UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

GLÍCIA MARIA DE BARROS ALVES

Avaliação da atividade expectorante/mucolítica e antioxidante do ácido fumarprotocetrárico isolado de *Cladonia verticillaris* (líquen) em camundongos Swiss *webster*

Recife
2012

GLÍCIA MARIA DE BARROS ALVES

Avaliação da atividade expectorante/mucolítica e antioxidante do ácido fumarprotocetrárico isolado de *Cladonia verticillaris* (líquen) em camundongos Swiss *webster*

Dissertação apresentada ao Programa de Pós-graduação como parte dos requisitos para obtenção do título de mestre em Bioquímica e Fisiologia da Universidade Federal de Pernambuco.

Orientador: Profº. Dr. Nicácio Henrique da Silva

Co-orientador: Profª. Drª. Maria Bernadete de Sousa Maia

Recife

2012

Alves, Glícia Maria de Barros

Avaliação da atividade expectorante/mucolítica e antioxidante do ácido fumarprotocetrático isolado de *Cladonia verticillaris* (líquen) em camundongos *Swiss webster*/ Glícia Maria de Barros Alves– Recife: O Autor, 2012.

59 folhas : il., fig.
Orientador: Nicácio Henrique da Silva
Coorientadora: Maria Bernadete de Sousa Maia
Dissertação (mestrado) – Universidade Federal de Pernambuco, Centro de Ciências Biológicas, Bioquímica e Fisiologia, 2012.

Inclui bibliografia e anexos

1. Líquens 2. Àcidos 3. Fitoterapia I. Silva, Nicácio Henrique da II. Maia, Maria Bernadete de Sousa (coorientadora) III. Título

579.7 CDD (22.ed.) UFPE/CCB- 2012- 234

GLÍCIA MARIA DE BARROS ALVES

Avaliação da atividade expectorante/mucolítica e antioxidante do ácido fumarprotocetrárico isolado de *Cladonia verticillaris* (líquen) em camundongos Swiss *webster*

Dissertação apresentada ao Programa de Pós-graduação como parte dos requisitos para obtenção do título de mestre em Bioquímica e Fisiologia da Universidade Federal de Pernambuco.

Aprovado em _____/_____/_____.

BANCA EXAMINADORA

_____
Profº Dr. Nicácio Henrique da Silva (Orientador)
Universidade Federal de Pernambuco

_____
Profª Drª Vera Lúcia de Menezes Lima (Examinador Interno)
Universidade Federal de Pernambuco

_____
Profª Drª Noemia Pereira da Silva Santos (Examinador Interno)
Universidade Federal de Pernambuco

_____
Profª Drª Maria Eliane Bezerra de Melo (Examinador Externo)
Centro de Pesquisas Aggeu Magalhães/FIOCRUZ

“Aprender é a única coisa de que a mente nunca se cansa, nunca se tem medo e nunca se arrepende” (DA VINCI, 2012).

Aos meus pais, Laura e Edvaldo, por sempre acreditarem em mim e a todos os meus familiares e amigos.

AGRADECIMENTOS

A Deus, o criador de todas as coisas, por sempre me guiar nos caminhos da vida.

Aos meus pais, irmãos, pelo apoio, convivência e lição de vida.

Ao meu orientador Profº Dr. Nicácio Henrique da Silva, do Departamento de Bioquímica da Universidade Federal de Pernambuco, pela prestimosa orientação científica, confiança, competência, incentivo, carinho tão bem transmitidos no decorrer do curso.

À Profª Dra Maria Bernadete de Souza Maia pela paciência, confiança, competência e apoio na co-orientação deste trabalho.

À Profª Dra Vera Lúcia de Menezes Lima pela confiança, apoio e dedicação, quando esteve na coordenação do mestrado.

Ao técnico do Laboratório de Produtos Naturais do Departamento de Bioquímica/UFPE, Sr. João Virgínio por ter me auxiliando nas horas em que eu mais necessitava.

A todos os professores do curso de extensão “Atualização em Bioquímica”.

Aos colegas dos Laboratórios de Produtos Naturais e de Farmacologia de Produtos Bioativos, muito obrigada pela amizade, paciência, compreensão e colaboração para a realização deste sonho.

À Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) pela concessão da bolsa de estudo utilizada como fonte financeira para a realização do presente trabalho.

RESUMO

O líquen *Cladonia verticillaris* apresenta metabólitos secundários bioativos como os ácidos fumarprotocetrárico (FUM) e protocetrárico (PRO). O gênero Cladonia apresenta atividade antitumoral, antiinflamatória, antipirética e é utilizada na medicina popular para tratar doenças do trato respiratório (irritação da garganta, tosse, asma e tuberculose). Este trabalho teve como objetivo avaliar a atividade expectorante/mucolítico e antioxidante do ácido fumarprotocetrárico em camundongos swiss albino. Métodos: O FUM foi extraído e purificado a partir do extrato acetônico de *C. verticillaris*. A fim de avaliar a atividade expectorante do FUM foi utilizado o método de quantificação de vermelho de fenol no lavado broncoalveolar. Quando este é administrado por via intraperitoneal em camundongos swiss. Os animais foram tratados com FUM (25, 50 ou 100 mg/kg por via oral ou intraduodenal e 12,5, 25 ou 50 mg/kg, por via intraperitoneal. Os grupos controles receberam pelas mesmas vias solução salina a 7,5 mL/kg ou ambroxol 1 mg/kg). A atividade antioxidante foi avaliada pelo método ácido tiobarbitúrico (TBARS) em tecido pulmonar dos camundongos, tratados com o FUM a 25, 50 ou 100 mg/kg por via oral), depois de receber a solução de LPS a 1 mg/kg via intrapleural. O mesmo protocolo foi adotado para os grupos controles (salina 7,5 mL/kg, por via oral ou N-acetilcisteina, 20mg/kg, por via oral). Resultados: O FUM em doses de 25 e 50mg/kg, administrada por via oral, promoveu um aumento dose-dependente do vermelho de fenol na lavagem broncoalveolar. A peroxidação lipídica (MDA medidos como equivalentes) foi reduzida em 50% em tecido pulmonar. Conclusão: Os resultados confirmam as propriedades expectorantes e antioxidantes do ácido fumarprotocetrárico produzido pelo líquen *Cladonia verticillaris.*

Palavras-chave: Ambroxol. N-acetilcisteína. Líquen. Antioxidante.

ABSTRACT

The lichen *Cladonia verticillaris* produce bioactive secondary methabolites as the acids fumarprotocetraric (FUM) and protocetraric (PRO). The *Cladonia* genus has demonstrate antitumoral, antiinflamatory and antipiretic activities and used in folk medicine to treat respiratory deseases (troat irritation, cough, asthma and tuberculosis). This work had the objective to evaluate the expectorante/mucolytic and antioxidant activity of the fumrprotocetraric acid in albino swiss mice. Methods: the FUM was extracted and purified from the acetonic extract of *C. verticillaris.* In order to evaluate the expectorante activity of FUM was used the phenol red method in quantitative scale in the no bronchoalveolar lavage fluid.When administered intraperitoneally in swiss mice.The animals were treated with FUM (25, 50 ou 100 mg/kg orally or intraduodenal and 12,5, 25 or 50 mg/kg, intraperitoneally) The control groups receive by the same via saline solution of 7,5 mL/kg or ambroxol 1 mg/kg). The antioxidante activity was evaluated with the TBARS method in pulmonar mouse tissue treated with the FUM at 25, 50 or 100 mg/kg orally) after receiving the LPS solution at 1 mg/kg intrapleural via. The same protocol was adopted to the control groups (salina 7,5 mL/kg, orally or N-acetilcisteine, 20mg/kg, orally). Results: The FUM in doses of 25 and 50mg/kg, administered orally, promoted increased dose-dependent of phenol red in the bronchoalveolar lavage. Lipid peroxidation (MDA measured as equivalent) was reduced by 50% in lung tissue. Conclusion: The results confirm the expectorant properties and antioxidants fumarprotocetraric acid produced by the lichen *Cladonia verticillaris.*

Keywords: Ambroxol. N-acetilcisteína. Lichen . Antioxidant.

SUMÁRIO

LISTA DE FIGURAS

Revisão de Literatura

Artigo

LISTA DE ABREVIATURAS

ATR	Atranorina
CCD	Cromatografia em camada delgada
CLAE	Cromatografia Líquida de Alta Eficiência
DRC	Doença Respiratórias Crônicas
FUM	Ácido fumarprotocetárico
I.P	Intraperitoneal
ID	Intraduodenal
LPS	Lipopolissacarídeo
MDA	medicina tradicional chinesa
MDA	Malonylaldeído
MS	Ministério da Saúde
MTC	Medicina Tradicional Chinesa
NAC	n-Acetilcisteína
OMS	Organização Mundial de Saúde
PRO	Ácido protocetrárico
TBARS	Ácido Tiobarbitúrico
V.O	Via oral

1 INTRODUÇÃO

Atualmente é crescente o interesse da sociedade pela utilização de fitoterápicos para o tratamento de uma grande diversidade de enfermidades, devido também, ao baixo custo desses produtos. A busca por novos agentes naturais pode gerar uma diversidade estrutural superior do que a química sintética convencional e que pode oferecer oportunidades significantes para a descoberta de novos compostos (BINDSEIL et al., 2001). O Brasil, com grandeza de seu litoral, de sua flora e sendo detentor da maior floresta equatorial e tropical úmida do planeta, não pode abdicar de sua vocação para os produtos naturais.

