UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

SCREENING DA ATIVIDADE ANTI-MICOBACTERIANA DE 28 EXTRATOS E TRÊS ÓLEOS ESSENCIAIS DE PLANTAS EXÓTICAS E NATIVAS BRASILEIRAS E AVALIAÇÃO DA TOXICIDADE AGUDA DA FRAÇÃO ATIVA

RAFAELE CARLA PIVETTA

DOURADOS - MS
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Dissertação apresentada à Universidade Federal da Grande Dourados – Faculdade de Ciências da Saúde, para obtenção do Título de Mestre em Ciências da Saúde.

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Lista de Siglas e Abreviaturas

AB	Alamar Blue
AIDS	Acquired Immunodeficiency Syndrome
ALT	Alanino amino transferase
AST	Aspartato amino transferase
ATCC	American Type Culture Collection
CD_3OD	Deuterated methanol
CDC	Center for Desease Control
CF	Cloroformic Fraction
$CHCl_3$	Clorofórmio
CIM	Concentração Inibitória Mínima
DL50	Dose Letal
DMSO	Dimethyl sulfoxide
DOTS	Directly Observed Treatment Short-Course
EAF	Ethil Acetate Fraction
EUA	Estados Unidos da América
FDA	Food and Drug Administration
ɣGT	Gama glutamil transferase
HIV	Human Imunodeficience Vírus
HMF	Hidro methanolic fraction
IS	Índice de Seletividade
LC-MS	Liquid crhomathograpy with mass spectophotometry
MDRTB	Tuberculose Multirresistente às Drogas
MS	Ministério da Saúde
MTT	Tetrazollium Based Assay
N.D.	Não descrito
NMR	Nuclear magnetic ressonancy
OADC	Oleic acid, bovine albumin, dextrose and catalase enrichment
OMS	Organização Mundial de Saúde
RBC	Red blood cell
REMA	Rezasurin Microtiter Assay
SINAN	Sistema de Informação de Agravos de Notificação

TB	Tuberculose
THF	Tetraidrofurano
TLC	Thin layer crhomatography
UFC	Unidades Formadoras de Colônias
WBC	White blood cell
WHO	World Health Organization
XDRTB	Tuberculose Extremamente Resistente às Drogas

Resumo

A recente emergência das cepas de Mycobacterium tuberculosis multidroga-resistente, extremamente resistente às drogas e totalmente resistente às drogas tem complicado o controle da tuberculose. Há uma necessidade urgente do desenvolvimento de novas moléculas originais, ativas e menos toxica do que as drogas anti-tuberculose. Plantas medicinais tem sido uma excelente fonte para o desenvolvimento de novas drogas. O objetivo deste estudo foi a avaliação da atividade anti-micobacteriana *in vitro* frente à *Mycobacterium tuberculosis* de 28 extratos alcoólicos e três óleos essenciais de plantas nativas e exóticas Brasileiras e posterior estudo bioguiado do fracionamento químico, isolamento dos constituintes e da toxicidade aguda *in vivo* do extrato ativo. Atividade anti-micobacteriana foi avaliada pelo método de redução da resazurina em microplacas (REMA). Para o estudo de toxicidade aguda utilizou-se modelo *in vivo* em camundongos Swiss fêmeas nas doses de 500, 1000 e 2000 $mg.kg^{-1}$ de peso corporal. A caracterização do extrato foi realizada por LC-MS, seguido do isolamento dos constituintes por métodos cromatográficos e identificação pela analise de dados espectroscópicos. Dos 28 extratos, o extrato metanólico obtido das folhas de *Annona sylvatica* apresentou atividade anti-micobacteriana, com CIM = 184,33 µg/mL e a FAE resultante do particionamento líquido-líquido teve CIM = 115,2 µg/mL. A caracterização do extrato por LC-MS identificou flavonóides e acetogeninas como principais constituintes. O estudo fitoquímico da FAE resultou no isolamento de quercetina, luteolina e almunequina. Dos compostos isolados da FAE, luteolina e almunequina foram os mais promissores com CIM de 236,8 µg/mL e 209,9 µg/mL, respectivamente. A administração aguda da fração FAE nas doses de 500, 1000 e 2000 $mg.kg^{-1}$ de peso corporal não promoveu sinais de toxicidade nos animais tratados. O extrato metanólico das folhas de *A. sylvatica*, FAE e os compostos isolados luteolina e almunequina são potencialmente ativos contra *M. tuberculosis*, não apresentando sinais de toxicidade aguda.

Palavras-chave: *Mycobacterium tuberculosis*, *Annona sylvatica*, redução da resazurina.

Abstract

The recent emergence of multidrug-resistant, extensively drug-resistant, and totally drug-resistant *Mycobacterium tuberculosis* strains have further complicated the control of tuberculosis. There is an urgent need of new molecules candidates to be developed as novel, active, and less toxic anti-tuberculosis drugs. Medicinal plants have been an excellent source of leads for the development of drugs The aim of this study was the evaluation of *in vitro* anti-mycobacterial activity against *Mycobacterium tuberculosis*. 28 alcoholic extracts and essential oils of three native and exotic and Brazilian plants . Further study of chemical fractionation, isolation of constituents and *in vivo* acute toxicity of the active extract. The anti-mycobacterial activity was evaluated through the resazurina reduction microtiter assay (REMA). For the acute toxicity study *in vivo* model was used in female Swiss mice at doses of 500, 1000 and 2000 $mg.kg^{-1}$ of body weight. The characterization of the extracts was performed by LC-MS, followed by the isolation of the constituents and identification by chromatographic analysis of spectroscopic data. Of the 28 extracts, the methanol extract obtained from the leaves of *Annona sylvatica* showed anti-mycobacterial activity with MIC = 184.33 µg/mL and the FAE resulting from liquid-liquid partitioning showed MIC = 115.2 µg/mL. The characterization of the extract by LC-MS identified flavonoids and acetogenins as main constituents. The phytochemical study of FAE resulted in the isolation of quercetin, luteolin and almunequin. Between the isolated compounds of the FAE, luteolin and almunequin were the most promising with MIC = 236.8 µg/mL and 209.9 µg/mL, respectively. Acute administration of FAE fraction in doses of 500, 1000 and 2000 $mg.kg^{-1}$ of body weight did not cause signs of toxicity in treated animals. The methanol extract of the leaves of *A. sylvatica*, FAE and isolated compounds luteolin and almunequina are potentially active against *M. tuberculosis*, showing no signs of acute toxicity.

Keywords: *Mycobacterium tuberculosis*, *Annona sylvatica*, resazurina reduction.

1 INTRODUÇÃO

A tuberculose é uma doença infecciosa, cujo agente etiológico é o bacilo *Mycobacterium tuberculosis*. A tuberculose pulmonar afeta os pulmões enquanto a forma extrapulmonar pode afetar outros sítios. O doente com a forma pulmonar da doença expele os bacilos no ar através da tosse. De forma geral, uma pequena parte das pessoas infectadas pelo bacilo desenvolve a doença, entretanto a probabilidade de desenvolvê-la é muito maior entre as pessoas infectadas pelo HIV (human immunodeficiency virus). A tuberculose é mais comum em homens em idade produtiva (OMS – TB Global Report 2012).

O tratamento para os casos de tuberculose consiste em seis meses de utilização de quatro drogas de primeira linha: isoniazida, rifampicina, etambutol e pirazinamida. Já o tratamento da tuberculose multidroga resistente – MDRTB (multidrug resistant tuberculosis), definida por resistência a isoniazida e rifampicina, é mais longo e requer drogas de elevados custo e toxicidade (a duração do regime de tratamento chega a vinte meses) [1-2].

O tratamento de longa duração, leva ao abandono e à emergência da MDRTB e XDRTB (extremely drug resistant tuberculosis) [3-4]. A MDRTB é considerada uma doença emergente, bem como as consequências do tratamento inadequado [5].

