UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

ANÁLISE TOXICOGENÉTICA E ATIVIDADE ANTIMICOBACTERIANA *in vitro* DO COMPOSTO FLAVONA

VANESSA VILAMAIOR DE SOUZA

DOURADOS MS
2016

VANESSA VILAMAIOR DE SOUZA

ANÁLISE TOXIGENÉTICA E ATIVIDADE ANTIMICOBACTERIANA *in vitro* DO COMPOSTO FLAVONA

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

Orientador: Dr. JULIO HENRIQUE ROSA

DOURADOS MS
2016

Dados Internacionais de Catalogação na Publicação (CIP).

S719a Souza, Vanessa Vilamaior de.
Análise toxicogenética e atividade antimicobacteriana *in vitro* do composto flavona. / Vanessa Vilamaior de Souza. – Dourados, MS : UFGD, 2016.
45f.

Orientador: Prof. Dr. Julio Henrique Rosa.
Dissertação (Mestrado em Ciências da Saúde) – Universidade Federal da Grande Dourados.

1. Toxicogenética. 2. Antimicobacteriana. 3. Flavona. I. Título.

CDD – 610.042

Ficha catalográfica elaborada pela Biblioteca Central – UFGD.

ATA DA DEFESA DE **DISSERTAÇÃO DE MESTRADO** APRESENTADA PELA CANDIDATA **VANESSA VILAMAIOR DE SOUZA**, ALUNA DO PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM CIÊNCIAS DA SAÚDE ÁREA DE CONCENTRAÇÃO "FARMACOLOGIA", REALIZADA NO DIA 19 DE AGOSTO DE 2014.

Aos dezenove dias do mês de agosto de dois mil e quatorze (19/08/2014), às 13h30min, em sessão pública, realizou-se, na sala cinco do Bloco da FCS da Universidade Federal da Grande Dourados, a Defesa de Dissertação de Mestrado intitulada **"ATIVIDADE ANTIMICOBACTERIANA, TOXICIDADE AGUDA, GENOTOXICIDADE E MUTAGENICIDADE DA FLAVONA (2-FENIL-4H-1-BENZOPIRANO-4-ONA) EM CAMUNDONGOS"** apresentada pela mestranda **VANESSA VILAMAIOR DE SOUZA**, do Programa de Pós-Graduação Mestrado em Ciências da Saúde, à Banca Examinadora constituída pelos professores **Dr. Júlio Henrique Rosa Croda/UFGD** (presidente/orientador), **Dra. Cândida Aparecida Leite Kassuya/UFGD** (membro titular), **Dr. Fábio Juliano Negrão/ UFGD e Dra. Alexeia Baruffati Grisolia** (membro titular). Iniciados os trabalhos, a presidência deu a conhecer a candidata e aos integrantes da Banca as normas a serem observadas na apresentação da Dissertação. Após a candidata ter apresentado a sua Dissertação, os componentes da Banca Examinadora fizeram suas arguições, que foram intercaladas pela defesa da candidata. Terminadas as arguições, a Banca Examinadora, em sessão secreta, passou aos trabalhos de julgamento, tendo sido a candidata considerada **APROVADA**, fazendo *jus* ao título de **MESTRE EM CIÊNCIAS DA SAÚDE**. Nada mais havendo a tratar, lavrou-se a presente ata, que vai assinada pelos membros da Banca Examinadora.

Dourados, 19 de agosto de 2014.

Dr. Júlio Henrique Rosa Croda _____

Dra. Cândida Aparecida Leite Kassuya _____

Dr. Fábio Juliano Negrão _____

Dra. Alexeia Baruffati Grisolia _____

ATA HOMOLOGADA EM: ___/___/_____, PELA PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA / UFGD.

Prof. Cláudio Alves de Vasconcelos
Pró-Reitor de Ensino de Pós-Graduação e Pesquisa
Matr. n. 0432923

Agradecimentos

Agradeço,

A Deus, por ter me dado forças, luz e sabedoria.

Aos meus pais Elis Regina Sousa Vilamaior e Antonio Alves de Souza pelos momentos de plenitude e apoio familiar incondicionais. A Vocês, minha eterna gratidão.

Aos meus irmãos Alexsandra Vila Maior de Souza e Geandro Vila Maior de Souza pelo incentivo e pela presença constante em minha vida. Agradeço em especial à minha irmã pela ajuda, incentivo, dedicação e paciência.

A professora Drª Candida Aparecida Leite Kassuya por fornecer auxilio quanto à realização de todo o trabalho desde o início do mestrado. Sou imensamente grata por tudo.

Aos professores doutores: Candida Aparecida Leite Kassuya, Fábio Juliano Negrão, Anelise Samara Nazari Formagio e Alexéia Barufatti Grisólia, pela composição da banca de defesa.

Ao professor Dr. Julio Henrique Rosa Croda pela orientação, auxílio durante o mestrado e paciência para trabalhar com algo que foge a sua área de atuação.

Ao professor Dr. Rodrigo Juliano Oliveira pela disposição em ajudar e por abrir as portas de seu laboratório, com todos os recursos necessários para realização de parte dos experimentos.

Aos meus companheiros nessa caminhada, que diga-se de passagem, foi árdua, que nos momentos mais difíceis estiveram ao meu lado sem até então, nem mesmo me conhecer, Flávio Henrique Souza de Araújo, Claudia Rodrigues Berno e Roberto. Muito obrigada pelo acolhimento.

Aos meus amigos Wesley Clovis Barbieri Mendonça e, Karine Sartori vocês moram no meu coração.

As minhas companheiras de mestrado Ana Claudia Piccinelli e Giseli Karenina Traesel obrigada pelo incentivo e pelos momentos de descontração.

As técnicas de laboratório do LPCS (Laboratório de Pesquisa de Ciências da Saúde) e da FCS (Urinálise e Anatomia Humana) que me auxiliaram em alguns experimentos e no fornecimento de reagentes Flora Martinez Figueira Moreira, Mariana Bento Tatara, Lujan Nunes Sanabria Aliatti, Débora Regina Hoff Brait, Priscilla Elly

Vieira Hattori.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de estudos de mestrado.

Dedicatória

Dedico aos meus pais Elis Regina e Antonio, meus irmãos Alexsandra e Geandro e a minha sobrinha Isabella.

Sumário

1 INTRODUÇÃO

A tuberculose (TB) é uma doença crônica infecciosa transmissível, causada pela bactéria de morfologia bacilar, *Mycobacterium tuberculosis* também conhecido como Bacilo de Koch. Vários aspectos devem ser considerados para a manutenção da TB em nosso meio destacando-se, a posição socioeconômica desfavorável, comorbidade de tuberculose HIV/AIDS, etilismo, diabetes, tabagismo, dependência química, dificuldade de acesso aos serviços de saúde, o envelhecimento da população, a urbanização desordenada e processos migratórios contribuíram de maneira significativa para que a doença ainda hoje não tenha sido erradicada (Barreto, 2009; Bignall, 1971).

A forma pulmonar da TB é a mais comum, o indivíduo pode adquiri-la a medida que entra em contato com o bacilo em suspensão emitido por um paciente portador. Cerca de um terço da população mundial está infectada com o *M. tuberculosis*, e a chance de desenvolver a forma ativa da doença é de 10% durante a vida (Barbosa e Costa, 2014).

A TB está concentrada em um grupo de 22 países, no ano de 2012 foi registrado um total de 8,6 milhões de pessoas que desenvolveram a doença, deste montante, 1,3 milhão evoluiu para óbito (WHO, 2013). No mesmo período, no Brasil, foram notificados 71.230 casos, a taxa de incidência foi de, 36.7/100.000 habitantes, colocando o Brasil na 17ª posição entre os países com alta carga da doença (Brasil, 2013; SINAN, 2014).

O tratamento da TB é prolongado, dura no mínimo seis meses. O uso inadequado e o abando do tratamento podem resultar no surgimento de cepas monorresistentes, caracterizadas pela resistência do bacilo a pelo menos um medicamento utilizado para o tratamento e cepas multidroga resistentes (MDR) que são resistentes às drogas isoniazida e rifampicina. A resistência é agravada quando as cepas se tornam resistentes a pelo menos rifampicina e isoniazida, uma quinolona (ofloxacina, levofloxacina, moxifloxacina) e um medicamento injetável de segunda linha (amicacina, canamicina, capreomicina), são então consideradas cepas extremamente resistentes (Arbex et al., 2010; Dalcolmo et al., 2007).

A elevação das taxas de incidência da TB e o surgimento de cepas resistentes ocasionou a necessidade da realização de novos ensaios de triagem, bem como, a síntese de novos fármacos e testes de susceptibilidade. Dessa forma, a indústria farmacêutica

procura a implementação de terapias medicamentosas que sejam eficientes tanto, na redução do período de tratamento quanto na eliminação de infecções persistentes (Warner e Mizrahi, 2004).

Dentro dos compostos utilizados como base para as novas formulações, as pesquisas apontam para a utilização de plantas medicinais, as quais, são fonte de compostos como os metabólitos secundários (fontes potencias de drogas) contidos em extratos e óleos essenciais com importância terapêutica (Dolly et al., 2012).

O conhecimento popular é responsável por propagar dentro das comunidades as propriedades terapêuticas das plantas, e é a partir das indicações populares que vários estudos químicos e farmacológicos têm início (Mathias et al, 2013). Nos últimos anos, a pesquisa sobre plantas medicinais tem atraído à atenção em todo o mundo por serem fonte de substâncias químicas que são potenciais substratos para síntese de novas drogas (Bellik et al., 2012).

Assim, se faz necessária à busca por drogas eficazes no tratamento de diversas doenças sendo necessários estudos acerca da segurança e eficácia de seu uso. A partir da pesquisa realizada pelo nosso grupo de pesquisa, o qual extraímos fase metanólica das folhas de *Annona sylvatica* (CIM = 184,33 mm/mL). A fração FAE (Fração Acetato de etila), resultante do fracionamento, teve CIM = 115,2 mm/mL. Detectou-se ainda a CIM do composto isolado luteolina (236,8 mm/mL) a qual pertence à classe das flavonas, em sua estrutura ocorre a ligação de hidroxilas nas posições C3, C4, C5 e C7.

O presente estudo teve como finalidade verificar a atividade antimicobacteriana *in vitro* de flavona sem nenhum radical de hidroxila (2-fenil-4H-1-benzopirano-4-ona) e análise toxicológica *in vivo* através dos testes de toxicidade aguda, genotoxicidade e mutagenicidade.

2 REVISÃO DA LITERATURA

2.1 Tuberculose: Doença e epidemiologia

A infecção tuberculosa é endêmica, causada pelo *Mycobacterium tuberculosis* ou bacilo de Koch pode afetar praticamente todos os órgãos, mas tem especial predisposição pelos pulmões, o que provavelmente se deva, a alta oxigenação do órgão e de o bacilo ser aeróbio estrito. A evolução ocorre em ciclos lentos, podendo apresentar as mais diversas complicações (Demachki et al., 2013; Pellenz, et al, 2011; Rodrigues et al., 2013).

A partir do momento que o bacilo adentra ao organismo, rapidamente acomete o pulmão que forma como resposta tubérculos e corpos cavernosos. Quando o indivíduo infectado tosse, espirra ou fala, expectora milhares de gotículas (1 a 10 µm de diâmetro) no ar, cada uma contém em média um quarto de bacilos, que podem se manter suspensos no ar por várias horas, podendo assim, serem aspirados e contaminarem outras pessoas. As vias aéreas são a principal forma de propagação da doença (Araujo et al, 2013, Carvalho et al., 2011). Outro meio de contágio é, a inoculação direta que ocorre através da pele lesionada, é mais comum em patologistas ou outros profissionais de laboratórios que manipulam tecidos infectados (Campos et al., 2011).

Após atingir os alvéolos pulmonares, os bacilos sofrem a ação do sistema imunológico. A resposta imunológica inata desencadeia uma resposta inflamatória que envolve os macrófagos alveolares locais e o recrutamento de neutrófilos e monócitos, aumento progressivo de linfócitos T e B, muitas vezes a infecção poderá ser eliminada por esse mecanismo (Capone et al., 2006).

Os macrófagos, neutrófilos e monócitos circundam os macrófagos infectados, células dentríticas e fibroblastos, formando o característico granuloma tuberculoso. O granuloma é o principal mecanismo que limita a disseminação da micobactéria, criando assim a interação da resposta entre os linfócitos T e os macrófagos ativados pelo infeferon-γ, os quais impedem a multiplicação do *Mycobacterium tuberculosis*, assim a doença pode apresentar longos períodos de latência, e reativar quando ocorrer algum desequilíbrio da reposta do sistema imunológico frente ao bacilo (Turolla e Nascimento, 2006).

O risco de doença pulmonar ativa é baixo após uma exposição ao organismo, mas

aumenta sob condições de estresse ou em um ambiente confinado no qual ocorrem exposições repetidas (San Pedro e Oliveira, 2013; Selig et al., 2012). Segundo estimativas, 1/3 da população mundial está infectada pelo *M. tuberculosis*, desse total 5 a 10% irá desenvolver a doença. Pacientes co-infectados TB/HIV existe um incremento de 10% ao ano de desenvolvimento de doença ativa. Nesses casos está recomendado o tratamento da tuberculose latente (Barbosa e Costa, 2014).

Os fatores que contribuem para o desenvolvimento da doença podem estar associados ao ambiente, ao hospedeiro (idade, sexo, estado nutricional, imunológico e doenças intercorrentes) e a linhagem do *M. tuberculosis* (Ferreira e Pinto, 2010; Vicentin et al., 2002).

Ainda podem predispor o desenvolvimento da doença fatores como: o abuso de drogas injetáveis, a infecção recente nos últimos 2 anos, a silicose, o diabetes *mellitus*, a gastrectomia, o uso prolongado com corticosteroide, a doença renal em estágio avançado, as síndromes de má absorção crônicas, ou baixo peso corporal (10% ou mais de peso abaixo do ideal) e o etilismo (Brasil, 2002; Brasil, 2005).