O líquen *Cladonia verticillaris,* comum no Nordeste brasileiro, contêm como compostos principais os ácidos fumarprotocetrárico e protocetrárico que demonstrou ação antitumoral (SANTOS et al., 1997), antiinflamatória aguda e crônica, antinociceptiva e antipirética (SANTOS, 2003). No entanto o mesmo possui composição química semelhante a *C. islandica*.

Através dos anos, os liquens têm sido utilizados com vários propósitos, como corantes, perfumes e medicamentos na medicina popular (MULLER, 2001). Em muitos países da Europa, o líquen Ce*traria islandica* é uma espécie também usada para tratar irritação da garganta laringite, tosse e afecções do trato respiratório na forma de xaropes expectorantes e pastilhas (INGÖLFSDÓTTIR, 2000).

As doenças respiratórias constituem importante causa de mortalidade em adultos e crianças no mundo. Segundo dados da Organização Mundial de Saúde (2004, 2005), estas doenças representam cerca de 8% do total de mortes em países desenvolvidos e 5% em países em desenvolvimento. Estima-se que quatro milhões de pessoas com doenças respiratórias crônicas (DRC) podem ter morrido prematuramente em 2005 e as projeções são de aumento considerável do número de mortes no futuro. Como estratégia para enfrentar esse problema de saúde no plano mundial, a OMS criou a

Global Alliance Against Chronic Respiratory Diseases (BRASIL. Ministério da Saúde, 2010).

Os expectorantes são usados para aliviar a tosse, aumentando a secreção das vias respiratórias, reduzindo a viscosidade tornando-a mais eficaz. O campo de medicamentos para o tratamento da tosse é relativamente escasso. O obstáculo para a investigação neste campo é a dificuldade de coleta de amostras do muco, uma vez que a produção deste é relativamente baixo em indivíduos sadios (KAGAN; LAVY; HOFFMAN, 2009).

Em virtude do exposto acima, levando em consideração a comprovada eficácia das substâncias liquênicas, notadamente do ácido fumarprotocetrárico e a disponibilidade de espécies produtoras deste composto, torna-se viável esta pesquisa da ação da atividade mucolítica e antioxidante.

2 REVISÃO DE LITERATURA

2.1 Líquens: generalidades

Os liquens são organismos resultantes da associação simbiótica entre um fungo (micobionte) e uma ou mais espécies de algas (fotobionte) para formar um talo no qual o fungo é exohabitante (HARKSWOTH; HILL, 1984). A simbiose entre estes organismos distintos, onde a alga, que é clorofilada e, portanto, capaz de realizar a fotossíntese e o fungo, aclorofilado e heterótrofo, conferem ao líquen um funcionamento não observado em nenhum outro grupo taxonômico (NASH III, 1996; CONTI, 2001).

As algas dos liquens são unicelulares pertencentes às divisões *Chlorophycophyta* e *Cyanophycophyta*. Os fungos, ou micobiontes, são na maioria *Ascomycota* e em menor proporção Basídio e *Deuteromycota*, formadores dos Asco, Basidio e Deuteroliquens (XAVIER-FILHO; RIZZINI, 1976; ALEXOPOULOS, 1979). A associação entre a alga e o fungo propicia a transferência de nutrientes entre os organismos, mantendo o equilíbrio de carbono, hidrogênio e outros elementos vitais (SEAWARD, 1977).

A morfologia do líquen é baseada na organização do talo liquênico em camadas bem definidas, onde o córtex superior é constituído por hifas entrelaçadas do fungo, possibilitando proteção à camada de algas que está posicionada logo abaixo, protegida por um feixe de hifas frouxas, a medula, seguida de um outro feixe de hifas, o cótex inferior (Figura 1) (HALLE-Jr., 1983). O talo do líquen pode se apresentar de cores e formas variadas, a depender da espécie e das substâncias que ele contém (THOMSON; PAVIA; MCNICOI, 1973).

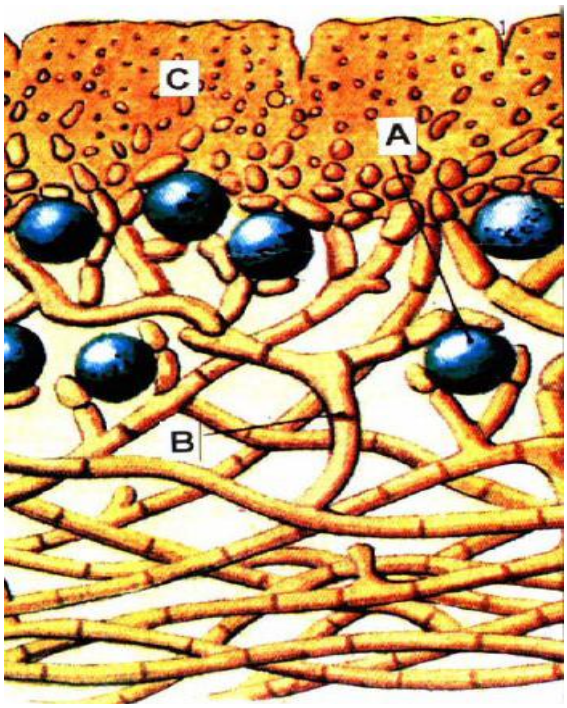


Figura 1- Modelo esquemático da anatomia de um talo liquênico.
A-Alga; B-Hifas medulares; C–Hifas corticais
Fonte: Pereira (1998)

O talo foliáceo ou folhoso é semelhante a uma folha sobre um substrato, e dele se destaca com facilidade, enquanto o talo crustáceo se impregna no substrato como se fosse uma mancha nele, sendo impossível destacá-lo. O talo arbustivo ou fruticoloso apresenta, como sugere o nome um arbusto, e pode ser ereto ou decumbente semelhante a uma planta epífita. Como substrato os liquens habitam desde rochas dos mais diversos tipos, a madeira queimada em decomposição, troncos vivos, folhas, solo, entre outros (SEAWARD, 1977; FEIGE; KREMER, 1979). Eles ocorrem também em condições ambientais naturais, variadas tais como temperaturas baixas, escuridão prolongada, seca e luz contínua. No entanto, em resposta a essas condições extremas, os liquens produzem substâncias características em boas concentrações, tais como depsídeos, depsidonas, dibenzofuranos, antraquinonas e ácidos graxos entre outros (SCHMITT; LUMBSCH, 2004).

2.2 Substâncias liquênicas

No metabolismo primário, a fotossíntese da alga liquênica fornece os hidratos de carbono necessários para o início da nutrição, e todas as reações metabólicas do líquen. Estes açúcares são repassados ao fungo, que não têm capacidade de sintetizá-los, e a partir desse transporte são sintetizadas as substâncias liquênicas, produtos finais do metabolismo secundário únicas desses seres, tais como, as depsídeos e depsidonas (CULBERSON, C.; CULBERSON, W.; JOHNSON, 1977; MACFARLANA; KERSHAW, 1978; NASH III, 1996).

As substâncias liquênicas ao serem sintetizadas no interior do fungo, são excretadas sob a forma de cristais, que de acordo com sua cor, transparência e forma, captam a radiação solar, selecionando o que lhe é útil. Em climas áridos e semi-áridos estes cristais funcionam como impermeabilizantes evitando a perda de umidade. Por este motivo, as substâncias liquênicas têm papel importante no mecanismo de adaptação do líquen aos ambientes mais diversos (LAWREY, 1986; PEREIRA, 1998).

Os liquens produzem substâncias resultantes do metabolismo secundário, denominadas substâncias liquênicas, antigamente designadas como “ácidos liquênicos”. Atualmente sabe-se que sua natureza é quase na totalidade fenólica e são essas substâncias responsáveis pela maioria dos benefícios dos liquens, tais como o antitumoral, antioxidante, antimicrobiana entre outros (XAVIER FILHO; RIZZINI, 1976). A produção das substâncias liquênicas se dá por três vias biossintéticas: a) acetato polimalonato, que forma os ácidos graxos, depsídeos, depsidonas, quinonas e debenzofuranos; b) ácido chiquímico que forma os pigmentos amarelos (compostos aromáticos) e c) ácido mevalônico que origina os terpenóides e esteróides. São ainda relatadas as vias dos aminoácidos e carboidratos verificando-se, nesta última, a biossíntese dos sacarídeos e polióis. Por outro lado, a maioria dos metabólitos secundários têm origem biossintética na via acetato polimalonato e, perfazem cerca de 10% do peso do talo seco (HALE-Jr., 1983; XAVIER-FILHO, 1989; NASH III, 1996).