As drogas utilizadas para o tratamento da MDRTB são menos efetivas, mais tóxicas, e mais caras do que as utilizadas no esquema básico, o que além de prolongar o tempo de tratamento, aumenta seus custos, e aumenta o risco de ocorrência de efeitos indesejáveis, diminuindo a adesão, e aumentando as taxas de falha do tratamento [6-8].

A emergência da multidroga resistência, reforça a necessidade urgente de se encontrar novas drogas que reduzam o tempo e a complexidade do tratamento, e que consigam erradicar as infecções persistentes. Apesar deste contexto, nenhuma nova droga foi desenvolvida nos últimos 40 anos [9]. A Jhonson & Jhonson anunciou a aprovação de uma nova droga pelo FDA (Food and Drug Administration) em 2013, o Sirturo, que deve ser utilizada em conjunto com as drogas clássicas para a erradicação de infecções persistentes. Porém a droga ainda deverá passar por ensaios de fase clínica, antes de ser disponibilizada para a população geral [10].

A medicina popular utiliza largamente as plantas medicinais como adjuvantes no tratamento de diversas doenças. Entretanto, sabe-se que as plantas possuem diversos metabólitos secundários com diferentes atividades biológicas [11]. Nas populações indígenas o uso de plantas para o tratamento e cura de doenças é largamente difundido, e os ensinamentos são repassados por xamãs e pajés de geração em geração. Etnobotânicos e etnofarmacologistas têm saído a campo, em busca destes conhecimentos, a fim de identificar plantas que possam levar ao desenvolvimento de novos medicamentos [12].

Devido ao lento crescimento das cepas de micobactérias, os testes que se baseiam no desenvolvimento das colônias ou na turbidez necessitam de um longo período de incubação, tornando-se mais longos e caros [13].

Recentemente, métodos mais rápidos, reprodutíveis e de baixo custo, em relação aos anteriormente citados, para a determinação da susceptibilidade às drogas vêm sendo desenvolvidos e descritos. São métodos colorimétricos, que se baseiam no uso de indicadores de Oxi-Redução, que produzem mudança de cor na presença de células viáveis de micobactérias [14], como Alamar Blue, Resazurin Reduction Assay, Tetrazolium based assay, entre outros [13, 15-17].

Devido ao grande impacto epidemiológico da tuberculose e suas consequências sociais e econômicas, e à estimativa de que menos de 10% da biodiversidade do planeta foram analisadas para algum tipo de atividade biológica [18] faz-se necessária a busca por novas drogas, sobretudo contra cepas resistentes às drogas de primeira e segunda linha, principalmente através de pesquisas e estudos sobre a utilização de plantas no desenvolvimento de novos compostos, fazendo-se importante a triagem de extratos de plantas ativos contra *M. tuberculosis*.

2 REVISÃO DA LITERATURA

A tuberculose, ainda hoje, representa um grave problema de saúde pública em todo o mundo, principalmente nos países em desenvolvimento [19-20]. Doença infecciosa e transmissível, a tuberculose é causada por uma bactéria de morfologia bacilar, o *M. tuberculosis,* também conhecido como Bacilo de Koch [21]. Possui evolução crônica e acomete principalmente os pulmões, mas pode afetar outros órgãos e tecidos [22].

Com a introdução da terapia baseada na utilização de antibióticos como a estreptomicina, ácido p-aminosalicílico, e a isoniazida, a partir da década de 40, observou-se uma queda nos índices de mortes por doenças infecciosas, inclusive por tuberculose [23]. Por volta nos anos 1980, os índices da doença estavam tão baixos, que se chegou a acreditar na sua erradicação, principalmente nos países desenvolvidos [24].

Após a década de 80, fatores como a epidemia de AIDS (acquired immunodeficiency syndrome), problemas socioeconômicos mundiais, movimentos migratórios, deficiências nos programas de controle e sistemas de saúde, além do surgimento das cepas de micobactérias resistentes, a tuberculose torna-se uma doença reemergente, com um aumento expressivo do número de casos nos países em desenvolvimento, e o reaparecimento nos países industrializados [25].

A Organização Mundial de Saúde (OMS) declarou em 1993, estado de emergência mundial, com a previsão de que em quatro anos, um terço da população mundial estaria infectada pelo *M. tuberculosis*, e destes, 5% desenvolveriam a doença ativa, ressaltando a necessidade de medidas efetivas para o controle da doença [20, 26].

O Brasil ocupa o 15º lugar na lista dos 22 países responsáveis por 80% dos casos de tuberculose em todo o mundo, ocorrendo 111.000 casos novos por ano e 6.000 óbitos. Segundo o SINAN – MS (Sistema de Informação de Agravos de Notificação – Ministério da Saúde), em 2010 o coeficiente de incidência da tuberculose no Brasil foi de 37,57/100.000 habitantes Fonte: MS/SVS - Sistema de Informação de Agravos de Notificação - SINAN [27-28].

A estratégia DOTS – "Directly Observed Treatment Short-Course" foi lançada em 1994, pela OMS, e engloba cinco elementos: vontade política, garantia da baciloscopia, aquisição e distribuição regular de medicamentos, tratamento assistido e regular sistema de

informações. Este plano foi criado na tentativa de minimizar a transmissão da doença, e possui metas como diagnosticar pelo menos 70% dos novos casos e curar 85% dos doentes, diminuindo a taxa de abandono, evitando o surgimento de bacilos resistentes e possibilitando um efetivo controle da tuberculose no país [20, 29].

2.1 Tuberculose Multidroga Resistente

Nas últimas décadas, entra em cena um problema importante, e de impacto negativo nos programas de controle da doença: a multidroga-resistência. Cepas de bacilos resistentes às drogas de primeira linha utilizadas para o tratamento (rifampicina e isoniazida) são chamadas de MDRTB, e aquelas resistentes às drogas de primeira e segunda linha, são consideradas extremamente resistentes: XDRTB [30-31].

A resistência está associada a fatores como terapia incorreta ou incompleta e abandono do tratamento por parte do paciente [32-33], além disso, aumento das aglomerações humanas, migração dos povos, aumento do número de imunossuprimidos e alta incidência de coinfecção com HIV, aumentam as chances de ocorrência da resistência aos antimicrobianos [24, 34-35].

No Brasil o regime de tratamento é baseado em quatro esquemas diferenciados de antibioticoterapia: Esquema I, Esquema I Reforçado, Esquema II e Esquema III, de acordo com a forma de tuberculose apresentada pelo paciente (Pulmonar, Extrapulmonar ou Meningoencefálica) ou para pacientes recidivantes.

Os medicamentos utilizados no tratamento da tuberculose incluem drogas de primeira escolha, e outras de atividade antibacteriana de amplo espectro. As drogas de primeira escolha são: isoniazida, rifampicina, pirazinamida e etambutol. Capreomicina, viomicina, cicloserina/terizidone, etionamida/protionamida, e ácido para-amino-salicílico podem ser utilizados para cepas resistentes. Outras indicações incluem os aminoglicosídeos, e as fluoroquinolonas. Os betalactâmicos, linezolida, clofazimina, claritromicina, dapsona, e metronidazol podem ser utilizados ocasionalmente para a MDRTB ou XDRTB [36-37].

O tratamento da tuberculose ativa requer quimioterapia combinada para evitar a ocorrência de seleção natural de cepas resistentes e mutações. Ao contrário de outras infecções bacterianas a combinação escolhida não se baseia nos mecanismos de complementação ou sinergismo. O regime inicial foi definido por aquilo que estava

disponível em meados do século 20, e depois foram incluídas as novas drogas desenvolvidas juntamente com as antigas. Acredita-se que diferentes esquemas, baseados em doses e frequências diferentes possam alcançar melhores resultados com as mesmas drogas [38].

A resistência às terapias disponíveis faz com que o tratamento seja mais difícil e caro, necessitando de drogas mais tóxicas e de efetividade ainda não comprovada, prolonga o tratamento e aumenta seus custos em até 100%, bem como aumenta os riscos de ocorrência de efeitos adversos, podendo levar a um aumento das taxas de abandono e falha do tratamento [4, 6, 8].