Estima-se que, no ano de 2012, ocorreram 8,6 milhões de casos incidentes, dos quais 400.000 seriam pacientes coinfectados com HIV e 1,3 milhão de óbitos entre pacientes não portadores de HIV (Lönnoroth et al., 2010; WHO 2013). No mesmo período, no Brasil, foram notificadas 71.230 pessoas, a taxa de incidência foi de aproximadamente de 36.7/ 100.000 habitantes e uma taxa de cura de 69,2% e a de abandono11,9% (Brasil, 2013; SINAN, 2014).

Os gastos financeiros são relevantes, visto que, a tuberculose dentre as doenças infecciosas, é a nona causa de internação e ocupa o vigésimo sétimo lugar em gastos com internação no Sistema Único de Saúde (SUS), e é a quarta causa de mortalidade no Brasil (Brasil, 2013). Estima-se que entre 2013 – 2015 serão necessários US$ 8 bilhões por ano nos países de renda média e baixa para o tratamento e controle da tuberculose, desse montante US$ 5 bilhões serão destinados para o tratamento da tuberculose comum, US$ 2 bilhões para o tratamento de MDR-TB e XDR-TB e US$ 1 bilhão para o tratamento de pacientes com HIV/TB.

2.2 Tratamento

A descoberta de novas drogas para o tratamento da tuberculose se iniciou no final dos

anos de 1940, nesse período houve a descoberta da estreptomicina (monoterapia). Com o surgimento de cepas resistentes fez-se necessário a descoberta de fármacos, como ácido para-aminossalicílico (PAS), isoniazida, pirazinamida, etionamida, etambutol e capreomicina (Mitchison, 1990).

Atualmente a terapia é baseada em: Rifampicina (R), Isoniazida (I), Pirazinamida (Z), Etambutol (E), Estreptomicina (S) e Etionamida (Et) - via oral, cujos os mecanismos estão descritos no quadro 1. A forma de tratamento é baseada em casos novos, falência terapêutica e recidiva: Esquema I (Básico), 2RHZ / 4RH, recomendado para todas as formas de tuberculose pulmonar e extrapulmonar. Esquema II, 2 RHZ/7RH, recomendado para a forma meningo encefálica da tuberculose; Esquema III (reforçado), 2RHZE/4RHE, recomendado nos casos de recidiva após cura ou retorno após abandono; esquema IV, 3SZEEt/9EEt, recomendado nos casos de falência de tratamento. A duração do tratamento é caracterizada no esquema I (6 meses), esquema II (9 meses), esquema III (6 meses) e esquema IV (12 meses) (Brasil, 2002).

Quando ocorre falência no esquema terapêutico básico, é proposto um esquema constituído por estreptomicina, etambutol, terizidona, pirazinamida e uma quinolona (levofloxacina ou ofloxacina). Na impossibilidade de se utilizar a estreptomicina, esta é substituída por amicacina. Em 2009 o etambutol foi incluído no esquema de tratamento, mais especificamente, na fase intensiva (engloba os dois primeiros meses) com o objetivo de diminuir a transmissibilidade e evitar o uso da rifampicina neste momento, para assim minimizar a resistência (Kritski, 2010; Reffino-Netto, 2001; Selig et al., 2012).

Quadro 1. Mecanismo de ação dos principais antimicobacterianos

Medicamento	Mecanismo de ação
Rifampicina	Liga-se de forma irreversível ao RNA-polimerase DNA-dependente, impedindo a produção de RNA e a síntese de Proteínas
Isoniazida	Quelação de íons cobre essenciais para a célula bacteriana; interfere também na enzima micolase-sintetase, importante na síntese de ácido micólico
Etambutol	Inibição da síntese de ácido nucleicos da célula bacteriana
Pirazinamida	Provavelmente semelhante a isoniazida
Estreptomicina	Se liga de forma irreversível ao ribossomo bacteriano, produzindo bloqueio ou alterações profundas na síntese de Proteínas
Etionamida	Age na enzima nicotinamida adenina-dinucleotídeo

Fonte: Bisaglia et al., 2013

2.3 Resistência micobacteriana

Uma preocupação recente, é a resistência dos bacilos frente ao tratamento, a resistência pode ser classificada como: monorresistente - o bacilo se torna resistente a pelo menos um medicamento utilizado para o tratamento ou polirresistente - o bacilo se torna resistente a mais de um medicamento, mas não à combinação de isoniazida e rifampicina.

As cepas MDR apresentam resistência a rifampicina, isoniazida, enquanto que, as cepas XDR apresentam resistência a pelo menos rifampicina e isoniazida, uma quinolona (ofloxacina, levofloxacina, moxifloxacina) e um medicamento injetável de segunda linha (amicacina, canamicina, capreomicina) (Conde et al., 2009; Dalcolmo et al., 2007).

As formas XDR-TB e MDR- TB não respondem ao tratamento preconizado de seis

meses com drogas de primeira linha, podendo levar dois anos ou mais para tratar com drogas menos eficazes, mais tóxicas e com custo mais elevado. Para o tratamento da forma MDR - falência do esquema básico e resistência à rifampicina e isoniazida, a terapia é composta por: Estreptomicina (S), etambutol (E), ofloxacina (O), pirazinamida (Z) e terizidona (T) (WHO, 2013).

A resistência do patógeno causador da tuberculose aos medicamentos utilizados no tratamento, constitui uma barreira para a obtenção do sucesso do esquema terapêutico adotado. Pode ocorrer por conta de um contato prévio do *M. tuberculosis* com o medicamento ou a falha do uso da medicação (principal fator para a formação da resistência). O contato inicial bacilo-fármaco ocorre por conta da administração de esquemas terapêuticos inadequados, irregularidade na administração do medicamento e o controle insatisfatório do indivíduo durante o tratamento (Conde et al., 2009; Pablos-Mendez et al., 2002).

Aproximadamente 3,7% dos pacientes portadores de tuberculose, possuem a forma resistente da doença. Em 2012, cerca de 450 mil pessoas teriam sido diagnosticadas com MDR-TB, o que corresponde a um aumento de 42% em relação ao ano anterior. A maioria dos casos esteve concentrado na China, Índia e Rússia. Embora os casos de XDR sejam mais raros, estudos apontam que aproximadamente 9,6% de MDR possuem características de XDR-TB (WHO, 2013).

Em 2013, no Brasil foram notificados 148 casos novos de monorresistência, 50 de polirresistência, 525 de multirresistência e 21 de resistência extensiva (Fraga et al., 2014). O aumento das taxas de incidência de MDR-TB e XDR-TB tem impulsionado a busca de novas alternativas farmacológicas efetivas que possam reduzir o período de tratamento, bem como minimizar as reações adversas causadas pela terapia.

2.4 Novas alternativas terapêuticas para o tratamento da tuberculose

Planta medicinal é qualquer espécie de planta que contenha substâncias que podem ser utilizadas para fins terapêuticos ou ainda possua princípios ativos que sirvam como precursores da síntese de novos medicamentos (Mitchison, 2004). As plantas e, por conseguinte os produtos naturais são utilizados para fins medicinais. Atualmente os fitoterápicos são responsáveis por cerca de 40% dos fármacos disponíveis no mercado, sendo 70% antimicrobianos e antitumorais (Farnsworth et al., 1985; WHO, 2009).

A busca de novos fármacos menos tóxicos e mais ativos para o tratamento da tuberculose tem estimulado os pesquisadores a investigar novos compostos para o tratamento desta doença (Calixto e Yunes, 2001). As propriedades terapêuticas dos produtos naturais vem sendo alvo de estudo há décadas, mas principalmente desde a descoberta e industrialização da aspirina e penicilina. Outros exemplos clássicos de plantas medicinais utilizadas como fármacos são: o ácido acetilsalicílico (aspirina) proveniente da planta *Salix alba* L. analgésico, antitérmico, anti-inflamatório e antiagregante plaquetário, a vincristina e a vimblastina da planta *Catharanthus roseus* utilizadas no tratamento de alguns tipos de câncer e ainda a digoxina e a digitoxina, potentes glicosídeos cardiotônicos extraídos de *Digitalis purpúrea* L. e a *D. lanata*, respectivamente (Andrade et al., 1997).

As plantas produzem metabólitos secundários que possuem características químicas variadas e são encontrados em grupos - famílias ou gêneros - de plantas, os produtos do metabolismo secundário constituem os chamados “produtos naturais”. Adicionalmente são utilizados em escala industrial para produção de inseticidas, corantes, flavorizantes e medicamentos (Newman e Cragg, 2007).

A diversidade química das plantas permite o isolamento de metabólitos farmacologicamente significativos, através de vias metabólicas secundárias elas produzem alcalóides, flavonóides, isoflavonóides, cumarinas, taninos, glicosídeos, poliacetilenos, terpenos e óleos, que, por vezes, são específicos a determinadas famílias, gêneros ou espécies. Estes complexos químicos podem estar presentes em diferentes partes da planta, por isso, é importante pesquisar a planta em sua totalidade, a fim de, identificar quais locais possuem maiores concentrações de ativos (Behling et al., 2004; Filho e Yunes, 1998).

2.5 Plantas e produtos naturais com atividade antimicobacteriana

As propriedades antimicobacterianas de plantas medicinais estão sendo cada vez mais analisadas em diferentes partes do mundo. Estudos anteriores demonstram sua atividade contra *M. tuberculosis*, como *Clavija procera* B. Stahl, *S. aintabensis* e *T. sibthorpii*, que apresentaram atividade contra estirpes resistentes (Rojas et al., 2006; Tülin et al., 2013). Assim como outras plantas também aprsentaram atividade antimicobacteriana tais como: *Faurea saligna* Harv, *Parinari curatellifolia* Planch ex Benth (Leon-Diaz et al., 2010), *Abelmoschus esculentus Moench* (Chimponda e Mukanganyama, 2010).

Aristolochia taliscana Gancho (Webster et al., 2010); *Securidaca longepedunculata* Fres, *Maerua edulis* (Gilg & Gilg-Ben.) DeWolf (Luo et al., 2011) *Acorus cálamo* L. var. americanus (Webster et al., 2010).

A maioria dos medicamentos sintéticos que estão disponibilizados no mercado possuem origem de produtos naturais (Veiga-Junior et al., 2005). Estima-se que 50% dos medicamentos utilizados para o tratamento de infecções sejam oriundos de produtos naturais ou semi- sintéticos, sendo 19,4% dos produtos naturais utilizados para síntese de medicamentos sintéticos (Pretto, 2005).

Algumas flavonas já foram isoladas e testadas frente cepas virulentas de *M. tuberculosis*, apresentando uma CIM considerável como: 3'-O-dimetoxi-5, 6,4 '-trihiidroxiflavona (MIC 200 mcg/mL) cirsimaritin (MIC de 50 ng/mL), eupatilin (MIC de 50 ng/mL), salvigenin (MIC 100 mcg/ml), (Warner e Mizraki, 2004), 7 - metoxiflavona (MIC 12,5-50 ug/ml) e 5,4 '-di-hidroxi-7-metoxiflavona (MIC 25)-50 ug/mL) (Castellar et al., 2011).

O nosso grupo de pesquisa da rede de pesquisa Pró Centro–Oeste verificou a atividade do extrato bruto e frações e compostos da *Annona sylvatica*. A fração hexânica apresentou atividade, assim foi possível isolar o composto ativo, a luteolina com uma MIC de 236,89 mcg/mL (Araujo et al., 204).

A apigenina (4’,5,7-triidroxiflavona) foi testada a fim de inibir a hialuronidase, utilizado como única fonte de carbono para a estirpe de *M. tuberculosis* H37Rv. Através desse estudo, verificou-se que a apigenina inibiu a atividade da hialuronidase, havendo assim a inibição da micobactéria (Hirayama et al., 2007). Provavelmente, o mecanismo de ação da flavona ocorra devido a essa inibição, uma vez que o efeito possa ser atribuído à ausência do substituinte (por exemplo, metoxilo e hidroxilo) no anel A e B, fazendo com que não haja bloqueio estérico efeito entre o grupo carbonilo na posição C-4 da flavona, fornecendo também uma maior interação eletrostática, hidrofóbicas, ligações de hidrogênio com os receptores. Fazendo com que haja ação proteínas e consequentemente inibindo algumas enzimas bacterianas e interferindo nas suas vias de síntese (Dornas et al., 2007).

2.5.1 Flavonóides

Entre a classe de compostos secundários presentes em plantas medicinais com propriedades farmacológicas merece destaque os flavonóides (polifenóis), que são compostos formados por um esqueleto de difenil propano (C6C3C6) e dois anéis

benzênicos ligados a um anel pirano, de forma livre (aglicona) ou ligados a açúcares (glicosídeos) (Behling et al., 2004) (Figura. 1) A classe é dividida em mais de 10 diferentes subclasses dentre elas os flavonóis, flavonas, isoflavonas, antocianinas e flavononas (Arts e Hollman, 2005).

Os flavonóides estão distribuídos no reino vegetal e podem estar presentes em todas as partes das plantas, desde as raízes até as flores e frutos. Distinguem-se entre si pela coloração, vários representantes possuem a cor amarela, e agem na atração de insetos para a polinização de plantas (Yao et al, 2004).

Para que as diferentes classes de flavonóides sejam formadas, ocorrem modificações estruturais, como: adição ou redução, metilação de grupos hidroxila ou do núcleo dos flavonóides, hidroxilação, dimerização (biflavonóides), glicosilação de grupos hidroxila (O-glicosídeos) ou em algum núcleo carbônico (C-glicosídeos) (Balasundram et al., 2006).

Os flavonóides têm atividade antioxidante, devido à capacidade de estabilizar radicais livres e espécies reativas de oxigênio, essas propriedades se devem aos grupos hidroxilas ligadas à estrutura do anel aromático. A ação antioxidante pode ser potencializada com a adição de grupos hidroxilas, caso ocorra ligação a glicosídeos pode ocorrer à redução da atividade antioxidante. Estes fatores contribuem para a deslocalização de elétrons nos núcleos aromáticos, concedendo a estabilidade do radical que passa a não ter energia suficiente para reagir (Galati e Brien, 2004).

