a clean surface. The distance travelled by the charcoal meal from the pylorus was measured. The entire length of the small intestine was measured and the percentage distance travelled by the charcoal plug along the small intestine (from the pylorus to the caecum) was estimated for the extract, control and the reference drug.

Acute oral toxicity

The acute oral toxicity was conducted in compliance with Costa-Silva *et al*,[16] with slight modifications. After acclimatization, eight rats were randomly divided into five groups. Group A, control group received the vehicle (saline + 3% DMSO) in a volume of 1 ml/100 g body weight by gavage; groups B–E: received MFM at a dose of 10, 100, 1000 or 2000 mg/kg, respectively.

All animals were observed individually for clinical signs of toxicity immediately and at 1, 2, 4, and 8 h after dosing. Observations were focused on changes in skin, fur, eyes, mucous membranes, respiratory system, autonomic and central nervous systems as well as somatomotor activity and behavioural patterns. The number of animals was noted after 24 h and then maintained for a further 14 days with a once daily observation. Animals were weighed on day 0, and on days 7 and 14. At the end of the study, all surviving animals were killed. Gross pathological examinations of all major internal organs such as heart, lungs, livers, kidneys were performed.

Subchronic toxicity

After acclimatization, eight rats were randomly divided into four groups. Group A, control group to which saline + 3% DMSO were administered orally; groups B–D received a daily dose of MFM at 100, 300 or 1000 mg/kg body weight, respectively, for 42 days. In each case the volume administered was 1 ml/100 g body weight. Each animal was marked and each day for 42 days its body weight, consumption of water and food were measured and behaviour observed.

Biochemical and haematological analyses

At the end of the study, all surviving animals were fasted overnight but with free access to water. Animals were anaesthetized by intraperitoneal injection with pentobarbital sodium (40 mg/kg). Blood samples were collected from the common cardiac puncture and used to measure haematological and biochemical parameters. The haematological parameters assessed included white blood cell count (WBC), differential leucocyte count, red blood cell count (RBC), haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC). The biochemical parameters assessed included glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, globulin, total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglyceride and total cholesterol.[17,18]

Histopathological analysis

On day 42 after blood collection for biological analysis, all the animals were killed and the principal vital organs were removed and macroscopically analysed. After macroscopic analysis, representative fragments of liver and kidneys were subsequently fixed in a 10% solution of buffered formalin

(pH 7.4) and enclosed in paraffin. Five-micrometer sections were obtained and coloured with haematoxylin–eosin for evaluation under an optical microscope.

Statistical analysis

For the in-vivo assays and biochemical analyses, values were presented as means ± standard error of mean (SEM). For the in-vitro antitumoral assays, the results were presented as mean ± standard deviation (SD) for at least two independent experiment performed in duplicate. Statistical differences between the treatments and the controls were tested by one-way analysis of variance followed by the Bonferroni test using the GraphPad Prism 4 statistic computer program. A difference in the mean values of $P < 0.05$ was considered to be statistically significant.

Results and Discussion

In-vitro antitumour activity

The antitumour activity of MFM was evaluated for three lineages of cancer cells: two leukaemic, HL60 (human promyelocytic leukaemia cells) and Jurkat (human T cell lymphoblast-like cell line), and a human breast adenocarcinoma cell line (MCF-7)

MFM (20 μg/ml) was cytotoxic against HL60 and Jurkat cells, with 89 and 83% growth inhibition, respectively. It is important to point out that for both cells the reduction of the cellular proliferation was closer to the reference drug (etoposide) (Figure 1a).

To propose a mechanism of cytotoxic action of MFM, its ability to induce apoptotic death was evaluated (Figure 1b). The method described by Nicoletti *et al.*[12] used here is based on the principle that the apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Apoptotic nuclei appear in the analysis as a broad hypodiploid DNA peak, which was easily discriminable from the narrow peak of cells with normal (diploid) DNA content. This simple and reproducible method should prove useful for assessing apoptosis of specific cell populations in heterogeneous tissues. The results presented here clearly demonstrated that MFM did not induce an increase of subdiploid DNA content (DNA fragmentation) in HL60 and Jurkat cells when compared with the respective control (DMSO 0.05%).

Thus, MFM induced nonapoptotic cell death, based upon the evidence that it reduced cell viability but it did not induce a much lower level of DNA fragmentation compared with etoposide, the positive control. This suggested that MFM at the tested concentration (20 μg/ml) may have induced another kind of cell death. It is well described that different substances can induce cytotoxicity. Drug sensitivity is likely correlated with the accumulation of apoptotic and nonapoptotic cell deaths, which may influence overall tumour response in anticancer treatment.[19] Nonapoptotic cell death is mainly attributed to autophagy. Death-inducing agents such as tumour necrosis factor (TNF) and staurosporin induced cytotoxicity without producing apoptotic changes. Mechanisms of apoptotic and nonapoptotic cell death induced by anticancer treatment have provided critical information not only for understanding tumour response in terms of signal transduction pathways of cell death, but also for creating an opportunity to design targeting therapy for promoting cell death.[20]

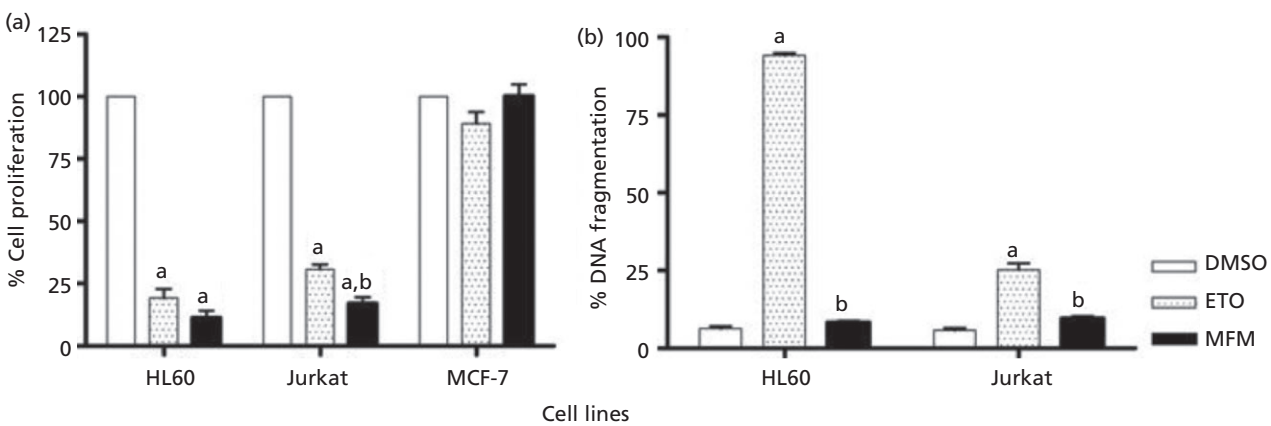

Figure 1 Effect of the methanolic extract of the aerial parts of *Mitracarpus frigidus* on cell proliferation (a) and on DNA content of tumour cell lines (b). HL60, Jurkat and MCF-7 cell lines were incubated with 20 μg/ml methanolic extract of the aerial parts of *M. frigidus* (MFM) or with 0.05% dimethyl sulfoxide (DMSO) or 14 μM etoposide (ETO) for 24 h. Each datum represents mean ± SD for at least two independent experiments performed in duplicate. [a]Statistically different from negative control (DMSO). [b]Statistically different from positive control (ETO) (analysis of variance followed by Bonferroni, $P < 0.05$).

Laxative and intestinal motility effects

The results of the laxative and of the intestinal motility effects caused by MFM are shown in Figure 2.

To evaluate the laxative activity, faeces were examined 8 and 16 h after treatment. In this study, an oral administration of MFM significantly increased the production of faeces 0–8 h after its administration (Figure 2a). The laxative activity of MFM was similar to that of the reference drug, bisacodyl. Importantly, treated animals showed few pasty stools, without however, the appearance of watery stools. Likewise, after 16 h, although all groups increased the amount of faeces produced, no difference in the weight of stools was observed among the different treatments, except for the group treated with saline (Figure 2b). This probably occurred because after 8 h of treatment, animals were allowed free access to food.

Regarding the effect on intestinal motility, the groups treated with MFM showed a significant increase in the pathway of the coal compared with the group treated with saline and bisacodyl (Figure 2c). Furthermore, we observed no relationship with the dose administered. The significant difference between the activity of MFM and the reference drug was probably due to the fact that bisacodyl only began to exert its activity between 6 and 12 h after administration.

Free and anthraquinone *C*- and *O*-glycosides were detected in MFM. The contents of total anthraquinones (total aglycones + total glycosides) and total anthraquinones glycosides were 7.3 ± 0.03 and 5.4 ± 0.05% w/w calculated as emodin, respectively. The laxative effect of anthraquinones is well known, which suggested that these compounds could, in part, be involved in the activity found. The laxative effect is caused by two independent mechanisms, namely changes in colonic motility leading to an accelerated large intestinal transit, and alterations in colonic absorption and secretion resulting in fluid accumulation.[21,22] Both mechanisms are dependent on an interaction of the laxative with the colonic epithelium. Anthraquinone laxatives are used in medicinal practice for diagnostic procedures such as a barium enema, which requires a clean colon, and sometimes for the short-term treatment of constipation.[23]

Toxicological evaluation

Acute toxicity

No toxic symptoms or death were observed in any of the animals and they lived up to 14 days. An autopsy at the end of the experimental period revealed no apparent changes in any

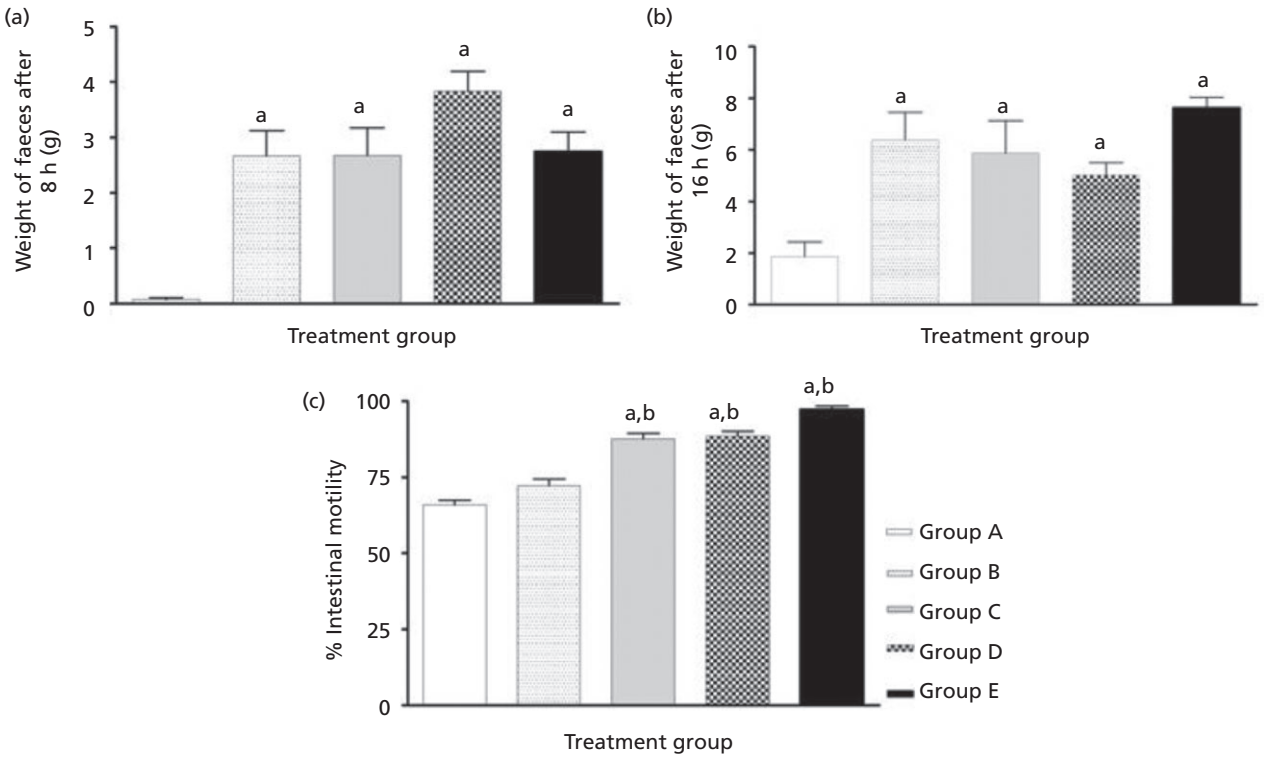

Figure 2 Laxative activity (a, b) and intestinal motility effect (c) of the methanolic extract of the aerial parts of *Mitracarpus frigidus* in rats. Group A, saline; group B, bisacodyl; group C, 100 mg/kg methanolic extract of the aerial parts of *M. frigidus* (MFM); group D, 300 mg/kg MFM; group E, 1000 mg/kg MFM. The values shown are mean ± SEM ($n = 8$). [a]Statistically different from negative control (group A). [b]Statistically different from positive control (group B) (analysis of variance followed by Bonferroni, $P < 0.05$).

organs. There were no changes either in the corporal weight or in the weight of the principal organs and all animals exhibited a gain in body weight.

Therefore, the minimum lethal dose (LD50) of MFM to male Wistar rats was greater than 2000 mg/kg. This result characterized MFM as being of low toxicity, as described by Larini,[24] which classified oral toxicity as extremely toxic (LD50 less than 25 mg/kg), highly toxic (LD50 between 100 and 500 mg/kg), moderately toxic (LD50 between 500 and 2000 mg/kg) and low toxicity (LD50 above 2000 mg/kg).

Subchronic toxicity

Treatment evolution

The effect of the oral administration of MFM on water consumption, food intake and body weight gain were evaluated daily during subchronic treatment (Figure 3). There was an increase in water consumption in the groups treated with MFM, and this increase was directly proportional to the administered dose (Figure 3a). On the other hand, the groups treated with MFM showed a reduction in food intake (Figure 3b). Figure 3c shows the average body weight gain of animals accumulated every two weeks. A significant decrease in the groups C and D was observed in the first two weeks when compared with the control group. Thereafter, a stabilization of body weight gain was observed for all groups.

Table 1 represents the final weight of the animals and of the principal organs. No significant difference was found when comparing the groups treated with control, except of liver weight in the group treated with 1000 mg/kg MFM.

Biochemical analyses

The biochemical parameters after subchronic administration of MFM at different doses are presented in Table 2. The AST, ALT, ALP and total bilirubin, direct and indirect levels showed no significant changes between the groups treated with MFM and the vehicle. However, in the group treated with 1000 mg/kg MFM there was a significant increase in AST level. AST, ALT, ALP and total direct and indirect bilirubin are important parameters for evaluating hepatoprotective functions and biliary system.[25] The increase in AST level is very nonspecific, since this aminotransferase is abundant in cardiac tissues, skeletal muscles, brain and kidney.[26]

To assess the protein profile analyses on total protein, albumin, globulin and albumin and globulin ratio (A/G) were performed. As shown in Table 2, a significant decrease in total protein and globulin levels of all groups treated with MFM was observed when compared with the vehicle group.

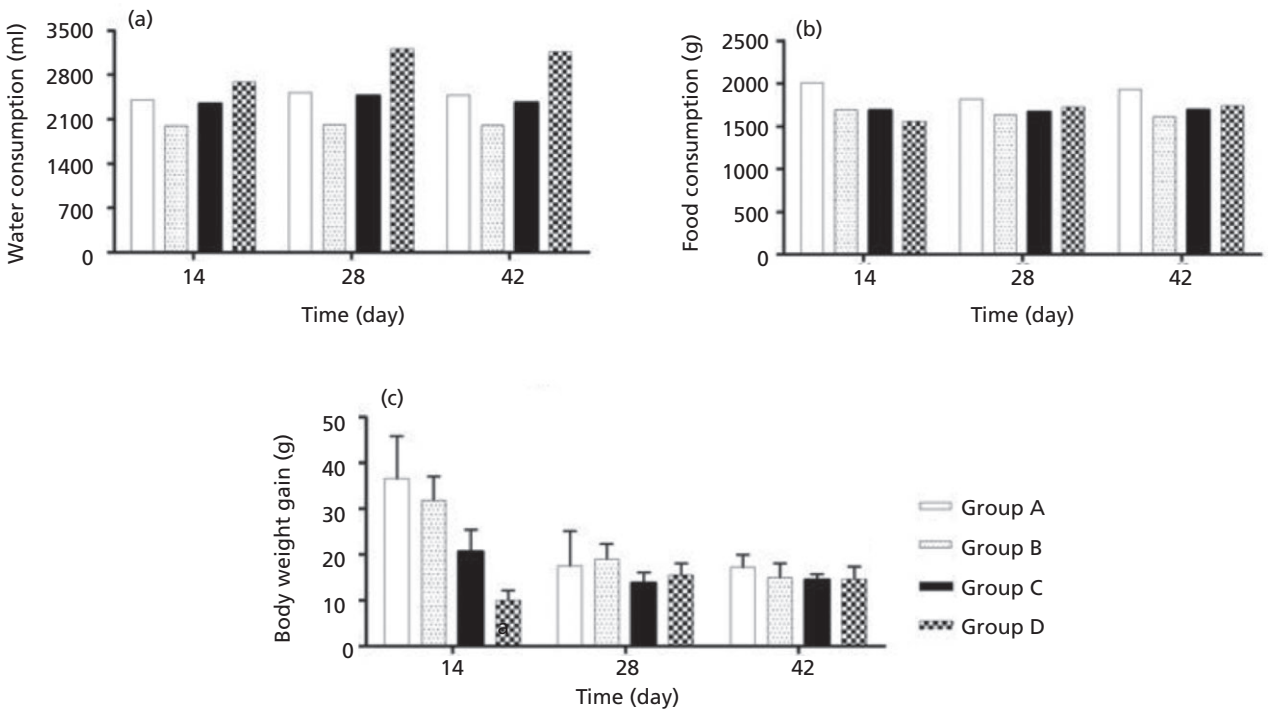

Figure 3 Consumption of water and food, and body weight gain of animals accumulated every two weeks after treatment with methanolic extract of the aerial parts of *Mitracarpus frigidus*. Group A, saline; group B, 100 mg/kg methanolic extract of the aerial parts of *M. frigidus* (MFM); group C, 300 mg/kg MFM; group D, 1000 mg/kg MFM. The values shown are mean ± SEM ($n = 8$). [a]Statistically different from negative control (group A) (analysis of variance followed by Bonferroni, $P < 0.05$).

Table 1 Body weight of animals and their principal organs after 42 days of treatment with the methanolic extract of the aerial parts of *Mitracarpus frigidus*

Body weight of animals and principal organs	Group A Saline	Group B 100 mg/kg	Group C 300 mg/kg	Group D 1000 mg/kg
Mean weight of animals (g)	310.4 ± 7.4	288.5 ± 8.3	281.8 ± 4.3	285.0 ± 9.0
Heart (g)	1.1 ± 0.04	1.1 ± 0.01	1.2 ± 0.05	1.3 ± 0.08
Liver (g)	9.3 ± 0.4	9.1 ± 0.3	9.5 ± 0.1	10.7 ± 0.2[a]
Right kidney (g)	1.1 ± 0.05	1.2 ± 0.03	1.2 ± 0.02	1.2 ± 0.05
Left kidney (g)	1.1 ± 0.06	1.2 ± 0.03	1.2 ± 0,03	1.3 ± 0.06

The values shown are mean ± SEM ($n = 8$). [a]Statistically different from negative control group (group A) (analysis of variance followed by Bonferroni, $P < 0.05$).

Table 2 Biochemical and haematological parameters after 42 days of treatment with the methanolic extract of the aerial parts of *Mitracarpus frigidus*

Biochemical and haematological parameters	Group A Saline	Group B 100 mg/kg	Group C 300 mg/kg	Group D 1000 mg/kg
Glucose (mg/dl)	154.8 ± 12.0	142.0 ± 3.5	136.6 ± 6.9	120.2 ± 4.8[a]
Total protein (g/dl)	7.0 ± 0.2	4.9 ± 0.6[a]	5.3 ± 0.6	4.1 ± 0.4[a]
Albumin (g/dl)	3.8 ± 0.3	3.4 ± 0.4	4.0 ± 0.2	4.0 ± 0.2
Globulin (g/dl)	3.2 ± 0.4	1.8 ± 0.5	1.5 ± 0.5[a]	0.8 ± 0.3[a]
A/G	1.2 ± 0.3	1.5 ± 0.3	5.2 ± 2.2[a]	6.5 ± 3.0[a]
ALP (U/l)	34.6 ± 2.4	29.3 ± 7.5	34.8 ± 7.2	25.2 ± 7.2
AST (U/l)	3.8 ± 0.2	3.8 ± 0.3	7.0 ± 2.3	15.7 ± 1.3[a]
ALT (U/l)	16.7 ± 7.4	13.2 ± 1.6	14.9 ± 1.2	24.3 ± 1.0
Total bilirubin (mg/dl)	1.1 ± 0.01	0.7 ± 0.01	0.6 ± 0.01	0.5 ± 0.01
Direct bilirubin (mg/dl)	0.67 ± 0.01	0.3 ± 0.01	0.4 ± 0.02	0.3 ± 0.01
Indirect bilirubin (mg/dl)	0.42 ± 0.01	0.4 ± 0.01	0.2 ± 0.01	0.2 ± 0.02
Urea (mg/dl)	45.7 ± 3.0	39.3 ± 3.4	30.9 ± 5.2	50.8 ± 5.0
Creatinine (mg/dl)	0.7 ± 0.1	1.6 ± 0.6[a]	1.6 ± 0.8[a]	1.3 ± 0.2[a]
Total cholesterol (mg/dl)	92.9 ± 4.9	41.8 ± 9.0[a]	47.2 ± 8.7[a]	51.1 ± 6.3[a]
Triglycerides (mg/dl)	86.7 ± 48.4	116.7 ± 19.1	136.0 ± 10.8	160.0 ± 25.0
RBC (10^6/μl)	5.6 ± 0.2	5.2 ± 0.3	5.1 ± 0.1	4.5 ± 0.3
Haemoglobin (g/dl)	17.0 ± 3.3	21.1 ± 2.3	23.8 ± 0.9	20.7 ± 1.2
Haematocrit (%)	48.4 ± 0.7	41.7 ± 3.2	46.3 ± 0.7	42.3 ± 1.7
MCV (fl)	86.8 ± 0.8	80.6 ± 4.1[a]	90.3 ± 0.8	94.2 ± 4.1
MCHC (g/dl)	35.1 ± 0.8	50.6 ± 4.0[a]	51.4 ± 0.8[a]	48.8 ± 4.0[a]
MCH (pg)	30.4 ± 0.1	40.8 ± 0.1[a]	46.4 ± 0.1[a]	46.0 ± 0.1[a]
WBC (10^3/μl)	3.2 ± 0.4	3.3 ± 0.2	4.2 ± 0.2[a]	4.2 ± 0.2[a]
Basophil (%)	1.0 ± 0.25	1.0 ± 0.01	1.0 ± 0.01	1.0 ± 0.01
Eosinophil (%)	4.8 ± 0.6	3.2 ± 0.5	2.7 ± 0.3[a]	2.8 ± 0.3[a]
Monocyte (%)	16.0 ± 3.6	12.7 ± 2.7	10.5 ± 1.3	9.0 ± 1.5
Neutrophil (%)	42.8 ± 8.6	47.8 ± 4.6	46.0 ± 2.7	49.3 ± 1.3
Lymphocyte (%)	35.3 ± 4.6	35.3 ± 3.4	41.5 ± 3.3	39.5 ± 0.5

The values shown are mean ± SEM ($n = 8$). [a]Statistically different from negative control group (group A) (analysis of variance followed by Bonferroni, $P < 0.05$). A/G, albumin and globulin ratio; ALP, alkaline phosphatase; AST, aspartate aminotransferase: ALT, alanine aminotransferase; RBC, red blood cell count; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; MCH, mean corpuscular haemoglobin; WBC, white blood ell count.

However, these results did not mean any kind of toxicity of MFM, as only the enhancement in the level of serum proteins is an indication of tissue injury and reflection of hepatic toxicity.[27] The histological analyses of the liver revealed no significant change, as shown by a normal lobular architecture and portal-space containing arterioles, venules and bile ducts (Figure 4a and c).

The renal function was assessed by blood urea nitrogen and creatinine.[25] Animals treated with MFM presented a moderate increase in creatinine levels, however this trend was not observed for urea levels (Table 2). The increase observed may have been clinically insignificant as the reference values of creatinine were 0.39–2.29 mg/dl.[28] Urea and creatinine are compounds derived from proteins, which are eliminated

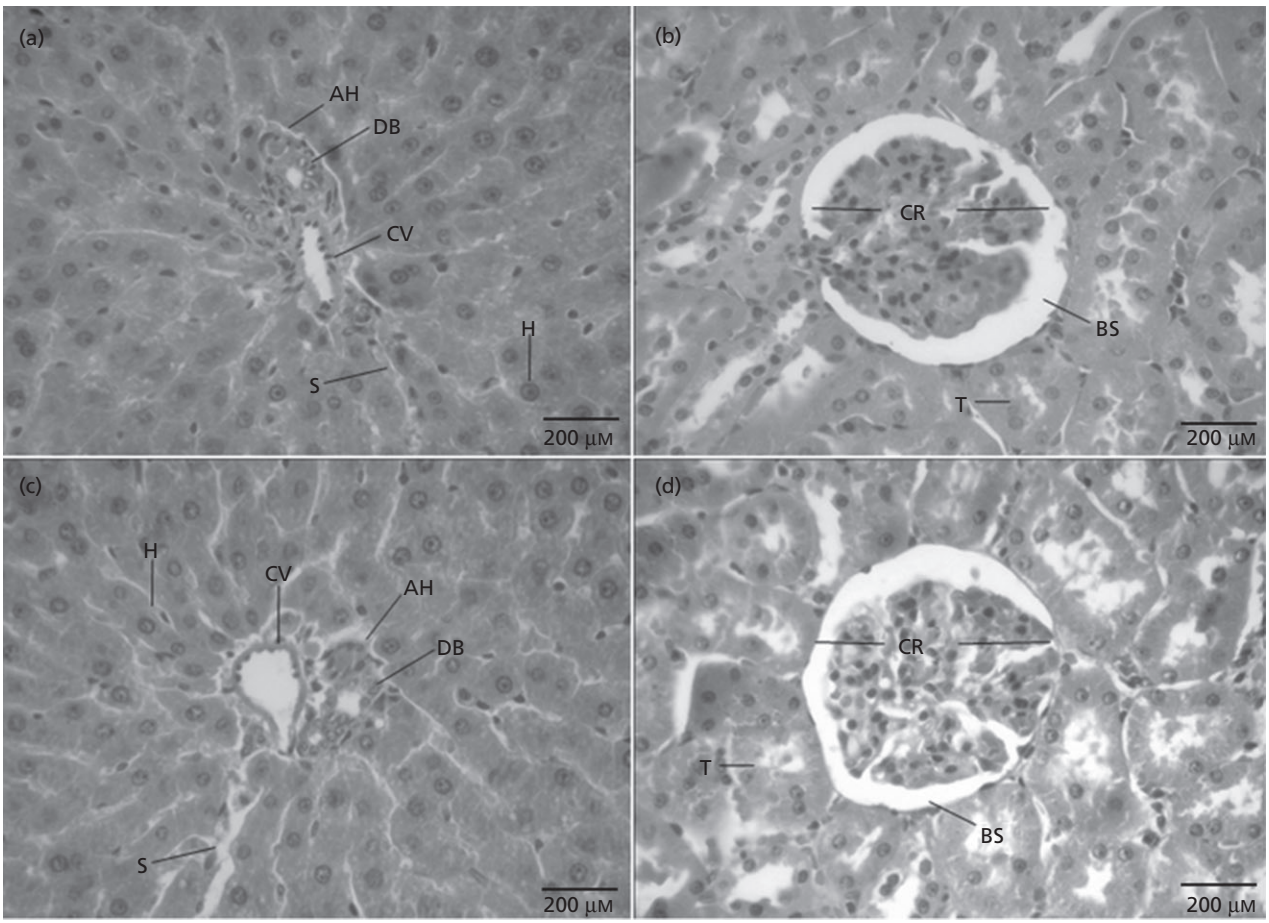

Figure 4 Photomicrographs of the liver and kidney from rats treated with saline (a and b, respectively) or 1000 mg/kg methanolic extract of the aerial parts of *Mitracarpus frigidus* (c and d, respectively) in a subchronic oral toxicity evaluation. Evaluation lasted for 42 days. Cross-sections were stained with haematoxylin and eosin. The liver cross-section shows (at magnification 40x) hepatic artery (AH), bile duct (DB), central vein (CV), sinusoids (S), hepatocytes (H), all clearly conserved. Cross-section of kidney shows (at magnification 40x) renal corpuscles (CR), tubules (T) and Bowman's space (BS), all conserved.

by the kidney. When their blood levels are high, there is nitrogen retention that may provoke renal diseases. Satyanarayana *et al*.[29] reported that renal damage was obtained only when creatinine and urea increased concomitantly. Moreover, in the histological analysis, no alterations in kidney morphology were observed. In all cases, renal cortex and renal corpuscles were preserved, as well as all types of tubules (Figure 4b and d). Finally, MFM was unlikely to affect hepatic and renal function.

Besides these parameters, cholesterol and triglycerides were analysed to determine the serum lipid profile of groups throughout the experiment. Table 2 shows the significant decrease in cholesterol levels in the MFM-treated groups when compared with control group. On the other hand, there was no significant change in triglycerides levels between groups. Cholesterol and triglycerides are parameters associated with coronary artery diseases. Moreover, these parameters are used for evaluation of hyperlipidaemia.[26]

Regarding the values of glucose, a reduction was observed in the group treated with 1000 mg/kg MFM. According to Harkness and Wagner,[30] in rodents glycaemic levels present large physiological fluctuations ranging between 50 and 135 mg/dl, so the glycaemic changes observed were within the reference range and may have been due to physiological fluctuations and not to treatment with MFM.

Haematological analyses

Analysis of blood parameters is relevant to risk evaluation as the changes in the haematological system have a higher predictive value for human toxicity when the data are translated from animal studies.[31] The haematological profile of treated

rats showed no significant difference with the control group, except for WBC and eosinophil which increased and decreased in the groups treated with MFM 300 and 1000 mg/kg, respectively. An increase in WBC directly indicated the strengthening of the organism defence.[32,33] This elevation in total leucocyte count suggested that MFM may have contained biologically active principles that had the ability to boost the immune system.

Conclusions

MFM presented a laxative potential probably due to the presence of free and glycosidic anthraquinones. Moreover, MFM presented cytotoxic activity against leukaemic cells which indicated a potential antitumour activity. Further experiments are necessary to elucidate the cytotoxic mechanism induced by MFM. It is important to point out that this study has provided valuable data on the acute and subchronic oral toxicity profile of *M. frigidus* that should be very useful for any future in-vivo and clinical study of this plant.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Araújo CAC, Leon LL. Biological activities of *Curcuma long* L. *Mem Inst Oswaldo Cruz* 2001; 96: 723–728.
2. Coutinho MR *et al*. Partial purification of anthocyanins from *Brassica oleracea* (red cabbage). *Sep Sci Technol* 2004; 39: 3769–3782.
3. Bisignano G *et al*. Antimicrobial activity of *Mitracarpus scaber* extract and isolated constituents. *Lett Appl Microbiol* 2000; 30: 105–108.
4. Germano MP *et al*. Hepatoprotective properties of *Mitracarpus scaber* (Rubiaceae). *J Pharm Pharmacol* 1999; 51: 729–734.
5. Pereira ZV *et al*. Rubiaceae Juss. da Reserva Florestal Mata de Paraíso, Viçosa, MG, Brasil. *Acta Bot Bras* 2006; 20: 207–224.
6. Fabri RL *et al*. *Mitracarpus frigidus* aerial parts exhibited potent antimicrobial, antileishmanial, and antioxidant effects. *Bioresour Technol* 2009; 100: 428–433.
7. Matos FJA. *Introdução à Fitoquímica Experimental*. Fortaleza: EUFC, 1997.
8. Sakulpanich A, Gritsanapan W. Extraction method for high content of anthraquinones from *Cassia fistula* pods. *J Health Res* 2008; 22: 167–172.
9. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55–63.
10. Monks A *et al*. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 1991; 83: 757–766.
11. Ulukaya E *et al*. Interference by anticancer chemotherapeutic agents in the MTT – tumor chemosensitivity assay. *Chemotherapy* 2004; 50: 43–50.
12. Nicoletti I *et al*. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; 139: 271–279.
13. Capasso F *et al*. Laxatives and the production of autacoids by rat colon. *J Pharm Pharmacol* 1986; 38: 627–629.
14. Jansen P, Jageneau AH. A new series of potent analgesics. Part I – Chemical structure and pharmacological activity. *J Pharm Pharmacol* 1957; 9: 381–400.
15. Wong CL, Wai MK. Effects of aspirin and paracetamol on naloxone reversal or morphine-induced inhibition of gastrointestinal propulsion in mice. *Eur J Pharmacol* 1981; 73: 11–19.
16. Costa-Silva JH *et al*. Acute and subacute toxicity of the *Carapa guianensis* Aublet (Meliceae) seed oil. *J Ethnopharmacol* 2008; 116: 495–500.
17. Silva EJR *et al*. Toxicological studies on hydroalcohol extract of *Calendula officinalis* L. *Phytother Res* 2007; 21: 332–336.
18. Lahlou S *et al*. Acute and chronic toxicity of a lyophilised aqueous extract of *Tanacetum vulgare* leaves in rodents. *J Ethnopharmacol* 2008; 117: 221–227.
19. Lan TM *et al*. Anticancer medicinal plant, *Epipremnum pinnatum* (L.) Engl. chloroform extracts elicited both apoptotic and non-apoptotic cell deaths in T-47D mammary carcinoma cells. *KMITL Sci Tech J* 2007; 7: 24–43.
20. Kim R *et al*. The role of apoptotic or nonapoptotic cell death in determining cellular response to anticancer treatment. *Eur J Surg Oncol* 2006; 32: 269–277.
21. Leng-Peschlow E. Dual effect of orally administered sennosides on large intestine transit and fluid absorption in the rat. *J Pharm Pharmacol* 1986; 38: 606–610.
22. Wanitschke R. Influence of rhein on electrolyte and water transfer in the isolated rat colonic mucosa. *Pharmacology* 1980; 20: 21–26.
23. Van Gorkom BAP *et al*. The effect of sennosides on colonic mucosal histology and bowel preparation. *Z Gastroenterol* 1998; 36: 13–18.
24. Larini L, ed. *Toxicologia*, 1st edn. São Paulo: Manole, 1987: 301.

25. Widmann L *et al*. *Interpretação clínica de exames laboratoriais*, 2nd edn. São Paulo: Manole, 2001: chapter 11.
26. Henry JB, ed. *Diagnósticos Clínicos e Tratamentos por Métodos Laboratoriais*, 19a edn. São Paulo: Manole, 1999: chapter 13.
27. Emerson FS *et al*. Toxic effects of crude extract of *Plumbago rosea* (Rokta chitraka). *J Ethnopharmacol* 1993; 38: 79–84.
28. Matsuda H *et al*. Immunology and hematology. In: Krinke GJ, ed. *The Laboratory Rat*. London: Academic Press, 2000: 437–446.
29. Satyanarayana PS *et al*. Quercetin, a bioflavonoid, protects against oxidative stress-related renal dysfunction by cyclosporine in rats. *Methods Find Exp Clin Pharmacol* 2001; 34: 175–181.
30. Harkness SE, Wagner JE. *Biologia e clínica de coelhos e roedores*. São Paulo: Livraria Roca, 1993.
31. Olson H *et al*. Concordance of toxicology of pharmaceuticals in humans and animals. *Regul Toxicol Pharmacol* 2000; 32: 56–67.
32. Chang-Gue S *et al*. Induction of hemopoiesis by saenghyuldan, a mixture of Ginseng radix, Paeoniae radix, and hominis placenta. *Acta Pharmacol Sin* 2003; 24: 120–126.
33. Stanley OA *et al*. Toxicity studies in rats fed nature cure bitters. *Afr J Biotechnol* 2005; 4: 72–78.

5.2 O artigo a seguir refere-se a atividade citotóxica, leishmanicida e antimicrobiana de psicorubrina, uma piranonaftoquinona isolada das partes aéreas de *Mitracarpus frigidus.*

Artigo 2

ANTITUMOR, ANTIBIOTIC AND ANTILEISHMANIAL PROPERTIES OF THE PYRANONAPHTHOQUINONE PSYCHORUBRIN FROM *Mitracarpus frigidus*

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Antitumor, Antibiotic and Antileishmanial Properties of the Pyranonaphthoquinone Psychorubrin from *Mitracarpus frigidus*

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ABSTRACT

The bioactivity guided fractionation of the dichloromethane extract of *Mitracarpus frigidus* afforded the pyranonaphthoquinone psychorubrin. This compound, hitherto unknown in the genus *Mitracarpus*, had its biological activity evaluated against one panel of bacteria and two fungi, three tumor cell lines (HL60, Jurkat and MCF-7) and four *Leishmania* species. Its identity was confirmed unambiguously by ^{1}H, ^{13}C, ^{1}H-COSY, IR and UV-Vis spectroscopy and mass spectrometry. Psychorubrin displayed a very promising antitumor with IC_{50} of 4.5, 5.6 and 1.1 μM for HL60, Jurkat and MCF-7 cell lines, respectively. Antimicrobial activity, mainly against *Cryptococcus neoformans* (MIC of 87.3 μM) was observed. A pronounced antileishmanial potential was also verified with IC_{50} varying from 1.7 to 2.7 μM for the *Leishmania* species tested. This is the first report of the presence of pyranonapthoquinones in the *Mitracarpus* genus, which may serve as a chemotaxonomical marker.

Key words: *Mitracarpus frigidus*, psychorubrin, pyranonaphthoquinone, Rubiaceae.

INTRODUCTION

The genus *Mitracarpus*, known in the vernacular as the girdlepods, consists of no fewer than 50 species (Arruda and Gomes 1996). However, there is very little information in the literature regarding the constituents of *Mitracarpus spp. Mitracarpus scaber* Zucc. ex Schult. & Schult. F. is the most widely studied species and is employed in traditional medicine in West Africa for headaches, toothache, amenorrhoea, dyspepsia, hepatic and veneral diseases, leprosy, and in the treatment of skin diseases (Bisignano et al. 2000). Previous studies have reported the isolation of gallic acid, 3,4,5-trimethoxybenzoic acid, 4-methoxyacetophenone, 3,4,5-trimethoxy-acetophenone, kaempferol-3-O-rutinoside, rutin and psoralen (Bisignano et al. 2000) as well as ursolic

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and oleanolic acids from this species (Gbaguidi et al. 2005). With particular interest is the presence of the biologically active napthaquinone derivatives pentalongin (Moulis et al. 1992), its hydroquinone diglycoside, harounoside (Harouna et al. 1995), and benz[g]isoquinoline-5,10-dione (Okunade et al. 1999) in *M. scaber*. Several patents (Greff 1998, Spindler and Urbanec 2009, Tsuji and Nakanishi 2010) have been solicited using extracts of *M. scaber* as a skin lightening component and it may be the presence of these and other napthaquinones that are responsible for this action. Another species of *Mitracarpus*, *M. villosus* (Sw.) DC was studied and found to contain stigmasterol, 24-methylcholesta-5-en-3*β*-ol, and ursolic acid (Ekpendu et al. 2001).

Psychorubrin **(1)** (Figure 1) was first described in *Psychotria rubra* (Rubiaceae), a Chinese folk medicine which demonstrated activity against KB cells (Hayashi et al. 1987). Other authors erroneously attributed a different structure for psychorubrin to a napthoquinone isolated from *P. camponutans* which showed strong *in vitro* activity against brine shrimp, KB cells, and chloroquine-resistant *P. falciparum* (Jacobs et al. 2008). This compound was later shown to be psychorubrin by means of total synthesis. Three total syntheses of psychorubrin have also been reported (Kesteleyn et al. 1999, Bulbule et al. 2003, Nguyen and Kimpe 2004).

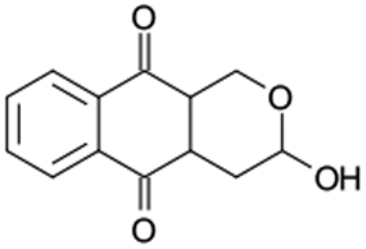

Figure 1 - The structure of psychorubrin **(1)** isolated from the aerial parts of *Mitracarpus frigidus*.

To the best of our knowledge, *Mitracarpus frigidus* (Willd. ex Roem. & Schult.) K. Shum, an annual shrub commonly found in Brazil, has never been subjected to phytochemical investigation, except a recently published paper (Fabri et al. 2009). In that research article, we describe the antitumor, antimicrobial and antileishmanial properties of crude extracts of *M. frigidus*. Upon the discovery of strong biological activity of the dichloromethane (CH_2Cl_2) extract, the authors sought to isolate the compound or compounds responsible for this activity.

MATERIALS AND METHODS

Plant Material

Mitracarpus frigidus aerial parts were collected in Juiz de Fora, state of Minas Gerais, Brazil, in May, 2009. The plant was identified by Dra. Tatiana Konno from the Ecological and Socio-environmental Core of Macaé/UFRJ. A voucher specimen (CESJ 46076) was deposited at the Leopoldo Krieger Herbarium at the Universidade Federal de Juiz de Fora.

Extraction and Isolation

Preparation of the crude CH_2Cl_2 extract was previously described in detail (Fabri et al. 2009). Purification of this extract was performed in the following manner: the CH_2Cl_2 extract (4.8 g) was chromatographed on a 74 x 4 cm column of silica gel (70-230 mesh) with a gradient of increasing polarity (CH_2Cl_2, CH_2Cl_2-EtOAc, EtOAc, EtOAc-MeOH, MeOH) to obtain a total of twenty fractions. The fractions obtained were analyzed by thin layer chromatography on silica gel 60 F_{254} (Merck) using hexane: EtOAc, 60:40, v/v and hexane: EtOAc, 30:70 v/v as the mobile phase for fractions F_1 to F_{10} and fractions F_{11} to F_{20}, respectively. Identification was made with a UV lamp (254 and 365 nm) and with vanillin: sulfuric acid followed by heating. The fractions were pooled and concentrated on a rotary film evaporator under reduced pressure. While preparing the fractions for HPLC analysis (*vide infra*), we proceeded to dissolve the samples in methanol. Upon addition of methanol to fraction

7, an off-white solid precipitated. This was filtered and dried under vacuum to yield 100 mg (2.08% w/w) of the title compound **(1)**. The sample was homogeneous by TLC with an Rf of 0.57 (hexane:EtOAc 7:3) and revealed as a brownish-orange spot with vanillin-sulfuric acid.

Structural Elucidation

^{1}H-NMR and COSY (300 MHz) and ^{13}C-NMR (75 MHz) spectra were recorded on a Bruker DRX spectrometer using the residual solvent peak ($CHCl_3$) as reference. The IR spectrum was recorded as a KBr pellet on a Bomem B102 FT spectrophotometer. The UV spectrum was acquired in MeOH on a Shimadzu UV160 spectrophotometer. The EI mass spectrum was obtained on a Hewlett-Packard 5973 MSD spectrometer by direct insertion in the positive ion mode (70 eV).

HPLC Analysis

During the course of our investigations of the crude extract, it was necessary to develop an HPLC method suitable for determining the purity of the various column fractions which were obtained. These analyses were performed on an Agilent Technologies 1200 Series, with a PDA detector and automatic injector. The column employed was a Zorbax SB-18; 250 x 4.6 mm, 5 μm particle size. A linear gradient of a binary solvent system, A:B, which varied from 0 to 100% B ran at a flow rate of 1 mL.min^{-1} over 30 minutes where A consists of acetonitrile: H_2O, 5:95, pH adjusted to 4.0 with H_3PO_4 and B consists of acetonitrile: H_2O, 90:10, pH adjusted to 4.0 with H_3PO_4. The mobile phase was returned to its original composition over the course of 30 minutes, and additional 10 minutes were allowed for the column to re-equilibrate before injection of the next sample. The sample volume was 10 μL at a concentration of 1 mg.mL^{-1} and the temperature was maintained at 25°C during the analysis. Detection was performed simultaneously at 210, 230, 254 and 280 nm.

Biological Assays

Human Tumor Cells

Three human tumor cell lines were used, Jurkat (human immortalized line of T lymphocyte), HL60 (human promyelocytic leukemia) and MCF-7 (breast cancer). HL60 and Jurkat cells were kindly furnished by Dr. Gustavo Amarante-Mendes (Universidade de São Paulo, Brazil). MCF-7 was generously provided by Alfredo Goes (Universidade Federal de Minas Gerais, Brazil). All lineages were maintained in the logarithmic phase of growth in RPMI 1640 supplemented with 100 IU.mL^{-1} penicillin and 100 μg.mL^{-1} streptomycin enriched with 2mM of L-glutamine and 10% of fetal bovine serum. All cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air. The media were changed twice weekly and they were regularly examined. All cell lines were used between 16 and 30 passages.

Parasites

Four *Leishmania* species for in vitro screening were used: *L. amazonensis* (IFLA/Br/67/PH8), *L. major* (MRHO/SU/59/P), *L. braziliensis* (MHOM/Br/75/M2903) and *L. chagasi* (MHOM/Br/74/PP75). Promastigotes of *L. amazonensis* and *L. braziliensis* were cultured in Warren's medium (brain heart infusion- BHI- plus hemin and folic acid), promastigotes of *L. major* were maintained in BHI medium, and promastigotes of *L. chagasi* were maintained in Medium 199, both supplemented with 10% fetal bovine serum at 24°C.

Antitumor and Antileishmanial Assays

Cell viability was estimated by measuring the rate of mitochondrial reduction of tetrazolium–dye (MTT). MTT is a standard colorimetric assay, in which mitochondrial activity was measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells

only (Mosmann 1983). To evaluate the antitumor activity, the tumor cell lines (Jurkat, HL60 and MCF-7) were inoculated at 50,000 cells per well. The plates were pre-incubated for 24 h at 37°C to allow cells adaptation prior to the addition of the test compounds. The half maximal inhibitory concentration (IC_{50}) was determined over a range of concentrations of a freshly prepared solution of (1) (100.0; 50.0; 25.0; 12.5; 6.25; 3.12; 1.56; 0.78; 0.39 μM) tested for 48 h in an atmosphere of 5% CO_2 and 100% relative humidity. Control groups included treatment with 0.1% DMSO (negative control) and etoposide 14 μM (positive control). All compounds were tested in triplicate, in three independent experiments (Monks et al. 1991). The antileishmanial activity was performed according to a previously described method (M'Bongo et al. 1997, Braga et al. 2007). Briefly, promastigotes from a logarithmic phase culture were suspended to yield 2 million cells.mL^{-1} (*L. amazonensis*) or 3 million cells.mL^{-1} (*L. chagasi*, *L. braziliensis* and *L. major*) after Neubauer chamber counting. The test was performed in 96-well microtiter plates maintained at 24°C. The assays were performed in triplicate. The parasites were exposed to increasing concentrations of (1) (100.0; 50.0; 25.0; 12.5; 6.25; 3.12; 1.56; 0.78; 0.39 μM) for 72 h at 24°C, for the determination of IC50 values. Controls with DMSO were also performed. Amphotericin B was used as the reference drug.