2.3 Atividade biológica dos liquens

Os liquens são utilizados pela população de diversas partes do mundo como plantas medicinais e por produzirem substâncias oloríferas (óleos essenciais), ou produtos de degradação que geram perfumes muito apreciados. *Evernia furfuracea* era empregada para embalsamar cadáveres no Egito antigo. Fragmentos deste líquen foram encontrados em um vaso da 17ª dinastia egípcia, aproximadamente no século XVII a.C. Os liquens na medicina popular, eram designados como “liquens verdadeiros” aqueles que Dioscorides, cirurgião do exército de Nero, indicava para tratamento de doenças, de acordo com a semelhança que tivessem com a enfermidade, ou os órgãos afetados. Esta era a “Doutrina dos Sinais” (HALE-Jr., 1983).

Outros liquens como *Lobaria pulmonaria* e *Cetraria islandica* também são relatados para uso medicinal desde o século XVII, sendo incluídos em inúmeras farmacopéias até o século passado. O uso de *Usnea longissima* como expectorante e para o tratamento local de ferimentos é relatado na China. Além disso, há relatos que *Usnea* sp é usado a mais de 50 anos com sucesso para o tratamento da tuberculose. Na Finlândia, o extrato aquoso a quente obtido dos chamados musgos das renas (*Cladonia alpestris, C. rangiferina* ou *C. sylvatica*) é tradicionalmente usado como medicamento para tuberculose (HALE-Jr., 1983).

A *Cladonia verticillaris* pertencente a classe dos Ascomycetos, ordem Lecanorales e família Cladoniaceae, foi classificada por Vanio em 1894, o qual a designou cladoniforme (AHTI; XAVIER-FILHO, 1993; NASH III, 1996). No talo de *C. verticillaris* já foi detectada a presença de vários compostos, entre eles o orcinol, β-metil orcinol, atranorina, os ácidos evérnico, protocetrárico e fumarprotocetrárico (XAVIER-FILHO et al., 1984; PEREIRA, 1989), além dos produtos intermediários da biossíntese deste penúltimo, como ácido hipoprotocetrárico e seu aldeído (AHTI; XAVIER-FILHO, 1993; PEREIRA et al., 1999).

Os ácidos fumarprotocetárico (FUM), protoliquesterínico, a α-metileno-γ-lactona, e o β-metil-orcinol são considerados os metabólicos secundários com maior atividade biológica da *C. Islandica* (ÖGMUNDSDOTTIR et al., 1998). O FUM produzido apenas por liquens é classificado como depsidona. Este composto liquênico possui dois anéis aromáticos (A e B) e um heterociclo resultante de uma ligação éter e éster (Figura 2A*).* O FUM possui no anel B uma molécula de ácido fumárico adicionada por reação de esterificação direta do grupo –CH_2OH ácido protocetrárico (PRO) (Figura 2B) (HONDA; VILEGAS, 1998).

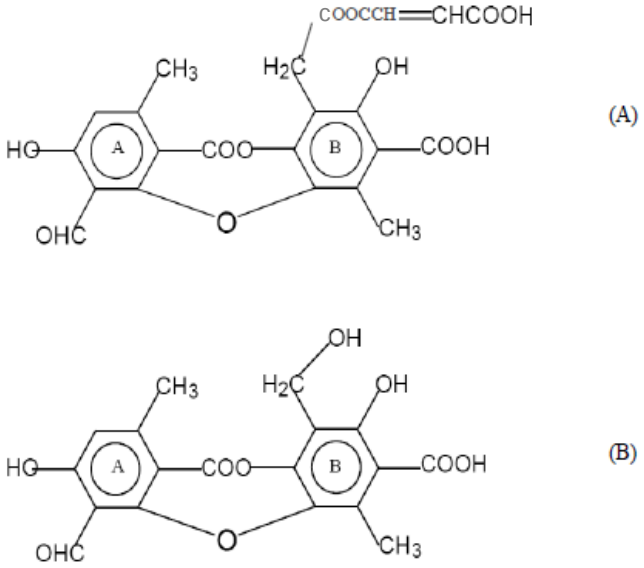


Figura 2 - Fórmulas estruturais dos Ácidos fumarprotocetrárico (A) e do protocetrárico (B)
Fonte: Pereira (1998)

As depsidonas derivadas do líquen *C. verticillaris* que dão origem aos ácidos fumaprotocetrárico e protocetrárico mostram-se efetivas como fotoprotetores (FERNANDEZ et al., 1996); como inibidores da integrase do HIV-1, que é uma enzima responsável por inserir o DNA viral no cromossomo do hospedeiro (NEAMATI et al., 1997) e como inibidores da lipoxigenase-5 de leucócito de porco, que é uma enzima responsável por catalisar o primeiro passo da transformação do ácido araquidônico em

leucotrienos, desempenhando importante função em uma variedade de processos patofisiológicos em humanos, particularmente nos inflamatórios (INGÓLFSDÓTTIR et al., 1996).

2.4 Produtos naturais e os fármacos sintéticos

A Medicina Tradicional Chinesa (MTC) é uma das alternativas a qual utiliza, ervas medicinais há centenas de anos para tratar doenças respiratórias, com menos efeitos colaterais quando comparada com os fármacos sintéticos (SHANG et al., 2010). Por isso, é importante pesquisar novos produtos bioativos para o tratamento da tosse através da MTC (AKAH et al., 2003; CHU et al., 2007; YANG et al., 2008). A tosse é um dos sintomas comuns associados com muitas doenças respiratórias como: asma, bronquite crônica, pneumonia, dentre outras (IRWING; MADISON, 2000); (GE; LIU; SU, 2009). Atualmente, a tosse pode ser controlada através da utilização de fármacos específicos. Por este motivo, há uma crescente demanda no que concerne à pesquisa de novos fármacos, para o tratamento da tosse e de suas complicações.

O Ambroxol (trans-4-{(2-amino-3,5-dibromofenil-metil) - amino} - ciclohexanol). (Figura 3a) é um expectorante de estimulação brônquica, que estimula a síntese e secreção de partículas do surfactante pulmonar. Este fármaco é um metabólito da bromexina (NOWAK et al., 1994) que promove muco intratraqueal. Além da atividade farmacológica, o ambroxol é conhecido como um agente antioxidante e para deprimir a quimiotaxia e a produção de radicais livres e citosinas em macrófagos ativados (YANG et al., 2002).

N-acetilcisteína (NAC) (Figura 3b) é considerado um importante agente terapêutico sendo normalmente utilizado na prática clínica. É um dos compostos mais amplamente investigados e tem efeitos benéficos em condições clínicas em que os radicais livres estão envolvidos (CORTIJO et al., 2001; SERRANO et al., 2002; HAGIWARA; SHII; KITAMURA, 2000; YILDIRIM et al., 2005). O efeito potencial de NAC reduz H_2O_2, alterando o equilíbrio pulmonar oxidante-antioxidante (MACNEE, 2001). O

uso de NAC sozinho podem ter limitações ou apresentar efeitos pró-oxidantes, devido à facilidade com que ele interage com o ferro (RITTER et al., 2004). Diante disso, a utilização de um quelante de ferro pode melhorar a resposta à utilização do NAC (PINHO et al., 2005).

A Guaifenesina (Figura 3c) é um expectorante amplamente utilizado por via oral originalmente aprovado pelo Food and Drug Administration em 1952. É comum o seu uso para tratar a tosse e infecções do trato respiratório superior. No entanto, o benefício clínico da guaifenesina não está elucidado e os resultados de estudos clínicos são controversos. Robinson et al. (1977) relataram que guaifenesina foi superior ao placebo na melhora da gravidade e frequência da tosse causada por infecção respiratória superior (ROBINSON; CUMMINGS; DEFFENBAUGH, 1977). Em contraste, Kuhn et al. (1982) não encontraram qualquer benefício do tratamento da guaifenesina nesta condição. Thomson, Paiva e Mcnicoi (1973) relataram nenhum efeito da droga sobre a freqüência da tosse em pacientes com bronquite crônica. No entanto, estudo realizado por Parvez et al. (1996) em pacientes com doença pulmonar obstrutiva crônica foi demonstrado que a administração da guaifenesina resultou em uma maior redução na intensidade média da tosse em comparação com placebo.