Os flavonóides possuem atividade anti- hemolítica, anticarcinogênica, peroxidação lipídica, formação de radicais superóxido e apoptose celular (Duthie e Dobson, 1999; Lee et al., 2003; Undeger et al., 2004). Além disso, têm propriedades de modulação do reparo do DNA, antialérgicas, antimicrobianas, vasoprotetoras, antitumorais e anti-inflamatórias (Erlund, 2004; Harbone, 1999; Martens e Mithofer, 2005; Miean e Mohamed, 2001; Muanda et al., 2010).

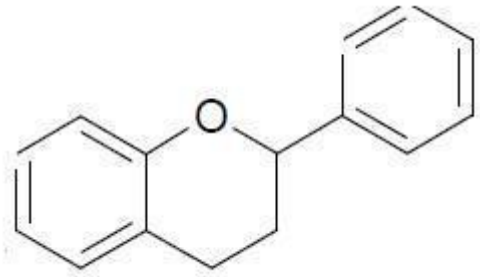

Figura 1. Estrutura química dos flavonoides (Balasundram et al, 2006).

2.5.2 Flavona

2.5.2.1 Propriedades químicas

A flavona (2-fenil-4H-1-benzopirano-4-ona) pertence ao grupo dos flavonóides, contém um anel característico e não há nenhum substituinte em sua estrutura (Figura 2), é encontrada em plantas como a salsa, o endro, e alguns grãos de cereais (Canivenc – Lavier et al., 1996; Choi et al., 2002).

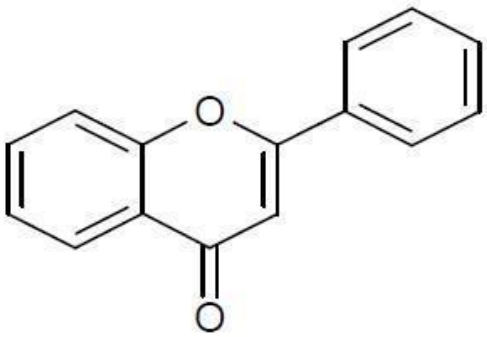

Figura 2. Estrutura química da flavona (Martens e Mithofer, 2005)

As flavonas são divididas em subgrupos o que depende da presença ou ausência de hidroxilação, *O*-metilação, metilação e isoprenilação, existe assim, uma infinidade de combinações estruturais, é possível que atualmente existem mais de 300 flavonas com ausência de açúcar na estrutura e pelo menos 500 glicosadas (Ullmannova e Popescu, 2007).

A salsa é uma erva que contém elevada concentração da flavona apigenina (5,7,4'-triidroxiflavona) e de crisoeriol (3'- *O*- metiluteolina). A apigenina é encontrada em grãos

de cereais, ervas e alguns vegetais (Middleton et al., 2000). A luteolina (5,7,3',4'-tetraidroxoflavona) outra flavona encontrada em abundância está presente no brócolis, cenoura e cebola. A pimenta vermelha e o aipo são as maiores fontes tanto de apigenina quanto de luteolina (Kromhout, 2001; Peterson e Dwyer, 1998).

Algumas flavonas metoxiladas conferem sabor amargo a frutas cítricas, como a nobiletina (5,6,7,8,3',4'- hematoxiflavona), sinesetina (5,6,7,3',4'- pentametoxiflavona), tangeritina (5,6,7,8,4'- pentametoxiflavona) (Van Zanden et al., 2005).

A síntese das flavona ocorre por duas vias diferentes que utilizam o mesmo substrato (Figura 3).

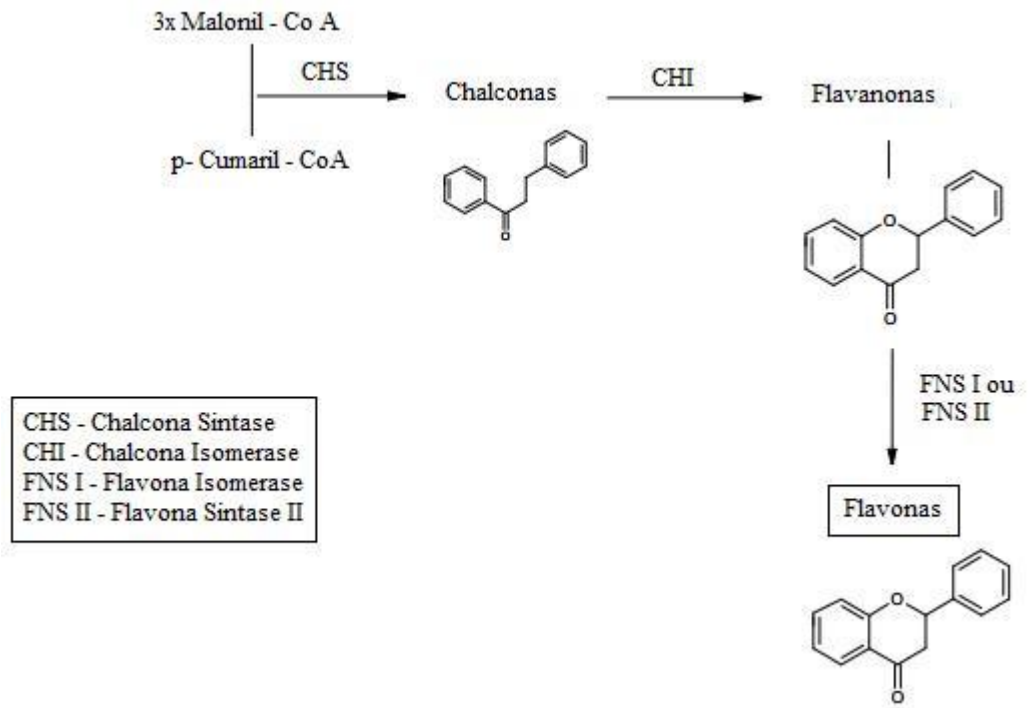

Figura 3. Esquema adaptado da síntese da flavona (Harbone, 1999).

2.5.2.2 Atividades biológicas

As propriedades terapêuticas da flavona dependem de sua estrutura e orientação dos grupos na molécula. Pequenas quantidades da substância pode agir como estimulante cardíaco e dentro do grupo das flavonas algumas são antibacterianas, antialérgicas, calmantes, e ainda, há aquelas que inibem ou estimulam sistemas de algumas enzimas como a Glutationa S-Transferase, UDP-Glicuroniltransferase 1 e isoformas 1A e 2B do citocromo P450 responsáveis pela biotransformação de xenobióticos (Fotsis et al., 1998).

Segundo estudos, a flavona pode afetar uma variedade de genes responsáveis pelo controle da proliferação celular e a resposta a danos ao DNA, por exemplo, como ocorre no processo de apoptose - células malignas expostas a flavona tendem a passar por esse processo. Células normais quando lesionadas podem recuperar sua capacidade proliferativa quando expostas a esse metabólito (Caltagirone et al., 2000).

O potencial terapêutico da flavona faz desta, um possível alvo para aplicação na área farmacêutica (Galati e Brien, 2004). Estudos epidemiológicos sugerem que, uma alta ingestão de flavona pode estar associada a uma redução de alguns tipos de câncer como, por exemplo, câncer do pulmão e do cólon, além da inflamação crônica e da osteoporose (Bektic et al., 2006; Collins e Franzblau, 1997; Palomino et al., 2002).

Células neurais com estresse oxidativo tiveram maior sobrevida quando expostas ao extrato de *Scutellaria baicalensis* (solidéu-de-baicak) que apresenta em sua composição quatro flavonas: wogonia (5,7-driidroxi-8-metoxiflavona), baicaleína (5,6,7-triidroxiflavona), skullpflavona I (5,2'-diidroxi-7,8-dimetoxiflavona), skullpflavona II (5,6'-diidroxi-6,7,8,2'-tetrametoxiflavona), nestes estudos as flavonas teriam apresentado o efeito de sequestro direto de espécies reativas de oxigênio (Ahmed et al., 1994).

A epigenina, luteolina, nobiletina e a tangeritina, tem atividades antimutagênica e inibem o crescimento de algumas células cancerosas (carcinoma de células escamosas) (Kromhout, 2001).

As flavonas epigenina e a luteolina atuam inibindo a atividade da proteína de resistência a multidroga em 47 e 53% respectivamente, a epigenina na dose de 80 µl mol. L^{-1} inibem alguns tipos de câncer como o de cólon, pele, tireóide e células de leucemia e ainda pode prevenir e tratar a doença proliferativa prostática. A epigenina inibe a topoisomerase I e a luteolina promove apoptose em células malignas a medida que causa danos ao DNA (Fotsis et al., 1998; Kromhout, 2001; Peterson e Dwyer, 1998; Van Zanden et al., 2005).

2.6 Testes in vitro

Novas metodologias que visam a triagem da viabilidade celular tem sido implementadas, as metodologias mais modernas são REMA (Resazurin Reduction Microtiter Assay) e MTT (dimethyl thiazolyl diphenyl tetrazolium salt) dentre outros, diferem-se da turbidimetria e da contagem de UFC (Unidade formadora de colônia) por

serem mais rápidas, terem um custo menor e um alto rendimento (Mosmann, 1983).

Para o ensaio do REMA é utilizada como substância reveladora a Resazurina, que tem potencial REDOX (Óxido-Redução), com mudança colorimétrica é um indicador de fluorescência que indica o metabolismo celular. A análise de proliferação de células serve para avaliar a viabilidade celular e auxilia na descoberta de drogas (Adoijan e Buchbauer, 2010).

O corante resazurina é um indicador de viabilidade celular que utiliza o poder redutor natural de células vivas para converter resazurina para a molécula fluorescente, resorufina. A resazurina é um composto não-tóxico, permeável que é de cor azul e não fluorescente. Ao entrar em contatos com as células é reduzida a resorufina, que produz fluorescência vermelho brilhante. As células viáveis convertem a resazurina em resorufina, gerando, assim, uma medida quantitativa da viabilidade e citotoxicidade (Ahmed et al., 1994).

O teste MTT é utilizado para micobactérias e avalia a ação antimicobacteriana de compostos isolados frente a uma cepa padronizada, e determina a quantidade de cepas viáveis. Nesta técnica o azul de tetrazólio liga-se a enzimas desidrogenases mitocondriais formando um substrato cromogênico o qual é um indicador de oxi-redução (Mosmann, 1983).

2.7 Toxicologia de produtos naturais

As plantas medicinais são utilizadas para o tratamento de enfermidades mesmo havendo fármacos sintéticos utilizados para essas mesmas doenças. Mas, assim como ocorre com as drogas sintéticas é importante cautela na utilização destas plantas, uma vez que, podem ser dotadas de substâncias tóxicas que podem levar a morte (Ferreira e Pinto, 2010).

Alguns dos efeitos tóxicos de substâncias presentes em plantas são: i) hepatotóxicos como apiol, safrol, lignanas e alcaloides pirrolizidínicos; ii) a ação tóxica renal que pode ser provocada por terpenos e saponinas e ii) algumas dermatites ocasionadas por espécies com abundância de lactonas sesquiterpênicas. Um exemplo é o confrei (*Symphytum officinale L.*) tradicionalmente utilizado pela população com cicatrizante por conta da alantoína presente, contudo, a plantas também apresenta alcaloides pirrolizidínicos que além de hepatotóxicos são comprovadamente carcinogênicos (Behling et al., 2004).

Os óleos essenciais obtidos das plantas medicinais também podem ter propriedades

tóxicas, alguns provenientes de frutos cítricos têm alto índice de defurano cumarinas em sua constituição e como consequência possuem atividade fotossensibilizante, os óleos da canela, funcho e alho tem um nível alto de cinamaldeído e podem causar reação alérgica de contato, o óleo da noz-moscada pode ocasionar alucinações o que se deve provavelmente pela presença da miristicina e da elemicina (Adorjan e Buchbauer, 2010).

No Brasil os estudos de toxicidade pré-clínica para fitoterápicos são normatizados pela Resolução N° 90/04 da Agência Nacional de Vigilância Sanitária que é baseada nas normas preconizadas pela OMS que por sua vez recomenda que sejam realizados estudos de toxicidade aguda e de genotoxicidade quando houver uso contínuo e prolongado do medicamento em humanos (ANVISA, 2004; Turolla e Nascimento, 2006; WHO, 1997).

Testes *in vivo* são importantes para que seja feita a observação dos efeitos dos extratos das plantas nos modelos animais utilizados. Destaca-se, o modelo utilizado de toxicidade aguda. Os testes de toxicidade aguda visam estabelecer um estudo que determina qual é a espécie mais sensível e o índice de letalidade, os extratos, compostos ou frações são administrados em uma ou várias doses em um período de 24 horas (Brasil, 1996). A maioria dos estudos pré-clínicos de produtos sintéticos ou naturais envolve a utilização de parâmetros bioquímicos, hematológicos e anatomopatológicos (Oliveira et al., 2010).

2.8 Genotoxicidade e mutagenicidade

Os agentes genotóxicos apresentam a capacidade de interagir com o DNA, formando alterações oxidativas ou rompendo a fita de DNA, o que compromete sua replicação e a transmissão genética. Normalmente a lesão é reparada pelo próprio organismo ou as células são eliminadas, quando a infecção é persistente, pode ocorre mutação (alterações hereditárias), que pode se perpetuar nas células filhas durante o processo de replicação, nesses casos o agente causador é denominado mutagênico (Eastmond et al., 2009).

Os ensaios de genotoxicidade desempenham um papel significativo na síntese de novos fármacos, devem ser efetuados nas fases iniciais, a fim de presumir uma possível atividade genotóxica e/ou carcinogênica e para subsidiar na obtenção de novas estruturas químicas com menor atividade tóxica (Freitas et al., 2013; Gollapudi e Krislma, 2000). Os ensaios de genotoxicidade *in vivo* detectam a genotoxicidade e a potencial

carcinogenicidade de agentes químicos ou físicos. Esses danos podem ser avaliados através dos ensaios: Cometa, micronúcleo, apoptose e fagocitose.

