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VANESSA PIETROWSKI BALDIN

Atividade anti-*Mycobacterium tuberculosis* do óleo essencial e substâncias isoladas de *Tetradenia riparia* (Hochst.) Codd (Lamiaceae)

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Orientadora: Profª Drª Rosilene Fressatti Cardoso
Co-Orientadora: Profª Drª Regiane Bertin de Lima Scodro

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Profª Drª Rosilene Fressatti Cardoso
Universidade Estadual de Maringá (Presidente)

Profª Drª Katiany Rizzieri Caleffi Ferracioli
Universidade Estadual de Maringá

Profª Drª Patrícia de Souza B. de Mendonça
Universidade Estadual de Maringá

Profª Drª Vanessa da Silva Carrara
Universidade Estadual de Londrina

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“Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.”

Charles Chaplin

Atividade anti-*Mycobacterium tuberculosis* do óleo essencial e substâncias isoladas de *Tetradenia riparia* (Hochst.) Codd (Lamiaceae)

RESUMO

A tuberculose (TB) é uma doença infectocontagiosa causada por bacilos do complexo *Mycobacterium tuberculosis.* O tratamento recomendado consiste no uso de isoniazida (INH), rifampicina (RIF), pirazinamida (PZA) e etambutol (EMB). A busca por compostos ativos derivados de plantas é uma abordagem para a descoberta de novos medicamentos. *Tetradenia riparia* (Hochst.) é uma importante planta medicinal nativa do sul da África que possui atividade antimicrobiana e acaricida relatada na literatura. O objetivo do presente estudo foi avaliar a atividade anti-*Mycobacterium tuberculosis* pelo método *Resazurin Microtiter Assay Plate* (REMA) e a citotoxicidade pelo ensaio por *Alamar blue* do óleo essencial de *Tetradenia riparia* bem como das substâncias puras 6,7-dehidroroileanona e 9β,13β-epoxi-7-abieteno. O resultado desse trabalho está apresentado no artigo: "Anti-*Mycobacterium tuberculosis* activity of essential oil and pure compounds isolated from *Tetradenia riparia* (Hochst.) Codd (Lamiaceae)". A concentração inibitória mínima (CIM) para *M. tuberculosis* do óleo essencial de *T. riparia* (OETr) e para as substâncias puras 6,7-dehidroroileanona (Tr1) e 9β,13β-epoxi-7-abieteno foi de 61,5 µg/mL, 31,25 µg/mL e > 250 µg/mL, respectivamente. A concentração citotóxica para 50% das células (CC_{50}) foi > 100 µg/mL para o OETr e Tr1 e os valores de índice de seletividade (IS) foram > 1,6 e > 3,2 para o OETr e Tr1, respectivamente. Nossos estudos mostraram boa atividade do óleo essencial de *T. riparia* e da substância pura 6,7-dehidroroileanona contra *M. tuberculosis* sendo considerados candidatos promissores para estudos adicionais como fármacos anti-TB.

Palavras-chave: *Mycobacterium tuberculosis*. *Tetradenia riparia*. Atividade antimicobacteriana. Citotoxicidade.

Anti-*Mycobacterium tuberculosis* activity and cytotoxicity of essential oil and pure compounds isolated from *Tetradenia riparia* (Hochst.) Codd (Lamiaceae)

ABSTRACT

Tuberculosis (TB) is a contagious infectious disease caused by *Mycobacterium tuberculosis* complex. The recommended treatment is the use of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB). The search for plant derived active compounds is an approach to drug discovery. *Tetradenia riparia* (Hochst.) is an important medicinal plant native to southern Africa and has antimicrobial activity and acaricide reported in the literature. Thus, the aim of this study was to evaluate the anti-*Mycobacterium tuberculosis* activity by Resazurin Microtiter Assay Plate (REMA) method and the cytotoxicity by Alamar blue assay for essential oil of leaves and pure compounds (6,7-dehydroroyleanone and 9β,13β-epoxy-7-abietene) isolated from *Tetradenia riparia*. The result of this work is presented in the article, "Anti-*Mycobacterium tuberculosis* activity and cytotoxicity of essential oil and pure compounds isolated from *Tetradenia riparia* (Hochst.) Codd (Lamiaceae)". The minimal inhibitory concentration (MIC) found for the essential oil of *T. riparia* (OETr) and the pure compounds 6,7-dehydroroyleanone (Tr1) and 9β,13β-epoxy-7-abietene (Tr2) were 61.5 µg/mL, 31.25 µg/mL and > 250 µg/mL, respectively. The cytotoxic concentration to 50% of the cells (CC_{50}) was > 100 µg/mL for OETr and Tr1 and SI values were > 1.6 and > 3.2 for OETr and Tr1, respectively. Our study found that the EOTr and Tr1 showed anti-*M. tuberculosis* activity and it encourage us to consider these compounds as promising candidate for additional studies as anti-TB drugs.

Keywords: *Mycobacterium tuberculosis*. *Tetradenia riparia*. Antimicrobial activity. Citotoxicity.

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SUMÁRIO

CAPÍTULO I

1. INTRODUÇÃO

1.1 Tuberculose

A tuberculose (TB) é uma doença infectocontagiosa causada por bacilos do complexo *Mycobacterium tuberculosis* (*Mycobacterium tuberculosis*, *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium pinnipedii, Mycobacterium caprae* e *Mycobacterium canettii*), sendo *Mycobacterium tuberculosis* a espécie mais importante clinicamente. Apesar de seu principal agente causador, *M. tuberculosis*, ter sido descoberto em 1882, a TB ainda hoje é um grave problema de saúde pública mundial. Afeta geralmente os pulmões, mas também pode ocorrer em outros órgãos do corpo e provocar problemas de saúde entre milhões de pessoas a cada ano[1]. A apresentação da TB na forma pulmonar, além de ser mais frequente, é também a mais relevante para a saúde pública, pois é a forma pulmonar, especialmente a bacilífera, a responsável pela manutenção da cadeia de transmissão da doença[2].

Seu desenvolvimento e evolução são dependentes de fatores como aglomerados humanos, a desnutrição e a baixa resistência imunológica, estando este último fator intimamente relacionado com casos de infecção provocada pelo vírus da imunodeficiência humana (HIV). Indivíduos co-infectados com HIV e *M. tuberculosis* possuem 26 vezes mais chances de desenvolver a TB[1].

