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ESTUDO DA ATIVIDADE DE BOMBAS DE EFLUXO DE *Mycobacterium tuberculosis* APÓS EXPOSIÇÃO À PIPERINA E FÁRMACOS ANTITUBERCULOSE

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Maringá como requisito para a obtenção do título de Doutora em Ciências da Saúde.
Área de concentração: Doenças Infecciosas e Parasitárias
Orientadora: Profª. Drª. Rosilene Fressatti Cardoso.

FOLHA DE APROVAÇÃO

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"Ninguém crea que lhe baste a leitura sem a unção, a especulação sem a devoção, a investigação sem a admiração, a atenção sem a alegria, o trabalho sem a piedade, a ciência sem a caridade, a inteligência sem a humildade, o estudo sem a graça divina e a pesquisa sem a sabedoria inspirada por Deus"

São Boaventura, doutor da Igreja

Estudo da atividade de bombas de efluxo de *Mycobacterium tuberculosis* após exposição à piperina e fármacos antituberculose

RESUMO

A tuberculose (TB) é uma doença infectocontagiosa, causada principalmente por *Mycobacterium tuberculosis* e está entre as principais causas de morte no mundo. A resistência aos fármacos disponíveis para o tratamento é uma ameaça contínua. As combinações de fármacos e a associação com compostos inibidores de bombas de efluxo (IBEs) surgem como expectativa para burlar esse mecanismo de resistência do bacilo. Nesse contexto, este estudo teve por objetivo**,** fazer uma revisão sobre a substância piperina (PIP) e sua atividade contra o bacilo da TB, além de propor uma avaliação de sua ação combinada aos fármacos de primeira linha no tratamento da TB. Os resultados desse estudo estão apresentados em um artigo de revisão sistemática, um artigo e um manuscrito contendo estudos experimentais. No artigo "Promising Anti-Tuberculosis Activity of Piperine Combined with Antimicrobials: a Systematic Review" foi desenvolvida pelo método PRISMA e os artigos selecionados mostraram que a PIP desempenha um papel importante na inibição de bombas de efluxo (BEs) em micobactérias e modula o sistema imunológico, especialmente quando combinado com outros fármacos anti-TB (por exemplo, rifampicina (RIF)). A proposta do artigo "*In vitro* Combinatory Activity of Piperine and Anti-Tuberculosis Drugs in *Mycobacterium tuberculosis*", foi de fazer uma triagem da ação da PIP combinada aos fármacos de primeira linha na cepa de referência *M. tuberculosis* e testar as combinações sinérgicas em isolados clínicos pelo método de *resazurin drugs combination microtiter assay* (REDCA), além de avaliar o potencial de PIP como IBE por meio do ensaio de acúmulo de brometo de etídeo. Os resultados confirmaram o potencial de PIP como IBE e que a combinação sinérgica de PIP com RIF ou estreptomicina (SM) se mostra promissora, sendo eficiente até em isolados clínicos com diferentes perfis de resistência. Esses resultados nos encorajaram para a realização do estudo que resultou no manuscrito "Piperine activity on efflux pumps and morphology of *Mycobacterium tuberculosis*", que reporta o perfil transcricional de genes que codificam para BEs em *M. tuberculosis* H_{37}Rv e as mudanças morfológicas celulares após exposição as combinações promissoras estabelecidas anteriormente. Na análise do perfil transcricional foi possível observar que, quando exposto às combinações de fármacos, o bacilo tem sua expressão de BEs reduzida em relação a exposição do fármaco isolado, favorecendo a proposta de usar a PIP como um fármaco adjunto ao tratamento. Esse trabalho contribuiu no entendimento da relação entre PIP e BEs e sugere que a combinação não só com RIF, mas também com SM, pode ser promissora ao tratamento da TB, além de guiar a realização de trabalhos futuros envolvendo a PIP.

Palavras-Chave: Tuberculose, Bomba de Efluxo, Sinergismo, Piperina, Rifampicina, Estreptomicina.

Study of the efflux pumps activity of *Mycobacterium tuberculosis* after exposure to piperine and antituberculosis drugs

ABSTRACT

Tuberculosis (TB) is an infectious disease, mainly caused by *Mycobacterium tuberculosis* and is among the leading causes of death in the world. Drug resistance available for treatment is a continuing threat. Combinations of drugs and the association with inhibitory compounds of efflux pumps (IEPs) appear as an expectation to circumvent this mechanism of bacillus resistance. In this context, this study aimed to review the piperine substance (PIP) and its activity against the TB bacillus, in addition to proposing an evaluation of its combined action to first-line drugs in the treatment of TB. The results of this study are presented in a systematic review paper and a paper and a manuscript containing experimental studies. In the article "Promising Anti-Tuberculosis Activity of Piperine Combined with Antimicrobials: Systematic Review" was developed by the PRISMA method and the selected articles showed that PIP plays an important role in the inhibition of efflux pumps (EPs) in mycobacteria and modulates the system especially when combined with other anti-TB drugs (e.g rifampicin (RIF)). The purpose of this paper "*In vitro* Combinatory Activity of Piperine and Anti-Tuberculosis Drugs in *Mycobacterium tuberculosis*", was to screen the action of combined PIP on first line drugs in the *M. tuberculosis* reference strain and to test the synergistic combinations in clinical isolates trials using the resazurin drugs combination microtiter assay method (REDCA), in addition to evaluating the potential of PIP as IBE through the ethidium bromide accumulation assay. The results confirmed the potential of PIP as IBE and that the synergistic combination of PIP with RIF or streptomycin (SM) is promising, being efficient even in clinical isolates with different resistance profiles. These results encouraged us to carry out the study that resulted in the manuscript "Piperine activity on efflux pumps and morphology of *Mycobacterium tuberculosis*", which reports the transcriptional profile of genes coding EPs in *M. tuberculosis* H_{37}Rv and the cellular morphological changes after exposure to combinations previously established. In the analysis of the transcriptional profile, it was possible to observe that, when exposed to the drug combinations, the bacillus has its EP expression reduced in relation to the exposure of the isolated drug, favoring the proposal to use PIP as a drug adjunct to the treatment. This work contributed to the understanding of the relationship between PIP and EPs, suggests that the combination not only with RIF, but also with SM, can be promising to treatment of TB, besides guiding future work involving PIP.

Keywords: Tuberculosis, Efflux Pump, Synergism, Piperine, Rifampicin, Streptomycin.

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LISTA DE SIGLAS

ABC	ATP-binding cassete superfamily
a.C	Antes de Cristo
AIDS	Síndrome da imunodeficiência adquirida
BAAR	Bacilo álcool-ácido resistente
BE	Bomba de efluxo
EMB	Etambutol
CCCP	Carbonil cianeto *m*-clorofenilhidrazona
CIM	Concentração inibitória mínima
HIV	Vírus da imunodeficiência Humana
IBE	Inibidor de bomba de efluxo
INH	Isoniazida
MATE	*Multidrug and toxic compounds extrusion family*
MDR-TB	Tuberculose multiresistente
MFS	*Major facilitator superfamily*
M. tuberculosis	*Mycobacterium tuberculosis*
OMS	Organização Mundial da Saúde
PAS	Ácido para-aminosalicílico
PIP	Piperina
PZA	Pirazinamida
REDCA	*Resazurin drugs combination microtiter assay*
REMA	*Resazurin microtiter assay*
RIF	Rifampicina
RND	*Resistance-Nodulation-cell Division family*
RR-TB	Tuberculose resistente à rifampicina
qPCR	Reação em cadeia da polimerase em tempo real
SMR	*Small Multidrug Resistance*
SM	Estreptomicina
TB	Tuberculose
VP	Verapamil

SUMÁRIO

CAPÍTULO I

1. INTRODUÇÃO

1.1. Tuberculose: História, Aspectos Gerais e Epidemiologia

A tuberculose (TB) é uma doença infectocontagiosa muito antiga, causada principalmente pelo bacilo *Mycobacterium tuberculosis*. Acredita-se que esse bacilo seja anterior ao próprio homem, sucedendo formas ainda mais elementares de vida microscópica (BERTOLLI FILHO, 2001). Estudos comprovam a presença do bacilo em múmias egípcias datadas de 2.400 a.C. (TRIPATHI et al., 2004). Hipócrates, Celsius e Galeno, foram importantes personagens nas primeiras descobertas envolvendo TB. Eles observavam e descreviam os sintomas dos pacientes e acreditavam na importância do descanso e ar fresco como sendo essenciais no tratamento desta doença. Essas observações estabeleceram os fundamentos do raciocínio clínico que persistiram, quase inalterados, até o advento dos tempos modernos, confirmando o grau de sofisticação alcançado pela medicina da antiguidade (BERTOLLI FILHO, 2001).

Durante o século XVI a TB foi reconhecida como uma doença contagiosa na região do Mediterrâneo, e denominada como “tísica”, termo esse importado da Índia que significa emagrecimento ou depauperação do corpo. As características anatômicas e patológicas da TB foram relatadas no tratado Opera Medica de Silvius, em 1679 e foi seguida da descrição de achados patológicos da TB miliar por Manget, em 1702 (BERTOLLI FILHO, 2001).

Mais tarde, entre o final do século 18 e início do 19, ocorreu a revolução industrial na Inglaterra, e a doença ficou conhecida como praga branca e foi a principal causa de morte na Europa e Estados Unidos. Em toda a história das conquistas territoriais, das colonizações, onde o homem civilizado chegou, levou também o bacilo causador da TB, contaminando os nativos, os quais, sem defesas imunitárias, foram em grandes contingentes dizimados. Vale lembrar que na colonização do Brasil vieram jesuítas e colonos, na maioria tuberculosos, para cá atraídos e destacados pelos "benefícios do clima ameno". Eles infectaram os índios, contaminando-os em massa, na primeira fase da colonização (ROSEMBERG, 1999; TRIPATHI et al., 2004).

O termo tuberculose foi criado em 1839, por Schoenlein, que aproveitou a raiz "tubérculo", nome dado ao nódulo lesional por Sylvios Deleboe em 1680. Em 1882, estudos

do cientista Robert Koch levou à descoberta do bacilo *M. tuberculosis* como agente causador da doença, também conhecido como bacilo de Koch (MURRAY; SCHRAUFNAGEL; HOPEWELL, 2015).

O bacilo causador da TB faz parte do gênero *Mycobacterium*, o nome *myco* é proveniente do crescimento semelhante aos dos fungos filamentosos em meio de cultura liquido. As bactérias desse gênero são consideradas bacilos álcool-ácido resistentes (BAAR), possuem forma de bastonete, são aeróbios, imóveis e não produtores de esporos. A parede celular das micobactérias é semelhante as de bactérias Gram positivas, porém possui suas peculiaridades, como uma composição rica em lipídeos, com à presença de ácidos micólicos, sendo hidrofóbica e com isso torna-se resistente a desinfetantes e corantes laboratoriais (DAFFÉ; DRAPER, 1998).

Apesar de ser uma doença antiga e curável, a alta mortalidade e morbidade associada à TB são aspectos que caracterizam a doença como um importante problema de saúde pública em todo o mundo. No ano de 2016, a incidência de TB no mundo foi aproximadamente 10,4 milhões de casos, equivalentes a 140 casos por 100.000 habitantes. Houve cerca de 1,3 milhões de mortes por TB e mais 374.000 mortes entre portadores de HIV. Globalmente, a proporção de pessoas que desenvolveram TB e morreram da doença foi de 16 %. A TB é uma das dez principais causas de morte em todo o mundo. Nos últimos 5 anos, tem sido a principal causa de morte por um único agente infeccioso, acima do HIV / AIDS (WHO, 2017).

A TB causada por bacilo resistente a fármacos é uma ameaça persistente, com 490.000 casos de TB multidrogarresistente (MDR-TB) emergentes em 2016 e 110.000 casos adicionais suscetíveis à isoniazida, mas resistentes à rifampicina (RR-TB). Os países com maior número de casos MDR / RR-TB (47% do total global) foram a China, Índia e a Rússa (WHO, 2017).

Esse quadro epidemiológico fez com que a Organização Mundial da Saúde (OMS) redefinisse a classificação de países prioritários para o período de 2016 a 2020. Três são as listas prioritárias, definidas segundo os critérios epidemiológicos: número de casos de TB; MDR-TB; e coinfecção TB/HIV (Figura 1). O Brasil, que ainda permanece entre os 20 países que apresentam mais casos da doença, encontra-se em duas dessas listas, ocupando a 20ª posição na classificação de carga da doença e a 19ª quanto à coinfecção TB/HIV.

O último relatório do Ministério da Saúde, divulgado em 2016, aponta que no Brasil, apesar de o número de casos ter sido reduzido em cerca de 20% nos últimos 10 anos, ainda são notificados aproximadamente 70 mil casos novos de TB e ocorrem 4,5 mil mortes em decorrência da doença a cada ano (BRASIL, 2017)

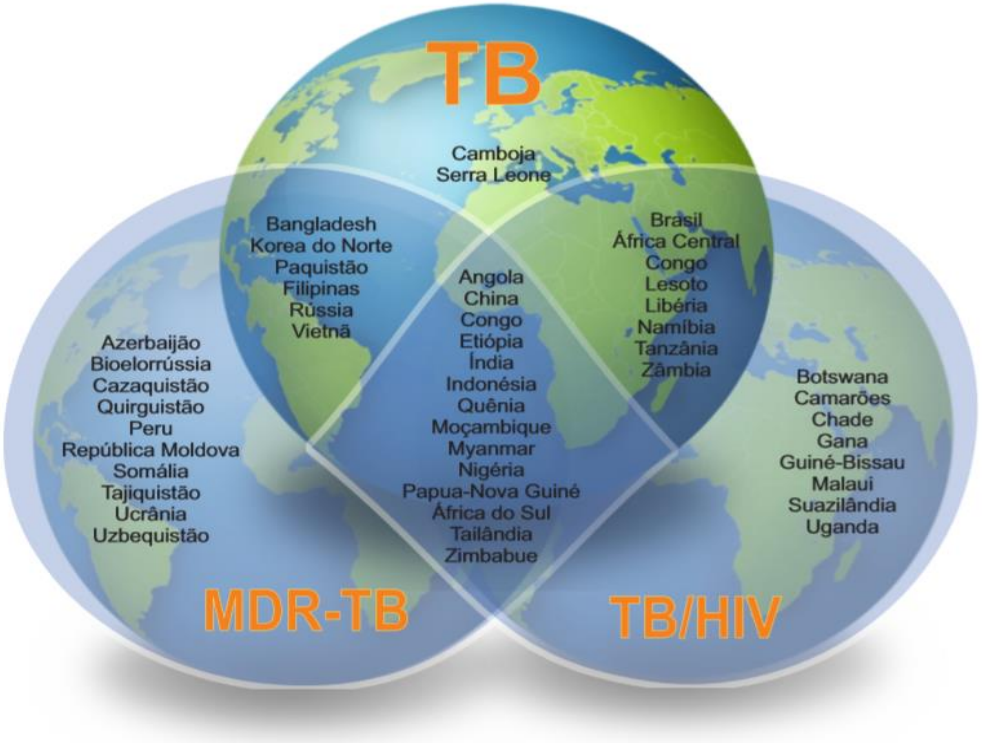

Figura 1. Grupos de países com alta incidência de tuberculose no mundo e as suas áreas de sobreposições.
Fonte: Adaptado de WHO (2017).

1.2. Transmissão, sintomas e diagnóstico da tuberculose

A TB é disseminada quando indíviduos com doença na forma ativa, ao tossir, falar ou espirrar, expulsam gotículas (aerossóis) contendo o bacilo para o meio ambiente. A proximidade com indivíduos doentes, assim como os ambientes fechados e pouco ventilados favorecem o contágio. Mesmo entrando em contato com o bacilo, a pessoa pode não desenvolver a doença e a probabilidade de desenvolver TB é maior entre as pessoas infectadas com o HIV, e entre as pessoas afetadas por fatores de risco, como a subnutrição, diabetes, tabagismo e consumo de álcool. A doença normalmente afeta os pulmões (TB pulmonar), mas também pode afetar outros órgãos como rins, ossos, meninges, entre outros (TB extrapulmonar) (JEREB et al., 2003; WHO, 2017).

Os principais sintomas da TB pulmonar incluem tosse por mais de duas semanas, produção de escarro, febre, sudorese, cansaço, dor no peito, falta de apetite, emagrecimento e nos casos mais avançados, pode aparecer escarro com sangue. O diagnóstico torna-se

complexo em alguns casos pois a presença de sintomas isolados se confundem com outras doenças, como um resfriado por exemplo (WHO, 2017).

Os métodos baciloscopia, cultura (padrão ouro) e teste de susceptibilidade aos antimicobacterianos podem ser realizados para o diagnóstico laboratórial dos casos suspeitos de TB pulmonar, caracterizados por sintomatologia clínica ou pelo exame de imagem do tórax (BAMMANN et al., 2010). A baciloscopia é um método simples, rápido, de baixo custo e seguro para elucidação diagnóstica, permite detectar de 60 % a 80 % dos casos de TB pulmonar, o que é importante do ponto de vista epidemiológico, já que os casos bacilíferos são os responsáveis pela manutenção da cadeia de transmissão da doença. Esse exame consiste na análise microscópica de materiais de provaveis sítios da doença, como o escarro, corados pelo método Ziehl-Neelsen (BRASIL, 2011).

A cultura é o método considerado “padrão ouro”, por ter elevada especificidade e sensibilidade no diagnóstico da TB. Nos casos pulmonares com baciloscopia negativa, a cultura do escarro pode aumentar em até 30% um diagnóstico positivo da doença. Os métodos clássicos para cultura de micobactérias utilizam a semeadura da amostra clínica em meio líquido como Middlebrook 7H9 ou meio de cultura sólidos como Löwenstein-Jensen e Ogawa-Kudoh. Porém a desvantagem da cultura é o tempo de detecção do crescimento bacteriano que varia de 14 a 30 dias, podendo se estender por até oito semanas (BRASIL, 2011).

Existe ainda uma nova opção de diagnóstico laboratorial, o Gene Xpert® MTB/RIF, que é um método molecular que consiste na extração e amplificação de ácidos nucléicos por reação em cadeia da polimerase em tempo real (qPCR). É uma alternativa automatizada, simples, rápida e de fácil execução nos laboratórios. O teste detecta simultaneamente *M. tuberculosis* e a presença de resistência à RIF, diretamente do escarro, em aproximadamente, duas horas. Esse método além de aumentar a detecção de TB em casos de pacientes portadores de HIV, comparado com a baciloscopia, ele auxilia no diagnóstico de MDR-TB, direcionando melhor o tratamento. Por todas essas qualidades é recomendado pela OMS e atualmente tem apoio para ser implantado em países com alta prevalência da doença (WHO, 2017).

O teste de susceptibilidade aos fármacos pode ser realizado por diferentes metodologias, porém, no Brasil, o método recomendado pelo Ministério da Saúde é o método das proporções (BRASIL, 2011).