For both assays, after 4 h of the end of incubation of cells with different compounds, 20 μL of MTT solution (5 mg.mL^{-1} in phosphate-buffered saline) were added to each well, the supernatant was removed and 200 μL of 0.04M HCl in isopropyl alcohol were added to dissolve the formazan crystal. The optical densities (ODs) were evaluated in a spectrophotometer at 590 nm (antitumor assay) and 570 nm (antileishmanial assay). Controls included drug-containing medium (background) and drug-free complete medium. Drug-free complete medium was used as control (blank) and was treated in the same way as the drug-containing media. Interactions between compounds and media were estimated on the basis of the variations between drug-containing medium and drug-free medium to avoid false-positive or false-negative results.

DNA Fragmentation Assay

Cell cycle status and quantification of DNA fragmentation (hypodiploid DNA-content) were performed by propidium iodide (PI) staining according to Nicoletti et al. (1991) Cells were treated with psychorubrin **(1)** 43 μM in a 5% CO_2/95% air-humidified atmosphere at 37°C for 24 h. After drug incubation, the cells were centrifuged and re-suspended in hypotonic fluorochrome solution – HFS (50 μg.mL^{-1} PI in 0.1% sodium citrate plus 0.1% Triton X-100). The samples in HFS were incubated at 4°C during 4 h and immediately analyzed by flow cytometry. The PI fluorescence of 20,000 individual nuclei was measured using a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems). The DNA content of cell lines was analyzed by FlowJo software (TreeStar Inc).

Antimicrobial Assay

Microbial Strains

The samples were evaluated against a panel of microorganisms, including the bacterial strains *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), *Salmonella enterica sorovar typhimurium* (ATCC 13311), *Shigella sonnei* (ATCC 11060), *Klebsiella pneumoniae* (ATCC 13866), Escherichia coli (ATCC 10536), *Bacillus cereus* (ATCC 11778), and the yeasts *Candida albicans* (ATCC 18804) and *Cryptococcus neoformans* (ATCC 32608). Bacterial strains were cultured overnight at 37°C in Mueller Hinton agar (MHA). Yeasts were cultured for 48 h at 30°C in Sabouraud dextrose agar (SDA).

Serial Dilution Assay for Determination of the Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) of each extract was determined by using broth microdilution techniques as described previously for bacteria and yeasts, respectively (Perez et al. 1990, NCCLS 2002). MIC values were determined in RPMI 1640 buffered to a pH 7.0 with MOPS for yeasts and Mueller Hinton broth (MHB) for bacteria. Yeasts were cultured at 30°C for 48 h in SDA and bacteria were cultured overnight at 37°C for 24 h in MHA. Stock solution of **(1)** was diluted two-fold from 2170 to 10.9 μM (final volume = 80 μL) and a final DMSO concentration ≤ 1%. Then, 100 μL of RPMI or MHB were added onto microplates. Finally, 20 μL of 10^6 CFU.mL^{-1} (according to McFarland turbidity standards) of standardized yeasts and bacterial suspensions were inoculated onto microplates and the test was performed in a volume of 200 μL. Plates were incubated at 30°C for 48 h for yeasts and 37°C for 24 h for bacteria. The same tests were performed simultaneously for growth control (RPMI + yeast and MHB + bacteria) and sterility control (RPMI or MHB + psychorubrin). The MIC values were calculated as the highest dilution showing complete inhibition of tested strain. Chloramphenicol and amphotericin B were used as standard antimicrobial agents for bacteria and yeast, respectively.

STATISTICAL ANALYSIS

The IC_{50} values for antitumor and antileishmanial activities were determined using Prism 5.0 (GraphPad Software Inc.). Data were presented as median and 95% confidence interval. Statistical differences between the treatments and the control were evaluated by ANOVA test. To express the DNA content, each data point represented mean ± SD from at least two independent experiments performed in duplicate. Statistical differences between the treatments and the control were evaluated by ANOVA test followed by Bonferroni ($P > 0.05$).

Psychorubrin (3-hydroxy-3,4-dihydro-1H-benzo[g]isochromene-5,10-dione, **1)**: amorphous off-white powder; R_f = 0.57 (hexane:EtOAc 7:3), silica gel 60 F_{254} ; UV λ_{max} nm (MeOH) 245, 250 (sh), 262, 333; IR (KBr) ν 3409, 2925, 1666, 1297 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.13-8.05 (2H, m), 7.76 – 7.70 (2H, m), 5.51 (1H, t), 4.81 (1H, dt), 4.71 (1H, dt), 2.84 (1H, dq), 2.74 (1H, dq); ^{13}C NMR ($CDCl_3$, 75 MHz) 183.7, 183.3, 141.5, 139.4, 134.1, 134.0, 132.2, 132.0, 126.7, 126.4, 90.9, 57.9, 28.2; EIMS, *m/z* (relative intensity, %) 230 (5.1) [M+], 212 (17.1) [M^+ - H_2O], 184 (21.2) [M^+ - HCO_2H], 173 (4.9), 156 (9.2), 149 (16.3), 128 (7.0), 77 (15.8), 57 (46.5), 41 (100).

RESULTS AND DISCUSSION

Under the conditions used in the HPLC method, psychorubrin eluted as a narrow peak at 12.6 minutes and had a chromatographic purity of 98%.

Our spectral data is in excellent agreement with that reported for both the natural and synthetic psychorubrin **(1)** (Hayashi et al. 1987, Kesteleyn et al. 1999, Bulbule et al. 2003, Nguyen and Kimpe 2004, Jacobs et al. 2008). Pyranonaphthoquinones are a diverse family of naturally occurring 1*H*-naphtho[2,3-c]pyran-5,10-diones, which are found in bacteria, fungi, aphids and higher plants (Thomson 1971).

Some of these presented antimicrobial, antiparasitic and anticancer properties (Lee et al. 1996, Wang et al. 1998, Krishnan and Bastow 2000). Psychorubrin **(1)** were also isolated from *Psychotria rubra* and *P. camponutans* that also belong to the Rubiaceae family (Hayashi et al. 1987). The fact that it was not reported outside this genus is an indication of its chemotaxonomical importance.

A literature search revealed few reports regarding to its biological activity although significant cytotoxicity against KB tumor cells has been previously reported (Hayashi et al. 1987).

In our study, we demonstrated (Table I) that this compound is toxic against some tumor cells, including leukemia (HL60 and Jurkat), and solid tumors such as breast cancer (MCF-7). The antitumor activity observed in this study is supported by previous scientific reports on biological activities of other pyranonaphthoquinone derivatives as eleutherin, *β*-lapachone, and its structural isomer, *α*-lapachone. The mechanism of topoisomerase II inhibition by those three derivatives was examined systematically with respect to the steps of the catalytic cycle of the enzyme. Etoposide, a topoisomerase inhibitor, was used as a control (Krishnan and Bastow 2000).

We investigated if the cytotoxic effects of psychorubrin (1) observed in our experiments were associated with its potential to induce cell death by apoptosis. We used the method described by Nicoletti et al. (1991) which is based on the principle that the apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Apoptotic nuclei appeared as a broad hypodiploid DNA peak which was easily discriminable from the narrow peak of cells with normal (diploid) DNA content. This simple and reproducible method should prove useful for assessing apoptosis of specific cell populations in heterogeneous tissues. Our results (Figure 2) clearly demonstrated that psychorubrin induced an increase of subdiploid DNA content (DNA fragmentation) in HL60 and MCF-7 cell when compared with their respective control (DMSO 0.05%). This effect was cell type dependent. To the resistant lineage MCF-7, psychorubrin (1) (43 μM) demonstrated a pro-apoptotic potential better than etoposide (14 μM), the clinical drug used as positive control in our experiments, possessing pro-apoptotic activities against several cancers.

In vitro cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals. Different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed (Fotakis and Timbrell 2006). In this context, the DNA content analysis revealed a different toxicity profile for psychorubrin compared with the MTT method. This effect can be attributed to the fact that MTT evaluates the energetic function of mitochondria that is preserved during the course of apoptosis. When the damage level to mitochondria is mild or moderate, mitochondria can still generate sufficient energy for initiation and execution of the apoptotic process

TABLE I
Antitumor and antileishmanial activity of psychorubrin (1).

Samples	Antitumor activity[a] IC_{50} (μM)			Antileishmanial activity[a] IC_{50} (μM)			
	HL60	Jurkat	MCF-7	La	Lc	Lb	Lm
Psychorubrin	4.5	5.6	1.1	2.7	1.7	1.7	2.4
	(4.1-5.0)	(5.1-6.1)	(0.4-2.6)	(2.0-3.0)	(1.0-2.2)	(0.9-2.1)	(1.6-3.1)
Amphotericin B[b]	-	-	-	0.9	1.9	0.1	0.3
				(0.2-1.7)	(1.7-2.4)	(0.05-0.2)	(0.1-0.5)
Etoposide[c]	0.5	2.5	> 50	-	-	-	-
	(0.3-0.9)	(0.9-7.3)					

[a] La – *Leishmania amazonensis*; Lc – *Leishmania chagasi*; Lb – *Leishmania braziliensis*; Lm – *Leishmania major*. Data are presented as median and 95% confidence interval (in parentheses).
[b,c] Positive controls.

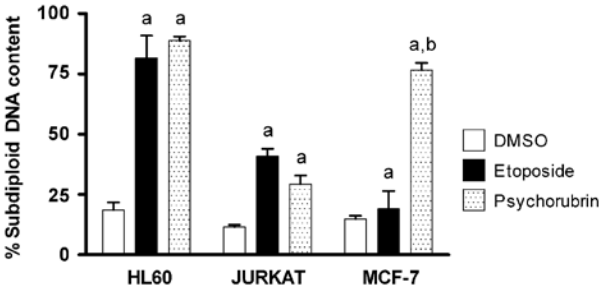

Figure 2 - Effect of psychorubrin **(1)** on DNA content of tumor cell lines. HL-60, Jurkat e MCF-7 cells were incubated with 43 μM psychorubrin or with 0.05% DMSO for 24 h. DNA content was assayed by PI stain and flow cytometry analysis. Etoposide (14μM), positive control is demonstrated. Each data point represents mean ± SD from at least two independent experiments performed in duplicate. [a]Statistically different from negative control (DMSO). [b]Statistically different from positive control (etoposide) (ANOVA followed by Bonferroni, $P > 0.05$).

(Yang et al. 2010). The increase of subdiploid DNA content (DNA fragmentation) observed in HL60 and MCF-7 cells after psychorubrin treatment suggested that this compound activates the apoptosis pathway. Many chemotherapeutic strategies interfere with important steps in cell cycle progression such as DNA replication and repair. They act as DNA polymerase inhibitors, topoisomerase inhibitors, or DNA alkylating agents to impede cancer progression and induction of apoptosis as a secondary effect (Li et al. 2006). Apoptosis is accompanied by the shrinkage and fragmentation of both cells and their nuclei, loss of microvilli, and extensive degradation of chromosomal DNA. Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. Molecular characterization of this process demonstrated that the chromosomal DNA cleavage is caspase-dependent, in particular caspase 3 cleaves ICAD to dissociate the CAD:ICAD complex, allowing CAD to cleave chromosomal DNA (Nagata 2000). Despite the cell mechanism involved on DNA fragmentation induced by psychorubrin in HL60 and MCF-7 cells were not described in the present work, our results suggest that this compound has a potential antitumoral activity, inducing the apoptotic pathway.

Psychorubrin **(1)** also presented an interesting antileishmanial activity with IC_{50} values above 3 μM against promastigote forms of different *Leishmania* species (Table I). Leishmaniasis is a debilitating disease caused by protozoan parasites of the genus Leishmania, which affects an estimated 12 million people worldwide. The discovery of new lead compounds for leishmaniasis is therefore a pressing concern for global health programs (Sanchez et al. 2010).

We also demonstrated that psychorubrin **(1)** displayed a broader spectrum of antimicrobial activity, most particularly against *C. neoformans* (MIC of 10 μM), (Table II), an opportunistic yeast and etiological agent of cryptococcosis. Cryptococcosis is an opportunistic systemic mycosis that involves the central nervous system, especially in immunocompromised patients (Perfect and Casadevall 2002). Cryptococcosis is currently treated with amphotericin B/azoles as the main therapeutic choice (Van der Horst et al. 1997). Nevertheless, the high lethality rate among immunocompromised patients indicates that new therapeutic options are necessary.

TABLE II
Antimicrobial activity of psychorubrin (1).

Test microorganisms	MIC[a]	S[b]
Bacteria		
Staphylococcus aureus	340.3	193.4[c]
Escherichia coli	340.3	48.3[c]
Salmonella typhimurium	340.3	3.1[c]
Shigella sonnei	340.3	3.1[c]
Klebsiella pneumoniae	680.6	3.1[c]
Bacillus cereus	170.2	12.1[c]
Pseudomonas aeruginosa	680.6	48.3[c]
Yeasts		
Candida albicans	340.3	0.09[d]
Cryptococcus neoformans	87.3	0.05[d]

[a] Minimal inhibitory concentration (μM).
[b] Standard antimicrobial agents (μM): [c] Chloramphenicol; [d] Amphotericin B.

In conclusion, the results obtained in this work reinforce that pyranonapthoquinones are an important class of natural products for investigation in the search of new bioactive compounds. In this regard, psychorubrin **(1)** displayed a very promising antitumor and antimicrobial activity, mainly against *C. neoformans*. A pronounced antileishmanial potential was also observed. Furthermore, this is the first report of the presence of pyranonapthoquinones in the *Mitracarpus genus*, which may serve as a chemotaxonomical marker.

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RESUMO

O fracionamento biomonitorado do extrato diclorometânico de Mitracarpus frigidus forneceu a piranonaftoquinona psicorubrina. Essa substância, até então desconhecida no gênero *Mitracarpus*, teve sua atividade biológica avaliada contra várias bactérias e dois fungos, três linhagens de células tumorais (HL60, Jurkat e MCF-7) e quatro espécies de *Leishmania*. Sua estrutura foi confirmada por meio de ^{1}H, ^{13}C, ^{1}H-COSY, IR e UV-Vis e espectrometria de massas. Psicorubrina exibiu uma atividade antitumoral promissora com CI50 de 4,5, 5,6 e 1,1 μM para HL60, Jurkat e MCF-7, respectivamente. Atividade antimicrobiana, principalmente contra *Cryptococcus neoformans* (CIM de 87,3 μM), foi observada. Um pronunciado potencial leishmanicida também foi verificado com CI_{50} variando de 1,7 – 2,7 μM para as diferentes espécies de *Leishmania* testadas. Este é o primeiro relato da presença de piranonaftoquinonas no gênero *Mitracarpus*, que poderão ser úteis como marcadores quimiotaxonômicos.

Palavras-chave: *Mitracarpus frigidus*, psicorubrina, piranonaftoquinona, Rubiaceae.

REFERENCES

Arruda RCO and Gomes DMS. 1996. Anatomia foliar de Mitracarpus frigidus (Wild.) K. Schum. var. salzmannianus (DC) K. Schum. e *Mitracarpus lhorzkianus* Cham (Rubiaceae), São Paulo: Herbarium Bradeanum, 14 p.

Bisignano G, Sanogo R, Marino A, Aquino R, D'Angelo V, Germano MP, de Pasquale R and Pizza C. 2000. Antimicrobial activity of *Mitracarpus scaber* extract and isolated constituents. Lett Applied Microbiol 30: 105-108.

Braga FG, Bouzada MLM, Fabri RL, Matos MO, Moreira FO, Scio E and Coimbra ES. 2007. Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. J Ethnopharmacol 111: 396-402.

Bulbule VJ, Koranne PS, Munot YS, Borate HB and Deshpande VH. 2003. Simple synthesis of two naphthoquinone antibiotics psychorubrin and pentalongin. Synthetic Commun 33: 587-594.

Ekpendu TOE, Adesomoju AA and Okogun JI. 2001. Chemical studies of *Mitracarpus villosus* (Sw.) DC - a medicinal rubiaceous weed. J Chem Soc Nigeria 26: 69-74.

Fabri RL, Nogueira MS, Braga FG, Coimbra ES and Scio E. 2009. *Mitracarpus frigidus* aerial parts exhibited potent antimicrobial, antileishmanial, and antioxidant effects. Bioresource Technol 100: 428-433.

Fotakis G and Timbrell JA. 2006. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett 160: 171-177.

Gbaguidi F, Accrombessi G, Moudachirou M and Quetin-Leclercq J. 2005. HPLC quantification of two isomeric triterpenic acids isolated from Mitracarpus scaber and antimicrobial activity on *Dermatophilus congolensis*. J Pharm Biomed Anal 39: 990-995.

Greff D. 1998. Cosmetic and pharmaceutical compositions for beautifying and lightening skin containing *Mitracarpus extracts*. PCT Int Appl WO 9805299.

Harouna H, Faure R, Elias R, Debrauwerm L, Saadou M, Balansard G and Boudon G. 1995. Harounoside a pentalongin hydroquinone diglycoside from *Mitracarpus scaber*. Phytochemistry 39: 1483-1484.

Hayashi T, Smith FT and Lee KH. 1987. Antitumor agents. 89. Psychorubrin, a new cytotoxic naphthoquinone from *Psychotria rubra* and its structure-activity relationships. J Med Chem 30: 2005-2008.

Jacobs J, Claessens S and de Kimpe N. 2008. First straightforward synthesis of 1-hydroxy-3,4-dihydro-1H-benz[g]isochromene-5,10-dione and structure revision of a bioactive benz[g]isochromene-5,10-dione from *Psychotria camponutans*. Tetrahedron 64: 412-418.

Kesteleyn B, de Kimpe N and Van Puyvelde L. 1999. Synthesis of two naphthoquinone antibiotics pentalongin and psychorubrin. Synthesis 11: 1881-1883.

KRISHNAN P AND BASTOW KF. 2000. Novel mechanisms of DNA topoisomerase II inhibition by pyranonaphthoquinone derivatives eleutherin, α-lapachone, and β-lapachone. Biochem Pharmacol 60: 1367-1379.

LEE H, HONG SS AND KIM YH. 1996. Synthesis and in vitro evaluation of 3-substituted-1-azaanthraquinones. Bioorg Med Chem Lett 6: 933-936.

LI W, LAM MS, BIRKELAND A, RIFFEL A, MONTANA L, SULLIVAM ME AND POST JM. 2006. Cell-based assays for profiling acticity and safety properties of cancer drugs. J Pharmacol Toxicol Methods 54: 313-319.

M'BONGO N, LOISEAU P, LAWRENCE F, BORIES C, CRACIUNESCU DG AND ROBERT-GERO M. 1997. *In vitro* sensivity of *Leishmania donovani* to organometallic derivatives of pentamidine. Parasitol Res 83: 515-517.

MONKS A, SCUDIERO D, SKEHAN P, SHOEMAKER R, PAULL K, VISTICA D, HOSE C, LANGLEY J, CRONISE P AND VAIGRO-WOLFF A. 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer I 83: 757-766.

MOSMANN T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Immunol Methods 65: 55-63.

MOULIS C, PELISIER J, BAMBA D AND FOURASTÉ L. 1992. Pentalongin, antifungal naphthoquinoid pigment from Mitracarpus scaber. Proceeding of the 2nd International Congress on Ethnopharmacology, July 2-4, 1992, Uppsala, Sweden.

NAGATA S. 2000. Apoptotic DNA fragmentation. Exp Cell Res 256: 12-18.

NCCLS - NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS. 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2 - P. National Committee for Clinical Laboratory Standards. Wayne, PA.

NGUYEN VT AND DE KIMPE N. 2004. Synthesis of pyranonaphthoquinone antibiotics involving the ring-closing metathesis of vinyl ether. Tetrahedron Lett 45: 3443-3446.

NICOLETTI I, MIGLIORATIM G, PAGLIACCIM MC, GRIGNANIM F AND RICCARDI C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139: 271-279.

OKUNADE AL, CLARK AM, HUFFORD CD AND OGUNTIMEIN BO. 1999. Azaanthraquinone: an antimicrobial alkaloid from Mitracarpus scaber. Planta Med 65: 447-448.

PEREZ C, PAULI M AND BAZERQUEM P. 1990. An antibiotic assay by the well agar method. Acta Biol Med Exp 15: 113-115.

PERFECT JR AND CASADEVALL A. 2002. Cryptococcosis. Infect Dis Clin North Am 16: 837-874.

SANCHEZ LM, LOPEZ D, VESELY BA, TOGNA GD, GERWICK WH, KYLE DE AND LININGTON RG. 2010. Almiramides A-C: Discovery and Development of a New Class of Leishmaniasis Lead Compounds. J Med Chem 53: 4187-4197.

SPINDLER R AND URBANEC SJ. 2009. Improved skin brightening compositions. PCT Int. Appl. WO 2009/099911.

THOMSON RH. 1971. Naturally Occuring Quinones. 2nd ed., London: Academic, 282 p.

TSUJI D AND NAKANISHI H. 2010. Skin-conditioning compositions containing fucoxanthin and hydroquinone derivative and cosmetics, skin-lightening compositions, and cosmetic creams containing them. JP 2010/209023.

VAN DER HORST CM ET AL. 1997. Treatment of Cryptococcal Meningitis Associated with the Acquired Immunodeficiency Syndrome. N Engl J Med 337: 15-21.

WANG W, LI T, MILBURN R, YATES J, HINNANT E, LUZZIO MJ, NOBLE SA AND ATTARDO G. 1998. Novel 1,3-disubstituted-5,10-dihydro-5,10-dioxo-1*H*-mbenzo[*g*] isochromene-3-carboxamides as potent antitumor agents. Bioorg Med Chem Lett 8: 1579-1584.

YANG W, HU B, SUN J, ZHAI S AND DONALD H. 2010. Death mode-dependent reduction in succinate dehydrogenase activity in hair cells of aging rat cochleae. Chin Med J 123: 1633-1638.

5.3 O artigo a seguir descreve a composição química e as atividades antimicrobiana, leishmanicida e antioxidante do óleo essencial das partes aéreas de *Mitracarpus frigidus.*

Artigo 3

ESSENTIAL OIL OF *Mitracarpus frigidus* AS A POTENT SOURCE OF BIOACTIVE COMPOUNDS

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Essential oil of *Mitracarpus frigidus* as a potent source of bioactive compounds

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ABSTRACT

In our previous work (Fabri et al. 2009), we showed that different extracts of *Mitracarpus frigidus* had significant antibacterial, antifungal and leishmanicidal activities. In order to increase our knowledge about this species, this work assesses the chemical composition and the *in vitro* biological activity of its essential oil. Thus, the essential oil obtained by hydrodistillation of the aerial parts of *M. frigidus* was analyzed by GC/MS. Among several compounds detected, 11 were identified, being linalool and eugenol acetate the major components. The essential oil exhibited a moderate antibacterial effect against *Staphyloccocus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* (MIC 250 μg/mL). On the other hand, it showed a strong antifungal effect against Cryptoccocus neoformans (MIC 8 μg/mL) and Candida albicans (MIC 63 μg/mL). Expressive activity against *L. major* and *L. amazonensis* promastigote forms with IC_{50} values of 47.2 and 89.7 μg/mL, respectively, were also observed. In addition, the antioxidant activity was investigated through DPPH radical-scavenging and showed a significative activity with IC_{50} of 38 μg/mL. The cytotoxicity against *Artemia salina* was moderate with LC_{50} of 88 μg/mL. The results presented here are the first report on the chemical composition and biological properties of *M. frigidus* essential oil.

Key words: *Mitracarpus frigidus*, essential oil, antimicrobial, antileishmanial, cytotoxicity.

INTRODUCTION

Some *Mitracarpus* species have ethnopharmacological importance as they are used in folk medicine for various purposes. For example, *M. scaber* is widely employed in traditional medicine in West Africa for headaches, toothache, amenorrhea, dyspepsia, hepatic diseases and leprosy. Among those folkloric uses, the juice of the plant is applied topically for the treatment of skin diseases (Dalziel 1936, Kerharo and Adam 1974).

The potential antimicrobial activity of the aerial parts extracts of some *Mitracarpus* species against bacterial and mould strains, like *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Cryptococcus neoformans* were already reported (Irob and Daramola 1993,

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1994, Sanago et al. 1996). Also, in our previous work, we showed that *M. frigidus* extracts had a significant antibacterial, antifungal and leishmanicidal activities (Fabri et al. 2009). So, in order to increase our knowledge about *M. frigidus*, this work deals with the chemical composition and the *in vitro* activity against Gram-positive and Gram-negative bacteria and yeasts strains, promastigote forms of *Leishmania* species and cytotoxicity against *Artemia salina* of the essential oil of this specie. Its *in vitro* antioxidant activity was also determined.

MATERIALS AND METHODS

Plant Material

M. frigidus aerial parts were collected in Juiz de Fora, Minas Gerais, Brazil, in May, 2006. Dr. Tatiana Konno identified the plant. A voucher specimen (CESJ 46076) was deposited at the Herbarium Leopoldo Krieger of the Universidade Federal de Juiz de Fora.

Hydrodistillation of Volatile Oils

Essential oil was obtained by means of hydrodistillation (3 h) of the dried plant material using a Clevenger type apparatus. Oil was dried and stored at 4°C until tested and analyzed.

Collection of Volatiles by Solid Phase Micro-Extration (SPME)

Fifty microliters from the 20 mg/mL solutions were transferred to a 2 mL glass vial and the solvent was removed under vacuum (speedVac®, SC250 model, ThermoSavant, U.S.A) for 18 h, 30°C and 10 millibar. The vial was closed with a cap sealed Teflon coated septum (Supelco, U.S.A) and placed in a heat block adjusted to 90°C. A SPME fiber (PDMS/DVB™ 65 μm, SUPELCO, U.S.A) was inserted with a manual holder through the septum and left in the headspace for 30 min. Immediately after that, the holder with the saturated fiber was analyzed by means of injection on gas chromatography (GC) injection port. Before use, the fiber was preconditioned at 230°C during 30 min in the GC injector port.

Gas Chromatography/Mass Spectrometry Analysis

Gas Chromatography/Mass Spectrometry (GC-MS) analyses were performed on a Shimadzu QP-5050A (SHIMADZU, JP) instrument, equipped with a PTE™-5 column (30 m, 0.25 mm, 0.25 μm, Supelco, USA), using helium as the carrier gas. The following conditions were employed for all analysis: helium at 22.3 mL/min; injector temperature maintained at 230°C; the oven at 80°C during 3 minutes and then heated to 300°C at 7°C/min, holding for 5 min at 300°C. The split valve was closed during the first minute of injection and then opened, with a 1:10 ratio. The mass detector was set to scan from 50 to 500 *m*/*z*, at a rate of 2 scans per second. Data acquisition and handling was done via CLASS 5000 Shimadzu software.

Analysis of the Raw Data using AMDIS Software (Automated Mass Spectral Deconvolution and Identification System)

Raw data files were analyzed by Automated Mass Deconvolution and Identification System software (AMDIS), version 2.1, supplied by National Institute of Standards and Technology (NIST, USA). Retention Index (RI) in the range of 900 to 3,000 was generated from the analysis of a standard mixture containing hydrocarbons C9 to C30 (Fluka, U.S.A).Elucidation of the compounds was done by means of the NIST MS Search 2.0 Program (NIST/EPA/NIH Mass Spectral Library, version 2002) and on the basis of comparison of retention indices determined according Van Den Dool and Kratz (1963) for each constituent, as well as, previously described by Adams (2007).

Microbial Strains

The sample was evaluated against a panel of microorganisms, including *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), *Salmonella enterica* sorovar Typhimurium (ATCC 13311), *Shigella sonnei* (ATCC 11060), *Klebsiella pneumoniae* (ATCC 13866), *Escherichia coli* (ATCC 10536), *Bacillus cereus* (ATCC 11778), *Micrococcus luteus* (ATCC 10054), *Enterococcus faecalis* (ATCC 51299), *Enterobacter cloacae* (ATCC 10699), *Streptococcus pyogenes* (ATCC 10096), *Candida albicans* (ATCC 18804) and *Cryptococcus neoformans* (ATCC 32608). Bacterial strains were cultured overnight at 37°C in Mueller Hinton agar (MHA). Yeasts were cultured for 48 h at 30°C in Sabouraud dextrose agar (SDA).

Serial Dilution Assay for Determination of the Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by using broth microdilution techniques for bacterial and yeasts (NCCLS 2002 Perez et al. 1990). MIC values were determined in RPMI 1640 (Sigma) buffered to a pH 7.0 with MOPS (Sigma) for yeasts and Mueller Hinton broth (MHB) for bacteria. Yeasts were cultured at 30°C for 48 h in SDA and bacteria were cultured overnight at 37°C for 24 h in MHA. Sample stock solutions were two-fold diluted from 5,000 to 2.5 μg/mL (final volume = 80 μL) and a final DMSO concentration ≤ 1%. Then, 100 μL of RPMI or MHB were added onto microplates. Finally, 20 μL of 10^6 CFU/mL (according to McFarland turbidity standards) of standardized yeasts and bacterial suspensions were inoculated onto microplates and the test was performed in a volume of 200 μL. Plates were incubated at 30°C for 48 h for yeasts and 37°C for 24 h for bacteria. The same tests were performed simultaneously for growth control (RPMI + yeast and MHB + bacteria) and sterility control (RPMI or MHB + essential oil). The test was performed in duplicate. The MIC values were calculated as the highest dilution showing complete inhibition of tested strain.

DPPH Radical Scavenging Assay

The free radical scavenging activity of sample solutions in methanol was determined based on their ability to react with stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (Govidarajan et al. 2003). The essential oil at various concentrations (7.8 to 250 μg/mL) was added to a 152 μM solution of DPPH in methanol. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 517 nm. The antioxidant activity of essential oil was expressed as IC_{50} (inhibitory concentration), which was defined as the concentration (in μg/mL) of sample required to inhibit the formation of DPPH radicals by 50 %. α-Tocopherol and rutin were used as positive control.

Antileishmanial Assay

Three species of *Leishmania* were used in this study: *L. chagasi* (MHOM/Br/74/PP75), *L. amazonensis* (MHOM/Br/75/Josefa) and *L. major* (MRHO/SU/59/P). Anti-leishmanial activity was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method based on tetrazolium salt reduction by mitochondrial dehydrogenases (Braga et al. 2007). Briefly, promastigotes of *L. amazonensis* and *L. major* were cultured in Warren's medium (brain heart infusion plus hemin and folic acid) and promastigotes of *L. chagasi* were maintained in Medium 199, both supplemented with 10 % fetal bovine serum at 24°C. The screening was performed in 96-well microtiter plates maintained at 24°C. The essential oil solution in DMSO was added in a serial solution in the wells (11 to 108 μg/mL). Then a parasite suspension from a logarithmic phase

culture was suspended to yield 2 million cells/mL (*L. amazonensis* or *L. major*) or 3 million cells/mL (*L. chagasi*) after Neubauer chamber counting. Controls with DMSO and without plants samples were performed. All the tests were performed in triplicates. The viability of promastigotes was assayed after a three day incubation period with addition of MTT. The reaction was stopped with HCl in isopropyl alcohol and the optical densities were evaluated in a spectrophotometer at 570 nm (Multiskan MS microplate reader, LabSystems Oy, Helsink, Finland). The results were expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) and the percentage of inhibition of parasite growth. Amphotericin B was used as the standard drug.

Cytotoxicity Assay

Brine shrimp lethality bioassay (Meyer et al. 1982) was carried out to investigate the cytotoxicity of the essential oil. Brine shrimp (*Artemia salina* Leach) eggs were hatched in a beaker filled with sea water under constant aeration. After 48 h, the nauplii were collected by pipette and were counted macroscopically in the stem of the pipette against a lighted background. Solutions of the essential oil were made in seawater containing 1 % DMSO, at varying concentrations (10 to 1,000 μg/mL) and incubated in triplicate vials with 10 brine shrimp larvae. After 24 h of incubation, the nauplii were examined against a lighted background, with a magnifying glass and the number of survivors in each vial were counted and noted. Both positive (thymol) and negative (sea water containing 1 % DMSO) control assays were carried out in order to verify the susceptibility of A. salina under assay conditions employed.

Statistical Analysis

The IC_{50} for antioxidant activity was calculated by Grafit 5. The IC_{50} for leishmanicidal activity and cytotoxicity were calculated by Probit analysis. Both were expressed as mean ± standard error (SE). The inhibition percentages for leishmanicidal activity were analyzed by Graph Pad Prisma 4. Statistical differences between the treatments and the control were evaluated by ANOVA test.

RESULTS

Chemical Composition

Water-distillation of the dried aerial parts of *M. frigidus* yielded 0.01 % (v/w) of an orange oil. Chemical analysis of the oil's components resulted in the identification of 12 known components (Table I). Linalool (29.29%) and eugenol acetate (15.85%) were the major constituents of the essential oil, followed by 5-hydroxy-isobornyl isobutyrate (8.41%), 5-methyl-1-undecene (7.69%) and methyl salicylate (6.55%).

Antimicrobial Activity

The essential oil of *M. frigidus* exhibited a moderate antibacterial effect against *Staphyloccocus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* (MIC 250 μg/mL) (Table II). On the other hand, the oil showed a strong antifungal effect against *Cryptoccocus neoformans* (MIC 8 μg/mL) and *Candida albicans* (MIC 63 μg/mL). It was considered that if the extracts displayed a MIC less than 100 μg/mL, the antimicrobial activity was good; from 100 to 500 μg/mL, moderate; from 500 to 1,000 μg/mL, weak; over 1,000 μg/mL the extract was considered inactive (Holetz et al. 2002).

Antileishmanial Activity

The effect of the essential oil of *M. frigidus* on the viability of promastigotes of *L. major*, *L. amazonensis* and *L. chagasi* was tested. The essential oil showed expressive activity against *L. major* and *L. amazonensis* promastigote forms with IC_{50} values of 47.2 ± 4.0 and 89.7 ± 8.6 μg/mL, respectively. Those forms were susceptible to the essential oil

TABLE I
Chemical constituents, retention index and relative concentration of compounds of essential oil of *Mitracarpus frigidus* analyzed by GC-MS.

No	Compound	Retention time (min)	% relative	Retention index	Molecular Mass
1	7-Octen-4-ol	4.07	5.03	850.8	128
2	5-Hydroxy-isobornyl isobutyrate	5.0	8.41	1033.9	276
3	5-methyl-1-Undecene	6.39	7.69	1092.1	168
4	Linalool	6.53	29.29	1098.9	154
5	*β*-Phenylethyl alcohol	6.85	3.79	1113.5	122
6	Cucumber aldehyde	7.63	3.55	1149.4	138
7	*trans*-2-Nonenal	7.77	4.83	1155.6	140
8	Methyl salicylate	8.61	6.55	1194.3	152
9	8-Isobutyryloxy-isobornyl isobutyrate	9.38	4.66	1230.1	281
10	Not known	9.87	5.99	1253.0	341
11	Eugenol acetate	12.04	15.85	1357.6	206
12	Damascenone	12.58	4.36	1384.7	190

TABLE II
Antimicrobial activity of *Mitracarpus frigidus* essential oil.

Test microorganisms	MIC (µg/mL)[a)]	S[b)]
Bacteria		
Staphylococcus aureus	250	62.5[c)]
Escherichia coli	500	15.6[c)]
Salmonella typhimurium	1,000	1.0[c)]
Shigella sonnei	1,000	1.0[c)]
Klebsiella pneumoniae	500	1.0[c)]
Bacillus cereus	250	3.9 [c)]
Pseudomonas aeruginosa	250	15.6[c)]
Enterobacter cloacae	250	31.3[c)]
Enterococcus faecalis	1,000	31.3[c)]
Streptococcus pyogenes	500	15.6[c)]
Yeasts		
Candida albicans	63	0.08[d)]
Cryptococcus neoformans	8	0.04[d)]

[a)] Minimum inhibitory concentration.

[b)] Standard antimicrobial agents: [c)] Chloramphenicol; [d)] Amphotericin B.

at 108 µg/mL with 97% and 86% cell inhibition, respectively (Figure 1). Interestingly, *L. major* presented the greatest dose-dependent relationship. On *L. chagasi*, the percentage of inhibition did not varied significantly between the concentrations used. The percentage of inhibition presented by reference drug Amphotericin B at 10 µg/mL was 60 %, 90% and 86% for *L. major*, *L. amazonensis* and *L. chagasi* promastigotes, respectively.

Antioxidant Activity

The antioxidant activity of the essential oil of *M. frigidus* was investigated through DDPH radical-scavenging and showed a moderate activity with IC_{50} of 38 ± 8 µg/mL. The reference controls α-tocopherol and rutin presented IC_{50} of 0.2 ± 0.1 and 3 ± 1.8 µg/mL, respectively.

Cytotoxicity

The cytotoxicity against *Artemia salina* was moderate with LC_{50} 88 ± 10 µg/mL. The reference control thymol showed LC_{50} of 1.4 ± 0.7 µg/mL.

DISCUSSION

The essential oil exhibited a moderate antibacterial and a strong antifungal effect against *Cryptoccocus neoformans* and Candida albicans. Also, it showed expressive activity against *Leishmania major* and *L. amazonensis* promastigote forms.

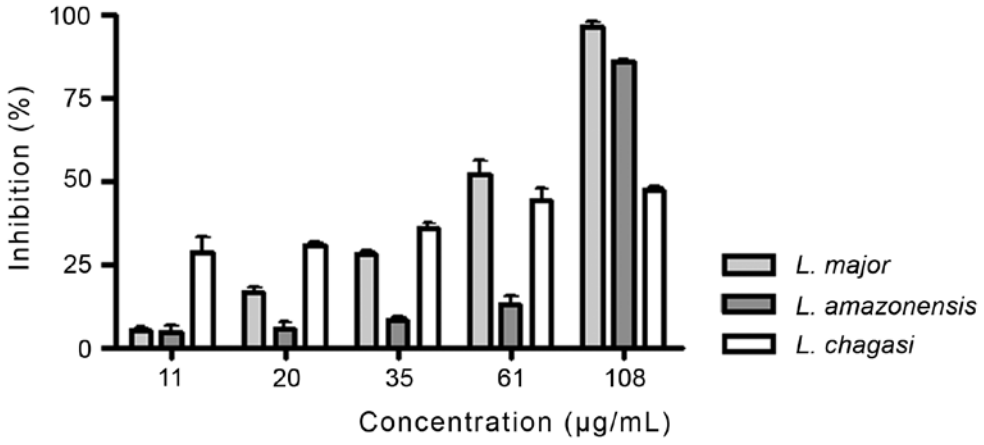

Figure 1 - Effect of the essential oil from *M. frigidus* on growth of *L. major*, *L. amazonensis* and *L. chagasi* promastigote forms. Parasites were treated with 11 to 108 µg/mL essential oil. Each bar represents the mean ± standard deviation of three different experiments.

Linalool and eugenol acetate were the major constituents of the essential oil. Linalool is a monoterpene commonly found in the essential oils of some aromatic plants. It is also obtained as a by-product in the industrial synthesis of vitamin E (Ohashi et al. 1997). Its biological activity, including antioxidant, antimicrobial, anti-inflammatory, anesthetic and antitumor was already reported (Ghelardini et al. 1999, Mazzanti et al. 1998, Letizia et al. 2003, Dadasoglu et al. 2011). Also, the ability to inhibit the development of the mosquito larvae of *Aedes aegypt* has been attributed to linalool (Gottlieb et al. 1981). Eugenol acetate is a derivative of eugenol, which is employed as antimicrobial, anti-inflammatory, anesthetic, anti-septic, antioxidant, repellent agent, and in cosmetics and condiments (Lahlou 2004).

Cryptoccocus neoformans and *Candida albicans* are opportunistic pathogens commonly associated with disease in immunocompromised hosts. *C. neoformans* causes systemic disease, and cryptococcal infection is usually acquired by inhalation of fungal cells and can be limited to the lung or disseminate to the central nervous system, causing meningoencephalitis (Rodrigues et al. 1999). *C. albicans* can cause local and systemic infection and it is found normally in the buccal and vaginal regions (Zhang and Lewis 1997). The lipophylicity of the essential oil constituents could explain their antimicrobial activity, a characteristic that allows the partition of these compounds in lipids of cell membrane and mitochondria, increasing their permeability and leading to leakage of cellular contents (Cowan 1999). According to other authors, essential oil constituents can also act on cellular proteins located in cytoplasm membranes, including the ATPases, by their accumulation in the lipid double layer and the consequent destruction of lipid-protein interaction (Ultee et al. 2002, Burt 2004). Alternatively, a direct interaction of lipophylic compounds may occur with the hydrophobic portions of proteins (Juven et al. 1994, Sikkema et al. 1995). However, due to the large number of different chemical groups present in essential oils, it is likely that its antimicrobial activity is not related to a specific mechanism of action (Carson et al. 2002, Kalemba and Kunicka 2003, Skadamis and Nychas 2001).

Leishmaniasis is a chronic disease that can assume different fatal clinical forms ranging from selfhealing cutaneous to progressive mucocutaneous infection, and potentially visceral leishmaniasis (Tripathi et al. 2007). According to the World

Health Organization (2004), leishmaniasis currently threatens 350 million people around the world and it is estimated that 2 million new cases occur each year. A common feature of volatiles compounds is their hydrophobic nature. Several studies addressing the action mode of such compounds usually point to cell membranes as the primary target as the essential oils, in general, have a passive entry through the membrane, leading to an increase of membrane permeability (Bakkali et al. 2008).

Antioxidant activity possibly proceeds from the presence of phenolic compounds such as methyl salicylate in the oil. The brine shrimp lethality assay is based on the ability to kill laboratory-cultured *Artemia salina* nauplii brine shrimp and it is considered to be one of the most useful tool for the preliminary assessment of general toxicity (Maclaughlin 1991). LC_{50} values < 250 μg/mL are considered significant for plant samples and had the potential for futher investigation (Rieser et al. 1996).

The results presented here are the first report on the chemical composition and biological properties of the *Mitracarpus* essential oil.

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RESUMO

Em nosso trabalho anterior (Fabri et al. 2009), mostramos que diferentes extratos de *Mitracarpus frigidus* apresentaram atividades antibacteriana, antifúngica e leishmanicida significativas. Com o objetivo de aprofundar o conhecimento sobre essa espécie, esse trabalho objetiva identificar os constituintes químicos e avaliar as atividades biológicas *in vitro* de seu óleo essencial. Dessa forma, o óleo essencial das partes aéreas de *M.frigidus* foi obtido por hidrodestilação e analisado por CG/EM. Entre os compostos detectados, 11 foram identificados, sendo linalol e o acetato de eugenol os componentes majoritários. O óleo essencial de *M. frigidus* exibiu moderada atividade antibacteriana contra *Staphyloccocus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* e *Enterobacter cloacae* (CIM 250 μg/mL). Por outro lado, apresentou forte efeito antifúngico contra Cryptoccocus neoformans (CIM 8 μg/mL) e *Candida albicans* (CIM 63 μg/mL). Expressiva atividade contra as formas promastigostas de *Leishmania major* e *Leishmania amazonensis* com valores CI_{50} de 47,2 e 89,7 μg/mL, respectivamente, também foi observada. Além disso, a atividade antioxidante foi investigada através do ensaio com o radical DPPH e este apresentou uma significativa atividade com CI_{50} de 38 μg/mL. A citotoxidade contra *Artemia salina* foi moderada com CL_{50} de 88 μg/mL. Os resultados aqui apresentados são o primeiro relato sobre a composição química e propriedades biológicas do óleo essencial *M. frigidus*.

Palavras-chave: *Mitracarpus frigidus*, óleo essencial, antimicrobiano, leishmanicida, citotoxidade.

REFERENCES

ADAMS RP. 2007. Identification of essential oil components by gas chromatograpy/ quadrupopole mass spectroscopy. Allured Publishing Corporation, Carol Stream, IL, USA, 804 p.

BAKKALI F, AVERBECK S, AVERBECK D AND IDAOMAR M. 2008. Biological effects of essential oils - a review. Food Chem Toxicol 46: 446-475.

BRAGA FG, BOUZADA MLM, FABRI RL, MATOS MO, MOREIRA FO, SCIO E AND COIMBRA ES. 2007. Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. J Ethnopharmacol 111: 396-402.

BURT S. 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. Int J Food Microbiol 94: 223-253.

CARSON CF, MEE BJ AND RILLEY TV. 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage and salt tolerance assays and eletron microscopy. Antimicrob Agents Ch 46: 1914-1920.

COWAN MM. 1999. Plant products as antimicrobial agents. Clin Microbiol Rev 12: 546-582.

DADASOGLU F, AYDIN T, KOTAN R, CAKIR A, OZER H, KORDALI S, CAKMAKCI R, DIKBAS N AND METE E. 2011. Antibacterial activities of extracts and essential oils of three origanum species against plant pathogenic bacteria and their potential use as seed disinfectants. J Plant Pathol 93: 271-282.

DALZIEL JM. 1936. Useful plants of West Tropical Africa. London: Crown Agents for the Colonies, 612 p.

FABRI RL, NOGUEIRA MS, BRAGA FG, COIMBRA ES AND SCIO E. 2009. *Mitracarpus frigidus* aerial parts exhibited potent antimicrobial, antileishmaninal, and antioxidant effects. Bioresource Technol 100: 428-433.

GHELARDINI C, GALEOTTI N, SALVATORE G AND MAZZANTI G. 1999. Local anaesthetic activity of the essential oil of *Lavandula angustifolia*. Planta Med 65: 700-703.

GOTTLIEB OR, KOKETSU M, MAGALHÃES MT, MAIA JGD, MENDES AI, DA SILVA ML AND WILBERG VC. 1981. Óleos essenciais da Amazônia. VII. Acta Amazonica 11: 143-148.

GOVIDARAJAN R, RASTOGI S, VIJAYAKUMAR M, SHIRWAIKAR A, RAWAT AKS, MEHROTRA S AND PUSHPANGADAN P. 2003. Studies on the antioxidant activities of *Desmodium gangeticum*. Biol Pharm Bull 26: 1424-1427.

HOLETZ FB, PESSIN GL, SANCHES NR, CORTEZ DAG, NAKAMURA CV AND FILHO BPD. 2002. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Mem Inst Oswaldo Cruz 97: 1027-1031.

IROB ON AND DARAMOLA SO. 1993. Antifungal activities of crude extracts of *Mitracarpus villosus* (Rubiaceae). J Ethnopharmacol 40: 137-140.

IROB ON AND DARAMOLA SO. 1994. Bactericidal properties of crude extracts of *Mitracarpus villosus*. J Ethnopharmacol 42: 39-43.

JUVEN BJ, KANNER J, SCHVED F AND WEISSLOWICZ H. 1994. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J Appl Bacteriol 76: 626-631.

KALEMBA D AND KUNICKA A. 2003. Antibacterial and antifungal properties of essential oils. Curr Med Chem 10: 813-829.

KERHARO J AND ADAM JG. 1974. La Pharmacopée Sénegalaise Traditionnelle: Plantes Médicinales Et Toxiques. Paris: Vigot Frères.