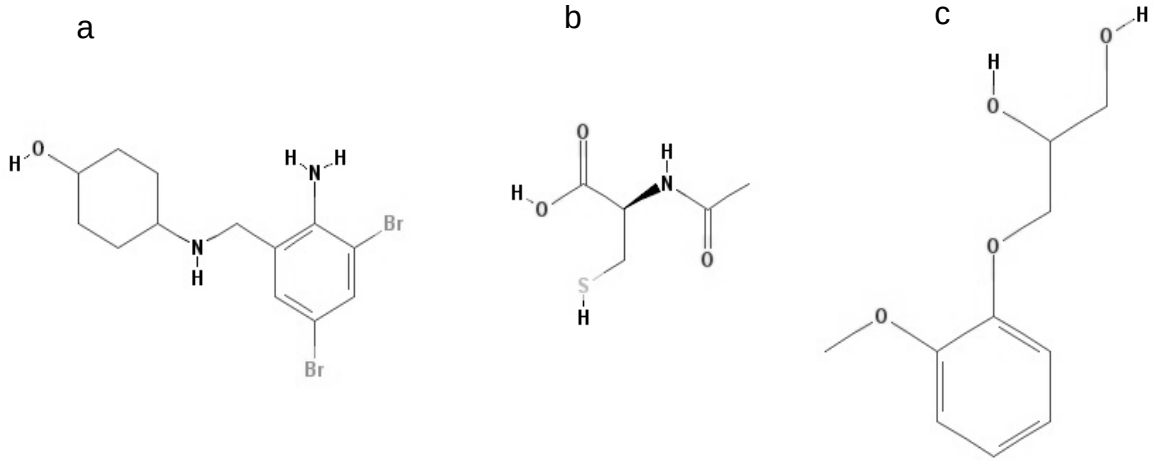


Figura 3 – Fórmulas estruturais: a) Ambroxol, b) N-Acetilcisteína e c) Guaifenesina
Fonte: Ambroxol (2012); N-Acetilcisteína (2012); Guaifenesina (2012)

2.5 Modelo para avaliar atividade mucolítica/expectorante através da quantificação de vermelho de fenol

Existem vários métodos para a avaliar a produção e excreção do muco respiratório.Por exemplo, exames radiológicos, e teor de sólidos ou íons. No entanto, essas metodologias são relativamente complexas, uma vez que pode exigir equipamento especial. Além disso, volume significante de muco se faz necessário para se efetuar essas avaliações sendo mais complicado obter este volume em modelos experimentais (BOLSER, 2006).

A abordagem experimental utilizada nesta pesquisa foi baseada na administração por via intraperitoneal do vermelho de fenol que em parte é absorvido sistemicamente e então, excretado nas vias respiratórias onde podem ser coletados e quantificados. Este método oferece a vantagem de avaliar a função secretora do sistema respiratório como um todo. Foi mostrado que a concentração de vermelho fenol no fluido do trato respiratório é capaz de agir como um marcador para secreção de mucina e água, e que esta secreção pode ser influenciada pela administração de vários fármacos com atividade expectorante (ENGLER, 1984).

2.6 Radicais livres, estresse oxidativo e antioxidantes

Radicais livres são moléculas orgânicas ou inorgânicas e ou átomos que contêm um ou mais elétrons não emparelhados, com existência independente (HALLIWELL, 1994). Essa configuração faz dos radicais livres moléculas altamente instáveis, com meia-vida curtíssima indo de minutos a nanossegundos. A presença dos radicais é crítica para a manutenção de muitas funções fisiológicas normais (POMPELLA, 1997). Algumas espécies de radicais livres: Espécies reativas de oxigênio – superóxido ($O_2^{\bullet -}$), radical hidroxila ($^{\bullet}OH$), peróxido de hidrogênio (H_2O_2), radical peroxila ($ROO^{\bullet}$), hidroperóxido orgânico (ROOH), oxigênio singleto ($^1O_2$), ozônio (O_3); Espécies reativas de nitrogênio: óxido nítrico ($NO^{\bullet}$), peroxinitrito ($ONOO^-$), ácido peroxidonitroso (ONOOH) e dióxido de nitrogênio (NO_2) (ARUOMA, 1994).

Os radicais livres podem ser gerados no citoplasma, nas mitocôndrias ou na membrana e o seu alvo celular (proteínas, lipídeos, carboidratos e DNA) está relacionado com o seu sítio de formação (ANDERSON, 1996; YU; ANDERSON, 1997).

A formação de radicais livres *in vivo* ocorre via ação catalítica de enzimas, durante os processos de transferência de elétrons que ocorrem no metabolismo celular e pela exposição à fatores exógenos (cigarro, dieta, medicamentos, ozônio). Contudo, na condição de pró-oxidante a concentração desses radicais pode aumentar devido à maior geração intracelular ou pela deficiência dos mecanismos antioxidantes (CERUTTI, 1991, 1994). O desequilíbrio entre moléculas oxidantes e antioxidantes que resulta na indução de danos celulares pelos radicais livres tem sido chamado de estresse oxidativo.

A ocorrência de um estresse oxidativo moderado, freqüentemente é acompanhada do aumento das defesas antioxidantes enzimáticas, mas a produção de uma grande quantidade de radicais livres pode causar danos e morte celular (ANDERSON, 1996; SIES, 1993). Os danos oxidativos induzidos nas células e tecidos têm sido relacionados com a etiologia de várias doenças, incluindo doenças degenerativas tais como as cardiopatias, aterosclerose e problemas pulmonares (AMES; SHIGENAGA; HAGEN, 1993; WITZUM, 1994; ROY; KULKARNI, 1996; STAHL; SIES, 1997).

O H_2O_2 não é considerado um radical livre verdadeiro, mas é capaz de atravessar a membrana nuclear e induzir danos na molécula de DNA por meio de reações enzimáticas (ANDERSON, 1996).

A produção contínua de radicais livres durante os processos metabólicos levou ao desenvolvimento de muitos mecanismos de defesa antioxidante para limitar os níveis intracelulares e impedir a indução de danos (SIES, 1993). Os antioxidantes são agentes responsáveis pela inibição e redução das lesões causadas pelos radicais livres nas células.

Uma ampla definição de antioxidante é “qualquer substância que, presente em baixas concentrações quando comparada a do substrato oxidável, atrasa ou inibe a oxidação deste substrato de maneira eficaz” (SIES; STAHL, 1995). Os antioxidantes podem ser classificado em enzimático (superóxido dismutase, catalase, e glutationa peroxidase ou os seus precursores) ou não enzimática (vitaminas C, E,beta-caroteno) (JANKOV; NEGUS; TANSWELL, 2001.

Knekt et al. (1997) encontraram uma relação inversa entre o consumo de flavonóides na dieta e o desenvolvimento de tumores em indivíduos na faixa etária de 50 anos e não-fumantes. Os pesquisadores observaram que entre as muitas fontes de flavonóides da dieta, o consumo de maçãs apresentou os melhores resultados na prevenção do desenvolvimento de tumores no pulmão.

3 REFERÊNCIAS

AKAH, P. A. et al. Evaluation of the anti-asthmatic property of Asystasia gangetica leaf extracts. **J. ethnopharmacol**., Limerick, v. 89, n. 1, p. 25-36, Nov. 2003.

AHTI, T. S.; XAVIER-FILHO, L. The lichen family Cladoniaceae in Paraíba, Pernambuco and Sergipe, northeast Brazil. **Tropical Bryology**, [S. l.], v. 7, p. 55-70, 1993.

ALEXOPOULOS, C. J.; MIMS, C. W. **Introductory micology**. New York: J. Wilwy, 1979. 632 p.

AMBROXOL. Disponível em: <http://pt.wikipedia.org/wiki/Ambroxol>. Acesso em: 20 jun. 2012.

AMES, B. N.; SHIGENAGA, M. K.; HAGEN, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. **Proc. Natl. Acad. Sci. U. S. A**., Washington, v. 90, n. 17, p. 7915-7922, Sept. 1993.

ANDERSON, D. Antioxidant defences against reactive oxygen species causing genetic and other damage. **Mutation Research**, Amsterdam, v. 350, n. 1, p. 103-108, Feb. 1996.

ARUOMA, O. I. Free radicals and antioxidant strategies in sports. **J. Nutr. Biochem**., Stoneham, v. 5, n. 8, p. 370-381, Aug. 1994.

BINDSEIL, K. U. et al. Pure compound libraries; a new perspective for natural product based drug discovery. **Drug Discov. Today**, Kidlinton, v. 6, n. 16, p. 840-847, Aug. 2001.

BOLSER, D. C. Cough suppressant protussive therapy and pharmacologic-ACCP evidence-based clinical practice guidelines. **Chest**, Chicago, v. 129, p. 238s–49s, Jan. 2006. Suplemento 1.

BRASIL. Ministério da Saúde. Secretaria de Atenção à Saúde. Departamento de Atenção Básica. **Doenças respiratórias crônicas**. Brasília, DF: Ministério da Saúde, 2010. 142 p.

CERUTTI, P. A. Oxidant stress and carcinogenesis. **European Journal of Clinical Investigation**, Oxford, v. 21, n. 1, p. 1-5, Feb. 1991.

_____. Oxy-radicals and cancer. **Lancet**, London, v. 344, n. 8926, p. 862-863, Sept. 1994.

CHU, X. P. et al. In vitro andin vivo evaluation of the anti-asthmatic activities of fractions from Pheretima. **J. ethnopharmacol**., Limerick, v. 111, n. 3, p. 490-495, May 2007.

CONTI, M. E.; CECHETTI, G. Biological monitoring of atmospheric in Five zones of córdoba city employing the transplanted lichen Usnea sp. **Envir. Pollut**., Barking, v. 114, p. 471-492, 2001.