1.3. Tratamento da tuberculose

Apesar de eficiente, o tratamento é longo e apresenta efeitos colaterais. Geralmente, após duas a três semanas de tratamento, a maior parte dos pacientes deixam de ser bacilíferos, diminuindo a possibilidade de transmissão da doença. O desaparecimento de alguns sintomas leva muitas vezes ao abandono do tratamento pelo paciente, fato preocupante, pois favorece a seleção de bacilos resistentes (BRASIL, 2011).

O primeiro antimicrobiano efetivo no tratamento da TB foi a estreptomicina (SM) (Figura 2), introduzida em 1944, seguida pelo ácido para-aminosalicílico (PAS). Ambos foram moderadamente efetivos, mas tiveram efeitos colaterais significativos e, em cada um dos casos houve desdobramento da resistência aos medicamentos. Por conseguinte, um importante acerto clínico do Conselho de Pesquisa Médica Britânica (BMRC) documentou o valor superior do tratamento combinado em comparação com a estreptomicina SM ou PAS isoladamente. Além disso, o ensaio do BMRC levou a um axioma de tratamento: nunca tratar a TB ativa com um único agente antimicrobiano (MURRAY; SCHRAUFNAGEL; HOPEWELL, 2015).

A isoniazida (INH) foi introduzida em 1952 e até hoje é considerada um dos fármacos mais eficiente na eliminação bacilar. Em 1965, a rifampicina (RIF) e em 1968 o etambutol (EMB) passaram a integrar a terapia e a pirazinamida (PZA) passou a compor a poliquimioterapia da doença em 1970 (MURRAY; SCHRAUFNAGEL; HOPEWELL, 2015).

Um tratamento eficiente depende da capacidade do fármaco em eliminar os bacilos em seus diferentes estágios dentro do indivíduo contaminado. Atualmente, o tratamento recomendado pela OMS para TB ativa é realizado em duas etapas. A primeira denominada fase intensiva, com administração diária de INH, RIF, EMB e PZA com a duração de dois meses (Figura 2). A segunda, chamada de fase de manutenção, tem duração de quatro meses, e a administração de INH e RIF, com o objetivo de reduzir a chance de falhas no tratamento e a possibilidade de recorrência da doença (BRASIL, 2011; WHO, 2017).

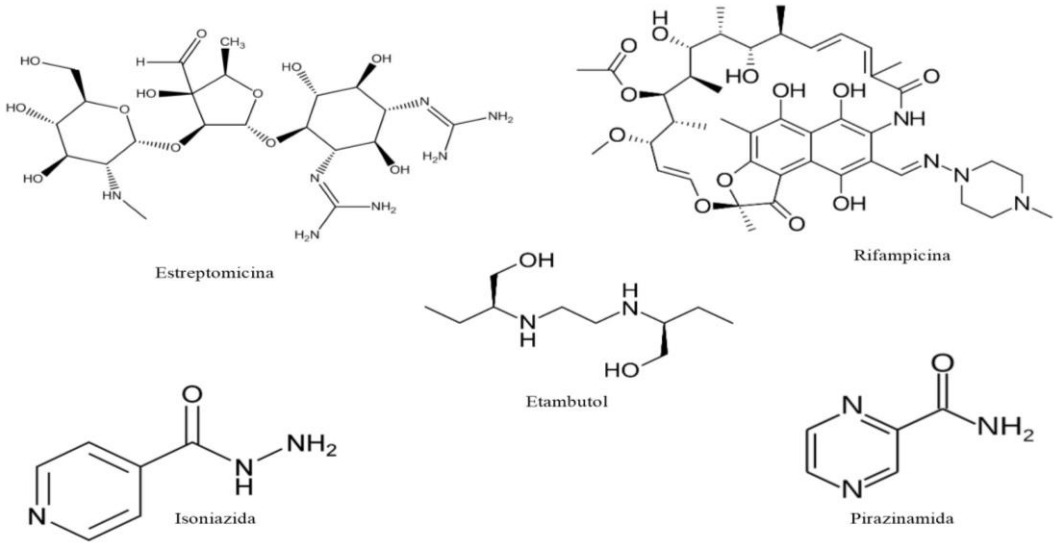

Figura 2. Estrutura química dos principais fármacos utilizados no tratamento da TB.

Uma possível explicação para a prevalência da doença é o desenvolvimento de *M. tuberculosis* com resistência, que pode ser atribuída ao abandono do tratamento pelo paciente ou até falhas no fornecimento adequado dos medicamentos. Os pacientes portadores de bacilos resistentes a INH e RIF constituem um grupo de doentes classificados como portadores de TB resistente a múltiplos fármacos (MDR-TB). Pacientes portadores de TB extensivamente resistente (XDR-TB), que são aqueles pacientes portadores de bacilos resistentes a INH, RIF, uma fluoroquinolona e aos fármacos injetáveis de segunda linha, devem ser encaminhados aos centros de referência terciários, pois necessitam de esquemas especiais e individualizados (WHO, 2017; ZUMLA et al., 2012).

1.4. Mecanismos de resistência

As micobactérias são intrinsecamente resistentes a uma gama de antimicrobianos, de amplo espectro, como: penicilinas, cefalosporinas e tetraciclinas. A constituição da parede celular, que possui baixa permeabilidade à vários compostos devido ao alto conteúdo lipídico, justifica essa resistência intrínseca. (DE ROSSI; AINSA; RICCARDI, 2006). Outra característica importante e natural das micobactérias é a presença de alguns receptores e produção de enzimas que impedem a ação dos antimicrobianos (CALGIN et al., 2013; CAMPOS, 1999).

A resistência aos fármacos utilizados no tratamento da TB, pode ser decorrente de mutação natural ou induzida por ações do meio em que o bacilo se encontra. A presença de mutações espontâneas no genoma de *M. tuberculosis*, que em algumas situações ocorrem em genes alvo dos fármacos, podem levar à perda de função de importantes proteínas (por exemplo, proteína ativadora de pró-fármaco), alteração física do alvo (especificamente do sítio de ligação do fármaco), além de inativação enzimática do fármaco utilizado na terapia (GREEN; GARNEAU-TSODIKOVA, 2013).

Entretanto, em certa parcela dos isolados clínicos, a resistência não pode ser explicada pela presença de mutações em genes já relacionados com resistência a fármacos. Já foi observado que isso ocorre em 30 % dos isolados resistentes à INH e 5 % dos isolados resistentes à RIF, sugerindo a existência de outros mecanismos de resistência (SILVA, P.E. et al., 2011; JIANG et al., 2008; SILVA, P.E. et al, 2001).

Recentemente, ficou reconhecido que sistemas de efluxo ativo é de grande importância na composição da resistência bacteriana aos fármacos. Este mecanismo é mediado por BE, que são transportadores ativos associados à membrana que promovem o extrusão de compostos tóxicos, incluindo antimicrobianos, das células (SINGH et al., 2011; ZECHINI; VERSACE, 2009) (Figura 3).

1.5. Bombas de efluxo e Inibidores

Bombas de efluxo (BEs) bacterianas estão distribuídas em 5 famílias: *ATP-binding cassete superfamily* (ABC) e *Major Facilitator Superfamily* (MFS). As outras três famílias são menores e de desenvolvimento mais recente: *Small Multidrug Resistance* (SMR), *Resistance-Nodulation-cell Division family* (RND) e *Multidrug and Toxic Compounds Extrusion Family* (MATE) (DE ROSSI; AINSA; RICCARDI, 2006).

As BEs da família “ABC transporters” fazem o transporte de moléculas em geral (íons, aminoácidos, peptídeos, antimicrobianos, polissacarídeos e proteínas), e são dependentes da hidrólise de ATP (adenosina trifosfato) para o seu funcionamento. Além da função de efluxo, essa familia tem grande importância na função importadora de moléculas, e para isso é indispensável a presença da proteína ligante de substrato (SBP – “Substrate Binding Protein”), que se encontra associada à membrana citoplasmática por meio de uma cauda lipídica em *M. tuberculosis* (AMES, 1993; BRAIBANT; GILOT; CONTENT, 2000). Estudos relacionados com essa família descreveram algumas BE como por exemplo Rv1456c, Rv1457c, Rv1458c, Rv1217c, Rv1218c, Rv1819 como responsáveis pela diminuição da

susceptibilidade de isolados MDR a determinados fármacos. (HAO et al., 2011; JIANG et al., 2008; PAN et al., 2012).

A outra grande família de BEs é a “MFS transporters”, que são responsáveis pelo efluxo de açúcares simples, oligossacarídeos, inositóis, fármacos, aminoácidos, ésteres organofosfatados, metabólitos do ciclo de Krebs, além de uma variedade de ânions e cátions inorgânicos, utilizando a força motriz de prótons para o funcionamento (DE ROSSI; AINSA; RICCARDI, 2006). A participação dessa família de BE na resistência, em linhagens micobacterianas, aos fármacos já foi reportada, sendo identificadas principalmente as BEs: Rv1258c, Rv1410c, Rv2459 (DE ROSSI; AINSA; RICCARDI, 2006; GUPTA et al., 2010; JIANG et al., 2008; MACHADO et al., 2012; RODRIGUES et al., 2012).

A família “RND transporters” é menor do que as anteriormente citadas, porém assim como as MFS são comuns na parede celular das bactérias. As BEs RND são responsavés por transportar várias classes de antibióticos, compostos anti-sépticos, corantes e detergentes (ZECHINI; VERSACE, 2009). Rodrigues et al (2012) relacionaram a BE Rv2942 com resistência a fármacos (RODRIGUES et al., 2012) .

Diferentes das demais famílias, que podem ocorrer em outros microrganismos, as “SMR transporters” são encontradas apenas em bactérias e estão envolvidas no efluxo de fármacos de peso molecular pequenos, catiônicos e lipofílicos. A BE codificada pelo gene *Rv3065* foi relatado *M. tuberculosis*. (RODRIGUES et al., 2012; ZECHINI; VERSACE, 2009). A família “MATE” é pequena, tem sido descrita em algumas bactérias, mas não é comum em micobacterias (ZECHINI; VERSACE, 2009).

As BEs conferem baixos níveis de resistencia, em relação com os altos níveis de resistência conferidos por mutações em genes alvos dos fármacos, porém a superexpressão de múltiplos genes de BEs em isolados clínicos resistentes pode causar a redução da eficiência dos fármacos (SZUMOWSKI et al., 2013; WEBBER; PIDDOCK, 2003).

Sabendo que a resistência em micobactérias pode ser mediada por BEs uma estratégia eficiente seria a busca por compostos capazes de contornar esse sistema. Tem sido demonstrada a existência de substâncias capazes de inibir as BEs *in vitro*, fazendo com que as concentrações inibitórias mínimas (CIM) de alguns antimicobacterianos diminuam consideravelmente quando em combinação com esses inibidores, como, por exemplo: verapamil (VP), reserpina, clorpromazina, tioridazina e recentemente a piperina (PIP) (ESCRIBANO et al., 2007; GUPTA et al., 2010b; RODRIGUES et al., 2012).

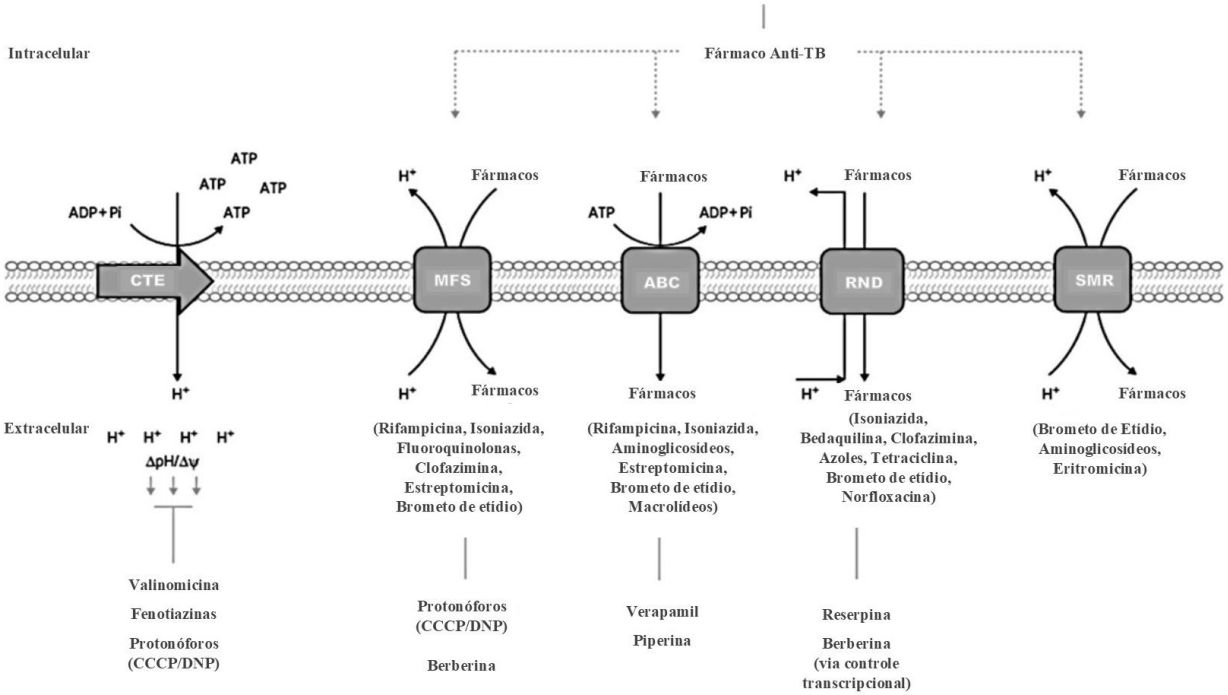

Figura 3. Representação do mecanismo de ação do inibidor da bomba de efluxo e o efeito sobre a terapia Anti-TB. CTE, cadeia de transporte de elétrons.

Fonte: Adaptado de PULE et al (2016)

1.6. Piperina

A PIP [1- [5- [1,3-benzodioxol-5-il] -1-oxo-2,4, pentadienilo]piperidina] (Figura 4) é um composto orgânico e o principal alcalóide pungente presente principalmente na pimenta preta (*Piper nigrum*) também conhecida como pimenta do reino. Essa espécie de pimenta é uma especiaria nativa da Índia e considerada um tempero culinário bastante popular que tem sido usado desde os tempos antigos.

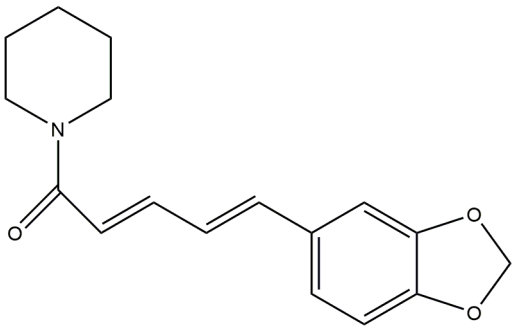

Figura 4. Fórmula molecular da piperina

Estudos com PIP relatam sua atividade como antiinflamatório, antifúngico, analgésico, antipirético, antioxidante, anticancerígeno entre outros (KHAJURIA et al., 1998; MOON et al., 2016; MURUNIKKARA et al., 2012; PARMAR et al., 1997; SELVENDIRAN et al., 2005; SELVENDIRAN; PRINCE VIJEYA SINGH; SAKTHISEKARAN, 2006; ZHAI et al., 2016).

Recentemente a PIP tem sido associada ao sistema de efluxo, mostrando ser um potencial inibidor de bomba de efluxo (IBE). Sua atividade como IBE foi primeiramente relatada em *Staphylococcus aureus* (KUMAR et al., 2008; SANGWAN et al., 2008), depois em micobacterias como *M. smegmatis* e *M. tuberculosis* (JIN et al., 2011; SHARMA et al., 2010). Em *Mycobacterium smegmatis* apresentou um efeito inibidor comparável a VP e carbonil cianeto *m*-clorofenilhidrazona (CCCP), que estão entre os inibidores mais estudados (JIN et al., 2011).

Ainda em relação ao *M. tuberculosis*, PIP também parece ter efeitos imunorregulatórios em camundongos. O tratamento com PIP aumentou a proliferação de células T e B e citocinas Th1 e aumentou a ativação de macrófagos, que são muito importantes para combater a multiplicação do bacilo. A piperina em combinação com o RIF melhorou significativamente o efeito terapêutico do RIF e resultou em uma redução da carga bacteriana nos pulmões em comparação com o RIF sozinho (SHARMA et al. 2014).

Por ser um composto natural, acessível e de atividades promissoras, principalmente no caso de doenças infeciosas, como TB, a PIP precisa ser melhor estudada, para desvendar detalhes do seu mecanismo de ação, e suas contribuições quando associada a outros compostos e outros fármacos.

2. JUSTIFICATIVA

A TB continua sendo um importante problema de saúde pública em muitos países devido, principalmente, ao surgimento de isolados clínicos resistentes aos fármacos disponíveis para o tratamento da doença (WHO, 2017). Mesmo com o tratamento eficaz para a TB sensível, os fármacos disponíveis, na maioria das vezes, têm efeitos colaterais e é um tratamento relativamente longo. Esses fatores contribuem para o abandono do regime de tratamento pelos pacientes, o que pode levar a seleção e a disseminação de bacilos resistentes (LAURENZO; MOUSA, 2011).

Nesse contexto, o desenvolvimento de novos fármacos ou a combinação dos já existentes, são estratégias essenciais para o futuro controle de bacilos resistentes no mundo (ISLAM et al., 2017). Estudos mostram que as BEs conferem um papel importante na resistência a um ou vários compostos em micobactérias. Os IBEs podem contribuir, consideravelmente, para diminuição das CIMs de alguns antimicobacterianos quando administrados em combinação com esses fármacos (ESCRIBANO et al., 2007; GUPTA et al., 2013; RODRIGUES et al., 2012). Desta forma, existe a necessidade de compreender melhor esses mecanismos de efluxo e a ação desses IBEs, afim de burlar estes mecanismos de resistência aos fármacos estabelecidos pela micobactéria e introduzir terapias mais eficazes e seguras para o paciente.

Considerando os poucos estudos envolvendo a combinação de fármacos antituberculose e IBE em cepa de referência de *M. tuberculosis* H_{37}Rv e isolados clínicos, o presente trabalho visa triar as combinações dos fármacos antituberculose que estão na primeira linha de tratamento, incluindo a SM, em associação com um IBE, a PIP. Esse trabalho irá somar para o entendimento do mecanismo de ação da PIP, contribuindo em futuros estudos envolvendo desenho molecular de novos fármacos, além de levantar hipóteses para uma possível alternativa no tratamento de TB. Nosso trabalho se apresenta como um dos pioneiros na avaliação do sinergismo entre diferentes fármacos e PIP e do efeito de combinações promissoras na expressão gênica das principais BEs relacionadas com a resistência em *M tuberculosis.*

3. OBJETIVOS

3.1. Geral

Realizar uma revisão sistemática e avaliar o efeito da combinação de fármacos anti-TB com PIP a expressão gênica de BEs e na morfologia de *M. tuberculosis.*

3.2. Específicos

- Determinar a concentração inibitória mínima da PIP e dos fármacos antituberculose (RIF, INH, ETB e SM) pelo método de REMA em *M. tuberculosis* $H_{37}Rv$;
- Determinar a ação combinatória entre o IBE e os fármacos antituberculose (RIF, INH, ETB e SM) pelo método REDCA em *M.tuberculosis* $H_{37}Rv$ e isolados clínicos;
- Determinar a atividade como IBE da PIP em *M. tuberculosis* $H_{37}Rv$ por brometo de etídio;
- Analisar a expressão relativa dos principais genes relacionados com BE em *M. tuberculosis* $H_{37}Rv$ após a exposição as combinações de PIP e fármacos antituberculose que apresentaram ação sinérgica;
- Analisar as alterações morfológicas de *M. tuberculosis* $H_{37}Rv$ após exposição as combinações de PIP e fármacos antituberculose que apresentaram ação sinérgica, por MEV.