LAHLOU M. 2004. Methods to study the phytochemistry and bioactivity of essential oils. Phytother Res 18: 435-444.

LETIZIA CS, COCCHIARA J, LALKO J AND API AM. 2003. Fragrance material review on linalool. Food Chem Toxicol 41: 943-964.

MACLAUGHLIN JL. 1991. Crown gall tumours on potato discs and brine shrimps lethalithy: two simple bioassays for higher plant screening and fractionation. In: Hostettmann K (Ed), METHODS IN BIOCHEMISTRY. ASSAYS FOR BIOACTIVITY. London: Academic Press, p.1-32.

MAZZANTI G, BATTINELLI L AND SALVATORE G. 1998. Antimicrobial properties of the linalool-rich essential oil of *Hyssopus officinalis* L. var. decumbens (Lamiaceae). Flavour Fragr J 13: 289-294.

MEYER BN, FERRIGNI NR, PUTNAM JE, JACOBSEN LB, NICHOLS DE AND MACLAUGHLIN JL. 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med 45: 31-34.

NCCLS - NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS. 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2 - P. National Committee for Clinical Laboratory Standards. Wayne, PA.

OHASHI ST, ROSA LS, SANTANA JA AND GREEN CL. 1997. Brazilian rosewood oil: sustainable production and oil quality management. Perf Flav 22: 1-5.

PEREZ C, PAULI M AND BAZERQUE P. 1990. An antibiotic assay by the well agar method. Acta Biol Med Exp 15: 113-115.

RIESER MJ, GU ZM, FRANG I, WOOD KV AND MACLAUGHLIN JL. 1996. Five novel mono-tetrahydrofuran ring acetogenins from seeds of *Annona muricata*. J Nat Prod 59: 100-129.

RODRIGUES ML, ALVIANO CS AND TRAVASSOS LR. 1999. Pathogenicity of *Cryptococcus neoformans*: virulence factors and immunological mechanisms. Microbes Infect 4: 293-301.

SANOGO R, GERMANÒ MP, DE PASQUALE R, KEITA A AND BISIGNANO G. 1996. Selective antimicrobial activities of *Mitracarpus scaber* Zucc against Candida and *Staphylococcus sp.* Phytomedicine 2: 265-268.

SIKKEMA J, DE BONT JAM AND POOLMAN B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59: 201-222.

SKANDAMIS PN AND NYCHAS GJE. 2001. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. J Appl Microbiol 91: 1011-1022.

TRIPATHI P, SINGH V AND NAIK S. 2007. Immune response to *Leishmania*: paradox rather than paradigm. FEMS Immunol Med Microbiol 51: 229-242.

ULTEE A, BENNIK MH AND MOEZELAAR R. 2002. The phenolic hydroxylgroup of carvacrol is essential for action against the food-bornepathogen Bacillus cereus. Appl Environ Microbiol 68: 1561-1568.

VAN DEN DOOL H AND KRATZ DJ. 1963. A generalization of the retention index system including liner temperature programmed gas-liquid partition chromatography. J Chromatograph 11: 463-467.

WHO - WORLD HEALTH ORGANIZATION. 2004. Report of the Scientific Working Group meeting on leishmaniasis.

ZHANG Y AND LEWIS K. 1997. Fabatins: new antimicrobial plant peptides. FEMS Microbiol Lett 149: 59-64.

5.4 O artigo a seguir relata o potencial antimicrobiano e leishmanicida da partição hexânica das partes aéreas de *Mitracarpus frigidus.*

Artigo 4

ANTIBACTERIAL AND LEISHMANICIDAL EFFECTS OF *Mitracarpus frigidus*

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Antibacterial and leishmanicidal effects of *Mitracarpus frigidus*

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ABSTRACT

In the present study it was investigated the antibacterial and leishmanicidal activities of the hexane extract of *Mitracarpus frigidus* aerial parts. The biological assays were evaluated against six strains of bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Escherichia coli* and *Enterobacter cloacae*), promastigote forms of four species of *Leishmania* (*L. major*, *L. braziliensis*, *L. chagasi* and *L. amazonensis*) and amastigote forms of *L. major*. Cytotoxicity against mammalian cells and phytochemical analysis of the major groups of phytoconstituents were also reported. The samples showed significant antimicrobial activity, mainly for *P. aeruginosa*, *S. dysenteriae* and *S. typhimurium* with MIC $<$ 100 µg/mL. A pronounced antileishmanial potential was also verified for promastigote and amastigote forms, being the samples more specific for the intracellular stage. Despite the samples showed some toxicity against mammalian cells, they were more selective for the intracellular parasite than to host cells. The most active samples showed the presence of steroids, terpenes and flavonoids.

Uniterms: *Mitracarpus frigidus*, *Leishmania major*, antibacterial, terpenes, flavonoids.

INTRODUCTION

In recent years, the frequency of microbial resistance and its association with serious infectious diseases have increased gradually (Jones, 2001). Many microorganisms have developed resistance against both the well-established conventional use of antibiotics as against the last generation of antibiotics, causing serious public health problems and economic loss (Austin, Kristinsson, Anderson, 1999; Prates, Bloch-Júnior, 2001). Not less important, leishmaniasis is a disease that causes serious public health problems in most tropical countries (Santos *et al*., 2008).

The use of medicinal plants for the treatment of many diseases is associated with folk medicine in different parts of the world (Araújo, Leon, 2001). Different cultures of the most distinguished countries, developed or not, know and use the therapeutic potential of plants in the treatment of diseases. These practices accompany men since prehistoric times and have evolved with him over the years (Coutinho *et al*., 2004).

The genus *Mitracarpus* is native to Brazil and is found mainly in tropical and subtropical countries. Some species of *Mitracarpus* have ethnopharmacological use reported, as *M. scaber* that is widely used in West African traditional medicine for headache, toothache, amenorrhea, dyspepsia, liver diseases, venereal diseases and leprosy. The juice of the plant is applied topically to dermatological diseases (Dalziel, 1936; Kerharo, Adam, 1974). Fabri *et al.* (2009) reported that extracts of *M. frigidus* aerial parts have significant leishmanicidal activity against *L. chagasi* and *L. amazonensis* promastigoste forms and growth inhibition of bacteria and fungi pathogenic strains.

So, the aim of this study was to evaluate the antibacterial and leishmanicidal potential and the *in vitro* citotoxicity of the hexane extract of *Mitracarpus frigidus* aerial parts. Furthermore, it was investigated their main groups of phytoconstituents.

MATERIAL AND METHODS

Plant material

The aerial parts of *Mitracarpus frigidus* (Reem Willd. ex Schult.) K. Schum. were collected in Juiz de Fora, Minas Gerais, Brazil, in May 2009. The voucher specimen was deposited in the Herbarium Leopoldo Krieger (CESJ 46,076) of the Federal University of Juiz de Fora.

Fractionation of the hexane extract

The hexane extract (MFH) (16 g) obtained from the methanolic extract of *M. frigidus* aerial parts (Fabri *et al.*, 2009) was fractionated using column chromatography on silica gel (Merck, 70-230 mesh ASTM) size 74 x 4 cm, with an increasing gradient elution (hexane, dichloromethane and MeOH). The fractions obtained were analyzed by thin layer chromatography (TLC) and grouped according to the chromatographic profile in 9 fractions (MFH1 to MFH9). These fractions were concentrated at reduced pressure using a rotary evaporator, weighed and kept refrigerated until the completion of the biological tests.

The hexane extract and fractions were analyzed by thin layer chromatography (TLC) on silica gel 60 F254 (Merck) to identify the chemical constituents. The mobile phases were: I) hexane: EtOAc, 70:30, v/v for MFH1-MFH4; II) hexane: EtOAc, 30:70, v/v for hexane extract and MFH5-MFH9. The plates were visualized under UV light at 254 and 365 nm and revealed with sulfuric acid and vanillin, as universal revealer; 10% KOH (Borntrager Reagent) for the identification of anthraquinones and coumarins; Dragendorff Reactive for alkaloids; NP/PEG for flavonoids; Liebermann-Burchard Reagent for steroids and triterpenes and 0.1% $FeCl_3$ for phenolic compounds and tannins.

Antimicrobial assay

Microbial strains

The samples were evaluated against a panel of microorganisms, *Staphylococcus aureus (*ATCC 6538*), Pseudomonas aeruginosa* (ATCC 15442*), Escherichia coli (*ATCC 10536*), Shigella dysenteriae (*ATCC 13313*), Salmonella*

enterica sorovar *typhimurium (*ATCC 13311*)* and *Enterobacter cloacae (*ATCC 10699*).* These strains were cultured overnight at 37 ºC in Mueller Hinton agar (MHA).

Serial dilution assay for determination of the minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of each sample was determined by using broth microdilution techniques as described by NCCLS (2002). MIC values were determined in Mueller Hinton broth (MHB). Bacteria were cultured overnight at 37 ºC for 24 h in Mueller Hinton Agar (MHA). Samples stock solutions were two-fold diluted from 5000 to 2.5 μg/mL (final volume = 80 μL) and a final DMSO concentration ≤ 1%. Then, 100 μL of MHB were added onto microplates. Finally, 20 μL of 10^6 CFU/mL (according to McFarland turbidity standards) of standardized bacterial suspensions were inoculated onto microplates and the test was performed in a volume of 200 μl. Plates were incubated at 37 ºC for 24 h. The same tests were performed simultaneously for growth control (MHB + bacteria) and sterility control (MHB + extract). Chloramphenicol (500 to 0.24 μg/mL) was using as positive control. The MIC values were calculated as the highest dilution showing complete inhibition of tested strain. The analyses were performed in duplicate.

Quantitative evaluation of antimicrobial activity

The antimicrobial activity of plant extracts may be expressed in different ways based on technique used. The agar diffusion method is commonly used as a preliminary test in the screening of plants for antimicrobial activity. While the micro-dilution method yields MIC values, the minimum concentration at which inhibition is observed (μg/mL). In this study other ways of expressing antimicrobial efficiency as comparative numerical values are used. Beside results being recorded in terms of MIC (μg/mL), percent activity values which demonstrate the total antimicrobial potency of particular extracts, and microbial susceptibility index (MSI), which is used to compare the relative susceptibility among the microbial strains were employed (Bonjar, 2004):

Percent activity (%) = (100 x No. of susceptible strains to a specific extract) / (Total nº of tested microbial strains). The percent activity demonstrates the total antimicrobial potency of particular extracts. It shows the number of microbial found susceptible to one particular extract.

Microbial susceptible index (MSI) = (100 x No. of extracts effective against each microbial strain) / (No. of total samples). MSI is used to compare the relative susceptibility among the microbial strains. MSI values ranges from ‘0’ (resistant to all samples) to ‘100’ (susceptible to all samples).

Antileishmanial assay

Parasites

Four *Leishmania* species for *in vitro* screening were used: *L. amazonensis* (IFLA/Br/67/PH8), *L. major* (MRHO/SU/59/P), *L. braziliensis* (MHOM/Br/75/M2903) and *L. chagasi* (MHOM/Br/74/PP75). Promastigotes of *L. amazonensis* were cultured in Warren’s medium (brain heart infusion – BHI – enriched by hemin and folic acid), promastigotes of *L. major* and *L. braziliensis* were maintained in BHI medium, and promastigotes of *L. chagasi* were maintained in Medium 199, both supplemented with 10% fetal bovine serum at 24 °C.

Promastigote forms

The antileishmanial activity was performed according to a previously described method (Braga *et al*., 2007). Briefly, promastigotes from a logarithmic phase culture were suspended to yield 2 x 10^6 cells/mL (*L. amazonensis*) and 3 x 10^6 cells/mL (*L. chagasi, L. braziliensis* and *L. major*) after Neubauer chamber counting. The test was performed in 96-well microtiter plates maintained at 24 °C. The analyses were performed in triplicate. The parasites were exposed to increasing concentrations of the samples (nonserial six dilutions: from 100 to 0.39 µg/mL) for 72 h at 24 °C. Controls with 0.5% DMSO were also performed. The results are expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) after a three day incubation period. Amphotericin B was used as reference drug.

Amastigote forms

Concerning the amastigotes *in vitro* model, inflammatory macrophages were obtained from BALB/c mice previously inoculated intraperitoneal with 2mL of 3% thioglycollate medium (Morais-Teixeira *et al*., 2008). Briefly, peritoneal macrophages were plated at 2 × 10^6 cells/mL on coverslips (13 mm diameter) previously

arranged in a 24-well plate in RPMI 1640 medium supplemented with 10% inactivated FBS and allowed to adhere for 24 h at 37 °C in 5% CO_2. Adherent macrophages were infected with *L. major* (MRHO/SU/59/P) promastigotes in the stationary growth phase using a ratio of 1:5 at 33 °C for 3 h. Non-internalized promastigotes were eliminated and solutions of tested compounds were added (nonserial five dilutions: from 50 to 1.2 µg/mL) and maintained at 33 °C in 5% CO_2 for 72 h. Slides were fixed and stained with Giemsa for parasite counting (optical microscopy, 1000x magnification). The parasite burden was evaluated by counting the intracellular parasite, uninfected and infected macrophages (at minimum 100 cells infected) in treated and untreated cultures and the survival index was obtained by multiplying the percentage of infected macrophages by the mean number of amastigote forms per infected cell (Santos *et al*., 2008). Control with 0.5% DMSO was also performed. The analyses were performed in triplicate.

Cytotoxicity against mammalian cells

Cytotoxicity was evaluated after 48 h incubation of murine macrophage-J774 with concentration ranging from 150 to 15 µg/mL of the samples. The viability of the macrophages was determined with the MTT assay and was confirmed by comparing the morphology of the control group via light microscopy (Carmo *et al*., 2011). Dose response curves were plotted (values expressed as percentage of control optical density) and CC_{50} values (50% inhibitory concentration) were obtained. The analyses were performed in triplicate.

Statistical Analysis

For the antileishmanial activity and cytotoxicity on macrophages, the IC_{50} and CC_{50} values, respectively, were carried out at 5% significance level ($p < 0.05$, CI 95%), using a nonlinear regression curve (GraFit Version 5 software). For *Leishmania* amastigote assays, the statistical analysis was performed with the software GraphPad Prism 4.

RESULTS AND DISCUSSION

Phytochemical analysis

The fractionation of the hexane extract yielded nine fractions (MFH1 - MFH9) which were weighed and analysed by TLC (Table I).

The TLC analysis identified the presence of triterpenoids in all samples tested, and steroids and flavonoids in the more polar samples (MFH6 - MFH9). Alkaloids and coumarins were found only in MFH6 and MFH9, respectively.

Table I

Antibacterial activity

The antibacterial activity was concentrated in MFH7 and MFH8 with MIC ≤ 125 µg/mL. *S. thyphimurium*, *P. aeruginosa*, *S. dysentereae* and *E. coli* were the most susceptible microorganisms (Table II). Importantly, for *S. dysenteriae*, the MIC value found to MFH8 was lower than that observed to the positive control (chloramphenicol).

The percent activity values and MSI were calculated for fractions with MIC < 1000 µg/mL. The percentage of antibacterial activity was greater for MFH8 with 100% activity, followed by MFH and MFH7 with 83%. The MSI revealed that *S. dysenteriae*, *S. typhimurium* and *E. cloacae* strains were susceptible to 83% of the samples tested, followed by *P. aeruginosa* (67%).

Table II

Previous studies have shown that substances with antibacterial activity from plants appear to be more active against Gram-positive than Gram-negative organisms (Herrera *et al*., 1996). The reason for this difference in sensitivity can be attributed to the morphological characteristics of these organisms. Gram-negative bacteria have an outer phospholipid layer with structural components of LPS. Gram-positive should be more susceptible to the action of substances because these have only an outer layer of peptidoglycan as effective barrier (Scherrer, Gerardt, 1971).

The broad spectrum of activity against Gram-negative bacteria found for the fractions can be attributed to the presence of the lipophilic substances, triterpenes and steroids, which may be related to lipophilic components of plant extracts. These components increase permeability and loss of cellular components, and a change variety of enzyme systems, including those involved in the production of cellular

energy and synthesis of structural components, inactivating or destroying genetic material (Bagamboula, Uyttendaele, Debevere, 2004). Flavonoids are a broad class of phenolics that are known to possess antimicrobial activity, essentially by enzyme inhibition of DNA gyrase (Cushnie, Lamb, 2005). These results are extremely important as Gram-negative bacteria such as *S. thyphimurium* and *P. aeruginosa* are responsible for a high incidence of infections. The most common infections in Intensive Care Unit (ICU) are those caused by Gram-negative such as *P. aeruginosa*, especially ventilator-associated pneumonia. At ICU of large hospitals, resistance to *P. aeruginosa* is 20 to 75% for beta-lactamic and aminoglycosides antibiotics and this is accompanied by cross-resistance (Arruda, 1998). *Salmonella* infections cause diarrhea, fever, abdominal pain, and people usually recover without treatment. However, some people like the elderly, children and those with compromised immune systems, the diarrhea may be so severe that the patient must be hospitalized. In these patients, the infection can spread from the intestine into the bloodstream and then to other body sites and can lead to death (Hohmann, 2001). *S. dysenteriae*, a gram-negative bacterium, is a significant cause of gastroenteritis in both developing and industrialized countries (Boumghar-Bourtchai *et al*., 2008). People infected with *Shigella* develop diarrhea, fever and stomach cramps starting a day or two after they are exposed to the bacterium. It is typically associated with mild self-limiting infection (DeLappe *et al*., 2003).

Antileishmanial activity and cytotoxicity

Protozoa parasites of the genus *Leishmania* cause visceral, cutaneous, and mucosal diseases in humans, which are collectively referred to as leishmaniasis. These diseases affect more than 12 million people worldwide and are responsible for high rates of mortality in tropical and subtropical countries. The drugs of choice for the treatment of leishmaniasis are pentavalent antimonials, but toxic side effects, limited efficacy to control parasite proliferation and drug resistance are frequently encountered (Santos *et al*., 2008; Cruz *et al*., 2009). Considering the side effects and the resistance that pathogenic protozoan parasites develop against these drugs, more attention should be given to the extracts and biologically active compounds isolated from plant species (Mendonça-Filho *et al*., 2004).

Table III shows the antileishmanial activity of MFH and fractions against the promastigotes forms of four *Leishmania* species: *L. amazonensis*, *L. braziliensis, L. major* and *L. chagasi*. MFH, MFH1 and MFH6 to MFH9 showed the best antiproliferative activities against *Leishmania* species. MFH and MFH9 showed the highest activity against the four species. In general, *L. major* promastigotes were the most sensitive parasite to the samples tested.

As *L. major* was the most sensitive strain for MFH and MFH6 to MFH9, *L. major*-infected peritoneal macrophage model was assayed in order to investigate if they were also active against the intracellular stage of the parasite, the amastigote forms (Table III and IV). All tested fractions showed a significant effect against the amastigote forms of *L. major*. MFH7 and MFH8 showed the best activity against intracellular amastigotes, with an IC_{50} value of 1.3 and 2.6 µg/mL, respectively. Despite these samples presented some toxicity against mammalian cells, MFH7 and MFH8 were 15.6 and 17.9 times more selective for the intracellular parasite than to the host cells, respectively (Table III). According to Muylder *et al.* (2011), values > 2 was the cut-off chosen to define a compound as more active against the intracellular amastigote stage; while a specificity value < 0.4 indicated a compound more active against promastigotes; compounds with specificity values between 0.4 and 2 were considered active against both stages. So, MFH7 and MFH8 were considered more active against amastigotes, the relevant parasite stage in clinical disease.

Table III

This is the first report of amastigote activity for *Mitracarpus frigidus* hexane extract against *L. major*. This form is related to the Old World cutaneous leishmaniasis (previously known as zoonotic or rural zoonotic cutaneous leishmaniasis) and can be found in several countries including the Eastern Mediterranean Region and Asia (WHO, 2010).

Table IV presents more information about the anti-amastigote activity of MFH and MFH6 to MFH9. When the parasites were treated with those fractions, a significant dose-dependent decrease of intracellular amastigote was observed by values of the survival index.

Phytoconstituents isolated from plants have shown potent antileishmanial activity, including terpenes, steroids and phenolic compounds like flavonoids (Sen, Chatterjee, 2011). Terpenes and steroids increased the production of NO in

Leishmania infected macrophages, along with directly targeting the parasite as evidenced by mitochondrial swelling and alterations in the organization of nuclear and kinetoplast chromatin (do Socorro *et al*., 2003). Furthermore, these compounds showed antileishmanial activity via targeting of DNA topoisomerases I and II, and preventing DNA cleavage, ultimately inducing apoptosis in parasites (Chowdhury *et al*., 2003). Flavonoids have been shown to inhibit the synthesis of parasite DNA via inhibition of topoisomerase II mediated linearization of kDNA minicircles, culminating in arresting of cell cycle progression (Mittra *et al*., 2000). Additionally, these compounds can chelate iron, which translates into a decreased availability of the iron dependent ribonucleotide reductase, a rate limiting enzyme for DNA synthesis (Sen *et al*., 2008). Extracts rich in flavonoids exhibited antileishmanial activity by increasing generation of reactive nitrogen intermediates that was further enhanced by the addition of IFN-γ (Gomes *et al*., 2010).

CONCLUSION

The results were encouraging, as *M. frigidus* showed significant antimicrobial and leishmanicidal activities. It is suggested that *M. frigidus* could be of use as a source of natural antimicrobial and leishmanicidal component for pharmaceutical industry. Active compounds are being isolated and elucidated for chemical characterization.

RESUMO

No presente estudo foram investigados as atividades antimicrobiana e leishmanicida do extrato em hexano das partes aéreas de *Mitracarpus frigidus*. Os ensaios biológicos foram avaliados contra seis cepas de bactérias (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Escherichia coli* e *Enterobacter cloacae*), formas promastigotas de quatro espécies de *Leishmania* (*L. major*, *L. braziliensis*, *L. chagasi* e *L. amazonensis*) e formas amastigotas de *L. major*. Citotoxidade contra células de mamíferos e triagem fitoquímica dos grupos majoritários de fitoconstituintes também foram reportados. As amostras apresentaram significativa atividade antimicrobiana, principalmente para *P. aeruginosa*, *S. dysenteriae* e *S. typhimurium* com CIM $<$ 100 µg/mL. Um pronunciado

potencial leishmanicida foi também verificado nas formas promastigotas e amastigotas, sendo que as amostras apresentaram especificidade para o estágio intracelular. Apesar destas amostras apresentaram certa toxidade contra células de mamíferos, estas foram mais seletivas para o parasita intracelular do que para as células hospedeiras. Esteroides, terpenos e flavonoides foram identificados nas amostras mais ativas.

Unitermos: *Mitracarpus frigidus*, *Leishmania major*, antibacteriano, terpenos, flavonoides.

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REFERENCES

ARAÚJO, C. A. C., LEON, L. L. Biological activities of *Curcuma long* L. *Mem. Inst. Oswaldo Cruz,* v.96, n.5, p.723-728, 2001.

ARRUDA, E. A. G. Infecção hospitalar por *Pseudomonas aeruginosa* multi-resistente: análise epidemiológica HC-FMUSP. *Rev. Soc. Bras. Med. Tro.,* v.31, n.5, p.503-504, 1998.

AUSTIN, D. J., KRISTINSSON, K. G., ANDERSON, R. M. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc. Natl. Acad. Sci. USA*, v.96, p. 1152-1156, 1999.

BAGAMBOULA, C. F., UYTTENDAELE, M., DEBEVERE, J. Antimicrobial and antioxidative activities of the essencial oils and methanol extracts of *Salvia cryptanha* (Montbret et Aucher ex Benth.) and *Salvia multicaulis* (Vahl.). *Food Chem.,* v.84, n.4, p.519-525, 2004.

BRAGA, F. G., BOUZADA, M. L. M., FABRI, R. L., MATOS, M. O., MOREIRA, F. O., SCIO, E., COIMBRA, E. S. Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. *J. Ethnopharmacol.,* v.111, p.396-402, 2007.

BONJAR, G. H. S. New approaches in screening for antibacterials in plants. *Asian J. Plant Sci*., v.3, p.55-60, 2004.

BOUMGHAR-BOURTCHAI, L. B., KURKDJIAN, P. M., BINGEN, E., FILLIOL, I., DHALLUIN, A., IFRANE, S. A, WEILL, F. X., LECLERCQ, R. Macrolide-Resistant *Shigella sonnei. Emerg. Infect. Dis.,* v.14, n.8, p.1297-1299, 2008.

CARMO, A. M. L., SILVA, F. M. C., MACHADO, P. A., FONTES, A. P., PAVAN, F. R., LEITE, C. Q., LEITE, S. R., COIMBRA, E. S., DA SILVA, A. D. Synthesis of 4-aminoquinoline analogues and their platinum(II) complexes as new antileishmanial and antitubercular agents. *Biomed. Pharmacother*., v.65, p.204-209, 2011.

CHOWDHURY, A. R., MANDAL, S., GOSWAMI, A., GHOSH, M., MANDAL, L., CHAKRABORTY, D., GANGULY, A., TRIPATHI, G., MUKHOPADHYAY, S., BANDYOPADHYAY, S., MAJUMDER, H. K. Dihydrobetulinic acid induces apoptosis in *Leishmania donovani* by targeting DNA topoisomerase I and II: implications in antileishmanial therapy. *Mol.Med.,* v.9, p.26-36, 2003.

COUTINHO, M. R., QUADRI, M. B., MOREIRA, R. F. P. M., QUADRI, M. G. N. Partial purification of anthocyanins from *Brassica oleracea* (red cabbage). *Sep. Sci. Technol.,* v.39, n.16, p.3769-3782, 2004.

CRUZ, A. K., DE TOLEDO, J. S., FALADE, M., TERRÃO, M. C., KAMCHONWONGPAISAN, S., KYLE, S., UTHAIPIBULL, C. Current treatment and drug discovery against *Leishmania* spp. and *Plasmodium* spp.: a review. *Curr. Drug Targets,* v.10, p.178-192, 2009.

CUSHNIE, T. P. T., LAMB, A. J. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Ag.*, v.26, n.5, p.343-356, 2005.

DALZIER, J. M. Useful Plants of West tropical Africa. London: The Crown Agents for Overareas Colonica, 1936.

DELAPPE, N., O'HALLORAN, F., FANNING, S., CORBETT-FEENEY, G., CHEASTY, T., CORMICAN, M. Antimicrobial resistance and genetic diversity of *Shigella sonnei* isolates from Western Ireland, an area of low incidence of infection. *J. Clin. Microbiol.,* v.41, n.5, p.1919-1924, 2003.

DO SOCORRO, S., ROSA, M. D. O. S., MENDONÇA-FILHO, R. R., BIZZO, H. R., DE ALMEIDA RODRIGUES, I., SOARES, R. M., SOUTO-PADRÓN, T., ALVIANO, C. S., LOPES, A. H. Antileishmanial activity of a linalool-rich essential oil from *Croton cajucara*. *Antimicrob. Agents Chemother.,* v.47, p.1895-1901, 2003.

FABRI, R. L., NOGUEIRA, M. S., BRAGA, F. G., COIMBRA, E. S., SCIO, E. *Mitracarpus frigidus* aerial parts exhibited potent antimicrobial, antileishmanial, and antioxidant effects. *Bioresource Technol.,* v.100, p.428-433, 2009.

GOMES, D. C., MUZITANO, M. F., COSTA, S. S., ROSSI-BERGMANN, B. Effectiveness of the immunomodulatory extract of *Kalanchoe pinnata* against murine visceral leishmaniasis. *Parasitology,* v.137, p.613-618, 2010.

HERRERA, R. M., PEREZ, M., MARTIN-HERRERA, D. A., LOPEZ-GARCIA, R., RABANAL, R. M. Antimicrobial activity of extracts from plants endemics to the Canary Islands. *Phytother. Res.*, v.10, p.364-366, 1996.

HOHMANN, E. L. Nontyphoidal salmonellosis. *Clin. Infect. Dis.,* v.32,n.2, p.263-269, 2001.

JONES, R. N. Resistance patterns among nosocomial pathogens - Trends over the past few years. *CHEST,* v.119, p.397-404, 2001.

KERHARO, J., ADAM, J. G. La Pharmacopée Sénegalaise Traditionnelle: Plantas Médicinales Et Toxiques. Paris: Editions Vigot Frères, 1974. p.692-693.

MENDONÇA-FILHO, R. R., RODRIGUES, I. A., ALVIANO, D. S., SANTOS, A. L. S., SOARES, R. M. A., ALVIANO, C. S., LOPES, A. H., ROSA, M. S. Leishmanicidal activity of polyphenolic-rich extract from husk fiber of *Cocos nucifera* Linn (Palmae). *Res. Microbiol.,* v.155, p.136-143, 2004.

MITTRA, B., SAHA, A., CHOWDHURY, A. R., PAL, C., MANDAL, S., MUKHOPADHYAY, S., BANDYOPADHYAY, S., MAJUMDER, H. K. Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. *Mol. Med.,* v.6, p.527-541, 2000.

MORAIS-TEIXEIRA, E., CARVALHO, A. S., COSTA, J. C. S., DUARTE, S. L., MENDONÇA, J. S., BOECHAT, N., RABELLO, A. *In vitro* and *in vivo* activity of meglumine antimoniate produced at Farmanguinhos-Fiocruz, Brazil, against *Leishmania* (Leishmania) *amazonensis*, L (L.) *chagasi* and L (Viannia) *braziliensis*. *Mem. Inst. Oswaldo Cruz,* v.103, p.358-362, 2008.

MUYLDER, G., ANG, K. K., CHEN, S., ARKIN, M. R., ENGEL, J. C., MCKERROW, J. H. A screen against Leishmania intracellular amastigotes: comparison to a promastigote screen and identification of a host cell-specific hit. *PLoS Negl. Trop. Dis.,* v.5, n.7, p.e1253, 2011.

NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS (NCCLS). Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2 - P. National Committee for Clinical Laboratory Standards. Wayne, PA., 2002.

PRATES MV, BLOCH-JÚNIOR C. Peptídeos antimicrobianos. *Biotecnol.*, v.23, p.30-36, 2001.

SANTOS, D. O., COUTINHO, C. E., MADEIRA, M. F., BOTTINO, C. G., VIEIRA, R. T., NASCIMENTO, S. B., BERNARDINO, A., BOURGUIGNON, S. C., CORTE-REAL, S., PINHO, R. T., RODRIGUES, C. R., CASTRO, H. C. Leishmaniasis treatment - a challenge that remains: a review. *Parasitol. Res*., v.103, p.1-10, 2008.

SCHERRER, R., GERHARDT, P. Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. *J. Bacteriol.*, v.107, p.718-735, 1971.

SEN, G., MUKHOPADHYAY, S., RAY, M., BISWAS, T. Quercetin interferes with iron metabolism in *Leishmania donovani* and targets ribonucleotide reductase to exert leishmanicidal activity. *J. Antimicrob. Chemother.*, v.61, p.1066- 1075, 2008.

SEN R, CHATTERJEE M. Plant derived therapeutics for the treatment of Leishmaniasis – review. *Phytomedicine*, v.18, p.1056-1069, 2011.

WORLD HEALTH ORGANIZATION (WHO). *Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases*. Technical report series no. 949. Geneva, 2010.

TABLE I – Yield (% w/w) and phytochemical screening of hexane extract (MFH) and fractions of *Mitracarpus frigidus*

Samples	Yield (% w/w)[a]	Phytocompounds[b]							
		St	Tr	Cm	An	Ph	Al	Fl	Ta
MFH	-	+	+	+	-	+	+	+	-
MFH1	4.7	-	+	-	-	-	-	-	-
MFH2	1.1	-	+	-	-	-	-	-	-
MFH3	2.7	-	+	-	-	-	-	-	-
MFH4	1.4	-	+	-	-	-	-	-	-
MFH5	2.2	-	+	-	-	-	-	-	-
MFH6	63.3	+	+	+	-	+	-	+	-
MFH7	10.9	+	+	-	-	+	-	+	-
MFH8	1.1	+	+	-	-	+	-	+	-
MFH9	3.4	+	+	-	-	+	+	+	-

[a] Yield in relation to the hexane extract (MFH).

[b] St – Sterols; Tr – Triterpenoids; Cm – Coumarins; An – Anthraquinones; Ph – Phenols; Al – Alkaloids; Fl – Flavonoids and Ta – Taninns.

TABLE II – Antibacterial activity of the hexane extract (MFH) and fractions of the *Mitracarpus frigidus*

Samples	Minimum inhibitory concentration – MIC (µg/mL)[a]					
	Sa	*Pa*	*Sd*	*St*	*Ec*	*Ecl*
MFH	250	>1000	250	500	500	250
MFH1	1000	500	500	500	1000	1000
MFH2	1000	1000	1000	1000	1000	1000
MFH3	>1000	>1000	1000	1000	>1000	1000
MFH4	>1000	>1000	>1000	>1000	>1000	>1000
MFH5	>1000	>1000	>1000	>1000	>1000	500
MFH6	1000	1000	1000	1000	1000	1000
MFH7	1000	500	250	125	125	500
MFH8	250	62.5	31.3	31.3	500	500
MFH9	1000	500	500	500	1000	500
Chl[b]	25	>100	50	3.12	1.56	3.12

[a]*Sa - Staphylococcus aureus*; *Pa - Pseudomonas aeruginosa*; *Sd - Shigella dysenteriae*; *St - Salmonella typhimurium*; *Ec - Escherichia coli*; *Ecl - Enterobacter cloacae*

[b]Chl - Chloramphenicol

TABLE III – Effects of hexane extract (MFH) and fractions of the *Mitracarpus frigidus* against *Leishmania* promastigote and amastigote forms and murine macrophages

Samples	Antileishmanial activity - IC_{50} (µg/mL)[a]					Peritoneal macrophages CC_{50} (µg/mL)[b]	SE[d]	SS[e]
	Promastigote				Amastigote			
	La	Lb	Lm	Lc	Lm			
MFH	58.5 ± 0.10	30.2 ± 1.24	23.9 ± 1.1	27.5 ± 0.44	6.6 ± 1.1	18.2 (13.9 – 27.8)	2.8	3.6
MFH1	44.1 ± 2.00	43.7 ± 1.88	> 100	34.5 – 19.5	-	90.3 (55.5 - 111.0)	-	-
MFH2	> 100	> 100	> 100	> 100	-	> 100	-	-
MFH3	> 100	> 100	> 100	> 100	-	> 100	-	-
MFH4	> 100	> 100	> 100	65.1 ± 0.08	-	> 100	-	-
MFH5	> 100	> 100	> 100	> 100	-	> 100	-	-
MFH6	> 100	47.4 ± 2.4	18.6 ± 3.03	32.7 ± 1.17	5.7 ± 2.0	29.4 (27.8 – 55.5)	5.2	3.3
MFH7	> 100	> 100	17.8 ± 0.3	36.7 ± 0.8	1.3 ± 0.2	20.3 (13.9 – 27.8)	15.6	13.7
MFH8	> 100	48.7 ± 0.85	28.6 ± 2.25	55.3 ± 0.3	2.6 ± 0.5	46.5 (27.8 – 55.5)	17.9	11.0
MFH9	60.6 ± 0.88	24.7 ± 0.66	16.6 ± 1.57	38.4 ± 0.43	3.7 ± 0.9	17.1 (13.9 – 27.8)	4.6	4.5
Amp B[c]	0.2 ± 0.009	0.1 ±0.02	0.3 ± 0.07	0.1 ± 0.004	0.3 ± 0.02	> 100	-	-

[a]La – *Leishmania amazonensis*; Lb – *L. braziliensis*; Lm – *L. major*; Lc – *L. chagasi*. Mean of triplicate assays ± SE

[b] 95% confidence limits in parenthesis

[c] Amphotericin B

[d]SE (Selectivity) - CC_{50} of macrophages / IC_{50} of amastigotes of *L. major*

[e]SP (Specificity) - IC_{50} promastigotes of *L. major* / IC_{50} amastigotes of *L. major*

TABLE IV – Effect of hexane extract (MFH) and active fractions on *Leishmania major* interiorized in peritoneal macrophage cells after 72 hours of treatment.

Samples		Survival Index for Amastigotes (µg/mL) (% Inhibition)			
		1.2	2.5	5.0	10.0
Control	257.3 ± 18.5	-	-	-	-
MFH	-	228.6 ± 1.7 (11)	232.8 ± 2.6 (15)	141.3 ± 1.0 (49)	112.8 ± 1.3 (59)
MFH6	-	198.3 ± 1.5 (23)	200.7 ± 0.3 (22)	171.7 ± 6.1 (33)	153.3 ± 1.8 (40)
MFH7	-	168.1 ± 1.5 (35)	143.2 ± 11.7 (44)	14.6 ± 0 (94)	9.0 ± 0.1 (97)
MFH8	-	170.7 ± 1.4 (33)	155.9 ± 0.8 (39)	24.2 ± 0.1 (91)	18.4 ± 1.6 (93)
MFH9	-	205. 3 ± 2.3 (26)	188.4 ± 0.7 (32)	151.7 ± 2.8 (41)	139.6 ± 6.9 (46)

[a]Survival Index – number of amastigotes per cells x % infected cells

[b]Data are presented as mean ± standard deviation

5.5 O artigo a seguir descreve o potencial citotóxico, leishmanicida e anti-inflamatório de dois triterpenos pentacíclicos isolados do extrato metanólico das partes aéreas de *Mitracarpus frigidus.*

Artigo 5

PENTACYCLIC TRITERPENOIDS FROM *Mitracarpus frigidus*: *IN VITRO* CITOTOXICITY AND LEISHMANICIDAL AND *IN VIVO* ANTI-INFLAMMATORY AND ANTIOXIDATIVE ACTIVITIES

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Pentacyclic triterpenoids from *Mitracarpus frigidus*: *In vitro* cytotoxic and leishmanicidal and *in vivo* anti-inflammatory and antioxidative activities

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Abstract

Objectives To isolate pentacyclic triterpenoids from *Mitracarpus frigidus* and evaluate their *in vitro* cytotoxic and leishmanicidal and *in vivo* anti-inflammatory and antioxidative activities.

Methods The identities of the compounds were confirmed by ^{1}H, $^{1}3C$, ^{1}H COSY, IR and UV-Vis spectroscopy and mass spectrometry. These compounds were evaluated *in vitro* against four tumor cell lines and four *Leishmania* species. Also the *in vivo* anti-inflammatory and antioxidative effects were evaluated using carrageenan-induced paw edema and carrageenan-induced peritonitis.

Key findings The fractionation of the hexane extract afforded two pentacyclic triterpenoids ursolic acid and methyl ursolate. Ursolic acid was active in all tumor cell lines (ED_{50} varying from 4.2 to 35.7 $\mu g.mL^{-1}$), and methyl ursolate was active only for HL60 cells (ED_{50} of 22.7 $\mu g.mL^{-1}$). An antileishmanial potential was verified for amastigote form of *L. major,* with IC_{50} values of 1.3 and 2.1 $\mu g.mL^{-1}$ for ursolic acid and methyl ursolate, respectively. They also presented anti-inflammatory effects as their oral administration significantly inhibited edema formation induced by carrageenan about 80% and also inhibited peritoneal leukocyte migration in mice about 85% at 1 $mg.kg^{-1}$.

Conclusions The results obtained reinforced that pentacyclic triterpenoids are an important class of natural products for investigation in the search of new bioactive compounds.

Keywords *Mitracarpus frigidus*; pentacyclic triterpenoids; ursolic acid; methyl ursolate; anti-inflammatory activity; antileishmanial activity

Introduction

The genus *Mitracarpus* Zucc., known as the girdlepods, consists of no fewer than 50 species.[1] However, there is very little information in the literature regarding their constituents and pharmacological activities. *Mitracarpus scaber* Zucc. is the most widely studied species and is employed in traditional medicine in West Africa for headaches, toothache, amenorrhoea, dyspepsia, hepatic and veneral diseases, leprosy, and for the treatment of skin diseases. [2] Previous studies had reported the isolation of harounoside[3], benz[g]isoquinoline-5,10-dione[4], gallic acid, 3,4,5-trimethoxybenzoic acid, 4-methoxyacetophenone, 3,4,5-trimethoxyacetophenone, kaempferol-3-*O*-rutinoside, rutin and psoralen[2] as well as ursolic and oleanolic acids from this species.[5] Another species of *Mitracarpus*, *M. villosus* (Sw.) DC was studied and found to contain stigmasterol, 24-methylcholesta-5-en-3β-ol and ursolic acid.[6]

However, the species *Mitracarpus frigidus* (Willd. ex Roem. & Schult.) K. Shum, an annual shrub commonly found in Brazil, has never been subjected to phytochemical investigation with the exception of had recently work from this laboratory published paper.[7] Then discovery of strong biological activity of the hexane extract, had to an attempt to isolate the compound(s) responsible for those activities.

Materials and Methods

Plant material

Mitracarpus frigidus aerial parts were collected in Juiz de Fora, State of Minas Gerais, Brazil, in May, 2009. A voucher specimen (CESJ 46076) was deposited at the Leopoldo Krieger Herbarium at the Universidade Federal de Juiz de Fora.

Extraction and isolation

Preparation of the crude hexane extract was previously described in detail.[8] Purification of this extract was performed in the following manner: the hexane extract (17 g) was chromatographed on a 75 x 5 cm column of silica gel (70-230 mesh) with a gradient of increasing polarity (hexane, hexane-CH_2Cl_2, CH_2Cl_2, CH_2Cl_2-MeOH, MeOH) to obtain a total of nine fractions. The fractions thus obtained were analyzed by thin layer chromatography on silica gel 60 F_{254} (Merck) using CH_2Cl_2: MeOH 90:10 (v/v) or CH_2Cl_2: MeOH 80:20 (v/v) as the mobile phase for fractions F_1 to F_5, and fractions F_6 to F_9, respectively. Identification was made with a UV lamp (254 and 365 nm) and with vanillin:sulfuric acid spray reagent followed by heating. The fractions were pooled and concentrated on a rotatory evaporator under reduced pressure. Fraction 7 (1.8 g) was chromatographed on a 54 x 2 cm column of silica

gel (70-230 mesh) with a gradient of increasing polarity (hexane, hexane-EtOAc, EtOAc, MeOH) to obtain the compounds **1** (210 mg - 1.3%) and **2** (20 mg - 0.12%), respectively.

Structural elucidation

^{1}H-NMR and COSY (300 MHz) and ^{13}C-NMR (75 MHz) spectra were recorded on a Bruker DRX spectrometer using the residual solvent peak (MeOH) as reference. The IR spectrum was recorded as a KBr pellet on a Bomem B102 FT spectrophotometer. The UV spectrum was acquired in MeOH on a Shimadzu UV160 spectrophotometer. The EI mass spectrum was obtained on a Hewlett-Packard 5973 MSD spectrometer by direct insertion in the positive ion mode (70 eV).

Cytotoxicity assay

Human tumor cell lines

Four human tumor cell lines were used, Jurkat (human immortalized line of T lymphocyte), HL60 (human promyelocytic leukemia), MCF-7 (breast cancer) and HCT (colorectal carcinoma). HL60 and Jurkat cells were kindly furnished by Dr. Gustavo Amarante-Mendes (São Paulo University, Brazil). MCF-7 was generously provided by Alfredo Goes (Federal University of Minas Gerais, Brazil). HCT was purchased from ATCC. All lineages were maintained in the logarithmic phase of growth in RPMI 1640 supplemented with 100 IU.mL^{-1} penicillin and 100 μg.mL^{-1} streptomycin enriched with 2 mM of L-glutamine and 10% of fetal bovine serum. All cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 and 95% air. The media were changed twice weekly and the cells were regularly examined. All cell lines were used between 16 and 30 passages.

Evaluation of cytotoxic effect against human tumor cell lines

Tumor cell lines (Jurkat, HL60, MCF-7 and HCT) were inoculated at 4 x 10^4 cells (MCF-7), 5 x 10^4 cells (HL60) and 1 x 10^5 cells (Jurkat and HCT) per well. The plates were pre-incubated for 24 h at 37 °C to allow adaptation of cells prior to the addition of the test compounds. All substances were dissolved in dimethyl sulfoxide (DMSO) prior to dilution. The half maximal effective dose (ED_{50}) was determined over a range of concentrations (nonserial six dilutions: from 50 to 1.5 μg.mL^{-1}). All cell cultures were incubated in a 5% CO_2/95% air-humidified atmosphere at 37 °C for 48 h. Negative control included treatment with 0.05% DMSO. Cell viability was estimated by measuring the rate of mitochondrial reduction of tetrazolium–dye (MTT). All compounds were tested in triplicate, in three independent experiments.[9] The cytotoxicity of etoposide and doxorubicin were evaluated under the same experimental conditions as positive controls.

DNA fragmentation assay

Cell cycle status and quantification of DNA fragmentation (hypodiploid DNA-content) were performed by propidium iodide (PI) staining according to Nicolleti *et al.*[10] Cells were treated with the samples at 20 µg/mL in a 5% CO_2/95% air-humidified atmosphere at 37 °C for 24 h. After drug incubation, the cells were centrifuged and ressuspended in hypotonic fluorochrome solution – HFS (50 µg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100). The samples in HFS were incubated at 4 ºC during 4 h and immediately analyzed by flow cytometry. The PI fluorescence of 20,000 individual nuclei was measured using a FACScalibur flow cytometer. Data were analyzed by FlowJo software (TreeStar Inc).

Antileishmanial assay

Parasites

Four *Leishmania* species were used for *in vitro* screening: *L. amazonensis* (IFLA/Br/67/PH8), *L. major* (MRHO/SU/59/P), *L. braziliensis* (MHOM/Br/75/M2903) and *L. chagasi* (MHOM/Br/74/PP75). Promastigotes of *L. amazonensis* were cultured in Warren's medium (brain heart infusion – BHI – enriched by hemin and folic acid), promastigotes of *L. major* and *L. braziliensis* were maintained in BHI medium, and promastigotes of *L. chagasi* were maintained in Medium 199, both supplemented with 10% fetal bovine serum at 24 °C.

Promastigote forms

The antileishmanial activity was performed according to a previously described method.[11] Briefly, promastigotes from a logarithmic phase culture were suspended to yield 2 x 10^6 cells.mL^{-1} (*L. amazonensis*) and 3 x 10^6 cells.mL^{-1} (*L. chagasi, L. braziliensis* and *L. major*) after Neubauer chamber counting. The test was performed in 96-well microtiter plates maintained at 24 °C. The analyses were performed in triplicate. The parasites were exposed to increasing concentrations of the samples (nonserial six dilutions: from 50 to 0.2 μg.mL^{-1}) for 72 h at 24 °C. Controls with 0.5% DMSO were also performed. The results were expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) after a three day incubation period. Amphotericin B was used as the positive control.