2.2 Extratos vegetais utilizados para o tratamento da tuberculose

A OMS alerta que são necessárias novas drogas para o tratamento da tuberculose [39-40]. A descoberta de novas drogas a partir de plantas medicinais fornece pistas importantes contra diversos alvos farmacológicos, como o câncer [41-43], AIDS [44-46], malária [47-49], doença de Alzheimer [50-52], e outras [53-55]. Diversas drogas de origem natural foram introduzidas recentemente no mercado em países desenvolvidos como os EUA (Estados Unidos das Américas) [56].

Indígenas têm utilizado plantas e seus extratos para o tratamento de doenças infecciosas desde antes da descoberta dos antibióticos. Um estudo realizado identificou mais de 80 plantas que vêm sendo utilizadas popularmente na Uganda para tratar a tuberculose [57].

No México foi realizada uma pesquisa baseada na literatura etnobotânica, que selecionou plantas utilizadas por curandeiros tradicionais para testar sua atividade contra o *M. tuberculosis* [58].

Enquanto grande parte do foco estava voltado para o desenvolvimento de compostos sintéticos para a inibição de micobactérias, as plantas vem provendo uma fonte representativa de compostos antibacterianos. Por exemplo, a *Clavija procera* B. Ståhl mostrou-se ativa contra *M. tuberculosis* e contra cepas resistentes [59], entre outras plantas já estudadas, como mostra a Tabela 1.

Tabela 1. Plantas testadas contra *Mycobacterium tuberculosis.*

Nome Científico	Atividade antimicobacteriana	CIM	País	Autor, ano
Abelmoschus esculentus Moench	Leve / Moderada	N.D.	Zimbabue	Chimponda, and Mukanganyama, 2010
Acorus calamus L. var. americanus (Raf.);	Potente	N.D.	Canadá	Webster, *et al*., 2010
Adhatoda vasica Ness.	Significante	N.D.	Índia	Ignacimuthu and Shanmugam, 2010
Aloe vera barbadensis Miller	Leve / Moderada	N.D.	Zimbabue	Chimponda, and Mukanganyama, 2010
Aralia nudicaulis L.;	Significante	N.D.	Canadá	Webster, *et al*., 2010
Aristolochia taliscana Hook	Potente	50 (µg/mL)	México	Rosalba León-Díaz *et al*., 2010
Dugandiodendron argyrotrichum Lozano	Significante	N.D.	Colômbia	Guzman *et al*., 2010
Faurea saligna Harv	Potente	250 (µg/disco)	Zimbabue	Chimponda, and Mukanganyama, 2010
Garcinia huillensis Welw	Leve / Moderada	N.D.	Zimbabue	Chimponda, and Mukanganyama, 2010
Heracleum maximum Bartr.;	Significante	N.D.	Canadá	Webster, *et al.,* 2010
Ocotea macrophylla Kunth	Significante	N.D.	Colômbia	Guzman *et al*., 2010
Piper eriopodon	Significante	N.D.	Colômbia	Guzman *et al*., 2010
Piper hispidum Kunth	Significante	N.D.	Colômbia	Guzman *et al*., 2010

CIM – Concentração Inibitória Minima
N.D. – Não descrita

Com o aumento da necessidade de novas drogas para o combate das cepas resistentes às drogas de primeira e segunda escolha, existe uma urgência no desenvolvimento de técnicas e ensaios para a triagem de novas drogas, que sejam mais rápidas, de baixo custo e de alto rendimento [13].

A descoberta de novas drogas depende de ensaios celulares para avaliar e triar novos compostos com atividade anti-tuberculose, e contra bacilos dormentes. Entretanto as pesquisas de novos candidatos a drogas são afetadas pelas limitações das técnicas usuais baseadas em UFC (Unidades Formadoras de Colônias) [15].

Devido ao lento crescimento do *M. tuberculosis*, o tempo de incubação dos testes de susceptibilidade aos antimicrobianos que são baseados em crescimento e contagem de colônias ou métodos de turbidimetria é muito longo [13].

Há algumas décadas entrou em cena um sistema semi-automatizado para a realização rápida de testes de susceptibilidade, BACTEC 460 TB (System Mycobacterial Culture Media). Apesar do alto rendimento, este sistema possui algumas desvantagens como o alto custo, e a utilização de radioisótopos, o que limita sua utilidade para triagens em massa [60-61].

2.3 Metodologias para a triagem de novas drogas

Novas abordagens não baseadas em turbidimetria ou contagem UFC, tem sido descritas [62-67]. Porém, a maioria destes testes deixa a desejar em pelo menos um ponto: rapidez, baixo custo ou alto rendimento, importantes para um ensaio de triagem em massa [13].

As metodologias mais modernas, baseadas na aplicação de corantes de oxi-redução, como Alamar Blue, REMA (Resazurin Reduction Microtiter Assay), MTT (dimethyl thiazolyl diphenyl tetrazolium salt) e outros, são em geral, mais rápidos, eficientes e baratos, possibilitando a triagem de um grande número de amostras. O Alamar Blue é um corante de oxi-redução, indicador geral de crescimento e viabilidade celular, passando de azul não fluorescente para rosa fluorescente após a redução [68]. Assim o crescimento celular pode ser mensurado através de um espectrofotômetro [13]. A reação de redução da resazurina ocorre de acordo com a Figura 1.

O ensaio colorimétrico da resazurina permite um teste de susceptibilidade rápido para micobactérias em condição aeróbia. Neste teste, a forma oxidada do corante é azul e não fluorescente, e é reduzida através da ação das células viáveis a forma de resorufina, que é rosa e fluorescente [69-70].

De acordo com o programa de seleção de novas drogas contra a tuberculose desenvolvido como parte de um programa de pesquisa pelo National Institutes of Healf dos EUA, devem ser utilizados modelos *in vitro* e *in vivo* para encontrar compostos promissores a novos agentes anti-tuberculose [71].

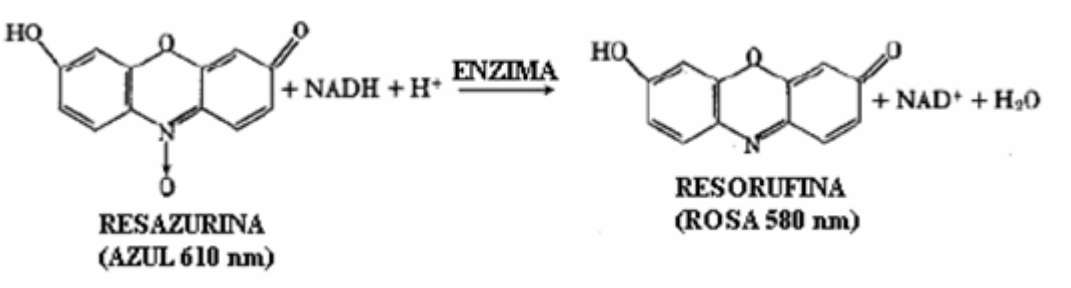

Figura 1. Reação química de redução da resazurina para resorufina

Nos ensaios *in vitro* determina-se a concentração inibitória mínima (CIM) de cada amostra frente aos *M. tuberculosis* H37Rv ATCC 27294, através da técnica Rezasurin Reduction Microtiter Assay (REMA) [70]. A etapa seguinte consiste na avaliação da citotoxidade - DL_{50} (dose letal), determinando-se a maior concentração capaz de permitir a viabilidade de 50% das células frente a culturas de macrófagos da linhagem J774 [72]. Na sequencia, determina-se um Índice de Seletividade (IS), através da razão entre o DL_{50} e a CIM. Um IS superior a 10 indica que a amostra analisada pode ser aplicada na concentração 10 ou mais vezes a CIM, sem apresentar citotoxicidade [71]. Compostos com IS $\geq$ 10 são considerados promissores e a próxima etapa consiste na avaliação da atividade intracelular do composto frente a macrófagos infectados com *M. tuberculosis* [73].