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CAPÍTULO II

Artigo 1: " Promising Anti-Tuberculosis Activity of Piperine Combined with Antimicrobials: a Systematic Review"

Promising Anti-Tuberculosis Activity of Piperine Combined with Antimicrobials: a Systematic Review

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Running Title: Anti-mycobacterial activity of piperine

Abstract

Piperine, a bioactive compound from *Piper nigrum* and *Piper longum*, has shown promissing activity as efflux pump inhibitor and as adjunct in treatment of tuberculosis. The present systematic review investigated scientific studies of the activity of piperine against mycobacteria, with a focus on its mechanism of action, drug interactions, and anti-mycobacterial activity. A broad and rigorous literature search of three electronic databases (PubMed, Web of Knowledge, and LILACS) was performed according to the PRISMA statement. We considered studies that were published up to December 01, 2017. Google Scholar was also searched to increase the number of publications. We searched for articles using the search terms "piperine" and "*Mycobacterium* spp." The search yielded a total of 225 articles. After removing duplicate publications, 208 publications remained. Of these, we evaluated the full text of 13 articles. After applying the inclusion criteria, eight studies were included in the present systematic review. The results of the systematic review showed that piperine has promising anti-TB activity, mainly when combined with antimicrobials, and plays an important role as an efflux pump inhibitor.

Keywords: Antimicrobials, Drug Synergism, Efflux Pumps, *Mycobacterium* spp., Piperine, Tuberculosis.

Introduction

In 2016, 6.3 million new cases of TB were reported. TB is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS.[1] Although the incidence of TB is slowly decreasing, roughly one-third of the world's population has been infected by the bacilli.[1,2]

Most antibiotics used for the treatment of TB are useless in the short term because of unique characteristics of *Mycobacterium tuberculosis*. The ideal treatment for TB should kill active bacilli, control latent organisms, and prevent the emergence of resistant strains.[3] The current treatment for TB often induces side effects, which is a likely reason why some patients eventually discontinue their treatment before it is completed and resistant strains can arise. [4]

Resistance to anti-TB drugs has been mainly attributed to spontaneous mutations in specific genes of *M. tuberculosis*. However, efflux pumps (EPs) may also initially impart natural tolerance and resistance to one or more compounds. [3,5–8]

The antimicrobial activity of natural products is a research area with great potential for the development of new technologies to combat TB especially in countries that have wide biodiversity, such as Brazil and India. [9]

Piperine (PIP) is a bioactive compound of *Piper nigrum* and *Piper longum* and possible alternative for the treatment of TB. Studies have suggested that it is an efflux pump inhibitor (EPI) and may increase the efficacy of some antimicrobials. [10–12] PIP has also been reported to have antiinflammatory [13], antimicrobial [14,15], antifungal [16], analgesic, antipyretic, antioxidant [17,18], anticarcinogenic effects. [19] It may also be able to increase the bioavailability of some drugs [20–22] and increase the activation of detoxification enzymes. [23] Our research question was whether piperine has some anti-mycobacterial activity. Could piperine potentiate the effect of antimicrobials? Thus, we investigated the activity of PIP against mycobacteria, with a focus on

its mechanism of action, drug interactions and anti-mycobacterial activity by a systematic review.

Methods

We performed rigorous search in electronic databases according to the PRISMA statement [24] using the search terms "piperine" and "*Mycobacterium* spp."

Data sources and research

We searched the PubMed, Web of Knowledge, and LILACS databases for articles that were published up to December 01, 2017. A flowchart of the search is presented in figure 1.

To identify target publications in PubMed, Medical Subject Heading (MeSH) terms were applied. Six independent researchers (Group 1: LAH, JVPS, ALA, SSNV, ILEB, and PCH) conducted searches to define and identify the largest number of MeSH terms to ensure high sensitivity for identifying relevant scientific publications. All disagreements were resolved by consensus. One expert (JJVT) was invited to ensure MeSH terms quality and accuracy. In the first phase of the study, the researchers from Group 1 focused on the titles and abstracts of the publications that were identified in each database. In PubMed, the MeSH terms were organized into three blocks: block 1 ("Tuberculosis" OR "Mycobacterium" OR "Gram-Positive Bacteria"), block 2 ("Piper" OR "Antitubercular" OR "Piperidines" OR "Polyunsaturated Alkamides/Pharmacology" or "Benzodioxoles"), and block 3 ("ATP-Binding Cassette Transporters" OR "Multidrug Resistance-Associated Proteins" OR "Gene Expression Regulation, Bacterial"). For the Web of Knowledge database, a Topic Search (TS) was applied, which ensured the sensitivity of the search. In the LILACS database, we searched "Piper/química" and "*Mycobacterium*". Other publications were retrieved in Google Scholar using free terms, and relevant articles were selected.

Inclusion and exclusion criteria

Studies that evaluated the PIP and *Mycobacterium* spp. interactions were included in the study. Using database filtering features and by manually checking the abstracts, publications were validated. Published English, Portuguese or Spanish articles up to December 01, 2017, either *in vitro* or *in vivo* assays, were included.

Reviews, comparative studies, case reports, editorials, editors' comments, news, guidelines, and interviews were excluded (Figure 1). Articles with no mention about PIP activity against *Mycobacterium* spp. in their summaries/abstract were also excluded.

Evaluation of quality

The second phase of the present systematic review was to evaluate the selected abstracts publications screened. The full-text articles were retrieved in PDF format, randomly arranged and distributed to the six independent researchers from Group 1 for validation to avoid bias with regard to interest and selection. Disagreement concerning the inclusion or exclusion was solved by consensus. In the third phase, the selected papers were randomized and distributed to five independent judges (Group 2: RFC, KRCF, RBLS, VLDS and PAZCS). The final number of publications (*n*) reflected the quantitative and qualitative aspects of the articles that were determined by consensus among Groups 1 and 2. To increase the breadth of the present review, the bibliographies of the selected articles were also searched to identify original articles that might not have been retrieved in the previous search phases.

Data extraction

With support from a specialist (JJVT), Group 1 researchers extracted data from the selected articles. The characteristics of the studies that comprised the systematic review were highlighted, including authors, year, *Mycobacterium* species, susceptibility profile, sample

type, and methods and a table was constructed to display these characteristics. After consensus was reached among the researchers from Group 1, the researchers from Group 2 also analyzed the table. Group 2 independently validated the publications. Disagreements among Groups 1 and 2 were solved by consensus.

Results and Discussion

To our knowledge, this is the first systematic review that addresses PIP activity in mycobacteria, focusing on its mechanism of action, drug interactions and anti-mycobacterial activity. Although a meta-analysis would be relevant to evaluate the subject, the publications selected for this systematic review did not present the adequate profile to perform this method.

The initial database search yielded 225 articles that were identified using the keywords predetermined by the researchers from Group 1. After removing duplicate articles, 208 remained. Of these, the full-text of 13 articles were evaluated. Eight of these met the established inclusion criteria.

Most of the studies were performed with *Mycobacterium* spp. clinical isolates and reference strains, conducted in India and published from 2001 to 2012. The main objectives of these studies were to evaluate the minimum inhibitory concentration (MIC) of PIP, time-kill curves, synergism and the inhibition of efflux pumps (Table 1).

Minimum inhibitory concentration

Seven of the eight articles selected evaluated the antimycobacterial activity of PIP or its effects in combination with other antimicrobials. The studies tested the activity of PIP that was obtained from different extracts and reported MICs that ranged from 50 to >100 μg/mL against the *M. tuberculosis* reference strain $H_{37}Rv$ and higher (e.g., 3,000 μg/mL) for *Mycobacterium smegmatis*. [25]

Raja et al. [26] tested six ofloxacin (OFL) resistant *M. tuberculosis* isolates, by microplate alamar blue assay (MABA), which showed PIP MIC 50 mg/L. Singh et al.[25] tested different extracts obtained from the fruit of *Piper longum L*., by a modified Kirby-Bauer disk diffusion method, and found *M. smegmatis* GN/ms-43 inhibition growth were 12.33 ± 0.57, 15.66 ± 1.52, and 11.66 ± 0.57 mm for the chloroform, ethyl-acetate, and methanol fractions, respectively. This preliminary assay revealed that the ethyl-acetate fraction had the best inhibitory activity against *M. smegmatis*. Piperine crystals were purified from the ethyl acetate fraction and showed MICs 3,000 and 39 mg/L, in multidrug-resistant *M. smegmatis* GN/ms-43 and *M. tuberculosis* GN/mt-75, respectively. Other Gram-negative and -positive bacteria were tested, and growth inhibition was observed (MICs ranged from 14 to 180 mg/L). Working with the reference strain *M. smegmatis* mc^2 155, Jin et al. [27] found that PIP had moderate antimycobacterial activity (MIC 128 mg/L) by broth dilution. On the other side, Balakrishnan et al. [28] observed no significant inhibitory activity of PIP against *M. smegmatis*, despite having tested concentrations up to 50 mg/L.

By tetrazolium microplate assay (TEMA), Patilaya et al. [29] showed that n-hexane, ethyl-acetate, and water leaf extracts of *P. nigrum* had activity against *M. tuberculosis* $H_{37}Rv$ with MICs 50, 25 and 100 mg/L, respectively. Sharma et al. [12] evaluated the activity of PIP against two *M. tuberculosis* isolates (one rifampicin monoresistant and one multidrug-resistant) and the reference strain $H_{37}Rv$ and found MICs > 100 mg/L for both of the isolates. Rukachaisirikul et al. [30] tested a dimer of PIP (chabamide) obtained from n-hexane fraction of *Piper chaba Hunter* and found a MIC 12.5 mg/L against *M. tuberculosis* $H_{37}Rv$.

According to Gu et al. [31] crude extracts that have MICs ≤ 128 mg/L and pure compounds with MICs ≤ 64 mg/L are promising and warrant further studies of their antimycobacterial activity. The studies that were included in this systematic review showed that PIP alone has low antimycobacterial activity. Studies that evaluated the effects of PIP and its structural analogues

on other bacteria, such as *Staphylococcus aureus*, have reported similar results, with MICs > 100 mg/L. [10]

Time-kill curve assay

Among the selected articles, only two evaluated the actions of PIP using time-kill assay. Patilaya et al. [29] evaluated the effects of an ethyl-acetate fraction of *P. nigrum* in *M. tuberculosis* $H_{37}Rv$ and observed a marked decrease in colony forming units (CFU) on day 2 of PIP exposure. The CFU continued to decrease until on the 5 day and remained stable to the end of treatment, showing a 98.92% reduction in CFU compared with the control group not exposed to PIP. Sharma et al. [12] performed a time-kill curve assay with exposure of a PIP and RIF combination also observed reduction in CFU.

Drugs combinations studies and efflux extrusion

Considering PIP has low antimycobacterial activity, some studies have evaluated its activity combined with antimicrobials. Sharma et al. [12] tested the effect of PIP plus RIF, by checkerboard assay, and found 4 and 8-fold reductions of RIF MIC against $H_{37}Rv$ and RIF-resistant isolates, respectively. Also, observed PIP acts as EPI, once ethidium bromide (EtBr) MIC decreased 8- and 32-fold in *M. tuberculosis* $H_{37}Rv$ and in a RIF-resistant *M. tuberculosis* isolate, respectively. Efflux pump extrusion is the only known resistance mechanism of EtBr [12]. Similar results were reported by Jin et al [27] with 2- and 4-fold reductions of the EtBr MIC in *M. smegmatis* mc^2 155. The ability of PIP to reduce the EtBr MIC was comparable to the effects of chlorpromazine and reserpine, which are known to be eflux pumps inhibitor (EPIs), and was in a concentration-dependent manner. [27]

Sharma et al. [12] tested the effects of RIF (0.5 mg/L) combined with PIP (25 mg/L) in *M. tuberculosis* $H_{37}Rv$, and observed a >3-log reduction in CFUs on day 8 of treatment,

whereas RIF alone had a similar effect only at a higher concentration (1 mg/L). Considering the concentration-dependent effect of RIF, the RIF plus PIP combination appears to be promising in combat the bacillus. In the same study, the Rv1258c induced gene expression upon exposure to RIF, determined by quantitative real-time polymerase chain reaction, was observed in resistant *M. tuberculosis* isolate. The authors suggested, after applying molecular docking model study that PIP may bind to the protein coded by this gene. PIP was shown to bind to the Arg141 residue of the protein with greater affinity, among other possible interactions. The authors suggested that such binding may be responsible for the RIF-induced inhibition of efflux out of the cell by the Rv1258c efflux pump. Consistent with this finding, a previous study by our group found Rv1258c overexpression that was induced by exposure to 0.5×MIC of RIF plus verapamil in *M. tuberculosis* H_{37}Rv. Verapamil is a well-known EPI that is used as a positive control in studies that develop new EPI molecules. [32,33]

Raja et al. [26] observed a 4-fold decrease in the OFL MIC when combined with PIP in six OFL-resistant *M. tuberculosis* isolates. These results seemed to us that PIP has synergic activity with other drugs by acting as an EPI. Similar results were found when PIP was combined with another fluoroquinolone, ciprofloxacin, in *Staphylococcus aureus.* [10,11] The combination of PIP with extracts of *Catharanthus roseus* L. resulted in a fractional inhibitory concentration index (FICI) 0.06 in *M. tuberculosis*.[26]

Another action of PIP combined with RIF is its ability to completely inhibit the transcriptional activity of RNA polymerase in *M. smegmatis* mc² 155 and in a RIF-resistant strain. To characterize this inhibition, a molecular docking study was performed using RNA polymerase structures from *Thermus aquaticus* and *Escherichia coli*. Both, RIF and PIP can be accommodated in the binding pocket of the RNA polymerase enzyme by stacking, which may enhance the inhibitory activity of RIF mainly in RIF-resistant *M. tuberculosis* isolates. [28]

Scanning electron microscopy

Patilaya et al. [29] performed a scanning electron microscopy study of *M. tuberculosis* $H_{37}Rv$ cells exposed to ethyl acetate fraction of *P. nigrum* containing PIP. They observed slim, shrunken, wrinkled, and empty bacilli after day 2 of exposure. The cells also appeared to stick to together because of the presence of a "web-like mess", which might have been attributable to release of the cytoplasmic contents of ruptured cells. In a similar study, Caleffi-Ferracioli et al. [32] observed the same morphological changes in *M. tuberculosis* cells (i.e., wrinkled and rounded cells) after exposure to 0.5×MIC of verapamil. These morphological changes that were induced by PIP and verapamil are probable attributable to their ability to inhibit bacterial EPs.

Post-antibiotic effects and immunological modulation by piperine

The post-antibiotic effect of RIF alone and combined with PIP in *M. tuberculosis* $H_{37}Rv$ was investigated by Sharma et al. [12] They found that 1 mg/L PIP concentration-dependently prolonged the post-antibiotic effect of RIF from 48 to 72 h. Sharma et al. [34] also investigated the immunological effects of PIP *in vitro* and *in vivo*. For the *in vitro* analysis, T- and B-lymphocytes were extracted from the spleen and peritoneal macrophages in BALB/c mice. The *in vivo* analysis used cells from mice that were infected with *M. tuberculosis* $H_{37}Rv$. The *in vitro* study found that PIP significantly increased the proliferation of T- and B-lymphocytes, increased the production of Th1 cytokines (i.e., interferon-γ [IFN-γ] and interleukin-2 [IL-2]), did not alter the Th2 response (i.e., IL-4) in lymphocytes, and significantly increased the production of nitric oxide in macrophages. PIP increased the production of IFN-γ and IL-2 in lymphocytes in mice that were treated with PIP before and after infection, did not alter IL-4 production, significantly increased the CD4+ and CD8+ cell populations, reduced the number of CFU in the lungs in treated mice, and caused a 2-fold increase in the gene expression of IFN-γ and IL-2 [34].

Conclusion

The findings of this systematic review showed that PIP plays an important role in the inhibition of EPs and modulates the immune system, especially when combined with other anti-TB drugs (e.g., RIF). The mechanisms of action of PIP should be further explored to develop possible alternative treatments for TB.

Strengths and limitations

The present systematic review searched three databases and Google Scholar. This strategy allowed us to increase the sensitivity and accuracy of the publications that were retrieved. The main findings were analyzed and organized in a table based on consensus by the researchers. The relatively few number of publications on the topic hinder wider discussions and definitive conclusions. However, the inclusion of articles that were published in English, Portuguese and Spanish helped increase the range of the study.

Drug efflux systems in mycobacteria are a worthy area of investigation that may allow the development of new therapeutic options for TB with greater safety and efficacy. PIP, the bioactive compound that is contained in *P. nigrum* and *P. longum*, is a promising EPI and has beneficial effects on several other biological processes.

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Conflict of Interest:

None to declare.

