UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

ATIVIDADE ANTIMICOBACTERIANA, TOXICIDADE AGUDA, GENOTOXICIDADE E MUTAGENICIDADE DA FLAVONA (2-FENIL-4H-1-BENZOPIRANO-4-ONA) EM CAMUNDONGOS

VANESSA VILAMAIOR DE SOUZA

DOURADOS MS
2014
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Orientador: Dr. Julio Henrique Rosa Croda

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Agradeço

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

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

Aos meus irmãos Alexsandra e Geandro pelo incentivo e pela presença sempre constante em minha vida. Gostaria de agradecer especialmente minha irmã por toda a ajuda, incentivo, dedicação e paciência.

A professora Drª Candida Kassuya por toda ajuda e paciência desde o início do mestrado. Sou imensamente grata por tudo.

Aos professores doutores: Candida Kassuya, Fábio Negrão, Anelise Samara e Alexéia Barufatti, pela composição da banca de defesa.

Ao professor Dr. Julio Henrique pela orientação e ajuda.

Ao professor Dr. Rodrigo Juliano pela disposição em ajudar, por abrir as portas de seu laboratório.

Aos meus companheiros nessa caminhada que me auxiliaram quando necessário Flávio Henrique Cláudia Berno e Roberto. A vocês, muito obrigada pelo acolhimento, ajuda e paciência.

Aos meus amigos e colegas de mestrado Wesley Barbieri, Karine Sartori, Ana Claudia Piccinelli e Giseli Traesel obrigada pelo incentivo, pelos momentos de descontração e ajuda.

Aos técnicos do LPCS e da FCS que me ajudaram com experimentos e reagentes Flora Martinez, Mariana Tatara, Lujan Sanabria Débora Brait, Priscilla Ely
Dedico aos meus pais Elis Regina e Antônio

Aos meus irmãos Alexsandra e Geandro

A minha sobrinha Isabella
Sumário

1 INTRODUÇÃO ............................................................................................................. 1

2 REVISÃO DE LITERATURA ....................................................................................... 3
  2.1 Tuberculose: Doença e epidemiologia ................................................................. 3
  2.2 Tratamento ........................................................................................................... 4
  2.3 Resistência micobacteriana ................................................................................ 6
  2.4 Novas alternativas terapêuticas para o tratamento da tuberculose ............... 7
  2.5 Produtos naturais com atividade antimicobacteriana ....................................... 8
      2.5.1 Flavonóides ................................................................................................... 8
      2.5.2 Flavona ....................................................................................................... 10
          2.5.2.1 Propriedades químicas ..................................................................... 10
          2.5.2.2 Atividades biológicas ...................................................................... 11
  2.6 Testes in vitro ....................................................................................................... 12
  2.7 Toxicologia de produtos naturais ....................................................................... 13
  2.8 Considerações sobre a genotoxicidade ............................................................... 14

3 OBJETIVOS .................................................................................................................. 16
  3.1 Objetivo Geral ...................................................................................................... 16
  3.2 Objetivos Específicos .......................................................................................... 16

4 REFERÊNCIAS BIBLIOGRÁFICAS ............................................................................. 17

5 ANEXO I ...................................................................................................................... 23
  5.1 Artigo Científico .................................................................................................. 23

ANEXO II ............................................................................................................................ 44

ANEXO III .......................................................................................................................... 63
Flavonóides são compostos polifenólicos encontrados em algumas plantas medicinais, estudos científicos demonstram que, alguns flavonóides tem atividades anti-inflamatória, anticarcinogênica e antioxidante. A flavona (2-fenil-4H-1-benzopirano-4-ona) é um flavonóide com atividade de estimulação cardíaca, antibacteriana, antialérgica e de inibição ou estimulação de enzimas. Dessa forma, esse trabalho avaliou os efeitos antimicobacterianos da flavona in vitro sobre a cepa H37Rv ATCC 27294. Foi realizado também os testes de toxicidade aguda, genotoxicidade e mutagenicidade com a flavona in vivo. A concentração inibitória mínima (CIM) da flavona foi mensurada nas concentrações de 0.98 a 250 µg/mL. Modelos experimentais de toxicidade aguda, genotoxicidade e mutagenicidade foram desenvolvidos em camundongos fêmea por via oral, sendo a flavona administrada por gavagem no ensaio de toxicidade aguda nas doses de 175, 560, 1792 e 2000 mg/Kg e em 175, 560, 1792 mg/Kg nos ensaios de genotoxicidade e mutagenicidade. Foram observados sinais hipocráticos bem como, foram efetuadas análises bioquímicas, hematológicas, genotóxicas (ensaio cometa, micronúcleo) e mutagênicas (fagocitose). A flavona apresentou atividade antimicobacteriana de CIM = 31,25 µg/mL. Nos dados obtidos verificou-se aumento no recrutamento de macrófagos para o tecido esplênico houve uma média de 55% de células fagocitadas, quando comparado ao controle negativo com 37% de células fagocitadas, estes dados demonstram que a flavona poderia desempenhar atividade imunoestimulatória. Nos testes in vivo, não foram observados sinais de toxicidade e nem alterações nos parâmetros bioquímicos, hematológicos, genotóxicos e mutagênicos. A análise macroscópica de órgãos como: rins, fígado e pulmões não apresentou nenhuma diferença significativa entre os grupos. Desta forma, o presente estudo demonstra a flavona apresenta atividade antimicobacteriana in vitro (eficácia contra Mycobacterium tuberculosis). Além disso, a flavona não é tóxica nos modelos realizados nesse trabalho. Esses resultados juntos levam a demonstração da eficácia da flavona contra micobactérias e a segurança de um composto presente na natureza.

**Palavras-chave:** Atividade antimicobacteriana, flavona, toxicidade, mutagenicidade
Abstract

Flavonoids are polyphenolic compounds found in medicinal plants. Scientific studies show that some flavonoids have anti-inflammatory, anticarcinogenic and antioxidant activities. The flavone (2-phenyl-4H-1-benzopyran-4-ona) is a flavonoid with cardiac, antibacterial and antiallergic stimulating activity as well as inhibition or stimulation of enzymes. Thus, this study evaluated the antimycobacterial effects of flavone in vitro on the strain H37Rv ATCC 27294. Tests of acute toxicity, genotoxicity and mutagenicity with flavone in vivo were also performed. The minimum inhibitory concentration (MIC) of flavone was measured at concentrations of 0.98 to 250 µg/mL. Experimental models of acute toxicity, genotoxicity and mutagenicity were developed in female mice and they were orally administered with a flavone by gavage in acute toxicity test at doses of 175, 560, 1792 and 2000 mg/ Kg and 175, 560, 1792 mg/ Kg in the tests of genotoxicity and mutagenicity. Hippocratic signals were observed as well as biochemistry, hematology, genotoxic (comet assay, micronucleus) and mutagenic (phagocytosis) analysis were performed. The flavone showed antimicrobial activity of MIC = 31.25 µg/mL. In the data there was an increase in the recruitment of macrophages into the splenic tissue was an average 55% phagocytosis of cells compared to the negative control in 37% phagocytosed cells, these data demonstrate that the flavone could play immunostimulatory activity. In the in vivo tests, no signs of toxicity and no changes in biochemical, hematological, genotoxic and mutagenic parameters were observed. The macroscopic analysis of organs such as kidneys, liver and lungs showed no significant difference between groups. Thus, this study demonstrates for the flavone has antimicrobial activity in vitro (efficacy against Mycobacterium tuberculosis). In addition, the flavone is not toxic models performed in this work. These results together lead to demonstration of efficacy of flavone against mycobacteria and the safety of a compound present in nature.