A TB é uma doença curável em aproximadamente 100 % dos casos, desde que o bacilo seja sensível aos medicamentos anti-TB e que sejam obedecidos os princípios básicos da poliquimioterapia medicamentosa e a adequada operacionalização do tratamento. A associação medicamentosa adequada, a dosagem correta e o uso por tempo suficiente são importantes para o tratamento e evitam a persistência bacteriana e o desenvolvimento de resistência aos fármacos, assegurando a cura do paciente[2].

1.2 Epidemiologia da tuberculose

Em 2014, ocorreram 9,6 milhões de novos casos de TB, sendo 5,4 milhões em homens; 3,2 milhões em mulheres e 1,0 milhões em crianças. Globalmente, estima-se que no mesmo ano ocorreram 1,5 milhões de mortes por TB (1,1 milhões dentre pessoas HIV-negativas e 390.000 casos entre pessoas HIV-positivas). Segundo a Organização Mundial de Saúde (OMS), o número de mortes por TB é inaceitavelmente elevado, pois com um diagnóstico precoce e tratamento correto, quase todos os indivíduos com a doença podem ser curados[1].

O Brasil está entre os 22 países que concentram 80 % da TB mundial. Em 2014, foram notificados 81.512 casos no Brasil e taxa de mortalidade de 2,6/100.000 habitantes, com prevalência de 52/100.000 hab. A TB e a AIDS (Síndrome da Imunodeficiência Adquirida) estão como uma das principais causas de morte no mundo. O número de mortes por HIV em 2014 foi estimado em 1,2 milhões, sendo que 390.000 casos foram atribuídos a TB[1].

1.3 Tratamento

O primeiro antimicrobiano efetivo no tratamento da TB foi a estreptomicina (SM), introduzida em 1944. Na primeira linha de fármacos anti-TB mais eficaz, a isoniazida (INH) tornou-se disponível em 1952, a rifampicina (RIF) em 1965, o etambutol (ETB) passou a integrar a terapia da TB em 1968 e a pirazinamida (PZA), apesar de sintetizada em 1936, só passou a compor a poliquimioterapia da doença em 1970. A partir da década de 80 foi recomendado para casos novos de TB o uso de isoniazida (INH), rifampicina (RIF), pirazinamida (PZA) e etambutol (EMB)[3]. Devido ao aumento dos casos de resistência aos medicamentos utilizados, o Ministério da Saúde propôs um novo sistema de tratamento da TB, implementado a partir do segundo semestre de 2009, passando a adotar a formulação de quatro fármacos (INH, RIF, PZA e EMB) em um único comprimido (dose fixa combinada) como estratégia para aumento de adesão ao tratamento e facilidade operacional pela redução do número de comprimidos a serem ingeridos[4].

Em 1993, a OMS declarou a TB estado de emergência mundial, passando a recomendar a estratégia DOTs (*Directly Observed Treatment Short-Course*) como resposta global para o controle da doença. O tratamento diretamente observado (TDO), onde o profissional treinado passa a observar a tomada da medicação do paciente desde o início do

tratamento até a sua cura, é um elemento chave dessa estratégia, que visa ao fortalecimento da adesão do paciente ao tratamento e à prevenção do aparecimento de cepas resistentes aos medicamentos, reduzindo os casos de abandono e aumentando a probabilidade de cura[5]. Após isso, o Brasil sinalizou sua posição frente às novas perspectivas do problema com marcos pontuais, como o Plano Emergencial para Controle da Tuberculose, lançado em 1994 pelo Ministério da Saúde[6]. O Programa Nacional de Controle da Tuberculose (PNCT) é integrado na rede de Serviços de Saúde e está subordinado a uma política de programação das suas ações com padrões técnicos e assistenciais bem definidos, garantindo desde a distribuição gratuita de medicamentos e outros insumos necessários até ações preventivas e de controle do agravo objetivando reduzir a morbidade, mortalidade e transmissão da TB[7].

1.4 Resistência aos fármacos anti-TB

A seleção e a propagação de isolados de *M. tuberculosis* resistentes aos fármacos clássicos usados na terapia anti-TB, diminuem a eficácia do tratamento e comprometem os avanços no controle da TB[8], o que torna a doença um grande problema de saúde pública em muitos países[1].

Os bacilos resistentes a pelo menos INH e RIF são denominados multidroga resistente (MDR). Nestes casos, os indivíduos portadores de bacilos MDR (TB-MDR) devem ser tratados com medicamentos de segunda linha (amicacina, canamicina, ou capreomicina). Estes pacientes apresentam mais efeitos colaterais causados pelos medicamentos e de forma geral este esquema terapêutico é mais caro e exige regimes duradouros de 18 a 24 meses. No entanto, estes fármacos são menos eficazes do que os de primeira linha, além de apresentar taxa de cura de 50 % a 60 %, em comparação com 95 % a 97 % quando da utilização do esquema padrão[9]. Os pacientes portadores de TB extensivamente resistente (TB-XDR) apresentam uma forma de TB em que o bacilo é resistente a INH, RIF, a uma fluoroquinolona e a um fármaco de segunda linha injetável. Estima-se que 9,7 % dos indivíduos com TB-MDR são TB-XDR[1].

1.5 *Tetradena riparia* (Lamiaceae)

A família Lamiaceae, pertencente ao grupo das angiospermas, é rica em espécies aromáticas usadas como ervas culinárias, medicamentos populares e aromas perfumados[10].

Inclui cerca de 300 gêneros e 7 500 espécies. São exemplos dessa família as espécies mirra (*Tetradenia riparia*)[11], alecrim (*Rosmarinus* sp.), sálvia (*Salvia* sp.), orégano (*Origanum* sp.), tomilho (*Thymus* sp.), manjericão (*Ocimum* sp.), manjerona (*Marjorana* sp.), menta (*Mentha* sp.), segurelha (*Satureja* sp.), dentre outras[12].

Tetradenia riparia (Hochst.) é uma importante planta medicinal nativa do sul da África, anteriormente conhecida como *Iboza riparia*. Porém, após minuciosos estudos, a posição sistemática dessa espécie foi revista e verificou-se que as características apresentadas pelos exemplares se adequavam ao gênero *Tetradenia*, modificando a denominação para *Tetradenia riparia*[13]. Essa espécie é tradicionalmente utilizada na África do Sul no tratamento de tosse, hidropisia, diarreia, febre, dores de cabeça, dores de dente, malária, tosse e dores de garganta. Suas folhas são também utilizadas no tratamento de hemoptise[10].