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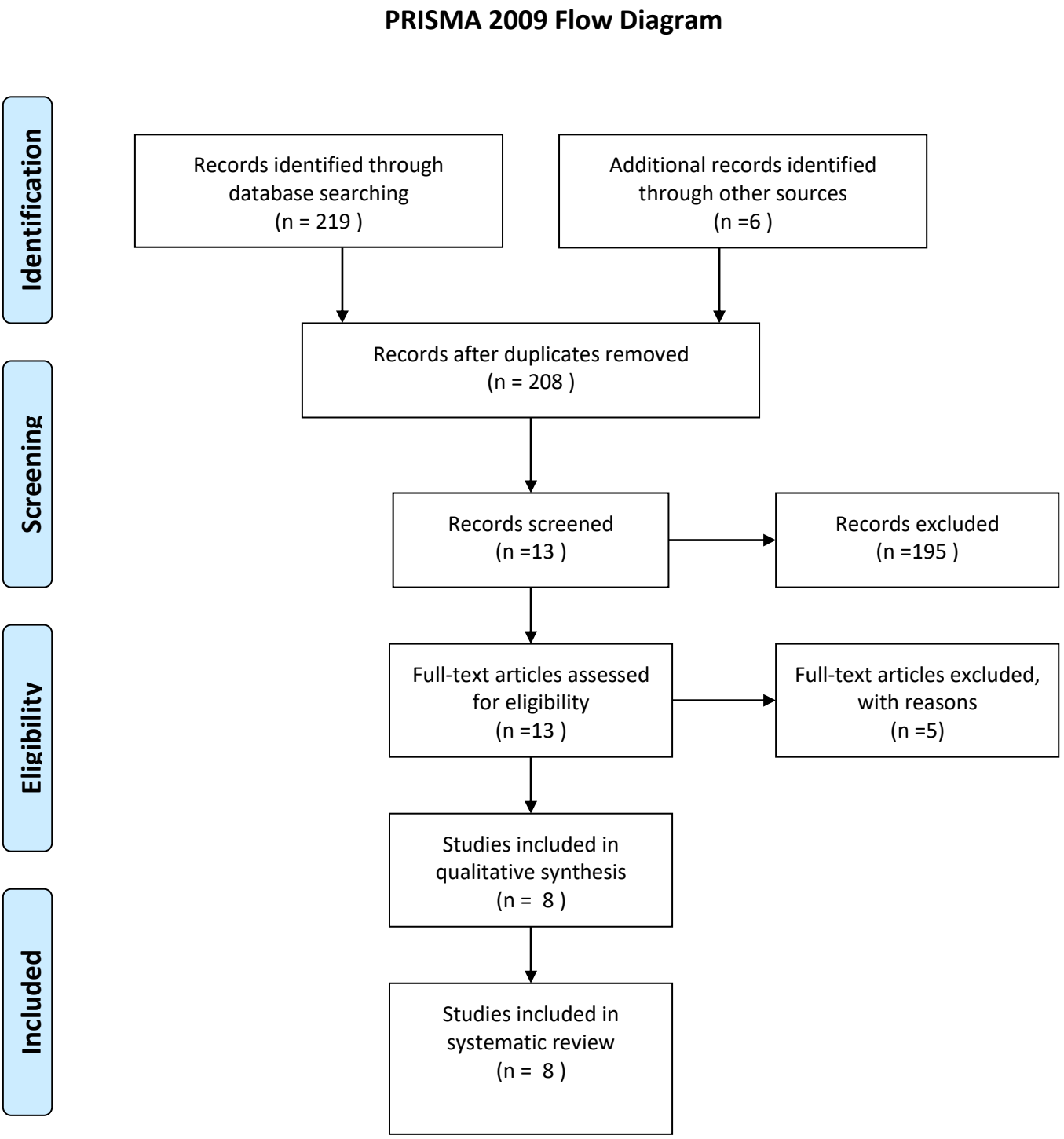

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

Figure 1. Data search and Extraction (PRISMA flow chart)

Table 1 Characteristics of included studies

Reference	*Mycobacterium* Species	Strains profile	Sample	Methods for evaluation	Main findings of PIP	Conclusion
Sharma et al., 2014	*Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294)	Pan-susceptility	BALB/C infection; Culture	Cell proliferation assay; Cytokine estimation by ELISA; MØ nitrite production - Griess. Protective efficacy of PIP in mice infection: 1)FC; 2)VCFUC; 3) RT-qPCR	Modulates the Th1 response, increases lymphoproliferation and NO production by MØ; The combination PIP + RIF showed a 1.4-0.8 log reduction on bacterial load at mice lungs, when compared to RIF alone.	The up-regulation of Th1 immunity by PIP can be synergistically combined with RIF to improve it's therapeutic efficacy in immunocompromised TB patients.
Jin et al., 2011	*Mycobacterium smegmatis* mc2 155 (ATCC 700084)	Pan-susceptility	Culture	EtBr Accumulation and Efflux Assay; MIC determination; Modulation assay.	PIP had a moderate anti-mycobacterial activity compared with the other positive-control EPIs (RES, CHL, VP, CCCP); exhibited a stronger EtBr efflux inhibitory effect in *M. smegmatis*.	PIP was shown to be a potential inhibitor of the intrinsic EP system in mycobacteria.
Sharma et al., 2010	*Mycobaterium tuberculosis* $H_{37}Rv$ (ATCC 27294), RIF-resistant mutant and MDR clinical isolate	Pan-susceptility, RIF resistant and MDR	Clinical isolates; Culture	Checkerboard ; TK; Selection of resistant mutants *in vitro*; Post-antibiotic effect;) RT-qPCR analysis of putative EP protein; DS	PIP reduced the MIC and improves the bactericidal activity of RIF in all bacilli tested; in the presence of RIF, *M. tuberculosis* RIF-R showed a 3.6-fold overexpression of Rv1258c.	PIP plays a significant role in the inhibition of Rv1258c and may be useful in augmenting the antimycobacterial activity of RIF in a clinical setting.
Rukachaisirikul et al., 2002	*Mycobacterium tuberculosis* ($H_{37}Ra$ strain)	Pan-susceptility	Culture	MABA	Chabamide (PIP dimer) exhibited antituberculosis activity agains *M. tuberculosis*.	The novel PIP dimer, named chabamine, isolated from stems of *P. chaba* has antituberculosis activity.
Balakrishnan et al., 2001	*Mycobacterium smegmatis* mc2155	RIF resistant and susceptility	Culture	Viable CFU count; Evaluation of RNA polymerase activity.	The combination of RIF + PIP caused a complete inhibition of growth of *M. smegmatis*. The mix completely abolished the transcriptional activity of RIF-R RNA polymerase.	PIP enhances the binding ability of RIF to RNA polymerase.
Singh et al., 2011	*Mycobacterium tuberculosis* GN/mt-75, *Mycobacterium smegmatis* GN/ms-43	MDR	Clinical isolate	MIC: different dilutions into Müeller-Hinton broth medium and Löwenstein-Jensen .	PIP was more efficient against *M. tuberculosis* than *M. smegmatis*.	PIP has activity against the multidrug resistant strains of *Mycobacterium*, specially *M. tuberculosis*.
Patilaya et al., 2012	*Mycobacterium tuberculosis* $H_{37}Rv$ ATCC 25618	Pan-susceptility	Culture	TEMA; TKC; Scanning Electron Microscopy	Ethylacetate fraction of *P. nigrum* containing PIP reduced the CFU count and caused significant morphological changes during the 7-day period study.	The ethylacetate fraction of *P. nigrum* leaf has potential activiy against *M. tuberculosis*.
Raja et al., 2015	*Mycobacterium tuberculosis*	Resistant to Ofloxacin	Clinical isolates	MABA; Checkerboard;	PIP showed potent anti-mycobacterial activity and EP inhibit activity against *M. tuberculosis*.	The biossay of PIP showed potent anti-TB activity.

MABA - Microplate Alamar Blue Assay; TEMA - Tetrazolium Microplate Assay; ELISA - Enzyme-Linked Immunosorbent Assay; FC - Flow Cytometry; RIF - Rifampicin; PIP - Piperine; VCFUC- Viable Colony-forming Unity Count; EtBr - Ethidium Bromide; Ly - Lymphocyte; NO - Nitric Oxide; MØ- Macrophages; EP - Efflux pump; MIC - Minimum inhibitory concentration; MDR - Multidrug resistant; TB - Tuberculosis; RES - Reserpine; VP - Verapamil; CHL - Chlorpromazine; CCCP - Carbonyl Cyanide m-Chlorophenyl Hydrazone; TK - Time-Kill Curve; DS - Docking Studies; RIF-R: Rifampicin Resistant isolates.

Supplementary file 1: Checklist PRISMA and other systematic review files

+Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3,4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	N/A
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	No registration of protocol
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4,5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4,5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	N/A

Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	N/A
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	N/A
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	N/A
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	N/A
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6 and Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	7-11
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	N/A
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	N/A
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	N/A
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	Table 1
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	11
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	11
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	No funding

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

SUPPLEMENTARY FILE 2: **MESH TERMS**

PubMed Database **Mesh terms definition**	**Web of science Database** **Strategy Ti (title)**
Stage I - General Mesh terms	**Stage I- Search the General terms as Ti (title) strategy**
# Gram-Positive Bacteria; # Mycobacterium; # Tuberculosis; # Piper; # piperine [Supplementary Concept]; # Antitubercular agents; # Antibiotics, Antitubercular; # Anti-BacterialAgents/metabolism*; #Anti-Bacterial Agents/therapeutic use; # Plant oils; # Drug synergism; # Piperidines; # Polyunsaturated alkamides; # benzodioxoles; # Enzyme Inhibitors; # Membrane Transport Proteins; # ATP-Binding Cassette Transporters; # Multidrug Resistance-Associated Proteins; # Cytokines; # Gene Expression Regulation, Bacterial*	Piperine [ti] mycobacterium [ti]; Piperine [ti] mycobacterium; Piperine mycobacterium [ti]; Piperine mycobacterium; Piperine [ti] mycobacterium tuberculosis [ti]; Piperine mycobacterium tuberculosis [ti]; Piperine [ti] mycobacterium tuberculosis; Piperine mycobacterium tuberculosis; Piperidine [ti] mycobacterium [ti]; Piperidine mycobacterium; Piperidine [ti] mycobacterium tuberculosis [ti]; Piperidine mycobacterium tuberculosis [ti]
Stage II- Mesh boolean terms	**Stage II- Ti (Title)**
Group I – *Tuberculosis OR* Mycobacterium; *OR Gram-Positive Bacteria* Grupo II – Piper; # Antitubercular agents; #Piperidines; # Polyunsaturaed alkamides/pharmacology; # Benzodioxoles Grupo III - # ATP-Binding Cassette Transporters; # Multidrug Resistance-Associated Proteins; # Gene expression regulation, bacterial	Piperine [ti] mycobacterium tuberculosis [ti]; Piperidine [ti] mycobacterium tuberculosis [ti]
Stage III - Mesh terms finals	**Stage III- Ti (Title finals)**
Group I – *Tuberculosis [MesH]* OR *Mycobacterium [MesH]* OR *Gram-Positive Bacteria [Mesh]* *AND* Grupo II – *Piper [Mesh]* OR *Antitubercular agents* [Mesh] OR *Piperidines [Mesh]* OR *Polyunsaturaed alkamides/pharmacology [Mesh]* OR *Benzodioxoles [Mesh]* AND Grupo III - *ATP-Binding Cassette Transporters [Mesh]* OR *Multidrug Resistance-Associated Proteins [Mesh]* OR *Gene expression regulation, bacterial [Mesh]*	Group I TI = [*Tuberculosis*] OR TI = [Mycobacterium] OR *TI = [Gram Positive Bacteria]* Grupo II – TI = [*Piper]* OR TI = [*Antitubercular agents*] OR TI = [*Piperidines]* OR TI = [*Polyunsaturaed alkamides/pharmacology]* OR TI = [*Benzodioxoles]* Grupo III - TI = [ATP-Binding Cassette Transporters] OR TI = [Multidrug Resistance-Associated Proteins] OR TI = [Gene expression regulation]
IV - **Busca por termo livre**	

1. Piperine [ti] mycobacterium [ti] 2. Piperine [ti] mycobacterium 3. Piperine mycobacterium [ti] 4. Piperine mycobacterium 5. Piperine [ti] mycobacterium tuberculosis [ti] 6. Piperine mycobacterium tuberculosis [ti] 7. Piperine [ti] mycobacterium tuberculosis 8. Piperine mycobacterium tuberculosis 9. Piperidine [ti] mycobacterium [ti] 10. Piperidine mycobacterium 11. Piperidine [ti] mycobacterium tuberculosis [ti] 12. Piperidine mycobacterium tuberculosis [ti] 13. Piperidine mycobacterium tuberculosis	http://www.ncbi.nlm.nih.gov/sites/myncbi/1BC9ldImHQLAF/collections/50732379/public/
LILACS Database **Busca por Termos Livres**	
"piper/quimica" and "*Mycobacterium*"	

Supplementary file 3: Included studies for the review

Balakrishnan V, Varma S, Chatterji D. Piperine augments transcription inhibitory activity of rifampicin by severalfold in Mycobacterium smegmatis. *Curr Sci* 2001; 80: 1302–5.

Jin G, Zhang J, Guo N, *et al.* The plant alkaloid piperine as a potential inhibitor of ethidium bromide efflux in Mycobacterium smegmatis. *J Med Microbiol* 2011; 60: 223–9.

Patilaya P, Ibrahim P, Ismail Z. Effects of Standardized Fractions of Piper nigrum on the growth of Mycobacterium tuberculosis cells. 2012; 1: 6–12.

Raja A, Abdul Kapur M FM, S MS. In vitro studies on Efflux pump Inhibition of Catharanthus roseus and piperine against ofloxacin resistant M . tuberculosis. 2015; 4: 32–7.

Rukachaisirikul T, Prabpai S, Champung P, Suksamrarn A. Chabamide, a novel piperine dimer from stems of Piper chaba. *Planta Med* 2002; 68: 853–5.

Sharma S, Kumar M, Sharma S, Nargotra A, Koul S, Khan IA. Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of Mycobacterium tuberculosis. *J Antimicrob Chemother* 2010; 65: 1694–701.

Sharma S, Kalia NP, Suden P, *et al.* Protective efficacy of piperine against Mycobacterium tuberculosis. *Tuberculosis* 2014; 94: 389–96

Singh C, Singh SK, Nath G, Rai NP. Anti-mycobacterial activity of Piper longum L. fruit extracts against multi drug resistant Mycobacterium Spp. *Int J Phytomedicine* 2011; 3: 353–61.

Supplementary file 4: Potential Studies Excluded

POTENTIAL STUDIES EXCLUDED

Nº	First Author	Year of study	Reason
1	Abul, A. T.	1998	Not reached goals
2	Ackart, D. F.	2014	Not reached goals
3	Affolabi, D.	2007	Not reached goals
4	Ahmed, A.	2015	Review
5	Alahari, A.	2009	Not reached goals
6	Alland, D.	2000	Not reached goals
7	Amaral, L.	2007	Review
8	Ando, H.	2011	Not reached goals
9	Anurag, M.	2009	Not reached goals
10	Arora, N.	2012	Review
11	Balganesh, M.	2010	Not reached goals
12	Balganesh, M.	2012	Not reached goals
13	Barh, D.	2013	Abstract not available
14	Barrios-Payan, J. A.	2010	Review
15	Bernuci, K. Z.	2016	Abstract not available
16	Betts, J. C.	2003	Not reached goals
17	Bhakta, S.	2016	Not reached goals
18	Bhattacharya, C. P.	1988	Abstract not available
19	Bhatter, Purva D.	2016	Not reached goals
20	Bisson, G. P.	2012	Not reached goals
21	Bolhuis, M. S.	2013	Not reached goals
22	Boshoff, H. I.	2004	Not reached goals
23	Brzostek, A.	2009	Not reached goals
24	Burian, J.	2012	Review
25	Caceres, N. E.	1997	Not reached goals
26	Caleffi-Ferracioli, K. R.	2016	Not reached goals
27	Calgin, M. K.	2013	Not reached goals
28	Campen, R. L.	2015	Not reached goals
29	Carta, A.	2007	Not reached goals
30	Chen, S.	2014	Not meet the purpose of the study
31	Chen, Si	2013	Not reached goals
32	Clarke, S. R.	2001	Not reached goals
33	Clemente-Soto, A. F.	2014	Not reached goals
34	Colangeli, R.	2005	Not reached goals
35	Combourieu, B.	2000	Not reached goals
36	Crowe, A.	2011	Not reached goals
37	De Keijzer, J.	2014	Not reached goals
38	De Knegt, G. J.	2013	Not reached goals

39	De Lima, A. M.	2011	Not reached goals
40	Denkin, S.	2005	Not reached goals
41	Dhar, N.	2010	Not reached goals
42	Diaz, L. E.	2012	Not reached goals
43	Domenech, P.	2001	Not reached goals
44	Dubnau, E.	1996	Not reached goals
45	Dudley, M.Z.	2016	Not reached goals
46	Dutta, N. K.	2011	Review
47	Eldholm, V.	2015	Not reached goals
48	Evangelopoulos, D.	2014	Not reached goals
49	Ferriz, J. M.	2010	Not reached goals
50	Fu, L. M.	2006	Not reached goals
51	Fu, L. M.	2007	Not reached goals
52	Fu, L. M.	2007	Not reached goals
53	Garima, K.	2015	Not reached goals
54	German, N.	2008	Not reached goals
55	Grau, T.	2012	Not reached goals
56	Gu, L.	2015	Not reached goals
57	Gupta, A. K.	2010	Not reached goals
58	Gupta, R. K.	2009	Not reached goals
59	Hao, P.	2011	Not reached goals
60	Hartkoorn, R. C.	2007	Not reached goals
61	Hasan, S.	2014	Not reached goals
62	He, L.	2015	Not reached goals
63	Heym, B.	1997	Not reached goals
64	Hong, W.	2013	Review
65	Hu, J.	2015	Not reached goals
66	Huang, D.	2013	Not reached goals
67	Hussain, K.	2009	Not reached goals
68	Huygens, F.	2005	Not reached goals
69	Jagannathan, V.	2010	Not reached goals
70	Jayaswal, S.	2010	Not reached goals
71	Jeeves, R. E.	2015	Not reached goals
72	Jiang, X.	2008	Not reached goals
73	Kalia, N. P.	2012	Not reached goals
74	Kamal, A.	2005	Not reached goals
75	Karabanovich, G.	2014	Not reached goals
76	Karabanovich, G.	2016	Not reached goals
77	Kaur, P.	2009	Not reached goals
78	Kim, S. H.	2012	Not reached goals
79	Kim, Y. H.	2006	Not reached goals
80	Kishan, J.	2003	Not reached goals
81	Korkegian, A.	2014	Not reached goals
82	Koul, A.	2008	Not reached goals

83	Koul, A.	2014	Not reached goals
84	Kumar, A.	2008	Not reached goals
85	Kurniawan, Y. N.	2014	Not reached goals
86	Kurniawan, Y. N.	2016	Not reached goals
87	Laurentiz, R. S.	2015	Not reached goals
88	Lee, R. E.	2014	Not reached goals
89	Li, G.	2015	Not reached goals
90	Li, H	2016	Not reached goals
91	Li, Y.	2010	Not reached goals
92	Liang J	2012	Not reached goals
93	Liu, F.	2014	Not reached goals
94	Liu, H.	2014	Review
95	Liu, J.	2012	Review
96	Liu, Y.	2016	Not reached goals
97	Lopes, M. A.	2014	Not reached goals
98	Lopez, A.	2001	Not reached goals
99	Machado, D.	2016	Not reached goals
100	Makarov, V.	2009	Not reached goals
101	Malkhed, V.	2011	Not reached goals
102	Malkhed, V.	2014	Not reached goals
103	Mata, Rachel	2004	Not meet the purpose of the study
104	McIlleron, H.	2007	Review
105	McLean, K. J.	2008	Review
106	Meena, L. S.	2015	Review
107	Mehra, R.	2016	Not reached goals
108	Mehra, S.	2010	Not reached goals
109	Mohamad, Suriyati	2011	Not reached goals
110	Mokaddas, E.	2015	Not reached goals
111	Mullin, S.	2004	Not reached goals
112	Murunikkara, V.	2012	Not reached goals
113	Nargotra, A.	2009	Not reached goals
114	Newell, K. V.	2006	Not reached goals
115	Ngaimisi, E.	2011	Not reached goals
116	Niki, M.	2012	Not reached goals
117	Nixon, M. R.	2014	Not reached goals
118	Nosova, E. Y.	2013	Not reached goals
119	Novakova, R.	2010	Not reached goals
120	O'Sullivan, D. M.	2008	Not reached goals
121	Olaleye, O.	2010	Not reached goals
122	Ollinger, J.	2013	Not reached goals
123	Pandey, R.	2012	Not reached goals
124	Pardieu, C.	2015	Not reached goals
125	Park, Y. K.	2012	Not reached goals
126	Pinault, L.	2013	Not reached goals