Amastigote forms

Concerning the amastigotes *in vitro* model, inflammatory macrophages were obtained from BALB/c mice previously inoculated intraperitoneally with 2 mL of 3% thioglycollate medium.[12] Briefly, peritoneal macrophages were plated at 2 × 10^6 cells.mL^{-1} on coverslips (13 mm diameter) previously arranged in a 24-well plate in RPMI 1640 medium

supplemented with 10% inactivated FBS and allowed to adhere for 24 h at 37 °C in 5% CO_2. Adherent macrophages were infected with *L. major* (MRHO/SU/59/P) promastigotes in the stationary growth phase using a ratio of 1:5 at 33 °C for 3 h. Non-internalized promastigotes were eliminated and solutions of the compounds were added (nonserial five dilutions: from 25 to 0.5 $\mu g.mL^{-1}$) and maintained at 33 °C in 5% CO_2 for 72 h. Slides were fixed and stained with Giemsa for parasite counting (optical microscopy, 1000x magnification). The parasite burden was evaluated by counting the intracellular parasite, uninfected and infected macrophages (at minimum 100 cells infected) in treated and untreated cultures, and the survival index was obtained by multiplying the percentage of infected macrophages by the mean number of amastigote forms per infected cell.[13] A control with 0.5% DMSO was also performed. The analyses were performed in triplicate.

Cytotoxicity against mammalian cells

Cytotoxicity was evaluated after 48 h incubation of murine macrophage-J774 with concentration ranging from 125 to 7.5 $\mu g.mL^{-1}$of the samples. The viability of the macrophages was determined with the MTT assay and was confirmed by comparing the morphology of the control group via light microscopy.[14] Dose response curves were plotted (values expressed as percentage of control optical density) and CC_{50} values (50% cytotoxic concentration) were obtained. The analyses were performed in triplicate.

Anti-inflammatory assay

Animals

Female Swiss albino mice weighing between 20 - 25 g, or male Wistar albino rats of 200 - 250 g, were used for the present study. Animals were housed in groups of five under standard laboratory conditions of temperature 25 ± 2 °C and 12/12 h light/dark cycle. They were provided with standard pellets and tap water *ad libitum*. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals. The study was approved by the Brazilian College of Animal Experimentation (Protocol n° 022/2012).

Carrageenan-induced rat paw edema

Rats were divided into seven groups of six animals each. Group A: negative control (0.6% sodium lauryl sulfate); Group B: dexamethasone – 1 $mg.kg^{-1}$; Group C: indomethacin – 10 $mg.kg^{-1}$; Group D: ursolic acid – 1 $mg.kg^{-1}$; Group E: ursolic acid – 0.5 $mg.kg^{-1}$; Group F: methyl ursolate – 1 $mg.kg^{-1}$and Group G: methyl ursolate – 0.5 $mg.kg^{-1}$. One hour after the oral treatment, acute paw edema was induced by injecting 0.1 mL of 1% carrageenan in

0.9% saline. Paw volume was measured with the help of plethysmometer by the mercury displacement method at 0 and 3 hours.[15] The percentage inhibition of paw edema in treated groups was then calculated using the formula: Percentage inhibition = (1 – Vt/Vc) X 100, where Vt = is the edema volume in the drug treated; Vc = is the edema volume in the control group.

Subsequently, liver tissues were taken at the third hour. The whole liver tissue was rinsed in ice-cold normal saline and immediately placed in buffer pH 7.4 (140 nM KCl + 20 nM phosphate) of the same volume and finally homogenized at 4 °C. Then, the homogenate was centrifuged at 11,270 g for 5 min. The supernatant was obtained for the malondialdehyde (MDA) and antioxidant enzyme catalase activity assays. All tests were performed in triplicate.

MDA assay

Malondialdehyde (MDA) was evaluated by the thiobarbituric acid reacting substance (TBARS) method.[16] In brief, MDA has reacted with thiobarbituric acid at a high temperature (100 °C) and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm. MDA levels were expressed as nmol.mg^{-1} protein.

Catalase activity

Decomposition of H_2O_2 in the presence of catalase was measured at 240 nm according to a standard procedure.[17] Catalase activity was defined as the amount of enzyme required to decompose 1 nmol of H_2O_2 per minute, at 37 °C. Results were expressed as µmol.µg protein^{-1}.

Carrageenan-induced mice peritonitis

Inflammation was induced by the modified method of Griswold *et al*.[18] Mice were divided into seven groups of six animals each. Group A: negative control (0.6% sodium lauryl sulfate); Group B: dexamethasone – 1 mg.kg^{-1}; Group C: indomethacin – 10 mg.kg^{-1}; Group D: ursolic acid – 1 mg.kg^{-1}; Group E: ursolic acid – 0.5 mg.kg^{-1}; Group F: methyl ursolate – 1 mg.kg^{-1}and Group G: methyl ursolate – 0.5 mg.kg^{-1}. The treatments were administered orally, one hour prior to the induction of peritonitis. After one hour, carrageenan (0.25 mL, 0.75% w/v in saline) was injected intraperitonially. Four hours later, the animals were euthanized by cervical dislocation, and 2 mL of heparinized PBS was injected into the peritoneal cavity. Following a gentle massage, peritoneal exudates were removed. The total leukocyte count was determined in a Neubauer chamber and the differential cell count was determined. The percentage of leukocyte inhibition was calculated using the following

formula: % of leukocyte inhibition = (1- T/C) X 100, where T represents the treated groups' leukocyte count and C represents the treated control group leukocyte count. Inhibition of neutrophil migration was calculated by the following equation: Inhibition of neutrophil migration = (1- NT/NC) X 100, where NT = neutrophil counts of treated groups and NC = neutrophil counts of the control group.

Statistical analysis

For the *in vivo* assays, values were presented as means ± standard error of mean (SEM). For the *in vitro* cytotoxicity and antileishmanial assays, the ED_{50} and IC_{50} values were obtained graphically from dose–effect curves using Prism 5.0 (GraphPad Software Inc). Statistical differences between the treatments and the control were evaluated by the ANOVA test followed by the Bonferroni test. A difference in the mean values of $P < 0.05$ was considered to be statistically significant.

Ursolic acid (**1**) *3β-Hydroxy-urs-12-en-28-oic acid,*. MP 264 °C; HR-EIMS *m/z* 456.6 (456.6 calcd. for $C_{30}H_{48}O_3$); ^{1}H-NMR (CD_3OD), 400 MHz): δ 5.22 0(1H, t, *J* = 3.5 Hz, H-12), 3.15 (1H, dd, *J* = 11.0, 4.8 Hz, H-3α), 2.20 (1H, d, *J* = 11.4 Hz, H-18), 0.96 (6H, s, H-23 and H-25), 1.10, 0.85, and 0.77 (each 3H, s, H-27, H-26 and H-24), 0.97(3H, d, *J* = 6.4 Hz, H-30), 0.88 (3H, d, *J* = 6.5 Hz, H-29); ^{13}CNMR (CD_3OD, 100 MHz): δ 29,3 (C-1), 27.9 (C-2), 79.8 (C-3), 39.9 (C-4), 56.8 (C-5), 19.5 (C-6), 34.4 (C-7), 40.8 (C-8), 48.6 (C-9), 37.5 (C-10), 24.4 (C-11), 126.9 (C-12), 139.7 (C-13), 43.3 (C-14), 29.3 (C-15), 25.4 (16), 48.1(C-17), 53.8 (C-18), 40.5 (C-19), 40.5 (C-20), 34.5 (C-21), 37.7 (C-22), 28.8 (C-23), 16.4 (C-24), 16.0 (C-25), 17.9 (C-26), 24.1 (C-27), 181.7 (C-28), 17.7 (C-29) and 21.6 (C-30).

Methyl ursolate (**2**) *3β-hydroxy-urs-12-en-28-oic acid, methyl ester.* MP 252 °C; HR-EIMS *m/z* 471.7 (471.7 calcd. for $C_{31}H_{50}O_3$); ^{1}H-NMR (CD_3OD), 400 MHz): δ 5.22 (1H, t, *J* = 3.4Hz, H-12), 3.36 (s, 3H, OCH_3 of ester), 3.15 (1H, dd, *J* = 11.0, 4.8 Hz, H-3α), 2.20 (1H, d, *J* = 11.2 Hz, H-18), 0.96 (6H, s, H-23 and H-25), 1.10, 0.85, and 0.77 (each 3H, s, H-27, H-26 and H-24), 0.97(3H, d, *J* = 6.4 Hz, H-30), 0.88 (3H, d, *J* = 6.5 Hz, H-29); ^{13}CNMR (CD_3OD, 100 MHz): δ 29,3 (C-1), 28,0 (C-2), 79.8 (C-3), 39.9 (C-4), 56.8 (C-5), 19.5 (C-6), 34.4 (C-7), 40.8 (C-8), 48.6 (C-9), 37.5 (C-10), 24.4 (C-11), 126,9 (C-12), 139.7 (C-13), 43.3 (C-14), 29.3 (C-15), 25.4 (16), 48.1(C-17), 54.4 (C-18), 40.5 (C-19), 40.5(C-20), 34.5 (C-21), 37.7 (C-22), 28.8 (C-23), 16.3 (C-24), 16.0 (C-25), 17.9 (C-26), 24.1 (C-27), 181.7 (C-28), 17.7 (C-29) , 21.6 (C-30) and 49,0 (OCH_3 of ester).

Results and Discussion

The fractionation of the hexane extract of *M. frigidus* aerial parts resulted in the isolation and characterization of ursolic acid (**1**) and methyl ursolate (**2**) (Figure 1), two pentacyclic triterpenoids isolated for the first time from this species.

The structures were confirmed by spectral comparison data with literature data.[7,19-20]

gure 1

Even though some biological activities had previously been reported, [20,21-27] this study proposed to investigate novel therapeutic potential for those triterpenoids.

In vitro antitumor activity

In the study, it was demonstrated that **1** was active for all the tumor cell lines tested with ED_{50} values varying from 4.2 to 35.7 $\mu g.mL^{-1}$, and **2** was active only for HL60 cells with an ED_{50} value of 22.7 $\mu g.mL^{-1}$ (Table 1). Cytotoxic activity had also been reported elsewhere, [28-31] however, to the best of our knowledge, for **2** it is the first time this activity was reported for Jurkat, MCF-7 and HCT cell lines.

It was also investigated if the cytotoxic effects found for those triterpenoids were

Table 1

associated with their potential to induce cell death by apoptosis. The method described by Nicoletti *et al*.[10] is based on the principle that the apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, by the loss of nuclear DNA content. Apoptotic nuclei appear as a broad hypodiploid DNA peak which was easily distinguished from the narrow cluster of cells with normal (diploid) DNA content. This simple and reproducible method should prove useful for assessing the apoptosis of specific cell populations in heterogeneous tissues.

The results clearly demonstrated that **1** and **2** induced an increase of subdiploid DNA content (DNA fragmentation) in all cells when compared to the control (0.05% DMSO) which suggested that these compounds activated the apoptosis pathway. This cytotoxic effect was more significant for Jurkat, MCF-7 and HCT cell lines, when compared to the control drugs (Figure 2).

gure 2

Pro-apoptotic effects were previously described for **1**.[22,31] In BGC-803 cells, DNA fragmentation induced by **1** was related to the activation of caspase-3, -8, and -9, and the expression of caspase-3 and -8 was elevated in tumor cells from xenograft treated with ursolic acid.[23]

Many chemotherapeutic strategies interfere with important steps in cell cycle progression, such as DNA replication and repair. They may act as DNA polymerase inhibitors, topoisomerase inhibitors, or DNA alkylating agents preventing cancer progression and inducing apoptosis as a secondary effect.[32]

Antileishmanial activity

Protozoa parasites of the *Leishmania* genus cause visceral, cutaneous, and mucosal diseases in humans, which are collectively referred as leishmaniasis. These diseases affect more than 12 million people worldwide and are responsible for high rates of mortality in tropical and subtropical countries.[13] The drugs of choice for the treatment of leishmaniasis are pentavalent antimonials, but toxic side effects, have limited efficacy to control parasite proliferation and drug resistance is frequently encountered.[33] Considering the side effects and the resistance which pathogenic protozoan parasites develop against these drugs, more attention should be given to the extracts and biologically active compounds isolated from plant species.[34,35]

Table 2 shows the antileishmanial activity of **1** and **2** against the promastigotes forms of four *Leishmania* species: *L. amazonensis*, *L. braziliensis, L. major* and *L. chagasi*. The highest activity was found against *L. major* with IC_{50} values of 4.3 and 2.9 $\mu g.mL^{-1}$, for **1** and **2**, respectively. However, the results obtained for those triterpenoids contrasted with the data previously reported by Peixoto *et al*.,[20] who evaluated the antileishmanial activity of those compounds against the promastigote forms of *L. amazonensis*.

Mallavdhani *et al*.[36] related that lipophilicity is an important parameter in the development of leishmanial agents. **2** seems to increase the leishmanicidal activity against promastigote forms due to its higher lipophilicity compared to **1**.

Table 2

As *L. major* was the most sensitive strain for both triterpenoids, the *L. major*-infected peritoneal macrophage model was used in order to investigate if they were also active against the intracellular stage of the parasite, the amastigote forms (Table 2 and 3). Compounds **1** and **2** showed a significant effect against the intracellular amastigote forms of *L. major* with IC_{50} values of 1.3 and 2.1 $\mu g.mL^{-1}$, respectively. Despite the triterpenoids presenting some toxicity against mammalian cells, they were 7.5 and 5.6 times more selective for the intracellular parasite than for the host cells, respectively, demonstrating specificity for amastigote forms (Table 2). According to Muylder *et al*.,[37] values > 2 was the cut-off chosen to define a compound as more active against the intracellular amastigote stage; while a specificity value < 0.4 indicated that a compound was more active against promastigotes. Compounds with specificity values between 0.4 and 2 were considered active against both stages. So, both compounds were considered more active against amastigote than promastigote forms, the relevant parasite stage in the clinical disease.

Table 3 presents more information about the anti-amastigote activity of the triterpenoids. When the parasites were treated with **1** and **2**, a significant dose-dependent decrease of intracellular amastigotes was observed. The results corresponded to an

Table 3

inhibition of survival index of 99.5, 98.3, 98.3 and 16.5% for **1**, and 97.6, 98.1, 70.1 and 6.0% for **2**, at the doses of 20.0, 10.0, 5.0 and 2.5 μg.mL^{-1}, respectively.

This is the first report of the amastigote activity for those compounds against *L. major* which is related to Old World cutaneous leishmaniasis (previously known as zoonotic or rural zoonotic cutaneous leishmaniasis) and which can be found in several countries including the Eastern Mediterranean Region and Asia.[38]

Anti-inflammatory activity

The acute anti-inflammatory effects of **1** and **2** on carrageenan-induced edema in the hind paws of rats are shown in Table 4. Carrageenan-induced paw edema remained even 3 h after infection into the subplantar region of rat paw. The triterpenoids showed a significant reduction in the paw edema volume in relation to the negative control and the maximum inhibition percentage was observed 30 min after the edema formation (Table 4). Although both triterpenoids showed anti-inflammatory activity greater than dexamethasone and indomethacin, the reference drugs, **1** at the doses of 1 and 0.5 mg.kg^{-1}, exhibited the greatest anti-inflammatory effect, with edema inhibition of 80-90% all long the experiment. Carrageenan-induced paw edema as an *in vivo* model of inflammation has been commonly used to evaluate the anti-edematous effect of natural products.[39] Edema formation in the carrageenan-induced paw edema model is a biphasic response. In the early phase, 0-2 h after carrageenan injection, there is a release of histamine, serotonin, and bradykinin on vascular permeability. The inflammatory edema reaches its maximum level at the third hour[40] due to the potentiating effect of bradykinin on mediator release and prostaglandins, producing edema after mobilization of the leukocytes.[41,42] In this study, the triterpenoids and positive controls showed anti-inflammatory effects in carrageenan-induced rat paw edema in both phases, suggesting that the significantly higher anti-inflammatory activity may be due to the inhibition of mediators of inflammation, such as histamine and serotonin released during the first phase, and prostaglandins and bradykinin released during the second phase of inflammation.

able 4

Some studies indicated that inflammatory effect induced by carrageenan is consorted with free radicals.[43] The carrageenan-induced inflammatory response has been associated to neutrophil infiltration and the production of neutrophil-derived free radicals, for instance superoxide, hydroxyl radicals and hydrogen peroxide, as well as due to the release of other neutrophils-derived mediators.[44] A previous study had established that malondialdehyde (MDA) production is due to free radical attack in the plasma membrane.[45] Hence, carrageenan-induced inflammation results in an accumulation of MDA[46], which is used to evaluate the free radical generation in liver tissues after inflammatory states.[44]

Therefore, in order to explore the effects of antioxidant defenses on the acute inflammation process in liver tissues, the MDA and the antioxidant enzyme catalase levels were evaluated. It was observed that MDA level decreased significantly in the animals treated with **1** and **2** (Figure 3a), indicating a reduction of oxidative stress as a result of anti-lipoperoxidative activity.

Catalase is a highly reactive enzyme, reacting with hydrogen peroxide to form water and molecular oxygen, and can form methanol, ethanol, formic acid or phenols by donating hydrogen.[47] In the present study, it was observed that **1** and **2** decreased catalase activity (Figura 3b). During normal body conditions, a balance exists between free radicals and the endogenous antioxidants, but in a traumatic state, like inflammation, the balance diminishes and reactive oxygen metabolites increase dramatically. The increased generation of oxygen free radicals in the extracellular space is verified in the inflammatory state in which the relatively low concentrations of catalase increase the susceptibility of extracellular components to oxygen radical injury and may stimulate chemotaxis for other inflammatory cells.[48] In contrast, these findings showed that catalase activity was decreased by carrageenan injection, which was probably due to the inhibition of inflammation indicated by the triterpenoids.

Mouse carrageenan peritonitis was also used to evaluate anti-inflammatory activity. Compounds **1**and **2** also inhibited peritoneal leukocyte migration (Table 5).

igure 3

Leukocyte aggregation at the site of inflammation is a fundamental event in the inflammatory process. Intraperitonial injection of carrageenan leads to inflammation of the peritoneum as a result of macrophages in the carrageenan insulated tissue.[39] It was followed by an influx of neutrophils and mononuclear leukocytes, with increased levels of plasma/peritoneal fluid chemoatractants and with the sequential appearance of exudate proinflammatory cytokines.[49] In this investigation, **1** and **2** inhibited the carrageenan-induced leukocyte migration in the peritonitis model in mice, and significantly reduced the migration of neutrophils, indicating their anti-inflammatory action by inhibiting chemotaxis (Table 5). Compound **1** is already known as a potent anti-inflammatory agent in rodents[50-52], however, this is the first report of the *in vivo* anti-inflammatory activity of **2**.

Conclusions

able 5

The results obtained reinforced that pentacyclic triterpenoids are an important class of natural products for investigation in the search of new bioactive compounds. In this regard, ursolic acid and methyl ursolate displayed a very interesting cytotoxic, leishmanicidal, anti-inflammatory and antioxidative activities. This is also the first report of those triterpenoids in

Mitracarpus frigidus. For the experiment are in progress to explore the significance of the biological acitivities.

Declarations

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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References

1. Arruda RCO, Gomes DMS*. Anatomia foliar de Mitracarpus frigidus (Willd.) K. Schum. var. salzmannianus (DC) K. Schum. e Mitracarpus lhorzkianus Cham (Rubiaceae).* São Paulo: Herbarium Bradeanum*,* 1996: p. 14.

2. Bisignano G *et al.* Antimicrobial activity of *Mitracarpus scaber* extract and isolated constituents. *Lett Appl Microbiol* 2000; 30: 105-108.

3. Harouna H *et al.* Harounoside a pentalongin hydroquinone diglycoside from *Mitracarpus scaber. Phytochemistry* 1995**;** 39: 1483-1484.

4. Okunade AL *et al.* Azaanthraquinone: an antimicrobial alkaloid from Mitracarpus *scaber*. *Planta Med* 1999**;** 65: 447-448.

5. Gbaguidi F *et al.* HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis*. *J Pharmaceut Biomed Anal* 2005; 39: 990-995.

6. Ekpendu TOE *et al.* Chemical studies of *Mitracarpus villosus* (Sw.) DC - a medicinal rubiaceous weed. *J Chem Soc Nigeria* 2001; 26: 69-74.

7. Numata A *et al.* Cytotoxic triterpenes from Chinese medicine, Goreishi. *Chem Pharm Bull* 1989; 37: 648-651.

8. Fabri RL *et al. Mitracarpus frigidus* aerial parts exhibited potent antimicrobial, antileishmanial, and antioxidant effects. *Bioresour Technol* 2009; 100: 428-433.

9. Monks A *et al.* Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 1991; 83: 757-766.

10. Nicoletti I *et al.* A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; 139: 271-279.

11. Braga FG *et al.* Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. *J Ethnopharmacol* 2007; 111: 396-402.

12. Morais-Teixeira E *et al. In vitro* and *in vivo* activity of meglumine antimoniate produced at Farmanguinhos-Fiocruz, Brazil, against *Leishmania* (Leishmania) *amazonensis*, *L.* (L.) *chagasi* and *L*. (Viannia) *braziliensis*. *Mem Inst Oswaldo Cruz* 2008; 103: 358-362.

13. Santos AO *et al.* Effect of Brazilian copaiba oils on *Leishmania amazonensis*. *J Ethnopharmacol* 2008; 120: 204-208.

14. Carmo AML *et al.* Synthesis of 4-aminoquinoline analogues and their platinum(II) complexes as new antileishmanial and antitubercular agents. *Biomed Pharmacother* 2011; 65: 204-209.

15. Winter C.A *et al.* Carrageenan-induced inflammation in hind paws of the rat as an assay for anti-inflammatory drugs*. Proc Soc Exp Biol Med* 1962; 111: 544-547.

16. Nakhai LA *et al.* Benefits of *Zataria multiflora* Boiss in experimental model of mouse inflammatory bowel disease. *Evid Based Complement Alternat Med* 2007; 4: 43-50.

17. Aebi H. Catalase. *Methods Enzymol* 1984; 105: 121-126.

18.Griswold DE *et al.* SK&F, 86002: a structurally novel anti-inflammatory agent that inhibits lipoxygenase- and cyclooxygenase-mediated metabolism of arachidonic acid. *Biochem Pharmacol* 1987; 36: 3463-3470.

19. Ishikawa T *et al.* Evaluation of gastroprotective activity of *Plinia edulis* (Vell.) Sobral (Myrtaceae) leaves in rats. *J Ethnopharmacol* 2008; 118: 527-529.

20. Peixoto JA *et al.* Antileishmanial activity of the hydroalcoholic extract of *Miconia langsdorffii*, isolated compounds, and semi-synthetic derivatives. *Molecules* 2011; 16: 1825-1833.

21. Takechi M, Tanaka Y. Structure-activity relationships of synthetic methyl ursolate glycosides. *Phytochemistry* 1993; 34(3): 675-677.

22. Es-Saady D *et al.* Effects of ursolic acid and its analogues on soybean 15-lipoxygenase activity and the proliferation rate of a human gastric tumour cell line. *Mediators Inflamm* 1994; 3(3): 181-184.

23. Hollósy F *et al.* Activation of caspase-3 protease during the process of ursolic acid and its derivative-induced apoptosis. *Anticancer Res* 2001; 21(5)**:** 3485-3491.

24. Saravanan R *et al.* Protective effect of ursolic acid on ethanol-mediated experimental liver damage in rats. *Life Sci* 2006; 78: 713-718.

25. da Silva Filho AA *et al. In vitro* antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from *Baccharis dracunculifolia* D.C. (Asteraceae). *Fitoterapia* 2009; 80: 478-482.

26. Passero LFD *et al.* Anti-leishmanial effects of purified compounds from aerial parts of *Baccharis uncinella* C. DC. (Asteraceae). *Parasitol Res* 2011; 108: 529-536.

27. Schwaiger S *et al.* Identification and pharmacological characterization of the anti-inflammatory principal of the leaves of dwarf elder (*Sambucus ebulus* L.). *J Ethnopharmacol* 2011; 133(2-4): 704-709.

28. Chen IH *et al.* Cytotoxic triterpenoids from the stems of *Microtropis japonica. J Nat Prod* 2009; 72(7): 1231-1236.

29. Meng YQ *et al.* Synthesis and anti-tumor activity of ursolic acid derivatives. *Yao Xue Xue Bao* 2011; 46(5): 556-560.

30. Prasad S *et al.* Ursolic acid, a pentacyclic triterpene, potentiates TRAIL-induced apoptosis through p53-independent up-regulation of death receptors: evidence for the role of reactive oxygen species and JNK. *J Biol Chem* 2011; 286(7): 5546-5557.

31. Wang X *et al.* Ursolic acid inhibits proliferation and induces apoptosis of cancer cells *in vitro* and *in vivo*. *J Biomed Biotechnol* 2011; 2011: 1-8.

32. Li W *et al.* Cell-based assays for profiling activity and safety properties of cancer drugs. *J Pharmacol Toxicol Methods* 2006; 54: 313-319.

33. Cruz AK *et al.* Current treatment and drug discovery against *Leishmania* spp. and *Plasmodium* spp.: a review. *Curr Drug Targets* 2009; 10: 178-192.

34. Mendonça-Filho RR *et al.* Leishmanicidal activity of polyphenolic-rich extract from husk fiber of *Cocos nucifera* Linn (Palmae). *Res Microbiol* 2004; 155: 136-143.

35. Rosa MSS *et al.* Antileishmanial activity of a linalool-rich essential oil from *Croton cajucara*. *Antimicrob Agents Ch* 2003; 47: 1895-1901.

36. Mallavadhani UV *et al.* Antimicrobial activity of some pentacyclic triterpenes and their synthesized 3-*O*-lipophilic chains. *Biol Pharm Bull* 2004; 27: 1576-1579.

37. Muylder G *et al.* A screen against Leishmania intracellular amastigotes: comparison to a promastigote screen and identification of a host cell-specific hit. *PLoS Negl Trop Dis* 2011; 5(7): e1253.

38. World Health Organization - WHO. *Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases.* Geneva: Technical report series no. 949, 2010.

39. Vyas BA *et al.* Evaluation of anti-inflammatory activities of n-butanol extract from *Pergularia daemia* in experimental animal models. *J Pharm Res* 2010; 3(12): 3039-3044.

40. Kirkova M *et al. In vivo* effects of indomethacin. I. Activity of antioxidant enzymes and lipid peroxidation. *Gen Pharmacol* 1992; 23: 503-507.

41. Olajide OA *et al.* Effects of the aqueous extract of *Bridelia ferruginea* stem bark on carrageenan-induced oedema and granuloma tissue formation in rats and mice. *J Ethnopharmacol* 1999; 66: 113-117.

42. Arulmozhi DK *et al.* Pharmacological investigation of *Sapindus trifolius* in various *in vitro* and *in vivo* models of inflammation. *Indian J Pharmacol* 2005; 37: 96-102.

43. Dawson J *et al.* Comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *Int J Tiss React* 1991; 13: 171-185.

44. Lu TC *et al.* Analgesic and antiinflammatory activities of the methanol extract from *Pogostemon cablin*. *Evid Based Complement Alternat Med* 2011; *2011*: 1-9.

45. Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic Biol Med* 1990; 9: 515-540.

46. Bilici D *et al.* Protective effect of melatonin in carrageenan-induced acute local inflammation. *Pharmacol Res* 2002; 46: 133-139.

47. Matés JM, Sánchez-Jiménez F. Antioxidant enzymes and their implications in pathophysiologic processes. *Front Biosci* 1999; 4: 339-345.

48. Halici Z *et al.* Amiodarone has anti-inflammatory and anti-oxidative properties: An experimental study in rats with carrageenan-induced paw edema. *Eur J Pharmacol* 2007; 566: 215-221.

49. Meade CJ *et al.* The role of polyphosphoinositides and their break down products in A23187 induced release of arachidonic acid from rabbit polymorphonuclear leukocytes. *J Biol Chem* 1996; 23: 425-436.

50. Liu J. Pharmacology of oleanolic acid and ursolic acid. *J Ethnopharmacol* 1995; 49: 57-68.

51. Ali MS *et al.* Ursolic acid: potent inhibitor of superoxides produced in the cellular system. *Phytother Res* 2007; 21: 558-561.

52. Ikeda Y *et al.* Ursolic acid: An anti- and pro-inflammatory triterpenoid. *Mol Nutr Food Res* 2008; 52: 26-42.

Table 1 Cytotoxic activity of ursolic acid (**1**) and methyl ursolate (**2**) isolated from *Mitracarpus frigidus*

Samples	Cytotoxic activity (ED_{50} $\mu g.mL^{-1}$)[a]			
	HL60	Jurkat	MCF-7	HCT
Ursolic acid	13.6	4.2	20.8	35.7
	(9.7 - 19.1)	(2.5 - 5.1)	(15.6 - 27.8)	(26.5 - 47.5)
Methyl ursolate	22.7	> 50	> 50	> 50
	(14.3 - 36.0)			
Etoposide[b]	0.3	1.5		
	(0.2- 0.5)	(0.5 - 4.3)		
Doxorubicin[b]			0.5	1.8
			(0.05 - 9.4)	(0.4 - 7.9)

[a]Data are presented as median and 95% confidence interval (in parentheses)

[b]Positive controls

Table 2 Antileishmanial activity and cytotoxicity of ursolic acid (**1**) and methyl ursolate (**2**) isolated from *Mitracarpus frigidus*

Samples	Antileishmanial activity (IC_{50} $\mu g.mL^{-1}$)[a]							
	Promastigote forms				Amastigote forms	Peritoneal macrophages (CC_{50} $\mu g.mL^{-1}$)[b]	SE[d]	SS[e]
	La	Lb	Lm	Lc	Lm			
Ursolic acid	39.3 ± 0.3	15.0 ± 0.4	4.3 ± 0.2	27.9 ± 1.5	1.3 ± 0.1	9.7 (7.7 - 14.7)	7.5	3.3
Methyl ursolate	27.1 ± 0.6	4.7 ± 0.04	2.9 ± 0.04	12.1 ± 1.4	2.1 ± 0.2	11.8 (10.7 - 20.5)	5.6	1.4
AmB[c]	0.2 ± 0.009	0.1 ± 0.02	0.3 ± 0.07	0.1 ± 0.004	0.27 ± 0.02	> 100	-	-

[a]La – *Leishmania amazonensis*; Lb – *L. braziliensis*; Lm – *L. major*; Lc – *L. chagasi*

[a,b]Data are presented as mean ± standard deviation

[c]AmB (amphotericin B) - Positive control

[d]SE (Selectivity) - CC_{50} of macrophages/IC_{50} of amastigotes of *L. major*

[e]SP (Specificity) - IC_{50} promastigotes of *L. major* / IC_{50} amastigotes of *L. major*

Table 3 Effect of ursolic acid (**1**) and methyl ursolate (**2**) on *Leishmania major* interiorized in peritoneal macrophage cells after 72 hours of treatment

Samples	Survival Index for Amastigotes (% Inhibition)				
		2.5 μg.mL^{-1}	5.0 μg.mL^{-1}	10.0 μg.mL^{-1}	20.0 μg.mL^{-1}
Control	585.0 ± 17.4	-	-	-	-
Ursolic acid	-	488.7 ± 36.1 (16.5)	10.0 ± 0.4 (98.3)	10.0 ± 0.7 (98.3)	2.8 ± 0.05 (99.5)
Methyl ursolate	-	550.0 ± 49.2 (6.0)	174.3 ± 12.4 (70.1)	11.3 ± 2.8 (98.1)	13.8 ± 0.4 (97.6)

[a]Survival Index – number of amastigotes per cells x % infected cells

[b]Data are presented as mean ± standard deviation

Table 4 Effect of ursolic acid (**1**) and methyl ursolate (**2**) isolated from *Mitracarpus frigidus* on carrageenan-induced rat paw edema

Treatment	Dose ($mg.kg^{-1}$)	Time, paw volume in mL (% inhibition)				
		0h	½h	1h	2h	3h
Vehicle	-	0.07 ± 0.01	0.32 ± 0.05	0.38 ± 0.11	0.60 ± 0.1	0.58 ± 0.04
Dexamethasone	1	0.04 ± 0.02	0.19 ± 0.05[a] (41)	0.16 ± 0.03[a] (58)	0.23 ± 0.03[a] (62)	0.29 ± 0.02[a] (50)
Indomethacin	10	0.06 ± 0.03	0.11 ± 0.02[a] (65)	0.10 ± 0.04[a] (74)	0.20 ± 0.04[a] (67)	0.24 ± 0.05[a] (59)
Ursolic acid	1	0.01 ± 0,01	0.03 ± 0.01[a,b,c] (91)	0.04 ± 0.01[a,b] (90)	0.06 ± 0.04[a,b,c] (90)	0.09 ± 0.04[a] (85)
Ursolic acid	0.5	0.06 ± 0.03	0.03 ± 0.002[a,b,c] (91)	0.07 ± 0.01[a] (82)	0.12 ± 0.03[a] (80)	0.10 ± 0.01[a] (83)
Methyl ursolate	1	0.03 ± 0.004	0.03 ± 0.02[a,b,c] (91)	0.06 ± 0.02[a] (84)	0.11 ± 0.02[a] (82)	0.13 ± 0.02[a] (78)
Methyl ursolate	0.5	0.03 ± 0.01	0.05 ± 0.03[a,b,c] (84)	0.20 ± 0.07[a] (47)	0.23 ± 0.08[a] (62)	0.47 ± 0.1[b,c] (19)

Values represent mean ± S.E.M. (n = 6)

[a]Statistically different from the negative control (vehicle) (ANOVA followed by Bonferroni, $P < 0.05$) [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, $P < 0.05$)

Table 5 Effect of ursolic acid (**1**) and methyl ursolate (**2**) isolated from *Mitracarpus frigidus* on leukocyte migration and neutrophil migration in peritoneal exudation in carrageenan-induced mice

Treatment	Dose ($mg.kg^{-1}$)	Leukocytes ($10^5.mL^{-1}$)	Leukocyte inhibition (%)	Neutrophils ($10^5.mL^{-1}$)	Neutrophil inhibition (%)
Vehicle	-	3.5 ± 0.76	-	2.8 ± 0.7	-
Dexamethasone	1	0.52 ± 0.009[a]	85	0.31 ± 0.05[a]	89
Indomethacin	10	0.58 ± 0.23[a]	83	0.44 ± 0.18[a]	84
Ursolic acid	1	0.40 ± 0.11[a]	88	0.25 ± 0.06	93
Ursolic acid	0.5	0.63 ± 0.009[a]	82	0.50 ± 0.09	82
Methyl ursolate	1	0.54 ± 0.14[a]	84	0.35 ± 0.09	88
Methyl ursolate	0.5	1.80 ± 0.22[a,b,c]	48	1.16 ± 0.06[a,b,c]	58

Values represent mean ± S.E.M. (n = 6)

[a]Statistically different from the negative control (vehicle) (ANOVA followed by Bonferroni, $P < 0.05$) [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, $P < 0.05$)

Legends for figures

Figure 1 The structures of ursolic acid (**1**) and methyl ursolate (**2**) isolated from the aerial parts of *Mitracarpus frigidus.*

Figure 2 Effect of ursolic acid (**1**) and methyl ursolate (**2**) on DNA content of tumor cell lines. HL-60, Jurkat, MCF-7 e HCT cells were incubated with samples (20 μg.mL^{-1}) or with control (0.05% DMSO) for 24 h. DNA content was assayed by PI stain and flow cytometry analysis. A) Etoposide (8 μg.mL^{-1}) and B) doxorubicin (5 μg.mL^{-1}), positive controls are demonstrated. Each data point represents mean ± SD from at least two independent experiments performed in duplicate. [a]Statistically different from negative control (0.05% DMSO). [b]Statistically different from positive control (etoposide) [c]Statistically different from positive control (doxorrubicin) (ANOVA followed by Bonferroni, $P < 0.05$).

Figure 3 Effects of ursolic acid (**1**) and methyl ursolate (**2**) and positive controls (dexamethasone and indomethacin) on changes in activities of the A) malondialdehyde (MDA) accumulation and B) catalase enzyme in carrageenan-induced paw edema (3rd hour) in rats. [a]Statistically different from the negative control (vehicle). [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, $P < 0.05$).

Figure 1

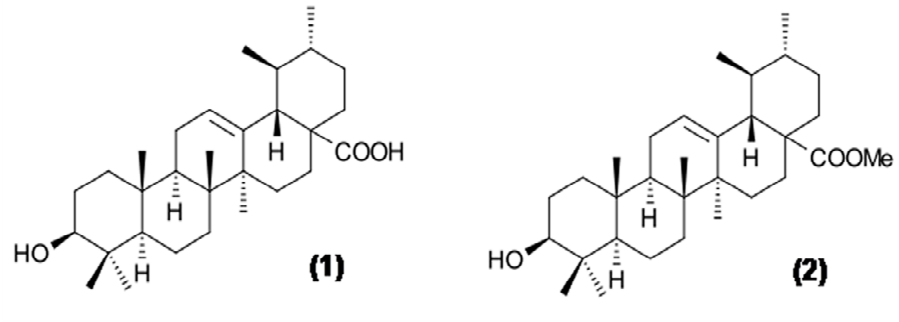


Figure 2

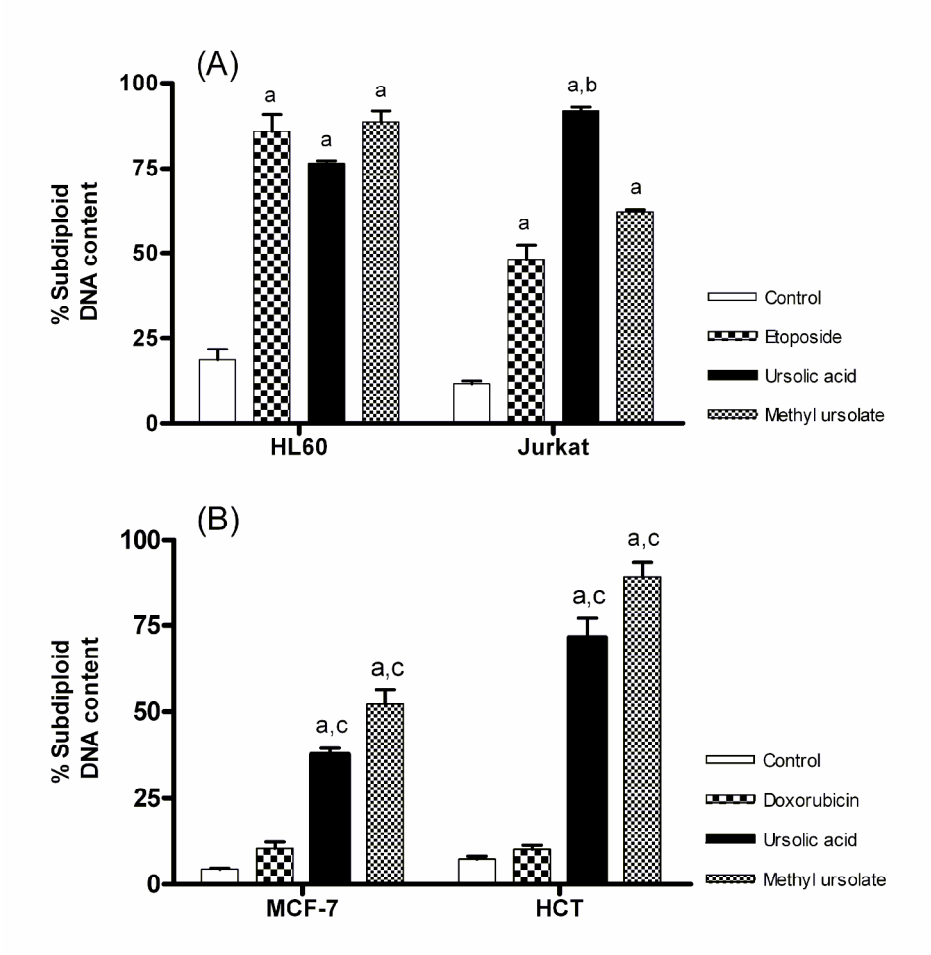
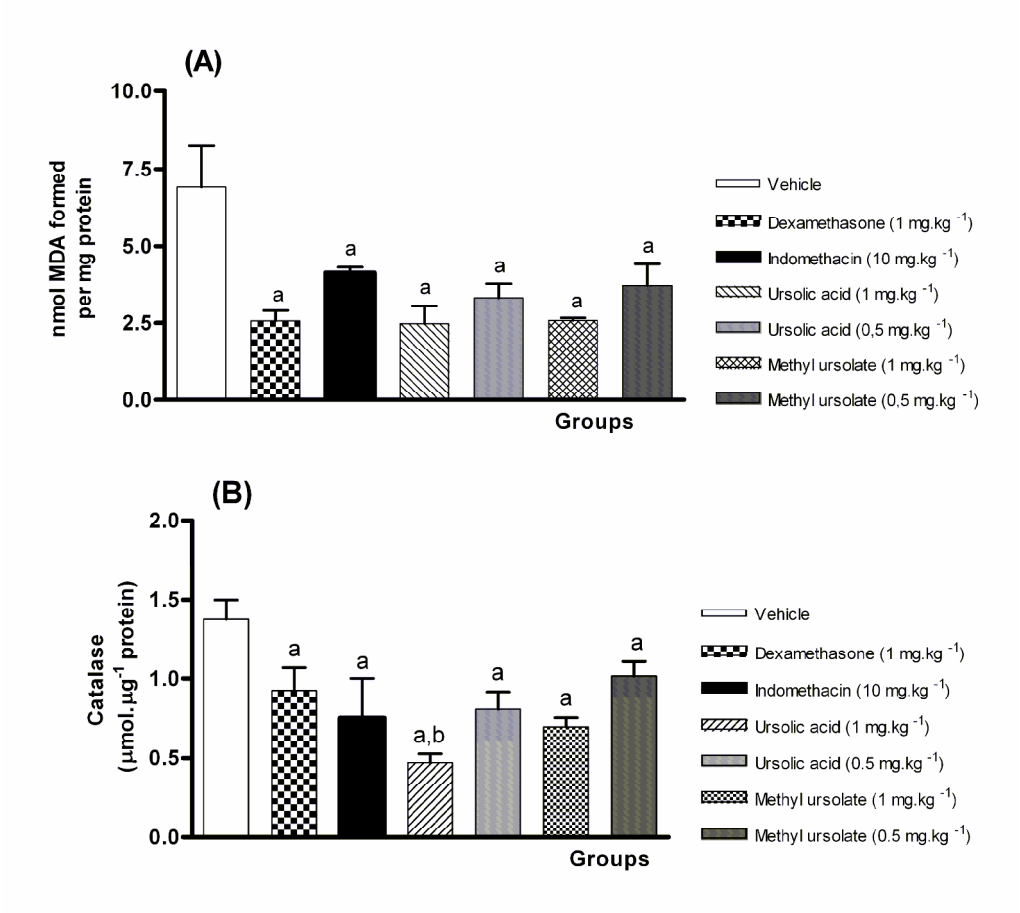


Figure 3


(A)
nmol MDA formed per mg protein
10.0
7.5
5.0
2.5
0.0
a
a
a
a
a
a
Groups
Vehicle
Dexamethasone (1 mg.kg -1)
Indomethacin (10 mg.kg -1)
Ursolic acid (1 mg.kg -1)
Ursolic acid (0,5 mg.kg -1)
Methyl ursolate (1 mg.kg -1)
Methyl ursolate (0,5 mg.kg -1)
(B)
Catalase (µmol.µg-1 protein)
2.0
1.5
1.0
0.5
0.0
a
a
a,b
a
a
a
Groups
Vehicle
Dexamethasone (1 mg.kg -1)
Indomethacin (10 mg.kg -1)
Ursolic acid (1 mg.kg -1)
Ursolic acid (0.5 mg.kg -1)
Methyl ursolate (1 mg.kg -1)
Methyl ursolate (0.5 mg.kg -1)

5.6 O artigo a seguir refere-se ao potencial esquistossomicida *in vitro* e *in vivo* do extrato metanólico das partes aéreas de *Mitracarpus frigidus.*

Artigo 6

EFFECTS OF THE MEDICINAL PLANT SPECIES *Mitracarpus frigidus* ON ADULT SCHISTOSOMA MANSONI WORMS

Submetido a Memórias do Instituto Oswaldo Cruz

Effects of the medicinal plant species *Mitracarpus frigidus* on adult *Schistosoma mansoni* worms

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Abstract

To evaluate the *in vitro* and *in vivo* schistosomicidal properties of the methanolic extract of aerial parts *Mitracarpus frigidus* (MFM). For the *in vitro* experiment, four pairs of adult worms, obtained from infected mice, were exposed to different concentrations of MFM (100 to 400 µg/mL) for 24 and 48 h and analyzed under an inverted microscope. For the *in vivo* experiment, mice were inoculated with cercariae and, 20 days after infection, MFM in concentrations of 100 and 300 mg/kg was administered orally for the following 25 days. Mice were euthanized after 60 days. Hematological, biochemical and parasitological parameters were also determined. MFM showed *in vitro* schistosomicidal activity, exhibiting the opening of the gynaecophoral canal of some male schistosomes, and the presence of contorted muscles, vesicles and darkening of the paired worms skin. *In vivo* experiments showed that MFM treatments (100 and 300 mg/kg) significantly reduced total worm count, as praziquantel, showing a decrease in liver and spleen weight. Also, a significant reduction in granuloma density was observed in the groups treated with MFM. MFM treatment demonstrated significant *in vitro* and *in vivo* schistosomicidal activities, and did not cause alterations in the liver function of either infected or non-infected mice.

Key words: *Mitracarpus frigidus*; *Schistosoma mansoni*; schistosomiasis.

INTRODUCTION

Schistosomiasis, an infection caused by trematode worms of the genus *Schistosoma*, is considered one of the most significant neglected tropical diseases in the world (Hotez et al. 1996). It is estimated that 779 million people are at risk for schistosomiasis, with 230 million infected in 77 countries and territories (Steinmann et al. 2006; WHO 2012).

The current treatment is based on the use of praziquantel and oxamniquine (Cioli et al. 1995; Cioli et al. 2004). Those drugs are effective against all species of schistosome, however, they do not prevent re-infection, are inactive against juvenile schistosomes, and have only a limited effect on the already developed liver and spleen lesions (Fallon & Doenhoff 1994; Stelma et al. 1995; Ismail et al. 1999). Praziquantel has a key role in population based disease control programs in most endemic countries (Salvioli et al. 1997; Araújo et al. 2008; WHO 2012). The *in vitro* mechanism of action of this drug on adult *S. mansoni* worms has been well-described in the literature. This drug can cause muscle contraction and promote the immediate death of adult worms, miracidia and primary sporocysts (Couto et al. 2010; Almeida et al. 2011). However, the worryingly small portfolio of treatment options and the inevitability of resistance now that mass-administration programmes are in effect (Penido et al. 1999; Ribeiro-dos-Santos et al. 2006), and the hemorrhage caused by this drug in the host lung tissue, as well as abdominal pain and diarrhea (Flisser & Meclaren 1989; Kabatereine et al. 2003), reinforce the need to develop new, safe and effective schistosomicidal drugs. In this regard, the search for bioactive natural products against the schistosome has been intensified to establish future strategies to control schistosomiasis (Ndamba et al. 1994; Molgaard et al. 2001; Sanderson et al. 2002; Mohamed et al. 2005).