Em toda a África e Madagascar, espécies de *Tetradenia* são utilizadas medicinalmente devido à presença de óleos essenciais[14]. A produção de óleos essenciais nas plantas está geralmente associada à presença de glândulas secretoras especializadas, tais como tricomas glandulares, ductos de óleo e resinas[15]. Os óleos essenciais são misturas complexas de substâncias voláteis, lipofílicas, geralmente odoríferas e líquidas, estão relacionados com diversas funções necessárias à sobrevivência vegetal e exercem importante papel na defesa contra micro-organismos[16].

1.6 Atividade antimicrobiana de *Tetradenia riparia*

Vários estudos já relataram as propriedades antimicrobianas de extratos de *T. riparia*. Van Puyvelde et al. (1994)[17] verificaram atividade anti-*M. tuberculosis* de extrato bruto de folhas de *T. riparia* com concentração inibitória mínima (CIM) de 500 µg/mL. Njau et al. (2014)[18] observaram atividade antimicrobiana de extratos de *T. riparia* contra *Escherichia coli*, *Staphylococcus aureus* e *Enterococcus faecalis* com CIM variando de 1,25 a 5,00 mg/mL. Ndamane et al. (2013)[19] verificaram propriedade antimicrobiana de diferentes extratos de *T. riparia* contra *S. aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Micrococcus kristinae*, *E. faecalis, E. coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Klebsiella pneumoniae* e *Serratia marcescens* em concentrações que variam de 1,0 a 10,0 mg/mL.

Na literatura, o óleo essencial de *T. riparia* apresenta moderada atividade antimalárica[20], atividade repelente contra *Anopheles gambiae*[21] e propriedade inseticida[22]. Gazim et al. (2010)[11] verificaram boa atividade antimicrobiana do óleo essencial de *T. riparia*

contra *S. aureus*, *B. subtilis* e *C. albicans* com valores de CIM variando de 15,6 a 31,2 μg/mL, 7,8 a 15,6 μg/mL e 31,2 a 62,4 μg/mL, respectivamente. Em outro estudo, Gazim et al. (2011)[23] verificaram alta atividade acaricida do óleo essencial de *T. riparia* contra o carrapato *Rhipicephalus (Boophilus) microplus*. Melo et al. (2015)[24] verificaram a atividade do óleo essencial de *T. riparia* contra bactérias cariogênicas apresentado valores de CIM entre 31,2 e 500 μg/mL.

O óleo essencial de *T. riparia* é uma mistura complexa de terpenóides, entre monoterpenos, sesquiterpenos e diterpenos (hidrocarbonetos ou oxigenados), sendo os sesquiterpenos oxigenados a classe mais representativa da sua composição[11]. Segundo Van Dunkel et al. (1990)[22] o óleo essencial de *T. riparia* possui aproximadamente 200 componentes.

Os terpenos frequentemente são chamados de compostos isoprenóides e são classificados de acordo com o número de unidades de isopreno que os mesmos contêm, entre monoterpenos, sesquiterpenos, diterpenos, sesterpenos, triterpenos e tetraterpenos[25]. Muitos são os estudos que demonstram atividade antimicobacteriana de terpenóides de plantas. Em revisão realizada por Cantrell et al. (2001)[26] observou-se atividade moderada a significante de 110 derivados terpenóides de plantas, contra *M. tuberculosis*.

Roileanonas são diterpenos do tipo abietano tricíclicos com uma porção hidroxiquinona, vastamente distribuída através da família Lamiaceae[27]. Entre os diterpenos dessa classe, o 6,7-dehidroroileanona (Figura 1) apresenta atividade anti-*M. tuberculosis* relatada na literatura. Rijo et al. (2010)[27] verificaram atividade inibitória dessa substância, isolada de *Plectranthus grandidentatus*, para o crescimento de *M. tuberculosis* sensível e MDR, com CIM de > 25 e ≤ 12,5 μg/mL, respectivamente. Essa mesma substância também apresentou atividade contra o cupim *Reticulitermes speratus*[28]. Segundo Gazim et al. (2014)[29] o diterpeno 6,7-dehidroroileanona apresenta alto potencial antioxidante. Outro diterpeno, 9β,13β-epoxi-7-abieteno, isolado do óleo essencial de *T. riparia*, foi primeiramente descrito por Gazim et al. (2014)[29] (Figura 2).

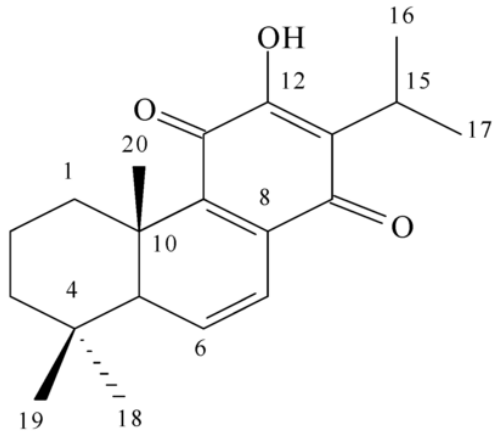

Figura 1: Estrutura química do 6,7-dehidroroileanona [29].

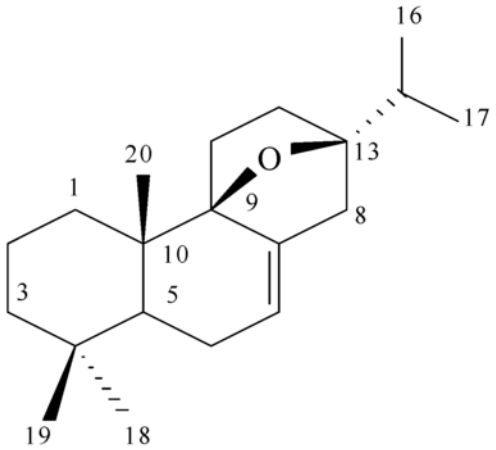

Figura 2: Estrutura química do 9β,13β-epoxi-7-abieteno[29].

Outro diterpeno isolado de folhas de *T. riparia*, 8(14),15-sandaracopimaradieno-7α,18-diol, apresentou importante atividade antimicrobiana contra *B. subtilis* e *Streptococcus pyogenes* com CIM de 12,5 µg/ml. Também apresenta atividade contra algumas bactérias Gram-negativas, como *Proteus vulgaris, Shigella dysenteriae* e *Pseudomonas solanacearum* com CIM de 25 µg/mL[30].