127	Podder, B.	2015	Not reached goals
128	Poupin, P.	1999	Not reached goals
129	Poupin, P.	1999	Not reached goals
130	Puniya, B. L.	2013	Not reached goals
131	Pym, A. S.	2001	Not reached goals
132	Rachmawaty, F. J.	2015	Abstract not available
133	Radmacher, E.	2005	Not reached goals
134	Ramon-Garcia, S.	2009	Not reached goals
135	Rand, L.	2009	Not reached goals
136	Rao, M.	2012	Not reached goals
137	Ravishankar, S.	2015	Not reached goals
138	Remuinan, M. J.	2013	Not meet the purpose of the study
139	Rienksma, R. A.	2014	Review
140	Ritacco, F. V.	2005	Not reached goals
141	Rodriguez-Castillo, J. A.	2015	Not reached goals
142	Rüegg, T.	2006	Not reached goals
143	Rukachaisirikul, T.	2004	Not reached goals
144	Rukachaisirikul, Thitima	2004	Not reached goals
145	Russo, F.	2015	Not reached goals
146	Ryndak, M.	2008	Review
147	Saikolappan, S.	2015	Not reached goals
148	Sandbhor, U.	2002	Not reached goals
149	Sangwan, P. L.	2008	Not reached goals
150	Sarathy, J.	2013	Not reached goals
151	Sayahi, H.	2008	Abstract not available
152	Schmalstieg, A. M.	2012	Not reached goals
153	Schneider, C. Z.	2008	Not reached goals
154	Scodro, R. B. D.	2015	Not reached goals
155	Scodro, R. B. L.	2012	Not reached goals
156	Scodro, R. B. L.	2013	Not reached goals
157	Scodro, R. B. L.	2013	Not reached goals
158	Seagar, A. L.	2012	Not reached goals
159	Sharma, K.	2006	Not reached goals
160	Sherman, D. R.	1996	Not reached goals
161	Sholto-Douglas-Vernon, C.	2005	Not reached goals
162	Singh, A. K.	2009	Not reached goals
163	Singh, V. K.	2006	Not reached goals
164	Smith, C. V.	2003	Review
165	Song, N.	2016	Not reached goals
166	Sun, A. H.	2009	Not reached goals
167	Sun, G.	2012	Not reached goals
168	Szumowski, J. D.	2013	Not reached goals
169	Tekwu, E. M.	2012	Not reached goals

170	Telenti, A.	1997	Not reached goals
171	Tudo, G.	2010	Not reached goals
172	Tuntiwachwuttikul, P.	2006	Not reached goals
173	Tyagi, J. S.	2002	Review
174	Tyagi, P.	2015	Not reached goals
175	Uttayamakul, S.	2012	Not reached goals
176	Valle, D. L.	2016	Not reached goals
177	Vasconcelos, I. B.	2010	Not reached goals
178	Verma, V. C.	2011	Not reached goals
179	Vilcheze, C.	2005	Not reached goals
180	Waddell, S. J.	2004	Not reached goals
181	Wakamoto, Y.	2013	Not reached goals
182	Wallis, R. S.	2013	Review
183	Walter, N. D.	2015	Not reached goals
184	Wang, C.	2013	Not reached goals
185	Wang, K.	2013	Not reached goals
186	Wanigasekera, A.	2001	Not reached goals
187	Wei, J.	2014	Not reached goals
188	Weiner, M.	2010	Not reached goals
189	Wilson, M.	1999	Not reached goals
190	Wilson, T.	1998	Not reached goals
191	Wolfe, L. M.	2013	Not reached goals
192	Worley, M. V.	2014	Review
193	Yang, M.	2015	Not reached goals
194	Yu, G.	2015	Not reached goals
195	Zhao, Q. J.	2011	Review

Artigo 2: "*In vitro* Combinatory Activity of Piperine and Anti-Tuberculosis Drugs in *Mycobacterium tuberculosis*"

IN VITRO COMBINATORY ACTIVITY 0F PIPERINE AND ANTI-TUBERCULOSIS DRUGS IN *Mycobacterium tuberculosis*

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Abstract

Tuberculosis (TB) is an important public health problem worldwide and the emergence of multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB worsened the global context. The resistance in *Mycobacterium tuberculosis*, the causative agent of TB, can partially derive from efflux pumps (EPs) activity in plasma membrane. Due to the recent discovery of piperine (PIP), an organic alkaloid compound, increasing the bioavailability of various drugs, the current assay evaluated the combined activity of PIP and anti-TB drugs in susceptible and resistant *M. tuberculosis* clinical isolates. The minimum inhibitory concentrations for isoniazid, rifampicin, ethambutol, streptomycin and PIP were determined by resazurin microtiter assay and the combined effects of anti-TB drugs with PIP determined by resazurin drug combination microtiter assay and time-kill curve. The efflux pump inhibitor activity of PIP was determined by bromide accumulation assay and cytotoxicity carried out in VERO cells and J774.A1 macrophages. PIP showed to have EPI activity and RIF + PIP and SM + PIP combinations showed synergistic effect, but low effect in enhancing the killing in *M. tuberculosis* H_{37}Rv and in the clinical isolates studied, which had different resistance profiles. Future studies are needed to further clarify the importance of PIP as an adjunctive drug in the therapy against TB.

Keywords: Tuberculosis, Efflux Pump, Inhibitor Efflux Pump, Piperine, Rifampicin, Streptomycin, Synergism.

1. Introduction

Tuberculosis (TB) is an infectious disease caused mainly by *Mycobacterium tuberculosis*. It is the ninth leading cause of death worldwide. From 2012 to 2016 it has been the leading cause of death from a single infectious agent, ranking above human immunodeficiency virus/acquired immunodeficiency syndrome [1] .

Although effective, the treatment for TB is long and anti-TB drugs can cause substantial side effects. These factors increase the problem of adherence to chemotherapy and patients frequently abandon the treatment. This, consequently, opens up the possibility for secondary resistance to anti-TB drugs and the spread of resistant bacilli, which in turn leads to primary resistance (i.e., the presence of resistant *M. tuberculosis* in patients with no history of prior treatment) [2,3] .

The emergence of multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB are worrisome in the global context. According to the World Health Organization, an estimated 4.1% of new cases and 19% of previously treated cases globally were MDR in 2016. In the same year, XDR-TB was reported by 123 member countries of the World Health Organization [1].

Resistance to anti-TB drugs has been mainly attributed to the presence of spontaneous mutations in the *M. tuberculosis* genome. However, in a number of clinical isolates, the condition of resistance could not be explained by the presence of the known mutations in specific genes, which occurs in 30% of isoniazid (INH)-resistant and 5% of rifampicin (RIF)-resistant isolates, suggesting other mechanisms [3,4], such as efflux pumps (EPs) activities [5–8].

Efflux pumps are membrane-associated active transporters that promote the extrusion of toxic compounds, including antibiotics, from the cells. The activities of EPs can confer natural resistance to one or more drugs in mycobacteria [9] . Bacterial EPs are distributed in 5 families, two of which are the main ones, since they are found in large numbers and more ancestors: "ATP-binding cassette superfamily" (ABC) and "Major Facilitator Superfamily" (MFS). The other three families are smaller and of more recent development: Small Multidrug Resistance (SMR), Resistance-Nodulation-cell Division family (RND) and Multidrug and Toxic Compounds Extrusion Family (MATE) [9]. Recently, EPs have been recognized as an important component of the challenges in TB treatment, and the administration of inhibitors of EPs, in combination with available anti-TB drugs, may be an effective strategy while there is no development of new drugs [10]. Such compounds would have the ability to block bacterial EPs and, thus, prevent the lowering of drugs concentrations inside the bacteria

[11,12]. Such compounds as verapamil (VP), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), reserpine, valinomycin, chlorpromazine, thioridazine and piperine (PIP) were shown to act in EPs, *in vitro* and *in vivo*, causing decrease in the minimum inhibitory concentrations (MICs) of some antimycobacterial drugs when combined with these compounds and shortening treatment time and adverse reactions [10–17], respectively.

Piperine is an organic alkaloid compound that is found in *Piper nigrum* and *Piper longum*. It has been reported to have antiinflammatory [14,15], antimicrobial [16,17], antifungal [18], analgesic, antipyretic, antioxidant [19] and anticancer [20] effects and can enhance the bioavailability of some drugs [13,21]. It was also reported to inhibit the NorA EP in *Staphylococcus aureus* [22,23] and *Rv1258c* EP in *M. tuberculosis* [13]. Recently, the Indian Institute of Integrative Medicine (Jammu), marketed in India in November 2009 in a public-private partnership with Cadila Pharmaceutical Ltd (Ahmedabad), developed a formulation that contained a fixed dose combination of RIF (200 mg), INH (300 mg) and PIP (10 mg) named Risorine. A clinical trial in human was undergone with the combination Risorine plus ethambutol and pirazinamide for treatment of drug-susceptible pulmonary TB [24]. According to this study, the use of Risorine showed to be highly effective and well tolerated in the treatment of patients with drug susceptible pulmonary TB, reducing the time of treatment and the adverse effects of the current treatment. To our knowledge, few studies *in vitro* have reported the synergistic activity of PIP with classical anti-TB drugs in *M. tuberculosis* clinical isolates and, even though human clinical trials using low dose RIF and INH have been conducted, this subject deserves more attention. Thus, the aim of the present study was to evaluate, *in vitro*, the combined activity of PIP and anti-TB drugs in *M. tuberculosis* H_{37}Rv, a reference strain, and clinical isolates with different resistance profiles.

2. Materials and methods

2.1. Bacterial culture

M. tuberculosis H_{37}Rv (ATCC 27294) and nine clinical isolates with different resistance profiles (Table 1) were obtained from the bacteriology laboratory of the State University of Maringá. Bacilli were cultured at 35°C for 15 days in Middlebrook 7H9 (Difco Laboratories, Detroit, MI, USA) supplemented with 10% (v/v) oleic acid-catalase-albumin-dextrose enrichment (OADC; BBL/Becton Dickinson, Sparks, MD, USA), to which 0.2% glycerol (v/v) and 0.025% (v/v) Tween 80 were added.

2.2. Anti-TB drugs and efflux pump inhibitor

All of the drugs were obtained from Sigma (St. Louis, MO, USA). Streptomycin (SM), ethambutol (EMB) and INH were prepared in distilled water. Piperine was prepared in dimethylsulfoxide (DMSO; Synth, Diadema, SP, Brazil) and RIF in methanol:water (1:10, v/v). Further dilutions were prepared in OADC-supplemented Middlebrook 7H9 at the following concentration ranges: SM (0.01-4 µg/ml), EMB (0.06-32 µg/ml), INH (0.002-50 µg/ml), RIF (0.00012-200 µg/ml) and PIP (7.812-500 µg/ml). The final DMSO and methanol concentrations had no effect on *M. tuberculosis* growth.

2.3. Minimum inhibitory concentration and checkerboard assay

The MICs of anti-TB drugs and PIP was determined by the resazurin microtiter assay (REMA) as described by Palomino et al. [25]. MIC was defined as the lowest drug concentration that was able to inhibit the visual color change of 0.01% resazurin (Acros, Morris Plains, NJ, USA) from blue to pink.

The combined activity of the anti-TB drugs (INH, EMB, RIF or SM) and PIP was determined by the resazurin drug combination microtiter assay (REDCA) [26] relative to the resistance profile of each clinical isolate. The fractional inhibitory concentration (FIC) was determined using the criteria that were established by Pillai et al. [27] and adapted by Coelho et al. [6]. However, we only evaluated the individual FICs for the antibiotics and not the sum of the FICs; therefore, FIC index ("FIC antibiotic") was calculated as the quotient between the MIC of the drug combination and the MIC of the antibiotic alone. The results were interpreted as the following: FIC $\leq$ 0.25 (synergism), FIC > 0.25 < 2 (indifference) and FIC > 2 (antagonism). Thus, an individual FIC $\leq$ 0.25, indicative of a four-fold reduction, was assumed as synergy [6]. We considered FIC > 0.25 as indifferent activity due to the inherent variability of the method.

2.4. Time-kill curve assay

The time-kill curve assay was performed for the *M. tuberculosis* reference strain $H_{37}Rv$ and two clinical isolates (7-SM monoresistant and 71A-MDR). The isolates and drug combinations were chosen based on the checkerboard results. The bacilli were first grown in OADC-supplemented Middlebrook 7H9 to 1 McFarland standard turbidity (3×10^8 colony-forming units [CFU]/ml) for 15 days at 35°C [28]. The cell suspensions were adjusted to a final concentration of ~$5x10^5$ CFU/mL in OADC-supplemented Middlebrook 7H9. Individual drugs (RIF, SM and PIP) and drug combinations (PIP+RIF and PIP+SM) at 0.5×MIC values

were added to the mycobacterial culture and incubated at 35°C with shaking at 96 rotations per minute for 10 days [29]. Aliquots (0.1 ml) were removed on the initial day of the experiment and then on the first, third, fifth, seventh, and tenth days of incubation and serially diluted (10^{-1}, 10^{-3}, and 10^{-5}) in OADC-supplemented Middlebrook 7H9 to avoid drug carryover. An aliquot (15 µl) of each dilution was plated on OADC-supplemented Middlebrook 7H11 [28]. The plates were incubated at 35°C for 21 days, and the colonies were counted. The time-kill curve assays were performed three times in independent experiments. The results are expressed as the mean of the three independent experiments. Synergism was defined as a ≥ 2 $\log_{10}$ reduction of CFU/ml for the drug combination relative to the single agent. A CFU/ml reduction between 2 $\log_{10}$ and 1 $\log_{10}$ was considered additive [30].

2.5. Efflux assay: ethidium bromide accumulation

The ethidium bromide (EtBr) accumulation assay was performed in *M. tuberculosis* H_{37}Rv, previously cultured in Middlebrook 7H9-OADC. The culture was rinsed with phosphate-buffered saline (PBS; pH 7.4) and the optical density at 600 nm (OD_{600}) was adjusted to 0.4. Afterwards, 0.25 µg/ml EtBr (0.5×MIC) plus 62.5 µg/ml PIP (0.5×MIC) were added to the bacterial suspension. Verapamil was used as the EPI control in all of the assays. Fluorescence relative to EtBr-loaded cells was determined in a VICTOR2 D fluorometer (PerkinElmer, Santa Clara, CA, USA), with 530/25 as the excitation wavelengths and 590/20 nm as the detection wavelengths. The relative fluorescence values were normalized to the EtBr fluorescence background. The relative final fluorescence (RFF) was determined using the following formula: $(RF_{assay} - RF_{ref}) / RF_{ref}$, where RF_{assay} is relative fluorescence at the last time point (minute 60) of the EtBr accumulation assay with EPIs, and RF_{ref} is the relative fluorescence at the last time point of the EtBr accumulation assay without EPIs [6,31].

2.6. Cytotoxicity assay

For the cytotoxicity assay, VERO epithelial cells (ATCC CCL81) and J774.A1 macrophages were cultured in Dulbecco's modified essential medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) and RPMI-1640 medium, respectively, both supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Life Technologies, NY, USA). The VERO cells were detached using 0.05% trypsin solution (Gibco, Life Technologies, Canada) and macrophages were detached with a cell scraper. The cell concentrations were then adjusted to 5×10^5 cells/ml and the plates were incubated for 24 h to allow cell adhesion. Piperine was

first dissolved in DMSO and then subjected to two-fold serial dilution from 250 to 3.12 µg/ml in culture medium. Afterwards, 100 µl of PIP were added to 96-well culture plates, and the plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. After 24 h, the plates were washed twice with PBS, and 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) was added [32] and left for 4 h of incubation while protected from light. Then, 150 µl of DMSO were added, and absorbance was read at 550 nm using a spectrophotometer plate reader (ASYS Expert Plus, Asys Hitech GMBH, Eugendorf, Austria). The cellular conversion of MTT into a formazan product was used to evaluate the cytotoxicity (IC_{50}) of PIP against VERO cells and macrophages. IC_{50} value was defined as the concentration of PIP that was required to inhibit 50% of cell proliferation in relation to the control. The selectivity index (SI) was calculated as IC_{50} / PIP MIC [33].

3. Results

The MICs of PIP, RIF, INH, EMB and SM against *M. tuberculosis* H_{37}Rv were 125, 0.004, 0.06, 2 and 0.25 µg/ml, respectively. For the *M. tuberculosis* clinical isolates, the MICs of PIP, RIF, INH, EMB and SM ranged from 31.2 to 125 µg/ml, 0.0039 to 50 µg/ml, 0.3 to 12.5 µg/ml, 8 to 16 µg/ml and 0.25 to 1 µg/ml, respectively (Table 2).

The FICs values obtained for PIP + anti-TB drug combinations are shown in Table 2. Synergism was observed in 5/10 (50%) and 7/10 (70%) of the clinical isolates (FIC $\leq$ 0.25), including *M. tuberculosis* H_{37}Rv for PIP+RIF and PIP+SM, respectively.

The time-kill curve assay was performed in *M. tuberculosis* H_{37}Rv to determine the bactericidal effect of the RIF (0.002 µg/ml) + PIP (62.5 µg/ml) and SM (0.125 µg/ml) +PIP (62.5 µg/ml) combinations, compared to RIF and SM alone. At RIF 0.5×MIC, the CFU failed to reach a ≥ 2 $\log_{10}$ decrease during the 10-day study period (Fig. 1), which was expected due to the RIF subdosage used in the study. The RIF+PIP combination at 0.5×MIC produced a $\leq$ 1.5 $\log_{10}$ CFU/ml decrease compared to RIF alone. When *M. tuberculosis* H_{37}Rv was exposed to the SM+PIP combination, a 2 $\log_{10}$ CFU/ml decrease was observed during the 10-day study period (Fig. 1).

The time-kill curve assays that were performed with *M. tuberculosis* 7 [SM (0.5 µg/ml) + PIP (62.5 µg/ml)] and *M. tuberculosis* 71A [RIF (25 µg/ml) + PIP (62.5 µg/ml)] clinical isolates indicated decrease of 1.5 $\log_{10}$ CFU/ml and no significant decrease relative to the drug alone, respectively (Fig. 2a, b).

The 0.25 μg/ml concentration (0.5×MIC) of EtBr did not affect *M. tuberculosis* viability, which presented influx-efflux equilibrium. EtBr efflux was inhibited by PIP, with RFF of 0.46 for PIP and 0.43 for VP (i.e., the control drug that was used in the assay; Fig. 3).

In the cytotoxicity assay, PIP had an IC_{50} of 183.33 μg/ml (SI = 1.46) and 18.5 μg/ml (SI = 0.14) for VERO cells and J774.A1 macrophages, respectively.