Ensaios *in vivo* têm a função de confirmar a investigação em modelos animais, determinando-se primeiramente a dose simples de tolerância máxima da amostra em ratos ou camundongos [74]. Verifica-se ainda a capacidade do candidato à droga anti-tuberculose de reduzir a carga bacilar nos pulmões de animais infectados [71].

3 OBJETIVOS

3.1 Objetivo Geral

Caracterizar a ação antimicobacteriana *in vitro* e a toxicidade aguda *in vivo* de extratos de plantas através do método de redução da resazurina (REMA).

3.2 Objetivos Específicos

Realizar uma triagem de extratos vegetais contra *M. tuberculosis*.

Estabelecer os valores de CIM destes extratos.

Identificar e avaliar as frações e principais compostos responsáveis pela ação antimicobacteriana do extrato ativo.

Avaliar a toxicidade aguda *in vivo* dos extratos/frações ativos.

Traçar um comparativo entre a atividade do extrato bruto, frações e compostos.

4 REFERÊNCIAS BIBLIOGRÁFICAS

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5. ANEXOS

5.1 Artigo Científico

Title: Evaluation of the anti-*Mycobacterium tuberculosis* activity and *in vivo* acute toxicity of *Annona sylvatica*

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ABSTRACT

The recent emergence of extensively multidrug-resistant *Mycobacterium tuberculosis* strains has further complicated the control of tuberculosis. There is an urgent need for the development of new molecular candidates anti-tuberculosis drugs. Medicinal plants have been an excellent source of leads for the development of drugs. The aim of this study was to evaluate the *in vitro* activity of 28 alcoholic extracts and essential oils of native and exotic Brazilian plants against *Mycobacterium tuberculosis* and to further study these extracts through chemical fractionation, the isolation of their constituents, and an evaluation of the *in vivo* acute toxicity of the active extracts. The anti-mycobacterial activity of these extracts and their constituent compounds was evaluated using the resazurin reduction microtiter assay (REMA). To investigate the acute toxicity of these extracts *in vivo*, female Swiss mice were treated with the extracts at doses of 500, 1000 and 2000 $mg \cdot kg^{-1}$ of body weight. The extracts were characterized by LC-MS, and the constituents were isolated and identified by chromatographic analysis of spectroscopic data. Of the 28 extracts, the methanol extract obtained from the leaves of *Annona sylvatica* showed anti-mycobacterial activity with an minimal inhibitory concentration (MIC) of 184.33 µg/mL, and the ethyl acetate fraction (EAF) resulting from liquid-liquid partitioning of the *A. sylvatica* extract showed an MIC of 115.2 µg/mL. The characterization of this extract by LC-MS identified flavonoids and acetogenins as its main constituents. The phytochemical study of the *A. sylvatica* EAF resulted in the isolation of quercetin, luteolin, and almunequin. Among the compounds isolated from the EAF, luteolin and almunequin were the most promising, with MICs of 236.8 µg/mL and 209.9 µg/mL, respectively. The acute administration of the EAF fraction in doses of 500, 1000, and 2000 $mg \cdot kg^{-1}$ of body weight did not cause signs of toxicity in the treated animals.

Keywords: *Mycobacterium tuberculosis*, *Annona sylvatica*, resazurin reduction toxicity, luteolin, almunequin

INTRODUCTION

Tuberculosis remains an important public health problem and a major cause of death worldwide; it is responsible for approximately one million deaths every year [1]. Due to the reduced effectiveness of current drugs resulting from the emergence of multidrug-resistant strains (MDRTB) and co-infection with HIV, there is an urgent need to develop new natural or synthetic anti-tuberculosis drugs [2,3].

The use of medicinal plants is important throughout the world, especially in traditional or alternative medicine. The search for active plant-derived compounds is a modern approach to drug discovery, especially in tropical regions with abundant flora. In underdeveloped or developing countries, medicines derived from plants are important weapons against serious diseases. Traditional medicine has enabled the treatment of common illnesses in approximately 60 to 80% of the world population [4,5]. The extraction of compounds from plants and the testing of the biological activity of those extracts and/or compounds represent the first steps toward identifying natural products or semi-synthetic derivatives that may provide new anti-tuberculosis drugs [6].

Research in the area of natural products has intensified, especially in the search for compounds and plant species that are active against *Mycobacterium tuberculosis* and with the development of easier, faster, and safer screening techniques [7,8,9,10,11,12,13,14,15,16]. Previous studies have demonstrated that several plants are active against *M. tuberculosis*, such as *Clavija procera* B. Ståhl, which has been shown to be active even against resistant strains [8]; *Abelmoschus esculentus* Moench; *Faurea saligna* Harv; *Parinari curatellifolia* Planch ex Benth [9]; *Maerua edulis* (Gilg & Gilg-Ben.) DeWolf; *Securidaca longepedunculata* Fres.; *Tabernaemontana elegans* Stapf; *Zanthoxylum capense* (Thunb.) Harv. [10]; *Aristolochia taliscana* Hook [11]; and *Acorus calamus* L. var. americanus [12].

According to the screening program for new anti-tuberculosis drugs developed by the National Institutes of Health of the United States, all compounds must be used in both *in vitro* and *in vivo* models to evaluate their potential as anti-tuberculosis agents [17].

This study was conducted as a preliminary *in vitro* screening of 28 plant extracts and three essential oils against *M. tuberculosis* using the REMA assay. The characterization of the active extract by LC-MS, the isolation of the main constituents of this extract, and the subsequent evaluation of the anti-*M. tuberculosis* activity and *in vivo* acute toxicity of these extracts were also performed.

METHODS

Plant material. Different species of Asteraceae, Anacardiaceae, Annonaceae, Bignoniaceae, Euphorbiaceae, Fabaceae, Gesneriaceae, Malvaceae, Meliaceae, Myrtaceae, Rubiaceae, Sapindaceae, and Tropaeolaceae were collected in Dourados, Mato Grosso do Sul, and Maringá and Curitiba, Paraná, Brazil. The plants were identified by Dr. Armando Carlos Cervi, Dr. Maria Conceição de Souza, and Dr. Zefa Valdevina Pereira. A voucher specimen of each species was identified and deposited in the herbarium of each institution (Table 1).

Preparation of extracts. The plants were air-dried at room temperature and ground with a pestle and mortar. Approximately 300 g of each sample was then exhaustively extracted by macerating the sample with 1.5 L of the appropriate solvents (Table 1) at room temperature (five times for each species at 48 h intervals). The crude extract was then isolated by the evaporation of the solvent under vacuum on a rotary evaporator. For subsequent studies, the extracts were diluted in DMSO (dimethyl sulfoxide) at 10,000 μg/mL.

Fractionation and compound isolation. An extract that exhibited potential activity against *Mycobacterium tuberculosis* was dissolved in methanol:water (1:1) and partitioned with chloroform and ethyl acetate to yield the chloroform fraction (CF), ethyl acetate fraction (EAF), and hydromethanol fraction (HMF) after the evaporation of the solvents on a rotary evaporator. The resulting EAF (2.3g) was applied to a chromatographic column on silica gel and eluted with a mixture of chloroform:methanol in increasing polarity, yielding 54 fractions of 10 mL each. After a thin-layer chromatography (TLC) comparison, the fractions with similar TLC patterns were grouped into ten sub-fractions. Sub-fraction 5 (173 mg) was fractionated on a Sephadex LH-20 column using H2O, H2O:MeOH 1:1, and MeOH; this process yielded two known flavonoids (6.5 mg and 5.8 mg). A series of experiments was conducted to isolate the acetogenin. Sub-fraction 8 (126 mg) was purified by flash chromatography on silica gel to yield almunequin (8.4 mg).