1.7 Óleos essenciais com atividade anti-*Mycobacterium tuberculosis*

Estudo realizado por Araujo et al. (2014)[31], avaliaram atividade anti-*M. tuberculosis* de três óleos essenciais (*Annona sylvatica*, *Trichilia silvatica* e *Schinus tereebinthifolius*) e

observaram CIM > 250 μg/mL. Recentemente, Andrade-Ochoa et al. (2013)[32] reportaram atividade antimicobacteriana do óleo essencial de *Cuminum cymium* (cominho), *Eugenia caryophyllata* (alho), *Cinnamomum verum* (canela), *Laurus nobilis* (louro) e *Pimpinella anisum* (anis), sendo os óleos de cominho e canela os mais ativos contra *M. tuberculosis* (CIM 12,5 μg/mL), seguido do óleo essencial de alho (CIM 25 μg/mL) e de louro e anis (CIM 100 μg/mL). A diferença na ação antibacteriana dos óleos essenciais pode ocorrer devido a complexidade de sua constituição química, estando relacionada com a presença de uma substância ativa ou um conjunto delas[33].

1.8 Concentração inibitória mínima (CIM)

O método *Resazurin Microtiter Assay Plate* (REMA) consiste em uma modificação da técnica *Microplate Alamar Blue Assay* (MABA) previamente descrita por Collins & Franzblau (1997)[34] e realizada por Palomino et al. (2002)[35]. Com esta técnica é possível determinar a CIM de compostos capazes de inibir a proliferação bacteriana. Utiliza como revelador de crescimento bacteriano a resazurina, um corante de oxido-redução que em sua forma oxidada apresenta cor azul e não fluorescente e quando reduzido a resofurina apresenta cor rosa e altamente fluorescente. Posteriormente, a resofurina é reduzida para hidroresofurina (incolor e não fluorescente)[36].

1.9 Determinação da citotoxicidade *in vitro*

A determinação da citotoxicidade *in vitro* é um procedimento importante na pesquisa de substâncias menos tóxicas e seletivas com atividade antimicrobiana. É fundamental que a substância estudada atue seletivamente contra a bactéria, porém seja inócua ou pouco tóxica ao hospedeiro[37]. Para a leitura de viabilidade celular podem ser utilizados diversos corantes, dentre eles resazurina[38], sulforrodamina B (SRB)[39], brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazólio (MTT)[40], 2,3-bis(2-metóxi-4-nitro-5-sulfofenil)-2H-tetrazolium-5-carboxanilide (XTT)[41] e 3-(4,5-dimetiltiazol-2-il)-5-(3-carboximetoxifenil)-2-(4-sulfofenil-2Htetrazólico (MTS)[42].

O indicador de oxidação-redução Alamar Blue® tem sido usado para a determinação colorimétrica da viabilidade e proliferação das células. Consiste em um ensaio simples comparado com outros métodos para avaliar a viabilidade celular, além de reprodutível,

econômico e não-tóxico. A forma oxidada do Alamar Blue entra no citosol, é convertida por enzimas mitocondriais e a coloração do meio muda de azul para rosa fluorescente nas células viáveis[43].

Diferentes tipos de ensaios foram desenvolvidos e são utilizados para a medição da viabilidade ou a citotoxicidade *in vitro*. Em ensaios para avaliar a citotoxicidade de óleos essenciais extraídos de plantas, algumas linhagens celulares como macrófagos J774.A1[44], macrófagos J774.G8[45] células de primata Vero[46] e macrófagos peritoneais murinos[41,44] tem sido usadas por inúmeros autores.

Para estimar uma possibilidade de relação entre o ensaio *in vitro* e a passagem segura para ensaio *in vivo* de compostos sintéticos ou naturais foi determinado o Índice de Seletividade (IS). IS é determinado pela razão da concentração do composto que é capaz de permitir a viabilidade de 50% dos macrófagos (CC_{50}) sob o valor da CIM encontrada[47]. Orme (2001)[48] considera promissoras para o desenvolvimento de fármacos anti-TB, substâncias que apresentam IS $\geq$ 10. A determinação da citotoxicidade é chave na descoberta de substâncias promissoras anti-tuberculose, e estudos de selectividade devem ser realizados no processo de descoberta[49].

2. JUSTIFICATIVA

O aumento da resistência de *M. tuberculosis* aos fármacos disponíveis, associado à carência de novos medicamentos para o tratamento da doença torna-se uma preocupação mundial e mostra a necessidade de estudos à procura de novos fármacos para o tratamento da TB. Diante disso, o interesse no estudo da composição de extratos de plantas e ação de substâncias puras, bem como, de fitoterápicos tem aumentado expressivamente nos últimos anos. Uma vez determinada a seletividade e atividade de determinada substância, a mesma poderá servir como modelo para síntese de substâncias análogas mais potentes e seletivas, que podem ser obtidas mais facilmente e talvez a custos menores. Isto sem dúvida tem motivado muitas indústrias farmacêuticas a investir em estudos envolvendo plantas.

Os óleos essenciais obtidos de *T. riparia* já se mostraram promissores em ação antimicrobiana[13,32]. Porém, até o momento não existe um estudo da ação deste óleo em *M. tuberculosis.* Nesse sentido, propomos avaliar a ação anti-*M. tuberculosis* do óleo essencial e de duas das substâncias puras obtidas a partir do óleo daquela espécie, na procura de novos candidatos a fármacos para estudos adicionais.

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar a atividade anti-*Mycobacterium tuberculosis* e a ação citotóxica do óleo essencial e substâncias puras de folhas de *Tetradenia riparia*.

3.2. Objetivos Específicos

- Determinar a CIM do óleo essencial e das substâncias pura 6,7-dehidroroileanona e 9β, 13β-epoxi-7-abieteno obtidas de folhas de *T. riparia* frente à cepa de referência *M. tuberculosis* H_{37}Rv (ATCC 27294), utilizando a técnica de REMA;
- Determinar a CIM, dos compostos que apresentarem melhor atividade, frente aos isolados clínicos de *M. tuberculosis* sensíveis e resistentes aos fármacos anti-TB, utilizando a técnica de REMA;
- Avaliar a citotoxidade *in vitro* do óleo essencial e das substâncias pura 6,7-dehidroroileanona e 9β, 13β-epoxi-7-abieteno em macrófagos peritoneais murino;
- Determinar o Índice de Seletividade (IS) do óleo essencial e das substâncias pura 6,7-dehidroroileanona e 9β, 13β-epoxi-7-abieteno.