4. Discussion

The recent use of PIP as bioenhancer in a formulation containing reduced concentration of RIF and INH for the treatment of drug-susceptible pulmonary TB, which was effective and achieved good clinical cure rates, reducing the time of treatment and the adverse effects of the current treatment [24], deserves attention in bringing additional light on PIP activity, including the combination of PIP with old and classical anti-TB drugs in *M. tuberculosis*.

Our *in vitro* results showed PIP exerts potentiating effects on the activity of RIF and SM, but not on INH and EMB against *M. tuberculosis* H_{37}Rv and *M. tuberculosis* clinical isolates. As the clinical isolates used in our study had a marked resistance profile by the presence of mutations in specific genes that are known to cause resistance to the classical anti-TB drugs, PIP did not reverse their resistance phenotype, just lessened it to some extent. The observed effect of PIP was independent of the presence of mutations in specific genes in *M. tuberculosis* clinical isolates. The synergism between PIP and RIF, observed by checkerboard assay REDCA in the present study, corroborates with Sharma et al. [13] findings, who also observed synergism, by broth checkerboard method in microtiter plates, with this combination against *M. tuberculosis* H_{37}Rv, RIF-resistant *M. tuberculosis* laboratory mutants and MDR *M. tuberculosis* clinical isolates.

The present study also provides additional novel findings, in which synergism between PIP and SM was observed in the *M. tuberculosis* reference strain, being observed effect was similar in susceptible and MDR clinical isolates. The aminoglycoside SM was first used to treat TB in 1946. However, resistance to SM was observed as soon as 1948. *M. tuberculosis* was found to develop resistance to SM because of its sole usage for TB treatment. The synergistic effect between PIP and SM that was observed in the present study raises the possibility that SM added of PIP could be, in some cases, useful as treatment for resistant TB. This draws attention to further studies of combinations of available drugs, including those that

are no longer used for the treatment of TB (e.g., SM), which may be a quick and economical strategy for helping patients who are infected with drug-resistant TB.

In *M. tuberculosis* $H_{37}Rv$, the time-kill curve assay performed with SM + PIP study (0.125 μg/ml SM and 62.5 μg/ml PIP equivalent to 0.5×MIC) showed that this combination is reflective of enhanced bacilli killing, compared to SM assay, according to the criteria of Bhusal et al. [30] (≥ 2 $\log_{10}$ CFU/ml decrease). The time-kill curve assay performed with RIF + PIP study (0.002 μg/ml RIF and 62.5 μg/ml PIP equivalent to 0.5×MIC) showed no enhanced bacilli killing effect (≤ 1.5 $\log_{10}$ CFU/ml decrease) compared with RIF alone at 10 days of exposure. Sharma et al. [13]observed a 3 $\log_{10}$ CFU/ml decrease for the RIF + PIP combination at 8 days of exposure. However, the above authors used PIP and RIF at higher concentrations (0.5 mg/l RIF and 25 mg/l PIP) than those used in the present study (0.002 μg/ml RIF, 0.125 μg/ml SM and 62.5 μg/ml PIP equivalent to 0.5×MIC). The comparison between our results and Sharma et al. suggests that the effects of the PIP + RIF combination appear to be concentration dependent.

The time-kill curve assays for clinical isolate 7, which was SM-monoresistant and for the MDR 71A showed the SM (0.5 μg/ml) + PIP (62.5 μg/ml) and RIF (25 μg/ml) + PIP (62.5 μg/ml) combinations, respectively, did not enhance bacilli killing. The observed effect can be due to the continued suppression of bacilli growth under sub inhibitory drugs concentrations used in our study.

The EtBr accumulation assay showed PIP could have potential activity inhibiting intrinsic EP system in *M. tuberculosis*. The RFFs for PIP were similar to VP, which is currently the most promising inhibitor of EPs in *M. tuberculosis* [6,12,13,23,28]. As PIP showed to have comparable EPI activity as VP, its effect could also arise from indirect membrane effects, such as it is observed with VP [34], promoting improved anti TB drugs uptake.

To further evaluate the potential of PIP as a possible adjunct therapeutic agent to combat TB, we performed *in vitro* cytotoxicity assays in VERO epithelial cells and J774.A1 macrophages. PIP showed to be more selective for *M. tuberculosis* than for the infected cell (SI = 1.46 and 0.14 for VERO cells and macrophages, respectively). Initial reports raised questions about the safety of PIP as a food additive, but such evidence was controversial. Subsequent studies established the safety of black pepper or its active constituent, piperine, in several animal studies [15,35].

Overall, PIP could be promising as a possible adjunctive drug for the treatment of TB. Therapeutic strategies that employ anti-TB drugs in combination with a suitable EPI could have some advantages over conventional therapy for special cases of resistance. In addition, it could be used to seek a stronger activity or safer effect when anti-TB drugs are co-administered with PIP.

5. Conclusion

The results confirmed the EPI activity of PIP and its modulatory effect on the activity of RIF and SM. RIF + PIP and SM + PIP combinations showed synergism effect, but low effect in enhancing the killing in *M. tuberculosis* $H_{37}Rv$ and in the clinical isolates studied with different resistance profiles. Future studies are needed to further clarify the importance of PIP as an adjunctive drug in the therapy against TB.

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Transparency declarations section

None to declare.

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Table 1. Susceptibility profile and genotypic characterization of *Mycobacterium tuberculosis* clinical isolates.

Strain/ Isolates	12/24 Loci MIRU	SPOLIGOTYPING	Susceptibility Profile	Mutations		
				rpoB	***KatG***	***inhA***
$H_{37}Rv$	22322513333245622242 35	777777377750771	S	-	-	-
47s	123323132321	777777777760771	S	-	-	-
1193	NP	NP	S	-	-	-
7	125326153222224274434132	747777507450771	SM^R	-	-	-
45	224326133323223463525211	776177400000171	INH^R/RIF^R	Ser 531 Leu	Ser 315 Thr	-
64 A	124325163322	677737607760771	INH^R/RIF^R	Ser 531 Leu	-	-
109	224326153325	776177607760771	INH^R/RIF^R	-	Ala 109 Val	-
3614	224225163321	677737607760771	$INH^R/RIF^R/EMB^R$	Ser 531 Leu	-	C- to T at -15 Ile 21 Thr
71A	225313153323	777777777720771	$INH^R/RIF^R/EMB^R$	Ser 531 Leu	Ser 315 Thr	-
19	224327153324	776177607760771	$INH^R/RIF^R/EMB^R$	Ser 531 Leu	Ser 315 Thr	-

NP: not performed, - no mutations, RIF: rifampicin, INH: isoniazid, EMB: ethambutol, SM: streptomycin, ^{*R}: resistance, Ser: serine, Leu: leucine, Ala: alanine, Thr: threonine, C: Cytosine, T: Thymine, (-) unrealized.

Table 2. Minimum inhibitory concentration (MIC) and drug combination activity of Rifampicin (RIF), Streptomycin (SM), Isoniazid (INH) and ethambutol (EMB) with Piperine (PIP) in *M. tuberculosis* $H_{37}Rv$ and clinical isolates.

Strain/ Isolates	Susceptibility Profile	MIC (µg/mL)					FIC			
		PIP	RIF	SM	INH	EMB	RIF/PIP	SM/PIP	INH/PIP	EMB/PIP
H37Rv	S	125	0.004	0.25	0.06	2	**0.25**	**0.24**		
47S	S	62.5	0.004	0.25	-	-	0,5	**0.24**	-	-
1193	S	125	0.003	0.25	-	-	**0.23**	**0.12**	-	-
7	SM^R	125	0.004	1	-	-	0.5	**0.25**	-	-
45	INH^R/RIF^R	31.2	31.2	0.25	4	-	1	0.48	1	-
64[a]	INH^R/RIF^R	31.2	50	0.5	12.5	-	0.5	**0.24**	1	-
109	INH^R/RIF^R	31.2	50	0.25	0.36	-	**0.25**	**0.24**	0.5	-
71[a]	$INH^R/RIF^R/EMB^R$	125	50	1	12.5	8	**0.12**	**0.25**	1	1
3614	$INH^R/RIF^R/EMB^R$	62.5	25	0.5	6.25	16	**0.12**	**0.25**	1	1
19	$INH^R/RIF^R/EMB^R$	31.2	50	1	3	8	**0.12**	0.5	1	1

FIC: Fractional inhibitory concentration, (-) unrealized.

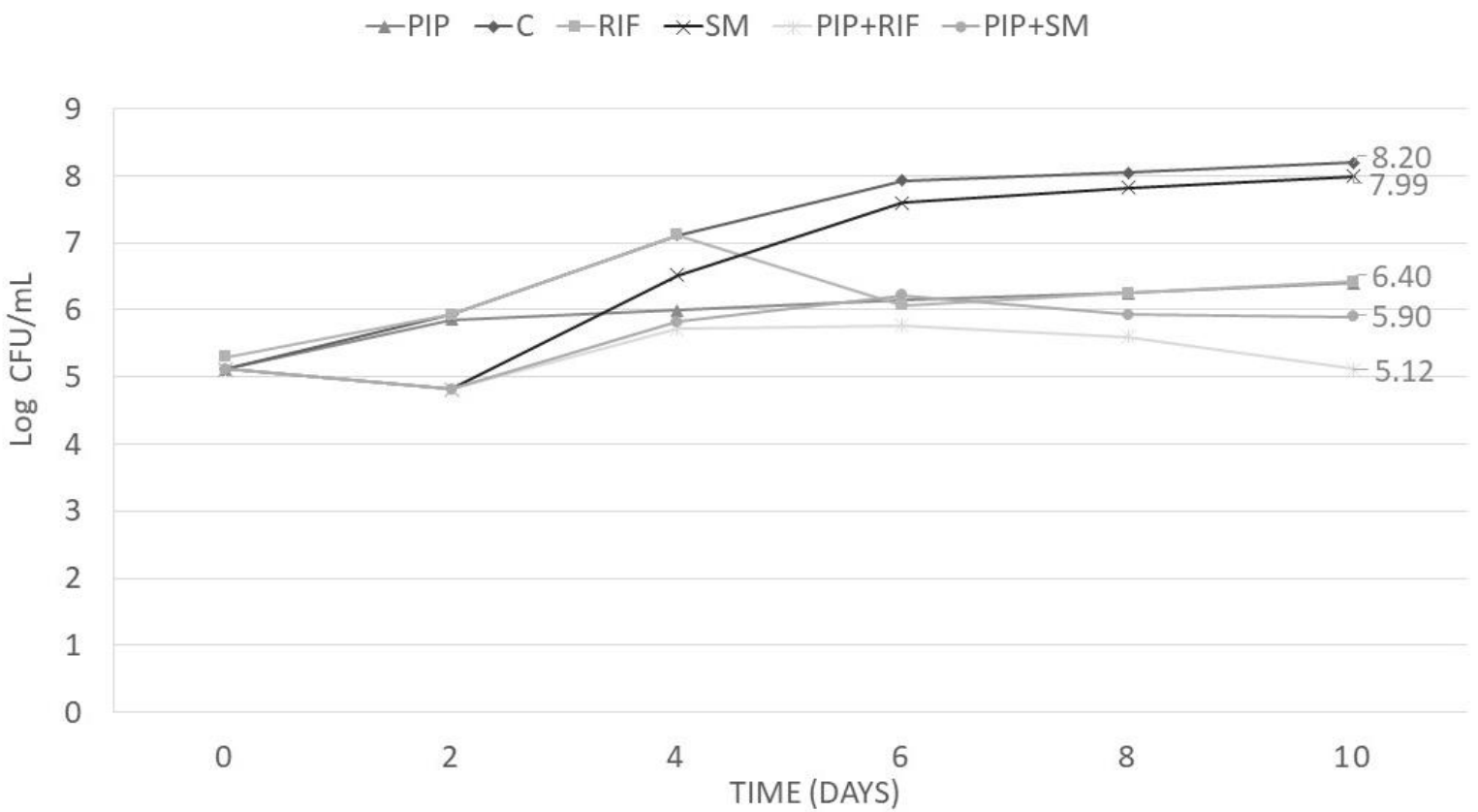

Fig. 1. Time kill curve of *Mycobacterium tuberculosis* using 0.5 x MIC of Rifampicin (RIF) and Streptomicin (SM) and Piperine (PIP) alone and in combinations (RIF+PIP) (SM+PIP) for 10 days. Each data point (days 0, 2, 4, 6, 8 and 10) represents the mean number of viable bacterial cell counts in duplicate experiments. CTL; Control of bacterial growth in the absence of drugs. C; Control of bacterial growth in the absence of drugs.

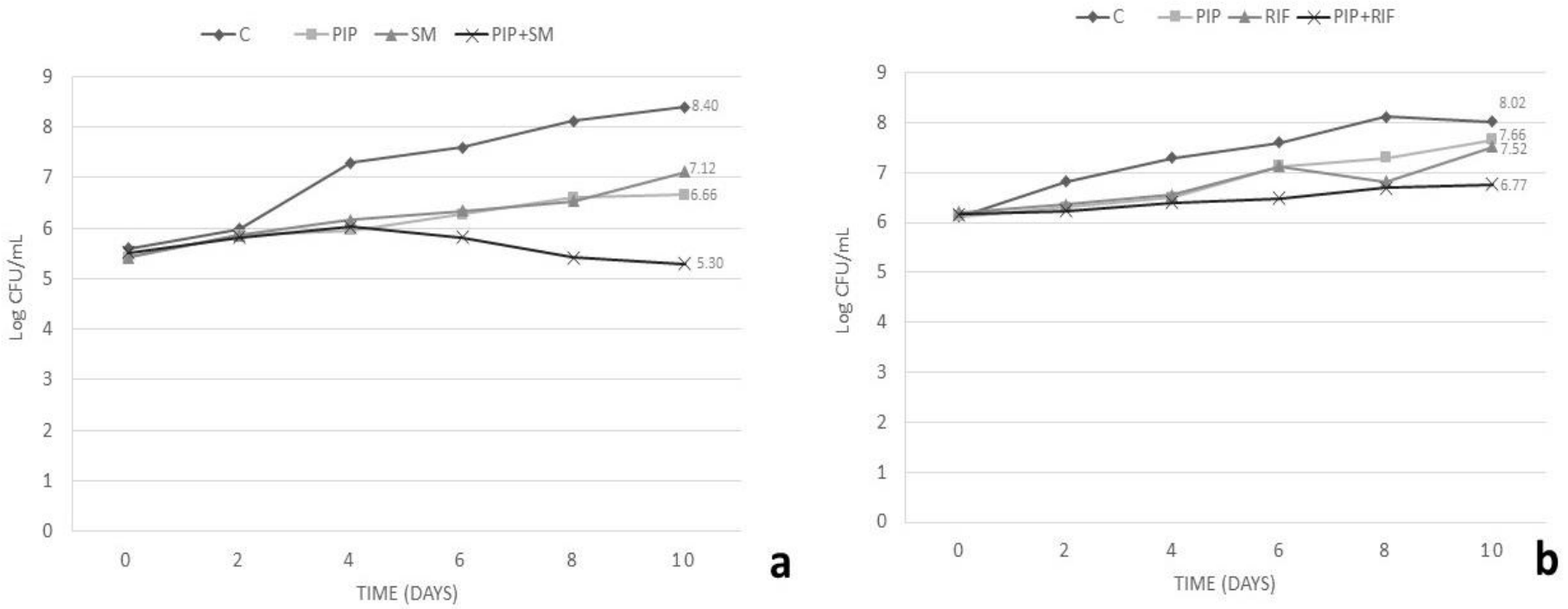

Fig. 2. Time-kill curve results. **a**: *Mycobacterium tuberculosis* clinical isolate 7, using 0.5 x MIC Streptomicin (SM) and Piperine (PIP) alone and in combinations (SM+PIP) for 10 days. **b**: *Mycobacterium tuberculosis* clinical isolate 71A, using 0.5 x MIC Rifampicin (RIF) and Piperine (PIP) alone and in combinations (RIF+PIP) for 10 days. Each data point (days 0, 2, 4, 6, 8 and 10) represents the mean number of viable bacterial cell counts in duplicate experiments. C; Control of bacterial growth in the absence of drugs.

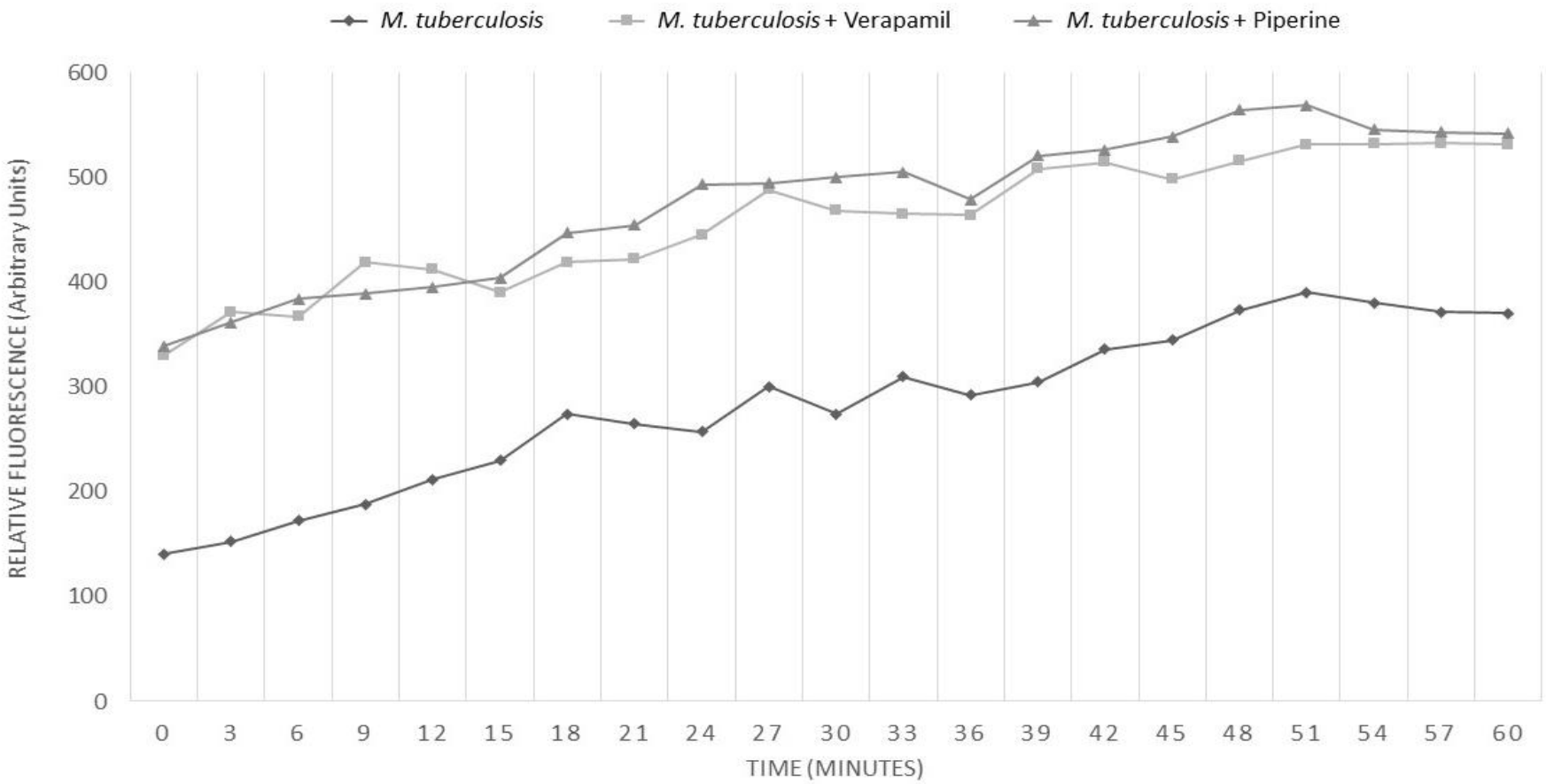

Fig. 3. Accumulation of ethidium bromide (EtBr) by *Mycobacterium tuberculosis* H_{37}Rv by fluorometry. The assays were conducted with or without an efflux pump inhibitor (EPI). Relative fluorescence was obtained by normalizing the data to the background fluorescence of EtBr. The efflux of EtBr was inhibited by verapamil (VP) and Piperine at 0.5 x MIC. Relative final fluorescence (RFF) was calculated for each EPI.