The isolated compounds were identified by an analysis of their nuclear magnetic resonance (NMR) data. NMR measurements were conducted on a Varian Mercury Plus BB spectrometer operating at 300 MHz for ^{1}H and 75.5 MHz for ^{13}C using CD_3OD and $CDCl_3$ as the solvents and tetramethylsilane (TMS) as the internal standard. The ^{1}H NMR and ^{13}C

NMR data are in agreement with the reported data for quercetin [18], luteolin [19], and almunequin [20,21]:

Quercetin ^{1}H NMR (300 MHz, CD_3OD) δ ppm: 7.65 (1H, d, J = 1.8 Hz), 7.00 (1H, d, J = 8.9; 2.0 Hz), 6.9 (1H, d, J = 8.9 Hz), 6.43 (1H, d, J = 2.1 Hz), 6.20 (1H, d, J = 2.1 Hz). ^{13}C NMR (75.5 MHz, CD_3OD) δ ppm: 158.5, 135.6, 179.4, 163.0, 99.9, 166.2, 94.8, 159.3, 104.7, 123.5, 117.6, 145.8, 149.3, 116.5, 123.1.

Luteolin ^{1}H NMR (300 MHz, CD_3OD) δ ppm: 7.43 (1H, d, J = 2 Hz), 6.68 (1H, d, J = 8.4; 1.8 Hz), 6.87 (1H, d, J = 8.4 Hz), 6.39 (1H, d, J = 2.1 Hz), 6.20 (1H, d, J = 2.1 Hz). ^{13}C NMR (75.5 MHz, CD_3OD) δ ppm: 158.5, 135.6, 179.4, 163.0, 99.9, 166.2, 94.8, 159.3, 104.7, 123.5, 117.6, 145.8, 149.3, 116.5, 123.1.

Almunequin ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.99 (1H, d, J = 1.2 Hz), 4.99 (1H, *dq,* J = 7.0; 1.2 Hz), 3.84 (5H, m), 3.60 (1H, m), 3.41 (2H, m), 2.25 (2H, t, J = 6.8 Hz), 2.00-1.60 (4H, m), 1.60-1.20 (m, CH_2), 1.40 (3H, d, J = 6.9 Hz, CH_3), 0.87 (3H, t, J = 6.8 Hz, CH_3); ^{13}C NMR (75.5 MHz, $CDC1_3$): 176.4, 148.8, 134.2, 83.2, 82.1, 81.9, 79.3, 77.3, 74.4, 72.0, 71.8, 37.3, 37.0, 35.6, 32.3, 29.5, 29.3,29.1, 28.6, 28.34, 26.1, 25.6, 31.8, 27.3, 25.1, 22.5, 22.0, 19.1, 14.0.

Essential oil extraction. Fresh leaves of *A. sylvatica, Trichilia silvatica,* and *S. terebinthifolius* were subjected to steam distillation for 3 h using a Clevenger-type apparatus. The oil was dried by anhydrous sodium sulfate and preserved in a sealed vial at 4°C until analysis.

LC-MS analysis of the methanolic extract of A. sylvatica. Studies using liquid chromatography coupled with mass spectrometry (LC-MS) were performed using a quad MS system spectrometer (Bruker, Bremen, Germany). Mass spectrometry was carried out in positive mode, and negative ionization (ESI) was performed using a mass/charge (m/z) ratio range from 60 to 1000. The sample was analyzed on an analytical LC system (Varian) with a ternary solvent fitted with an automatic sample, a diode array detector (PDA), and a mass spectrometer (Bruker). The LC column was a Luna C-18 column (25 cm × 4.6 mm; particle size, 5 μm) (Phenomenex, Torrance, CA, USA) with a small pre-column (2.5 cm × 3 mm) containing the same filling used to protect the analytical column. The flow rate was 1.0 mL/min, and an injected volume of 10 L was used for each analysis. All liquid chromatographic analyses were performed at 22°C. The elution was conducted using a

solvent gradient of 0.1% formic acid:acetonitrile (85:15, v/v); the elution required 40 min to reach 30% formic acid and 70% acetonitrile and was then returned to the initial conditions in exactly 5 min.

Anti-M. tuberculosis activity. The anti-*M. tuberculosis* activity of the extracts, essential oils, and compounds was determined using the REMA method [22]. *M. tuberculosis* H_{37}Rv ATCC 27294 was grown for 15 days in Middlebrook 7H9 broth (Difco) supplemented with OADC enrichment (BBL/Becton-Dickinson) containing oleic acid, albumin, dextrose, and catalase; 0.5% glycerol as a carbon source; and 0.5% Tween 80 to prevent clumping. Suspensions were prepared, and the turbidity was adjusted to a McFarland no. 1 standard.

Stock solutions of the tested extracts were prepared in DMSO, and dilutions to obtain final concentrations ranging from 0.98 to 250 μg/mL were prepared in Middlebrook 7H9 broth supplemented with oleic acid, albumin, dextrose, and catalase (OADC enrichment, BBL/Becton-Dickinson, Sparks, MD, USA). Isoniazid, rifampicin, streptomycin, and ethambutol were solubilized according to the manufacturers' recommendations (Difco Laboratories, Detroit, MI, USA) and used as positive control drugs.

After further dilutions to reach the final bacterial suspension concentration, 100 μL of the inoculum was added to each well of a 96-well microtiter plate containing the extracts. The assays were set up in duplicate. The plates were incubated for 7 days at 37°C, and after this incubation, 30 μL of 0.1 mg/mL resazurin was added. The wells were read for color change and fluorescence in a SPECTRAfluor Plus microfluorimeter (TECAN) (excitation/emission with 530/590 nm filters, respectively) after 24 h. The MIC (minimum inhibitory concentration) was defined as the lowest concentration resulting in a 90% growth inhibition of *M. tuberculosis*. The MIC values of isoniazid (0.06 μg/mL), rifampicin (0.03 - 0.06 μg/mL), streptomycin (0.25 μg/mL), and ethambutol (2.0 - 4.00 μg/mL) were determined in a single plate as standards [23].

A sample with an MIC value < 250 μg/mL was defined as active against *M. tuberculosis*, and further analysis was applied [24].

Animals and acute toxicity tests. Adult female Swiss mice (19 to 24 g) from the Federal University of Grande Dourados were maintained at a controlled temperature (23°C) and humidity (50% - 60%) with a constant 12 h light-dark cycle and free access to food and

water. The experimental procedures were in accordance with the Ethical Principles in Animal Research and approved by the Committee for Ethics in Animal Experimentation at the Federal University of Grande Dourados (Protocol no. 005/2010).

The acute toxicity studies were conducted according to OECD (Organization for Economic Cooperation and Development) Guideline 425 [25] and ANVISA (Brazilian Health Surveillance Agency) guidelines. After 12 h of fasting, the animals were divided into four groups. The treatments were performed by single oral administration as doses of 0, 500, 1000, and 2000 mg/kg of body weight of the EAF. The animals were observed for signs of toxicity over 14 days. Behavioral parameters, mortality, the weight of the animals, and the amount of water and feed were analyzed.

After 14 days of treatment, the animals were weighed and anesthetized (ketamine and xylazine, 25 and 10 mg/kg, respectively). Blood samples were collected with and without anticoagulant (heparin sodium, Cristália). The blood samples were used to determine the hematological parameters (total and differential leukocyte count, hematocrit, hemoglobin, and erythrocyte count), and the serum samples were used for biochemical analysis (aspartate aminotransferase – AST, alanine aminotransferase – ALT, gamma glutamyl transferase – γ-GT, urea, and creatinine) [25,26]. The biochemical parameters were determined by spectrophotometry (Gold Analysis kits).

Subsequently, the animals were euthanized, and the vital organs (lung, liver and right kidney) were removed and weighed (absolute and relative weight). For the histopathological analysis of these organs, the samples were fixed in 10% buffered formalin, and the tissues were processed by conventional techniques in 5-mm-thick paraffin slices. Slides were prepared and stained with hematoxylin and eosin for light microscopy examination. The evaluated parameters were reversible (degeneration) and irreversible (necrosis and apoptosis) cell damage, leukocyte infiltration, congestion, blood extravasation, and fibrosis.

The data were evaluated using an analysis of variance with an F-test, with $p<0.05$ defined as significant.