Key words: Antimycobacterial activity, tuberculosis, flavone, toxicity, mutagenicity.
Listas de siglas e abreviaturas

ANVISA - Agência Nacional de Vigilância Sanitária
AIDS - Síndrome de Imunodeficiência Adquirida
CEUA - Comitê de Ética em Uso de Animais
CIM - Concentração Inibitória Mínima
COBEA - Colégio Brasileiro em Experimentação Animal
E - Etambutol
Et - Etionamida
HIV - Vírus da Imunodeficiência Adquirida
I - Isoniazida
OMS - Organização Mundial da Saúde
R - Rifampicina
REDOX - Redução-Oxidação
REMA – Resazurin Reduction Microtiter Assay
S - Estreptomicina
SUS - Sistema Único de Saúde
TB - Tuberculose
MDR - Cepas Multidroga Resistentes
MTT – Dimethyl Thiazolyl Diphenyl Tetrazolium Salt
XDR - Cepas Extremamente Resistentes
UFC - Unidade Formadora de Colônia
Z - Pirazinamida
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 [1,2].

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 [3].

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 [4]. 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 [5,6].

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 (XDR) [7,8].

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. [9]
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 de importância terapêutica [10].

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 [11] Nos últimos anos, a pesquisa sobre plantas medicinais tem atraído a atenção em todo o mundo por serem fonte de substâncias químicas que são potenciais substratos para síntese de novas drogas [12].

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 µg/mL). A fração FAE (Fração Acetado de etila), resultante do fracionamento, teve CIM = 115,2 µg/mL. Detectou-se ainda a CIM do composto isolado luteolina (236,8 µg/mL) a qual pertence a 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 DE 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 aerobio estrito. A evolução ocorre em ciclos lentos, podendo apresentar as mais diversas complicações [13, 14, 15].

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 [16, 17]. 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 [18].

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 eliminda por esse mecanismo [19].

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 infereferon-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 resposta do sistema imunológico frente ao bacilo [20].

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 [21, 22]. Segundo estimativas, 1/3 da população mundial está infectada pelo *M. tuberculosis*, desse total 5 a 10% irá desenvolver a doença. Pacientes co-infetados TB/HIV existe um incremento de 10% ao ano de desenvolvimento de doença ativa. Nesses casos está recomendado o tratamento da tuberculose latente [24].

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* [25, 26].

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 mal absorção crônicas, ou baixo peso corporal (10% ou mais de peso abaixo do ideal) e o etilismo [27,28].

Estima-se que, no ano de 2012, ocorreram 8,6 milhões de casos incidentes, dos quais 400.000 seriam pacientes coinfetados com HIV e 1,3 milhão de óbitos entre pacientes não portadores de HIV [29, 30]. 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% [31, 32].

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 [5]. 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 [33].
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 meningencefálica 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) [28].

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 [34,35,36].

Quadro 1. Mecanismo de ação dos principais antimicobacterianos [37]

<table>
<thead>
<tr>
<th>Medicamento</th>
<th>Mecanismo de ação</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicina</td>
<td>Liga de forma irreversível ao RNA-polimerase DNA-dependente, impedindo a produção de RNA e a síntese de proteínas</td>
</tr>
<tr>
<td>Isoniazida</td>
<td>Quelação de íons cobre essenciais para a célula bacteriana; interfere também na enzima micolase-sintetase, importante na síntese de ácido micólico</td>
</tr>
<tr>
<td>Etambutol</td>
<td>Inibição da síntese de ácido nucleicos da célula bacteriana</td>
</tr>
<tr>
<td>Pirazinamida</td>
<td>Provavelmente semelhante a isoniazida</td>
</tr>
<tr>
<td>Estreptomicina</td>
<td>Se liga de forma irreversível ao ribossomo bacteriano, produzindo bloqueio ou alterações profundas na síntese de proteínas</td>
</tr>
<tr>
<td>Etionamida</td>
<td>Age na enzima nicotinamida adenina-dinucleotídeo</td>
</tr>
</tbody>
</table>
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). [7,35].

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 efiacles, 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) [30].

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 [38,39].

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 raras, estudos apontam que aproximadamente 9,6% de MDR possuem características de XDR-TB [30].

Em 2013, no Brasil foram notificados 148 casos novos de monorresistência, 50 de polirresistência, 525 de multirresistência e 21 de resistência extensiva [40]. 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 [41]. 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 [42,43].

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 [44]. 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 [45].

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 [46].

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 [47,48].
2.5 Plantas e produtos naturais com atividade antimicobacteriana


A maioria dos medicamentos sintéticos que estão disponibilizados no mercado possuem origem de produtos naturais [55]. 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 [56].

Algumas flavonas já foram isoladas e testadas frente cepas virulentas de *M. tuberculosis*, apresentando uma CIM considerável como: 3′-O-dimetoxi-5, 6,4 ′-trihidroxiflavona (MIC 200 mcg / mL) cirsimaritin (MIC de 50 ng / mL), eapatilin (MIC de 50 ng / mL), salvigenin (MIC 100 mcg / ml), [9], 7 - metoxiflavona (MIC 12,5-50 ug / ml) e 5,4 ′-di-hidroxi-7-metoxiflavona (MIC 25-50 ug / mL) [57].

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 [58].

A apigenina (4′,5,7-triroidroxiflavona) 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 [59]. 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 [60].

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) [61] (Figura. 1) A classe é dividida em mais de 10 diferentes subclasses dentre elas os flavonóis, flavonas, isoflavonas, antocianinas e flavononas [62].

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 [63].

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) [64].

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 [65].

Os flavonóides possuem atividade anti- hemolítica, anticarcinogênica, peroxidação lipídica, formação de radicais superóxido e apoptose celular [66,67,68]. Além disso, têm propriedades de modulação do reparo do DNA, antialérgicas, antimicrobianas, vasoprotetoras, antitumorais e anti-inflamatórias [69,70,71,72,73].
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 [74,75].

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 existam mais de 300 flavonas com ausência de açúcar na estrutura e pelo menos 500 glicosadas [76].

A salsa é uma erva que contém elevada concentração da flavona apigenina (5,7,4’-triidroxiflava) e de criscoeriol (3’- $O$- metiluteolina). A apigenina é encontrada em grãos de cereais, ervas e alguns vegetais [77]. A luteolina (5,7,3’,4’-tetraidroxoflava) outra

**Figura 1.** Estrutura química dos flavonóides [64].

**Figura 2.** Estrutura química da flavona [72]
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 [78,79].

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) [80].

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

![Figura 3. Esquema adaptado da síntese da flavona [71].](image)

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 [81].

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 [82]

O potencial terapêutico da flavona faz desta, um possível alvo para aplicação na área farmacêutica. 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 [83,84,85].

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-driidroxı-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 [86].

A epigenina, luteolina, nobiletina e a tangeritina, tem atividades antimutagênica e inibem o crescimento de algumas células cancerosas (carcinoma de células escamosas) [78].

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 [78,79,80,81].

**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 [87].

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 [88].
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 [86]. Assim foi possível determinar a concentração inibitória mínima (CIM) da flavona frente ao *M. tuberculosis* H37Rv ATCC 27294.

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 [87].

### 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 [26].

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 [48].

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 fotosensibilizante, 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 [88].
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 [20, 89,90].

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 [91] A maioria dos estudos pré-clínicos de produtos sintéticos ou naturais envolve a utilização de parâmetros bioquímicos, hematológicos e anatomopatológicos [92].