Mitracarpus frigidus (Willd. ex Roem. & Schult.) K. Shum is a species of the family Rubiaceae found throughout South America and, in Brazil, this species can be found in all states (Pereira et al. 2006). Its methanolic extract (MFM) revealed the presence of flavonoids, tannins, alkaloids, terpenes and quinones, and showed antimicrobial, leishmanicidal, cytotoxic and laxative activities. Moreover, MFM revealed no toxicity signs in rat models (Fabri et al. 2009; Fabri et al. 2012a). Recently, the pyranonaphthoquinone psychorubrin, was firstly isolated from this species (Fabri et al. 2012b).

However, there is no scientific report available in the literature on the anti-*Schistosoma mansoni* activity of *M. frigidus*. In view of this, the present study aimed to investigate the *in vitro* and *in vivo* shistosomicidal activity of the *M. frigidus* methanolic extract (MFM) in *Schistosoma mansoni*-infected mice. Furthermore, hematological, biochemical and parasitological parameters were also determined.

MATERIALS AND METHODS

Plant material and extraction

Mitracarpus frigidus aerial parts, collected in Juiz de Fora, Minas Gerais, Brazil, in May, 2011, were identified by Dr. Tatiana Konno from the Nucleus of Ecology and Socio-Environmental Development of Macaé/ Federal University of Rio de Janeiro. A voucher specimen (CESJ 46076) was deposited at the Herbarium Leopoldo Krieger of the Federal University of Juiz de Fora. Oven-dried and powdered aerial parts of the plant (1000 g) were extracted by maceration with methanol (5 x 2000 mL) for five days at room temperature and the methanolic extract (MFM) was obtained by evaporation (yield 10% w/w).

In vitro studies with *Schistosoma mansoni*

Swiss mice were individually infected subcutaneously with 100 cercariae/animal of the LE strain of *Schistosoma mansoni* (FIOCRUZ, Belo Horizonte, Brazil) in order to obtain the adult worms. The type of infection realized was bisexual, resulting in adult male and female worms. At the end of 50 days, the infected animals were euthanized using a solution of 3% sodium pentobarbital (30 µL/animal), and the worms were obtained by hepatic portal system perfusion according to the technique described by Smithers and Terry (1965). These studies were approved by the Ethical Committee of the Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil, protocol number 017/2009.

Viability assay

All the procedures conducted after worm extraction were made under aseptic conditions, including equipment and solutions. The worms were washed in RPMI-1640 medium to remove the perfusion detritus. After washing, four live worm pairs and showing intense motility were transferred to each well of a 24-well culture plate containing 4 ml of RPMI-1640 medium supplemented with 5% of fetal calf serum and 100 µg/mL of penicillin/streptomycin (Araújo et al. 2007). The pairs were exposed to increasing concentrations of MFM (100, 200 and 400 µg/mL), and the worms were kept in contact with the extracts for 24 h. In the first experiment, the analyses were performed 6 and 24 h after the addition of 200 or 400 µg/mL of MFM, and 24 h after removal of the extracts. In the second experiment, the analyses were conducted 24 h after the addition of 100 or 200 µg/mL of MFM, and 24 and 48 h after their removal. After removal of the extracts, the worms were washed three times with the culture medium and then maintained in culture. In both experiments, the negative control

group was included, comprising four pairs of worms in each well**,** in the presence of 1% DMSO (v/v) in 0.9% NaCl solution. During the entire assay, the worms were maintained in an incubator at 37 ºC and an atmosphere containing 5% of CO_2. Four independent experiments were performed. Analyses were carried out using an inverted Olympus microscope and photographed with digital camera Canon.

In vivo **schistosomicidal analyses**

Experimental design

Eighty female Swiss mice, weighing between 20 and 30 g, were used in this experiment. Forty mice were maintained without infection ,and the remainder subjected to infection with approximately 50 cercariae/animal (LE strain of *Schistosoma mansoni* BH) as described by Araújo et al. (2008) The mice were divided into eight groups (n = 10): four groups treated and non-infected, and four groups treated and infected. To evaluate the possible toxicity of the different treatments, non-infected animals were divided into: (A) negative control group treated with a 1% DMSO (v/v) in 0.9% NaCl solution, (B) positive control group treated with praziquantel (200 mg/kg), (C) and (D) groups treated with MFM at 100 and 300 mg/kg diluted with saline + 1% DMSO, respectively. The infected animals were divided into: (E) negative control group treated with a DMSO 1% (v/v) in NaCl 0.9% solution, (F) positive control group treated with praziquantel (200 mg/kg), (G) and (H) groups treated with MFM at 100 and 300 mg/kg diluted with saline + 1% DMSO, respectively. All of the treatments started on the twentieth day after infection. MFM and the negative control were administered in one daily dose for 25 days. The positive control, praziquantel, was administered in a single dose. At the end of the

treatment period, the animals were maintained for a period of 15 days, completing 60 days of infection.

Determination of parasite load and biochemical and hematological parameters

After 60 days of cercarial exposure, all animals were weighed, anesthetized and blood samples were collected for assessment of biochemical and hematological parameters. Immediately following, the animals were euthanized, and the adult worms recovered from the portal and mesenteric veins by perfusion. In addition, the liver and spleen were removed and weighed.

The measurement of biochemical parameters was performed using commercial kits (BIOCLIN® and LABTEST®), and included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein, albumin and globulin. Hematological parameters (total and specific leukocytes count) were also performed.

Histological analysis

Transverse sections of all liver lobes of infected mice (n = 5 per group) were collected, fixed in 4% buffered formaldehyde solution and embedded in paraffin. Sections of 5-10 µm were stained with haematoxylin and eosin (H&E). For the evaluation of granuloma density, and stained slides were observed using bright field microscopy and all granulomas containing central viable eggs were quantified. All evaluations were blind performed by two different observers (Phyrrho et al. 2004). The area of hepatic granuloma was determined in histological sections from 20 to 30 granulomas per animal, containing central viable eggs, randomly chosen. The

granuloma area was manually delimited, captured by a CCD camera using bright field microscopy and automatically processed with IMAGE-PROPLUS.

Statistical analysis

Values are presented as means ± SEM. Statistical differences between the treatments and the controls were tested by one-way analysis of variance (ANOVA), followed by the Bonferroni test using the “GraphPad Prism 4” statistic computer program. A difference in the mean values of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

In vitro studies with *Schistosoma mansoni*

The profile of the damage caused by the exposure of the adult worms of *S. mansoni* to medicinal plants extracts can be determined through the observation of reduced motility, incapacity of adhesion in the culture plate by sucker cup, and tegument darkening (Araújo et al. 2008; Magalhães et al. 2009; Magalhães et al. 2010; Almeida et al. 2011). When the motility is lost, the worms can be considered dead (Araújo et al. 2007).

The morphological characteristics of the paired worms of *S. mansoni* maintained in culture medium with 1% DMSO (v/v) in 0.9% NaCl solution (negative control group), and in the presence of MFM (100 μg/ml) are shown in Figure 1. After 48 h exposure, the pairs of worms in the control group (Figure 1A and B) continued mating and showing active movements, without lesions in the tegument, with the presence of eggs in the culture medium. On the other hand, after 24 h of exposure to MFM at concentrations of 100, 200 and 400 μg/mL, the worms showed complete

paralysis, including the loss of movement of the suction cups with darkening in the tegument and the death of all parasites. Figure 1 (C, D, E and F) shows the morphological changes occurring after exposure of pairs of adult worms to MFM at 100 μg/mL. These changes included the opening of the gynecophoral canal of some males (Figure 1C), and the presence of males and females with contorted muscles, darkening of the skin (Figure 1D), and the presence of vesicles in some skin formation (Figure 1 E and F). This type of damage was also observed at the concentrations of 200 and 400 μg/ml of MFM (data not shown).

Therefore, due to the promising activity observed for MFM against adult worms for *the in vitro* experiments, in vivo studies were performed to observe its therapeutic potential and toxicity.

ure 1

***In vivo* schistosomicidal activity**

In order to investigate the effect of MFM treatment on body weight gain of both normal and *S. mansoni*-infected mice, body weight was measured after 60 days of treatment. No significant difference was observed between infected and normal mice (Table I). *S. mansoni* infection is caused by cercariae penetration in the human skin and the symptoms are due to eggs that migrate to the liver, being secreted by worms living in the mesenteric and portal veins which leads to hepatosplenomegaly (Sanderson et al. 2002; Jatsa et al. 2009; Mata-Santos et al. 2010; Abdul-Ghani et a. 2011). Therefore, in order to examine the effect of MFM on hepatosplenomegaly, the liver and spleen were excised from dissected mice after perfusion, weighed and the relative weight percentage was calculated. As shown in Table I, there was no significant difference in the liver and spleen relative weights among the non-infected mice groups. Although the infected mice presented an increase in liver and spleen

weights, the groups treated with MFM (Group G and H) showed a significant decrease in both liver and spleen relative weights when compared to the respective negative control (Group E). Those results indicated that MFM treatment alleviated hepatosplenomegaly induced by *S. mansoni* infection.

ıle I

In addition, a significant reduction in granuloma density was observed in the groups treated with MFM (Group G and H) when compared with the respective negative control (Group E). These results were comparable to that found for the mice treated with praziquantel (Table I and Figure 2).

Histological examination of the H&E stained liver sections showed that the granulomas of the infected negative control group (Group E) were composed of central ova surrounded by inflammatory cells associated with laminated layers of fibrous tissue at the periphery. In addition, severe necrosis was observed in the hepatic tissue (Figure 2A). On the other hand, the granulomas of the infected, treated mice (Groups F, G and H) were observed as a concentric focus of mononuclear and polymorphonuclear cells around the egg, and the laminated layers of fibrous connective tissue nearly disappeared. Minimal microvascular changes, and no hepatocyte necrosis were observed in the liver sections of those mice (Figure 2B, C and D).

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ure 2

In order to evaluate the *in vivo* schistosomicidal effect of MFM, 60 days after cercarial exposure, the adult worms of *S. mansoni*-infected mice were recovered from the portal and mesenteric veins by perfusion and counted. As shown in Table II, MFM treatments (100 and 300 mg/kg) significantly reduced total worm count (69 and 58%, respectively), as well as the reference group treated with praziquantel (49%) when compared with the control group. MFM reduced worm liver and mesentery burden to the extent of 91 and 65% at 100 mg/kg, and by 65 and 58% at 300 mg/kg,

respectively. By the other side, praziquantel reduced the liver and mesentery worm burden in 48 and 51%, respectively.

There are relatively few reports in the literature that show *in vivo* schistosomicidal activity of plant extracts (Hamed & Hetta 2005; El-Shenawy et al. 2006). For example, artemether, an artemisinin derivative, used as a prophylactic agent against schistosomiasis japonica in China, at a concentration of 400 mg/kg, was able to reduce 60% of total worms during six days of treatment, in an experimental model (Abdul-Ghani et a. 2011). El-Shenawy et al. (2006) demonstrated that an alcoholic extract of *Cleome droserifolia* (Forssk.) Del. branches reduced 33% of worm burden at a concentration of 310 mg/kg. On the other hand, the ethanolic extract of *Nigella sativa* L., described in folk medicine as possessing hepatoprotective and antiprotozoal properties, was not able to reduce the number of worms after experimental infection (El-Shenawy et al. 2008).

ble II

In order to evaluate the ameliorative effect of MFM treatment on liver pathology induced by *S. mansoni* infection, the levels of total protein content and ALT, AST, ALP activity were measured in the serum. Total protein content of non-infected mice treated with MFM and praziquantel (Groups B, C and D) was comparable to the respective control group (Group A) (Table III). On the other hand, the treated and infected mice (Groups F, G and H) presented a protein content much lower than the non-infected groups. A decrease in total serum protein in the infected animals is attributed to the liver damage caused by infection (Guyton & Hall 2000; El-Shenawy et al. 2006).

The level of globulin increased significantly in the infected group treated with MFM at 100 mg/kg (Group G) compared to the negative control (Group E) and the praziquantel treated (Group F) groups. This increase in globulin level may represent

a responsive mechanism enhancing the immunity of the host (El-Shenawy & Soliman 2002). However, there was no significant difference in albumin levels among the infected groups. The A/G ratio content for the infected mice treated with MFM at 100 mg/kg (group G) is comparable to the negative control group (Group E), and lower than the praziquantel treated group (Table III).

AST, ALT, ALP levels in the infected treated mice (Groups F, G and H) were significantly lower than those of the respective control group (Group E). These observations could be attributed to the reduction in hepatic granuloma and fibrosis, as well as the absence of necrotic liver tissue, in the infected, treated mice. For non-infected mice, these enzymes remained at normal levels (Table III and Figure 2). The results indicated that the administration of MFM did not cause alterations in the liver function of either infected or the non-infected mice.

Evaluation of cell profile during infection is one of the strategies to assess which cells are stimulated by events which modify the inflammatory process. The mechanism of selective recruitment of leukocytes to the inflamed tissue is related to chemostatic factors (Larocca et al. 2004). As depicted in Table III, there was a significant decrease in total leukocyte count in the infected treated mice (Groups F, G and H) when compared to the respective control group (Group E). Although MFM was not able to reduce the granulomatous area, it reduced the number of liver worms and consequently, the recruitment of leukocytes.

The specific leukometry showed that MFM did not significantly affect the basophil, neutrophil, lymphocyte, or eosinophil count in the non-infected, treated mice (Groups B, C and D), compared to the respective control (Group A). The neutrophil counts of the infected, treated mice (Groups F, G and H) were significantly increased, but the eosinophil and monocyte counts were significantly decreased,

compared with the negative control (Group E). Therefore, eosinophil and monocyte counts are usually increased in helmintic diseases, those results are in agreement with the reduction of the worm burden caused by MFM.

ole III

CONCLUSIONS

It was demonstrated that *Mitracarpus frigidus* may be of further interest in schistosomiasis treatment, as it promoted a considerable improvement of disease severity reducing significantly the parasite load without altering liver function. Future studies are aimed at the determination of the active principles.

REFERENCES

Abdul-Ghani R, Loutfy N, Sheta M, Hassan A 2011. Artemether shows promising female schistosomicidal and ovicidal effects on the Egyptian strain of *Schistosoma mansoni* after maturity of infection. Parasitol Res 108(5): 1199-1205.

Almeida GT, Amaral MS, Beckedorff FC, Kitajima JP, Demarco R, Verjovski-Almeida S 2011. Exploring the *Schistosoma mansoni* adult male transcriptome using RNA-seq. Exp Parasitol (in press).

Araújo SC, Mattos AC, Teixeira HF, Coelho PM, Nelson DL, de Oliveira MC 2007. Improvement of in vitro efficacy of a novel schistosomicidal drug by incorporation into nanoemulsions. Int J Pharm 337: 307-315.

Araujo N, Mattos ACA, Coelho PMZ, Katz N 2008. Oxamniquine, praziquantel and lovastatin association in the experimental schistosomiasis mansoni. Mem Inst Oswaldo Cruz 103(5): 450-454.

Cioli D, Pica-Mattoccia L, Archer S 1995. Antischistosomal drugs: past, present and future? Pharmacol Ther 68(1): 35-85.

Cioli D, Botros SS, Wheatcroft-Francklow K, Mbaye A, Southgate V, Tchuenté LA, Pica-Mattoccia L, Troiani AR, El-Din SH, Sabra AN, Albin J, Engels D, Doenhoff MJ 2004. Determination of ED_{50} values for praziquantel in praziquantel-resistant and -susceptible *Schistosoma mansoni* isolates. Int J Parasitol 34(8): 979-987.

Couto FF, Coelho PM, Araújo N, Kusel JR, Katz N, Mattos AC 2010. Use of fluorescent probes as a useful tool to identify resistant *Schistosoma mansoni* isolates to praziquantel. Parasitology 137(12): 1791-1797.

El-Shenawy NS, Soliman MFM 2002. On the interaction between induced *Diabetes mellitus* and Schistosomiasis: mechanism and protection. Egypt J Hosp Med 8: 18-31.

El-Shenawy NS, Soliman MF, Abdel-Nabi IM 2006. Does *Cleome droser*ifolia have anti-schistosomiasis mansoni activity? Rev Inst Med Trop Sao Paulo 48(4): 223-228.

El Shenawy NS, Soliman MF, Reyad SI 2008. The effect of antioxidant properties of aqueous garlic extract and *Nigella sativa* as anti-schistosomiasis agents in mice. Rev Inst Med Trop S Paulo 50(1): 29-36.

Fabri RL, Nogueira MS, Braga FG, Coimbra ES, Scio E 2009. *Mitracarpus frigidus* aerial parts exhibited potent antimicrobial, antileishmanial, and antioxidant effects. Bioresour Technol 100: 428-433.

Fabri RL, de Oliveira Aragão DM, Florêncio JR, Cardoso GM, de Souza-Fagundes EM, Castanon MC, Scio E 2012a. *In vivo* laxative and toxicological evaluation and *in vitro* antitumor effects of *Mitracarpus frigidus* aerial parts. J Pharm Pharmacol 64: 439-448.

Fabri RL, Grazul RM, Carvalho LO, Coimbra ES, Cardoso, GMM, Souza-Fagundes EM, Da Silva AD, Scio E 2012b. Antitumor, antibiotic and antileishmanial properties

of the pyranonaphthoquinone psychorubrin from *Mitracarpus frigidus.* Ann Acad Bra Sci 84(4): 1081-1090.

Fallon PG, Doenhoff MJ 1994. Drug-resistant schistosomiasis, resistance to praziquantel and oxamniquine induced in *Schistosoma mansoni* in mice is drug specific*.* Am J Trop Med Hyg 51: 83-88.

Flisser A, Meclaren DJ. Effect of praziquantel treatment on lung stage larvae of *Schistosoma mansoni in vivo*. *Parasitology* 1989; 98: 203-211.

Hamed MA, Hetta MH. Efficacy of *Citrus reticulata* and Mirazid in treatment of *Schistosoma mansoni* 2005. Mem Inst Oswaldo Cruz 100(7): 771-778.

Guyton AC, Hall JE 2000. Textbook of Medical Physiology, 10th ed., WB Saunders, Philadelphia, 810-818.

Hotez PJ, Molyneux DH, Fenwick A, Ottessen E, Ehrlich Sachs S, Sachs JD 2006. Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria. PLOS Med 3(5): 576-584.

Ismail M, Botros S, Metwally A, William S, Farghally A, Tao LF, Day TA, Bennett JL*.* Resistance to praziquantel, direct evidence from *Schistosoma mansoni* isolated from Egyptian villagers 1999. Am J Trop Med Hyg 60: 932-935.

Jatsa HB, Ngo Sock ET, Tchuem Tchuente LA, Kamtchouing P 2009. Evaluation of the *in vivo* activity of different concentrations of *Clerodendrum umbellatum* Poir against *Schistosoma mansoni* infection in mice. Afr J Trad CAM 6(3): 216 – 221.

Kabatereine NB, Kemijumbi J, Ouma JH, Sturrock RF, Butterworth AE, Madsen H, Ornbjerg N, Dunne DW, Vennnervald BJ. Efficacy and side effects of praziquantel treatment in a highly endemic *Schistosoma mansoni* focus at Lake Albert, Uganda 2003. Trans R Soc Trop Med Hyg 97: 599-603.

Larocca RA, Souza BR, Marmol CHC, Silva DS, Brigoto OM, Rodrigues V, Aníbal FF *2004.* Avaliação do recrutamento celular no modelo experimental da esquistossomose mansônica. Revista UNIARA 15: 201-213.

Magalhães LG, Machado CB, Morais ER, Moreira EBC, Soares CS, Silva SH, Da Silva Filho AA, Rodrigues V 2009*. In vitro* schistosomicidal activity of curcumin against *Schistosoma mansoni* adult worms. Parasitol Res 104: 1197-1201.

Magalhães LG, Kapadia GJ, Tonuci LRS, Caixeta SC, Parreira NA, Rodrigues V, Da Silva Filho AA. *In vitro* schistosomicidal effects of some phloroglucinol derivatives from *Dryopteris* species against *Schistosoma mansoni* adult worms 2010. Parasitol Res 106*:* 395-401.

Mata-Santos HA, Lino FG, Rocha CC, Paiva CN, Castelo Branco MT, Pyrrho Ados S 2010*.* Silymarin treatment reduces granuloma and hepatic fribrosis in experimental schistosomiasis. Parasitol Res 107: 1429-1434.

Mohamed AM, Metwally NM, Mahmoud SS*.* Sativa seeds against *Schistosoma mansoni* different stages 2005. Mem Inst Oswaldo Cruz 100: 205-211.

Molgaard P, Nielsen SB, Rasmussen DE, Drummond RB, Makaza N, Andreassen J*.* Anthelmintic screening of Zimbabwean plants traditionally used against schistosomiasis 2001. J Ethnopharmacol 74: 257-264.

Ndamba J, Nyazema N, Makaza N, Anderson C, Kaondera KC 1994*.* Traditional herbal remedies used for the treatment of urinary schistosomiasis in Zimbabwe. J Ethnopharmacol 42: 125-132.

Penido ML, Coelho PM, Nelson DL. Efficacy of a new schistosomicidal agent 2-[(methylpropyl)amino]-1-octanethiosulfuric acid against an oxamniquine resistant *Schistosoma mansoni* isolate 1999. Mem Inst Oswaldo Cruz 94(6): 811-813.

Pereira ZV, Carvalho-Okano RM, Garcia FCP 2006. Rubiaceae Juss. da Reserva Florestal Mata de Paraíso, Viçosa, MG, Brasil. Acta Bot Bras 20(10): 207-224.

Pyrrho AS, Lenzi HL, Ramos JA, Moura-Neto R, Cachem FC, Santos da Silva C, Takiya CM, Gattass CR 2004. Dexamethasone treatment improves morphological and hematological parameters in chronic experimental schistosomiasis. Parasitol Res 92: 478-483.

Ribeiro-dos-Santos G, Verjovski-Almeida S, Leite LC 2006. Schistosomiasis - a century searching for chemotherapeutic drugs. Parasitol Res 99(5): 505-521.

Salvioli L, Renganathan E, Montresor A, Davis A, Behbehani K. Control of schistosomiasis - a global picture 1997. Parasitol Today 13: 444-448.

Sanderson L, Bartlett A, Whitfield PJ 2002. *In vitro* and *in vivo* studies on the bioactivity of a ginger (*Zingiber officinale*) extract towards adult schistosomes and their egg production. J Helminthol 76: 241-247.

Smithers SR, Terry RJ 1965. The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of the adult worms. Parasitology 55: 695-700.

Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J 2006. Schistosomiasis and water resources development, systematic review, metanalysis, and estimates of people at risk. Lancet Infect Dis 6: 411-425.

Stelma FF, Talla I, Sow S, Kongs A, Niang M, Polman K, Deelder AM, Gryseels B 1995. Efficacy and side effects of praziquantel in an epidemic focus of *Schistosoma mansoni*. Am J Trop Med Hyg 53: 167-170.

World Health Organization 2012. Schistosomiasis. Fact sheet N°115, Geneva, WHO.

Table I - Effects of *Mitracarpus frigidus* methanolic extract (MFM) treatment on body weight, relative organ weights and on granuloma formation, after 60 days of infection.

	Non-infected groups				Infected groups			
	Group A Negative control	Group B Praziquantel 200 mg/kg	Group C MFM 100 mg/kg	Group D MFM 300 mg/kg	Group E Negative control	Group F Praziquantel 200 mg/kg	Group G MFM 100 mg/kg	Group H MFM 300 mg/kg
Body weight (g)	26.5 ± 0.5	24.5 ± 0.7	24.8 ± 0.9	25.3 ± 0.6	28.0 ± 0.5	27.9 ± 0.5	29.8 ± 0.8	28.7 ± 0.9
Relative liver weights	4.9 ± 0.2	5.4 ± 0.2	5.4 ± 0.1	5.4 ± 0.2	12.8 ± 0.7	12.0 ± 0.4	10.7 ± 0.4[c]	11.0 ± 0.6[c]
Relative spleen weights	0.3 ± 0.04	0.3 ± 0.04	0.4 ± 0.03	0.5 ± 0.09	3.4 ± 0.2	2.8 ± 0.3	2.4 ± 0,1[c]	2.6 ± 0.2[c]
N[o] of granuloma	-	-	-	-	62.2 ± 1.6	45.1 ± 3.1[c]	49.7 ± 3.9[c]	45.0 ± 3.3[c]
Mean granuloma diameter (µm)	-	-	-	-	7.4 ± 0.3	8.8 ± 0.1[c]	9.4 ± 0.2[c]	9.2 ± 0.2[c]

The values shown are mean ± SEM (n = 10). [a]Statistically different from the non-infected, negative control group (A). [b]Statistically different from the non-infected, positive control group (B). [c]Statistically different from the infected, negative control group (E). [d]Statistically different from the infected, positive control group (F) (ANOVA followed by Bonferroni, $p < 0.05$)

Table II - Results obtained in mice experimentally infected with 50 ± 10 cercariae of *Schistosoma mansoni* (LE strain) treated with *Mitracarpus frigidus*, orally, after 60 days of infection.

	Worm distribution					
	Liver		Mesentery		Total	
Groups	Means of worms (M/F)	Reduction [c] (%)	Means of worms (M/F)	Reduction [c] (%)	Global means (M/F)	Global reduction[c] (%)
Negative control	4.6 ± 1.0	-	19.2 ± 1.4	-	23.0 ± 1.0	-
Praziquantel 200 mg/kg	2.4 ± 0.7[a]	48	9.4 ± 1.7[a]	51	11.8 ± 2.0[a]	49
MFM 100 mg/kg	0.4 ± 0.4 [a,b]	91	6.8 ± 1,6 [a]	65	7.2 ± 1.9 [a]	69
MFM 300 mg/kg	1.6 ± 0.8 [a]	65	8.0 ± 1,5 [a]	58	9.6 ± 1.7 [a]	58

The values shown are mean ± SEM (n = 8). [a]Statistically different from the negative control group. [b]Statistically different from the positive control group (prazinquantel) (ANOVA followed by Bonferroni, $p < 0.05$). [c]Percentage reduction (%) = {1 - (mean of worms in the negative control group / mean of worms in the groups treated)} x 100.

Table III - Effects of *Mitracarpus frigidus* methanolic extract (MFM) treatment on the biochemical and hematological parameters, after 60 days of infection**.**

	Non-infected groups				Infected groups			
	Group A Negative control	Group B Praziquantel 200 mg/kg	Group C MFM 100 mg/kg	Group D MFM 300 mg/kg	Group E Negative control	Group F Praziquantel 200 mg/kg	Group G MFM 100 mg/kg	Group H MFM 300 mg/kg
Total protein (g/dL)	13.2 ± 0.2	10.6 ± 0.3	12.5 ± 0.5	11.4 ± 0.5	5.1 ± 0.2	4.8 ± 0.2	5.7 ± 0.2	5.4 ± 0.2
Albumin (g/dL)	4.4 ± 0.4	3.0 ± 0.2[a]	2.3 ± 0.1[a,b]	1.8 ± 0.1[a,b]	2.8 ± 0.2	2.7 ± 0.2	2.7 ± 0.2	3.1 ± 0.2
Globulin (g/dL)	7.9 ± 1.4	7.6 ± 0.5	10.2 ± 0.6[a,b]	9.8 ± 0.4[a,b]	2.3 ± 0.2	2.0 ± 0.3	3.0 ± 0.3[c,d]	2.4 ± 0.3
A/G	0.5 ± 0.1	0.4 ± 0.05	0.2 ± 0.02	0.2 ± 0.02	1.3 ± 0.3	2.1 ± 0.6[c]	1.0 ± 0.1[d]	1.6 ± 0.3
ALP (U/L)	17.5 ± 1.5	22.7 ± 2.1	12.2 ± 1.4[b]	7.7 ± 1.9[a,b]	55.1 ± 5.0	38.7 ± 3.2[c]	38.7 ± 3.9[c]	41.7 ± 3.4[c]
AST (U/L)	10.9 ± 1.3	18.6 ± 4.3[a]	8.5 ± 1.4[b]	13.2 ± 3.1	44.6 ± 3.8	21.0 ± 3.5[c]	26.8 ± 1.9[c]	32.3 ± 3.0[c,d]
ALT (U/L)	17.9 ± 2.8	9.3 ± 1.6[a]	11.6 ± 2.7	6.3 ± 1.0[a]	33.3 ± 2.3	29.8 ± 3.1	4.2 ± 0.4[c,d]	14.6 ± 3.5[c,d]
Total leukocytes (10^3/µL)	5.1 ± 0.4	4.3 ± 0.4	4.8 ± 0.4	5.0 ± 0.4	8.4 ± 0.6	5.6 ± 0.7[c]	5.7 ± 0.4 [c]	5.2 ± 0.4 [c]
Basophil (%)	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1

Eosinophil (%)	2.1 ± 0.4	2.3 ± 0.2	1.9 ± 0.1	2.1 ± 0.1	20.4 ± 1.2	11.2 ± 1.3[c]	5.1 ± 1.0 [c,d]	6.5 ± 1.0 [c,d]
Monocyte (%)	9.4 ± 0.5	7.4 ± 0.7	5.4 ± 0.7[a]	5.2 ± 1.2[a]	19.6 ± 2.2	10.3 ± 1.3 [c]	4.6 ± 1.0 [c,d]	9.1 ± 1.4 [c]
Neutrophil (%)	43.3 ± 3.5	53.6 ± 2.5[a]	49.0 ± 1.4	49.7 ± 2.3	39.8 ± 2.6	59.4 ± 2.3 [c]	65.8 ± 2.0 [c]	65.4 ± 1.4 [c]
Lymphocyte (%)	42.4 ± 2.6	34.8 ± 2.5[a]	45.8 ± 2.7[b]	47.2 ± 2.0[b]	17.4 ± 2.5	13.9 ± 1.2	20.5 ± 1.5 [d]	16.9 ± 1.3

The values shown are mean ± SEM (n = 10). [a]Statistically different from the non-infected, negative control group (A). [b]Statistically different from the non-infected, positive control group (B). [c]Statistically different from the infected, negative control group (E). [d]Statistically different from the infected, positive control group (F) (ANOVA followed by Bonferroni, $p < 0.05$).

Legends for figures

Figure 1 - *In vitro* schistosomicidal activity of the *Mitracarpus frigidus* methanolic extract (MFM) at 100 µg/mL concentration after 24 hours of incubation. (A) Paired worms incubated in culture medium – the Arrow shows pairs united in gynecophoral canal; (B) Female worm incubated only with culture medium, showing eggs in the first stage of growth (arrow); (C) Male worm showing fully open gynecophoral canal (arrow); (D) Female worm showing contorted muscles; (E) and (F) Presence of vesicles in female worm tegument sections.

Figure 2 - Effects of the *Mitracarpus frigidus* methanolic extract on hepatic granuloma. At 60 days of infection, the hepatic tissues were collected and used for morphological study of the granulomatous area. All granulomas containing a central viable egg were measured and photographed. In (A) general aspects of the hepatic granulomas obtained from infected and untreated animals; (B) the infiltrate around the granuloma in treated animals with a single dose (200 mg/kg) of praziquantel is shown. In (C – 100 mg/Kg) and (D – 300 mg/kg) the granulomas from infected and treated animals after 20 days with different doses of the *M. frigidus* extract, showing that there are no changes in the structure and infiltrate granulomatous in this area.

Figure 1

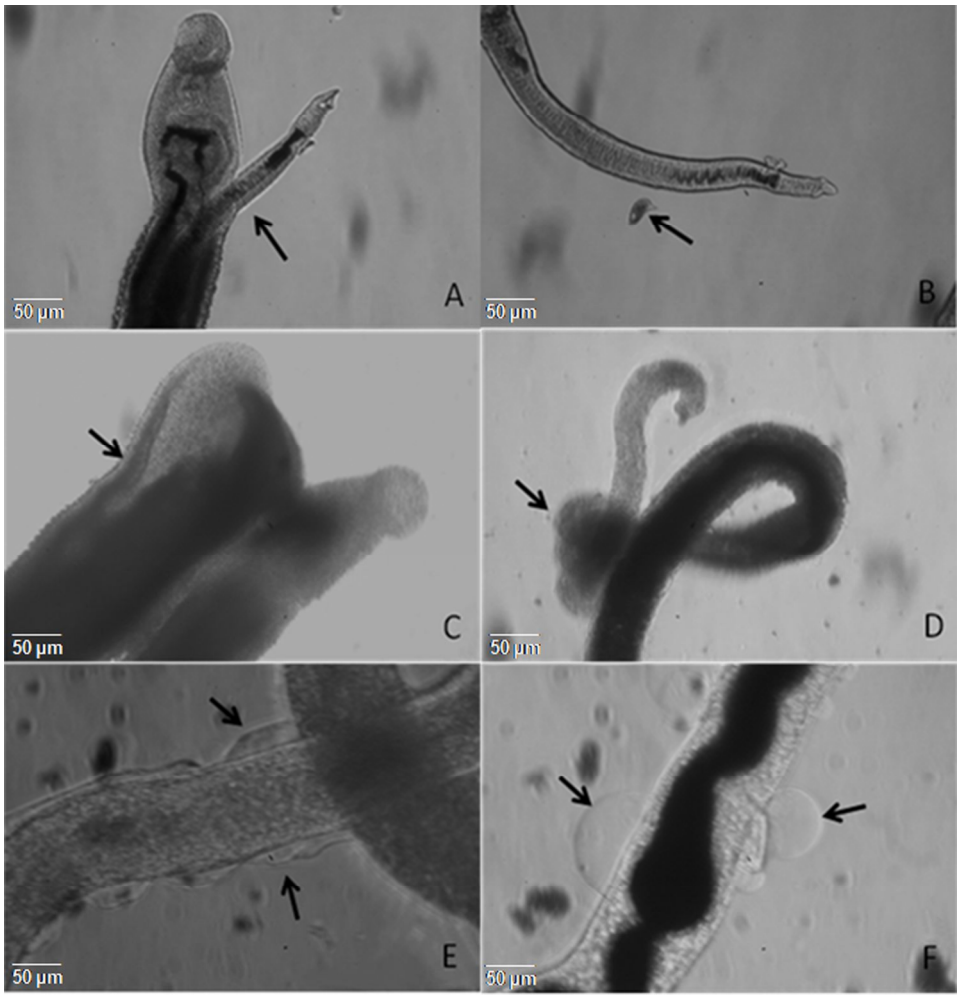
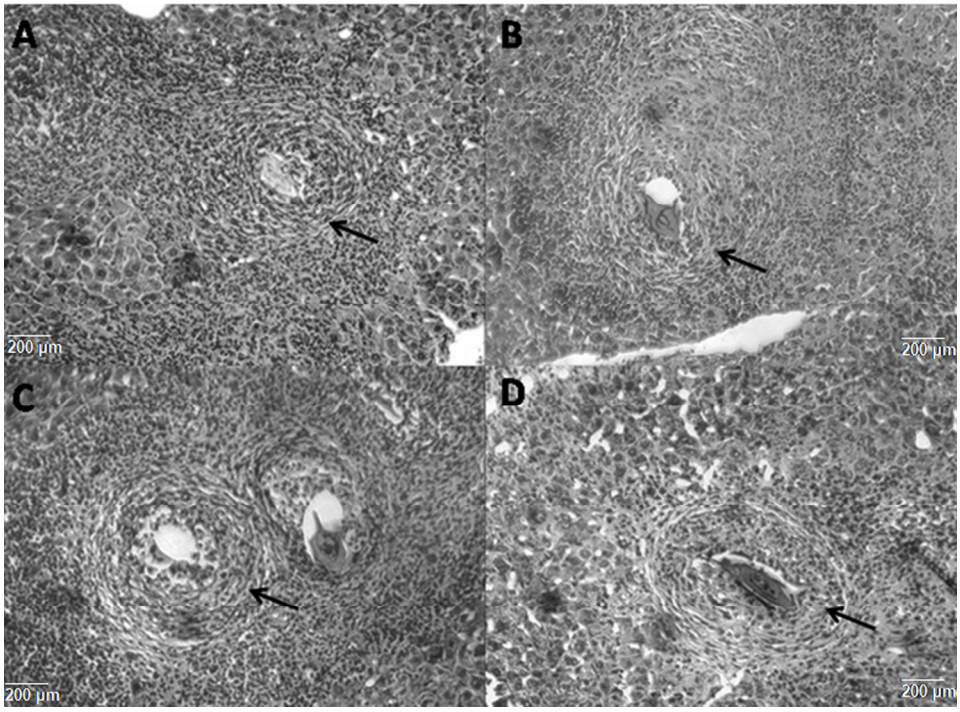


Figure 2



5.7 O artigo a seguir descreve a padronização e o potencial anti-inflamatório *in vivo* do extrato metanólico das partes aéreas de *Mitracarpus frigidus.*

Artigo 7

***Mitracarpus frigidus:* CHEMICAL STANDARDIZATION AND ANTI-INFLAMMATORY AND ANTIOXIDATIVE EFFECTS OF THE AERIAL PARTS METHANOLIC EXTRACT IN WELL-ESTABLISHED ANIMAL MODELS**

Manuscrito em preparação as ser submetido a *Phytomedicine*

Mitracarpus frigidus: Chemical standardization and anti-inflammatory and antioxidative effects of the aerial parts methanolic extract in well-established animal models

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Abstract

This study reports the standardization of the aerial parts of the methanolic extract of *Mitracarpus frigidus* (MFM) and its anti-inflammatory and antioxidative effects. The acute and chronic anti-inflammatory activity was tested by using carrageenan-induced paw edema and peritonitis, ear edema induced by croton oil and by ethyl phenylpropiolate (EPP) and cotton pellet granuloma. The antioxidative activity was assessed by using liver tissue malondialdehyde (MDA) accumulation, and also catalase and cyclooxygenase activities. The index of neutrophils infiltration into inflammation tissues was also determined by means of myeloperoxidase activity determination in ear tissues. The results revealed that MFM showed a more intense acute anti-inflammatory than chronic effects at both doses of 100 and 300 mg/kg when compared with the references drugs indomethacin (1 mg/kg) and dexametasone (10 mg kg) in all models tested. A strong antioxidative activity was also observed for MFM since its administration led to a decrease of some antioxidative markers as malondialdehyde (MDA), catalase and myeloperoxidase. MFM also inhibited COX in a selective manner for COX-2, when compared with indomethacin. MFM presented a potential anti-inflammatory activity against acute and chronic phases of inflammation. This activity may be related to the expressive antioxidative properties observed for MFM. COX blockade or inhibition of this enzyme expression, and other mechanisms, such as inhibition of inflammatory mediators were possibly involved in the effects observed.

Keywords: *Mitracarpus frigidus*; cyclooxygenase; lipoperoxidation; catalase; myeloperoxidase; inflammation

Introduction

Inflammation is a reaction of the tissue blood vessels against an aggressor agent, characterized by the access of liquids and cells to interstice (Gurib-Fakim, 2006). The inflammatory reaction symptoms include blush, heat, tumor, pain and loss of function (Dassoler et al., 2004). The use of anti-inflammatory agents is helpful as a therapeutic option but, unfortunately, the drugs currently available to treat inflammation are associated with several side effects and low efficacy, especially for chronic diseases (Gurib-Fakim, 2006).

Medicinal plants are widely used in traditional medicine of numerous countries to treat different inflammatory conditions and, in particular, skin inflammation processes. However, for many of these plants, the real efficacy and the relevant active principles are unknown. Consequently, experimental studies are needed to verify the pharmacological properties and to identify the active principles of these vegetable species (Sosa et al., 2002).

Mitracarpus frigidus (Willd. ex Roem. & Schult.) K. Shum belongs to the Rubiaceae family and it is found throughout South America including Brazil, where this species is distributed in all states (Pereira et al. 2006). The methanolic extract obtained from this plant (MFM) revealed the presence of flavonoids, tannins, alkaloids, terpenes and quinones, and showed antimicrobial, leishmanicidal, cytotoxic and laxative activities (Fabri et al. 2009; Fabri et al. 2012a). Recently, the pyranonaphthoquinone psychorubrin was firstly isolated from this extract (Fabri et al. 2012b). Moreover, MFM revealed no toxicity signs in rat models (Fabri et al. 2012a).

Based on these considerations, the present study was undertaken to evaluate the anti-inflammatory and antioxidative effects of the methanolic extract obtained from the aerial parts of *Mitracarpus frigidus* (MFM) in well-established animal models, and to identify some of its major compounds.

Materials and Methods

Plant material and extraction

Mitracarpus frigidus aerial parts were collected in Juiz de Fora, Minas Gerais, Brazil, in May, 2011. A voucher specimen (CESJ 46076) was deposited at the Herbarium Leopoldo Krieger of the Federal University of Juiz de Fora. Oven-dried and powdered aerial parts of the plant (1000 g) were extracted by maceration with methanol (5 x 2000 mL) for five days at room temperature and the methanolic extract (MFM) was obtained by evaporation (yield 10% w/w).

High Pressure Liquid Chromatography (HPLC) analysis

HPLC analysis was performed using an Agilent Technologies 1200 Series, with a PDA detector and an automatic injector. The column employed was a Zorbax SB-18; 250 x 4.6 mm, 5 µm particle sizes. Solvents that constituted the mobile phase was A (water pH adjusted to 4.0 with H_3PO_4) and B (acetonitrile). The elution conditions applied were: 0-20 min, 5-80% B and 20-30 min, 80-95% B. The mobile phase was returned to the original composition over the course of 30 minutes, and an additional 5 minutes were allowed for the column to re-equilibrate before injection of the next sample. The sample volume was 20 µL at a concentration of 1 mg/mL, the flow rate of 1 mL/min and the temperature was maintained at 25°C during the analysis. Detection was performed simultaneously at 210, 230, 254 and 280 nm. Four pure standards kaempferol, kaempferol-*O*-rutenoside, rutin and ursolic acid, previously identified in *Mitracarpus* genus (Bisignano et al., 2000; Gbaguidi et al., 2005), were used in this experiment as markers, and psychorubrin isolated from this species was also added (Fabri et al., 2012b). For all experiments, MFM and the standards were dissolved in methanol.

Quantitative analysis

Determination of the content of the compounds (kaempferol, rutin, psychorubrin and ursolic acid) in MFM was performed by the external standard method. Stock solutions of 5, 25, 50, 100, 200 and 500 µg/mL were used. Each determination was carried out in triplicate.

Animals

Female Swiss albino mice weighing 20 - 25 g or male Wistar albino rats with 200 - 250 g were used for the *in vivo* assays. Animals were kept under standard laboratory conditions of temperature (25 ° ± 2 °C) and light/dark cycles (12/12 h). They were provided with standard pellets and tap water *ad libitum*. During the experiments, animals were maintained according to the recommended laboratory ethical guidelines. The study was approved by the Brazilian College of Animal Experimentation (Protocol n° 022/2012).

Carrageenan-induced rat paw edema

Rats were divided into five groups of six animals each as follows: Group A: negative control (0.6% sodium lauryl sulfate); Group B: dexamethasone – 1 mg/kg; Group C: indomethacin – 10 mg/kg; Group D: MFM – 100 mg/kg; Group E: MFM – 300 mg/kg. One hour after the oral treatment, acute paw edema was induced by injecting 0.1 mL of 1% carrageenan in 0.9% saline. Paw volume was measured using plethysmometer by the mercury displacement method between 0 and 3 hours (Winter et al., 1962). The inhibition percentage (%) of paw edema in treated groups was then calculated using the formula: Inhibition (%) = (1 – Vt/Vc) X 100, where Vt = is the edema volume in the drug-treated group; Vc = is the edema volume in the control group.

At the third hour, animals were euthanized. Liver tissues were taken, rinsed in ice-cold normal saline and immediately placed in buffer pH 7.4 (140 nM KCl + 20 nM phosphate) of the same volume and finally homogenized at 4 °C. Then, the homogenate was centrifuged at 11,270 g for 5 min. The supernatant was obtained for the tissue malondialdehyde (MDA) accumulation, and catalase and cyclooxygenase activities. All tests were performed in triplicate.

Malondialdehyde (MDA) determination

MDA was evaluated by the thiobarbituric acid reacting substance (TBARS) method (Nakhai et al., 2007). Briefly, MDA reacted with thiobarbituric acid at a high temperature (100 °C) and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm. MDA levels were expressed as nmol/mg protein.

Catalase activity

Decomposition of H_2O_2 in the presence of catalase was measured at 240 nm in accordance with a standard procedure (Aebi, 1984). Catalase activity was defined as the amount of enzyme required to decompose 1 nmol of H_2O_2 per minute at 37 °C. Results were expressed as µmol/µg protein.

Cyclooxygenase activity (COX) assay

Liver tissue levels of total COX and its two isoforms (COX-1 and COX-2) were determined using a commercially available COX activity assay kit according to the manufacturer's instructions (Cayman No. 760151). The absorbance at 590 nm was measured on a microplate reader. The concentration of total COX was expressed as U/mL in tissue.

Carrageenan-induced mice peritonitis

Inflammation was induced by the modified method of Griswold et al. (1987). Mice were divided into five groups of six animals each (Groups A-E) as described previously. The oral treatments were performed one hour prior to the induction of peritonitis. After this time, carrageenan (0.25 mL, 0.75% w/v in saline) was injected intraperitonially. Four hours later, the animals were euthanized by cervical dislocation, and 2 mL of heparinized phosphate buffered saline (PBS) was injected into the peritoneal cavity. Posteriorly, a gentle massage was made and peritoneal exudates were removed. The total leukocyte count was performed in a Neubauer chamber and the differential cell determination was established. The percentage of leukocyte inhibition was calculated using the following formula: % of leukocyte inhibition = (1- T/C) X 100, where T represents the treated groups' leukocyte count and C represents the control group leukocyte count. Inhibition of neutrophil migration was calculated by the following equation: Inhibition of neutrophil migration = (1- NT/NC) X 100, where NT = neutrophil counts of treated groups and NC = neutrophil counts of the control group.

Ear edema induced by croton oil

The anti-inflammatory activity of MFM was evaluated by the ability of extract to prevent local inflammation induced by croton oil in rat ear (Rocha et al., 2008). MFM was tested both orally and topically. For oral treatment, rats were divided into five groups of six animals each as follows: (Groups A-E) as described previously. One hour after oral treatment, 50 µL of a fresh solution of croton oil (20 % in acetone v/v) were applied topically in the right ear and the same volume of acetone in the left ear of each animal. By the other side, the animals topically treated were divided into five groups of six animals each as follows: Group F: negative control (acetone); Group G: dexamethasone – 0.1 mg/50 µL; Group H: indomethacin – 0.1 mg/50 µL; Group I: MFM – 0.5 mg/50 µL; Group J: MFM – 1.0 mg/50 µL, diluted in acetone. Those animals were topically treated immediately after the application of croton oil. Four hours after treatment, all animals were sacrificed by cervical dislocation and identical discs of 6 mm, with the aid of a punch, were obtained from both the treated and the untreated ear. The discs were weighed and the weight difference of the right and the left ear discs was used as an index of the edema level. The smaller weight difference indicates a larger inflammation inhibition potential. The results were presented as mean ± S.E.M of weight differences within each group.