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CAPÍTULO II

MANUSCRITO: "ANTI-*Mycobacterium tuberculosis* ACTIVITY AND CYTOTOXICITY OF ESSENTIAL OIL AND PURE COMPOUNDS ISOLATED FROM *Tetradenia riparia* (HOCHST.) CODD (LAMIACEAE)"

ANTI-*Mycobacterium tuberculosis* ACTIVITY AND CYTOTOXICITY OF ESSENTIAL OIL AND PURE COMPOUNDS ISOLATED FROM *Tetradenia riparia* (HOCHST.) CODD (LAMIACEAE)

Vanessa Pietrowski Baldin[a]; Regiane Bertin de Lima Scodro[b]; Mariana Aparecida Lopes[a]; Claudia Terencio Agostinho Pires[a]; Zilda Cristiani Gazim[c]; Letícia Ferarrese[c]; Viviane dos Santos Faiões[d]; Eduardo Caio Torres-Santos[d]; Katiany Rizzieri Caleffi Ferracioli[b]; Vera Lúcia Dias Siqueira[a,b]; Diógenes Aparicio Garcia Cortez[e]; Rosilene Fressatti Cardoso[a,b*]

*Corresponding author. E-mail addresses: rfcardoso@uem.br

[a] Postgraduate Program in Biosciences and Physiopathology, State University of Maringá, Avenida Colombo, 5790, 87020-900, Maringa, Parana, Brazil

[b] Department of Clinical Analysis and Biomedicine, State University of Maringa, Avenida Colombo, 5790, 87020-900, Maringa, Parana, Brazil

[c] Chemistry Laboratory of Natural Products, Paranaense University, Praça Mascarenhas de Moraes, 4282, 87502-210, Umuarama, Parana, Brazil

[d] Laboratory of Biochemistry of Trypanosomatids e Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Av. Brasil, 4365, 21040-900, Rio de Janeiro, Brazil

[e] Postgraduate Program in Pharmaceutical Sciences, State University of Maringa, Avenida Colombo, 5790, 87020-900, Maringa, Parana, Brazil

ABSTRACT

Ethnopharmacological relevance: *Tetradenia riparia* (Lamiaceae) has antimicrobial, antimalarial, repellent, acaricidal, antileishmanial and insecticidal activities reported and has been traditionally used in popular medicine for treatment of some diseases. In Uganda, the leaves of *T. riparia* are used by tradicional medicine practioners to treat tuberculosis (TB).

Aim of the study: We aimed to evaluated the *in vitro* anti-*Mycobacterium tuberculosis* activity and cytotoxicity of essential oil and pure compounds isolated from leaves of *T. riparia*.

Materials and methods: We evaluated the in vitro anti-*M. tuberculosis* activity of essential oil and two pure compounds from leaves of *T. riparia* by resazurin microtiter assay plate (REMA). The cytotoxic activity was evaluated in murine peritoneal macrophages by Alamar Blue assay. Results were expressed as median concentration cytotoxicity (CC_{50}) and the selectivity index (SI) was calculated.

Results: The essential oil (EOTr), 6,7-dehydroroyleanone (Tr1) and 9β, 13β- epoxy-7-abietene (Tr2) showed anti-*M. tuberculosis* $H_{37}Rv$ activity with MIC (minimal inhibitory concentration) values of 62.5 µg/ml, 31.2 µg/ml and > 250 µg/mL, respectively. The EOTr and Tr1 exhibited activities against resistant and susceptible *M. tuberculosis* clinical isolates with MIC values from 31.2 to 62.5 µg/ml. The cytotoxicity results showed a selectivity index > 1.6 and > 3.2 for EOTr and Tr1, respectively.

Conclusions: Our study found that the EOTr and Tr1 showed anti-*M. tuberculosis* activity and it encourage us to consider these compounds as promising candidate for additional studies as anti-TB drugs.

Keywords: *Mycobacterium tuberculosis*. *Tetradenia riparia*. Essential oil. Antimycobacterial activity. Cytotoxicity.

1. Introduction

Tuberculosis (TB) is a global health problem and ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide. It was estimated, in 2014, there were approximately 9.6 million new TB cases and 1.5 million deaths (WHO, 2015). The global resurgence of TB and the development of drug resistance, multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* isolates is a threat to TB control, which demands the urgent development of new and more effective anti-TB drugs (Askun et al., 2013).

Natural products with biological activity have stimulated studies with plants (Graham et al., 2003). Plants and other natural materials have proved to be valuable sources of useful new antimycobacterial active molecules (Seidel and Taylor, 2004). *Tetradenia riparia* (Hochst.) Codd, a member of the Lamiaceae family, is known as "false myrrh" and is an important medicinal plant used in the native medicine in Rwanda (Africa) to treat a wide range of diseases (Phillipson and Steyn, 2008; Gazim et al., 2010; Van Puyvelde et al., 1986; Van Puyelde et al., 1994). In Rwanda, people cultivate and used for a variety of diseases as malaria, angina, yaws, helminths, gastroenteritis, dental abcesses and other disorders (Puyvelde et al., 1986). In Uganda, this plant is used to by tradicional medicine practioners (TMPs) to treat TB (Bunalema et al., 2014).

Tosun et al. (2004) reported the antymicobacterial activity of four plants from the Lamiaceae family. Several studies have reported activities of extracts obtained from *T. riparia* as antimicrobial (Boyli and Puyvelde, 1986; Van Puyvelde et al., 1994; Ndamane et al., 2013; Njau et al., 2014) and ascaricidal activities (Peter and Deogracious, 2006).

The pharmaceutical properties of aromatic plants are partially attributed to essential oils which are natural, complex, multi-component systems composed of terpenes, together with some non-terpenes (Edris, 2007). In studies with essential oil from *T. riparia,* it showed antimalarial (Campbell et al., 1997), repellent against *Anopheles gambiae* (Omolo et al., 2004), acaricidal (Gazim et al., 2011), antileishmanial (Demarchi et al., 2015; Cardoso et al., 2015) and insecticidal (Dunkel et al., 1990) activities.

The antimicrobial activity of essential oil from *T. riparia* (EOTr) has been reported. Recently, Melo et al. (2015) found the essential oil of *T. riparia* activity against cariogenic bacteria *Streptococcus mutans*, *Streptococcus mitis*, *Lactobacillus casei*, *Streptococcus sanuinis*, *Streptococcus sobrinus* and *Streptococcus salivarus* with MIC values between 31.2 to 500 μg/mL. Gazim et al. (2010) verify good antimicrobial activity of essential oil of *T.*

riparia against *S. aureus*, *Bacillus subtilis* and the pathogenic fungus *Candida albicans* with MIC values ranging from 15.6 to 31.2 μg/mL, 7.8 to 15.6 μg/mL and 31.2 the 62.4 μg/mL, respectively. In another study, York et al. (2012) showed the antimicrobial activity of essential oil from *T. riparia* against *Cryptococcus neoformans* (MIC 0.83 mg/mL), *Klebsiella pneumoniae* (4 mg/mL), *Moraxella catarrhalis* (5.33 mg/mL), *Mycobacterium smegmatis* (not susceptible at highest concentration tested) and *Staphylococcus aureus* (8 mg/mL).