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 ocorrer mutação (alterações hereditárias), que pode se perpetuar nas células filhas durante o processo de replicação, nesses casos o agente causador é denominado mutagênico [93].

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 [94,95]. 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 [96].
O teste de micronúcleo visa a quantificação de fragmentos cromossômicos ou de cromossomos intactos 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 [97]

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 [98,99]

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) [100]. 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 [100].

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 [101].
3. OBJETIVOS

3.1 Objetivo Geral

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

3.2 Objetivos Específicos

Avaliar o efeito antimicobacteriano \textit{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 “\textit{in vivo}” da flavona em camundongos através do ensaio cometa, teste do micronúcleo e fagocitose.
4 REFERÊNCIAS BIBLIOGRÁFICAS


5 ANEXO I

5.1 Scientific Article

Title: Evaluation of acute toxicity, antimicrobial activity, genotoxicity and mutagenicity of flavone in adult female mice.

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**ABSTRACT:** The compound flavone (2-phenyl-4H-1-benzopyran-4-ona) which belongs to the group of flavonoids has as one of its therapeutic properties the ability to stimulate or to inhibit the action of certain enzymes. This study aimed to evaluate the antimicrobial activity, the acute toxicity as well as genotoxic and mutagenic actions of the flavone. Hence, we performed MIC (minimum inhibitory concentration) and the acute toxicity test in female Swiss mice at doses of 175 mg/ Kg-1, 560 mg/ Kg-1, 1792 mg/ Kg-1 and 2000 mg/ Kg-1, which were administered in a single dose and at the end of 14 days the hematological and biochemical patterns were measured as well as the comet assays, the micronucleus test and phagocytosis. It was demonstrated that flavone has antimicrobial activity with MIC of 28.90 µg/mL. It has been observed that the treatment with flavone has no effect on weight gain, food and water consumption nor does it cause any change to the hematological and biochemical profiles. These results suggest that flavone has antymycobacterial activity. The data demonstrated absence of acute toxicity after a single-dose treatment. It was observed that in the comet and micronucleus assays flavone did not exert mutagenic and genotoxic activities. No changes were observed in the phagocytosis test nor in the hematological and biochemical analyzes. Thus, it can be inferred that flavone has no mutagenic nor genotoxic activity in the experimental model used.

**Key words:** Flavone, acute toxicity, antymycobacterial activity, mutagenicity, genotoxicity.
INTRODUCTION

Flavonoids (polyphenolic substances) are secondary metabolites active of plants, derived from the condensation of one molecule of cinnamic acid with three groups malonyl-CoA participants, forming a chalcone (C15), precursor of all flavonoids, which are subdivided into subclasses depending on the substitution and on the level of oxidation made on the C-ring [1]. Individual differences present in each subclass are result in variation in the number and arrangement of hydroxyl groups, as well as the nature and quantity of alkylations and/or glycosylation of these groups. Many of them are presented in the form glycosides, being the most common site C-3 and C-7 less frequent.

Flavone, an important subclass of flavonoids, characterized by aglicon with two phenolic rings A and B and a C ring with the presence of a carbonyl group at the 4-position and a double bond between positions 2 and 3 and no hydroxyl group at C-3 [2].

Studies have demonstrated that the presence of substituents in rings A and B could lead to more potent compounds with reduced toxicity. Numerous studies of flavones derivatives report a large spectrum of important pharmacological properties, including antimycobacterial and anticarcinogenics effects [3].

Our previous studies have identified that a hexane fraction of Annona sylvatica was active against M. Tuberculosis with a MIC of 115.2 mµ / mL [17]. Chromatographic studies showed the presence of two flavonoids, luteolin and quercetin. The luteolin is part of the class of flavones, which have numerous therapeutic properties, such as antimicrobial, antiinflammatory, antioxidant, immunomodulatory, among others [17].

Flavones have been an indispensable anchor for the development of new therapeutic agents. The majority of metabolic diseases are speculated to originate from oxidative stress, and it is therefore significant that recent studies have shown the positive effect of flavones on diseases related to oxidative stress. Due to the wide range of biological activities of flavones, the present study was investigating the antimicrobial activity in vitro, the acute toxicity as well as genotoxic and mutagenic actions in experimental models in mice of the flavone (2-phenyl-1-benzopyran-4-one).

Materials and Methods

The synthetic flavone (2-phenyl-4H-1-benzopyran-4-ona) used to perform the tests was purchased from Sigma-Aldrich® Co. LLC (St. Louis, MO, USA).
Minimum Concentration Inhibitory (MIC)

An aliquot of the *M. tuberculosis* H37Rv ATCC 27294 strain (700μL of the frozen strain) was inoculated 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 Incubator Shaker.

For the preparation of flavone mother solution (10 mg/ml) 10 mg of flavones was weighed and solubilized with 820μL of DMSO (Dimethylsulfoxide) then the solution was homogenized by vortexing until complete solubilization.

To prepare the mother solutions of the standard drugs Isoniazid (INH) and Rifampicin (RFP) (Difco Laboratories, Detroit, MI, USA) (10 mg/mL), 0,0150 g of the drugs were solubilized in sterile water and 1500μL of DMSO respectively, then the solutions were mixed by vortexing and filtered (filter/0.22 µm) . These drugs were used as positive control.

In a 96-well plate, the first column was used as sterility control (EC) and wells 2, 3, 4, 5, 6, 7, 8, 9 and 10 were used for pipetting the dilutions of flavones at concentrations of 250; 125; 62.5; 31.25; 15.63; 7.81; 3.91; 1.95 and 0.98 µg/mL respectively, in the 11th well in rows A, B, C and D was placed the positive control and in rows E, F, G and H the negative control, in all rows of column 12 was pipetted only the liquid medium used.

A bacterial solution containing 180 μL of the medium and 20 μL of the mother solution was prepared, 50 μL of this solution was withdrawn and pipetted in column 2 and from then 100 μL up to 10th column and wells A, B, C and D of column 11. In column 12 was pipetted only the mean to maintain moisture.

The plate was covered with plastic wrap and aluminum foil and left in the Shaker Incubator for 7 days at 36 °C, in the eighth day it was applied 30 μL of resazurin (1 mg of resazurin for 10 ml of water) and the covered plate was again placed in the Shaker Incubator for 24 hours, at the end of this period the wells were read in accordance with change of color.

MIC was defined as the lowest concentration resulting in growth inhibition of 90% of *M. tuberculosis*. A sample with the MIC <250 µg/mL was defined as active against *M. tuberculosis*. Assays were performed in duplicate.
Acute Toxicity

Animals

The 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 based on the guidelines of the Ethical Principles in Animal Research adopted by the Brazilian College on Animal Experimentation (COBEA). The experimental procedures were performed in accordance with the approval by the Ethics Committee on Animal Use (CEUA) of the Federal University of Grande Dourados (Protocol. 005/2010).

Doses and Treatment

The toxicity study was based on the protocol 425 from OECD (Organization for Economic Cooperation in Development) and the protocols established by ANVISA (Sanitary Surveillance Agency) [16,17]. According to the protocol established, 9 animals were used, each received a single oral administration of the compound flavone.

Initially one of the animal received a dose of 175 mg/kg and was observed at 30 minutes, 1h, 2h, 4h, 6h, 12h, 24h and 48 hours, 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 hours a fourth animal received a dose of 2000mg/kg. After the last dose administered no deaths were observed and according to protocol 4 more animals received 2000 mg/kg, the control group received the vehicle used for diluting flavones (hydroalcoholic solution 1:1).