CORTIJO, J. et al. Attenuation by oral N-acetylcysteine of bleomycin-induced lung injury in rats. **Eur. respir. j**., Copenhagen, v. 17, n. 6, p. 1228-35, Jun. 2001.

CULBERSON, C. F.; CULBERSON, W. L.; JOHNSON, A. **Second supplement to chemical and botanic guide of lichen products**. St. Louis: The American Bryological and Lichenological Society, 1977. 400 p.

DA VINCI, LEONARDO. **Frases de Leonardo da Vinci**. Disponível em: <http://pensador.uol.com.br/frases_de_leonardo_da_vinci/>. Acesso em: 20 jun. 2012.

ENGLER H, S. Tracheal phenol red secretion, a new method for screening mucosecretolytic compounds. **J. pharmacol. methods**, New York, v. 11, n. 3, p. 151-157, Jun. 1984.

FEIGE, G. B.; KREMER, B. P. Fliechten doppelwesen aus pilz und alge: Vorkommen, Lebensweise, Betismmung. **Kosmos**, Stuttgard, v. 302, p. 1-72, 1979.

FERNANDEZ, E. et al. Lichen metabolites as UV-B filters. **Cosmetic and Toiletries**, Carol Stream, v. 111, p. 69-74, Dec. 1996.

GE, Y. B.; LIU, J. Q.; SU, D. F. In vivo evaluation of the anti-asthmatic, antitussive and expectorant activities of extract and fractions from Elaeagnus pungens leaf. **J. ethnopharmacol**., Limerick, v. 126, n. 3, p. 538-542, Dec. 2009.

GUAIFENESINA. Disponível em: <http://en.wikipedia.org/wiki/Guaifenesin>. 20 jun. 2012.

HAGIWARA, S. I.; SHII, Y.; KITAMURA S. Aerosolized administration of Nacetylcysteine attenuates lung fibrosis induced by bleomycin in mice*.* **Am. j. respir. crit. care med**.,New York, v. 162, n. 1, p. 225-231, July 2000.

HAKSWORTH, D. L.; HILL, D. J. **The lichen forming fungi**. New York: Chapman & Hall, 1984. 158 p.

HALE-Jr., M. E. **The bilogy of lichens**. 3. ed. London: Edward Arnold, 1983. 90 p.

HALLIWELL, B. Free radicals and antioxidants: a personal view. **Nutrition Reviews**, New York, v. 52, n. 8, p. 253-265, 1994.

HONDA, N. K.; VILEGAS, W. A química dos liquens. **Química Nova**, São Paulo, v. 21, n. 6, p. 110-121, 1998.

INGÖLFSDÓTTIR, K. Bioactive compounds from Iceland moss. In: PAULSEN, B. S. (Ed.). **Bioactive carbohydrate polymers**. England: Kluwer Acedemic, 2000. p. 25-36.

INGÖLFSDÓTTIR, K. et al. Inhibitory effects of the lichen metabolite lobaric acid on arachidonate metabolism *in vitro*. **Phytomedicine**, Stuttgart, v. 2, n. 3, p. 243-246, Jan. 1996.

IRWING, R. S.; MADISON, J. M. The diagnosis and treatment of cough. **N. Engl. j. med**., Boston, v. 343, p. 1715-1721, Dec. 2000.

JANKOV, R. P.; NEGUS, A.; TANSWELL, A. K. Antioxidants as therapy in the newborn: some words of caution. **Pediatr. res**., Baltimore, v. 50, n. 6, p. 681-687, Dec. 2001.

KAGAN, L.; LAVY, E.; HOFFMAN, A. Effect of mode of administration on guaifenesin pharmacokinetics and expectorant action in the rat model. **Pulm. pharmacol. ther**., London, v. 22, n. 3, p. 260-265, Jun. 2009.

KNEKT, P. et al*.* Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. **American j. epidemiol**., Baltimore, v. 146, n. 3, p. 223-230, 1997.

KUHN, J. J. et al. Antitussive effect of guaifenesin in young adults with natural colds: objective and subjective assessment. **Chest**, Chicago, v. 82, n. 6, p. 713-718, 1982.

LAWREY, J. D. Biological role of lichen substances. **The Byrologist**, [S. l.], v. 89, p. 111-122, 1986.

MACFARLANE, J. D.; KERSHAW, K. A. Thermal sensitivity in lichens. **Science**, Washington, v. 201, n. 4357, p. 739-741, Aug. 1978.

MACNEE W, R. I. Is oxidative stress central the pathogenisis of chronic obstructive pulmonary disease? **Trends mol. med**., Oxford, v. 7, n. 2, p. 55-62, Feb. 2001.

MULLER, K. Pharmaceutically relevant metabolites lichens. **Appl. microbiol. biotechnol**., Berlin, v. 56, n. 1/2, p. 9-16, jun. 2001.

NASH III, T. H. **Lichen biology**. Cambridge: Cambridge University Press, 1996. 303 p.

N-ACETILCISTEÍNA. Disponível em: <http://pt.wikipedia.org/wiki/Acetilciste%C3%ADna>. Acesso em: 20 jun. 2012.

NEAMATI, H. et al. Depsidones as inhibitors of HIV-1integrase; discovery of through 3D databases searching. **J. med. chem**., Washington, v. 40, n. 6, p. 942-951, Mar. 1997.

NOWAK, D. et al. Antioxidant propertics of ambroxol. **Free radic. biol. med**., New York, v. 16, n. 4, p. 517-522, Apr. 1994.

ÖGMUNDSDÓTTIR, H. M. et al. Anti-proliferative effects of lichen-derived inhibitors of 5-lipoxygenase on malignant cell lines and mitogen-simulated lymphocytes. **J. pharm. pharmacol**., London, v. 50, n. 1, p. 107-115, Jan. 1998.

ORGANIZAÇÃO MUNDIAL DE SAÚDE. **Relatório mundial da Saúde 2004**: changing history. Geneva: WHO, 2004.

_____. _____. Geneva: WHO, 2005.

PARVEZ, L. V. M. et al. Evaluation of antitussive agents in man. **Pulm. pharmacol**., London, v. 9, n. 5/6, p. 299-308, Dec. 1996.

PEREIRA, E. C. **Influência da sazonalidade na dectação de atividade antimicrobiana de Cladonia e Cladina (líquen)**. 1989. 193 f. Dissertação (Mestrado em Criptógamos) – Universidade Federal de Pernambuco, Recife, 1989.

_____. Lichens from Brazilian Northeast (NE) – Studies and applications. In: MARCELI, M. P.; SEAWARD, M. R. D. (Ed.). **Lichenology in Latin America**. [S. l.]: Grupo Latino Americano de líquenólogos: International association for Lichenology, 1998. p. 65-70.

PEREIRA, E. C. et al. Production of lichen metabolites through cell immobilization by *Cladonia clathrata* Ahti & Xavier-Filho. **Phyton**, Horn, v. 39, n. 1, p. 79-89, 1999.

PINHO, R. A. et al. N-acetylcysteine and deferoxamine reduce pulmonary oxidative stress and inflammation in rats after coal dust exposure. **Environ. res**., New York, v. 99, n. 3, p. 355-360, Nov. 2005.

POMPELLA, A. Biochemistry and histochemistry of oxidant stress and lipid peroxidation. **Int. j. vitam. Nutr. res**., Bern, v. 67, n. 5, p. 289-297, 1997.

RITTER, C. et al. Effects of N-acetylcysteine plus deferoxamine in lipopolysaccharide-induced acute lung injury in the rat. **Crit. care med**., New York, v. 34,n. 2, p. 471-477, Feb. 2006.

_____. Treatment with N-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis. **Crit. care med**., New York, v. 32, n. 2, p. 342-349, Feb. 2004.

ROBINSON, R. E.; CUMMINGS, W. B.; DEFFENBAUGH, E. R. Effectiveness of guaifenesin as an expectorant – cooperative double-blind study. **Curr. ther res**., New York, v. 22, n. 2, p. 284-96, 1977.

ROY, P.; KULKARNI, A. P. Oxidation of ascorbic acid by lipoxygenase: effect of selected chemicals. **Food chem. toxicol**., Oxford, v. 34, n. 6, p. 563-570, Jun. 1996.

SANTOS, M. O. **Atividade antinociceptiva, antipirética e antiinflamatória do extrato bruto e do ácido fumarprotocetrárico isolado de *Cladonia verticillaris* (líquen)**. 2003. 82 f. Dissertação (Mestrado em Fisiologia) – Universidade Federal de Pernambuco, Recife, 2003.

SANTOS, N. P. et al. Efeito da sazonalidade na produção de metabólitos com ação antitumoral em *Cladonia verticillaris* (Líquen). **Rev. Univ. Amaz. Ser. Ciênc. Biol**., Manaus, v. 2, n. 2, p. 23-43, 1997.

SEAWARD, M. R. D. **Lichen ecology**. London: Academic Press, 1977. 550 p.