Manuscrito 3:"Piperine activity on efflux pumps and morphology of *Mycobacterium tuberculosis*"

Piperine activity on efflux pumps and morphology of *Mycobacterium tuberculosis*

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Short Running Title: Effect of piperine on *Mycobacterium tuberculosis*

Abstract

Mycobacterium tuberculosis, the causative agent of tuberculosis can presents resistance to anti-tuberculosis drugs as result of spontaneous gene mutations. Otherwise, drug resistance could not be explained by the presence of known mutations. Recently, efflux pumps (EPs) were recognized to be part of the resistance mechanism and compounds able to inhibit EPs could increase the efficacy of anti-tuberculosis drugs. The aim of this study was to evaluate how piperine alone or in combination with rifampicin or streptomycin work on on the regulation of EPs genes and on the cell morphology of *M. tuberculosis* H_{37}Rv. Piperine, rifampicin and streptomycin minimum inhibitory concentration was determined by Resazurin Microtiter Assay and drugs combination by Resazurin Drugs Combination Microtiter Assay, respectively. EPs regulation analysis were performed through RT-qPCR in eleven EPs genes. Scanning electron microscopy allowed us to obtain bacilli images for the evaluation of morphological changes. Piperine potentiated the activity of rifampicin and streptomycin against *M. tuberculosis* and significant difference in the EPs regulation, compared to control ($p \leq 0.01$), were observed in six EPs genes for PIP, three for rifampicin and eleven for streptomycin exposure. PIP was able to down-regulate three EPs genes when combined to rifampicin and all EPs genes tested in the bacilli exposed to the streptomycin combined with PIP. Scanning electron microscopy revealed bacilli with preserved cell structure but involved by a substance with cell disintegration aspect after exposure to piperine alone or combined to rifampicin or streptomycin. Further studies, including greater number of *M. tuberculosis* clinical isolates harboring different resistance patterns, are necessary to assess the intramycobacterial concentration-response relationship and mechanistic interactions involved in rifampicin or streptomycin plus piperine combination.

Keywords: Efflux Pumps Inhibitors, Gene regulation, Morphological changes, Drug combinations.

Abbreviations: TB, Tuberculosis; EPs, efflux pumps; RIF, rifampicin; SM, streptomycin; PIP, piperine; EPI, efflux pumps inhibitor; *M. tuberculosis*, *Mycobacterium tuberculosis*; RT-qPCR, Reverse transcription quantitative real time polymerase chain reaction.

Introduction

The high morbidity and mortality rates associated with tuberculosis (TB) characterize the disease as an important public health problem worldwide. Nowadays, the TB treatment is performed in two steps: an intensive phase during two months, in which isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) are used, , followed by for 4 months of INH and RIF (World Health Organization, 2017).

Mycobacterium tuberculosis presents intrinsic resistance to some drugs due to its rich mycolic acid cell wall and constitutive enzymes, which inactivate some drugs. This natural resistance can be aggravated by other situations such as selection of drug-resistant mutants by treatment failure, monotherapy, inadequate dosing regimens, poor patient compliance and interpatient pharmacokinetic variability (World Health Organization, 2017).

Resistance to specific anti-TB drugs is generally the result of spontaneous mutations in the of *M. tuberculosis* genome, which alter drug targets, prodrug-activating enzymes, or by altering the ribosomal units compromising the protein translation. However, in some clinical isolates, drug resistance can not be explained by the presence of known genetic mutations, occurring in 30% of INH-resistant isolates, 5% resistant to RIF and 30% resistant to streptomycin (SM), one of the first drug used in the treatment of TB (da Silva et al., 2011; Hlaing et al., 2017; Jiang et al., 2008).

Recently, drug resistance mediated by efflux pumps (EPs), which may confer resistance to one or more drugs, has been described in mycobacteria. To strengthen the subject described, sequential analysis of the genome showed that *M. tuberculosis* has multiple coding sequences for EPs (da Silva et al., 2011; Hao et al., 2011; Jiang et al., 2008; Rodrigues et al., 2012).

Phylogenetically, bacterial efflux transporters can be divided into five families: major family facilitator (MFS), family resistance-nodulation-division (RND), small family multidrug resistance (SMR), ATP family of binding cassette (ABC) and the family of multiple antibiotics and toxin extrusion (MATE) (Blair et al., 2014; Stavri et al., 2007; Zechini and Versace, 2009). The EPs described and well characterized in *M. tuberculosis* belong to the first four families cited above (da Silva et al., 2011; Hao et al., 2011; Jiang et al., 2008; Rodrigues et al., 2012).

Compounds with the ability of efflux pumps inhibitors (EPIs), in mycobacteria, have been demonstrated *in vitro*, inducing antimycobacterial minimum inhibitory concentration decrease when used in combinations (Gupta et al., 2013; Rodrigues et al., 2012; Szumowski

et al., 2013). In this sense, the administration of an EPI in combination with classical anti-TB drugs may be an effective strategy to improve TB treatment (Brake et al., 2018).

Piperine (PIP) (Fig.1) is a trans–trans isomer of 1-piperonyl-piperidine isolated from Piperaceae, such as black pepper (*Piper nigrum*) and long pepper (*Piper longum*). PIP has been commercially used as inhibitor of important enzymes for drug metabolism and also in the transport of metabolites and xenobiotics (Pule et al., 2016). Firstly, the EPI activity of PIP was reported in *Staphylococcus aureus* (Sangwan et al., 2008) and later in some mycobacteria species by increasing the efficacy of some antimicrobials, such as RIF. (Jin et al., 2011; Sharma et al., 2010).

In a first step of a study about EPIs as adjunctive drug in TB treatment, to understand the effect of EPIs plus anti-TB drugs combinations on the regulation of genes that codify for EPs in *M. tuberculosis* can bring light for some questions not answered yet about EPI activity in the bacillus.

Thus, knowing that RIF has its improved activity when combined to PIP (Sharma et al 2010) and that aminoglycosides, such as SM, can use efflux pumps as an intrinsic resistance mechanism (Hlaing et al., 2017) and regardless of the limited number of studies in mycobacteria, the aim of this study was to evaluate how PIP and RIF or SM plus PIP combinations work on the regulation of EPs genes and on the cell morphology of *M. tuberculosis*.

2. Material and methods

2.1. Bacterial culture

M. tuberculosis H_{37}Rv (ATCC 27294) was grown in Middlebrook 7H9 (Difco Laboratories, Detroit, MI, USA), supplemented with 10% (v/v) oleic acid-catalase-albumin-dextrose (OADC, BBL/Becton and Dickinson, Sparks, MD, USA), 0.2 % glycerol (v/v) and 0.025 % Tween 80 (v/v) for 15 days at 37 ºC.

2.2. Drugs

The anti-TB drugs RIF, SM and the EPI, PIP were purchased from Sigma (St. Louis, MO, USA). SM was prepared in distilled water, RIF in methanol:water (1:10, v/v) and PIP in dimethylsulfoxide (DMSO, Synth, Diadema/SP, Brazil). The tested drugs concentrations

range were 0.01 - 4 µg/ml, 0.00012 - 200 µg/ml and 7.812 - 500 µg/ml for SM, RIF and PIP, respectively.

2.3. MIC and drugs combination assays

RIF, SM and PIP minimum inhibitory concentrations (MIC) against *M. tuberculosis* H_{37}Rv were determined by Resazurin Microtiter Assay (REMA) (Palomino et al., 2002). MIC was determined as the lowest drug concentration that was able to inhibit the visual color change of 0.01% resazurin (Acros, Morris Plains, NJ, USA) from blue to pink.

The method Resazurin Drugs Combination Microtiter Assay (REDCA) evaluated the effect of combinations of each drug and the inhibitor against *M. tuberculosis* H_{37}Rv (Caleffi-Ferracioli et al., 2016). The fractional inhibitory concentration (FIC) was calculated as the quotient between the MIC of the drug combination and MIC of the antimicrobial alone (RIF and SM). The results were interpreted as FIC $\leq$ 0.25 (synergism), FIC > 0.25 < 2 (indifference), and FIC > 2 (antagonism). A FIC value $\leq$ 0.25, indicative of a four-fold reduction, was assumed as synergism (Coelho et al., 2015).

2.4. Efflux pump gene regulation

To access the impact of the drugs alone and in combination in the EP gene expression, we selected genes belonging to the main families of efflux transporters and related to intrinsic resistance in mycobacteria (Hao et al., 2011; Jiang et al., 2008; Machado et al., 2012; Rodrigues et al., 2012).

To study EP gene regulation, *M. tuberculosis* H_{37}Rv ~6-8 x 10^6 cfu (colony-forming unit)/mL) in OADC-supplemented Middlebrook 7H9 was exposed to ½ MIC PIP, RIF, SM alone and in combination (RIF+PIP and SM+PIP), in shaking at 100 rpm at 37 °C for 24h. A growth control without drugs was included in all assays.

Total RNA was extracted and purified by RNeasy Plus Mini Kit (Qiagen Biotechnology, Valencia, CA, USA) according to the manufacturer's instructions. DNA contaminants were removed by prior treatment with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA). RNA quantification was performed in Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with random primers (Invitrogen, Carlsbad, CA, USA) and qPCR was performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA). EP specific primers (Promega, Madison, WI, USA) are listed in Table 1. Every sample was run in triplicate, negative controls were added and melting curves were accessed. The 16S RNA (*rrs*) gene was used to normalize all

reactions. A reference assay was conducted in the absence of drugs. The relative quantification of target gene expression was calculated through $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Data analysis was performed using a one-way ANOVA test with SAS 9.0 software (SAS OnlineDoc 9, SAS Institute, Cary, NC, USA), followed by the Tukey post-hoc test ($p < 0.01$ and $p < 0.05$ were considered significant).

5. Scanning electron microscopy

After drug exposure, as previously described in the efflux pump gene regulation section, *M. tuberculosis* $H_{37}Rv$ growth was centrifuged and the cells were washed with phosphate buffered saline (PBS), pH 7.4. Cells were fixed with 2.5 % glutaraldehyde (Sigma-Aldrich, MO, USA) in 0.1 M cacodylate buffer (Electron Microscopy Science, Hatfield, PA, USA) for at least 2 h at 4 °C. Fixed cells were placed on a glass-lined poly-L-lysine carrier (Sigma-Aldrich, MO, USA), dehydrated with ethanol, subjected to critical drying in CO_2 and gold plated. The reading was performed on a Quanta 250 (Fei, OR, USA). An average of 20 to 30 micro-fields in each sample were selected by random scanning and photographed. Scanning electron microscopy (SEM) was performed in duplicate.

3. Results

Minimal inhibitory concentrations of PIP, RIF and SM against *M. tuberculosis* $H_{37}Rv$ were 125, 0.004 and 0.25 µg/ml, respectively. The FIC values obtained for RIF plus PIP and SM plus PIP combinations were 0.25 and 0.24, respectively.

The relative quantification of the transcript for 11 selected EPs genes in *M. tuberculosis* $H_{37}Rv$, after 24h exposure to PIP, RIF or SM alone and both combined to PIP can be observed in Fig. 2 and 3. In comparison to the control, a significant ($p \leq 0.01$) difference in gene expression was observed in six EPs genes (*Rv2942*, *Rv3065, Rv2846*, *Rv1456c*, *Rv1218c*, *Rv1819c*) for PIP, three (*Rv*2942, *Rv1456c*, *Rv1819c*) for RIF and eleven (*Rv2942*, *Rv3065*, *Rv2846 Rv1410c*, *Rv1258c*, *Rv1456c*, *Rv1457c*, *Rv1458c*, *Rv1218c*, *Rv1217c*, *Rv1819c*) for SM exposure.

Downregulation in three EPs genes (*Rv2942, Rv1456, Rv1819*) were observed in *M. tuberculosis* exposed to RIF + PIP (½ MIC), compared to RIF alone. For the bacillus exposed to SM + PIP (½ MIC), eleven EPs genes (*Rv2942*, *Rv3065*, *Rv2846 Rv1410c*, *Rv1258c*, *Rv1456c*, *Rv1457c*, *Rv1458c*, *Rv1218c*, *Rv1217c*, *Rv1819c*) were downregulated compared to SM alone exposure.

To evaluate the effects of RIF + PIP and SM + PIP combinations on *M. tuberculosis* cell morphology, SEM was conducted (Fig. 4). Typically, the rod-shaped *M. tuberculosis* drug-unexposed cells showed thin and slightly curved aspect with a smooth surface (Fig. 4A). After exposure to RIF and SM alone, discreet changes in bacteria morphology were observed (Fig. 4B and 4C). Exposure to PIP alone (Fig. 4D) revealed some entire bacilli involved by an elongated structure with cell disintegration aspect were observed. The combination of the drugs and the inhibitor (Fig. 4E and 4F) caused similar effects to those observed in the exposure to PIP, that being accentuated on the SM + PIP combination.

4. Discussion

Evidence suggests EPIs may restore anti-TB drug susceptibility in *M. tuberculosis* (Pule et al., 2016). However, the effect of some EPIs and drugs combinations, as RIF or SM with PIP, which is an EPI that has been shown effect comparable to reserpine, carbonyl m-chlorophenyl hydrazine cyanide and verapamil well-studied inhibitors, on the morphology and regulation of particular EPs genes is unclear.

The genome of *M. tuberculosis* H_{37}Rv reveals some genes encoding putative drug EPs (Cole, 1998) and some of these have been identified in conferring resistance to anti-TB drugs (da Silva et al., 2011; Hao et al., 2011; Jiang et al., 2008; Rodrigues et al., 2012). In the present study, the activity of RIF, SM, PIP alone and combined (RIF + PIP and SM + PIP) were evaluated in *M. tuberculosis* H_{37}Rv. For this, we selected 11 EPs genes belonging to four transporter families that were already associated with drug resistance in *M. tuberculosis*, RND, SMR, MFS and ABC (Jiang et al., 2008; Pasca et al., 2004; Zechini and Versace, 2009)

PIP exerted potentiating effects on the activity of RIF and SM in *M. tuberculosis* H_{37}Rv, observed by checkerboard assay. To the best of our knowledge, our research group has reported for the first time that the synergistic effect between PIP and SM (Hegeto et al., 2018), an aminoglycoside that is transported by the efflux system (Pule et al., 2016) is demonstrated in *M. tuberculosis* H_{37}Rv. The *M. tuberculosis* H_{37}Rv PIP (½ MIC) exposure induced changes in the regulation of 6 EPs genes ($p < 0.01$). However, a considerable down-regulation ($p < 0.001$) was observed in the *Rv1819* gene, which is classified as an ABC family transporter. The involvement of PIP in the inhibition of EPs was first demonstrated by the PIP connection to the EP coded by *Rv1258c* in the active site using docking method (Sharma et al., 2010). In our study, the *Rv1258c* had no change in its regulation when the bacillus was

exposed to ½ MIC PIP alone, although upregulation was observed in the bacillus exposed to RIF plus PIP combination.

From the 11 EPs genes tested in *M. tuberculosis* H_{37}Rv exposed to RIF (½ MIC), three (RND *Rv2942* and ABC *Rv1819c* and *Rv1456* families) showed significant ($p < 0.01$) increase in their regulation. Our results corroborate with those by Calleffi-Ferracioli et al. (Caleffi-Ferracioli et al., 2016) study, which also observed increased regulation in the same EPs gene after 72 h of RIF exposure at the same RIF concentration. The EPs up-regulation, in both studies, confirms the proposed theory by Calgin et al. (Calgin et al., 2013) that exposure of *M. tuberculosis* to antimicrobials can boost the expression of constitutive or inducible EPs and lead to an increase in the anti-TB drugs MICs and render resistant bacilli.

The RIF + PIP combination induced some changes in EPs genes regulation. Three of the 11 EPs genes draw attention, to mention *Rv2942* (RND family), *Rv1456* and *Rv1819* (ABC family), were down-regulated compared to the bacilli exposed to RIF alone.

Rifampicin was introduced in the treatment of TB in 1965 and is part of the multidrug therapy until today. Its mechanism of activity involves the inhibition of the DNA-dependent RNA polymerase, binding to the subunit of the enzyme interfering with protein synthesis and consequently death of RIF susceptible bacilli (Campbell et al., 2001). More recently, RIF was known to have EPs induction activity (Caleffi-Ferracioli et al., 2016) which is in agreement to the observed in our study by change in the regulation of *Rv1819* (ABC family) gene. In this sense, we can think that the RIF + PIP combination could contribute to intrabacillary concentration increase and improve the RIF activity against *M. tuberculosis*.

For the first time, to the best of our knowledge, the EPs genes regulation in *M. tuberculosis* exposed to SM alone and in combination with PIP was analyzed. In our study, all 11 EPs genes studied showed to be up-regulated after SM exposure.

SM, an aminoglycoside, was one of the first drug with bactericidal activity against *M. tuberculosis* and was introduced in the treatment of TB in 1944 (Marshall et al., 1948). However, high resistance to SM soon appeared and, currently, it is used in the treatment of TB cases when there is resistance to INH and/or RIF or when parenteral therapy is required (Pestka et al., 1965; World Health Organization, 2017). The aminoglycoside first bind to the bacilli cells surface and subsequently is transported through the cell wall by an oxidative energy-dependent process. Once inside the bacilli, the aminoglycoside binds to the 30S ribosomal unit and consequently causes defective protein synthesis or inhibition. In our study, it is clearly observed that all EPs genes studied showed to be up-regulated after SM exposure,

what was already expected, once SM has been put into disuse due to the early onset of rapid bacterial resistance when used as monotherapy.

By submitting *M. tuberculosis* H_{37}Rv to SM + PIP combination exposure, all EPs genes studied showed to be down-regulated, which were more pronounced in four, to mention *Rv1457*, *Rv1458*, *Rv1217* and *Rv1819* (all belonging to the ABC family). Our results with SM and PIP, bring expectation for the treatment of TB in patients in whom INH or RIF cannot be used.