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 in the first and the next 14 days after 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), 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 were assessed for appearance, color, size, weight and consistency.

Hematologic Assay

Hematologic analysis was performed through the determination of hematocrit and hemoglobin. Stained slides with hematologic May-Gruenwald-Giemsa method were prepared and examined under light where 100 cells were counted microscopy.

Biochemical Assay

After collection, the samples were centrifuged at 11,000 rpm and stored at -20 ° C for further analysis: Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Gamma Glutamyl Transferase, Urea and Creatinine [29]. Analyses were performed on Cobas Integra 400 plus.

Comet, Micronucleus and Phagocytosis Assays

Chemical agents, Animals and Experimental Design

The experiments were performed using female Swiss mice (n = 25, weighing 28-32g, aged approximately 60 days), provided by the Federal University of Mato Grosso do Sul (UFMS). The animals were kept in collective polypropylene cages under controlled lighting (12 hours light / 12 hours dark) and temperature (23 °C) conditions, receiving water and commercial food ad libitum. The procedures were performed based on the guidelines of the Ethical Principles in Animal Research adopted by the Brazilian College on Animal Experimentation (COBEA) and were 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 - Positive control: intraperitoneal injection of cyclophosphamide (100 mg/kg\textsuperscript{-1} of body weight, ip); Saline was orally administered.

Group 2 - Negative control: intraperitoneal injection of saline (0.1 mL/10g of body weight, ip); Saline was orally administered.
**Groups 3, 4 and 5:** Intraperitoneal injection of saline solution and flavone by oral route (0.1 mL/10g and 175, 560, 1792 mg / kg⁻¹ of body weight respectively, ip / vo);

The animals received a single dose of flavone. The vehicle used was 0.9% saline solution.

Following the administration of the compounds, 20 µL of peripheral blood were collected for the micronucleus test. The samples were collected at the times of: 24 hours (T1), 48 hours (T2) and 72 hours (T3), T1 and T3 samples were collected for further analysis of comet assay and differential count. After 72 hours the animals were anesthetized with ketamine and xylazine (25 mg/kg and 10 mg/kg respectively), and blood and organs were collected.

**Comet Assay**

After 24 hours of treatment with flavone, 20 µL of blood was collected by caudal puncture, was homogenized with 120 µL of LPM agarose (1.5%) at 37 °C and subsequently deposited in previously prepared slides with plain agarose (5%) and immediately covered with coverslips 24x60mm. 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 lysis solution recently prepared and kept at 4 °C for a period of at least 2 hours. 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% of sodium lauryl sarcosinate, 1,0 mL of Triton and 10,0 mL of DMSO) over a period of 1 hour at 4 °C under no light.

Afterwards, the slides were transferred to a horizontal electrophoresis tank, where it was 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 succeeded at 25 V to 30 mA (25V/cm) and the slides were then neutralized with a sequence of 3 cycles of 5 minutes 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 analyzes the slides were stained with 100mL of Ethidium Bromide
(0.002 mg / mL). Slides were observed with a fluorescence microscope in 40x objective and in excitation filter 515-560 nm.

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 nucleoid diameter; Class 2 – cells with tail size 1 and 2 times the diameter of the nucleoid; Class 3 - cells with tail 2 times superior the diameter of the nucleoid. Cells containing fully fragmented nucleoid were not calculated. At the end of the analysis it has been defined the score of each treatment, multiplying the number of nuclei counted in each class by the class value (0, 1, 2 or 3).

**Micronucleus Assay in Peripheral Blood**

After treatment, blood from the tail of each animal was collected at three different times (T1, T2 and T3). 20 µL 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 micronucleus and other nuclear changes were analyzed under fluorescence microscope with blue light (488 nm) using a 100x objective, 2,000 cells were observed.

**Splenic Phagocytosis Assay**

1/3 of the spleen was triturated with 100 µL of saline. Fifty µL of the suspension was placed in a previously prepared slide with 20µL of acridine orange (1mg/mL) and then they were covered with coverslips. The slides were maintained frozen until the time of the readings, therefore, it was made using a fluorescence microscope 400x, equipped with an excitation filter 420-490 nm and 520 nm barrier filter. Analyzes were conducted according to the protocol established by Hayashi, which evaluated the presence or absence of phagocytosis.

**Statistical Analysis**

Values were expressed as mean ± standard error of the mean (SEM). The differences between groups were determined by analysis of variance (ANOVA), followed, when the difference detected by the Tukey test.
RESULTS AND DISCUSSION

Minimum Inhibitory Concentration (MIC)

The MIC value of 2-phenyl-1-benzopyran-4-one against a strain M. tuberculosis H37Rv ATCC 27294 was 28.90 Mm/mL, isoniazid was 0.030 Mm/mL and rifampicin was 0.019 Mn/mL, values determined as standards.

Literature reports the effect of the six flavones against a strain of M. tuberculosis, with 3'-O-dimethoxy-5, 6,4 ' - trihydroxyflavone (MIC 200 µg/mL) cirsimaritin (MIC 50 µg/mL), eupatilin (MIC 50 µg/mL), salvigenin (MIC 100 µg/mL), [9], 7 - methoxyflavone (MIC 12.5-50 µg/mL) and 5,4 ' - dihydroxy-7-methoxyflavone (MIC 25-50 µg/mL) [10] and luteolin (MIC of 236.89 µg / mL) The apigenin was tested in order to inhibit hyaluronidase, used as only carbon source for M. tuberculosis H37Rv strains. The results showed that the activity of hyaluronidase is essential for the viability of the mycobacteria [8,9]. Probably the mechanism of action of flavone is due to hyaluronidase inhibition.

The flavone evaluated in this work showed highest activity in model tested, with those reported in the literature. The effect can be attributed the absence the substituent (e.g methoxyl and hydroxyl) in ring A and B, not causing an effect steric hindrance between the carbonyl group at the C-4 of the flavone, providing also greater eletrostatica interaction, hydrophobic, hydrogen bonds with the receivers. [10,11]

Acute Toxicity

For a substance to be characterized as toxic it must be done toxicological evaluations as required by regulatory agencies. Mortality is the most evident sign of toxicity, however, other aspects provide more subtle data of the adverse effects such as loss of body mass during the study period and clinical signs of toxicity (diarrhea, piloerection and behavior changes)[16, 17]

The animals used in the present study were exposed to flavone and did not exhibit clinical signs of toxicity in 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.
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.

In the biochemical analysis of the present study there was no significant differences between the animals studied. Thus, it was observed that after the acute exposure of animals to flavone any sign of toxicity was not detected.

**Genotoxicity and Mutagenicity**

The mutagenic effects of flavone were examined through the micronucleus assay in peripheral blood. With this technique it is possible to detect changes in DNA and/or damage to the mitotic spindle [20]

In Table 1 are expressed the results of the mutagenic effects evaluations in the acute treatment with flavone. There was no statistically significant difference (p> 0.05) between the negative control and treated groups. The data expresses the frequency of micronucleus in peripheral blood cells demonstrating the absence of mutagenic effects. The positive control group (cyclophosphamide) showed significant increase (p <0.05) in the number of micronucleus when compared to the negative control and treated groups (Table 1).

After 24, 48 and 72 hours of treatment with flavone it was observed a reduction in the number of micronucleus in the peripheral blood. The doses tested were statistically similar to the negative control group. These data demonstrate that the flavone has no mutagenic effects. Furthermore, the frequency of micronucleus decreased chronologically after treatment. This reduction can be attributed to the fact that the metabolization of flavone reduces its ability to induce DNA damage or can also be caused by its chemical structure, which usual in the class of flavonoids with effect on cancer chemoprevention and treatment [21, 22, 23].