SERRANO-MOLLAR, A. et al. P-selectin upregulation in bleomycin induced lung injury in rats: effect of N-acetyl-L-cysteine. **Thorax**, London, v. 57, n. 7, p. 629-34, Jul. 2002.

SCHMITT, I.; LUMBSCH, H. T. Molecular phylogeny of the Pertusariaceae supports secondary chemistry as an important systematic character set in lichen-forming ascomycetes. **Mol. phylogenet. evol**., San Diego, v. 33, n. 1, p. 43-55, Oct. 2004.

SHANG, J. H. et al. Pharmacological evaluationof Alstonia scholaris: anti-tussive, anti-asthmatic and expectorant activities. **J. ethnopharmacol**., Limerick, v. 129, n. 3, p. 293-298, Jun. 2010.

SIES, H. Strategies of antioxidant defence. **Eur. j. biochem**., Berlin, v. 215, n. 2, p. 213-219, Jul. 1993.

SIES, H.; STAHL, W. Vitamins E and C, b-carotene, and other carotenoids as antioxidants. **Am. j. clin. nutr**., Bethesda, v. 62, p. 1315-1321, Dec. 1995. Suplemento 6.

STAHL, W.; SIES, H. Antioxidant defence: vitamins E and C and carotenoids. **Diabetes**, New York, v. 46, p. S14-S18, Sept. 1997. Supplemento 2.

STEWART, D.; WAXMAN, K. Management of postoperative ileus. **Am. j. ther**., Philadelphia, v. 14, n. 6, p. 56156-56156,1988.

THOMSON, M. L.; PAVIA, D.; MCNICOl, M. W. A preliminary study of the effect of guaifenesin on mucociliary clearance from the human lung. **Thorax**, London, v. 28, n. 6, p. 742-747, Nov. 1973.

WITZUM, J. L. The oxidative hypothesis of atherosclerosis. **Lancet**, London, v. 344, n. 8926, p. 793-795, Sept. 1994.

XAVIER-FILHO, L. **Inibição fotooxidativa de β caroteno por cloroatranorina de *Parmelia tinctorun* Nyl**. 1989. 91 f. Tese (Professor Titular) – Labortaório de Tecnologia Farmacêutica, Universidade Federal da Paraíba, João Pessoa, 1989.

XAVIER-FILHO, L. et al. Chemotaxonomical study on cladonia calycantha and cladonia verticillaris- **Phyton**, Buenos Aires, v. 44, n. 2, p. 121-124, Oct. 1984.

XAVIER-FILHO, L.; RIZZINI, C. T. **Manual de líquenologia brasileiro**. Recife: UFPE, 1976. 431 p.

YANG, B. et al. Ambroxol suppresses influenza-virus proliferation in the mouse airway by increasing antiviral factor level. **Eur. respir. j**., Copenhagen, v. 19, n. 5, p. 952-958, May 2002.

YANG, E. J. et al. Inhibitory effects of Duchesnea chrysantha extract on ovalbumin-induced lung inflammation in a mouse model of asthma. **J. ethnopharmacol**., Limerick, v. 118, n. 1, p. 102-107, Jun. 2008.

YILDIRIM, Z. et al. Attenuation of bleomycin-induced lung fibrosis by oral sulfhydryl containing antioxidants in rats: erdosteine and N-acetylcysteine. **Pulm. pharm. ther**., London, v. 18, n. 5, p. 367-373, 2005.

YU, T. W.; ANDERSON, D. Reactive oxygen species--induced DNA damage and its modification: a chemical investigation. **Mutat. res**., Amsterdam, v. 379, n. 2, p. 201-210, Oct. 1997.

4 OBJETIVOS

4.1 Geral

Avaliar a atividade expectorante, mucolítica e antioxidante do ácido fumarprotocetrárico (FUM) em camundongos Swiss webster.

4.2 Específicos

a) obter os extratos etéreo e acetônico de *C. verticillaris*;
b) isolar, purificar, identificar e quantificar o FUM de *C. verticillaris;*
c) avaliar atividade mucolítica /expectorante do FUM de *C. verticillaris;*
d) avaliar a atividade antioxidante do FUM de *C. verticillaris.*

5 ARTIGO A SER PUBLICADO

à revista: PULMONARY PHARMACHOLOGY AND THERAPEUTICS

Impacto: 2.093

Qualis CAPES: B1

EXPECTORANT AND ANTIOXIDANT ACTIVITIES OF PURIFIED FUMARPROTOCETRARIC ACID FROM *Cladonia verticillaris* LICHEN ON MICE

Glícia Maria de Barros Alves, MSc[1]; André Martins Galvão, MSc[2], Eryvelton Souza Franco, MSc[3], Renata Miranda Gomes[1], Mônica Barroso Martins, MSc [1], Emerson Peter da Silva Falcão, PhD[4], Célia Maria Machado Barbosa de Castro, MD, PhD[5], Maria Bernadete de Souza Maia, PhD[3], Nicácio Henrique da Silva, PhD[1]

[1]Department of Biochemistry of the Universidade Federal de Pernambuco, Recife (PE) - Brazil.
[2] Post-Graduate Program (PhD level) in Biology applied to Health Science at Laboratory of Immunopathology Keizo Asami, Recife (PE), Brazil;
[3]Department of Physiology and Pharmacology of the Universidade Federal de Pernambuco, Recife (PE) - Brazil.
[4]Academic Center of Vitoria of the Universidade Federal de Pernambuco, Recife (PE) - Brazil.
[5]Department of Nutrition of the Universidade Federal de Pernambuco, Recife (PE), Brazil;

*Corresponding co-author: Maria Bernadete de Souza Maia University Federal of Pernambuco, Department of Physiology and Pharmacology , Avenida Professor Moraes Rego, s/n, Cidade Universitária, zip code: 50670-901, Recife - PE, Brazil. Phone: + 55 (81) 2126-8484 / Fax: + 55 (81) 2126-8484, email: mbsm@ufpe.br

ABSTRACT

The lichen *Cladonia verticillaris* produce bioactive secondary methabolites as the acids fumarprotocetraric (FUM) and protocetraric(PRO). The *Cladonia* genus has demonstrate antitumoral, antiinflamatory and antipiretic activities. I is used in folk medicine to treat respiratory deseases (troat irritation, cough, asthma and tuberculosis). This work had the objective to evaluate the expectorante and mucolytic activity of the fumrprotocetraric acid in albino swiss mice. Methods: the FUM was extracted and purified from the acetonic extract of *C. verticillaris.* In order to evaluate the expectorante activity of FUM was used the phenol red method in quantitative scale in the no bronchoalveolar lavage fluid.When administered intraperitoneally in swiss mice.The animals were treated with FUM (25, 50 ou 100 mg/kg orally or intraduodenal and 12,5, 25 ou 50 mg/kg, intraperitoneally) The control groups receive by the same via saline solution of 7,5 ml/kg or ambroxol 1 mg/kg). The antioxidante activity was evaluated with the TBARS method in pulmonar mouse tissue treated with the FUM at 25, 50 or 100 mg/kg orally) after receiving the LPS solution at 1 mg/kg intrapleural via. The same protocol was adopted to the control groups (salina 7,5 ml/kg, orally or N-acetilcisteine, 20mg/kg, orally). Results: The FUM in doses of 25 and 50mg/kg, administered orally, promoted increased dose-dependent of phenol red in the bronchoalveolar lavage. Lipid peroxidation (MDA measured as equivalent) was reduced by 50% in lung tissue. Conclusion: The results confirm the expectorant properties and antioxidants fumarprotocetraric acid produced by the lichen *Cladonia verticillaris.*

Keywords: Ambroxol, N-acetilcisteína, lichen, antioxidant.

INTRODUCTION

Respiratory diseases are major causes of morbidity and mortality in adults and children [1, 2]. Expectorants and similar drugs are widely prescribed drugs for the treatment of lung diseases, although their efficacy is still questioned [3-5]. Lichens are organisms consisting of fungi and algae or cyanobacteria, in a mutually beneficial relationship [6]. Lichens produces substances resulting from its metabolism, known as "lichen acids", which are responsible for the biological activities of lichen [7]. Currently it is known that the chemical nature of these substances is almost entirely phenolic [8-10]. Depsidonas produced by the lichen [11], *Cladonia verticillaris*, give rise to fumaprotocetraric (FUM) and protocetraric (PRO) acids, which has been shown to be photoprotector [12], inhibitors of integrase enzyme in HIV-1 [13] and inhibitor of 5-lipoxygenase in leukocytes [14].