O ensaio cometa é realizado em microgel, é empregado no processo de eletroforese e quantificação através da detecção de quebras das fitas do DNA, em células individuais, usando microscopia. A sensibilidade da versão alcalina é maior, pois possibilita a expressão máxima dos danos em fitas simples (Tice et al., 2000).

O teste de micronúcleo visa a quantificação de fragmentos cromossômicos ou de cromossomos inteiros que não estão acoplados ao grupamento de cromossomos de uma célula - provendo, um pequeno núcleo individual, chamado micronúcleo (MN). A análise detecta aberrações cromossômicas em organismos eucarióticos, aplicada na detecção de agentes que prejudicam tanto o processo de ligação dos cromossomos às microfibrilas do fuso como aqueles que induzem quebras cromossômicas (Maluf et al., 2001).

No processo de fagocitose, os corpos apoptóticos são retirados do tecido por macrófagos, esta sinalização ocorre devido a translocação da fosfatidilserina do lado interno para o lado externo da membrana “marcando” as células que deverão ser fagocitadas, a leitura do teste é feita de maneira visual através da leitura de lâminas coradas especificamente (Fenech, 2000; Nicholson e Thornberry, 1997).

Além do mecanismo de “marcação” outro mecanismo pode ser atribuído para o processo de fagocitose como por exemplo, o aumento da quantidade sérica de plaquetas, estas, aderem as áreas lesionadas. Quando ligadas, as plaquetas têm sua estrutura modificada fazendo que haja a expressão de fosfolipídios (carga negativa) e receptores de glicoproteínas, assim, ocorre a liberação de mediadores químicos, tais como, o tromboxano (aglomeração de plaquetas) (Rang et al., 2003). Durante a ativação das plaquetas inúmeros fatores quimiotáticos são liberados (fator de agregação plaquetária), necessários para o crescimento e reparação, recrutando células do tecido, tais como macrófagos (Rang et al., 2003).

O processo de fagocitose do *M. tuberculosis* por macrófagos alveolares desencadeiam fatores imunopatológicos da tuberculose. O macrófago possui a capacidade de fagocitar micobactéria e elimina-la pela circulação sanguínea ou linfática, porém se não o fizer, esta multiplica-se intracelularmente. Desencadeando lesão pulmonar ou áreas secundárias (Henry, 1999).

3 OBJETIVOS

3.1 Objetivo Geral

Avaliar o efeito antimicobacteriano *in vitro* e os efeitos toxicológicos da exposição aguda da flavona por meio de modelos experimentais *in vivo.*

3.2 Objetivos Específicos

- Avaliar o efeito antimicobacteriano *in vitro*;
- Avaliar a toxicidade sistêmica provocada pela exposição aguda da flavona, através da análise de sinais clínicos de toxicidade; parâmetros bioquímicos e hematológicos;
- Avaliar a genotoxicidade e mutagenicidade "*in vivo*" da flavona em camundongos através do ensaio cometa, teste do micronúcleo e fagocitose.

4 REFERÊNCIAS BIBLIOGRÁFICAS

Adorjan, B., Buchbauer, G., 2010. Biological properties of essential oils: an updated review. Flavour Frag. J. 25, 407-426.

Ahmed, S.A., Gogal Jr, R.M., Walsh, J.E, 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J. Immunol. Methods.170, 211-224.

Andrade, F.J.L., Melo-Diniz, M.F.F., Oliveira, R.A.G, 1997. Plantas que atuam no trato respiratório. In: Simpósio de Plantas Medicinais do Brasil. Anais. UFC, Fortaleza.

ANVISA, Agência Nacional de Vigilância Sanitária, 2004. Resolução- RE 90 Guia para a realização de estudos de toxicidade pré-clínica de fitoterápicos. Disponível em: <http://www.diariodasleis.com.br/busca/exibelink.php?numlink=1-9-34-2004-03-16-90.> Acesso: 01 de junho de 2014.

Araujo, K.M.F.A., Figueiredo, T.M.R.M., Gomes, L.C.F., Pinto, M.L., Silva, T.C, et al., 2013. Evolução da distribuição espacial dos casos novos de tuberculose no município de Patos (PB), 2001-2010. Cad. Saúde Colet. 21, 296-302.

Araujo, R.C.P, Neves, F.A.R., Formagio, A.S.N, Kassuya, C.A.L., Stefanello, M.E.A, et al., 2014. Evaluation of the anti-mycobacterium tuberculosis activity and in vivo acutetoxicity of annona sylvatic. BMC Complement. Altern. Med. 14, 209.

Arbex, M.A., Varella, M.C.L., Siqueira, H.R., Mello, F.A.F., 2010. Drogas antituberculose: interações medicamentosas, efeitos adversos e utilização em situações especiais - parte 1: fármacos de primeira linha. J. Bras. Pneumol. 36, 626-640.

Arts, I.C., Hollman, P.C., 2005. Polyphenols and disease risk in epidemiologic studies. Am. J. Clin. Nutr. 81, 317S-325S.

Balasundram, N., Sundram, K., Samman, S., 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chem. 99, 191-203.

Barbosa, I.R., Costa Í.C.C., 2014. Estudo epidemiológico da coinfecção tuberculose-HIV no nordeste do Brasil. Rev. Patol. Trop. 43, 27-38.

Barreto II, M.L., 2009. Características dos serviços de saúde associadas à adesão ao tratamento da tuberculose. Rev. Saúde Públ. 43, 998-1005.

Behling, E.B., Sendão, M.C., Francescato, H.D.C., Antunes, L.M.G., Bianchi, M.L.P., 2004. Flavonoid quercetin: general aspects and biological actions. Alim. Nutr. 15, 285-292.

Bektic, J., Guggenberger, R., Spengler, B., Christoffel, V., Pelzer, A., et al., 2006. The flavonoid apigenin inhibits the proliferation of prostatic stromal cells via the MAPK-pathway and cell-cycle arrest in G1/S. Maturitas. 55, Supplement 1, S37-846.

Bellik, Y., Boukraâ, L., Alzahrani, H.A., Bakhotmah, A.B., Abdellah, F., et al., 2012. Molecular mechanism underlying anti-inflammatory and anti-allergic activities of phytochemicals: an update. Molecules. 18, 322-35.

Bignall, J.R., 1971. Tuberculosis in England and Wales in the next 20 years. Postgrad. Med. J. 47, 759-762.

Bisaglia, J.B., Santussi, W.M., Guedes, A.G.M., Gomes, A.P., Oliveira, P.C., et al., 2003. Atualização terapêutica da tuberculose: principais efeitos adversos dos fármacos. Bol. Pneumol. Sanit. 11, 53-59.

Brasil, Ministério da Saúde, 2002. Manual técnico para o controle da tuberculose: Cadernos de atenção básica. Série A. Normas e Manuais Técnicos n. 148, Brasília, pp. 70.

Brasil, Ministério da Saúde, 2013. Secretaria de Vigilância Epidemiológica em Saúde. Departamento de Vigilância Epidemiológica. Situação da Tuberculose no Brasil – PNCT.Brasília: Ministério da Saúde. Brasília.

Brasil, Ministério da Saúde. Portaria nº 116, de 8 de agosto de 1996, DOU 12/08/96 – Dispõe sobre normas para estudo da toxicidade de produtos fitoterápicos. Secretaria Nacional de Vigilância Sanitária.

Brasil. Ministério da Saúde, 2005. Secretaria de Vigilância em Saúde. Tuberculose. In: Guia de vigilância epidemiológica. Ministério da Saúde, Brasília, pp. 732.

Calixto, J., Yunes, R., 2001. Plantas medicinais: Sob a ótica da química medicinal moderna. Chapecó, pp. 500.

Caltagirone, S., Rossi, C., Poggi, A., Ranelletti, F.O., Natali, P.G., et al., 2000. Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. Int. J. Cancer. 87, 595-600.

Campos, W.R., Campos, G.S., Miranda, S.S., 2011. Tuberculose intraocular. Rev. Bras. Oftalmol. 70, 437-451.

Canivenc-Lavier, M.C., Vernevaut, M.F., Totis, M., Siess, M.H., Magdalou, J., et al., 1996. Comparative effects of flavonoids and model inducers on drug-metabolizing enzymes in rat liver. Toxicology. 114, 19-27.

Capone, D., Jansen, J.M., Lopes, A.J., Soares, M.O., Pinto, R.S., 2006. Diagnóstico radiográfico e tomográfico da tuberculose pulmonar. Rev. Hosp. Univ. Pedro Ernesto. 5, 46-5.

Carvalho, E., Meglin, S.R., Valim, A.R., Possuelo, L.G., Becker, D., 2011. Tuberculose no presídio regional de Santa Cruz do Sul. In: Salão de Ensino e de Extensão. Anais... p. 220.

Castellar, A., Coelho, T.S., Silva, P.E.A., Ramos, D.F., Lourenço, M.C.S., et al., 2011. A atividade de flavonas e ácido oleanólico de *Lippia lacunosa* contra cepas de *Mycobacterium tuberculosis* sensíveis e resistentes. Rev. Bras. Farmacogn. 21, 835-840.

Chimponda, T., Mukanganyama, S., 2010. Antimycobacterial activities of selected medicinal plants from Zimbabwe against *Mycobacterium aurum* and *Corynebacterium glutamicum***.** Trop. Biomed. 27,595–610.

Choi, J., Conrad, C.C., Malakowsky, C.A., Talent, J.M., Yuan, C.S., et al., 2002. Flavones from Scutellaria baicalensis Georgi attenuate apoptosis and protein oxidation in neuronal cell lines. Biochim. Biophys. Acta. 1571; 201-210.

Collins, L., Franzblau, S.G., 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and *Mycobacterium avium*. Antimicrob. Agents Chemother. 41, 1004-1009.

Conde, M.B., Melo, F.A.F., Maques, A.M.C., Cardoso, N.C., Pinheiro, V.G.F., et al., 2009. III Diretrizes para tuberculose da sociedade brasileira de pneumologia e tisiologia. J. Bras. Pneumol. 35, 1018-1048.

Dalcolmo, M.P., Andrade, M.K.N., Picon, P.D., 2007. Tuberculose multirresistente no Brasil: histórico e medidas de controle. Ver. Saúde Públ. 41, 34-42.

Demachki, N.T.T., Serruya, T., Pontes, C.D.N., 2013. Relato de caso: tuberculose extrapulmonar de localização laríngea. In: 12º Congresso Brasileiro de Medicina da Família e Comunidade. Anais... 12, p. 929.

Dolly, G., Nidhi, S., Sagar, B., Shweta, R., Shisha, A., 2012. *Ocimum kilimandscharicum*: A systematic review. J. Drug. Deliv. Therapeut. 2, 45-52.

Dornas, W.C., Olivera, T., Rodrigues-das Dores, R.G., Santos, A.F., Nagem, T.J., 2007. Flavonóides:potencial terapêutico no estresse oxidativo. Rev. Ciên. Farm. Básc. Aplic. 28, 241-249..

Duthie, S., Dobson, V., 1999. Dietary flavonoids protect human colonocyte DNA from oxidative attack in vitro. Eur. J. Clin. Nutr. 38, 28-34.

Eastmond, D.A., Hartwig, A., Anderson, D., Anwar, W.A., Cimino, M.C., et al., 2009. Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme. Mutagenesis. 24, 341-349.

Erlund, I., 2004. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. Nutr. Re. 24, 851-874.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D., Guo, Z., 1985 Medicinal plants in therapy. Bull. World Health Organ. 63, 965.

Fenech, M., 2000. The in vitro micronucleus technique. Mutat. Res. 455, 81– 95.

Ferreira, V.F., Pinto, A.C., 2010. A Fitoterapia no mundo atual. Quím. Nova. 33, 1829-1829.

Filho, V.C., Yunes, R.A., 1998. Estratégias para a obtenção de compostos farmacologicamente ativos a partir de plantas medicinais: conceitos sobre modificação estrutural para otimização da atividade. Quím. Nova. 21, 99-105.

Fotsis, T., Pepper, M.S., Montesano, R., Aktas, E., Breit, S., et al., 1998. Phytoestrogens and inhibition of angiogenesis. Baillieres Clin. Endocrinol. Metab. 12, 649-666.

Fraga, A.C.P., Torrens, A.W., Lobo, A.P., Dantas, D.C.A., et al., 2014. O controle da tuberculose no Brasil: avanços, inovações e desafios. Bol. Epidemiol. 44, 2-13.

Freitas, T.M., Celso, F.B., Silva, T.G., Picada, J.N., 2013. Avaliação do efeito genotóxico do aripiprazol em camundongos. Rev. Inicia. Cient. ULBRA. 8, 21-29.

Galati, G., Brien, O.P.J., 2004. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radic. Biol. Med. 37, 287-303.

Gollapudi, B.B., Krishna, G., 2000. Practical aspects of mutagenicity testing strategy: an industrial perspective. Mutat. Res. 455, 21-28.

Harbone, J.B., 1999. Handbook of natural flavonoids. Wiley, p 44.

Henry, J.B., 1999. Diagnósticos clínicos e tratamentos por métodos laboratoriais. 19. ed. Manole, São Paulo.

Hirayama, Y., Yoshimura, M., Ozeki, Y., Sugawara, I., Udagawa, T., et al., 2009. Mycobacteria exploit host hyaluronan for efficient extracellular replication. Pathogens. 10, 1021- 1032.

Kritski, A.L., 2010. Emergência de tuberculose resistente: renovado desafio. J. Bras. Pneumol. 36, 157-158.

Kromhout, D., 2001. Diet and cardiovascular disease. J. Nutr. Health Aging. 5, 144-149.

Lee, J.C., Klim, J., Park, J.K., Chung, G.H., Jang, Y.S., 2003. The antioxidant, rather than prooxidant, activities of quercetin on normal cells:: quercetin protects mouse thymocytes from glucose oxidase-mediated apoptosis. Exp. Cell. Res. 291, 386-397.

Leon-Diaz, R., Meckes, M., Said-Fernandez, S., Molina-Salinas, G.M., Vargas-Villarreal, J., et al., 2010. Antimycobacterial neolignans isolated from *Aristolochia taliscana*. Mem. Inst. Oswaldo Cruz. 105**,** 45–51.