Ethics statement. These field studies did not involve endangered or protected species and no specific permits were required for the described studies. The studies performed with species of Annonaceae, Bignonaceae, Meliaceae, Fabaceae and *Myrcia* species were

collected in particular area, with access permitted the researchers to collect botanical material. The species of Asteraceae, Anacardiaceae, Tropaeolaceae and Malvaceae were collected in the Medicinal Plants Garden of the Federal University of Grande Dourados, and Gesneriaceae in the Municipal Botanical Museum of Curitiba. The Rubiaceae were collected in a Brazilian stretch of the Upper Paraná River, Porto Rico, park ecosystem components collection for scientific purposes. The work with Sinningia species had an access authorization to genetic patrimony given by CNPq (nr 010087/2012-5). Professor Armando C. Cervi (Federal University of Paraná) collected S. aggregata and S. canescens, and Clarice Bolfe Poliquesi (Municipal Botanical Museum of Curitiba) collected Sinningia allagophylla.

RESULTS AND DISCUSSION

Antimycobacterial activity. The species and tested parts of the plants used in the evaluation of anti-mycobacterial activity are shown in Table 1. Of the 28 samples, only the crude extract from *A. sylvatica* (MIC = 184.33 µg/mL) exhibited promising activity (Table 1).

Several studies and screens must be performed to develop a new drug for tuberculosis; therefore, many drug discovery studies ultimately fail. A study in Mozambique screened 75 extracts of medicinal plants used for the local treatment of symptoms related to tuberculosis and identified eight extracts with moderate to significant activity against *M. tuberculosis* H37Rv. Of these extracts, six showed MICs that were higher than those observed for *A. sylvatica* and the *A. sylvatica* EAF. One extract exhibited an MIC of 62 µg/mL, and another exhibited an MIC of 15 µg/mL [10]. Another study evaluated the anti-mycobacterial activity of the crude extract of *Byrsonima crassa* (leaves and bark) and obtained an MIC value of 62.5 µg/mL for the chloroform extract of the leaves. The chloroform extract of the bark presented an MIC of 312.25 µg/mL [27].

The fractionation of the extract by partitioning in different solvents provided the chloroform (CF), ethyl acetate (EAF), and hydromethanol (HMF) fractions, which were subsequently evaluated for their anti-mycobacterial activity. An evaluation of the MIC of these fractions (Table 2) revealed the potent activity of the EAF fraction, with an MIC value of 115.2 µg/mL.

The Brazilian flora is rich in plants of the family Annonaceae, which comprises approximately 120 genera and 2000 - 2200 species. This family is important as a source of

various edible fruits and seeds that can be used for the production of edible oils. The major components identified in members of the Annonaceae are typically acetogenins [28,29,30].

Annona sylvatica A.St.-Hill (formerly known as *Rollinia sylvatica* St.-Hil. Mart) is a native Brazilian plant found in Minas Gerais and São Paulo to Rio Grande do Sul. The leaves of *A. sylvatica* have been used mainly as an antipyretic in folk medicine [31]. There are no reports in the literature on the antimicrobial activity of this species, but studies have reported that the essential oil obtained from the leaves has anti-inflammatory activity and anticancer properties [32]. A chemical study reported the isolation of sylvatin from *Rollinia sylvatica* [33]. Preliminary LC-MS studies have shown that the major metabolites in the leaves of *A. sylvatica* are flavonoids and acetogenins (Figure 1).

The results of the present study indicate that the effects of the methanolic extract of *A. sylvatica* and ethyl acetate fraction may be associated with other components of the extract (which were not evaluated) or that there might be a synergism between the active isolated compounds.

Annonaceae acetogenins are secondary metabolites derived from polyketides and are structurally characterized by a long-chain terminal α, β-unsaturated methyl γ-lactone. This chain hydrocarbon generally contains one, two, or rarely three tetrahydrofuran (THF) rings. Acetogenins belong to two different classes: bis-tetrahydrofuran non-adjacent almunequin and dihydroalmunequin 2.5 and 2, and β-hydroxy-methyl-γ-lactone and laherrandurin otivarin. The acetogenins identified in the extract of *A. sylvatica* have been reported in other *Annona*, including *A. atemoya* and *A. cherimola* [20,34,35,36].

In this context, the present study revealed the potential anti-*M. tuberculosis* activity of the methanolic extract of *A. sylvatica*, which is still poorly characterized in phytochemical and pharmacological terms. To characterize the possible compounds responsible for this activity, we investigated the individual compounds in the methanolic extract of *A. sylvatica*; our chromatogram of the lyophilized extract (Figure 1) showed characteristic distributions of the flavonoids luteolin (m/z 286) (1) and quercetin (m/z 302) (2) and the acetogenins laherrandurin (m/z 624) (3), almunequin (m/z 638) (4), otivarin (m/z 640) (5), and 2,5 dihydroalmunequin (m/z 640) (6) (Figure 1). This is the first report of the chemical characterization of compounds from *A. sylvatica* leaves.

The isolated compounds evaluated for anti-mycobacterial activity are shown in Table 2. In the present study, we examined the effects of two flavonoids: luteolin and quercetin. Among the isolated compounds, luteolin and almunequin showed anti-*M.*

tuberculosis effects, with MIC values of 236.8 µg/mL and 209.9 µg/mL, respectively. Our data indicate that quercetin, unlike luteolin and almunequin, failed to exhibit anti-mycobacterial activity at low concentrations.

Luteolin isolated from the flowers of *Chromolaena odorata* showed weak activity (699.3 µM MIC) against *M. tuberculosis* [37]. However, luteolin isolated from *Ficus chlamydocarpa* showed stronger activity against *M. tuberculosis* and *M. smegmatis* (78.12 µg/mL MIC) [38]. Nevertheless, the luteolin isolated from the whole plant of *Gentianopsis paludosa* was inactive against these microorganisms [39]. Having established the inhibitory effects of luteolin on *M. tuberculosis,* we investigated whether this agent exhibits a structure-activity relationship in terms of its biological function. The isolated flavonoids belong to the flavone (luteolin) and flavon-3-ol (quercetin) classes. The structures of quercetin and luteolin share double bonds between C2 and C3 in ring C, the 3', 4' -diOH ring A groups, and the 7, 8 -diOH ring A groups. However, ring C in these compounds shows considerable variation, as it is a 3-OH group in quercetin. This variation shows that catechol groups could not confer appreciable activity when they were found on both ring A and ring B. From a structural point of view, this finding suggests that the 3-OH in quercetin results in an inactivation of the structure, which might be critical for *M. tuberculosis* survival. The observed results for quercetin are consistent with those observed for quercetin isolated from *Helichrysum melanacme* against *M. tuberculosis* [40]. Based on our data and those of others [40], we suggest that the absence of hydroxylation in the C3 in structure of the luteolin is required for anti-mycobacterial activity. These results may provide a basis for the further design of new anti-mycobacterial drugs.

The compound almunequin was very difficult to isolate. Almunequin is a C37 annonaceous acetogenin with a bis-tetrahydrofuranic structure containing hydroxyl groups and α, β-unsaturated γ-lactone methyl group. The structure-activity relationship for almunequin can be attributed the α, β-unsaturated lactone present at the long-chain terminal. The α, β-unsaturated lactones are a class of synthetic and naturally occurring compounds that exhibit a large spectrum of important pharmacological properties [41,42,43,44,45,46].

Acute toxicity. The EAF fraction of *A. sylvatica* produced significant weight gain in treated animals (Table 3). During treatment, no clinical signs of toxicity were observed, and no death was recorded. There were also no changes in food or water intake. The oral

administration of this fraction generally did not produce toxic effects on the behavior of adult female Swiss mice. Apart from the weight increase, no visible clinical signs of toxicity were observed.

There was no evidence for differences in physiological or behavioral responses between the control group and any of the treated groups at any time. There were also no differences in the consumption of food and water.