According to Gazim et al. (2010), the EOTr is a complex mixture of terpenoids, including monoterpenes, sesquiterpenes and diterpenes (hydrocarbons or oxygenated), the most representative class. In addition, Gazim et al. (2014) reported 6,7-dehydroroyleanone (Tr1) and 9β,13β-epoxy-7-abietene (Tr2), new natural products, isolated from EOTr. The Tr1 have antitermitic and antioxidant activities reported (Kusumoto et al., 2009; Gazim et al., 2014). Rijo et al., 2010 showed anti-*M. tuberculosis* activity of 6,7-dehydroroyleanone isolated from *Plectranthus grandidentatus* (Rijo et al., 2010).

Then, in the light of the increase of *M. tuberculosis* resistant to the available drugs associated with lack of new drugs for the treatment of the disease the present study aimed to determine the anti-*M. tuberculosis* activity and cytotoxicity of EOTr and pure compounds (Tr1 and Tr2) obtained from leaves of *T. riparia*.

2. Methods and Materials

2.1. Extraction and fractionating of essential oil from Tetradenia riparia

Leaves of *T. riparia* were collected in Umuarama, Parana, Brazil (−23°45'59 S, −53°19'30 W, 391 m). A voucher specimen was authenticated and deposited at the herbarium of the University Educational Paranaense (HEUP), under number 2502. The leaves were manually collected early in the morning, from 7:30 to 9:30, and the Essential oils were obtained from fresh leaves by hydrodistillation using a Clevenger-type apparatus and dried by Na_2SO_4. For the fractionating, 2g of essential oil of *T. riparia* was subjected to column chromatography with silica and eluted with pentane, pentane-dichloromethane (9:1, 8:2, 7:3 to 1:1), dichloromethane-pentane (3:7); dichloromethane, diclometano-methanol (9:1, 7:3 to 1:1) and methanol, which yielded 29 fractions. The essential oil was identified by GC-MS and GC-FID analysis and the fractions 16 and 17 were identified by 1H, 13C, DEPT, HSQC, HMBC and NOESY. Fraction 16 was elucidated as 9β,13β-epoxy-7-abietene (Tr2) and the

fraction 17 was elucidated as 6,7-dehydroroyleanona (Tr1), respectively (Gazim et al., 2014). The essential oil and two pure compounds isolated from *T. riparia* were used to evaluate the anti-*M. tuberculosis* activity and cytotoxicity.

2.2. Mycobacterial reference strain and clinical isolates

M. tuberculosis $H_{37}Rv$ (ATCC 27294) and 21 *M. tuberculosis* clinical isolates (eight susceptible, seven resistant to INH and six multidrug resistance (MDR) were used in the study. All clinical isolates were from the mycobacterial collection of the Laboratory of Medical Bacteriology, State University of Maringa, Parana, Brazil.

2.3. Mycobacterial Inoculum

The reference strain and clinical isolates were grown in Middlebrook 7H9 broth medium supplemented with oleic acid, albumin, dextrose and catalase (OADC) Enrichment (BBL/Becton-Dickinson, Sparks, MD, USA) for 15 days at 36 °C. Standardized inoculums, by visual comparison of turbidity equivalent to McFarland scale 1 ($3x10^8$ CFU/mL), were prepared for each isolate and diluted 1:20 in Middlebrook 7H9 supplemented with OADC Enrichment.

2.4. Determination of Minimal Inhibitory Concentration (MIC)

The anti-*M. tuberculosis* activity of the EOTr and pure compounds were determined in triplicate and by three independent Resazurin Microtiter Assay Plate (REMA) (Palomino et al., 2002). The assays were performed in 96-well sterile microplates. Firstly, stock solutions of the EOTr, Tr1 and Tr2 were prepared in Dimethyl sulfoxide (DMSO, SIGMA - Aldrich). After, two fold serial dilutions of EOTr, Tr1 and Tr2 to obtain final concentrations ranging from 250 to 0.98 µg/mL were prepared in Middlebrook 7H9 broth supplemented with OADC Enrichment. Then, 100 µL of the each standardized mycobacterial inoculum was added to each well of the microplate. The microplates were sealed and incubated at 36 ºC in normal atmosphere for 7 days. After this period, 30 µL of 0.02 % resazurin solution (Acros, Morris Plains, NJ, USA) were added to each well and the plates were reincubated at 36 ºC for 24 h for subsequent visual reading. A color change from blue to pink indicated mycobacterial

growth and the MIC was interpreted as the lowest concentration that prevented the color change. Medium, drug sterility and bacterial growth with and without 2.5 % (v/v) DMSO controls were included in all tests. Isoniazid (SIGMA - Aldrich) was used as the reference drug at concentrations from 1.0 to 0.007 µg/mL.

2.5. Cytotoxicity assay on murine peritoneal macrophages

Cytotoxicity colorimetric assay was assessed on murine peritoneal macrophages with Alamar Blue according to the manufacturer's instructions. Initially, the macrophages was isolated from peritoneal cavity of BALB/c male mice with cold RPMI 1640 medium (pH 7.2, supplemented with 10 % fetal bovine serum) and were plated ($1.0x10^6$ cells/well) and incubated for 1 h at 37 °C in a humidified 5 % CO_2 atmosphere. After 2 h of incubation, the culture was washed with RPMI and the EOTr and Tr1 solubilized in DMSO were added at different concentrations (0.39 to 100 µg/mL) and incubated for 72 h at 37 °C and 5% CO_2. After the incubation period, culture supernatant was removed and added 200 µL of PBS containing 22 µL of Alamar Blue. The cell viability was measured by fluorimetry (Spectra Max GEMINI XPS-Molecular Devices, Silicon Valley, USA) with excitation at 560 nm and 590 nm emission after 3 h of incubation. This trial was conducted at the Instituto Oswaldo Cruz (FIOCRUZ, Rio de Janeiro). The selectivity index (SI) was determined by the CC_{50}/MIC ratio for each compound tested (Protopopova, 2005).