Lönnroth, K., Kenneth, G.C., Chakaya, J.M., Chauhan, L.S., Floyd, K., 2010. Tuberculosis control and elimination 2010-50: cure, care and social development. Lancet. 375, 1814–29.

Luo, X., Pires, D., Ainsa, J.A., Gracia, B., Mulhovo, S., et al., 2011. Antimycobacterial evaluation and preliminary phytochemical investigation of selected medicinal plants traditionally used in Mozambique. J. Ethnopharmacol. 137,114– 120.

Maluf, S.W., Passo, D.F., Bacelar, A., Speit, G., Erdtmann, B., 2001. Assessment of DNA damage in lymphocytes of workers exposed to X-radiation using the micronucleus test and the comet assay. Environ. Mol. Mutagen. 38, 311-315.

Martens, S., Mithöfer, A., 2005. Flavones and flavone synthases. Phytochemistry. 66, 2399-2407.

Matias, E.F., Alves, E.F., Santos, B.S., Sobral, C.E., Alencar, F.J.V., et al., 2013. Biological activities and chemical characterization of *Cordia verbenacea* DC. as tool to validate the ethnobiological usage. Evid. Based. Complement. Alternat. Med. 164215, 164-215.

Middleton, E., Kandaswami, C., Theoharides, T.C., 2000. The effect of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. Pharmacol. Rev. 52, 673-751.

Miean, K.H., Mohamed, S., 2001. Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. J. Agric. Food. Chem. 49, 3106-3112.

Mitchison, D.A., 1990. Infectivity of patients with pulmonary tuberculosis during chemotherapy. Eur. Respir. J. 3, 385-386.

Mitchison, D.A., 2004. Antimicrobial therapy of tuberculosis: justification for currently recommended treatment regimens. Semin. Respir. Crit. Care Med. p. 307-315.

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

Muanda, F.N., Dicko, A., Soulimani, R., 2010. Assessment of polyphenolic compounds, in vitro antioxidant and anti-inflammation properties of *Securida calongipedunculata* root barks. C. R. Biol. 333, 663-669.

Newman, D.J., Cragg, G.M., 2007. Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. 70, 461-477.

Nicholson, D.W., Thornberry, N.A., 1997. Caspases: killer proteases.Trends Biochem. Sci. 22, 299 - 306.

Oliveira, L.P., Pinheiro, R.C., Vieira, M.S., Paula, J.R., Bara, M.T.F., et al., 2010. Atividade citotóxica e antiangiogênica de Punica granatum L., Punicaceae. Rev. Bras. Farmacogn. 20, 201-207.

Pablos-Mendez, A., Gowda, D.K., Frieden, T.R., 2002. Controlling multidrug-resistant tuberculosis and access to expensive drugs: a rational framework. Bull. World Health Organ. 80, 489- 500.

Palomino, J.C., Martin, A., Camacho, M., Guerra, H., Swings, J., et al., 2002. Resazurin microtier assay plate: simple and inexpensive method for deteccion of drug resistance *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 46, 2720-2722.

Pellenz, D.C., Silva, C.M.D., Seixas, L.A., Silva, F.C., 2011. Avaliação dos efeitos genotóxicos dos fármacos tuberculostáticos em células bucais de pacientes em tratamento. Rev. Cien. Fac. Edu. Mei. Amb. 2, 2-4.

Peterson, J., Dwyer, J., 1998. Flavonoids: Dietary occurrence and biochemical activity. J. Nutr. Res. 18, 1995-2018.

Pretto, J.B., 2005. Potencial antimicrobiano de extratos, frações e compostos puros obtidos

de algumas plantas da flora catarinense. 74f. Dissertação (Mestrado em Ciências Farmacêuticas) – Centro de Educação de Ciências da Saúde, Universidade do Vale do Itajaí, Itajaí, 2005.

Rang, H.P., Dale, M.M., Ritter, J.M., Moore, P.K., 2003. Farmacologia, 5ª ed. Guanabara Koogan S.A., Rio de Janeiro.

Rodrigues, A.R., Guimarães, J., Vieira, C., Alves, M., Vilarinho, S., et al., 2013. Otite média tuberculosa: A propósito de um caso clínico. Arq. Med. 27, 144-147.

Rojas, R., Caviedes, L., Aponte, J.C., Vaisberg, A.J., Lewis, W.H., Lamas, G., Sarasara, C., Gilman, R.H., Hammond, G.B., 2006. Aegicerin, the first oleanane triterpene with wide-ranging antimycobacterial activity, isolated from *Clavija procera***.** J. Nat. Prod. 69, 845–854.

Ruffino-Netto A (2001) Programa de controle da tuberculose no Brasil: situação atual e novas perspectivas. Inf. Epidemiol. SUS. 10: 129-138.

San Pedro, A., Oliveira, R.M., 2013. Tuberculose e indicadores socioeconômicos: revisão sistemática da literatura. Rev. Panam. Salud. Publ. 33, 294-301.

Selig, L., Geluda, K., Junqueira, T., Brito, R., 2012. A tuberculose no cotidiano médico eo efeito bumerangue do abandono. Cien. Saúde Colet.17, 113-122.

SINAN, Sistema de Informação de Agravos de Notificação, 2014. Tuberculose - Casos confirmados notificados no Sistema de Informação de Agravos de Notificação – Sinan Net, 2014. Disponível em: < http://dtr2004.saude.gov.br/sinanweb/tabnet/dh?sinannet/tuberculose/bases/tuberc brnet.Def >. Acesso em: 18.fev.2014.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., et al., 2000. Single cell gel/ Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Mutagenesis. 35, 206-221.

Tülin, A., Emmanuel, M.T., Fatih, S., Seyma, M., Hatice, A., 2013. Preliminary antimycobacterial study on selected Turkish plants (Lamiaceae) against Mycobacterium tuberculosis and search for some phenolic constituents. BMC Complement. Altern. Med. 13, 366-389.

Turolla, M.S., Nascimento, E.S., 2006. Informações toxicológicas de alguns fitoterápicos utilizados no Brasil. Braz. J. Pharm. Sci. 42, 289-306.

Ullmannova, V., Popescu, N.C., 2007. Inhibition of cell proliferation, induction of apoptosis, reactivation of DLC1, and modulation of other gene expression by dietary flavone in breast cancer cell lines. Cancer Detect. Prev. 31, 110-118.

Ündeğer, Ü., Aydin, S., Başaran, A.A., 2004. The modulating effects of quercetin and rutin on the mitomycin C induced DNA damage. Toxicol. Lett. 151, 143-149.

Van Zanden, J.J., Wortelboer, H.M., Bijlsma, S., Punt, A., Usta, M., et al., 2005. Quantitative structure activity relationship studies on the flavonoid mediated inhibition of multidrug resistance proteins 1 and 2. Biochem. Pharmacol. 69, 699-708.

Veiga-Junior, V.F., Pinto, A.C., Maciel, M.A.M., 2005. Plantas medicinais: cura segura? Quím. Nova. 28, 519-528.

Vicentin, G., Santo, A.H., Carvalho, M.S., 2002. Mortalidade por tuberculose e indicadores sociais no município do Rio de Janeiro. Ciênc. Saúde Colet. 7, 253-26.

Warner, D.F., Mizrahi, V., 2004. Mycobacterial genetics in target validation. Drug Discov. Today: Technologies. 1, 93-98.

Webster, D., Lee, T.D., Moore, J., Manning, T., Kunimoto, D., et al., 2010. Antimycobacterial screening of traditional medicinal plants using the microplate resazurin assay. Can. J. Microbiol. 56, 487–494.

WHO, World Health Organization, 1997. Safety in Health-Care Laboratories. pp. 148.

WHO, World Health Organization, 2002. Report – Global Tuberculosis Control: Surveillance, Planning, Financing.

WHO, World Health Organization, 2009. Report: Global Tuberculosis Control: Epidemiology, Strategy, Financing.

WHO, World Health Organization, 2013. Multidrug-resistant tuberculosis – 2013. Update. WHO.

Yao, L., Jiang, Y., Datta, N., Singanusong, R., Liu, X., et al., 2004. HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia. Food Chem. 84, 253-263.

5 ANEXOS

Toxicogenetic analysis and in vitro antimycobacterial activity of flavone compound

Vanessa Vilamaior de Souza[1], Flávio Henrique Souza de Araújo[2], Claudia Rodrigues Berno[2], Rodrigo Juliano Oliveira[2], Flora Martinez Figueira Moreira[1], Candida Aparecida Leite Kassuya[1], Julio Croda[1,3*].

1 Faculty of Health Sciences, Federal University of Grande Dourados, Dourados, MS, Brazil;

2 Federal University of Mato Grosso do Sul (UFMS), Campo Grande, MS, Brazil.

3 Oswaldo Cruz Foundation, Campo Grande, Brazil

Corresponding author: Faculdade de Ciências da Saúde, Universidade Federal da Grande Dourados, Dourados/MS, 79825-070, MS, Brazil, Phone: +55 67 3410-2321, Fax: +55 67 3410-2326. *E-mail adress: candida2005@gmail.com*

Abstract

Previous studies from our group have shown that the ethyl acetate extract fraction obtained from *Annona sylvatica* and the flavonoid luteolin showed anti-mycobacterial activity *in vitro*. As the compound flavone (2-phenyl-4H-1-benzopyran-4-one) belongs to the group of flavonoids and has close structural similarity to luteolin, we decided to examine the antimicrobial activity, acute toxicity as well as the genotoxic and mutagenic actions of this flavone. For this purpose, the resazurin reduction microtiter assay (REMA), the acute toxicity test as well as comet assays, the micronucleus test and phagocytosis assay were performed. The flavone showed anti-mycobacterial activity at a minimal inhibitory concentration (MIC) of 28.90 µg/mL while no signs of toxicity were detected in hematological and biochemical patterns analyzed by the acute toxicity test in female mice (the dose range was 175 to 2000 mg/Kg). In the micronucleus assay, the flavone did not exerted mutagenic activity. Splenic phagocytosis was increased by approximately 1.48-fold compared to that of the negative control. Our results show that the flavone does not have either acute toxicity or mutagenic activity and can act as an anti-mycobacterial agent and increase phagocytosis, which appears to enhance the immune response.

Key words: Flavone; acute toxicity; antimycobacterial activity; mutagenic activity; mice.

Introduction

Flavonoids obtained from medicinal plants or foods have been used for prevention and treatment of disease. For example, flavonoid-enriched diets have been associated with a reduced incidence and mortality of prostate cancer (Haddad et al., 2006). Dietary bioactive flavonoids have the potential to restore microglial cells to a more quiescent state (Johnson, 2015) and plants rich in flavonoids such as *Satureja aintabensis* and *Thymus sibthorpii* are capable of killing Mycobacteria strains (Askun et al., 2013).

No new treatment for tuberculosis has been developed in the last 20 years and new drugs or alternative therapeutics are needed. Extracts or compounds obtained from plants display anti-mycobacterial activity, for example: *Acorus calamus*, *Heracleum maximum* (Webster et al., 2010), *Parinari curatellifolia* (Chimponda and Mukanganyama, 2010), *Aristolochia taliscana* Gancho (León-Díaz et al., 2010); *Maerua edulis*, *Securidaca longepedunculata*, *Zanthoxylum capense*, and *Tabernaemontana elegans* (Luo et al., 2011).

Previous studies from our group have shown that the ethyl acetate extract fraction obtained from *Annona sylvatica*, and the flavonoid luteolin both showed anti-mycobacterial activity *in vitro* (Araujo et al., 2014). Also, several studies corroborated the possibility that flavonoids might provide "a model" for anti-tuberculosis drug design (Askun et al., 2013). Indeed flavonoid tests against micobacteria have shown that several flavonoids may be considered to be antimicobacterial. Flavonoids are classified as constitutive antimicrobial substances, recently termed as “Phytoanticipins,” especially those belonging to prenylated flavonoids and isoflavones, but not excluding other classes of compounds (Barron and Ibrahim, 1996).

With a successful of luteo lin against *M. tuberculosis in vitro*, we decided to test the flavone, a compound with structure similar to luteolina against *M. tuberculosis.* The safety study of isolated flavone is relevant because several material products in masked contain the flavone compound. Toxicological genetic, such as the micronucleus test and the comet assay, are tests widely accepted by international agencies, government institutons, ANVISA (Sanitary Surveillance Agency) and OECD (Organization for Economic Cooperation in Development) Test Guideline 487 (OECD 2010), Test Guideline 489 (OECD 2014).

For development of new chemicals or drug candidates prior to entering the market (Witte et al., 2007). Micronucleus is a moninvasive cytogenetic technique that can detect chromosomal mutations, wich are involved in risk of development of carcionogenesis (Fenech, 2000; Carbonari et al., 2005).

In the current work, the antimicobacterial activity flavones was demonstrated *in vitro*, and toxicity analysis was performed by testing for acute oral toxicity in mice as well by assaying the flavone for genotoxic and mutagenic actions in experimental mouse models..

Materials and Methods

The synthetic flavone (2-phenyl-4H-1-benzopyran-4-ona), middle Brook 7H9 culture medium, isoniazid, resazurin, cyclophosphamide, agarose, Ethidium Bromide, acridine orange used to perform the tests was purchased from Sigma-Aldrich® Co. LLC (St. Louis, MO, USA). Other drugs and reagents were of analytical grade.

Experiment 1: Minimum Concentration Inhibitory (MIC)

The Minimum Concentration Inhibitory assay was performed according to Araujo et al. (2014) with minor modifications described by Palomino et al. (2002). An aliquot of the *M. tuberculosis* H37Rv ATCC 27294 strain (700 µL of the frozen strain) was grown into 80 ml of Middle Brook 7H9 culture medium supplemented with 10% OADC (oleic acid, bovine albumin fraction V, dextrose and catalase) and 0.5% of glycerol (carbon source). Then, incubation was performed at 35 °C at 100 rpm in an Incubator Shaker. For all drugs, the first solution was prepared 10 mg/ml. The flavone and rifampicin solutions were prepared with DMSO while the isoniazid (INH) solution was made in sterile water. To conduct the tests, serial solutions of flavone (0.98 to 250 µg/ml) and of reference drugs (0.004 to 1 µg/ml) were prepared in supplemented Middlebrook 7H9 and a 100 µl of bacterial suspension (5 x 10^5 UFC/ml) was then added to each solution in a 96-well plate. Plates were incubated for 7 days at 37°C, after 30 µl of resazurin (Sigma-Aldrich) in sterile water (0.01%) was added to the whole plate, and the samples were incubated for 24 hours at 37ºC. At the end of this period, the plates were read by measuring the optical density at 492 nm. MIC was defined as the lowest concentration resulting in 90% growth inhibition of *M. tuberculosis*. A sample with MIC <250 µg/mL was defined as active against *M. tuberculosis*. Assays were performed in duplicate.