Myeloperoxidase assay

Myeloperoxidase activity was measured according to the modified method of Bradley et al. (1982). To prepare the tissue homogenates, ear edema tissues were ground with liquid nitrogen in a mortar. The discs were then treated with 1.0 mL of 10 mmol/L phosphate buffer (pH 6.0). The mixtures were homogenized and centrifuged using a refrigerated centrifuge at 1500 g for 10 min at 4 °C and the supernatants used for the determination of enzyme activity. Myeloperoxidase activity was determined by adding 100 µL of the supernatant to 190 µL of 10 mmol/L phosphate buffer (pH 6.0) and 100 µL of 1.5 mmol/L o-dianisidine hydrochloride containing 0.0005% (w/v) hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a UV-vis spectrophotometer. Myeloperoxidase levels in tissues were expressed as µmol/min/µg tissue.

Ear edema induced by ethyl phenylpropiolate (EPP)

Ethyl phenylpropiolate (EPP)-induced ear edema in rats to investigate the topical anti-inflammatory activity of the extract was assessed by the method described by Brattsand et al. (1982). MFM was tested both orally and topically. For oral treatment, rats were divided into five groups of six animals (Groups A-E) as described previously (Groups A-E). One hour after oral treatment, 50 µL of a fresh solution of EPP (20 % in acetone v/v) were topically applied in the right ear and the same volume of acetone in the left ear of each animal. On the other hand, the animals topically treated were divided into five groups of six animals each (Groups F-J) as described previously. Those animals were topically treated immediately after the application of EPP. One hour after treatment, all animals were euthanized by cervical dislocation and identical discs of 6 mm, with the aid of a punch, were obtained from both the treated and the untreated ear. The discs were weighed and the weight difference of the right and the left ear discs was used as an index of the edema level. The smaller weight difference indicates a larger inflammation inhibition potential. The results were presented as mean ± S.E.M of weight differences within each group.

Cotton pellet granuloma

The granulomas were developed in accordance to the method described by Naik et al. (1980). Rats were divided into five groups of six animals each (Groups A-E) as described previously. After one hour of oral administration, sterile cotton pellets weighing 10 mg were subcutaneously implanted in both axillae of rats under anesthesia. The treatments were daily given for 10 days. On the 11th day, rats were euthanized and the cotton pellets with the surrounding granulomas were resected out and their wet and dry weights were recorded.

Plasma MDA estimation

After 11 days of treatment of the cotton pellet-granuloma experiment, 2-4 mL of blood was collected from retro orbital plexuses from each animal using capillary tube, in a vial containing EDTA. Plasma was separated by centrifugation at 3000 g for 10 minutes. The sample was stored at -20 °C and used to estimate MDA levels. The reduced levels of MDA were taken as indicator of antilipoperoxidative activity

which can be taken as the index of the reduced oxidative stress. MDA levels were expressed as nmol/mg protein.

Statistical analysis

Statistical differences between the treatments and the control were evaluated by the ANOVA test followed by the Bonferroni test. A difference in the mean values of $P < 0.05$ was considered to be statistically significant.

Results and Discussion

HPLC assay

The HPLC chromatogram profile for MFM is shown in Figure 1. Five compounds were identified as kaempferol-*O*-rutenoside, rutin, kaempferol, psychorubrin and ursolic acid. The first compound was identified by its ultraviolet spectrum (Cardoso et al., 2005) and the others by external standard.

For better global positioning of any herbal drug it has become important to generate chemoprofiling data on the basis of maximum available number of markers for the extract (Malik et al. 2007). For this, the chemoprofiling data of MFM based on four markers (rutin, kaempferol, ursolic acid and psychorubrin) was performed for chemical standartization. Ursolic acid was found to be the most abundant compound with 275.3 ± 0.8 mg/g in MFM, while the contents of 56.8 ± 1.2, 21.5 ± 0.5 and 4.2 ± 0.2 mg/g were found for rutin, kaempferol and psychorubrin, respectively.

Figure 1

Carrageenan-induced paw edema

The acute anti-inflammatory effects of MFM on carrageenan-induced edema in the hind paws of rats are shown in Table 1. Carrageenan-induced paw edema remained even 3 h after infection into the subplantar region of rat paw. MFM showed a significant reduction in the paw edema volume in relation to the negative control and the maximum inhibition percentage was observed 1 h and 30 min after the edema formation for 100 and 300 mg/kg, respectively. Treatments with MFM showed similar activity compared with dexamethasone and indomethacin used as positive controls (Table 1).

able 1

Carrageenan-induced paw edema is a well-established *in vivo* model of inflammation and has been commonly used to evaluate the anti-edematous effect of natural products (Vyas et al., 2010). Edema formation, in this classical experiment, is a biphasic response. In the early phase, 0-2 h after carrageenan injection, there is a release of histamine, serotonin, and bradykinin on vascular permeability. The inflammatory edema reaches the maximum level at the third hour (Kirkov et al., 1992) due to the potentiating effect of bradykinin on mediator release and prostaglandins, producing edema after mobilization of the leukocytes (Arulmozhi et al., 2005, Olajide et al., 1999). In this study, MFM and positive controls showed anti-inflammatory effects in carrageenan-induced rat paw edema in both phases, suggesting that the significantly higher anti-inflammatory activity may be due to the inhibition of inflammatory mediators, such as histamine and serotonin released during the first phase, and prostaglandins and bradykinin released during the second phase of inflammation.

Some studies indicated that inflammatory effect induced by carrageenan is correlated with free radicals (Dawson et al., 1991). Carrageenan-induced inflammatory response has been associated with neutrophil infiltration and with the production of neutrophil-derived free radicals, for instance superoxide, hydroxyl radicals and hydrogen peroxide, as well as due to the release of other neutrophils-derived mediators (Lu et al., 2011). A previous study had established that malondialdehyde (MDA) production was due to the free radical attack in the plasma membrane (Janero, 1990). Hence, carrageenan-induced inflammation results in an accumulation of MDA (Bilici et al., 2002), and this data is used to evaluate the free radical generation in liver tissues after inflammatory states (Lu et al., 2011).

Therefore, in order to explore the effects of the antioxidant defenses on the acute inflammation process in liver tissues, the MDA and the antioxidant enzyme catalase levels were evaluated. In this step, it was observed that MDA level significantly decreased in the animals treated with MFM (Figure 2A), indicating a reduction of oxidative stress as a result of anti-lipoperoxidative activity.

Catalase is a highly reactive enzyme, reacting with hydrogen peroxide to form water and molecular oxygen, and can form methanol, ethanol, formic acid or phenols by donating hydrogen (Matés, Sanchez-Jimenez, 1999). During normal body conditions, a balance between free radicals and the endogenous antioxidants exists,

but in a traumatic state, like inflammation, this event diminishes and reactive oxygen metabolites dramatically increase. The increased generation of oxygen free radicals in the extracellular space is verified in the inflammatory state, in which the relatively low concentrations of catalase increase the susceptibility of extracellular components to oxygen radical injury, and may stimulate chemotaxis for other inflammatory cells (Halici et al., 2007). In the present study, it was observed that MFM decreased catalase activity, which was probably due to the inhibition of inflammation observed for MFM (Figure 2B).

ure 2

Since its identification in 1971, there have been many studies on the actions of cyclooxygenase (COX or prostaglandin H_2 synthase) and its inhibition (Vane, Botting, 1998), which has been associated with anti-platelet, antipyretic, anti-nociceptic, and anti-inflammatory effects (Seaver and Smith, 2004). There are two isoforms of COX, COX-1 and COX-2. COX-1 is constitutively produced by the body and is involved in protecting the gastrointestinal mucosal lining as well as maintaining kidney and platelet function (DeWitt, 1991), and COX-2 is involved with pain, inflammation, fever and possibly tumor growth (Fu et al., 1990).

Table 2 shows the effect of MFM on cyclooxygenase expression. The results suggested that MFM mechanism of action was probably due not only to COX direct blockade, since MFM (300 mg/kg) showed strong anti-inflammatory activity, but high total COX expression, unlike indomethacin (Table 1), a COX direct inhibitor (Vane, Botting, 1998). While MFM (100 mg/kg) response was similar to indomethacin, MFM (300 mg/kg) behavior was similar to dexamethasone, a steroid anti-inflammatory, which mechanism of action involves the inhibition of many inflammatory mediators synthesis (Masferrer et al., 1990). Furthermore, MFM (100 and 300 mg/kg) showed great inhibition of COX-2 isoform when compared with indomethacin. Most anti-inflammatory drugs available for therapy, such as indomethacin, acts in a non-selective manner, resulting in adverse reactions, mainly gastric disturbance which is due to COX-1 blockade (Fosslien, 1998).

able 2

Carrageenan-induced peritonitis

Mouse carrageenan peritonitis was also used to evaluate anti-inflammatory activity. MFM also inhibited peritoneal leukocyte migration (Table 3).

Leukocyte aggregation at the site of inflammation is a fundamental event in the inflammatory process. Intraperitonial injection of carrageenan leads to inflammation of the peritoneum as a result of macrophages in the carrageenan insulated tissue (Vyas et al., 2010). It was followed by an influx of neutrophils and mononuclear leukocytes, with increased levels of plasma/peritoneal fluid chemoatractants and with the sequential appearance of exudate proinflammatory cytokines (Meade et al., 1996). In this investigation, MFM (100 and 300 mg/kg) inhibited the carrageenan-induced leukocyte migration in the peritonitis model in mice, and significantly reduced the migration of neutrophils, indicating their anti-inflammatory action by inhibiting chemotaxis (Table 3).

ıle 3

Ear edema induced by croton oil

Croton oil-induced dermatitis in rats ear was used as a model of acute inflammation. The inflammatory reaction induced by the phorbol esters of croton oil is an acute response characterized by edema, neutrophil infiltration, prostaglandins production and increases in vascular permeability. The action of croton oil was believed to be involved in or to be dependent on arachidonic acid release and metabolism by both cyclooxygenase and lipoxygenase enzyme pathways (Bao et al., 2009). In this study, the anti-inflammatory effect of MFM was evaluated both for oral and topical treatments. As shown in Figure 3A and 3C, when used orally and topically, respectively, the anti-inflammatory effect of MFM was similar to that of the dexamethasone and indomethacin used as positive controls. Those results together suggested that MFM presented similar pharmacological properties of those of lipoxygenase and cyclooxygenase inhibitors (Hong et al., 2001).

In the present study, the changes of myeloperoxidase activity in ear tissues, an index of neutrophils infiltration into inflammation tissues, were also determined (Figure 3B and D). As can be observed from those figures, the orally and topically administration of MFM and positive controls, indomethacin and dexamethasone, significantly decrease the level of myeloperoxidase activity in comparison with negative control (vehicle). Myeloperoxidase is an enzyme found primarily in azurophilic granules of neutrophils, which is used as a marker for tissue neutrophil content and its inhibition implies the presence of anti-inflammatory activity (Bradley et al., 1982). Tissue myeloperoxidase activity is a sensitive and specific marker for

acute inflammation and reflects polymorphonuclear cell infiltration of the parenchyma (Paino et al., 2005).

gure 3

Ear edema induced by ethyl phenylpropiolate (EPP)

EPP causes instant irritation of the rat ear, which leads to fluid accumulation and edema characteristic of the acute inflammatory response. Suppression of this response is a likely indication of anti-inflammatory effect (Atta and Alkohafi, 1998). EPP causes a release of many inflammatory mediators such as kinin, serotonin (5-HT) and prostaglandins (PGs) (Brattsand et al., 1982). MFM (100 and 300 mg/kg) and positive controls (dexamethasone and indomethacin) exerted an inhibitory effect, both for oral and topical treatments, on the ear edema formation induced by EPP in comparison with negative control (vehicle) (Figure 4A and B).

ure 4

Cotton pellets-induced granuloma

The effects of MFM on the proliferative phase of inflammation are summarized in Figure 5. It was seen that MFM was responsible for anti-inflammatory effect, which would be calculated depending on the moist and dry weight of cotton pellets. According to these results, the antiproliferative effects of MFM (100 and 300 mg/kg) and dexamethasone were calculated as 19, 25 and 50% inhibition, respectively (Figure 5A). After they were dried, the antiproliferative effects were calculated on the basis of dry weight pellets. The inhibition of inflammation by MFM (100 and 300 mg/kg) and dexamethasone were established as 20, 28 and 48%, respectively (Figure 5B).

The cotton pellet granuloma method is widely used to evaluate the transudative and proliferative components of the chronic inflammation. The moist weight of the cotton pellet correlates with the transudate. The dry weight of the pellet correlates with the amount of the granulomatous tissue (Joseph et al., 2010). This effect may be due to the cellular migration to injured sites and accumulation of collagen and mucopolysaccharide (Monthana, 2011).

Also, an imbalance between free radical-generating and radical-scavenging systems results in oxidative stress and it is also documented in chronic inflammation (Robbin et al., 2007). MFM presented chronic anti-inflammatory effects, but although it was not so expressive, in oxidative stress model, MFM (100 and 300 mg/kg)

demonstrated significant reduction in MDA level in plasma, indicating reduction of oxidative stress by anti-lipoperoxidative activity (Figure 5C).

It is important to point out that the animals treated with indomethacin died after four days of treatment. Hematuria and melena were observed, suggesting gastric bleeding, probably due to COX-1 inhibition. The rats treated with MFM (100 and 300 mg/kg) did not show any gastric disturbance, which reinforces the possibility of MFM mechanism of action does not involve only COX inhibition. The animals treated with dexamethasone had a significant weight loss during the days of treatment, unlike the vehicle and MFM (Figure 5D), probably due to protein catabolism induced by corticosteroids, resulting in the reduction of muscle mass (Tomas et al., 1979). By the other side, animals treated with MFM presented normal weight gain.

ure 5

Conclusion

MFM presented a potential anti-inflammatory activity against acute and chronic phases of inflammation. This activity may be related to the expressive antioxidative properties observed for MFM. COX blockade or inhibition of this enzyme expression, and other mechanisms, such as inhibition of inflammatory mediators were possibly involved in the effects observed. Studies focusing the isolation and structure elucidation of anti-inflammatory and antioxidative active constituents from *Mitracarpus frigidus* are in progress.

Acknowledgments

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References

Aebi, H., 1984**.** Catalase. Method. Enzymol*.* 105, 121-126.

Arulmozhi, D.K., Veeranjaneyulu, A., Bodhankar, S.L., Arora, S.K., 2005. Pharmacological investigation of *Sapindus trifolius* in various *in vitro* and *in vivo* models of inflammation. Indian J. Pharmacol. 37, 96-102.

Atta, A.H., Alkohafi, A., 1998. Antinociceptive and anti-inflammatory effects of some Jordanian medicinal plants extracts. J. Ethnopharmacol. 60(2),117-124.

Bao, L., Yao, X., Xu, J., Guo, X., Liu, H., Kurihara, H., 2009. Effects of *Pithecellobium clypearia* Benth extract and its main components on inflammation and allergy. Fitoterapia 80, 349-353.

Bilici, D., Akpinar, E., Kiziltunc, A., 2002. Protective effect of melatonin in carrageenan-induced acute local inflammation. Pharmacol. Res*.* 46, 133-139.

Bisignano, G., Sanogo, R., Marino, A., Aquino, R., D'Angelo, V., Germanò, M.P., De Pasquale, R., Pizza, C., 2000. Antimicrobial activity of *Mitracarpus scaber* extract and isolated constituents. Lett. Applied Microbiol*.* 30, 105-108.

Bradley, P.P., Priebat, D.A., Christensen, R.D., Rothstein, G., 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J. Invest. Dermatol. 78, 206–209.

Brattsand, R., Thalen, A., Roempke, K., Kallstrom, L., Gruvstad, E., 1982. Influence of 16 alpha, 17 alpha-acetal substitution and steroid nucleus fluorination on the topical to systemic activity ratio of glucocorticoids. J. Steroid Biochem. 16(6), 779-786.

Cardoso, C.L., Silva, D.H.S., Castro-Gamboa, I., Bolzani, V.S., 2005. New biflavonoid and other flavonoids from the leaves of *Chimarrhis turbinata* and their antioxidant activities. J. Braz. Chem. Soc. 16(6B), 1353-1359.

Dassoler, M., Schwanz, M., Busseto, F., Moreira, E.A., Gutierrez, L., 2004. Perfil fitoquímico e ensaio farmacológico de *Averrhoa carambola* L. (Oxalidaceae). J. Bra. Fitomedicina 2, 4-8.

Dawson, J., Sedgwick, A.D., Edwards, J.C., Lees, P.A., 1991. Comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. Int. J. Tissue React., 13, 171-185.

DeWitt, D., 1991. Prostaglandin endoperoxidaxe synthase: regulation of enzyme expression. Biochim. Biophys. Acta 1083, 121-134.

Fabri, R.L., Nogueira, M.S., Braga, F.G., Coimbra, E.S., Scio, E., 2009. *Mitracarpus frigidus* aerial parts exhibited potent antimicrobial, antileishmanial, and antioxidant effects. Bioresour. Technol. 100, 428-433.

Fabri, R.L., de Oliveira, A.D.M., Florêncio, J.R., Cardoso, G.M., de Souza-Fagundes, E.M., Castanon, M.C., Scio, E., 2012a. *In vivo* laxative and toxicological evaluation and *in vitro* antitumor effects of *Mitracarpus frigidus* aerial parts. J. Pharm. Pharmacol. 64, 439-448.

Fabri, R.L., Grazul, R.M., Carvalho, L.O., Coimbra, E.S., Cardoso, G.M.M., Souza-Fagundes, E.M., Da Silva, A.D., Scio, E., 2012b. Antitumor, antibiotic and antileishmanial properties of the pyranonaphthoquinone psychorubrin from *Mitracarpus frigidus.* Ann. Acad. Bra. Sci. 84(4), 1081-1090.

Follien, E., 1998. Adverse effects of nonsteriodal anti-inflammatory drugs on the gastro-intestinal tract. Ann. Clin. Lab. Sci. 28, 67-81.

Fu, J.Y., Masferrer, J.L., Seibert, K., Raz, A., Needleman, P., 1990. The induction and suppression of prostaglandin H_2 synthase (cyclooxygenase) in human monocytes J. Biol. Chem. 265, 16737-16740.

.

Gbaguidi, F., Accrombessi, G., Moudachirou, M., Quetin-Leclercq, J., 2005. HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis*. J. Pharm. Biomed. Anal. 39, 990-995.

Griswold, D.E., Mrarshall, P.J., Webb, E.F., Godfrey, R., Newton, J., Diamartina, M.J., Sarau, H.M., Gleason, J.G., Poste, G., Hanna, N., 1987. SK&F, 86002: a structurally novel anti-inflammatory agent that inhibits lipoxygenase- and cyclooxygenase-mediated metabolism of arachidonic acid. Bioch. Pharmacol. 36, 3463-3470.

Gurib-Fakim, A., 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. Mol. Aspects Med. 27, 1-93.

Halici, Z., Dengiz, G.O., Obadasoglu, F., Suleyman, H., Cadirci, E., Halici, M., 2007. Amiodarone has anti-inflammatory and anti-oxidative properties: An experimental study in rats with carrageenan-induced paw edema. Eur. J. Pharmacol. 566, 215-221.

Hong, J.G., Smith, T.J., Ho, C.T., August, D.A., Yang, C.S., 2001. Effects of purified green and black tea polyphenols on cyclooxygenase and lipoxygenase dependent metabolism of arachidonic acid in human colon mucosa and colon tumor tissues. Biochem. Pharmacol. 62, 1175-1183.

Janero, D.R., 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic. Biol. Med. 9, 515-540.

Joseph, J.M., Sowndhararajan, K., Manian, S., 2010. Evaluation of analgesic and antiinflammatory potential of *Hedyotis puberula* (G. Don) R. Br. ex Arn. In experimental animal models. Food Chem. Toxicol. 48, 1876-1880.

Kirkova, M., Kassabova, T., Russanov, E., 1992. *In vivo* effects of indomethacin. I. Activity of antioxidant enzymes and lipid peroxidation. Gen. Pharmacol. 23, 503-507.

Lu, T.C., Liao, J.C., Hung, T.H., Lin, Y.C., Liu, C.Y., Chiu, Y.J., Peng, W.H., 2011. Analgesic and anti-inflammatory activities of the methanol extract from *Pogostemon cablin*. Evid. Based Complement. Alternat. Med. 2011,1-9.

Malik, F., Singh, J., Khajuria, A., Suri, K.A., Satti, N.K., Singh, S., Kaul, M.K., Kumar, A., Bhatia, A., Qazi, G.N., 2007. A standardized root extract of *Withania somnifera* and its major constituent withanolide-A elicit humoral and cell-mediated immune responses by up regulation of Th1-dominant polarization in BALB/c mice. Life Sci. 8(16), 1525-1538.

Masferrer, J.L., Ben, S.Z., Seibert, K., Needleman, P., 1990. Selective regulation of cellular cyclooxygenase by dexamethasone and endotoxin in mice. J. Clin. Invest. 86, 1375-1379.

Matés, J.M., Sánchez-Jiménez, F., 1999. Antioxidant enzymes and their implications in pathophysiologic processes. Front. Biosci. 4, 339-345.

Meade, C.J., Turner, G.A., Bateman, P.E., 1996. The role of polyphosphoinositides and their break down products in A23187 induced release of arachidonic acid from rabbit polymorphonuclear leukocytes. J. Biol. Chem. 23, 425-436.

Monthana, R.A.A., 2011. Anti-inflammatory, antinociceptive and antioxidant activities of the endemic Soqotraen *Boswellia elongata* Balf. F. and *Jatropha unicostata* Balf. F. in different experimental models. Food Chem. Toxicol. 49, 2594-2599.

Naik, V.R., Agshikar, N.V., Abraham, G.J.S., 1980. Analgesic and anti-inflammatory activity in alcoholic extracts of *Cucumis trigonus Roxburghii*: a preliminary communication. Pharmacol. 20, 52-56.

Nakhai, L.A., Mohammadirad, A., Yasa, N., Minaie, B., Nikfar, S., Ghazanfari, G., Zamani, M.J., Dehghan, G., Amshidi, H., Boushehri, V.S., Khorasani, R., Abdollahi, M., 2007. Benefits of *Zataria multiflora* Boiss in experimental model of mouse inflammatory bowel disease. Evid. Based Complement. Alternat. Med. 4, 43-50.

Olajide, O.A., Makinde, J.M., Awe, S.O., 1999. Effects of the aqueous extract of *Bridelia ferruginea* stem bark on carrageenan-induced oedema and granuloma tissue formation in rats and mice. J. Ethnopharmacol. 66, 113-117.

Paino, I.M.M., Ximenes, V.F., da Fonseca, L.M., Kanegae, M.P.P., Khalil, N.M., Brunetti, I.L., 2005. Effect of therapeutic plasma concentrations of non-steroidal anti-inflammatory drugs on the production of reactive oxygenspecies by activated rat neutrophils. Braz. J. Med. Biol. Res. 38, 543-551.

Pereira, Z.V., Carvalho-Okano, R.M., Garcia, F.C.P., 2006. Rubiaceae Juss. da Reserva Florestal Mata de Paraíso, Viçosa, MG, Brasil. Acta Bot. Bras. 20(10), 207-224.

Robbin, S.L., Cotran, R.S., Kumar, V., Collins, T., 2007. Pathological Basis of Disease. 7th ed, Har Cocert Asia, Saunders Company, pp. 73-74.

Rocha, F.F., Neves, E.M.N., Costa, E.A., Matos, L.G., Müller, A.H., Guilhon, M.S.P., Cortes, W.S., Vanderlinde, F.A., 2008. Evaluation of antinociceptive and anti-inflammatory effects of *Croton pullei* var. *glabrior* Lanj. (Euphorbiaceae). Braz. J. Pharmacog. 18, 344-349.

Seaver, B., Smith, J.R., 2004. Inhibition of COX isoforms by nutraceuticals. J. Herb. Pharmacother. 4(2), 11-18.

Sosa, S., Balick, M.J., Arvigo, R., Esposito, R.G., Pizza, C., Altinier, G., Tubaro, A., 2002. Screening of the topical antiinflammatory activity of some Central American plants. J. Ethnopharmacol. 81, 211-215.

Tomas, F.M., Munro, H.N., Young, V.R., 1979 Effect of glucocorticoid administration on the rate of muscle protein breakdown *in vivo* in rats, as measured by urinary excretion of NT-methylhistidine. Biochemical. J. 178, 139-146.

Vane, J.R., Botting, R.M. 1998. Mechanisms of action of anti-inflammatory drugs. Int. J. Tissue React. 20, 3-15.

Vyas, B.A., Vyas, R.B., Joshi, S.V., Santani, D.D., 2010. Evaluation of anti-inflammatory activities of n-butanol extract from *Pergularia daemia* in experimental animal models. J. Pharm. Res. 3(12), 3039-3044.

Winter, C.A., Risley, E.A., Nuss, G.W., 1962. Carrageenan-induced inflammation in hind paws of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. Med. 111, 544-547.

Table 1 – Effects of the methanolic extract from aerial parts of *Mitracarpus frigidus* (MFM) on carrageenan-induced rat paw edema.

Treatment	Dose (mg/kg)	Time, paw volume in mL (% inhibition)				
		0 h	1/2 h	1 h	2 h	3 h
Vehicle	-	0.24 ± 0.08	0.32 ± 0.05	0.38 ± 0.10	0.60 ± 0.10	0.55 ± 0.04
Dexamethasone	1	0.02 ± 0.01	0.19 ± 0.05[a] (41)	0.16 ± 0.03[a] (58)	0.23 ± 0.03[a] (62)	0.29 ± 0.02[a] (47)
Indomethacin	10	0.06 ± 0.03	0.11 ± 0.02[a] (66)	0.10 ± 0.04[a] (74)	0.20 ± 0.04[a] (67)	0.25 ± 0.05[a] (55)
MFM	100	0.11 ± 0.04	0.17 ± 0.04[a] (47)	0.12 ± 0.02[a] (68)	0.21 ± 0.03[a] (65)	0.25 ± 0.03[a] (55)
MFM	300	0.03 ± 0.01	0.06 ± 0.01[a,b,c] (81)	0.10 ± 0.04[a] (74)	0.13 ± 0.03[a,b] (78)	0.29 ± 0.05[a] (47)

Values represent mean ± S.E.M. (n = 6) [a]Statistically different from the negative control (vehicle) [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, $P < 0.05$)

Table 2 - Effect of methanolic extract from *Mitracarpus frigidus* (MFM) on cyclooxygenase (COX) expression in carrageenan-induced rat paw edema.

Treatment	Dose (mg/kg)	Total COX (U/mL)	COX-1 (%)	COX-2 (%)
Vehicle	-	25.5 ± 1.0	25 ± 0.8	75 ± 1.2
Dexamethasone	1	22.0 ± 1.4[c]	49 ± 1.2[a]	51 ± 0.7[a]
Indomethacin	10	12.7 ± 0.8[a,b]	45 ± 0.3[a]	55 ± 0.6[a]
MFM	100	13.5 ± 1.0[a,b]	54 ± 1.0[a,c,d]	46 ± 0.9[a,c]
MFM	300	20.9 ± 1.3[c]	55 ± 1.2[a,c,e]	45 ± 0.2[a,c]

Values represent mean ± S.E.M. (n = 6) [a]Statistically different from the negative control (vehicle) [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) [d,e]Statistically different for the COX-2 isoform expression to MFM 100 and 300 mg/kg, respectively (ANOVA followed by Bonferroni, $P < 0.05$).

Table 3 – Effect of methanolic extract from *Mitracarpus frigidus* (MFM) on leukocyte migration and neutrophil migration in peritoneal exudation in carrageenan-induced mice.

Treatment	Dose (mg/kg)	Leukocytes (10^5/mL)	Leukocyte inhibition (%)	Neutrophils (10^5/mL)	Neutrophil inhibition (%)
Vehicle	-	8.5 ± 0.6	-	5.9 ± 0.2	-
Dexamethasone	1	2.1 ± 0.4[a]	75.3	1.3 ± 0.3[a]	77.4
Indomethacin	10	2.3 ± 0.3[a]	73.5	1.4 ± 0.3[a]	75.8
MFM	100	1.8 ± 0.4[a]	78.8	1.2 ± 0.3[a]	78.9
MFM	300	1.4 ± 0.4[a]	83.5	0.9 ± 0.3[a]	83.4

Values represent mean ± S.E.M. (n = 6) [a]Statistically different from the negative control (vehicle) [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, $P < 0.05$).

Legend for figures

Figure 1 - HPLC chromatogram of *Mitracarpus frigidus* methanolic extract (MFM). The analysis was done in a linear gradient of a binary solvent system A (water pH adjusted to 4.0 with H_3PO_4):B (acetonitrile). The elution conditions applied were: 0-20 min, 5-80% B and 20-30 min, 80-95% B. It was run at a flow rate of 1 mL/min over 30 minutes, with an injection volume (“loop”) of 20 µL and UV detection was at 230 nm.

Figure 2 - Effects of MFM and positive controls (dexamethasone and indomethacin) on changes in activities of the A) malondialdehyde (MDA) accumulation and B) catalase enzyme in carrageenan-induced paw edema (3rd hour) in rats. [a]Statistically different from the negative control (vehicle). [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, $P < 0.05$).

Figure 3 - Effects of MFM and positive controls, dexamethasone and indomethacin: A and C) on the weight of ear administratered orally and topically, respectively; B and D) on changes in activity of the myeloperoxidase enzyme administratered orally and topically, respectively, in ear edema induced by croton oil in rats. [a]Statistically different from the negative control (vehicle). [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, $P < 0.05$).

Figure 4 - Effects of MFM and positive controls (dexamethasone and indomethacin) on the weight of ear administratered A) orally and B) topically in ear edema induced by EPP in rats. [a]Statistically different from the negative control (vehicle). [b,c]Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, $P < 0.05$).

Figure 5 - Effects of MFM and positive control (dexamethasone) on cotton pellets-induced granuloma in rats. A) Wet weight of cotton pellet (g); B) dry weight of cotton pellet (g); C) tissue MDA accumulation and D) weight gain of treatments. [a]Statistically

different from the negative control (vehicle). [b]Statistically different from the positive controls (dexamethasone) (ANOVA followed by Bonferroni, $P < 0.05$).

Figure 1

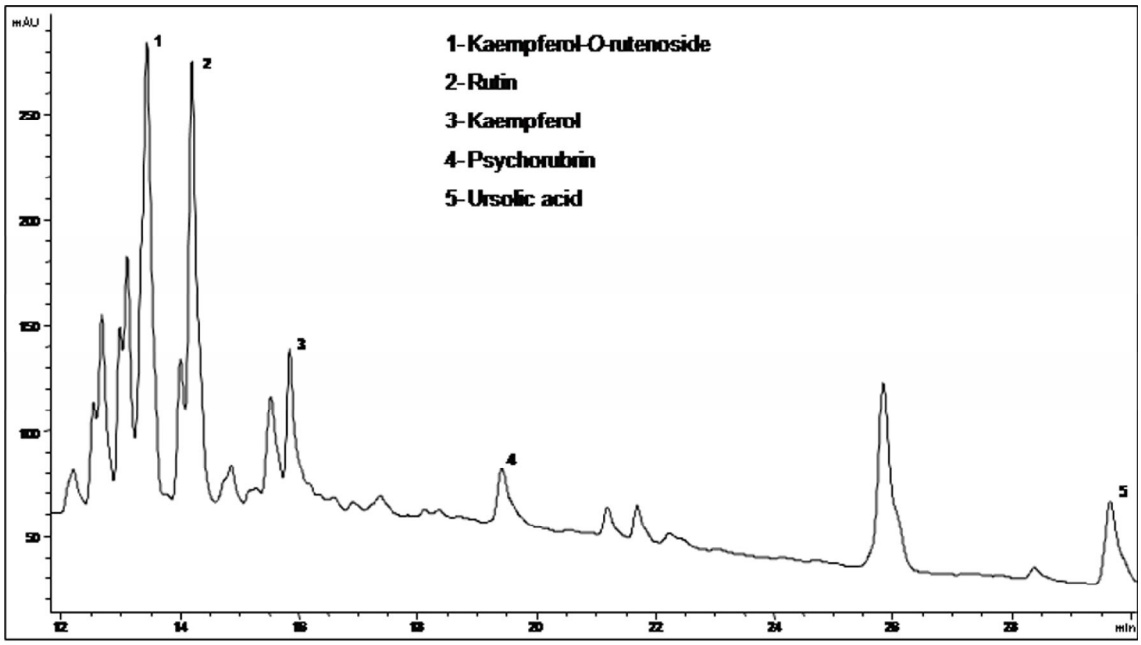
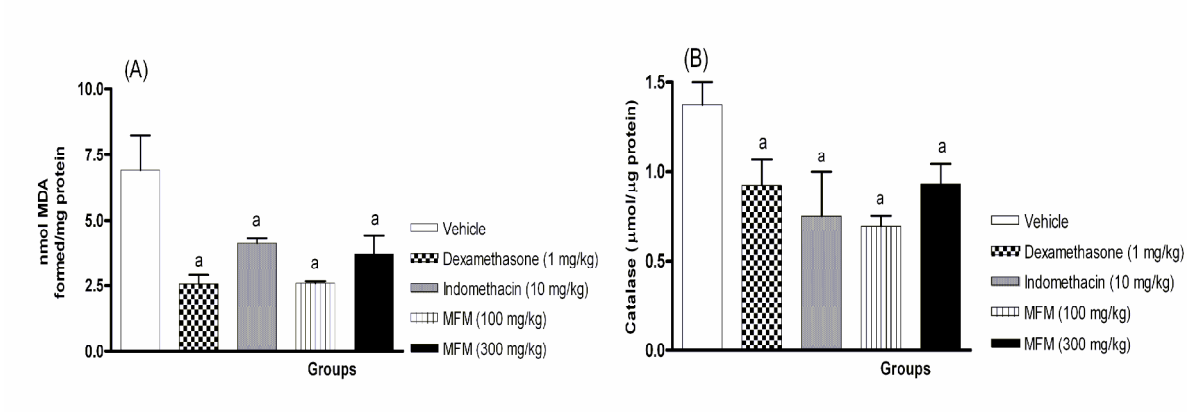


Figure 2



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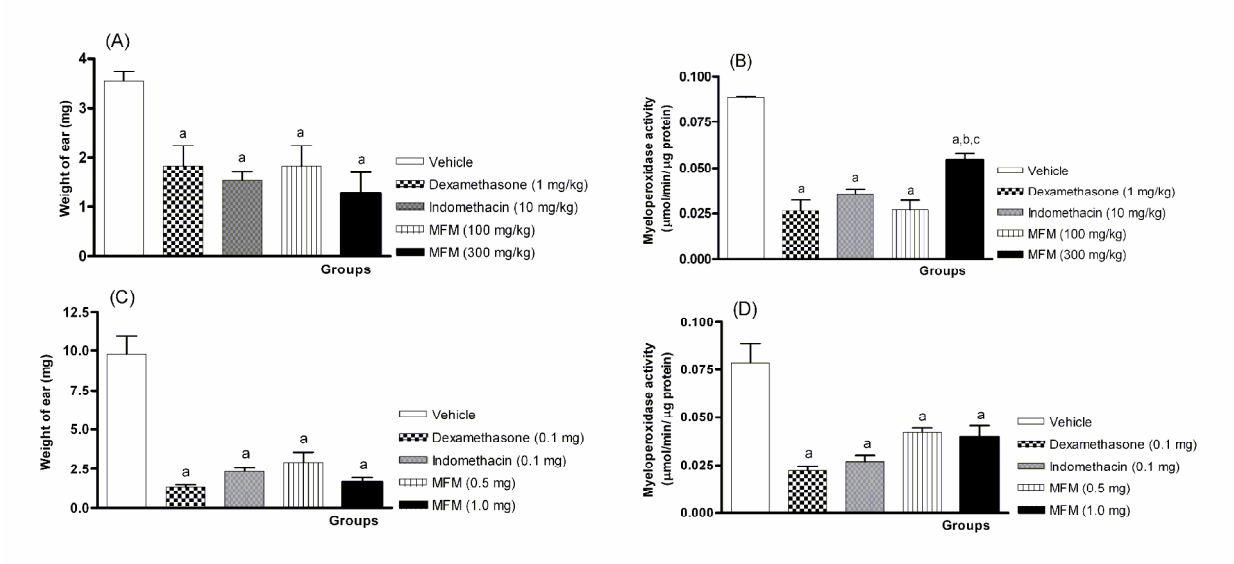


Figure4

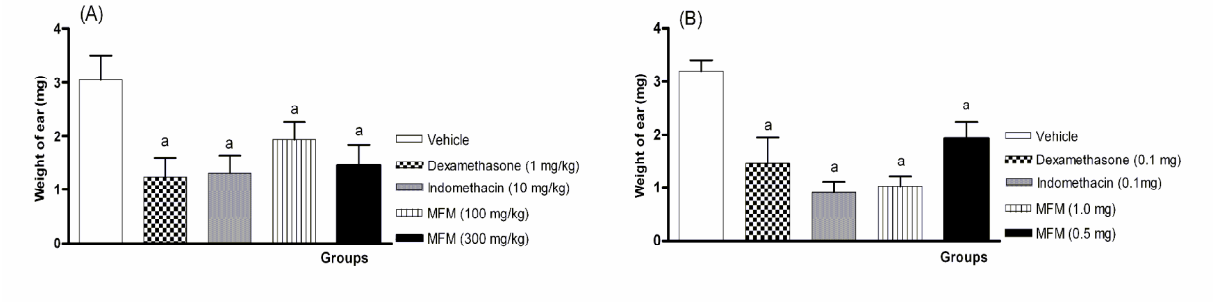
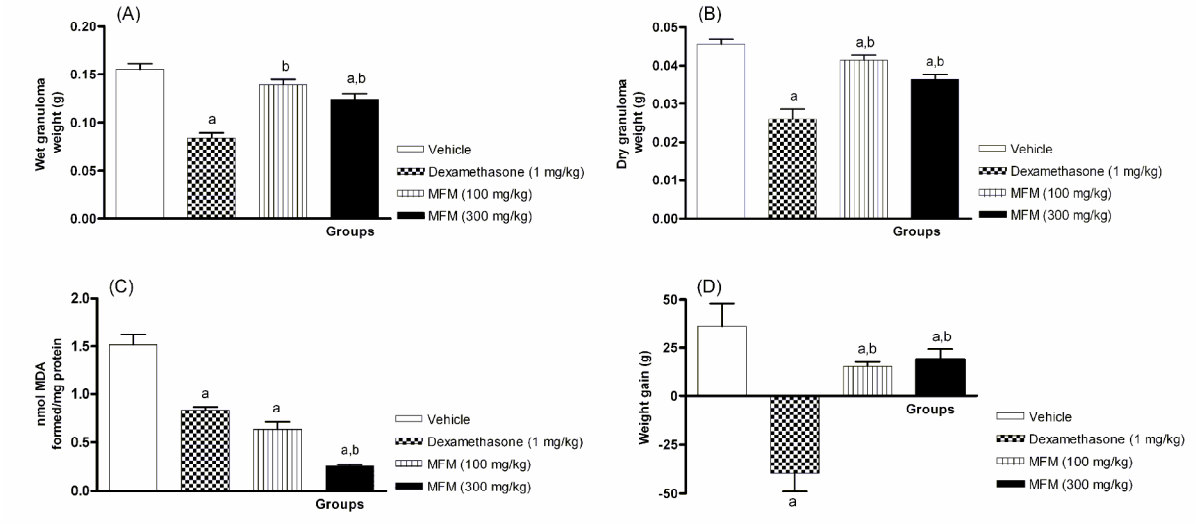


Figure 5



6 CONSIDERAÇÕES FINAIS E PERPECTIVAS

De acordo com os resultados encontrados nesse trabalho, podemos concluir que:

- A padronização química do extrato metanólico mostrou que o triterpeno pentacíclico, ácido ursólico, foi a substância majoritária com 275,3 ± 0,8 mg/g, seguido de dois flavonoides, rutina e campferol, e uma piranonaftoquinona, psicorubrina, com teores de 56,8 ± 1,2; 21,5 ± 0,5 e 4,2 ± 0,2 mg/g, respectivamente, que podem estar relacionadas com as atividades fármaco-biológicas. Além disso, foi detectado o flavonoide glicosilado, campferol-3-*O*-rutInosídeo.
- O extrato metanólico apresentou atividade esquistossomicida tanto *in vitro* quanto *in vivo* com considerável pela redução da carga parasitária. Destaca-se que no experimento *in vivo* não houve alterações na função hepática dos animais. Sendo assim, a espécie pode ser considerada uma alternativa de estudos para o tratamento da esquistossomose.
- O extrato metanólico apresentou atividade antiinflamatória aguda e crônica para os modelos testados, sendo a resposta aguda mais expressiva. A partir destes resultados, verificou-se que o mesmo inibe o processo de migração celular mediada pela inflamação e também diminui o processo oxidativo do organismo, evidenciado pela baixa concentração de MDA, catalase e mieloperoxidase. Em relação à expressão de ciclooxigenase, verificou-se que o extrato possui atividade inibitória maior contra a isoforma COX-2.
- O extrato metanólico apresentou atividade citotóxica contra células leucêmicas, HL60 e Jurkat. Em relação ao mecanismo de ação do extrato foi possível verificar que este não induz apoptose. Acredita-se, portanto, no potencial terapêutico deste extrato, porém para confirmar esta hipótese faz-se necessário a realização de testes *in vivo*.
- O extrato metanólico induziu aumento do peristaltismo intestinal e de produção de fezes. Estes resultados podem estar relacionados com a presença de antraquinonas detectou no extrato, que são amplamente utilizadas para este fim. Salienta-se a importância de novos estudos, que

avaliem a possibilidade de utilização desta planta em fitocomplexos para emagrecimento.

- A partir do estudo toxicológico agudo e subcrônico do extrato metanólico foi possível verificar que a planta tem baixa toxidade (DL_{50} > 2000 mg/kg) e que não apresentou significativas alterações bioquímicas e hematológicas durante 42 dias de experimento, principalmente nas concentrações de 100 e 300 mg/kg . Portanto, trata-se de uma planta que confere segurança, o que viabiliza a realização de testes farmacológicos pré-clínicos.
- Foram isoladas e elucidadas três substâncias bioativas do extrato metanólico, dois triterpenos (ácido ursólico e ursolato de metila) e uma naftoquinona (psicorubrina). Estas substâncias apresentam resultados promissores para atividade citotóxica, antimicrobiana, leishmanicida e antiinflamatória. Além disso, podem ser utilizadas como marcadores quimiotaxonômicos da espécie.
- A partição em hexano apresentou atividade antimicrobiana, especialmente para as amostras testadas, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella typhimurium* ATCC 13311 e *Shigella dysenteriae* ATCC 13313*,* e leishmanicida, tendo uma especificidade e seletividade para formas intracelulares (amastigotas). Além disso, foi observado que o extrato apresentou pouca toxidade contra macrófagos.
- Verificou-se, por meio da análise da composição química do óleo essencial, que as substâncias majoritárias foram linalol e acetato de eugenol. Além disso, foi observado que o óleo apresenta atividade antifúngica e leishmanicida, sendo, portanto, uma alternativa terapêutica para doenças relacionadas com estes organismos.

Pontanto com este estudo, a espécie *Mitracarpus frigidus*, que não possui relatos de uso popular, pode ser uma alternativa no tratamento de diversas doenças, como as provocadas por fungos, bactérias e parasitas, além de patologias relacionadas a processos inflamatórios. Para tanto, é necessário a realização de novos estudos, que possibilitem identificar o potencial desta espécie visando a obtenção de novos fitoterápicos.

7 ANEXOS

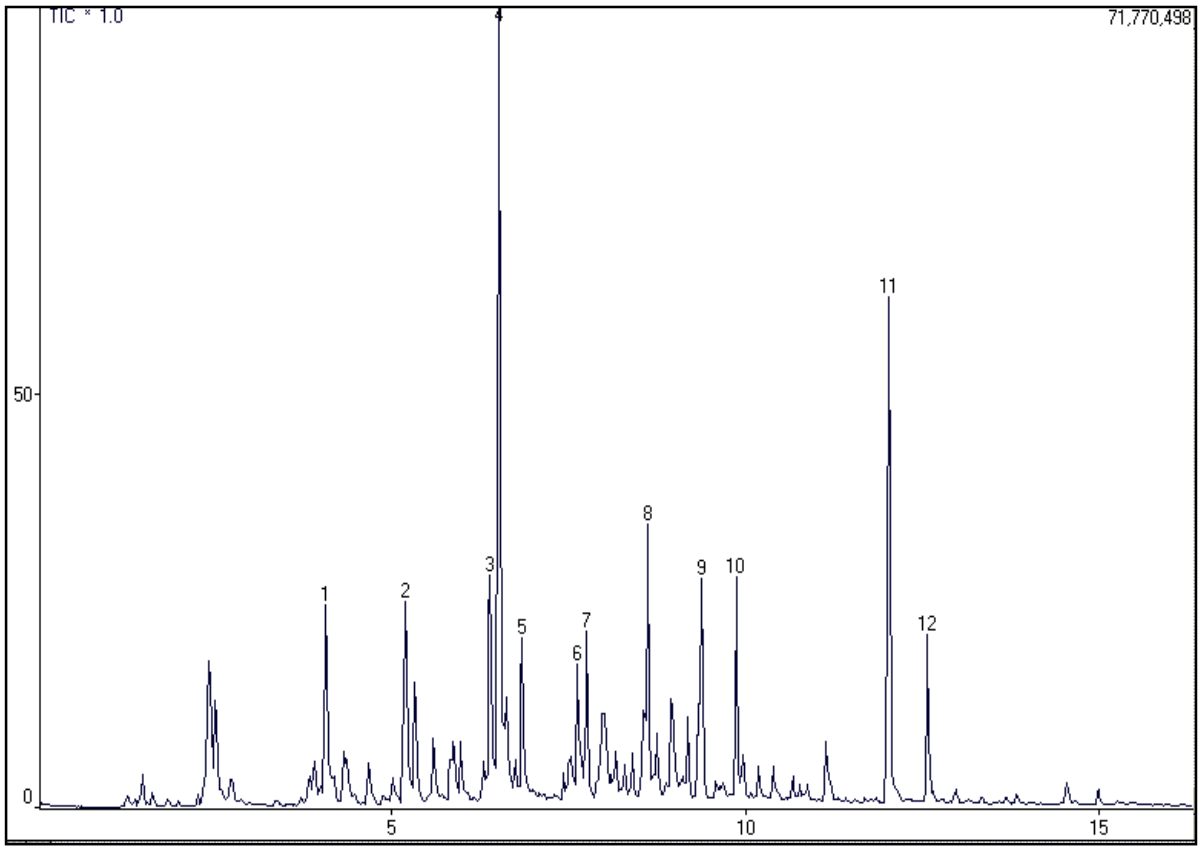
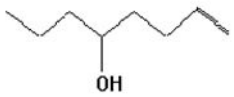
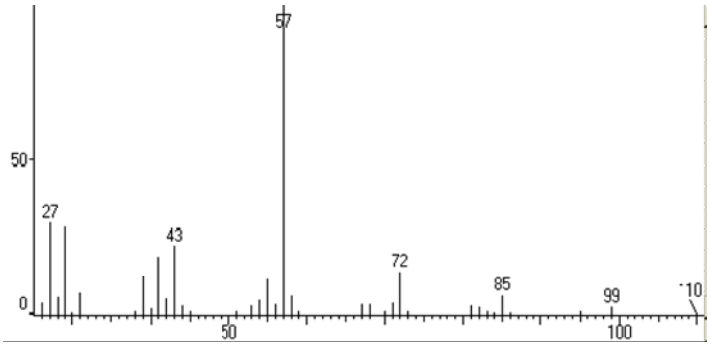


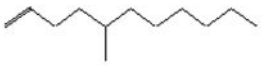
Figura 1 – Perfil cromatográfico do óleo essencial das partes aéreas de *M. frigidus* por CG/EM.