The hematologic parameters of the treated groups did not differ from those of the control group. A biochemical evaluation confirmed these results; there were no significant differences in the AST, ALT, or γ GT results between the control group and treated animals. These enzymes are liver function markers, and changes in these parameters may result from reversible or irreversible hepatocellular membrane damage. Changes in these markers are often associated with necrosis, cholestasis, hypoxia, hypoperfusion, inflammation, infectious agents and toxins, or excess lipid or glycogen deposition in hepatocytes [47]. The integrity of liver function was assessed by histopathological analysis of the liver, and we found no damage associated with hepatotoxicity. The effects of the acute administration of the EAF fraction of *A. sylvatica* on hematological and biochemical parameters are presented in Table 4.

The EAF fraction of *A. sylvatica* had no acute toxicity, as evidenced by the absence of relevant clinical signs in the toxicological screening and the absence of death throughout the observation period. Hippocratic screening such as this is often used for the preliminary screening of plants for toxicological and pharmacological activity. The EAF fraction also did not have any influence on the consciousness of the animals during the observed period. No effects on the motor coordination or reflexes of the treated animals were observed. At doses of 500, 1000, and 2000 mg/kg, the EAF fraction produced no dose-dependent changes in histopathology between the control group and the treated animals (Figure 2). Therefore, EAF has low toxicity at high doses in the short term. Additional studies are required to determine the safety of this fraction over a prolonged period.

The acute administration of the EAF produced no toxic effects in adult female Swiss mice. No visible clinical signs of toxicity, such as irritability, twisting, righting reflex, tremors, convulsions, breathing, weight loss, or death, were observed. In the present study, we did not observe an increase in the accumulation of urea or creatinine. These

results were also confirmed by histopathological analysis of the kidneys, which indicates that the acute oral administration of *A. sylvatica* does not induce nephrotoxicity.

Ethics statement. These field studies did not involve endangered or protected species and no specific permits were required for the described studies. The studies performed with species of Annonaceae, Bignonaceae, Meliaceae, Fabaceae and *Myrcia* species were collected in particular area, with access permitted the researchers to collect botanical material. The species of Asteraceae, Anacardiaceae, Tropaeolaceae and Malvaceae were collected in the Medicinal Plants Garden of the Federal University of Grande Dourados, and Gesneriaceae in the Municipal Botanical Museum of Curitiba. The Rubiaceae were collected in a Brazilian stretch of the Upper Paraná River, Porto Rico, park ecosystem components collection for scientific purposes. The work with Sinningia species had an access authorization to genetic patrimony given by National Research Council (CNPq, 010087/2012-5). Professor Armando C. Cervi (Federal University of Paraná) collected S. aggregata and S. canescens, and Clarice Bolfe Poliquesi (Municipal Botanical Museum of Curitiba) collected Sinningia allagophylla.

CONCLUSION

To the best of our knowledge, this is the first chemical characterization, evaluation of anti-tuberculosis activity, and analysis of the *in vivo* acute toxicity of the *A. sylvatica* methanolic extract. Our study demonstrates the potential anti-mycobacterial activity and the lack of *in vivo* acute toxicity of its isolated compounds.

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Table 1. Plants (family and specimen), popular name, tested part (solvent), popular indication, and MICs of the extracts tested in this study

Family	Specimen	Popular name	Tested part (solvent)	Popular indication	MIC(µg/mL)
Asteraceae	*Gochnatia polymorpha* Less. UPCB 30100[a]	Cambará	Bark (e)	Antitussive [48]	>500
Anacardiaceae	*Schinus terebinthifolius* Raddi. DDMS 4602[b]	Pimenta rosa, aroeirinha, Aroeira vermelha	L (m); F (hm), OE	Uterine inflammation [49]	>250
Annonaceae	*Annona crassiflora* Mart. DDMS4599[b]	Araticum do cerrado	L (m)	Antidiarrheal [50]	>250
	Annona coriacea Mart. DDMS186[b]		L (m)	Antidiarrheal [51]	>250
	Annona sylvatica St.Hill DDMS4600[b]	Araticum	L (m)	Antitussive, antipyretic, antispasmodic [32]	184.33
	Annona cacans Warm. DDMS	Araticum-cagão, Araticum-de-paca	L (m)		>250
	Annona dioica A.St.-Hill DDMS 4598[b]	Ata-rasteira, Marolo	L (m)	Antirheumatic, antidiarrheal, expectorant [36]	>250
	Duguetia furfuracea A.St.-Hill DDMS 166[b]	Araticum seco, Araticum-miúdo, Ata-do-mato	L (m)	Antidiarrheal, antispasmodic [52]	>250
Bignoniaceae	*Jacaranda decurrens* Cham.	Carobinha	L (m)	Astringent [53]	>250
Euphorbiaceae	*Alchornea glandulosa* Poepp. & Endl. HU7569[c]	Tamanqueiro, Tapiá, Tanheiro	L (m)	Antidiarrheal, anti-inflammatory, antirheumatic, anti-leprosy [54]	>250
Fabaceae	*Stryphnodendron adstringens* (Mart.) Coville DDMS152[b]	Barbatimão	L (m), S (hs)	Leucorrhoea, bleeding, wound cleaning [55]	>250
Gesneriaceae	*Sinningia aggregata* (Ker. Gawl.) Wiehler UPCB383[a]		Tuber (e)		>250
	Sinningia allagophylla (Mart.) Wiehler MBM313530[d]	Batata-do-campo ou Batata-de-perdiz	Tuber (e)	Antipyretic, diuretic, depurative [56]	>250
	Sinningia canescens (Mart.) Wiehler MBM363740[d]	Rainha do abismo	Tuber (e)		>250

Malvaceae	*Hibiscus sabdariffa* L. DDMS4593[b]	Vinagreira, Azedinha	L(e), C(e)	Antispasmodic, anti-inflammatory, antioxidant, diuretic, mild laxative [57]	>250
Meliaceae	*Trichilia silvatica* DC. DDMS4662[b]	Catiguá-branco, Catiguá, Rosa-branca	L(m), S(hs), C(m), OE	Anti-inflammatory [58]	>250
Myrtaceae	*Eugenia pyriformis* Cambess. UPCB 16741[a]	Uvaia	L (e)	Astringent, digestive, antitumoral, antimalarial, anti-inflammatory [59]	>250
	Myrcia obtecta (O. Berg) Kiaersk. var. obtecta UPCB50504[a]	Guamirim branco, Cambuí	L (e)		>250
	Myrcia laruotteana Camb. UPCB53303[a]	Cambuí	L (e)	Antidiarrheal [60]	>250
	Campomanesia adamantium (Cambess.) O.Berg DDMS[b]	Guabiroba-do-campo, Guabiroba-do-cerrado	L(m)	Antidiarrheal and anti-inflammatory [61]	>250
Rubiaceae	*Randia hebecarpa* Benth.	Limãozinho	L(m)		>250
	Geophila repens (L.) I.M. Johnst.	Cauá-pirí, Cauá-Pixi	L(m)		>250
	Psychotria brachybotrya DC.		L(e)		>250
	Palicourea crocea (Sw.) Roem. & Schult.		L(m)		>250
Sapindaceae	*Serjania hebecarpa* Benth.		L(m)		>250
	Urvillea ulmaceae Kunth.		L(m)		>250
Tropaeolaceae	*Tropaeolum majus* L.	Capuchinha	L(e), C(e), R(hs)		>250

[a] Herbarium of the Universidade Federal do Paraná – UFPR
[b] Herbarium of the Faculdade de Ciências Biológicas e Ambientais – UFGD/MS
[c] Herbarium of the Departamento de Biologia – UEM/PR.
[d] Herbarium of the Museu Botânico Municipal de Curitiba - PR
L = leaves; R = root (wood + bark); S = stem (wood + bark); C = capitulum; F = fruit.
Solvents: h, hexane; hs, 90% hydroethanolic solution; m, methanol; e, ethanol.