Experiment 2: Acute Oral Toxicity

Animals - Experiments were performed using female *Swiss* mice (28 - 32g, n = 9) provided by the Federal University of Mato Grosso do Sul (UFMS). Animals were maintained under a light-dark cycle of 12 h with controlled humidity (60-80%) and temperature (22 ± 1 ° C). Animals were acclimated to the experimental environment for at least 2 h before testing and were used only once during the experiment. The procedures were performed in accordance with approval by the Ethics Committee on Animal Use

(CEUA) of the Federal University of Grande Dourados (Protocol. 005/2010).

Doses and Treatment - Toxicity studies were based on protocol 425 from the OECD (OEDC, 2006; OEDC, 2008 and the protocols established by ANVISA (ANVISA, 2010). According to the established protocol, 9 animals were used and each received a single oral administration of the compound flavone. Initially one of the animals received a dose of 175 mg/kg and was observed at 30 minutes, 1, 2, 4, 6, 12, 24 and 48 h. After this period, a second animal received a dose of 560 mg/ kg, after 48 hours the third animal received a dose of 1792 mg/kg and after an additional 48 h a fourth animal received a dose of 2000 mg/kg. After the last dose administered no deaths were observed and according to the protocol, 4 more animals received 2000 mg/kg while the control group received the vehicle (0.1 % of tween 80, 0.2 % DMSO in saline 0.9%) used for diluting flavones. The animals fasted for 2 h before the administration of flavone with free access only to water; food was allowed 3 h after administration. The body weight of the animals was checked on the first day and on the next 14 days following administration. During the experimental period, the animals were observed daily for clinical aspects, including posture, seizures/tremors, consistency and appearance of the feces, eyelid closure, piloerection, appearance of skin and hair, stress, salivation eyes, behavior, body weight and consumption of food and water.

Evaluation of Acute Toxicity - Initially, the animals were anesthetized with ketamine and xylazine (25 mg/Kg and 10 mg/Kg, respectively) and after anesthesia, the animals were euthanized by cervical dislocation and the presence of macroscopic alterations in organs such as spleen, heart, liver, lungs and kidneys was assessed for appearance, color, size, weight and consistency.

Experiment 3: Genetic toxicological evaluations

Chemical Agents, animals and experimental design - The same animal was used for

comet, micronucleus and Splenic Phagocytosis Assay. Experiments were performed using female *Swiss* mice (n = 25, weighing 28-32g, aged approximately 60 days), provided by the UFMS. Procedures were also approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Grande Dourados (Protocol. 005/2010).

The animals were divided into the following groups: **Group 1 -** Control: intraperitoneal injection of saline (0.1 mL/10g of body weight, ip); Hydroalcoholic solution (1:1) was orally administered (0.1 mL/10g of body weight, vo); **Group 2 -** Positive control: intraperitoneal injection of cyclophosphamide (100 mg/kg of body weight, i.p.); Saline was orally administered; **Groups 3, 4 and 5:** Intraperitoneal injection of saline solution and flavone by the oral route (175, 560, 1792 mg/kg). The animals received a single dose of flavone. The vehicle (same solution used to dissolve flavone) used was 0.1 % of tween 80, 0.2 % DMSO in saline 0.9%.

Following the administration of the compounds, 20 µL of peripheral blood were collected for the micronucleus test at 24 hours (T1), 48 hours (T2) and 72 hours (T3) post injection. T1 samples were collected for comet assay. After 72 hours, the animals were anesthetized with ketamine and xylazine (25 mg/kg and 10 mg/kg, respectively) and peripheral blood for hematologic and biochemical analyses were collected on retrorbital Plexus. Following this, the animals were euthanized by cervical dislocation for organ harvesting.

Comet Assay - After 24 hours of treatment with flavone, 20 µl of blood was collected by caudal puncture and was homogenized with 120 µL of LPM agarose (1.5%) at 37 °C and was subsequently deposited on slides previously prepared with agarose (5%) and slides were immediately covered with coverslips (24x60 mm). The slides were kept at 4 ° C for 20 minutes to solidify the agarose. Thereafter, the slides were protected from light to prevent additional DNA damage.

After this period, the coverslips were removed and the slides submerged in recently prepared lysis solution and kept at 4 °C. The lysis solution is composed of (890 mL of stock lysis solution – 2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris, exact pH of 10.0 adjusted with solid NaOH, 890.0 mL of milli-Q water, 1.0 mL of Triton and 10.0 mL of DMSO) and submersion was over a period of 1 hour at 4 ° C in the dark.

Afterwards, the slides were transferred to a horizontal electrophoresis tank, which contained buffer solution with pH higher than 13.0 (300.0 mM of NaOH and 1.0 mM of EDTA at pH 13.0, which was prepared from a stock solution of NaOH 10.0 N and 200.0 mM of EDTA, pH 10.0), the slides were maintained in the tank for a period of 20 minutes at 4 ° C for DNA denaturation. Electrophoresis was carried out at 25 V and 30 mA (25 V/cm) and the slides were then neutralized with a sequence of 3 cycles of 5 minute immersion in buffer with pH 7.5 (0.4 M Tris - HCl) and then dried at room temperature and fixed in absolute ethanol for 10 minutes. For the DNA analyses, the slides were stained with 100µL of ethidium bromide (0.002 mg / mL). Slides were observed with a fluorescence microscope using the 40x objective and a 515-560 nm excitation filter. One hundred cells were analyzed and classified by length and intensity of the tail. Class 0 - no damage; Class 1 - cells with tail inferior to the nucleus diameter; Class 2 – cells with tail size 1 and 2 times the diameter of the nucleus; Class 3 - cells with tail 2 times superior the diameter of the nucleus. Cells containing fully fragmented nuclei were not included in the calculations. At the end of the analysis, the *score* of each treatment was determined by multiplying the number of nuclei counted in each class by the class value (0, 1, 2 or 3) (Navarro et al., 2014).

Micronucleus Assay of Peripheral Blood - After treatment, blood from the tail of each animal was collected at three different times (T1, T2 and T3). Twenty microliters of blood was deposited on slides previously prepared with acridine orange (1mg/ml). The slides were placed in a freezer at -20 º C. The count of polychromatic erythroblasts with

micronuclei was determined under a fluorescence microscope with blue light (488 nm) using a 100x objective and 2,000 cells were observed (Navarro et al., 2014).

Splenic Phagocytosis Assay - One third of the spleen was ground in a physiological solution. One hundred microliters of the suspension was placed on a previously prepared slide with 20µL of acridine orange (1mg/mL) and then slides were covered with coverslips. The slides were maintained frozen until the time of readings, which were made using a 400x fluorescence microscope, equipped with a 420-490 nm excitation filter and a 520 nm barrier filter. Analyzes were conducted according to the protocol established by Navarro et al. (2014), which evaluated the presence or absence of phagocytosis.

Statistical Analysis - The mean ± standard error (SEM) was calculated, and ANOVA/Tukey's tests were performed at $p < 0.05$ using GraphPad Prism software (version 3.02; Graph-Pad Software Inc., San Diego, CA, USA).

Results and discussion

The MIC value of flavone against a strain *M. tuberculosis* H37Rv ATCC 27294 was 28.90 µg/mL while values determined as standards were 0.030 mM/mL for isoniazid and 0.019 for rifampicin. Negative controls tested included myrtenol, limonene, cineol and camphor (others isolated substances from plants), which did not exhibit ability to kill *M. tuberculosis* at low concentrations.

Flavonoids are secondary metabolites obtained from the plant kingdom and which have a diversity of structures and biological activities. Depending on the basic structure, compounds belonging to flavonoid class may be divided into the flavone, flavonole and flavonone subclasses (Bravo, 1998). Because Araujo et al. (2014) showed that luteolin and

products of *A. sylvatica* have a potential role in killing *M. tuberculosis* without toxicity in acute toxicity, we focused our studies on flavone antimicobacterial activity and safety.

Other studies have reported the effects of the six natural flavones against a strain of *M. tuberculosis* with respective lowest *concentration* of an antimicrobial that will inhibit the growth of a microorganism (MIC): 3'-O-dimethoxy-5, 6,4 '-trihyidroxyflavone (MIC 200 µg/mL) cirsimaritin (MIC 50 µg/mL), eupatilin (MIC 50 µg/mL), salvigenin (MIC 100 µg/mL), 7-methoxyflavone (MIC 12.5-50 µg/mL) and 5,4 '-dihydroxy-7-methoxyflavone (MIC 25-50 µg/mL) (Castellar et al., 2011) and luteolin (MIC of 236.89 µg/mL) (Araujo et al., 2014). Also, synthetic flavones like 3,6-dimethoxy flavone, 6,2'-dimethoxy flavone (both with values higher than 100 µg/mL) and 6,3'-dimethoxy flavone (MIC 100 µg/mL) also demonstrated antimicobacterial activity (Jayshree et al., 2012**)**. The antimicobacterial activities of flavonoids and flavones have already been reported by (Lin et al., 2002; Brown et al., 2007, Sivakumar et al., 2007; Zheng et al., 2014). The results led Zheng et al. (2014) to conclude that flavonoids provide a basis for rational structural design and discovery of novel anti-tuberculosis drugs. Our study corroborates and extends this work and contributes another flavone with higher antimicobacterial activity than observed for other flavone compounds *in vitro*.

With regard to structure and activity, it seems that the flavone compound has the same structure as apigenin, luteonin and other flavones. All of them have pharmacologic structures suitable for the development of new drugs. When 3 or 4 hydroxyl groups are added to flavone, 4′,5,7-Trihydroxyflavone (apigenin) or 3′,4′,5,7-Tetrahydroxyflavone (luteolin), respectively, the antimicrobacterial activity is decreased in case of luteolin **(**Araujo et al., 2014) or extinguished in case of apigenin (Chen et al., 2010). Another consideration is the methoxy group; when methoxy groups were added to flavone

structures, the anti-tubercular activity was reduced or eliminated (Jayshree et al., 2012). In addition to the direct effect of flavones against *Mycobacterium tuberculosis***,** apigenin and quercetin are hyaluronidase inhibitors that also inhibited hyaluronan dependent growth of *M. tuberculosis (*Hirayama et al. (2009)). *M. tuberculosis increase of* hyaluronan synthase expression is a mechanism of mycobacteria pathogenisis according to Hirayama et al. (2009). These results lead us to suggest that flavone and its natural and synthetic derivatives can play an important role in protection against *M. tuberculosis*, but more studies are needed to evaluate their effectiveness *in vivo*.

Thus, studies of acute toxicity and genetic toxicological studies are required by the Brazilian Health Surveillance Agency (ANVISA) to regulate and register phytotherapic products in order to evaluate the safety of their use (ANVISA, 2004, OECD, 2008). In addition, flavonoids are considered to have relatively little toxicity when compared to compounds such as the alkaloid class of drugs. However, some flavonoids such asflavone, kaempferol, and other flavonoids have mutagenic properties (Middleton et al., 2000).

The animals used in the present study were exposed to flavone and did not exhibit clinical signs of toxicity at the doses administered and no significant changes were observed in water and food intake. Furthermore, the absolute and relative weight of the organs (liver, kidneys and lungs) showed no statistically significant difference (data not shown). Hematological analysis demonstrated that the values of hematocrit, hemoglobin, platelet count, erythrocytes and total and differential count of leukocytes in the treated animals were similar to the negative control, indicating that the product provided has no effect on circulating blood cells or their production (Table 1). The levels of neutrophils, eosinophils, monocytes, basophils and lymphocytes remained within the reference range, while platelets had an average of 366,000 in groups treated with flavone, and 348,000 and

326,000 in the positive control and negative control respectively (Table 1). The biochemical profile indicated a difference in aminotransferase enzyme and urea in the group treated with cyclophosphamide, indicating the occurrence of acute injury. Treated groups had results similar to the negative control group. There was no significant statistical difference in the levels of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Urea (U) and Creatinine (Cr) in the treated groups (Table 2). Thus, it was found that after the acute exposure of animals to flavone, no sign of toxicity was detected.

The intermediary dose of the flavone was genotoxic (increase of 1.36-fold compared to that of the negative control; $p>0.05$). However, the highest and the lowest doses did not show evidence of genotoxicity (Table 3). Thus, there is a tendency observed in Table 3 for an increase of DNA damage. It is likely that these lesions were not fixed in DNA because the same doses did not show a mutagenic (effect Table 4). Taken together, these data suggest that the genotoxic lesions may be capable of repair (Oliveira et al., 2015).

The genotoxic and mutagenic effects of flavone were examined respectively through the comet and micronucleus assays in peripheral blood. With these techniques it is possible to detect changes in DNA (*i.e.* single-strand breaks, double alkali-labile sites, cross-linking, loss of excision repair), clastogenic damage and / or damage to the mitotic spindle (Oliveira et al., 2007; Hermeto et al., 2015).

Table 4 presents the evaluation of the mutagenic effects following acute treatment with flavone. There was no statistically significant difference ($P> 0.05$) between the negative control and treated groups, demonstrating the absence of mutagenic effects. The positive control group (cyclophosphamide) showed a significant increase ($p <0.05$) in the number of micronuclei, of 6.22-, 4.39- and 4.09-fold at 24, 48 and 72h, respectively, compared to that of the negative control (Table 4).