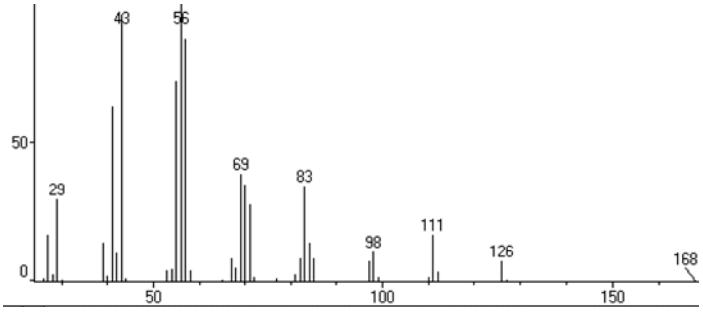
7-octen-4-ol



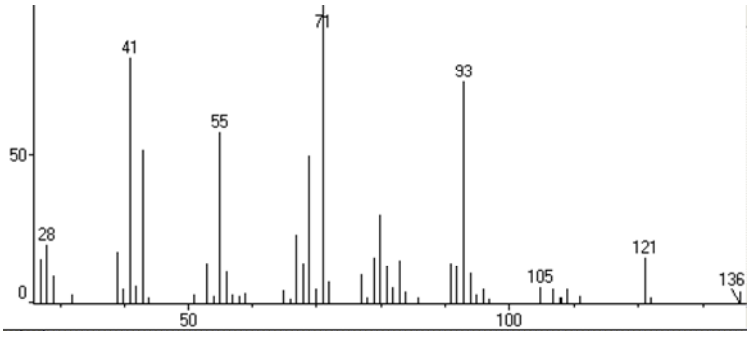


5-metil-1-Undeceno

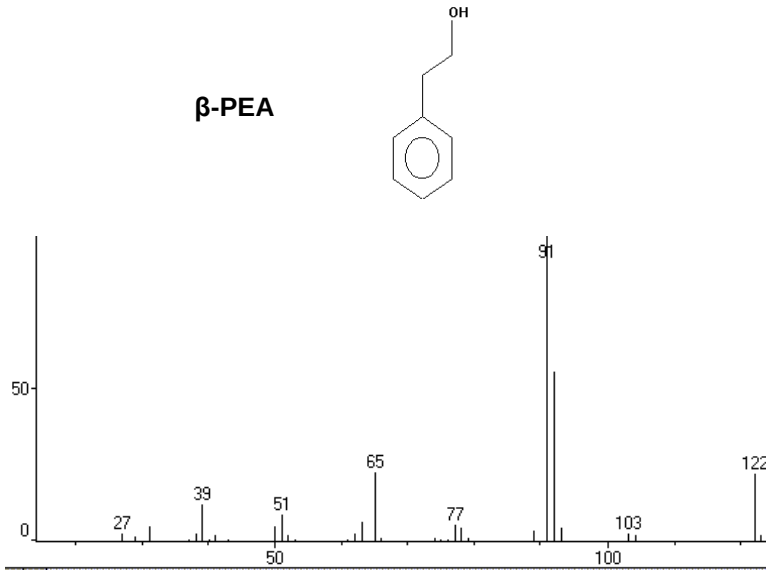




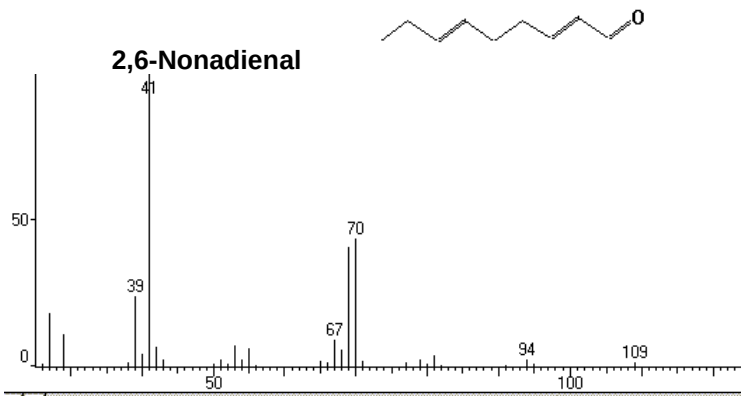
Linalol



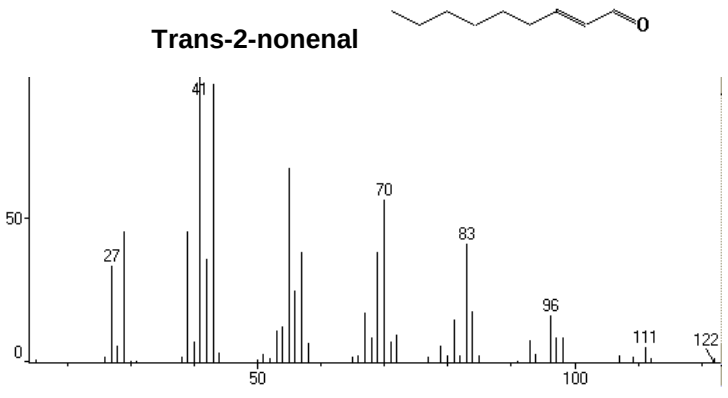
β-PEA



2,6-Nonadienal



Trans-2-nonenal



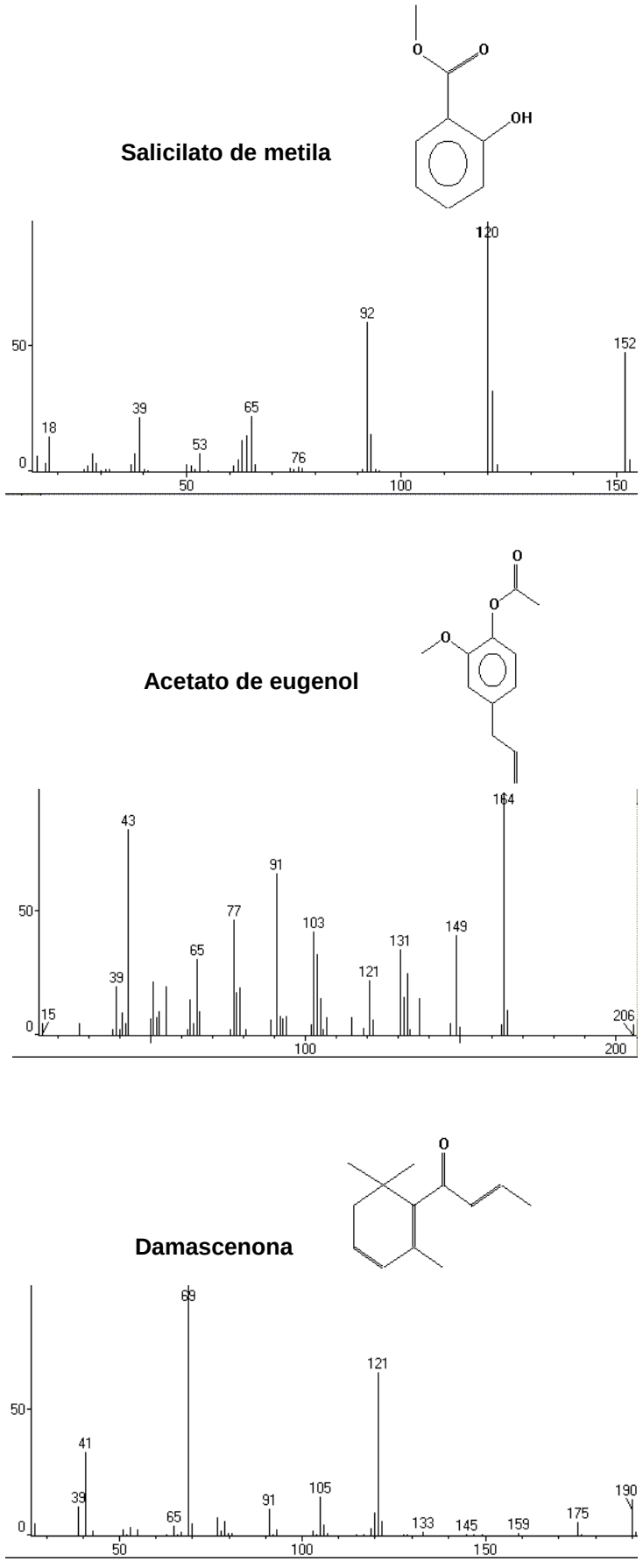
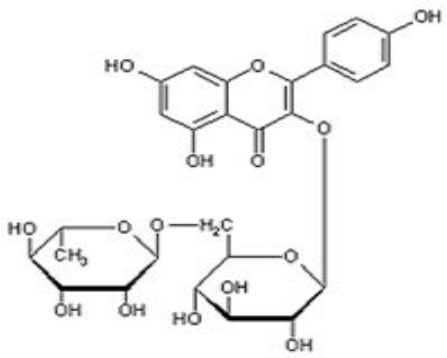
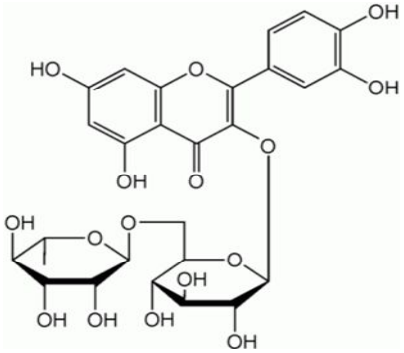


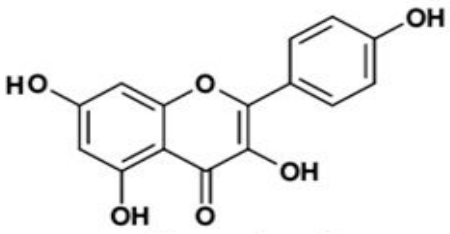
Figura 2 – Fórmula estrutural e o espectro de massa dos constituintes presentes no óleo das partes aéreas de *Mitracarpus frigidus.*



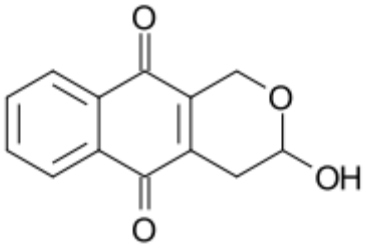
Campferol-3-*O*-rutenosideo



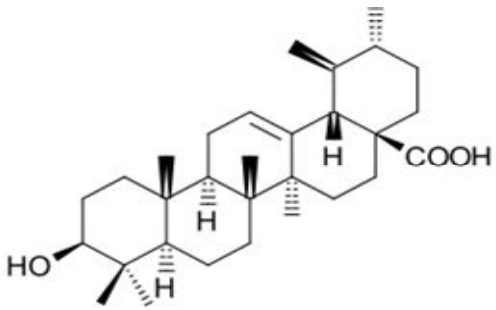
Rutina



Campferol



Psicorubrina

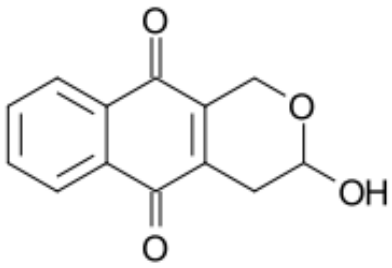


Ácido ursólico

Figura 3 – Compostos detectados por CLAE no extrato metanólico das partes aéreas de *Mitracarpus frigidus.*

⇒ **Substâncias isoladas de *Mitracarpus frigidus***

1- Psicorubrina (3-hidróxi-3,4-dihidro-1H-benzo[g]isocromeno-5,10-diona)



- Isolados 100 mg
- *m/z*: 230,1 calcd. para $C_{13}H_{10}O_4$
- UV (MeOH) $\lambda_{máx}$ (log ε): 245; 250; 262; 333 nm
- PF: 146,2 ^{o}C

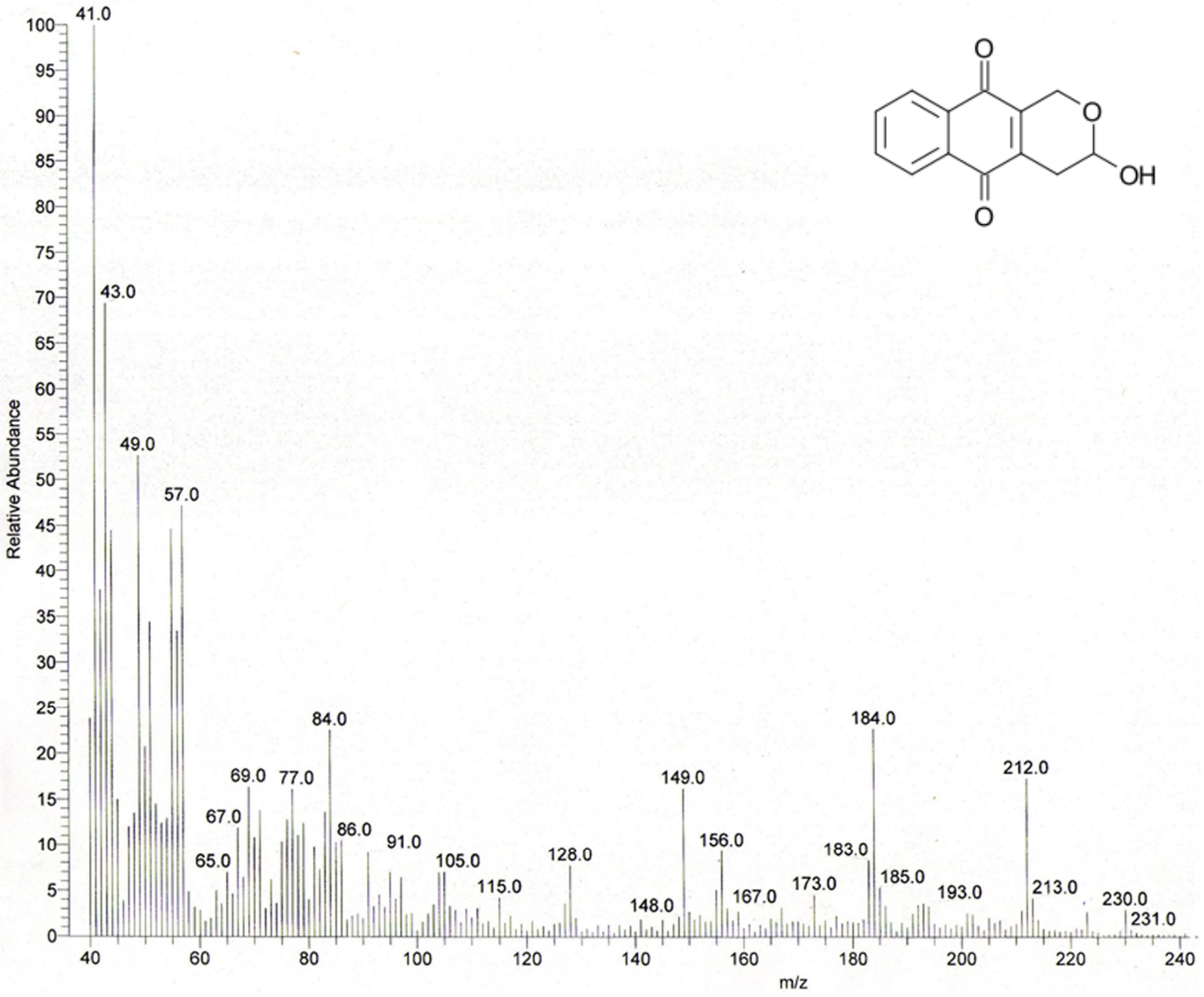


Figura 4 – Espectro de massa EI de psic orubrina.

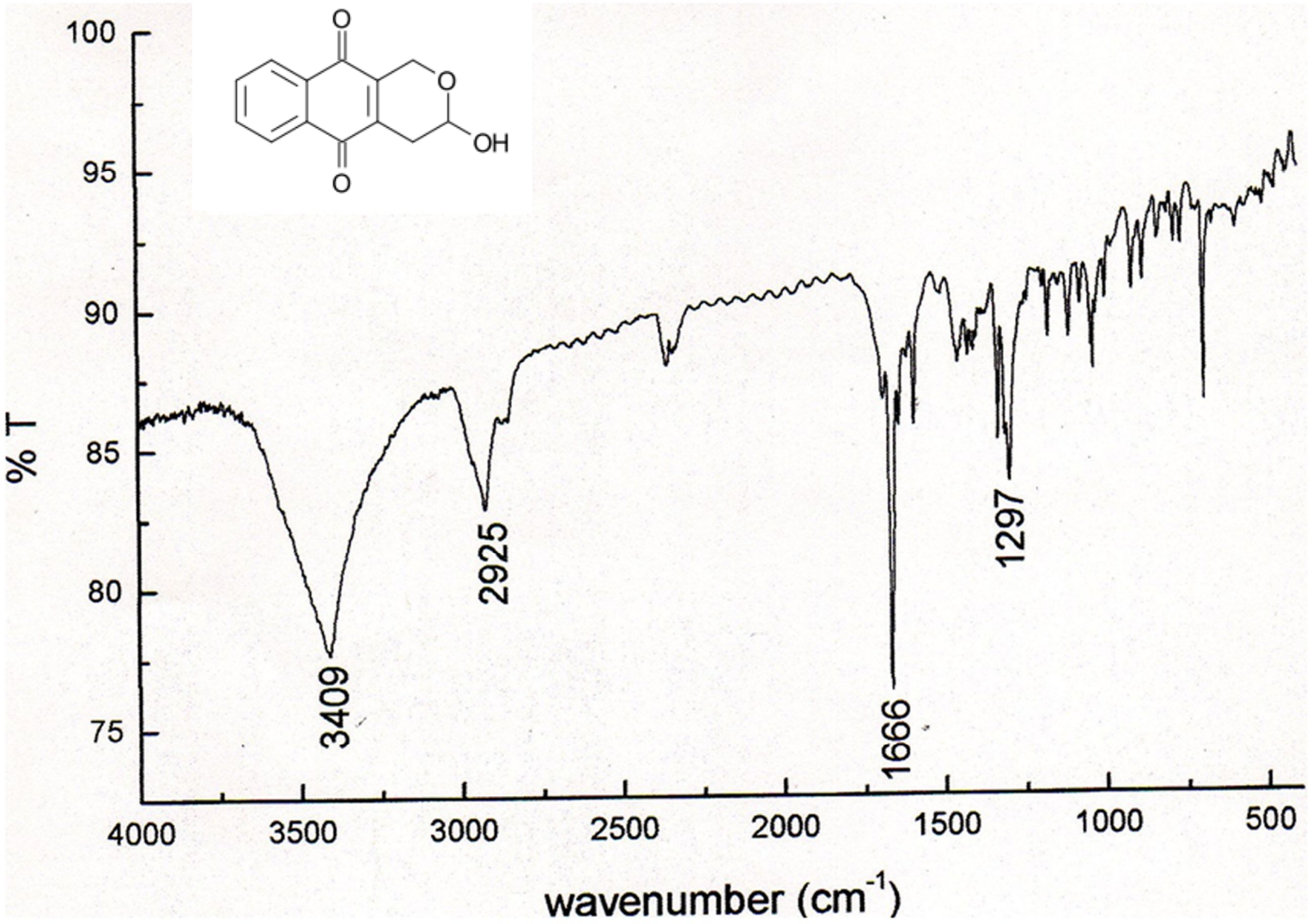


Figura 5 – Espectro de infravermelho de psicorubrina.

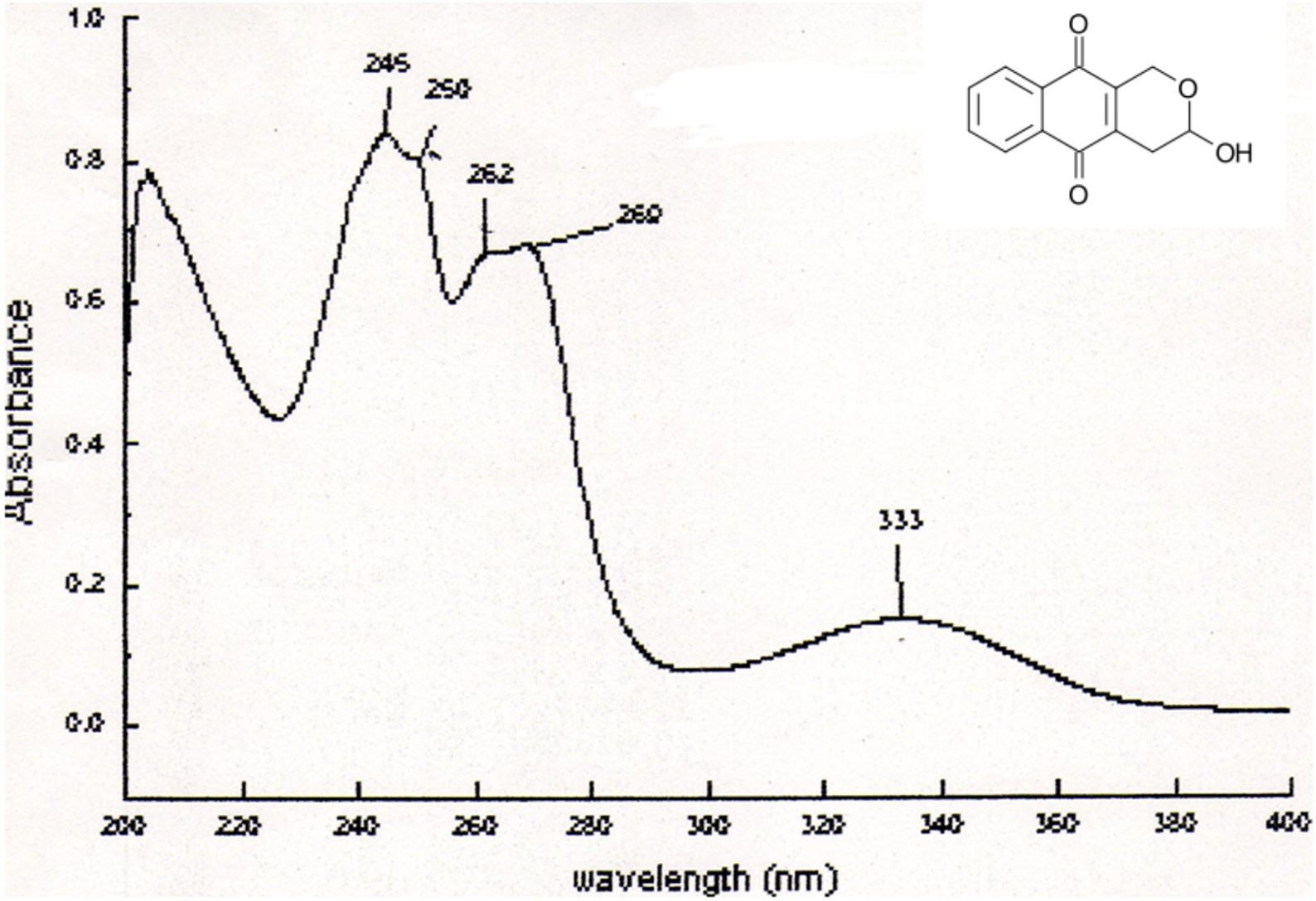


Figura 6 – Espectro de ultravioleta de psicorubrina.

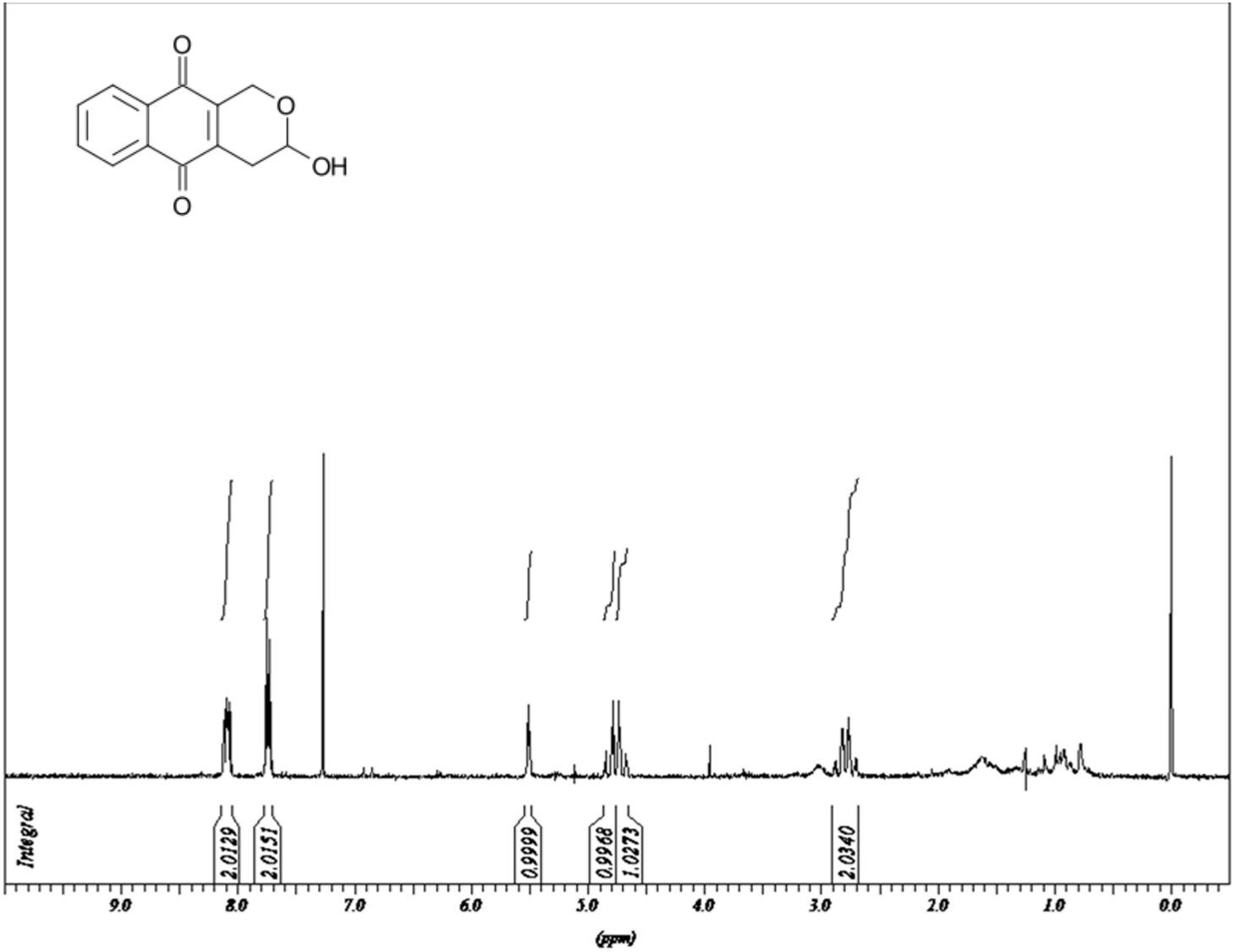


Figura 7 – Espectro de RMN de ^{1}H [$CDCl_3$, 300 MHz] de psicorubrina.

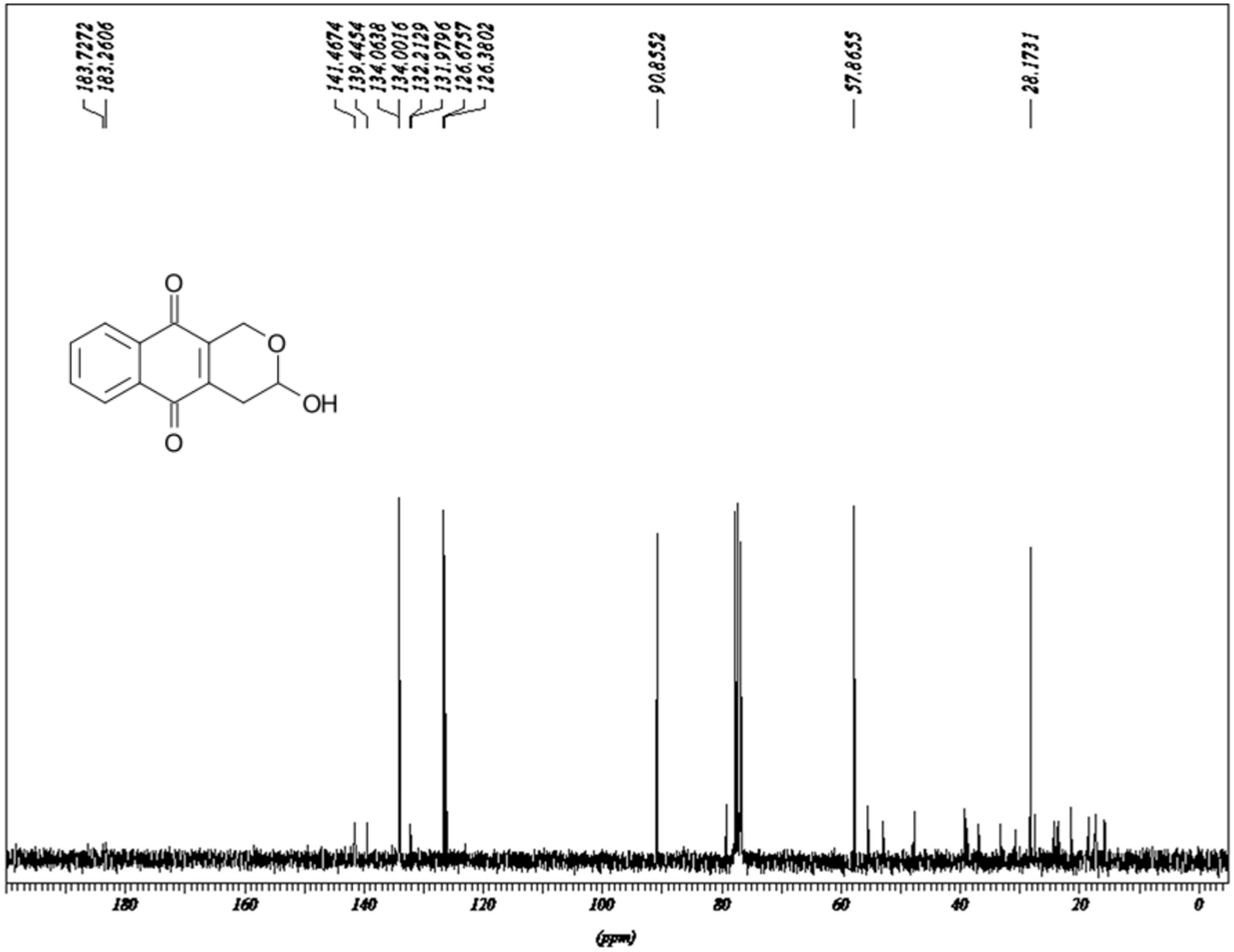


Figura 8 - Espectro de RMN de ^{13}C [$CDCl_3$, 75 MHz] de psicorubrina.

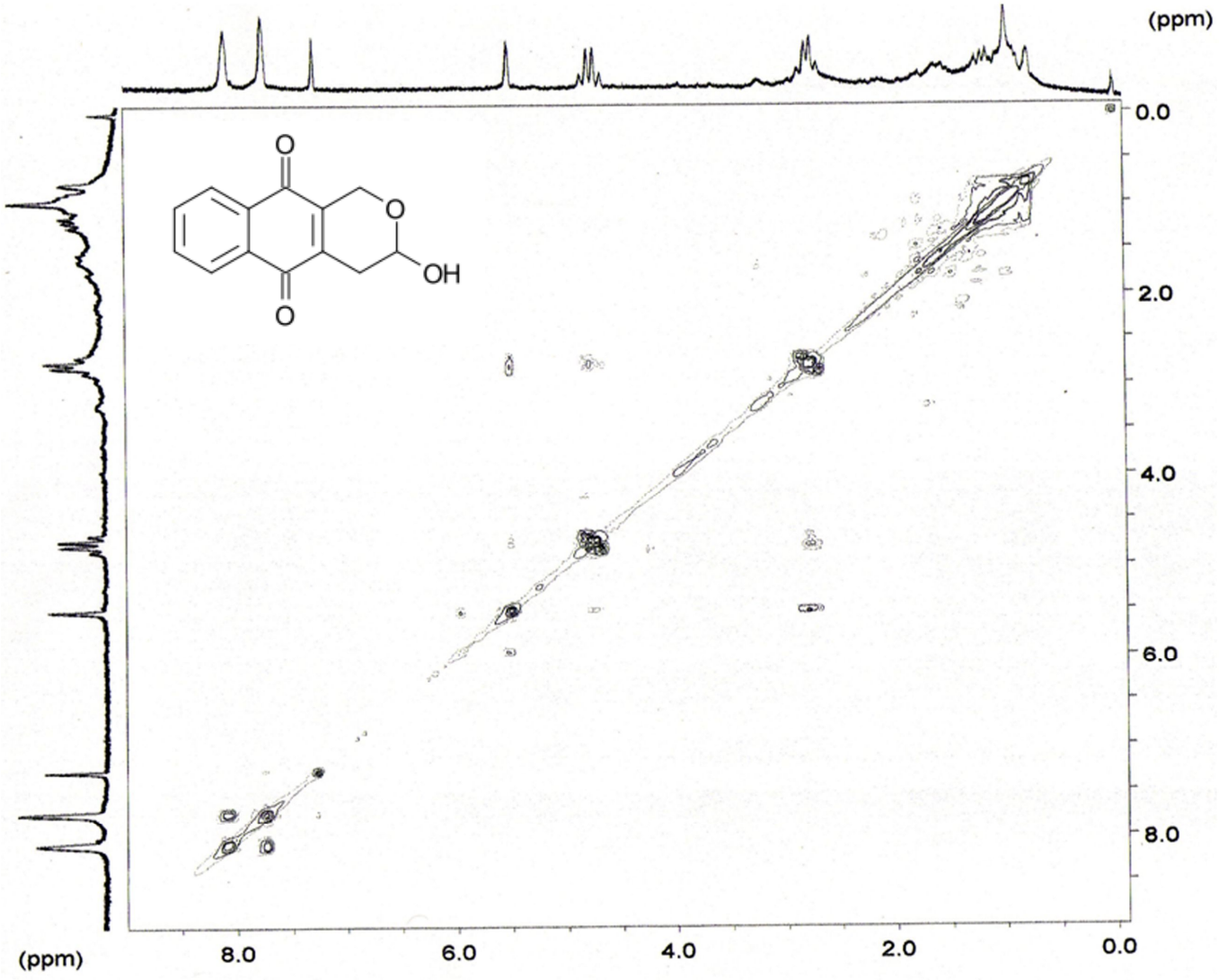
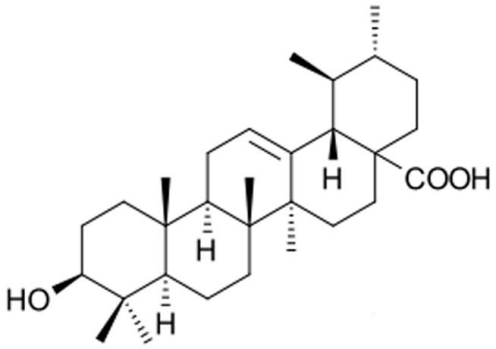


Figura 9 – Espectro de COSY [$CDCl_3$, 300 MHz] de psicorubrina.

2- Ácido ursólico (ácido 3*β*-hidróxi-urs-12-en-28-óico)



- Isolados 210 mg
- *m/z*: 456,6 calcd. para $C_{30}H_{48}O_3$
- UV (MeOH) $\lambda_{máx}$ (log ε): 215 nm
- PF: 264 °C

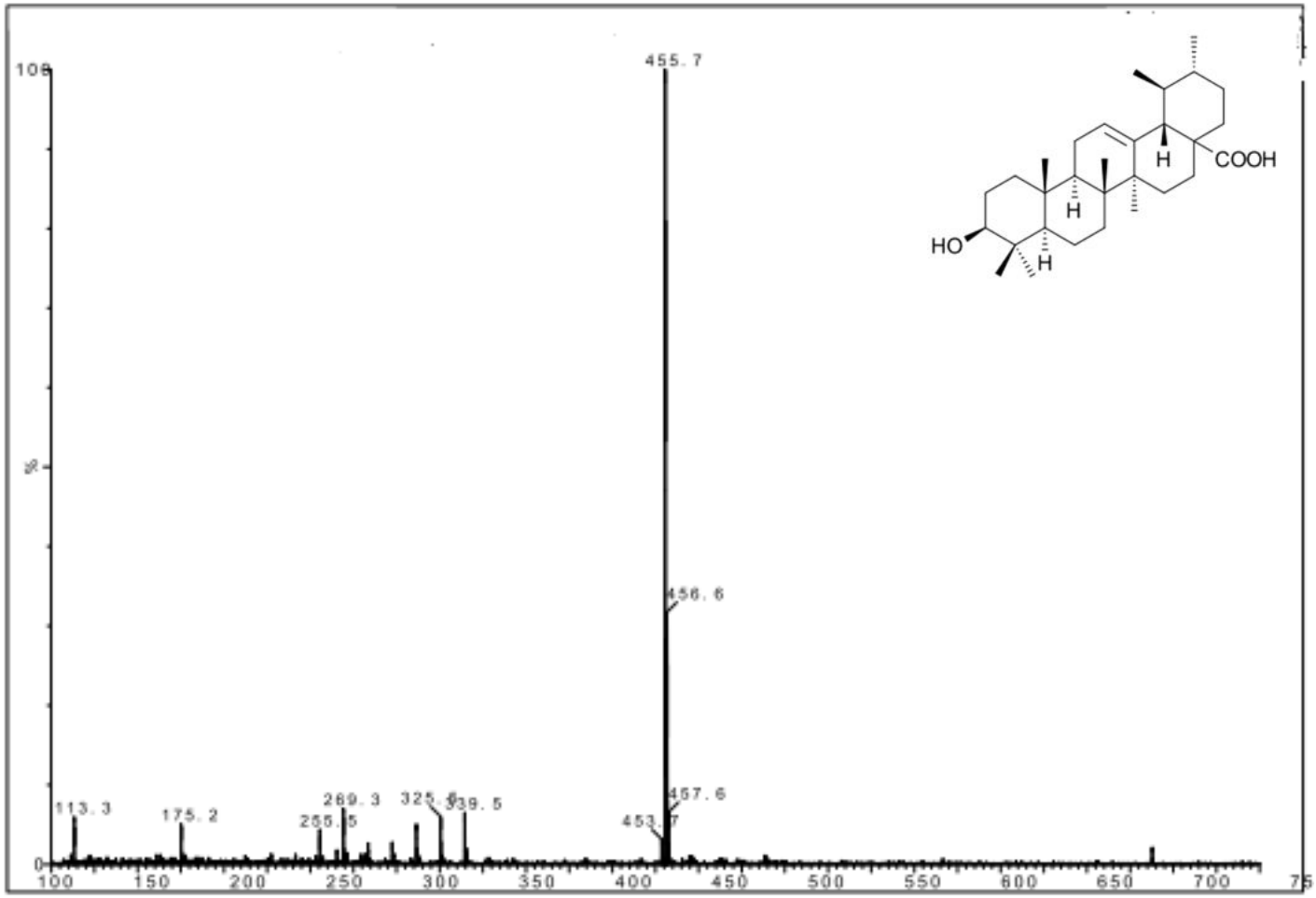


Figura 10 – Espectro de massa do ácido ursólico.

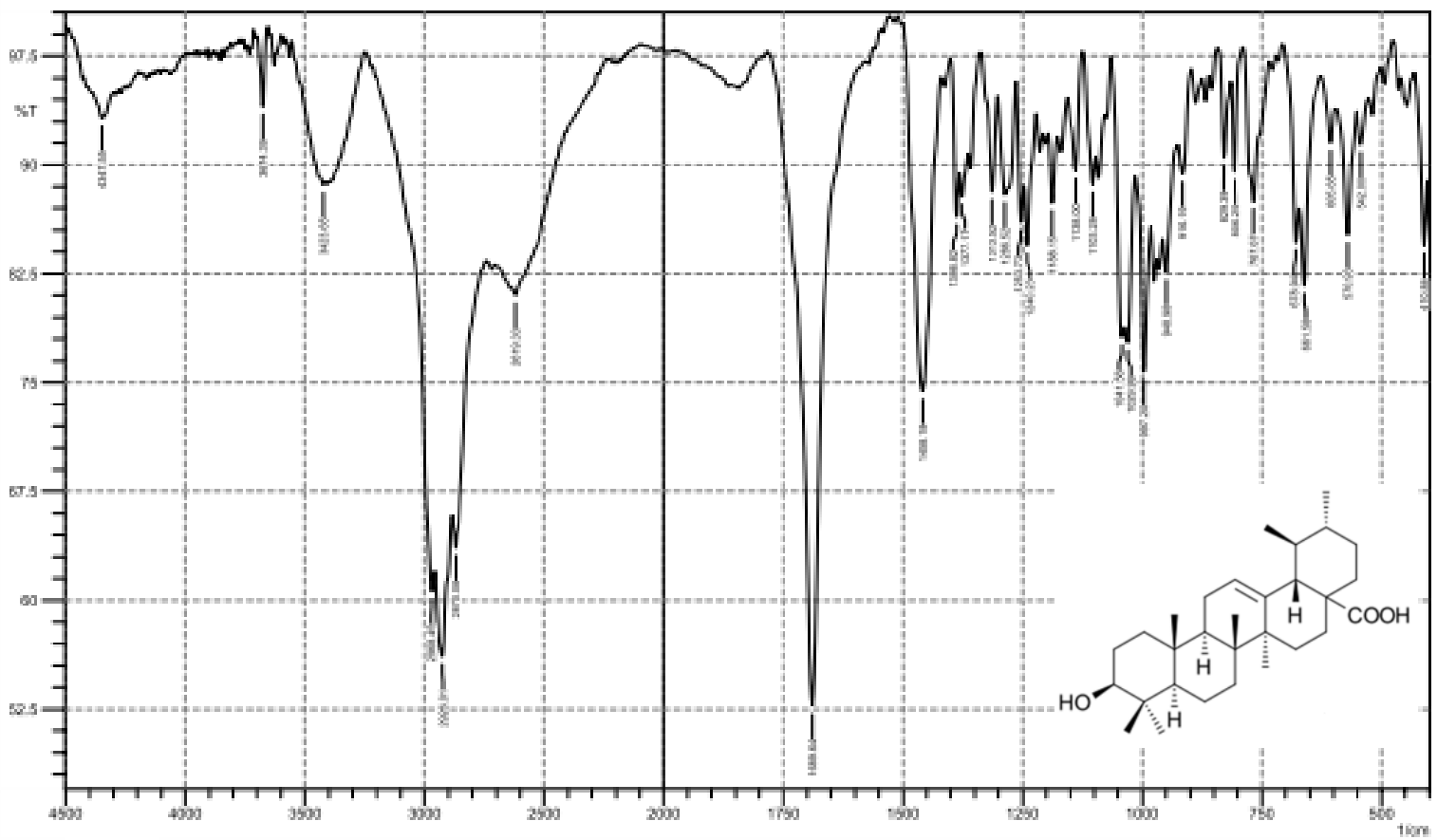


Figura 11 – Espectro de infravermelho do ácido ursólico.

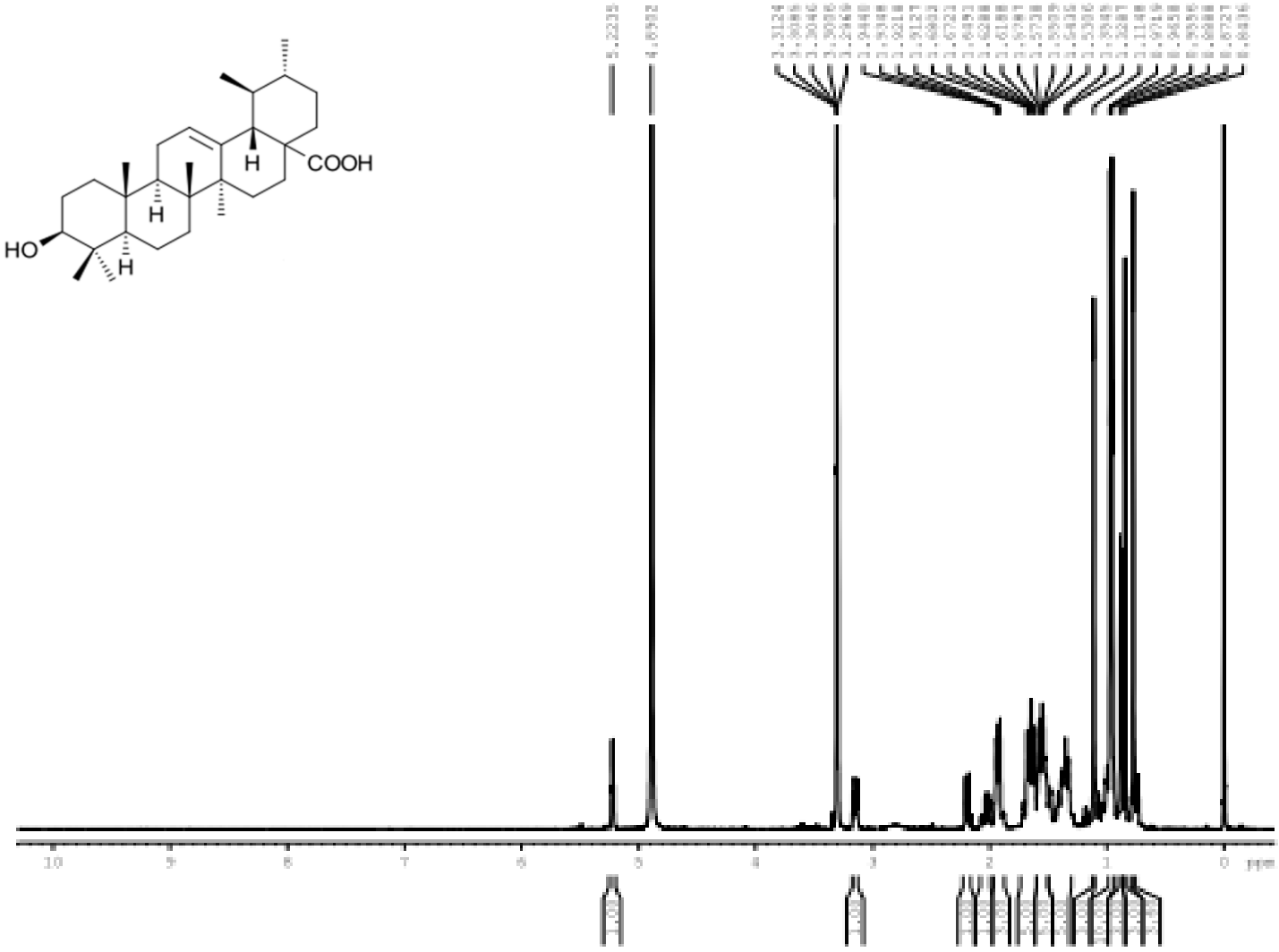


Figura 12 – Espectro de RMN de ^{1}H [MeOD, 300 MHz] do ácido ursólico.