Cladonia verticillaris, common in northeastern region of Brazil, has similar composition to *Cetraria islandica* [6-8]. *Cetraria islandica*, found in Europe in flat regions near the margin of lakes [15], is a species that has been used in the form of expectorants and cough syrups, to treat sore throat, cough and bronchitis [3, 16]. The *Cladonia verticillaris*, found in Brazil, belongs to the class Ascomycetos, order Lecanorales and family Cladoniaceae [15, 17]. Both species (*Cladonia verticillaris and Cetraria islandica*) have large concentrations of fumarprotocetraric acid, which have demonstrated biological actions, such as antitumor activity [18], antiinflammatory [19], antibacterial [20] and insecticide [21]. This study aimed to evaluate the antioxidant and expectorant activities of fumarprotocetraric acid produced by *Cladonia verticillaris.*

MATERIALS AND METHODS

Collection and storage of lichen

Samples of *Cladonia verticillaris* (200g) were collected in sand trays (flat regions, with gentle slopes, near ponds) in the City of Alhandra Paraíba, Paraíba State, Northeast Brazil (Latitude: -7.43933, Longitude: - 34.9136, 7 ° 26 '22 "South, 34 ° 54' 49" West), as previously described [22]. They were packed in paper bags and dried at room temperature (28°C). The lichen was identified by chemical and morphological characters of the talus, at the Laboratório de

Química de Produtos Naturais da Universidade Federal de Pernambuco and a voucher specimen was deposited in the Herbarium UFP - Geraldo Mariz, UFPE, Brazil, nº. 361638.

Obtention of organic extracts

Organic extracts were obtained from 60g of *C. verticillaris* fresh talus for sewage system at room temperature (28°C) as described previously [22]. The stem was initially milled and subjected to successive extractions with 250 mL of diethyl ether, under mechanical stirring for 1h, cooled to 4°C for 24h, followed by filtration. The residue was subjected to extraction with acetone in a similar manner to that described for diethyl ether. The extracts were evaporated by route evaporation in a water bath at 40°C until dry and then kept in a desiccator until constant weight of 3.6 g.

Extraction, identification and purification of fumarprotocetraric acid

Fumarprotocetraric acid was isolated from the acetone extract the high pure cristals were obtained after successive crystallizations, as previously described [7]. Samples were identified by thin layer chromatography (TLC) [23] and the purity were determined in apurified by high performance liquid chromatographer (HPLC), according to Legaz et al [22]. The organic extracts were also analyzed by high performance liquid chromatography (HPLC) using an Hitachi liquid chromatographer (655A-11, Tokyo, Japan) coupled to an UV detector (CG437-B) seted at 254 nm, using a C-18 reverse phase column MicroPack MCH-18 de 300×4mm, Berlin, Germany. The mobile phase consisted of methanol, deionized water, acetic acid (Merck KGaA, Darmstadt, Germany) in the proportion 80: 19.5: 0.5 v/v/v, a flow rate of 1 mL min^{-1}, injection vlume of 10 µL, 0.04 attenuation at room temperature (28ºC), according to the methodology described by Legaz et al; [22]. Dry residues were dissolved in methanol at 0.01 g dm^{-3} and injected onto a column. Quantization of FUM and PRO was achieved by injecting pure standards (FUM and PRO) in order to construct the calibration functions.

Animals and study design

Albino mice, Swiss Webster, male (25 to 40g), were provided by Laboratory of Imunopatologia Keizo Asami (Pernambuco, Brazil),placed in cages and kept under standard

environmental conditions (22°C, 12/12h light / dark cycle) fed with Labina diet (Purina, Brazil) and water ad libitum. Animals (n = 6 animals / group) were treated with FUM (dissolved in 0.9% saline) by oral route (25, 50 and 100mg/kg) intraduodenal (25, 50 and 100mg/kg) or intraperitoneally (12.5, 25, and 50 mg / kg). To access the duodenum of the small intestine and administer FUM, the animals were anesthetized with a combination of ketamine and xylazine (75 and 15 mg / kg, intraperitoneal, respectively). At the same time, three control groups were used: a) treated with Ambroxol, mainly known for its expectorant activity, (1 mg / kg, oral), b) 0.9% saline (7.5 ml / kg, oral) and c) N-acetylcysteine, mainly known for its antioxidant activity (NAC, 20 mg / kg, oral).

Pharmacological tests

Determination of the Expectorant activity of FUM

The expectorant activity was determined by measuring the phenol red, as described above [16]. Thirty minutes after treatment the animals received intraperitoneal, phenol red (10 mg / mL) in a dose of 200 mg / kg. After 30 min of administration, the animals were anesthetized, shaved on the anterior-superior neck and performed exposure of the trachea. Then, a tracheobronchial lavage using 2 mL 0.9% saline solution was done with a recovery of 1.0 mL. The lavage fluid collected was centrifuged at 1600 rpm (638 G) for 10 min and the supernatant (1 mL) was removed and added 0.5 mL of sodium hydroxide (NaOH N 00:01). The concentration of phenol red was measured spectrophotometrically at a wavelength of 535 nm and results were expressed as mg/mL. A standard curve (0,055 to 10 mg/mL) with phenol red was done to normalize the data.

Lipid peroxidation assay

As an index of the lipid peroxidation, we used the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction as previously described [24]. Animals were treated orally with FUM (25, 50 and 100 mg / kg) and compared to positive control group (treated with n-acetylcysteine-NAC, 20 mg / kg) or negative control (treated with 0.9% saline, 7.5 mL / kg). Thirty minutes after treatments, was administered intrapleural, 1 mg/kg of

lipopolysaccharide (LPS) from Escherichia coli 055; B5. After 60 minutes of the oxidative stress induction, induced after LPS, micewere euthanized with an overdose of anesthetic (ketamine 10%). Briefly, organs were homogenized in 50mM phosphate buffer using a Potter-Elvehjem homogenizer. An aliquot (200 µL) was mixed with trichloroacetic acid 15% (400 µL) and centrifuged 10 min (4000 x g) and the supernatant was mixed with equal volume of thiobarbituric acid 0.67%. This system was heated in boiling water bath for 15 min and the TBARS were determined by the absorbance at 535 nm. Protein content was assessed by Lowry assay [25], and results were expressed as nmol of malondialdehyde (MDA) equivalents/mg of protein [26].

Statistical analyze

Data were expressed as the mean value and error deviation. To analyze normality data was used Kolmogorov-Smirnov test and as parametric tests was used ANOVA test with Tukey as a post-test. All analyses were performed using the GraphPad Prism® 4.03 software, San Diego, California (USA), and p-values less than 0.05 were considered statistically significant.

RESULTS

Extraction, identification and purification of FUM

The FUM was isolated and purified from the acetonic extract of *C. verticillaris* by successive recrystallizations following the Asahia & Shibata [9] method modified by Pereira [10]. The samples were analyzed in thin layer chromatography (TLC) [11] and High pressure liquid chromatography (HPLC) [12].(Figure1).

Expectorant Activity

The FUM administered by oral route showed increasing actions of 5.03, 6.4 and 8.8 fold in the excretion of phenol red in comparison to saline group ($p < 0.001$). In relashinships to ambroxol group, the FUM showed increasing responses of 1.37, 1.7 and 2.42 fold in the phenol red excretion , respectively (Figure 2-I). The group treated with FUM by intraperitoneal route at concentrations of showed increasing of 6.39, 7.18 and 9.7 fold in the excretion of phenol red

when compared to saline group ($p < 0.001$). When compared to ambroxol group showed an increasing of 1.15 and 1.57, respectively (Figure 2-II). The intraduodenal route results showed an increasing on the excretion of phenol red of 2.5 and 4.6, respectively (Figure 2-III). When compared toambroxol group there was an increasing of 2.29 and 4.25 fold, respectively ($p < 0.001$).

Lipid peroxidation

The groups treated with FUM showed a decreasing in lipid peroxidation proportional to the tested concentrations (0.286 ± 0.035, 0.126 ± 0.008 and 0.306 ± 0.121 nmol MDA equivalents / mg protein). When compared to saline group (0.688 ± 0.048) and NAC group (0.691 ± 0.075 MDA equivalents nmol / mg protein), respectively (Figure 3). The results showed a reduction on lipid peroxidation of 58%, 81% and 55% compared to saline and NAC group.

DISCUSSION

The FUM biosynthesis in lichens, is preceded by the synthesis of atranorina and protocetraric acid, which has as intermediary compounds the hipoprotocetraric acid and its aldehyde [7, 9, 21]. By TLC, more than one band were found in acetonic extracts of *Cladonia Islandica* and those compounds are intermediary compounds of the synthetic pathway of FUM [7]. These results are also been observed in *Cladonia verticillaris*. In our study, the HPLC analyzes demonstrated that fumarprotocetraric acid was the major compound (95.12%) of the acetone extract in accordance with the TLC assays. These results are in accordance with previous studies, when the compounds were extracted from dry material with acetone followed by and HPLC analysis performed in a LiChrosorb RP-8 column, isocratic elution and UV detection [7].

The expectorants mechanism of action is not fully understood. In our expirements, when the fumarprotocetraric acid was administered by oral route there was an increasing more than 5.0 fold on the excretion of phenol red in comparison to saline group ($p < 0.001$) and in comparison to ambroxol group, the FUM showed an increasing more than 1.37 fold (Figure 2-I). The expectorant activity of some drugs is mediated, in part, by a reflex vagal stimulation initiated by the gastric mucosa after oral administration [27]. Misawa and Yanaura showed that Senega syrup (0.3 mL/kg) had no effect when given intravenously, yet, 2 mL/kg given orally increased the output of fluid within 5 minutes, thereby suggesting that the related secretagogic activity is due to

a reflex action following stimulation of the gastric mucosa [27]. Our study showed that the FUM, administered by oral route, at concentrations of 50 to 100mg/kg showed an increase on the excretion of the phenol red in comparison to ambroxol group. This fact could corroborate with the theory of vagal reflex, as described above.