Through 72h, no sign of mutagenic activity was observed and thus the data suggest that the flavone metabolites are also non-mutagenic. The same data did not show a dose-response correlation because the increase in the dose did not result in an increase in the frequency of micronuclei in the groups treated with the flavone. Also, data from these groups did not shown a linear reduction in the frequency of micronuclei after 72h, as observed in the positive control (Table 4). In this group, the results showed a relationship with cyclophosphamide metabolism, in which reducing the amount of the chemical and its metabolites (mutagens) in the stock reduced the frequency of DNA damage over 72 hours.

The decrease in the frequency of DNA damage caused by cyclophosphamide and its metabolites can be explained by the body's detoxification capacity, which can occur through NADPH-mediated oxidation by various aldehyde dehydrogenases (ALDH1A1 and ALDH3A1) or by another pathway, the conjugation of CP with glutathione by various glutathione S-transferases (GSTs; GSTA1, GSTM1, GSTP1 and GSTT1) (Pinto et al., 2009).

Immunomodulation was confirmed in the treatments with flavone and cyclophosphamide because of the increase in the splenic phagocytosis of approximately 1.63-, 1.46- 1.48- and 1.48-fold for the chemotherapy drug and the doses of 175, 560 e 1792 mg/kg, respectively, when compared to that in the negative control group ($p<0.05$) (Table 5). In the comet, micronuclei and splenic phagocytosis assays, a dose-response correlation was not observed. However, cells with genotoxic and mutagenic damage (clastogenic or aneugenic) can be removed from the circulatory system by the spleen (Table 5), which is a well-established and very important function of this organ (Lima et al., 2013).

Conclusion

Our results show that flavone does not have either acute toxicity or mutagenic activity and can act as an anti-mycobacterial agent and increase phagocytosis, which seems to change the immune response positively. These findings that flavones may have therapeutic applications against tuberculosis.

Acknowledgments

The authors are thankful to CAPES, CNPq, and FUNDECT for the financial support.

References

Araujo, R.C., Neves, F.A., Formagio, A.S., Kassuya, C.A., Stefanello, M.E., Souza, V.V., Pavan, F.R., Croda, J., 2014. Evaluation of the anti-mycobacterium tuberculosis activity and in vivo acute toxicity of Annona sylvatica. BMC Complement. Altern Med. 14, 209.

Askun, T., Tekwu, E.M., Satil, F., Modanlioglu, S., Aydeniz, H, 2013. Preliminary antimycobacterial study on selected Turkish plants (Lamiaceae) against *Mycobacterium tuberculosis* and search for some phenolic constituents. BMC Complement. Altern. Med., 13, 365.

Barron, D., Ibrahim, R.K., 1996. Isoprenylated Flavonoids: a review. Phytochem. 43, 921-982.

Bravo, L., 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56, 317–333.

Brazilian Health Surveillance Agency (ANVISA), 2004. Guide to toxicity studies conducting preclinical herbal. Available at: < http://www.diariodasleis.com.br/busca/exibelink.php?numlink=1-9-34-2004-03-16-90> Acess: June 01, 2014.

Brazilian Health Surveillance Agency (ANVISA), 2010. Guide for conducting non-clinical safety studies required for drug development. 1, 37.

Brown, A.K., Papaemmanouil, A., Bhowruth, V., Bhatt, A., Dover, B., Besra, G.S., 2007. Flavonoid inhibitors as novel antimycobacterial agents targeting Rv0636, a putative dehydratase enzyme involved in Mycobacterium tuberculosis fatty acid synthase II. Microbiol. 10, 3314-3322.

Carbonari, K., Gonçalves L., Roth, D., Moreira, P., 2005. Increased micronucleated cell frequency related to exposure to radiation emitted by computer cathode ray tube video display monitors. Genet. Mol. Biol. 28, 469-474.

Castellar, A., Coelho, T.S., Silva, P.E.A., Ramos D.F., Lourenço, M.C.S., Lage, C.L.S., Julião, L.S., Barbosa, Y.G., Leitão, S.G., 2011. The activity of flavones and oleanolic acid from *Lippia lacunosa* against susceptible and resistant *Mycobacterium tuberculosis* strains. Rev. Bras. Farmacogn. 215, 835-840.

Chen, L.W, Cheng, M.J., Peng, C.F., Chen, I.S., 2010. Secondary metabolites and antimycobacterial activities from the roots of Ficus nervosa. Chem. Biodivers. 7, 1814-1821.

Chimponda, T., Mukanganyama, S., 2010. Antimycobacterial activities of selected medicinal plants from Zimbabwe against *Mycobacterium aurum* and *Corynebacterium glutamicum*. Trop. Biomed. 27, 595-610.

Lima, P.L., Sugui, M.M., Petrício, A.I., Vilela, L.C., Pinto, A.V., Martins, P.R., Kaneno, R., Ribeiro, D.A., Salvadori, D.M., Ribeiro, L.R., 2013. Lentinula edodes (shiitake) modulates chemically induced mutagenesis by enhancing pitting. J. Med. Food. 16, 733-739.

Fenech, M., 2000. The in vitro micronucleus technique. Mutat. Res. 455: 81-95.

Haddad, A.Q., Venkateswaran, V., Viswanathan, L., Teahan, S.J., Fleshner, N.E., Klotz, L.H., 2006. Novel antiproliferative flavonoids induce cell cycle arrest in human prostate cancer cell lines. Prostate Cancer Prostatic. Dis. 9, 68-76.

Hermeto, L.C., Oliveira, R.J., Matuo, R., Jardim, P.H., DeRossi, R., Antoniolli, A.C., Deffune, E., Evaristo, T.C., Santana, Á.E., 2015. Evaluation of pH effects on genomic integrity in adipose-derived mesenchymal stem cells using the comet assay. Genet. Mol. Res. 14, 339-348.

Hirayama, Y., Yoshimura, M., Ozeki, Y., Sugawara, I., Udagawa, T., Mizuno, S., Itano, N., Kimata, K., Tamaru, A., Ogura, H., Kobayashi, K., Matsumoto, S., 2009. Mycobacteria exploit host hyaluronan for efficient extracellular replication. PLoS Pathog. 5, e1000643.

Jayshree, N., Narayanan, N., Sriram L., 2012. Antibacterial, antifungal and antimycobacterial studies on some synthetic dimethoxy flavones. *Asian* J. Pharm. Clin. Res. 5, 101-103.

Johnson, R.W, 2015. Feeding the beast: Can microglia in the senescent brain be regulated by diet? Brain Behav. Immun. 43, 1-8.

León-Díaz, R., Meckes, M., Said-Fernández, S., Molina-Salinas, G.M., Vargas-Villarreal, J., Torres, J., Luna-Herrera, J., Jiménez-Arellanes, A., 2010. Antimycobacterial neolignans isolated from *Aristolochia taliscana*. Mem. Inst. Oswaldo Cruz. 105, 45-51.

Lin, Y.M., Zhou, Y., Flavin, M.T., Zhou, L.M., Nie, W., Chen, F.C., 2002. Chalcones and flavonoids as anti-tuberculosis agents. Bioorg. Med. Chem. 10, 2795-2802.

Luo, X., Pires, D., Aínsa, J.A., Gracia, B., Mulhovo, S., Duarte, A., Anes, E., Ferreira, M.J., 2011. Antimycobacterial evaluation and preliminary phytochemical investigation of selected medicinal plants traditionally used in Mozambique. J. Ethnopharmacol. 137, 114-120.

Middleton, E. Jr, Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52, 673-751.

Navarro, S.D., Beatriz, A., Meza, A., Pesarini, J.R., Gomes, R. da S., Karaziack,C.B., Cunha-Laura, A.L., Monreal, A.C., Romão, W., Lacerda Júnior, V., Mauro, M. de O., Oliveira R.J., 2014. A new synthetic resorcinolic lipid 3-heptyl-3,4,6-trimethoxy-3H-isobenzofuran-1-one: evaluation of toxicology and ability to potentiate the mutagenic and apoptotic effects of cyclophosphamide. Eur. J. Med. Chem. 21, 132-42.

OECD, 2006. Test No. 407: Repeated dose 28-day oral toxicity study in rodents; updated with parameters for endocrine effects. In: OECD- Organization for Economic Cooperation and Development.

OECD, 2008. Test No. 425: Acute oral toxicity – up-and-down-procedure. In: OECD - Organization for Economic Cooperation and Development.

OECD, 2010. Test No. 487: In Vitro Mammalian Cell Micronucleus Test. In: OECD Guidance for the Testing of Chemicals. Paris, Organization for Economic Cooperation and Development.

OECD, 2014. Test No. 489: In Vivo mammalian alkaline comet assay. Guidelines for the Testing of Chemicals, Section 4OECD Publishing, Paris.

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

Oliveira, R.J., Matuo, R., da Silva, A.F., Matiazi, H.J., Mantovani, M.S., Ribeiro, L.R., 2007. Protective effect of beta-glucan extracted from Saccharomyces cerevisiae, against DNA damage and cytotoxicity in wild-type (k1) and repair-deficient (xrs5) CHO cells. Toxicol. In Vitro. 21, 41-52.

Palomino, J.C., Martin, A., Camacho, M., Guerra, H., Swings, J., Portaels, F., 2002. Resazurin microtier assay plate: simple and inexpensive method for deteccion of drug resistance *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 46, 2720-2722.

Pinto, N., Ludeman, S.M., Dolan, M.E., 2009. Drug focus: Pharmacogenetic studies related to cyclophosphamide-based therapy. Pharmacogen. 10, 897-903.

Sivakumar, P.M., Geetha Babu, S.K., Mukesh, D., 2007. QSAR studies on chalcones and flavonoids as anti-tuberculosis agents using genetic function approximation (GFA) method. Chem. Pharm. Bull. 55, 44-49.

Webster, D., Lee, T.D., Moore, J., Manning, T., Kunimoto, D., LeBlanc, D., Johnson, J.A., Gray, C.A., 2010. Antimycobacterial screening of traditional medicinal plants using the microplate resazurin assay. Can. J. Microbiol. 56, 487-494.

Witte, I., Plappert, U., de WH and Hartmann, A., 2007. Genetic toxicity assessment: employing the best science for human safe*y*t evaluation part III: the comet assay as an alternative to in vitro clastogenicity tests for early drug candidate selection. Toxicol. Sci. 97, 21-26.

Zheng, Y., Jiang, Gao, F., Song, J., Sun, J., Wang, L., Sun, X., Lu, Z., Zhang, H., 2014. Identification of plant-derived natural products as potential inhibitors of the Mycobacterium tuberculosis proteasome. BMC Complement. Altern. Med. 15, 14-400.

APÊNDICE

Table 1 – **Effect of flavone on hematological parameters**

Experimental group	Red cells	Hematocrit	Hemoglobin	Neutrophil	Lymphocyt
Negative Control	9.24 ± 0.39[a]	44.40 ± 4.83 [a]	14.46 ± 1.60 [a]	43.80 ± 3.49 [a]	56.20 ±3.49 [a]
Positive Control	9.66 ± 0.13[a]	46.60 ± 1.34 [a]	15.4 ± 0.55 [a]	53.00 ± 6.89 [a]	45.60 ±7.02 [a]
Flavone/175 mg/Kg	9.28 ± 0.19[a]	42.80 ± 1.92 [a]	14.2 ± 0.84 [a]	42.00 ± 2.54 [a]	57.60 ±3.04 [a]
Flavone/560 mg/Kg	9.56 ± 0.36[a]	44.80 ± 3.34 [a]	15.0 ± 1.22 [a]	46.00 ± 1.58 [a]	54.00 ±1.58 [a]
Flavone/1792 mg/Kg	9.34 ± 0.40[a]	43.40 ± 3.98 [a]	14.6 ± 1.14 [a]	49.00 ± 2.00 [a]	49.8 ± 2.94 [a]

Letters indicate statistically significant differences.

Table 2- Effect of flavone on biochemical parameters

Experimental group	Urea	Creatinine	Alkaline Phosphatase
Negative Control	47.80 ± 4.06[b]	0.36 ± 0.05[b]	117.40 ± 1.67[c]
Positive Control	69.00 ± 7.17[a]	0.50 ± 0.07[c]	115.80 ± 1.48[b]
Flavone/175 mg/Kg	44.40 ± 3.87[b]	0.28 ± 0.08[b]	115.80 ± 3.11[b]
Flavone/560 mg/Kg	44.00 ± 2.54[c]	0.30 ± 0.07[b]	114.60 ± 3.78[a]
Flavone/1792 mg/Kg	44.60 ± 2.71[b]	0.26 ± 0.05[a]	117.00 ±2.91[b]

Letters indicate statistically significant differences.

Table 3 - Effect of flavone total of injured cells and mean frequency between different classes for *Swiss* female mice.

Experimental group	Damaged cells	Damage classes				Escore
		0	1	2	3	
Control	13.60±0.51[a]	86.40 ± 0.51	13.00 ± 0.45	0.60 ± 0.25	0.00 ± 0.00	14.20 ± 0.66[a]
Cyclophosphamide	85.40 ±0.68[c]	14.60 ± 0.68	68.20 ± 0.58	13.80 ± 0.66	3.40 ± 0.40	106. 00 ± 1.64[c]
Flavone/175 mg kg	17.20±0.66 [ab]	82.80 ± 0.66	17.20 ± 0.66	0.00 ± 0.00	0.00 ± 0.00	17.20 ± 0.66 [ab]
Flavone/560mg kg	18.60±0.25[b]	81.40 ± 0.24	17.60 ± 0.40	1.00 ± 0.31	0.00 ± 0.00	19.60 ± 0.40[b]
Flavone/1792mg kg	17.00±1.58[ab]	82.20 ± 0.86	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	17.00 ± 1.58[ab]

Letters indicate statistically significant differences.