3. Results

The EOTr and Tr1, showed activity against *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) with MIC values 62.5 µg/mL and 31.2 µg/mL, respectively. The Tr2 compound showed low activity against *M. tuberculosis* $H_{37}Rv$ (MIC > 250 µg/mL) and it was not tested in the clinical isolates (Table 1).

Table 1

Anti-*Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) activity of essential oil (EOTr) and pure compounds (Tr1) isolated from *Tetradenia riparia*.

Essential oil and pure compounds	**MIC (µg/mL)**
	$H_{37}Rv$
EOTr	62.5
Tr1	31.2
Tr2	>250
INH	0.06

MIC: Minimum Inhibitory Concentration. INH: isoniazid.

The antimycobacterial activity of EOTr and Tr1 against susceptible and resistant *M. tuberculosis* clinical isolates are shown in Table 2 with MIC values ranged between 31.2 and 62.5 µg/mL, respectively.

Table 2

Minimal inhibitory concentration (MIC) of isoniazid, essential oil (EOTr) and 6,7-dehydroroyleanone (Tr1) isolated from *Tetradenia riparia* against *Mycobacterium tuberculosis* clinical isolates.

Isolates	Resistance	MIC µg/mL		
		INH	EOTr	Tr1
13638	Susceptible	0.03	62.5	31.2
TB27	Susceptible	0.03	62.5	31.2
47S	Susceptible	0.03	62.5	31.2
4851	Susceptible	0.03	62.5	31.2
24	Susceptible	0.03	62.5	31.2
01F	Susceptible	0.03	62.5	31.2
02F	Susceptible	0.03	62.5	31.2
03F	Susceptible	0.03	62.5	31.2
3614	INH^R, RIF^R, EMB^R, SM^R, ETH^R	4	62.5	31.2
73[a]	INH^R, RIF^R	4	31.2	31.2
71[a]	INH^R, RIF^R	4	62.5	31.2
04F	INH^R	4	62.5	31.2
12F	INH^R, RIF^R	8	31.2	31.2
14F	INH^R	8	31.2	31.2
16F	INH^R	8	31.2	31.2
23F	INH^R	4	62.5	31.2
26F	INH^R	8	31.2	31.2
45F	INH^R, RIF^R	8	62.5	31.2
57F	INH^R	4	62.5	31.2
19	INH^R, RIF^R, EMB^R	2	62.5	31.2
91	INH^R, SM^R	2	62.5	31.2

[R] Resistant; INH: isoniazid, EMB: ethambutol; RIF: rifampicin; SM: streptomycin; PZA: pyrazinamide; ETH: Etionamide.

In cytotoxicity assay with murine peritoneal macrophages, both the OETr and Tr1 showed CC_{50} > 100 µg/mL. The values of SI were > 3.2 and > 1.6, respectively (Table 3).

Table 3

Cytotoxicity and selectivity index of essential oil (EOTr) and 6,7-dehydroroyleanone (Tr1) isolated from *Tetradenia riparia,* on murine peritoneal macrophages.

Essential oil and pure compound	CC_{50} (µg/mL)	MIC (µg/mL)	SI
EOTr	>100	62.5	>1.6
Tr1	>100	31.2	>3.2

SI: selectivity index.

4. Discussion

Despite the advances in antibacterial chemotherapy against some microorganisms, this is not really true for *M. tuberculosis*. The number of drugs that is used to treat patients with TB has been the same since the beginning of chemotherapy for the disease. Complicating factor in the management of patients with TB has been the increased number of resistant *M. tuberculosis* clinical isolates worldwide. In this sense, the search for natural products that inhibit the growth of bacillus is an alternative to find new compounds with antimycobacterial activity and may be a new drug candidate to treat TB.

As the EOTr have been already showed to be promising against some others bacteria, we evaluated the activity of EOTr and two compounds isolated from this specie of plant, against *M. tuberculosis*. In the present study, we could observe a promising anti-*M. tuberculosis* activity of the EOTr and the isolated compound Tr1. The MIC values ranged from 31.2 to 62.5 µg/mL.

That we have knowledge, there is no clear definition, in the literature, about the ideal MIC value of essential oil against *M. tuberculosis* and this is the first report describing the anti-M. *tuberculosis* activity of EOTr from leaves of *T. riparia*. According to Bueno-Sanchez et al. (2009), a MIC value of essential oils to be a good candidate against mycobacteria should be < 100 µg/mL. A MIC value range of 100 - 200 µg/mL is considered a moderate candidate. Thus, the MIC values of EOTr and Tr1 obtained for susceptible and resistant *M. tuberculosis* clinical isolates classify them as good candidates for additional studies as antituberculosis drug.

The antimycobacterial activity reported here correlates to the reported MIC values for others essential oils of Lamiaceae family. Bueno-Sánchez et al. (2009) evaluated the activity

of essential oil from *Hyptis mutabilis* (Lamiaceae) and found MIC of 125 μg/mL against *M. tuberculosis* $H_{37}Rv$. Similar antimycobacterial findings were obtained by Bueno et al. (2011) with essential oils from *Salvia aratocensis* (Lamiaceae) and obtained MIC values 62.5 μg/mL against *M. tuberculosis* $H_{37}Rv$, ≤ 125 μg/mL for *M. tuberculosis* Beijing genotype strains and resistant strains and 79 to 397 μg/mL for nontuberculous mycobacterial isolates.

Essential oil obtained from *Anemia tomentosa* var. *anthriscifolia* (MIC 120 μg/mL), *Lantana trifolia* L. (80 μg/mL), *Lantana tucata* Lindl. (100 μmL), C*uminum cymium* (12.5 μg/mL), *Eugenia caryophyllata* (25 μg/mL), *Cinnamomum verum* (12.5 μg/mL), *Laurus nobilis* (100 μg/mL), *Pimpinella anisum* (100 μg/mL), *Cananga odorata*, *Swinglea glutinosa*, *Achyrocline alata* (62.5 μg/mL), *Piper auritum*, *Lippia origanoides*, *Lippia alba* and *Piper bogotense* (MIC between 100 and 200 μg/mL), *Croton cajucara* Benth. (MIC 4.88 μg/mL) and *Turnera diffusa* (62.5 μg/mL) have been reported in the literature as having potential activities against *M. tuberculosis* $H_{37}Rv$ (Andrade-Ochoa et al., 2013; Azevedo et al., 2013; Bueno-Sanchez et al., 2009; Bueno et al., 2011; Julião et al., 2009 and Pinto et al., 2009). *M. tuberculosis* presents rich cell wall of mycolic acid (lipophilic) which has affinity with the essential oil. According to Bhardwaj et al. (2013), essential oil behaves as lipophilic entity and passes through the cell wall and cytoplasmic membrane, causing a disruption of the cell wall.