The mechanism of RIF and SM activity are driven to the transcriptional machinery (Campbell et al., 2001; Pestka et al., 1965), without direct correlation to cell wall biosynthesis. Although, we know that some EPI modulates the effect of different anti-TB drugs and it is not fully elucidated on how such inhibition occurs. Recently Chen et al. (Chen et al., 2018) indicated a direct effect of verapamil, a potente EPI, on *M. tuberculosis* membrane energetics inducing a membrane stress response, and it was of our interest to carry out analyzes on *M. tuberculosis* cell morphology, by SEM, after PIP alone and the two combinations studied, RIF + PIP and SM +PIP exposure.

The bacilli morphological changes observed in our study were similar to those observed by Patilaya et al. (2012) after *M. tuberculosis* exposure to the ethylacetate fraction, containing PIP, obtained from *P. nigrum*. PIP treated cells showed morphological changes driven to the outer *M. tuberculosis* layers and the bacilli cells appeared to be linked to each other due to the presence of cells debris, which suggesting the release of the cytoplasmic content of lysed cells.

Speculation may be gained by that *M. tuberculosis* does not necessarily die from the accumulation of the drug inside but may be also by disturbance caused in the membrane, or even the sum these factors as observed by Chen et al. (2018) with verapamil. These speculations may raise the hypothesis that PIP may also have this disturbing effect on the membrane, since our results show that there was possible cell lysis after exposure with PIP alone and in combination with RIF or SM. Further studies, including greater number of *M. tuberculosis* clinical isolates harboring different resistance patterns, are necessary to assess the intramycobacterial concentration-response relationship and mechanistic interactions involved in RIF or SM + PIP combination.

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Transparency declarations section

None to declare.

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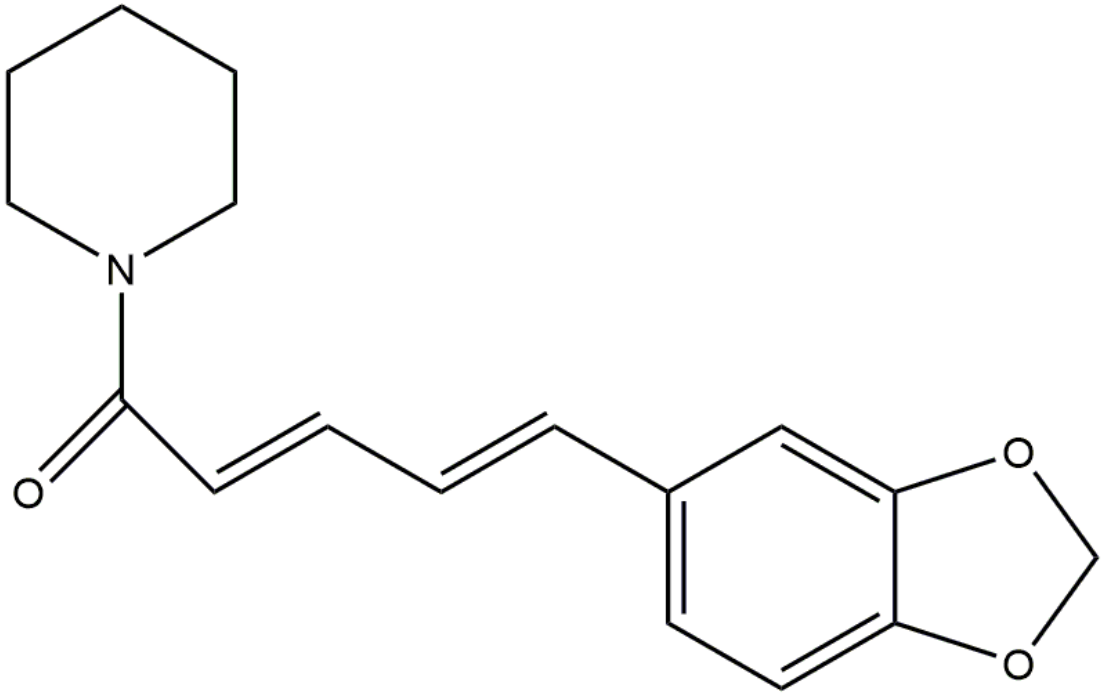

Fig. 1. Molecular structure of piperine

Table 1. Primers used to assess relative efflux pump gene expression by qPCR.

Efflux pump gene	**Sequences (5' – 3')**	**Transporter family**
Rv2942	Fw - TACCCAAGCTGGAAACAA	RND
	Rv - CCGTCAGAATAGAGGAACAG	
Rv3065	Fw - AACCAGCCTGCTCAAAAG	SMR
	Rv - CAACCACCTTCATCACAGA	
Rv2846	Fw - ATGGTAATGCCTGACATCC	MFS
	Rv - CTACGGGAAACCAACAAAG	
Rv1410c	Fw - AGTGGGAAATAAGCCAGTAA	MFS
	Rv - TGGTTGATGTCGAGCTGT	
Rv1258c	Fw - AGTTATAGATCG GCTGGATG	MFS
	Rv - GTGCTGTTCCCGAAATAC	
Rv1456c	Fw - GAGTCGCACCAGAATCGC	ABC
	Rv - TCGCTGTTGGTTGCCTAC	
Rv1457c	Fw - GTAGCACCGAGTCGTTTG	ABC
	Rv - ATCTCCACCGCATTCACC	
Rv1458c	Fw - CAGTCCAAGTACCTCAATG	ABC
	Rv - GCGATACGGGTCAATAAC	
Rv1218c	Fw - CCGCAAGGCGTCTAGTGAA	ABC
	Rv - TGGACCCGTTGATGGAAAA	
Rv1217c	Fw - CGGTGAGGTTGGCGTAG	ABC
	Rv - CGGTCGGAATCTGGAAA	
Rv1819c	Fw - CGGTGATTTCTTTCACAGC	ABC
	Rv - CCGACAGATTCCATCCATT	
16s RNA	Fw - CAAGGCTAAAACTCAAAGGA	
	Rv - GGACTTAACCCAACATCTCA	

Fw, forward. Rv, reverse

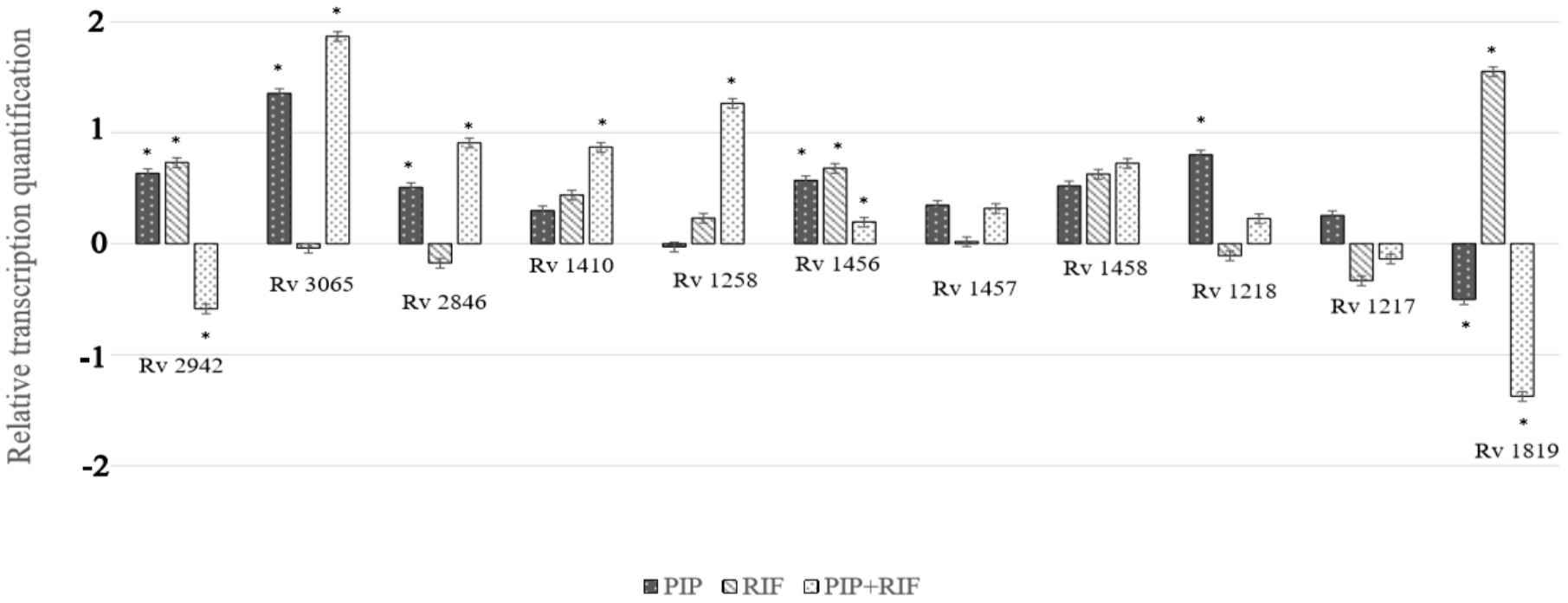

Fig. 2. Relative transcription quantification of 11 efflux pumps genes in *Mycobacterium tuberculosis* assessed by Rq-PCR after 24h of exposure to ½ MIC rifampicin (RIF), piperine (PIP) and PIP plus RIF combination. The results were normalized to 16s RNA and calculated by $2^{-\Delta\Delta Ct}$ method. **p* <0.01, compared with control *M. tuberculosis* growth in the absence of drugs. On logarithmic scale.

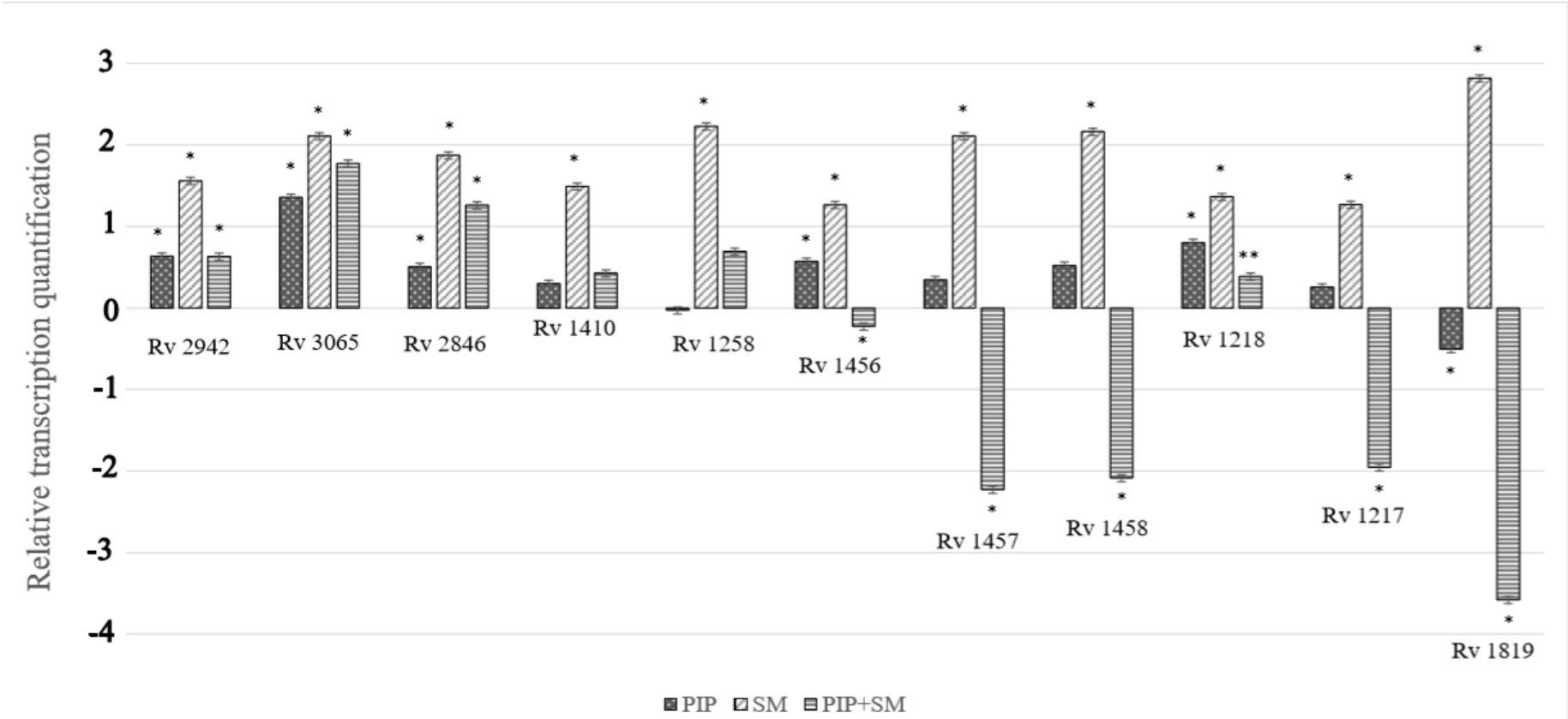

Fig. 3. Relative transcription quantification of 11 efflux pumps genes in *Mycobacterium tuberculosis* assessed by Rq-PCR after 24h of exposure to ½ MIC streptomycin (SM), piperine (PIP) and SM plus PIP combination. The results were normalized to 16s RNA and calculated by $2^{-\Delta\Delta Ct}$ method. *p <0.01 and ** p <0.05, compared with control *M. tuberculosis* growth in the absence of drugs. On logarithmic scale.

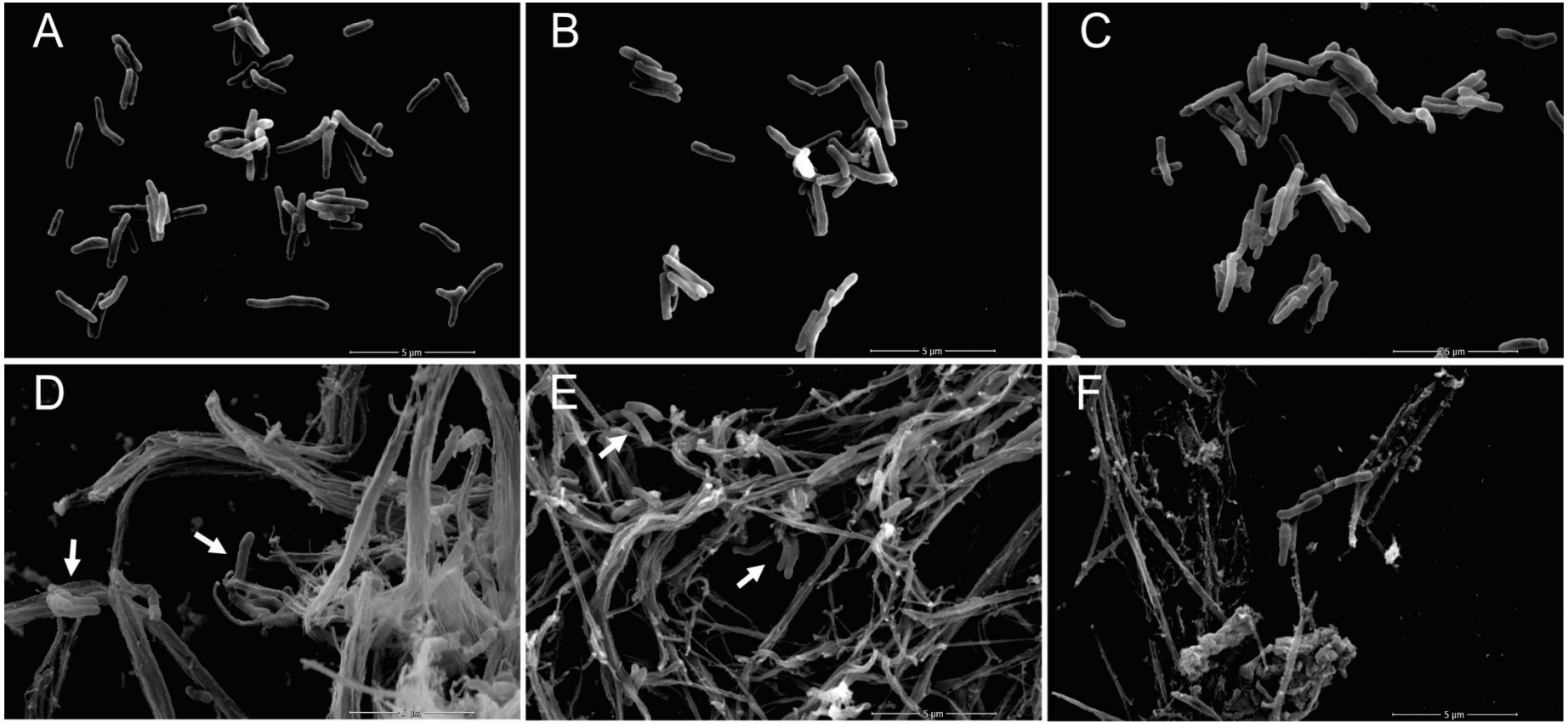

Fig. 4. Scanning electron micrograph of *Mycobacterium tuberculosis*, control without drugs exposure (A), after 24h of exposure to a sub-inhibitory concentration (½ MIC) of rifampicin (B), streptomycin (C), piperine (D), rifampicin plus piperine combination (E) streptomycin plus piperine combination (F)

CAPÍTULO III

1. CONCLUSÕES

- A revisão sistemática mostrou que PIP desempenha um papel importante na inibição de BEs e que tal composto é capaz de modular o sistema imunológico, especialmente quando combinado com outros fármacos anti-TB (por exemplo, RIF);
- A CIM de PIP encontrada através da metodologia de REMA indica que esse composto tem certa atividade antimicobacteriana;
- A combinação de PIP com fármacos é uma estratégia promissora para o combate ao bacilo da TB, sendo as melhores combinações PIP+RIF e PIP+SM;
- As combinações apresentam efeito sinérgico também em isolados clínicos com ou sem presença de mutações que causam resistência aos fármacos anti-TB.
- Uma vez exposta a essas combinações o bacilo tem a expressão de algumas BEs reduzida em relação a exposição dos mesmos fármacos isoladamente;
- A combinação de PIP+SM inibiu a expressão em maior número de BEs do que a combinação de PIP+RIF;
- A exposição de PIP sozinha ou de forma combinada altera a morfologia celular do bacilo.

2. PERSPECTIVAS

Diante dos resultados obtidos neste trabalho, a PIP se apresenta como um potencial EPI sendo candidato a fármaco adjunto na terapia de TB. Dessa forma, mais investigações devem ser feitas para que essa hipótese se concretize. Como alvo de investigações futuras em relação a essa interação da PIP com RIF e SM, pode-se realizar a analise da expressão gênica de BE em isolados clínicos, análise da expressão gênica de BEs após infecção de macrófagos, da produção de citocinas e avaliação da síntese proteica, além de explorar o campo da bioinformática no desenho e/ou modificações de fármacos ou até mesmo da PIP para melhor ação contra o bacilo da TB.