Table 2. MIC[a] of *A. sylvatica* fractions and isolated compounds against *M. tuberculosis* using the REMA assay

Fraction	MIC (μg/mL)
CF	> 250
EAF	115.2
HMF	> 250
Luteolin (1)	236.8
Quercetin (2)	> 250
Almunequin (4)	209.9

[a]: Values are means of duplicate samples

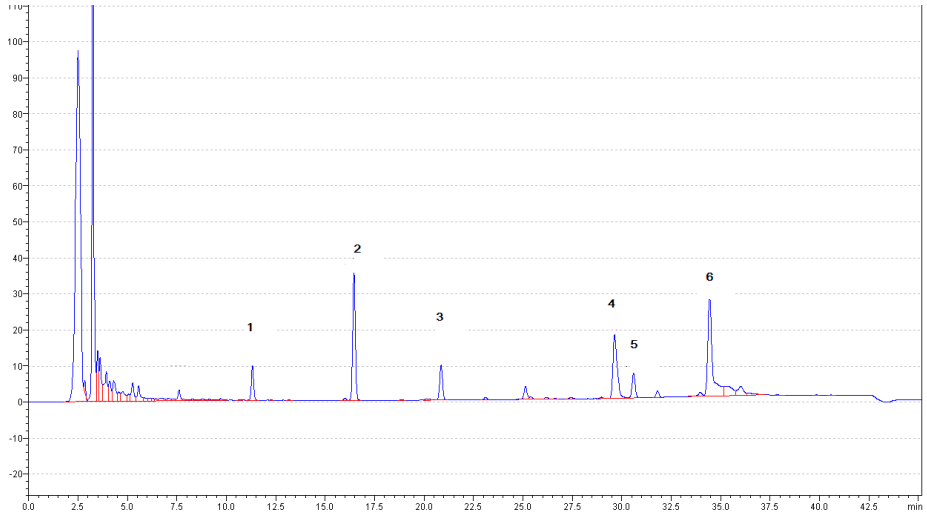

Figure 1. Chromatogram of the lyophilized extract of leaves of *A. sylvatica*

Table 3. Body weight and relative weights of organs of animals exposed to EAF fraction

Parameters	Control	500 mg kg^{-1}	1000 mg kg^{-1}	2000 mg kg^{-1}	p*
Corporeal weight	21.09 ± 1.31	23.24 ± 1.71	22.24 ± 0.64	23.08 ± 0.74	0.0205
Liver	6.31 ± 0.45	5.98 ± 0.32	5.96 ± 0.70	5.89 ± 0.55	0.5294
Lungs	0.64 ± 0.06	0.56 ± 0.03	0.67 ± 0.15	0.68 ± 0.07	0.1458
Heart	0.47 ± 0.06	0.42 ± 0.02	0.43 ± 0.03	0.48 ± 0.03	0.0666
Right kidney	0.53 ± 0.05	0.58 ± 0.02	0.57 ± 0.05	0.57 ± 0.02	0.1773
Left kidney	0.56 ± 0.03	0.56 ± 0.02	0.59 ± 0.04	0.54 ± 0.08	0.7158
Spleen	0.53 ± 0.19	0.43 ± 0.02	0.48 ± 0.03	0.46 ± 0.04	0.5074

Relative weight (%) of the liver, lung, heart, right kidney, left kidney and spleen of female Swiss mice treated with 500, 1000 and 2000 mg kg^{-1} of the EAF fraction of *A. sylvatica*. Data are expressed as the mean ± standard deviation SISVAR 5.3 [26]. (n=6) is the number of animals in each group. p*=5%.

Table 4. Biochemical and hematological parameters of Swiss mice exposed to the EAF fraction of *A. sylvatica* in the acute toxicity study

Parameters	Control	500 mg kg^{-1}	1000 mg kg^{-1}	2000 mg kg^{-1}	p*
Creatinine (mg/dL)	0.29 ± 0.03	0.29 ± 0.07	0.28 ± 0.06	0.28 ± 0.05	0.8100
Urea (mg/dL)	43.66 ± 4.27	44.33 ± 4.41	45 ± 5.03	42.5 ± 4.23	0.6391
AST (U/L)	76 ± 4.45	78 ± 5.91	77 ± 4.94	79 ± 4.99	0.9015
ALT (U/L)	52.5 ± 4.27	48.5 ± 4.37	50 ± 4.27	53 ± 4.75	0.1990
γ GT (U/L)	11 ± 1.41	11.5 ± 1.37	11 ± 1.26	12 ± 12.6	0.5218
RBC (x $10^6/mm^3$)	7.06 ± 0.12	7.11 ± 0.11	7.1 ± 0.10	7.15 ± 0.08	0.6161
HT (%)	45.33 ± 3.66	44.68 ± 3.55	44.5 ± 2.81	45.16 ± 3.9	0.9721
WBC (%)	8.16 ± 0.75	8.33 ± 1.03	8 ± 0.63	8.33 ± 0.81	0.4138
PLT (x $10^3/\mu L$)	916 ± 10.78	928 ± 9.77	928.66 ± 27	925.16 ± 13	0.5353
LYNF (%)	77 ± 1.63	71±1.36	77.83 ± 0.89	79 ± 2.06	0.4913
NEUT (%)	19.16 ± 1.02	20 ± 1.69	19 ± 0.13	17 ± 1.14	0.5783

Hematological and biochemical parameters of female Swiss mice treated acutely orally with vehicle (control) or the EAF fraction of *A. sylvatica* at doses of 500 mg kg^{-1} 1000 mg kg^{-1} and 2000 mg kg^{-1} Values represent the mean ± SD. (n=6) is the number of animals in each group. p*=5%.

AST = Aspartate aminotransferase

ALT = Alanine aminotransferase

γ GT = Gamma glutamyl transferase

RBC = Red Blood Cell

HT = Hematocrite

WBC = White Blood Cell

PLT= Platelets

LYNF = Lymphocytes

NEUT = Neutrophils

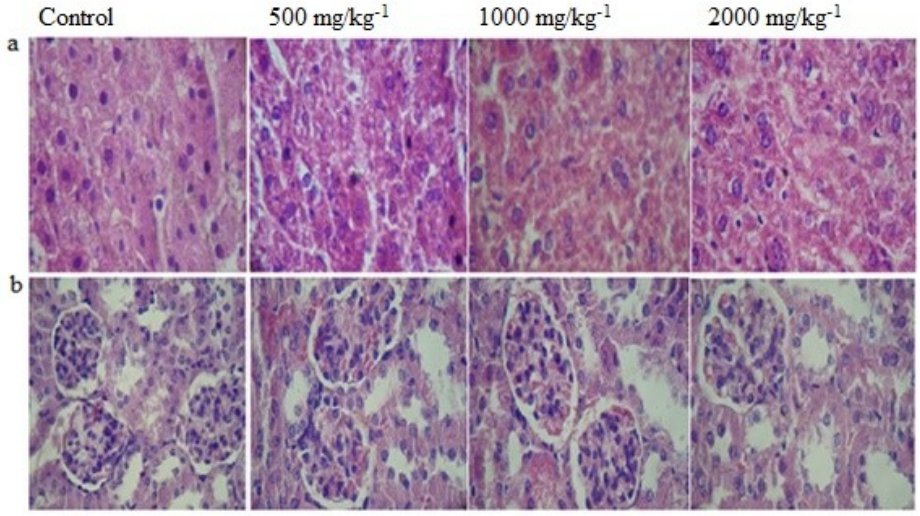

Figure 2. Histological analysis of acute toxicity at doses of 500, 1000 and 2000 mg/kg^{-1} of EAF fraction (hematoxylin – eosin staining, 100X magnification). a) Longitudinal section of liver; b) longitudinal section of kidney.

5.2 Normas da Revista

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This section may each be divided by subheadings or may be combined.

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This should clearly explain the main conclusions of the work highlighting its importance and relevance.

Acknowledgments

All acknowledgments (if any) should be included at the very end of the paper before the references and may include supporting grants, presentations, and so forth.

References

Authors are responsible for ensuring that the information in each reference is complete and accurate. All references must be numbered consecutively and citations of references in text should be identified using numbers in square brackets (e.g., “as discussed by Smith [9]”; “as discussed elsewhere [9, 10]”). All references should be cited within the text; otherwise, these references will be automatically removed.

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