Table 4 - Effect of flavone on micronucleus test

Experimental group			
	24 h (T1)	**48(T2)**	**72(T3)**
Control	7.4±0.93[a]	9.8±1.8[a]	8.4±1.36[a]
Cyclophosphamide	46±1.95[b]	43±1.34[b]	34.3±2.54[b]
Flavone/175 mg kg	8.4±1.03[a]	6±0.84[a]	6.2±1.07[a]
Flavone /560 mg kg	9.2±0.97[a]	5.4±0.6[a]	8.4±1.21[a]
Flavone/1792 mg kg	9±1.45[a]	8.2±0.86[a]	8.4±1.50[a]

REGULATORY TOXICOLOGY AND PHARMACOLOGY

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0273-2300

DESCRIPTION

Regulatory Toxicology and Pharmacology, Official Journal of the International Society for Regulatory Toxicology and Pharmacology, reports the concepts and problems involved with the generation, evaluation, and interpretation of **experimental animal and human data** in the larger perspective of the **societal** considerations of protecting human health and the environment. The journal is devoted to reports of significant developments, public opinion, scientific data, and ideas that bridge the gap between scientific information and the **legal** aspects of **toxicological** and **pharmacological regulations**.

The journal presents articles concerned with **regulatory decisions** and with the interpretation of scientific knowledge as influencing regulatory decisions. It is addressed to an International readership of scientists, program managers, research directors, attorneys, and business professionals.

Benefits to authors
We also provide many author benefits, such as free PDFs, a liberal copyright policy, special discounts on Elsevier publications and much more. Please click here for more information on our author services .

Please see our Guide for Authors for information on article submission. If you require any further information or help, please visit our support pages: http://support.elsevier.com

IMPACT FACTOR

2014: 2.031 © Thomson Reuters Journal Citation Reports 2015

ABSTRACTING AND INDEXING

EMBASE
Scopus
EMBiology

EDITORIAL BOARD

GUIDE FOR AUTHORS

Your Paper Your Way

We now differentiate between the requirements for new and revised submissions. You may choose to submit your manuscript as a single Word or PDF file to be used in the refereeing process. Only when your paper is at the revision stage, will you be requested to put your paper in to a 'correct format' for acceptance and provide the items required for the publication of your article.
To find out more, please visit the Preparation section below.

INTRODUCTION

Regulatory Toxicology and Pharmacology, the Official Journal of the International Society for Regulatory Toxicology and Pharmacology, matches reports in toxicology, pharmacology, epidemiology, and allied sciences, with reports on philosophical, legislative, legal, and public opinion issues that define public health, safety, and environmental regulations. International in scope, the journal speaks at all levels of science and policy to the community of research and development scientists, to business and legal decision makers, and to regulatory officials and legislative operators worldwide.

Types of paper

Ethical Treatment of Subjects. Submission of a manuscript implies that the authors warrant to have complied with institutional policies governing the ethical treatment of human subjects and animals, and that the authors are ready to share the original approval documents if so requested.

Peer Review. Submitted manuscripts not rejected at first screen will undergo a peer review process that occasionally could require substantial revision and may last several months, depending on the timeliness of author response.

Letters. A brief one-printed-page observation may be published under a "Letters" heading with the understanding that only one reply will be published in a rebuttal. A manuscript intended for publication in Letters or a rebuttal should be so designated when submitted for publication.

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see https://www.elsevier.com/publishingethics and https://www.elsevier.com/journal-authors/ethics.

Conflict of interest

Regulatory Toxicology and Pharmacology follows the ICMJE recommendations regarding conflict of interest disclosures. All authors are required to report the following information with each submission: All third-party financial support for the work in the submitted manuscript. All financial relationships with any entities that could be viewed as relevant to the general area of the submitted manuscript. All sources of revenue with relevance to the submitted work who made payments to you, or to your institution on your behalf, in the 36 months prior to submission. Any other interactions with the sponsor of outside of the submitted work should also be reported. Any relevant patents or copyrights (planned, pending, or issued). Any other relationships or affiliations that may be perceived by readers to have influenced, or give the appearance of potentially influencing, what you wrote in the submitted work.

As a general guideline, it is usually better to disclose a relationship than not. This information will be acknowledged at publication in a Transparency Document. Additional information on the ICMJE recommendations can be found at: http://www.icmje.org. The form for conflict of interest disclosure can be downloaded here, or at http://www.icmje.org/coi_disclosure.pdf (if this link does not display properly in your browser, please right-click the link and select "Save Target As..." or "Save Link as..." from the popup menu.)

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/postingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere

in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck http://www.elsevier.com/editors/plagdetect.

Written authorization by personal sources may be required at the Editor's discretion. Articles and any other material published in *Regulatory Toxicology and Pharmacology* represent the opinions of the authors, unless directly endorsed or authorized, should not be construed to reflect the opinions of the International Society of Regulatory Toxicology and Pharmacology, the Editors, or the Publisher. Original papers only will be considered.

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.
Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Article transfer service

This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. Please note that your article will be reviewed again by the new journal. More information about this can be found here: https://www.elsevier.com/authors/article-transfer-service.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, see https://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult https://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult https://www.elsevier.com/permissions.

For open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information see https://www.elsevier.com/OAauthoragreement). Permitted third party reuse of open access articles is determined by the author's choice of user license (see https://www.elsevier.com/openaccesslicenses).

Author rights

As an author you (or your employer or institution) have certain rights to reuse your work. For more information see https://www.elsevier.com/copyright.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some authors may also be reimbursed for associated publication fees. To learn more about existing agreements please visit https://www.elsevier.com/fundingbodies.

US National Institutes of Health (NIH) voluntary posting (" Public Access") policy. Elsevier facilitates author response to the NIH voluntary posting request (referred to as the NIH "Public Access Policy"; see http://www.nih.gov/about/publicaccess/index.htm) by posting the peer-reviewed author's manuscript directly to PubMed Central on request from the author, 12 months after formal publication. Upon notification from Elsevier of acceptance, we will ask you to confirm via e-mail (by e-mailing us at NIHauthorrequest@elsevier.com) that your work has received NIH funding and that you intend to respond to the NIH policy request, along with your NIH award number to facilitate processing. Upon such confirmation, Elsevier will submit to PubMed Central on your behalf a version of your manuscript that will include peer-review comments, for posting 12 months after formal publication. This will ensure that you will have responded fully to the NIH request policy. There will be no need for you to post your manuscript directly with PubMed Central, and any such posting is prohibited.

Open access

This journal offers authors a choice in publishing their research:

Open access

- Articles are freely available to both subscribers and the wider public with permitted reuse
- An open access publication fee is payable by authors or on their behalf e.g. by their research funder or institution

Subscription

- Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs (https://www.elsevier.com/access).
- No open access publication fee payable by authors.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution (CC BY)

Lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The open access publication fee for this journal is **USD 2400**, excluding taxes. Learn more about Elsevier's pricing policy: http://www.elsevier.com/openaccesspricing.

Green open access

Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our green open access page for further information (http://elsevier.com/greenopenaccess). Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form.

This journal has an embargo period of 12 months.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Contact information:

Regulatory Toxicology and Pharmacology
Editorial Office
525 B Street, Suite 1900
San Diego, CA 92101-4495, USA
Telephone: (619) 699-6275
Fax: (619) 699-6211
E-mail: rtp@elsevier.com

Experimental procedures

All animal experiments reported in submitted manuscripts should be carried out in accordance with the official guidelines for experimental animal treatment and reporting, as current in the countries where a reporting manuscript originated. Examples of these guidelines are the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC) or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Authors should detail in the manuscript how such guidelines have been followed.

Referees

Please submit the names and institutional e-mail addresses of several potential referees. For more details, visit our Support site. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

PREPARATION

NEW SUBMISSIONS

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process.
As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay-out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.

If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.
Divide the article into clearly defined sections.

Please ensure the text of your paper is double-spaced and has consecutive line numbering– this is an essential peer review requirement.

Figures and tables embedded in text

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

REVISED SUBMISSIONS

Use of word processing software

Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: https://www.elsevier.com/guidepublication). See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article Structure

Preparation of Manuscript.

Authors of scientific reports should make a point to validate whether the data measure what they claim to have measured or whether they could be distorted by adventitious interferences. Authors should also provide evidence that test and control conditions differ only on account of the experimental variables tested, or are not affected by spurious confounding conditions. Conclusions should focus on the most probable explanation of results, but should also endeavor to point out other less apparent but plausible conclusions. Submissions that are not scientific experimental reports, such as policy positions and reviews, should strive for range, logical sequence, clarity, and well-articulated conclusions.

The popularity of *Regulatory Toxicology and Pharmacology* makes it necessary to severely limit authors' discussions and data presentations in their manuscripts. Because the scope of environmental toxicology and pharmacology is so great, it is not possible to devote many pages to a single issue.

Use generic names of chemicals whenever possible. Proprietary names and trademarks should appear only to identify the source of the chemical, and subsequently only the generic name should be used. All abbreviations, other than those for standard units, should be defined in text or in a footnote. Abbreviations should be unpunctuated.

Manuscripts should be double-spaced and use continuous line numbering throughout the body of your manuscript only. Pages should be numbered consecutively and organized as follows:

The title page (p. 1) should contain the article title, authors' names and complete affiliations, footnotes to the title, and the address for manuscript correspondence (including e-mail address and telephone and fax numbers). Separate word counts should be provided for abstract, text, and references. A second page containing only the title of the paper should be submitted if the authors wish to obtain a blind peer review.

The abstract (p. 2) must be a single paragraph that summarizes the main findings of the paper in less than 200 words. After the abstract a list of up to 10 keywords that will be useful for indexing or searching should be included.

Format. Flexibility of format is allowed, given the mix of multidisciplinary scientific reports and of policy and review articles of interest to the journal. Clarity and brevity will be preferred.

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

- ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower- case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
- ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
- ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

The abstract should be fewer than 200 words.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 ×

13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See https://www.elsevier.com/graphicalabstracts for examples.

Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images and in accordance with all technical requirements: Illustration Service.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, or, maximum 20 words per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Provide up to 10 keywords.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork *Electronic artwork General points*

- Make sure you use uniform lettering and sizing of your original artwork.
- Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Indicate per figure if it is a single, 1.5 or 2-column fitting image.
- For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
- Please note that individual figure files larger than 10 MB must be provided in separate source files. A detailed guide on electronic artwork is available on our website: https://www.elsevier.com/artworkinstructions.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

Please do not:

- Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
- Supply files that are too low in resolution.
- Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations

are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. For further information on the preparation of electronic artwork, please seehttps://www.elsevier.com/artworkinstructions.

Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

References should be cited in the text by the first author's surname followed by et al. If only two authors are listed, both names should be cited. References should be listed alphabetically by name. Journal names should follow the style of the latest edition of the Chemical Abstracts Service Source Index. Only articles that have been published or are in press should be included in the references. Unpublished results or personal communications should be cited as such in the text.

Gerrity, T.R., Henry, C.J., 1990. Principles of Route-to-Route Extrapolation for Risk Assessment. Elsevier, New York.

LaPuma, P.T., Brain S. Rhodes, B.S., 2002. Chromate content versus particle size for aircraft paints. Regul. Toxicol. Pharmacol. 36, 318-324.

Mettam, O.R., Adams, L.B., 1999. How to prepare an electronic version of your article, in: Jones, B.B., Smith, R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281-304.

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

Most Elsevier journals have their reference template available in many of the

most popular reference management software products. These include all products that support Citation Style Language styles (http://citationstyles.org), such as Mendeley

(http://www.mendeley.com/features/reference-manager) and Zotero (https://www.zotero.org/), as well as EndNote (http://endnote.com/downloads/styles). Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:
http://open.mendeley.com/use-citation-style/regulatory-toxicology-and-pharmacology
When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference formatting

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication. Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, thenchronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:

Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations:
http://www.issn.org/services/online-services/access-to-the-ltwa/.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 150 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at

https://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at https://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary material

Supplementary material can support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Please note that such items are published online exactly as they are submitted; there is no typesetting involved (supplementary data supplied as an Excel file or as a PowerPoint slide will appear as such online). Please submit the material together with the article and supply a concise and descriptive caption for each file. If you wish to make any changes to supplementary data during any stage of the process, then please make sure to provide an updated file, and do not annotate any corrections on a previous version. Please also make sure to switch off the 'Track Changes' option in any Microsoft Office files as these will appear in the published supplementary file(s). For more detailed instructions please visit our artwork instruction pages at https://www.elsevier.com/artworkinstructions.

Data in Brief

Authors have the option of converting any or all parts of their supplementary or additional raw data into one or multiple Data in Brief articles, a new kind of article that houses and describes their data. Data in Brief articles ensure that your data, which is normally buried in supplementary material, is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. Authors are encouraged to submit their Data in Brief article as an additional item directly alongside the revised version of their manuscript. If your research article is accepted, your Data in Brief article will automatically be transferred over to *Data in Brief* where it will be editorially reviewed and published in the new, open access journal, *Data in Brief* (http://www.journals.elsevier.com/data-in-brief). The open access fee for *Data in Brief* is $500. For authors who submit in 2015 a reduced fee of $250 will apply. Please use the following template to write your Data in Brief: https://www.elsevier.com/dib-template.

Database linking

Elsevier encourages authors to connect articles with external databases, giving readers access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See https://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

- E-mail address
- Full postal address

All necessary files have been uploaded, and contain:

- Keywords
- All figure captions
- All tables (including title, description, footnotes) Further considerations
- Manuscript has been 'spell-checked' and 'grammar-checked'
- All references mentioned in the Reference list are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Internet)

Printed version of figures (if applicable) in color or black-and-white

- Indicate clearly whether or not color or black-and-white in print is required.

For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. Example of a correctly given DOI (in URL format; here an article in the journal *Physics Letters B*):
http://dx.doi.org/10.1016/j.physletb.2010.09.059
When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author, at no cost, will be provided with a personalized link providing 50 days free access to the final published version of the article on ScienceDirect. This link can also be used for sharing via email and social networks. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's WebShop (http://webshop.elsevier.com/myarticleservices/offprints). Authors requiring printed copies of multiple articles may use Elsevier WebShop's 'Create Your Own Book' service to collate multiple articles within a single cover (http://webshop.elsevier.com/myarticleservices/booklets).

AUTHOR INQUIRIES

You can track your submitted article at https://www.elsevier.com/track-submission. You can track your accepted article at https://www.elsevier.com/trackarticle. You are also welcome to contact Customer Support via http://support.elsevier.com.