Flavonoids inhibit hemolysis, lipid peroxidation, superoxide radicals formation, tumor cells and apoptosis inductors. Moreover, they have antioxidant and modulator of DNA repair properties [24, 25]

Table 2 demonstrates the level of DNA damage in cells of peripheral blood in the acute treatment. According to the results, no damage has occurred to DNA in the groups treated with flavone which shows the absence of genotoxic effects. Animals treated with
cyclophosphamide had a high rate of DNA damage in 106\% of cells (most Class 1), negative control group presented 14.6\% of injured cells (Class 0), treated groups presented 17, 2\% at the dose of 175 mg/kg⁻¹, 19\% at a dose of 560 mg/kg⁻¹ and 19\% at the dose of 1792 mg/kg⁻¹ (class 0), which indicate the absence of genotoxic activity at the doses tested.

The administration of cyclophosphamide increased phagocytosis (61, 20\%), the same had happened in the groups treated with flavones, with mean of 55.53\%. The negative control group showed an average recruitment of 37, 60\% (Table 3). Likely the mechanism of induction of phagocytosis is due to its ability immunomodulation of the immune system.

The hematopoietic system is a relevant to study installed conditions because it is susceptible to the action of toxic substances [18]. The frequency of neutrophils, eosinophils, monocytes, lymphocytes and basophils. The frequency of neutrophils, eosinophils, monocytes, basophils and lymphocytes remained within the reference range. While the platelets had an average of 366,000 in groups treated with flavone, 348,000 and 326,000 positive control negative control (Table 4).

The biochemical profile presented a difference in aminotransferase enzyme and the product urea in the group treated with cyclophosphamide, indicating that there was acute injury. Treated groups had similar results 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 5).

The mean values for the final and initial weights are shown in table 6 and the absolute and relative organ weights in Table 7. There were no statistically significant differences. Similarly, there were no significant alterations on the absolute and relative weight of vital organs (spleen, heart, liver, lungs and kidneys) (Table 6).
Conclusion

The data presented show that flavone could be a possible drug for the treatment of tuberculosis since they showed good antimicrobial activity and lack of toxicity. In addition, the data demonstrate a possible immunostimulatory activity in our model.
References Bibliographical

Table 1 - Total frequency and mean of the micronucleus assay in erythrocytes from the peripheral blood of *Swiss* female mice treated with single exposure of different doses of the compound flavone.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>24 h (T1)</th>
<th>48h(T2)</th>
<th>72h(T3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>46 ± 3.35 c</td>
<td>43 ± 3.00 c</td>
<td>34.4 ±5.68 c</td>
</tr>
<tr>
<td>Negative Control</td>
<td>7.4 ± 2.0736 a</td>
<td>9.8 ± 4.0249 b</td>
<td>8.4 ±3.0495 b</td>
</tr>
<tr>
<td>175 mg/Kg⁻¹</td>
<td>8.4 ± 2,3021 ab</td>
<td>6 ± 1,8708 ab</td>
<td>6.2 ±2,3874 a</td>
</tr>
<tr>
<td>560 mg/Kg⁻¹</td>
<td>9.2 ± 2,1679 b</td>
<td>5.4 ± 1,3416 a</td>
<td>8.4 ±2,7018 b</td>
</tr>
<tr>
<td>1792 mg/Kg⁻¹</td>
<td>9 ± 3,2403 ab</td>
<td>8.2 ± 1,9235 ab</td>
<td>8.4 ±3,3615 b</td>
</tr>
</tbody>
</table>

Negative Control - DMSO 1%; Ciclofosfamide – 100mg/Kg, i.p.; Different letters indicate statistically significant differences (p < 0.05; ANOVA-Tukey).
Table 2: Mean, ± S.E.M, total of injured cells and mean frequency between different classes for the anti-genotoxic test of flavones, comet assay in Swiss female mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Injured Cells</th>
<th>Classes of damage</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Negative Control</strong></td>
<td>13,60±0,51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86,40±0,51</td>
<td>13,00±0,45</td>
</tr>
<tr>
<td><strong>Positive Control</strong></td>
<td>85,40 ±0,68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14,60±0,68</td>
<td>68,20±0,58</td>
</tr>
<tr>
<td><strong>175 mg/Kg&lt;sup&gt;1&lt;/sup&gt;</strong></td>
<td>17,20±0,66 &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>82,80±0,66</td>
<td>17,20±0,66</td>
</tr>
<tr>
<td><strong>560 mg/Kg&lt;sup&gt;1&lt;/sup&gt;</strong></td>
<td>18,60±0,24 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>81,40±0,24</td>
<td>17,60±0,40</td>
</tr>
<tr>
<td><strong>1792 mg/Kg&lt;sup&gt;1&lt;/sup&gt;</strong></td>
<td>17,00±1,58 &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>82,20±0,86</td>
<td>0,00±0,00</td>
</tr>
</tbody>
</table>

Negatives Control - DMSO 1%; Ciclofosfamide – 100mg/Kg, i.p.; Different letters indicate statistically significant differences (p < 0,05; ANOVA-Tukey).
Table 3 – Frequency, mean and standard deviation referring to the splenic phagocytosis in female *Swiss* mice, treated with different doses of flavone o.r. i.p.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total of analyzed cells</th>
<th>Total of cells with evidence of phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value</td>
<td>Mean±S.E.M</td>
</tr>
<tr>
<td>Negative Control</td>
<td>500</td>
<td>188</td>
</tr>
<tr>
<td>Positive Control</td>
<td>500</td>
<td>306</td>
</tr>
<tr>
<td>175 mg/Kg^1</td>
<td>500</td>
<td>275</td>
</tr>
<tr>
<td>560 mg/Kg^1</td>
<td>500</td>
<td>279</td>
</tr>
<tr>
<td>1792 mg/Kg^1</td>
<td>500</td>
<td>279</td>
</tr>
</tbody>
</table>

Negative Control - DMSO 1%; Ciclofosfamide – 100mg/Kg^1 p.c i.p.; Different letters indicate statistically significant differences (p < 0.05; ANOVA- Tukey).
Table 4 - Differential count of groups treated with a single dose of flavone.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Red cells</th>
<th>Hematocrit</th>
<th>Hemoglobin</th>
<th>Neutrophil</th>
<th>Lymphocyte</th>
<th>Monocyte</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>9.66±0.134</td>
<td>46.6±1.341</td>
<td>15.4±0.547</td>
<td>53.6±8.9</td>
<td>45.6±7.02</td>
<td>1.40±0.54</td>
<td>348.8±71.394</td>
</tr>
<tr>
<td>Negative Control</td>
<td>9.24±0.391</td>
<td>44.4±4.827</td>
<td>14.46±1.599</td>
<td>43.8±3.49</td>
<td>56.2±3.49</td>
<td>0.2±0.447</td>
<td>326±69.932</td>
</tr>
<tr>
<td>175 mg/Kg⁻¹</td>
<td>9.28±0.192</td>
<td>42.8±1.923</td>
<td>14.2±0.836</td>
<td>42.2±5.4</td>
<td>57.6±3.04</td>
<td>0.4±0.894</td>
<td>360.8±30.474</td>
</tr>
<tr>
<td>560 mg/Kg⁻¹</td>
<td>9.56±0.357</td>
<td>44.8±3.346</td>
<td>15.1±1.224</td>
<td>46.1±5.8</td>
<td>54.1±5.8</td>
<td>0.0±0.00</td>
<td>347±42.332</td>
</tr>
<tr>
<td>1792 mg/Kg⁻¹</td>
<td>9.34±0.397</td>
<td>43.4±3.974</td>
<td>14.6±1.140</td>
<td>49.2±0.0</td>
<td>49.8±2.94</td>
<td>1.2±1.30</td>
<td>392.2±44.650</td>
</tr>
</tbody>
</table>