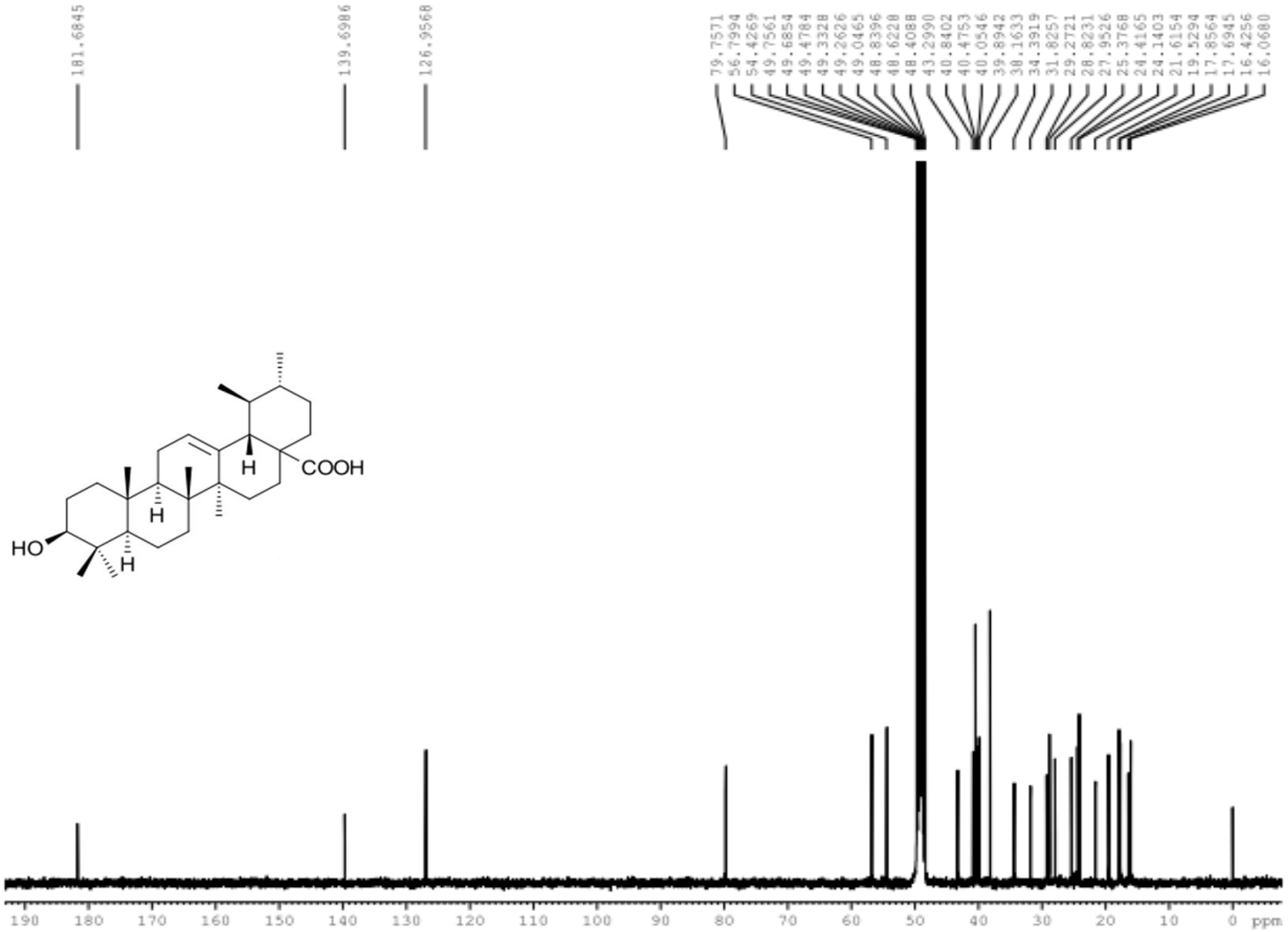


Figura 13 – Espectro de RMN de ^{13}C [MeOD, 75 MHz] do ácido ursólico.

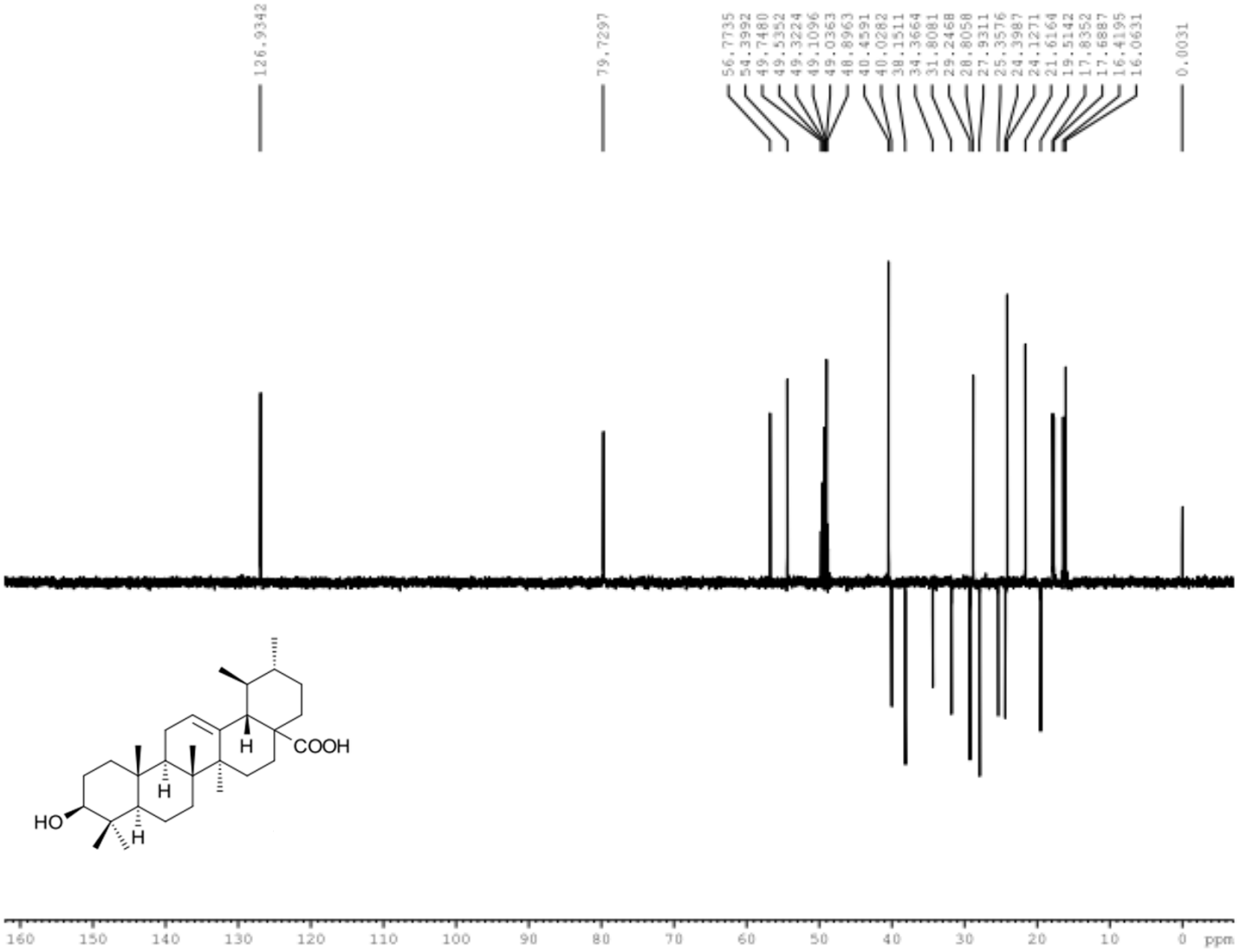


Figura 14 – Espectro de RMN de Dept 135 [MeOD, 75 MHz] do ácido ursólico.

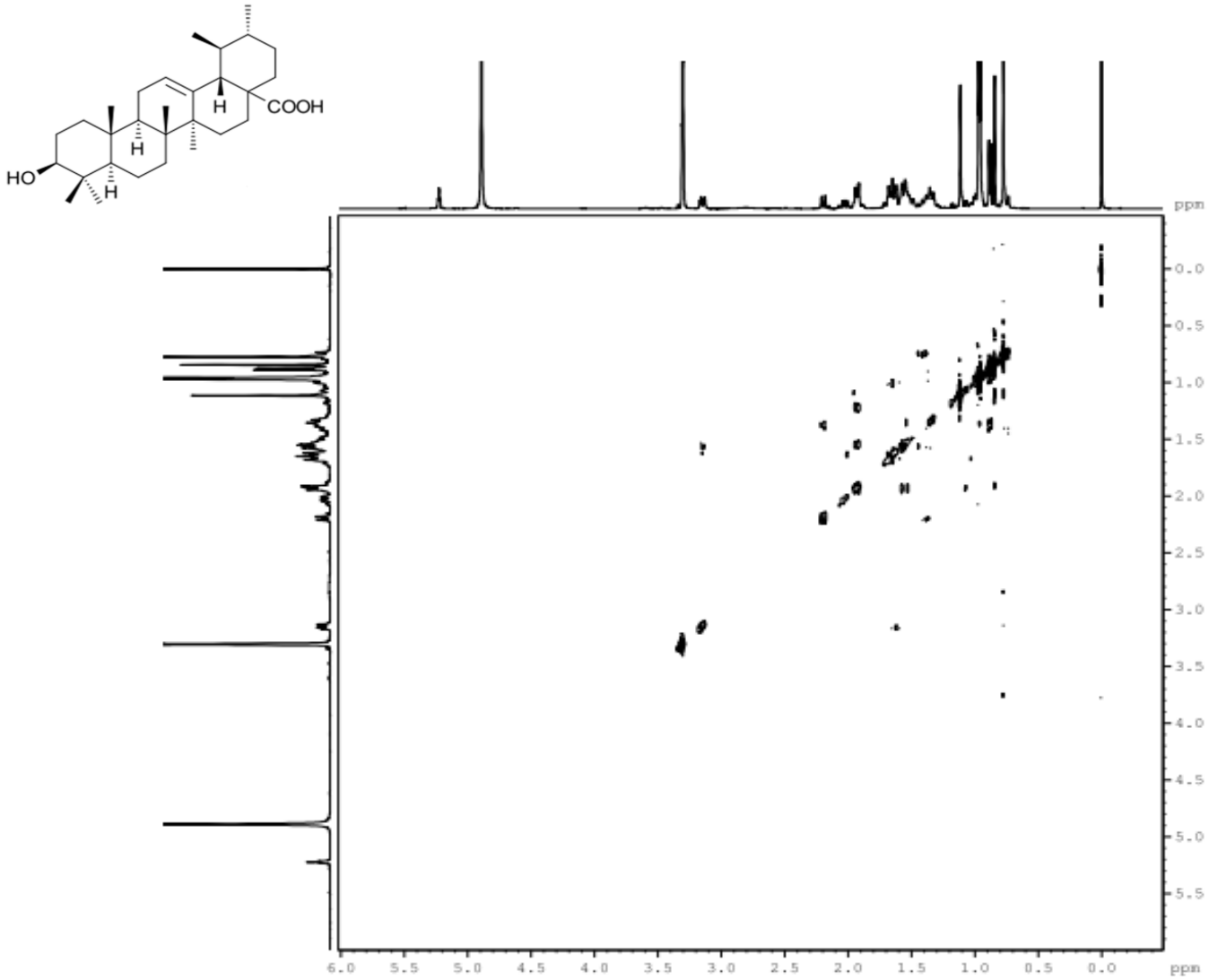


Figura 15 – Espectro de RMN de COSY [MeOD, 300 MHz] do ácido ursólico.

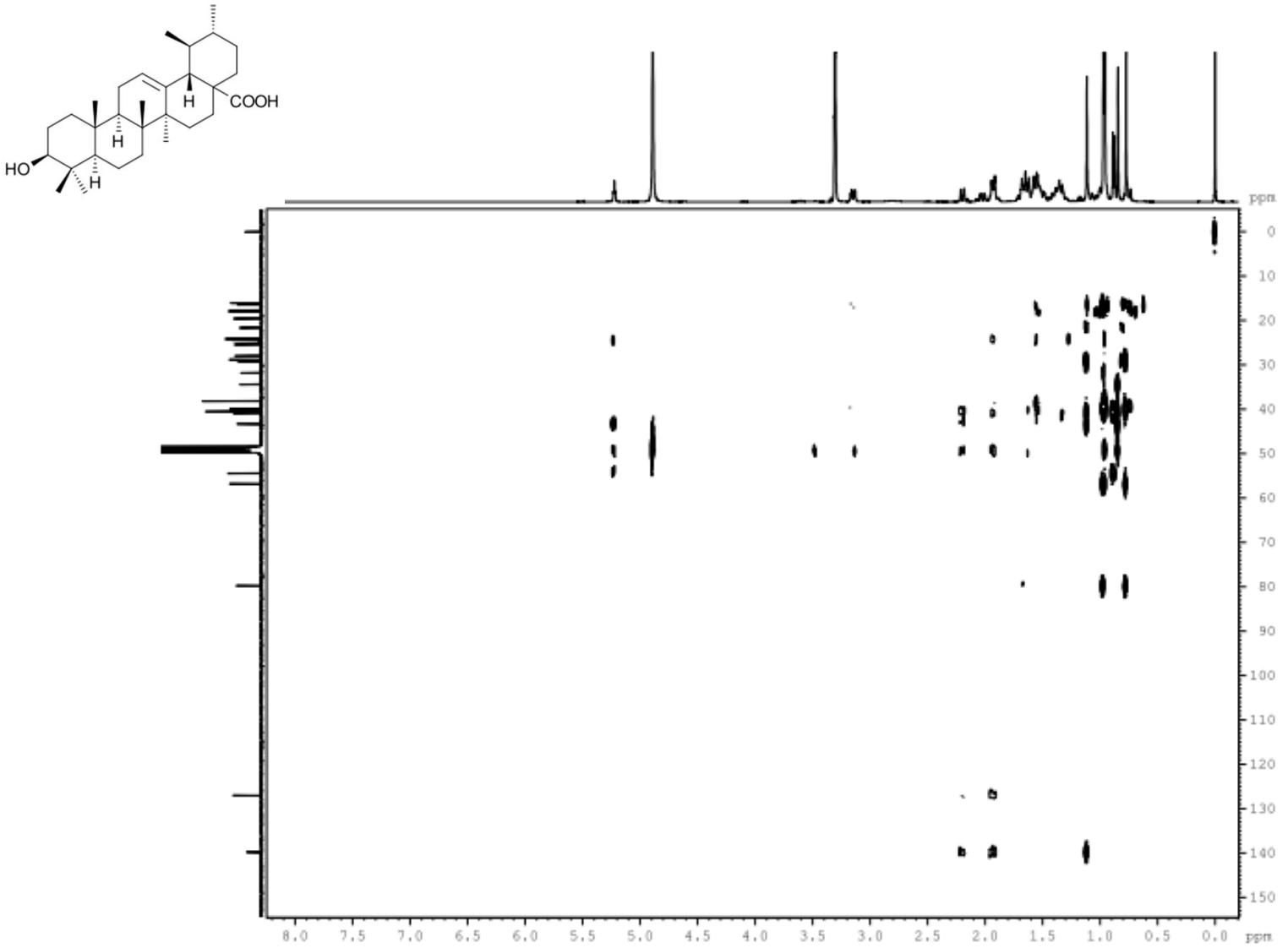


Figura 16 – Espectro de RMN de HMBC [MeOD, 300 MHz] do ácido ursólico.

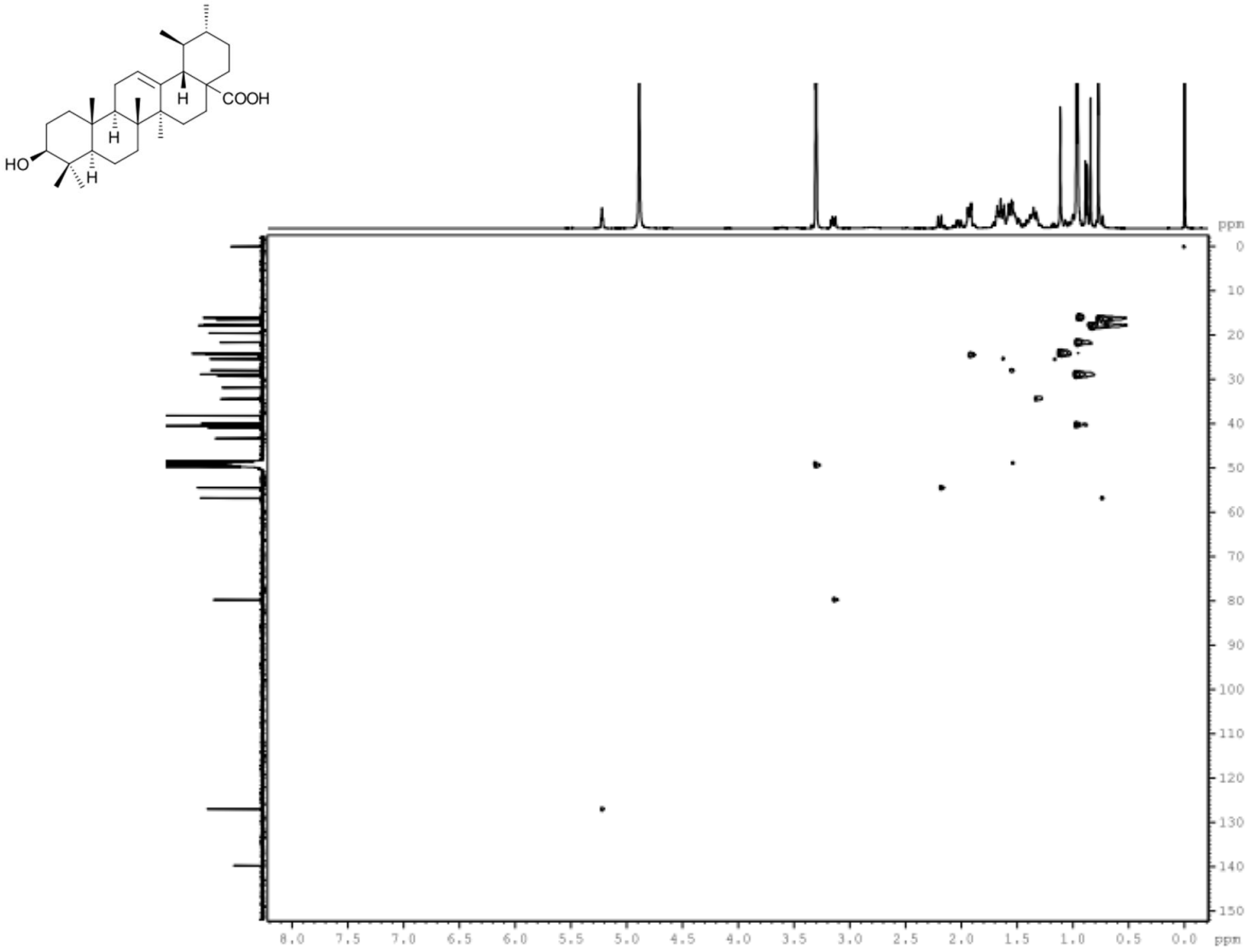
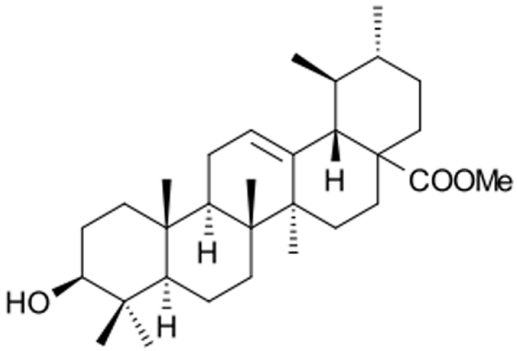


Figura 17 – Espectro de RMN de HSQC [MeOD, 300 MHz] do ácido ursólico.

3- Ursolato de metila (3*β*-hidróxi-urs-12-en-28-olato de metila)



- Isolados 20 mg
- *m/z*: 470,7 calcd. para $C_{31}H_{50}O_3$
- UV (MeOH) $\lambda_{máx}$ (log ε): 215 nm
- PF: 252 ^{o}C

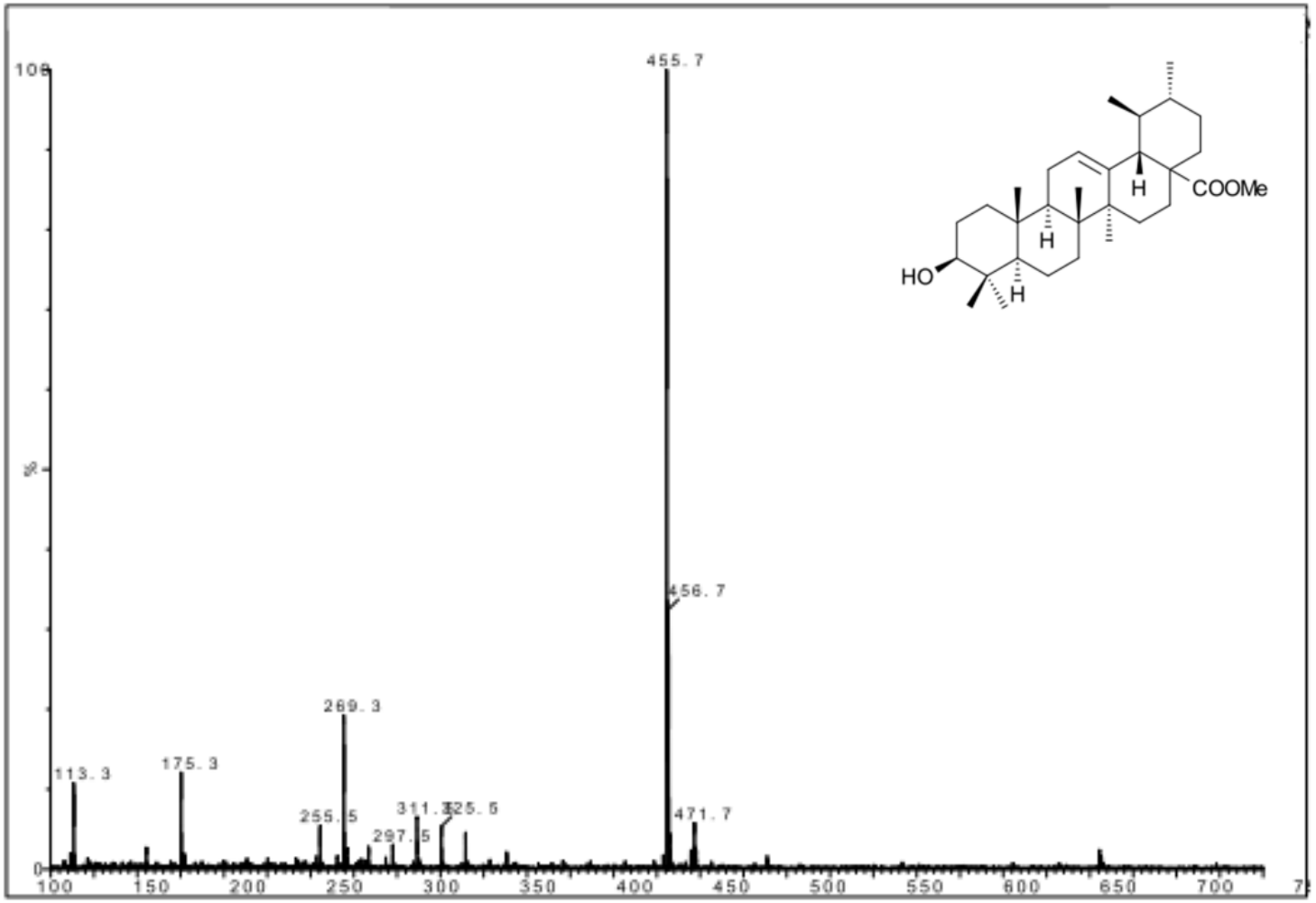


Figura 18 – Espectro de massa do ursolato de metila.

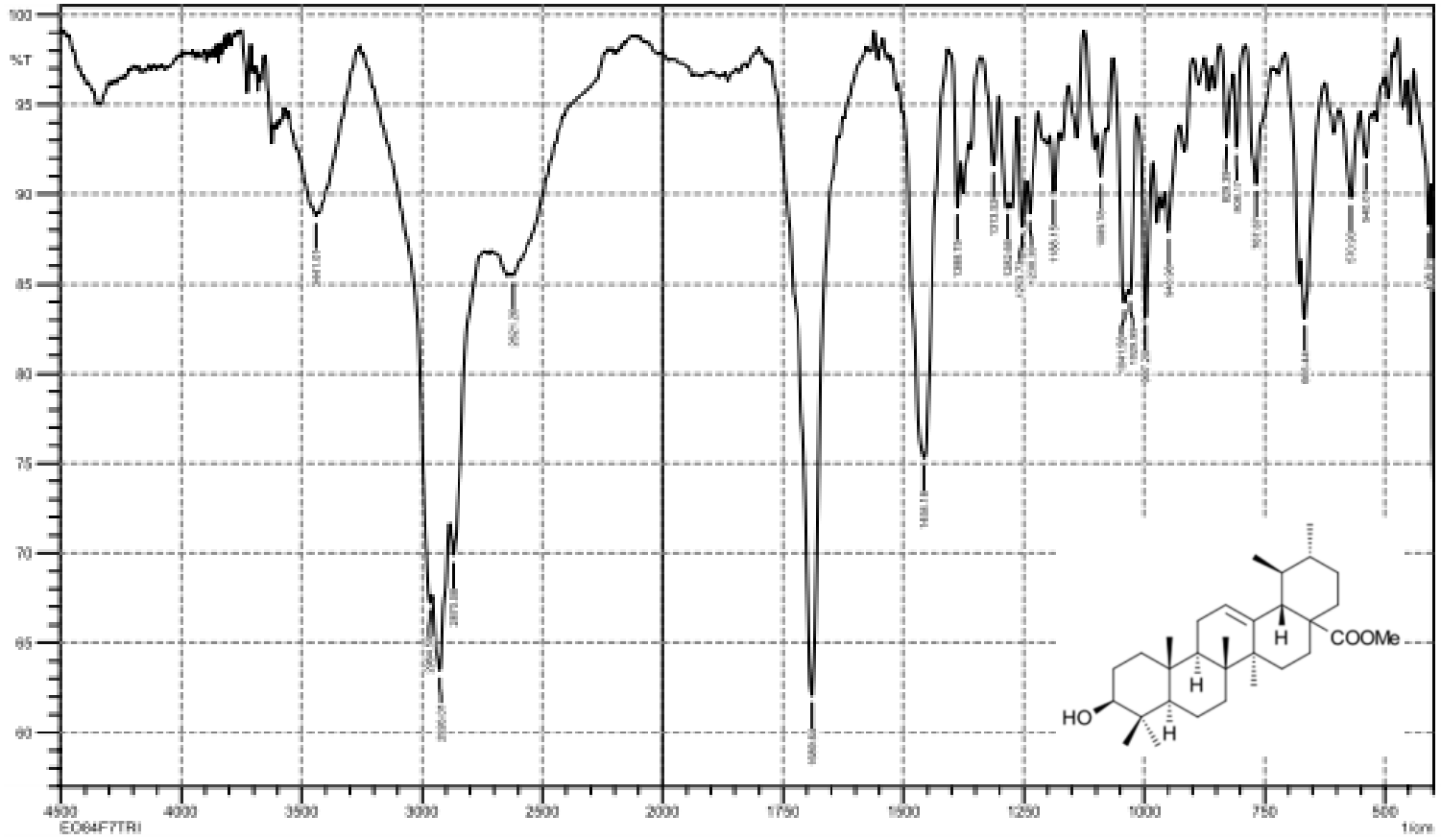


Figura 19 – Espectro de infravermelho do ursolato de metila.

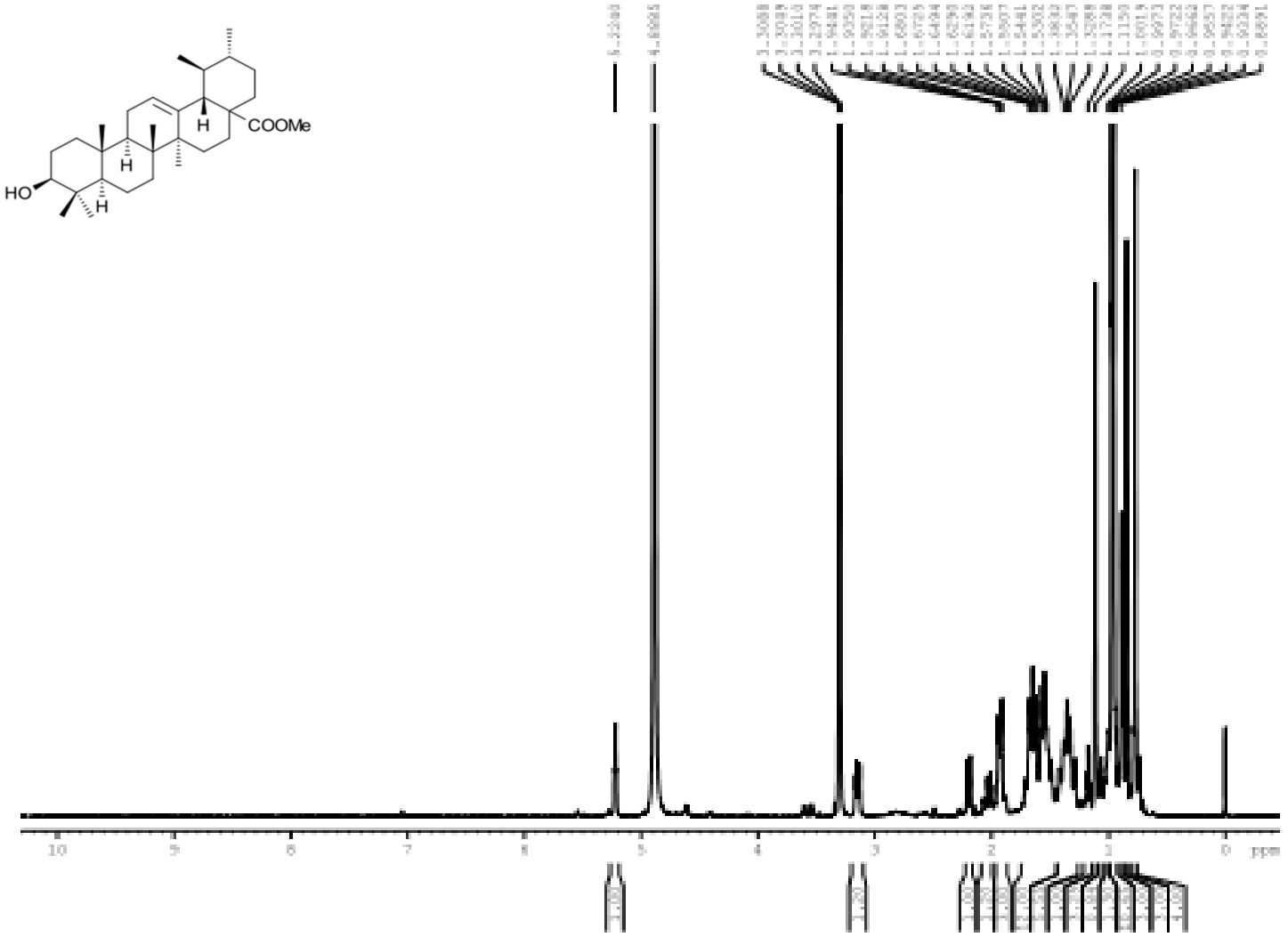


Figura 20 – Espectro de RMN de ^{1}H [MeOD, 300 MHz] do ursolato de metila.

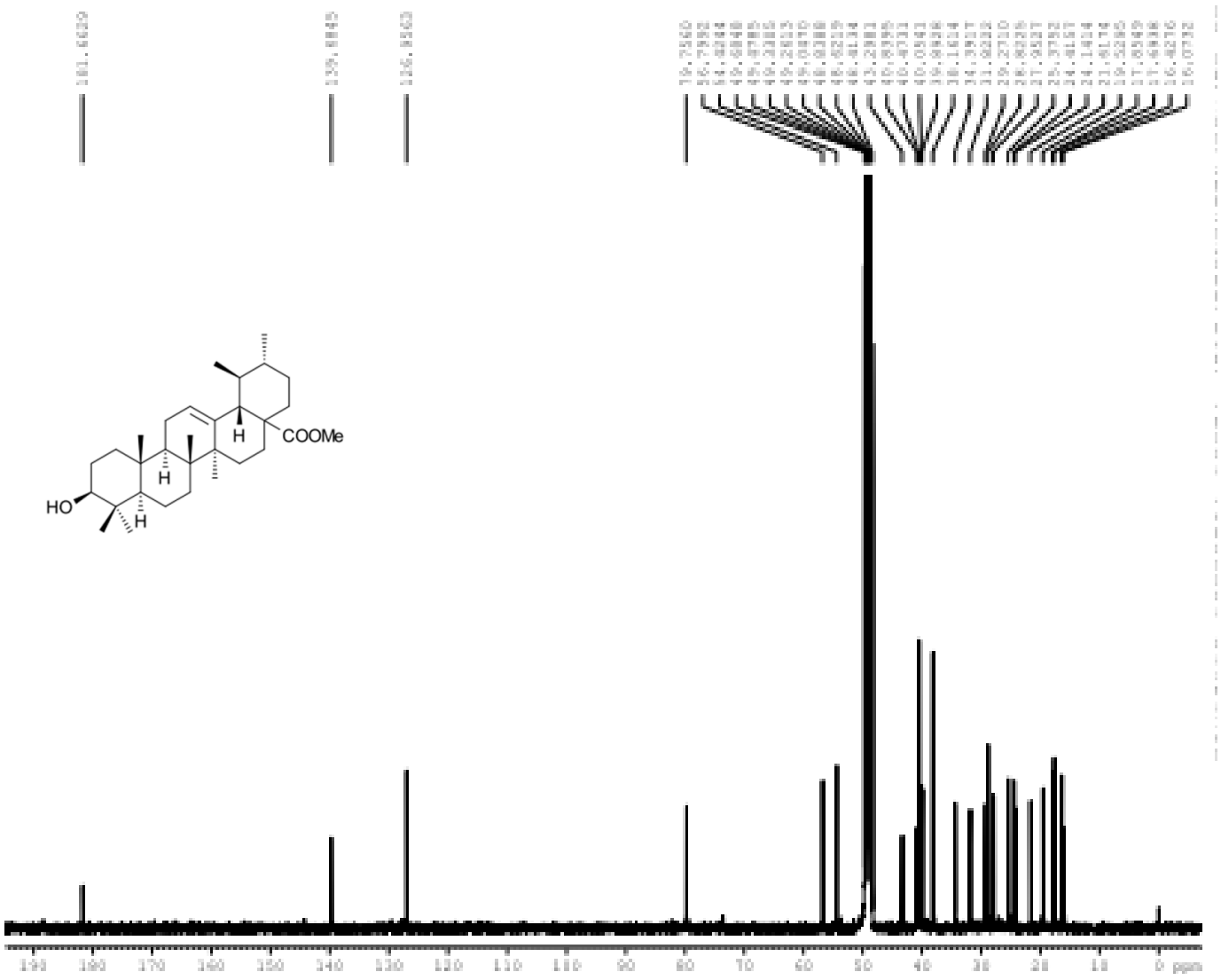


Figura 21 – Espectro de RMN de ^{13}C [MeOD, 75 MHz] do ursolato de metila.

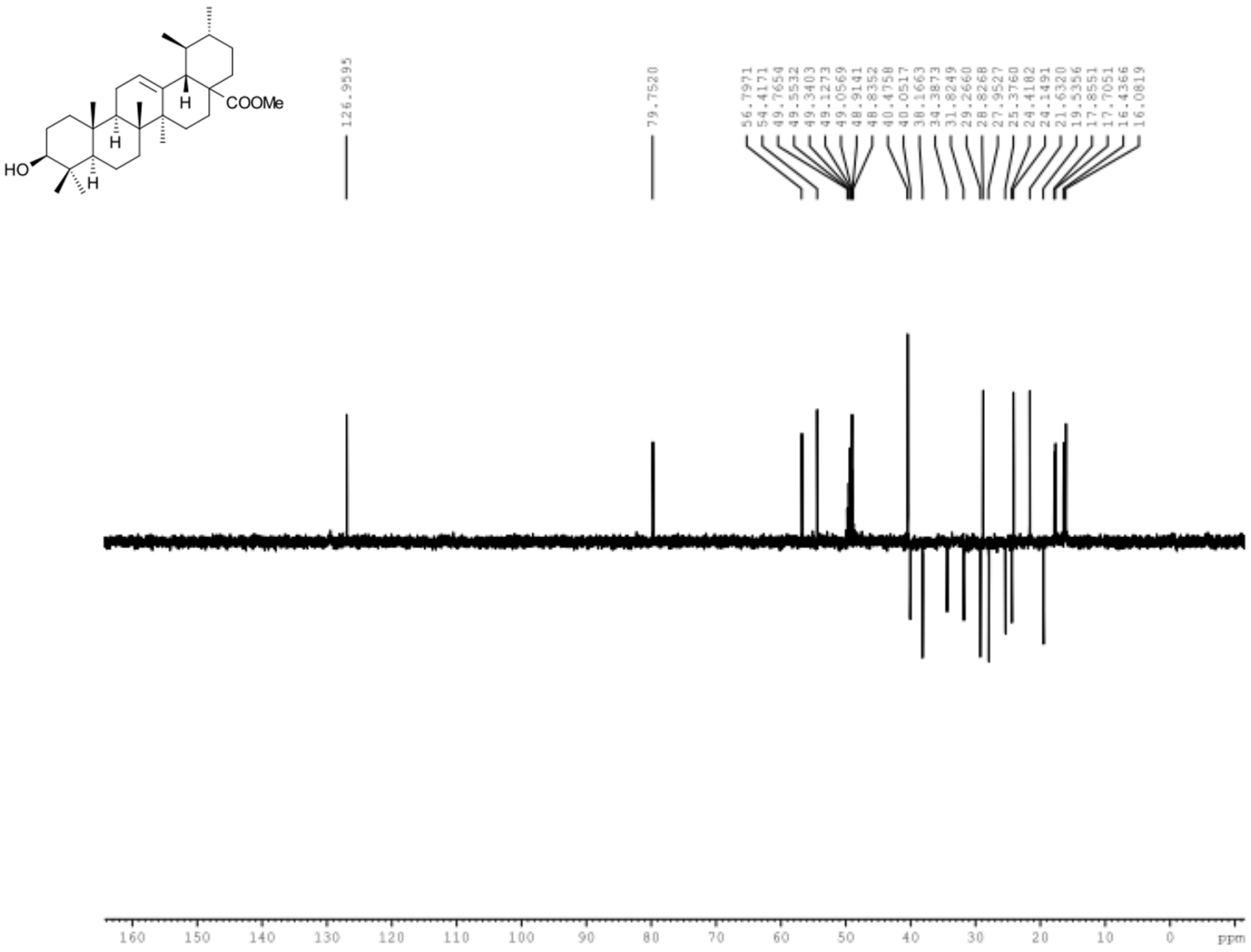


Figura 22 – Espectro de RMN de Dept 135 [MeOD, 75 MHz] do ursolato de metila.

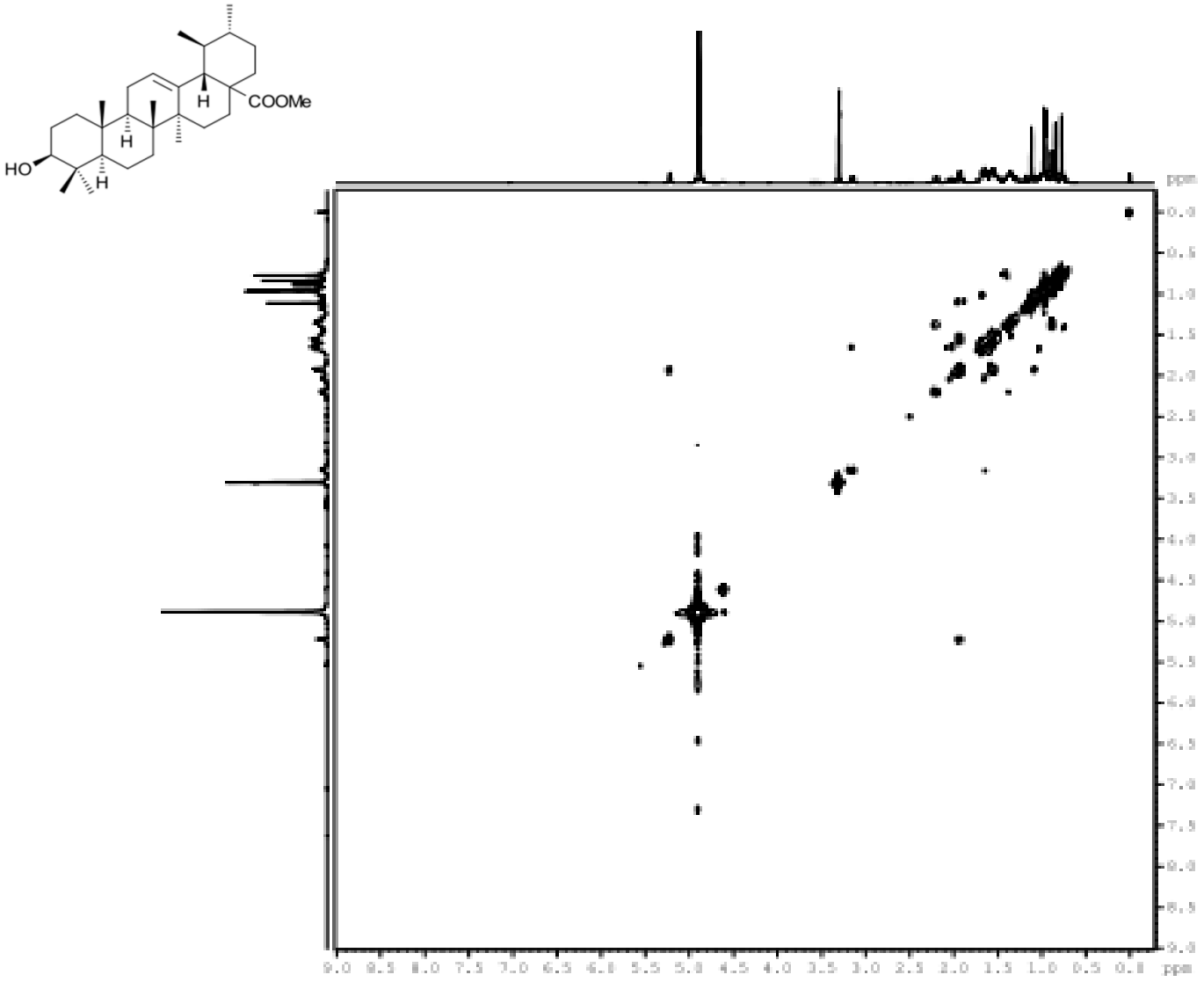


Figura 23 – Espectro de RMN de COSY [MeOD, 300 MHz] do ursolato de metila.

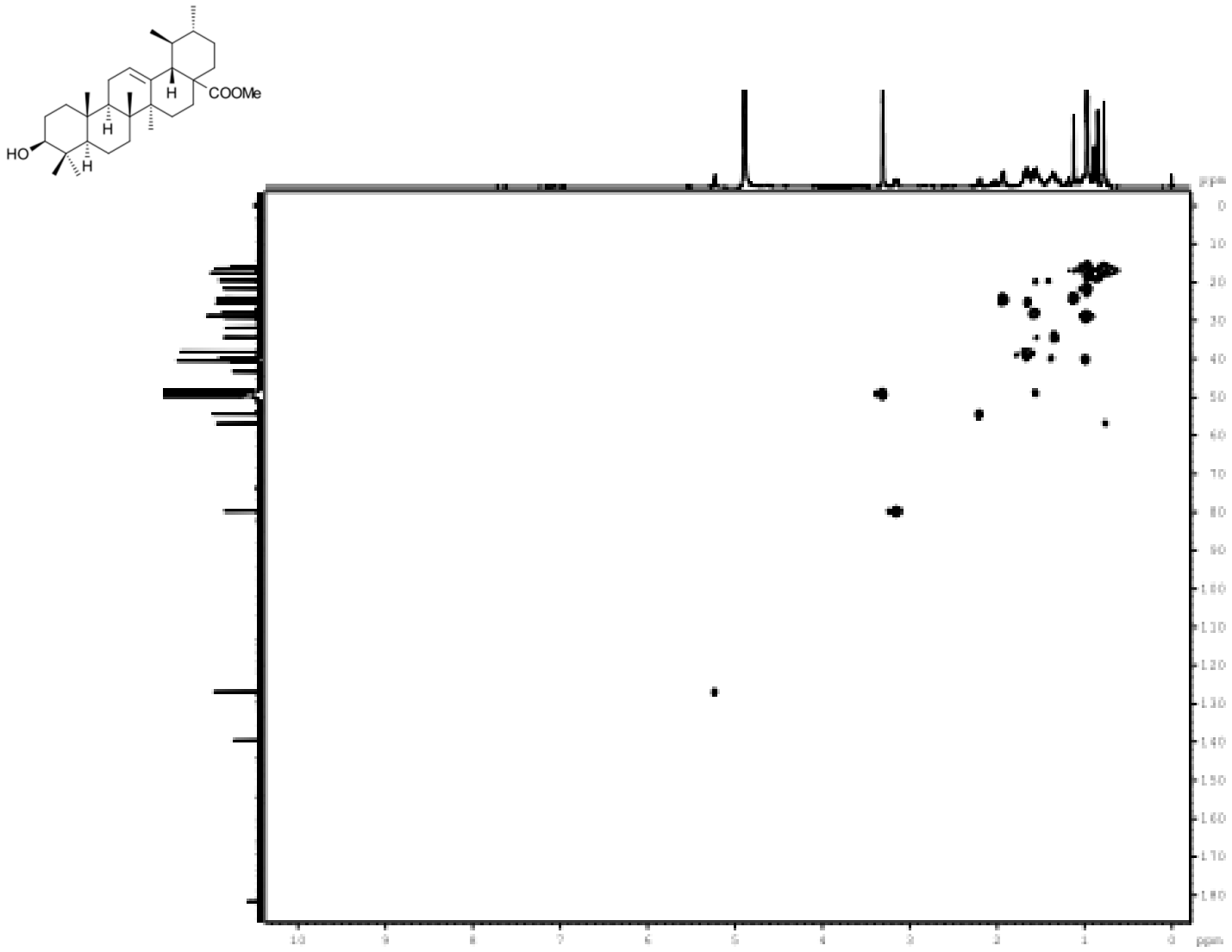


Figura 24 – Espectro de RMN de HSQC [MeOD, 300 MHz] do ursolato de metila.