In studies with natural products, some terpenes have already been evaluated for antimycobacterial activity (Cantrell et al., 2001). In some essential oils, the antimicrobial activity is due to presence of isoprenes such as monoterpenes, sesquiterpenes or related alcohols and phenols (Koroch & Zygadio, 2007). Van Puyvelde et al. (1994) also evaluated antimycobacterial activity of 8(14),15-sandaracopimaradiene,7α,18-diol isolated from leaves of *T. riparia*, and found MIC ranging from 25 to 100 μg/mL. This MIC value is similar to the diterpene (Tr1) analyzed in this study (MIC 31.25 μg/mL). Rijo et al. (2010), evaluated the activity of 6,7-dehydroroyleanone diterpene (Tr1), isolated from *Plectranthus grandidentatus* and found MIC values > 25 μg/mL for *M. tuberculosis* $H_{37}Rv$ and ≤ 12.50 μg/mL for only one MDR *M. tuberculosis* clinical isolate.

Although the MIC of EOTr and Tr1 showed high value compared to the isoniazid, the reference drug used in this study (MIC 0.03 μg/mL for *M. tuberculosis* $H_{37}Rv$), their MIC are comparable another first-line anti-TB drug, pyrazinamide with MIC 20-100 μg/mL (Higuchi et al., 2011).

In the search for active compounds with therapeutic potential against *M. tuberculosis* it is important to determine if the compound has cytotoxic effects on host cells. The SI values

obtained by assay *in vitro* indicate the relative safety of the tested compounds and the ratio of its activity and toxicity. According to Orme (2001) and Garcia et al. (2012) a compound is considered promising when presenting SI ≥ 10. In our study the cytotoxicity of EOTr and Tr1 were evaluated and the SI was determined by the CC_{50}/MIC ratio (Protopova et al., 2005). The EOTr and Tr1 showed to be more selective for the bacillus than for macrophages. Both (EOTr and Tr1) showed CC_{50} > 100 µg/mL with SI >1.6 and >3.2, respectively.

On the other hand, Demarchi et al. (2015) in studies with leishmanicidal activity and cytotoxicity of EOTr and Tr1 using XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide), found SI values of 5.67 and 0.22, respectively. Furthermore, Gazim et al. (2014) evaluated the cytotoxicity of EOTr and the Tr1 in tumor cell lines, HCT-8 (ileocecal colorectal adenocarcinoma), SF-295 (Human Glioblastoma Cells) and MDA-MB-435 (human breast carcinoma), and found high cytotoxic potential of the oil only for the two first cell line. The Tr1 showed to have no cytotoxic effect on the cells tested. Although the EOTr showed to be cytotoxic for human tumor cells, in our study, with peritoneal murine macrophages, the EOTr as well as the Tr1 showed low cytotoxic potential at the maximal concentration tested (100 µg/mL).

In conclusion, several studies with natural products have been made to find new antituberculosis drugs. In our study the MIC values of EOTr and Tr1 for resistant *M. tuberculosis* clinical isolates and the reference strain were equivalent for those obtained with susceptible isolates. Additionaly, the Tr1 obtained from *T. riparia* showed to be good candidate for additional studies to find new anti-*M. tuberculosis* molecules. Also, other compounds in the EOTr not studied here should be evaluated apart with the objective to find new antimycobacterial molecules and support the traditional use of that plant in medicinal popular to treat respiratory infections. The results of this study encourage us to consider these compounds as promising candidate for anti-TB drugs.

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CAPÍTULO III

4. CONCLUSÕES

Nossos estudos demonstraram que o óleo essencial de *Tetradenia riparia* e a substância pura 6,7-dehidroroileanona apresentaram boa atividade em *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) e em isolados clínicos sensíveis e resistentes aos fármacos anti-tuberculose com CIM de 62,5 e 31,25 μg/mL, respectivamente, sendo que o composto isolado (Tr1) apresentou menor citotoxicidade (IS > 3,2).

5. PERSPECTIVAS FUTURAS

São necessários estudos na área de proteômica e transcriptoma com a substância pura 6,7-dehidroroilenona, isolada do óleo essencial de *T. riparia*, em *M. tuberculosis* para elucidar os efeitos desta substância no bacilo. Também o isolamento de outras substâncias presentes no óleo essencial é de fundamental importância para a continuidade dos estudos na busca de mais moléculas bioativas contra *M. tuberculosis* além de estudos adicionais para avaliar o sinergismo da substância pura 6,7-dehidroroileanona com outros fármacos antituberculosos (*checkerboard).* Adicionalmente, o grupo de pesquisa pretende realizar derivações da substância Tr1 para viabilizar a obtenção de uma molécula com maior atividade contra o bacilo e que apresente baixa citotoxicidade para células de linhagem normal humana.

Anexo

JOURNAL OF ETHNOPHARMACOLOGY
An Interdisciplinary Journal Devoted to Indigenous Drugs
ISSN: 0378-8741

DESCRIPTION

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

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

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

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

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

AUDIENCE

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

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INTRODUCTION

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

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Types of paper

The *Journal of Ethnopharmacology* will accept the following contributions:

1. Original research articles - whose length is not limited and should include Title, Abstract, Methods and Materials, Results, Discussion, Conclusions, Acknowledgements and References. As a guideline, a full length paper normally occupies no more than 10 printed pages of the journal, including tables and illustrations.

2. Short Communications - whose average length is not more than 4 pages in print (approx. 2000-2300 words, including abstract and references). A maximum of 2 illustrations (figures or tables) is allowed. See paragraph below for description and format.
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used.

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Froede, T.SA. and Y.S. Medeiros, Y.S. (2008) Animal models to test drugs with potential antidiabetic activity. Journal of Ethnopharmacology 115: 173-183. Gertsch J. (2009) How scientific is the science in ethnopharmacology? Historical perspectives and epistemological problems. Journal of Ethnopharmacology, 122: 177-183.

Chan K., et al. (2012) Good practice in reviewing and publishing studies on herbal medicine, with special emphasis on traditional Chinese medicine and Chinese Materia Medica. Journal of Ethnopharmacology 140: 469-475.

Heinrich, M., Edwards. S., Moerman. D.E.. and Leonti. M. (2009), Ethnopharmacological field studies: a critical assessment of their conceptual basis and methods. J. Ethnopharmacol, 124: 1-17.

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