Negative Control - DMSO 1%; Ciclofosfamide – 100mg/Kg⁻¹ p.c i.p.; Different letters indicate statistically significant differences (p < 0.05; ANOVA- Tukey).
<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Urea</th>
<th>Creatinine</th>
<th>Alkaline Phosphatase</th>
<th>AST</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>69±7,1763</td>
<td>0,5 ±0,070</td>
<td>115,8 ±1,483</td>
<td>75 ±3,033</td>
<td>70,4 ±7,765</td>
</tr>
<tr>
<td>Negative Control</td>
<td>47,8±4,061</td>
<td>0,36±0,054</td>
<td>117,41,673</td>
<td>74,4±1,341</td>
<td>51,6±3,781</td>
</tr>
<tr>
<td>175 mg/Kg⁻¹</td>
<td>44,4±3,872</td>
<td>0,28±0,083</td>
<td>115,8±3,114</td>
<td>73,2±1,788</td>
<td>48±2,549</td>
</tr>
<tr>
<td>560 mg/Kg⁻¹</td>
<td>44±2,543</td>
<td>0,3±0,070</td>
<td>114,6±3,781</td>
<td>72,8±2,049</td>
<td>48,21,788</td>
</tr>
<tr>
<td>1792 mg/Kg⁻¹</td>
<td>44,6±271</td>
<td>0,26±0,054</td>
<td>117±2,915</td>
<td>72,4±1,140</td>
<td>49,8±2,489</td>
</tr>
</tbody>
</table>

Negative Control - DMSO 1%; Ciclofosfamide – 100mg/Kg⁻¹ p.c i.p.; Different letters indicate statistically significant differences (p < 0.05; ANOVA-Tukey).
Table 6. Initial and final body weight and weight gain.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Weight gain (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>28,742 ± 2,868</td>
<td>29,616 ± 3,326</td>
<td>0,87b</td>
</tr>
<tr>
<td>Negative Control</td>
<td>25,588 ± 1,5529</td>
<td>27,088 ± 1,982</td>
<td>1,50a</td>
</tr>
<tr>
<td>175 mg/Kg⁻¹</td>
<td>27,658 ± 1,8828</td>
<td>28,248 ± 1,931</td>
<td>0,59b</td>
</tr>
<tr>
<td>560 mg/Kg⁻¹</td>
<td>25,660 ± 1,1828</td>
<td>27,010 ± 2,021</td>
<td>1,35b</td>
</tr>
<tr>
<td>1792 mg/Kg⁻¹</td>
<td>27,006 ± 1,8500</td>
<td>27,170 ± 2,038</td>
<td>0,16c</td>
</tr>
</tbody>
</table>

Negative Control - DMSO 1%; Ciclofosfamide – 100mg/Kg⁻¹ p.c i.p.; Different letters indicate statistically significant differences (p < 0, 05; ANOVA-Tukey).
Table 7. Absolute and relative weight of animals and absolute weight of organs of the groups treated with flavone.

<table>
<thead>
<tr>
<th>Absolute weight (g)</th>
<th>Heart</th>
<th>Lung</th>
<th>Liver</th>
<th>Right kidney</th>
<th>Left kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>0.15±0.019</td>
<td>0.232±0.037</td>
<td>1.2960±0.214</td>
<td>0.178±0.03</td>
<td>0.186±0.023</td>
<td>0.166±0.018</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0.15±0.021</td>
<td>0.228±0.031</td>
<td>1.1660±0.168</td>
<td>0.176±0.01</td>
<td>0.176±0.028</td>
<td>0.156±0.042</td>
</tr>
<tr>
<td>175 mg/Kg⁻¹</td>
<td>0.15±0.019</td>
<td>0.262±0.040</td>
<td>1.3020±0.116</td>
<td>0.176±0.02</td>
<td>0.176±0.011</td>
<td>0.18±0.045</td>
</tr>
<tr>
<td>560 mg/Kg⁻¹</td>
<td>0.16±0.024</td>
<td>0.236±0.036</td>
<td>1.2580±0.122</td>
<td>0.184±0.01</td>
<td>0.182±0.019</td>
<td>0.16±0.045</td>
</tr>
<tr>
<td>1792 mg/Kg⁻¹</td>
<td>0.17±0.028</td>
<td>0.264±0.047</td>
<td>1.3460±0.211</td>
<td>0.18±0.031</td>
<td>0.196±0.042</td>
<td>0.172±0.010</td>
</tr>
<tr>
<td>Relative weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive Control</td>
<td>0.528±0.060</td>
<td>0.787±0.131</td>
<td>4.367±0.418</td>
<td>0.601±0.933</td>
<td>0.629±0.058</td>
<td>0.567±0.096</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0.581±0.046</td>
<td>0.839±0.077</td>
<td>4.300±0.480</td>
<td>0.651±0.707</td>
<td>0.648±0.083</td>
<td>0.576±0.155</td>
</tr>
<tr>
<td>175 mg/Kg⁻¹</td>
<td>0.551±0.036</td>
<td>0.926±0.122</td>
<td>4.620±0.448</td>
<td>0.625±0.096</td>
<td>0.626±0.069</td>
<td>0.641±0.174</td>
</tr>
<tr>
<td>560 mg/Kg⁻¹</td>
<td>0.593±0.088</td>
<td>0.873±0.109</td>
<td>4.657±0.279</td>
<td>0.685±0.085</td>
<td>0.675±0.068</td>
<td>0.593±0.163</td>
</tr>
<tr>
<td>1792 mg/Kg⁻¹</td>
<td>0.625±0.091</td>
<td>0.968±0.134</td>
<td>4.959±0.725</td>
<td>0.659±0.087</td>
<td>0.716±0.130</td>
<td>0.635±0.048</td>
</tr>
</tbody>
</table>

+/- Standard deviation
* Statistically significant difference (p <0.05; ANOVA / Tukey)
ANEXO II – NORMAS DA REVISTA
1. Format Requirements

*PLOS ONE* does *not* consider presubmission inquiries. All submissions should be prepared with the following files:

- Cover letter
- Manuscript, including tables and figure legends
- Figures (guidelines for preparing figures can be found at the [Figure and Table Guidelines](#))

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like "scientific editing service" or "manuscript editing service." Submissions are *not* copyedited before publication.

Submissions that do not meet the *PLOS ONE* Publication Criterion for language standards may be rejected.

**Cover Letter**

You should supply an approximately one page cover letter that:

- Concisely summarizes why your paper is a valuable addition to the scientific literature
- Briefly relates your study to previously published work
- Specifies the type of article you are submitting (for example, research article, systematic review, meta-analysis, clinical trial)
- Describes any prior interactions with PLOS regarding the submitted manuscript
- Suggests appropriate *PLOS ONE* Academic Editors to handle your manuscript (view a complete listing of our academic editors)
- Lists any recommended or opposed reviewers

Your cover letter should *not* include requests to reduce or waive publication fees. Should your manuscript be accepted, you will have the opportunity to include your requests at that time. See *PLOS ONE* Editorial Policy for more information regarding publication fees.

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*PLOS ONE* considers manuscripts of any length. There are no explicit restrictions for the number of words, figures, or the length of the supporting information, although we encourage a concise and accessible writing style. We will *not* consider monographs.

All manuscripts should be double-spaced and include line numbers and page numbers.

Manuscripts should begin with the ordered sections:

- Title
- Authors
• Affiliations
• Abstract
• Introduction and end with the sections of:
• Acknowledgments
• References
• Figure Legends
• Tables

Figures should not be included in the main manuscript file. Each figure must be prepared and submitted as an individual file. Find more information about preparing figures here.

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

There are no explicit requirements for section organization between these beginning and ending sections. Articles may be organized in different ways and with different section titles, according to the authors’ preference. In most cases, internal sections include:

• Materials and Methods
• Results
• Discussion
• Conclusions (optional)

PLOS ONE has no specific requirements for the order of these sections, and in some cases it may be appropriate to combine sections. Guidelines for individual sections can be found below.

Abbreviations should be kept to a minimum and defined upon first use in the text. Non-standard abbreviations should not be used unless they appear at least three times in the text.

Standardized nomenclature should be used as appropriate, including appropriate usage of species names and SI units.

PLOS articles do not support text footnotes. If your accepted submission contains footnotes, you will be asked to move that material into either the main text or the reference list, depending on the content.

Manuscript File Type Requirements

Authors may submit their manuscript files in Word (as .doc or .docx), LaTeX (as .pdf), or RTF format. Only RTF and .doc files can be used during the production process. Word files must not be protected.

LaTeX Submissions. If you would like to submit your manuscript using LaTeX, you must author your article using the PLOS ONE LaTeX template and BibTeX style sheet. Articles prepared in LaTeX may be submitted in PDF format for use during the review process. After acceptance, however, .tex files and formatting information will be required as a zipped file. Please consult our LaTeX guidelines for a list of what will be required.
Submissions with equations. If your manuscript is or will be in .docx format and contains equations, you must follow the instructions below to make sure that your equations are editable when the file enters production.

If you have not yet composed your article, you can ensure that the equations in your .docx file remain editable in .doc by enabling "Compatibility Mode" before you begin. To do this, open a new document and save as Word 97-2003 (*.doc). Several features of Word 2007/10 will now be inactive, including the built-in equation editing tool. You can insert equations in one of the two ways listed below.

If you have already composed your article as .docx and used its built-in equation editing tool, your equations will become images when the file is saved down to .doc. To resolve this problem, re-key your equations in one of the two following ways.

1. Use MathType to create the equation (recommended)
2. Go to Insert > Object > Microsoft Equation 3.0 and create the equation

If, when saving your final document, you see a message saying "Equations will be converted to images," your equations are no longer editable and PLoS will not be able to accept your file.

Back to top

2. Guidelines for Standard Sections

Title

Manuscripts must be submitted with both a full title and a short title, which will appear at the top of the PDF upon publication if accepted. Only the full title should be included in the manuscript file; the short title will be entered during the online submission process.

The full title must be 250 characters or fewer. It should be specific, descriptive, concise, and comprehensible to readers outside the subject field. Avoid abbreviations if possible. Where appropriate, authors should include the species or model system used (for biological papers) or type of study design (for clinical papers).

Examples:

- Impact of Cigarette Smoke Exposure on Innate Immunity: A Caenorhabditis elegans Model
- Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial

The short title must be 50 characters or fewer and should state the topic of the paper.

Back to top

Authors and Affiliations

All author names should be listed in the following order:

- First names (or initials, if used),
- Middle names (or initials, if used), and
- Last names (surname, family name)
  Each author should list an associated department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article.

  **This information cannot be changed after initial submission, so please ensure that it is correct.**

To qualify for authorship, a researcher should contribute to **all** of the following:

1. Conception and design of the work, acquisition of data, or analysis and interpretation of data
2. Drafting the article or revising it critically for important intellectual content
3. Final approval of the version to be published
   All persons designated as authors should qualify for authorship, and all those who qualify should be listed. Each author must have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments.

When a large group or center has conducted the work, the author list should include the individuals whose contributions meet the criteria defined above, as well as the group name.

One author should be designated as the corresponding author, and his or her email address or other contact information should be included on the manuscript cover page. This information will be published with the article if accepted.

See the *PLOS ONE* Editorial Policy regarding authorship criteria for more information.

Back to top

Abstract

The abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words
  Abstracts should **not** include:
  - Citations
  - Abbreviations, if possible

Back to top

Introduction

The introduction should:
• Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
• Define the problem addressed and why it is important
• Include a brief review of the key literature
• Note any relevant controversies or disagreements in the field
• Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Back to top

Materials and Methods

This section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

We encourage authors to submit detailed protocols for newer or less well-established methods as Supporting Information. Further information about formatting Supporting Information files, can be found here.

Methods sections of papers on research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the Reporting Guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

Methods sections of papers with data that should be deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers and version numbers, if appropriate. Accession numbers should be provided in parentheses after the entity on first use. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication. A list of recommended repositories for different types of data can be found here.

Methods sections of papers using cell lines must state the origin of the cell lines used. See the Reporting Guidelines for cell line research for more information.

Methods sections of papers adding new taxon names to the literature must follow the Reporting Guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

Back to top

Results, Discussion, and Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled “Results and Discussion”) or a mixed Discussion/Conclusions section (commonly labeled “Discussion”). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.
Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn. Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

*PLOS ONE* editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* [Publication Criteria](https://journals.plos.org/plosone/s/publication-criteria) for more information.

**Back to top**

**Acknowledgments**

People who contributed to the work but do not fit the *PLOS ONE* authorship criteria should be listed in the acknowledgments, along with their contributions. You must ensure that anyone named in the acknowledgments agrees to being so named.

Funding sources should **not** be included in the acknowledgments, or anywhere in the manuscript file. You will provide this information during the manuscript submission process.

**Back to top**

**References**

**General guidelines**

- Authors may cite any and all available works in the reference list.
- Authors may not cite unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”).
- If an article is submitted to a journal and also publicly available as a pre-print, the pre-print may be cited.
- If related work has been submitted to *PLOS ONE* or elsewhere, authors should include a copy with the submitted article as confidential supplementary information, for review purposes only.
- Authors should not state 'unpublished work' or 'data not shown,' but instead include those data as supplementary material or deposit the data in a publicly available database.
- Authors should not state 'unpublished work' or 'data not shown,' but instead include those data as supplementary material or deposit the data in a publicly available database.

**Reference formatting**

References must be listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, citations should be indicated by the reference number in brackets. Journal name abbreviations should be those found in the NCBI databases. A number of reference software companies supply PLOS style files (e.g., Reference Manager, EndNote).

References should be formatted as follows:

- **Published papers.** Hou WR, Hou YL, Wu GF, Song Y, Su XL, et al. (2011) cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda...

Note: Use of a DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.

- **Accepted, unpublished papers.** Same as above, but “In press” appears instead of the page numbers.


• **Multimedia. Examples: videos, movies, and TV shows.** Hitchcock A, producer and director (1954) Rear Window [Film]. Los Angeles: MGM.

Back to top

Tables

Tables should be included at the end of the manuscript. All tables should have a concise title. Footnotes can be used to explain abbreviations. Citations should be indicated using the same style as outlined above. Tables occupying more than one printed page should be avoided, if possible. Larger tables can be published as Supporting Information. Please ensure that table formatting conforms to our Guidelines for table preparation.

Back to top

Figure Legends

Figures should not be included in the manuscript file, but figure legends should be. Guidelines for preparing figures can be found here.

Figure legends should describe the key messages of a figure. Legends should have a short title of 15 words or less. The full legend should have a description of the figure and allow readers to understand the figure without referring to the text. The legend itself should be succinct, avoid lengthy descriptions of methods, and define all non-standard symbols and abbreviations.

Further information about figure legends can be found in the Figure Guidelines.

Back to top

Accession Numbers

All appropriate datasets, images, and information should be deposited in public resources. Please provide the relevant accession numbers (and version numbers, if appropriate). Accession numbers should be provided in parentheses after the entity on first use. Suggested databases include, but are not limited to:

- ArrayExpress
- BioModels Database
- Database of Interacting Proteins
- DNA Data Bank of Japan [DDBJ]
- DRYAD
- EMBL Nucleotide Sequence Database
- GenBank
- Gene Expression Omnibus [GEO]
- Protein Data Bank
- UniProtKB/Swiss-Prot
• ClinicalTrials.gov
   In addition, as much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

• Ensembl
• Entrez Gene
• FlyBase
• InterPro
• Mouse Genome Database (MGD)
• Online Mendelian Inheritance in Man (OMIM)
• PubChem
   Providing accession numbers allows linking to and from established databases and integrates your article with a broader collection of scientific information.

Back to top

Striking Images

Authors are encouraged to upload a “striking image” that may be used to represent their paper online in places like the journal homepage or in search results. The striking image must be derived from a figure or supporting information file from the paper, ie. a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows. If no striking image is uploaded, a figure from the paper will be designated as the striking image.

Please keep in mind that PLOS’s Creative Commons Attribution License applies to striking images. As such, do not submit any figures or photos that have been previously copyrighted unless you have express written permission from the copyright holder to publish under the CCAL license. Note that all published materials in PLOS ONE are freely available online, and any third party is permitted to read, download, copy, distribute, and use these materials in any way, even commercially, with proper attribution.

Care should be taken with the following image types in particular:

1. PLOS ONE is unable to publish any images generated by Google software (Google Maps, Street View, and Earth)
2. Maps in general are usually copyrighted, especially satellite maps
3. Photographs
4. Commercial or government images, slogans, or logos
5. Images from Facebook or Twitter
   Authors must also take special care when submitting manuscripts that contain potentially identifying images of people. Identifying information should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF).

For license inquiries, e-mail license [at] plos.org.
3. Specific Reporting Guidelines

Human Subject Research

Methods sections of papers on research using human subject or samples must include ethics statements that specify:

- The name of the approving institutional review board or equivalent committee(s). If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed.
- Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:
  - Why written consent could not be obtained
  - That the Institutional Review Board (IRB) approved use of oral consent
  - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual’s case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

**The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.**

For more information about PLOS ONE policies regarding human subject research, see the Publication Criteria and Editorial Policies.
Clinical Trials

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement website. Before the paper can enter peer review, authors must:

1. Provide the registry name and number in the methods section of the manuscript
2. Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as Supporting Information (which will be published alongside the paper, if accepted)
3. Include the CONSORT flow diagram as the manuscript's "Figure 1"
   Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

For more information about PLOS ONE policies regarding clinical trials, see the Editorial Policies.

Back to top

Animal Research

Methods sections of manuscripts reporting results of animal research must include required ethics statements that specify:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s) (where ethical approval is not required, the manuscript should include a clear statement of this and the reason why)
- Relevant details for efforts taken to ameliorate animal suffering

For example:

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

The organism(s) studied should always be stated in the abstract. Where research may be confused as pertaining to clinical research, the animal model should also be stated in the title.

We ask authors to follow the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information. Please note that inclusion of a completed ARRIVE Checklist will be a formal requirement for publication at a later date.
Observational and Field Studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

For more information about PLOS ONE policies regarding observational and field studies, see the Publication Criteria and Editorial Policies.

Cell Line Research

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate. Authors must also include the following information for each cell line:

For de novo (new) cell lines, including those given to the researchers a gift, authors must follow our policies for human subject research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended - e.g. by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis - and may be required during peer review or after publication.
Blots and Gels

Authors of manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with PLOS ONE's policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast
- All blots and gels that support results reported in the manuscript should be provided
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made

Back to top

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species
- The commercial supplier or source laboratory
- The catalogue or clone number and, if known, the batch number
- The antigen(s) used to raise the antibody
- For established antibodies, authors are encouraged to supply a stable public identifier from the Antibody Registry (www.antibodyregistry.org). Authors should also report the following experimental details:
  - The final antibody concentration or dilution
  - A reference to the validation study if the antibody was previously validated, and if not, details of how the authors validated the antibody for the applications and species used. Authors should consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

Back to top
Systematic Review/Meta-Analysis

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as Supporting Information and provide the registry number in the abstract.

If your article is a Systematic Review or a Meta-Analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flowchart as Figure 1 (required where applicable)
- Include the PRISMA checklist as Supporting Information

Meta-Analysis of Genetic Association Studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in “Systematic Reviews of Genetic Association Studies” by Sagoo et al.

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Back to top

Paleontology and Archaeology Research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.
If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

See the PLOS ONE Editorial Policies for more information regarding manuscripts describing paleontology and archaeology research.

Software Papers

Manuscripts describing software should provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

See the PLOS ONE Editorial Policies for more information about submitting manuscripts.

Database Papers

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

See the PLOS ONE Editorial Policies for more information about submitting manuscripts describing databases.

New Zoological Taxon

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni Fisher sp. nov. urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB
You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the Methods section, in a sub-section to be called "Nomenclatural Acts":

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories]. All PLOS ONE articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

New Botanical Taxon

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). In association with the International Plant Names Index (IPNI), the following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, "the description or diagnosis required for valid publication of the name of a new taxon" can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Solanum aspersum S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San
Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

_PLOS ONE_ staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase.

In the _Methods_ section, include a sub-section called "Nomenclature" using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a _PLOS ONE_ article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All _PLOS ONE_ articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

**New Fungal Taxon**

When publishing papers that describe a new fungal taxon name, _PLOS_ aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific fungal name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, "the description or diagnosis required for valid publication of the name of a new taxon" can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of a _PLOS ONE_ article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found [here](#).

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the _Results_ section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

*Hymenogaster huthii*. Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]
You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the Methods section, include a sub-section called "Nomenclature" using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies. In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)]. All PLOS ONE articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative Research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.
ANEXO III
Genotoxic and Mutagenic Effects of Diflubenzuron, an Insect Growth Regulator, on Mice

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Published online: 29 Oct 2013.

To cite this article: Aline Lima de Barros, Vanessa Vilamor de Souza, Stephanie Dynczuki Navarro, Silvia Aparecida Oesterreichen, Rodrigo Juliano Oliveira, Cândida Aparecida Leite Kassaya & Arielle Cristina Arena (2013) Genotoxic and Mutagenic Effects of Diflubenzuron, an Insect Growth Regulator, on Mice, Journal of Toxicology and Environmental Health, Part A: Current Issues, 76:17, 1003-1006

To link to this article: http://dx.doi.org/10.1080/15287354.2013.830585

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Evaluation of the anti-mycobacterium tuberculosis activity and in vivo acute toxicity of annona sylvatic


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ISSN 1472-6882
Article type Research article
Submission date 18 March 2014
Acceptance date 17 June 2014
Publication date 28 June 2014
Article URL http://www.biomedcentral.com/1472-6882/14/209

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