The group treated by intraperitoneal route showed an increasing more than 6.4 fold on the excretion of phenol red in comparison to saline group ($p < 0.001$) and in comparison to ambroxol group, the FUM showed an increasing more than 1.15 fold (Figure 2-II). When the FUM was administered by intraduodenal route, the results showed an increasing more than 2.5 fold in comparison to saline group (Figure 2-III) and in compariosn to ambroxol group there was an increasing more than 2.3 fold ($p < 0.001$). When the FUM was administered by intraperitoneal route (100 mg / kg) and intraduodenal route (50 mg / kg to 100 mg / kg), there was an increasing in the excretion of phenol red in comparison to ambroxol group. On this case, we noticed the existence of another mechanism of action of expectorants.

Hosoe and colleagues reported that the physico-chemical mechanisms are responsible for the expectorant activity because they reduce the rheology (viscosity) of airway mucus [28]. They suggest that a new homocysteine-derived expectorant (erdosteine) removes sputum by reducing its viscosity, and by promoting mucociliary transport and sustained enhancement of airway secretion. It also suppressed the chemical stimulation-induced cough reflex and plasma leakage into the airway. These changes in fluid mechanics can promote and facilitate the clearance of exogenous substances to the body [28]. Probably, the expectorant activity of FUM observed after administration, by intraperitoneal and intraduodenal routes, at concentration of 100 mg/kg, is connected with these mechanisms described above. The expectorant activity of FUM showed to be superior in almost all doses and routes as compared to saline group. However, this effect was not detected when administered by intraduodenal route at concentration of 25 mg / kg. This fact could be explained, in part, by the low concentration of FUM to induce any activity after this route administration. Another factor that could corroborate to this finding was reported by Lesniewska and colleagues who observed the occurrence of the reduction of intestinal myoelectric activity in rats after surgery [29]. Stewart and colleagues reported the existence of intestinal paralysis after abdominal surgery in humans [30]. All these factors together can be influenced in the reduction of expectorant activity of FUM when administered by intraduodenal route at a concentration of 25 mg / kg.

The lungs of rats treated with intrapleural administration of endotoxin (1 mg/kg of LPS) and oral administrations of FUM at concentrations of 25, 50 and 100mg/kg showed reductions in the lipid peroxidation. We found that the FUM at different concentrations significantly reduced lipid peroxidation by 58%, 81% and 55% respectively, in comparison to control groups (saline or NAC). We note, however, that the NAC had no antioxidant activity as expected, since its activity was similar to that observed in control saline. This result can be explained by the fact that the NAC was administered by oral route. According to Ritter and colleagues when NAC is administered by oral route, NAC can lose their reductive ability and could induces pro-oxidant effects, due its ability to interact with the iron [31].

CONCLUSIONS

The acetone used for extraction of fumaprotocetraric acid, showed to be effective. The FUM showed expectorant activity and its antioxidant activity exhibited a decreasing of lipid peroxidation endotoxin-induced.

ACKNOWLEDGEMENTS

This research was supported by grants from the Brazilian Research Council - The Brazilian Ministry of Education - CAPES (Post-graduate program) and Laboratory of Immunopathology Keizo Asami at the Universidade Federal de Pernambuco, Brazil.

Reference list

[1] Tesfaigzi Y., Meek P., Lareau S. Exacerbations of chronic obstructive pulmonary disease and chronic mucus hypersecretion. *Clinical and Applied Immunology Reviews* ;**6**:21-36;2006

[2] Parvez L., Vaidya M., Sakhardande A., Subburaj S., Rajagopalan T.G. Evaluation of Antitussive Agents in Man. *Pulmonary Pharmacology* ;**9**:299-308;1996

[3] Ziment I. Herbal Antitussives. *Pulmonary Pharmacology & Therapeutics* ;**15**:327-33;2002

[4] Dicpinigaitis P.V. Currently available antitussives. *Pulmonary Pharmacology & Therapeutics* ;**22**:148-51;2009

[5] Carr M.J. Antitussives: The pharmacological pipeline. *Pulmonary Pharmacology & Therapeutics* ;**22**:152-4;2009

[6] Stenroos S., Hyv+Ânen J., Myllys L., Thell A., Ahti T. Phylogeny of the Genus Cladonia s.lat. (Cladoniaceae, Ascomycetes) Inferred from Molecular, Morphological, and Chemical Data. *Cladistics* ;**18**:237-78;2002

[7] Gudj+¦nsd+¦ttir G.A., Ing+¦lfsd+¦ttir K. Quantitative determination of protolichesterinic- and fumarprotocetraric acids in Cetraria islandica by high-performance liquid chromatography. *Journal of Chromatography A* ;**757**:303-6;1997

[8] Cordeiro L.M.C., Reis R.A., Cruz L.M., Stocker-W+Ârg+Âtter E., MartinGrube, Iacomini M. Molecular studies of photobionts of selected lichens from the coastal vegetation of Brazil. *FEMS Microbiology Ecology* ;**54**:381-90;2005

[9] Nimis P.L., Skert N. Lichen chemistry and selective grazing by the coleopteran Lasioderma serricorne. *Environmental and Experimental Botany* ;**55**:175-82;2006

[10] Legaz M.E., Diaz-Santos E., Vicente C. Lichen substrates and urease production and secretion: A physiological approach using four Antarctic species. *Biochemical Systematics and Ecology* ;**14**:375-9;1986

[11] Nobrega N.d.A., Ribeiro S.M., Pereira E.C., Marcelli M., Martins M.C.B., Falcão E.P.d.S. et al. Produção de compostos fenólicos a partir de células imobilizadas do líquen Parmotrema andinum (M++ll. Arg.) Hale e avaliação de atividade antimicrobiana. *Acta Botanica Brasilica* ;**26**:101-7;2012

[12] Millot M., Di Meo F., Tomasi S., Boustie J.l., Trouillas P. Photoprotective capacities of lichen metabolites: A joint theoretical and experimental study. *Journal of Photochemistry and Photobiology B: Biology* ;**111**:17-26;2012

[13] Hirabayashi K., Iwata S., Ito M., Shigeta S., Narui T., Mori T. et al. Inhibitory effect of a lichen polysaccharide sulfate, GE-3-S, on the replication of human immunodeficiency virus (HIV) in vitro. *Chem Pharm Bull (Tokyo)* ;**37**:2410-2;1989

[14] Ogmundsdottir H.M., Zoega G.M., Gissurarson S.R., Ingolfsdottir K. Anti-proliferative effects of lichen-derived inhibitors of 5-lipoxygenase on malignant cell-lines and mitogen-stimulated lymphocytes. *J Pharm Pharmacol* ;**50**:107-15;1998

[15] Hauck M. Susceptibility to acidic precipitation contributes to the decline of the terricolous lichens Cetraria aculeata and Cetraria islandica in central Europe. *Environmental Pollution* ;**152**:731-5;2008

[16] Lin B.q., Li P.b., Wang Y.g., Peng W., Wu Z., Su W.w. et al. The expectorant activity of naringenin. *Pulmonary Pharmacology & Therapeutics* ;**21**:259-63;2008

[17] PINTADO A.N.A., VALLADARES F.E.R.N., SANCHO L.G. Exploring Phenotypic Plasticity in the LichenRamalina capitata: Morphology, Water Relations and Chlorophyll Content in North- and South-facing Populations. *Annals of Botany* ;**80**:345-53;1997

[18] Guti+®rrez-Pascual M., Vicente-Mart+¡n F.J., L+¦pez-Estebaranz J.L. Liquen escleroso y carcinoma escamoso. *Actas Dermo-Sifiliogr+íficas* ;**103**:21-8;2012

[19] Freysdottir J., Omarsdottir S., Ing+¦lfsd+¦ttir K., Vikingsson A., Olafsdottir E.S. In vitro and in vivo immunomodulating effects of traditionally prepared extract and purified compounds from Cetraria islandica. *International Immunopharmacology* ;**8**:423-30;2008

[20] Ramos D.B.M., Gomes F.S., Paiva P.M.G., Silva M.D.C., Coelho L.C.B.B. Antibacterial activity of easily purified lichen lectin from Cladonia verticillaris. *Journal of Biotechnology* ;**150, Supplement**:453-4;2010

[21] Silva M.D.C., S+í R.A., Napole+úo T.H., Gomes F.S., Santos N.D.L., Albuquerque A.C. et al. Purified Cladonia verticillaris lichen lectin: Insecticidal activity on Nasutitermes corniger (Isoptera: Termitidae). *International Biodeterioration & Biodegradation* ;**63**:334-40;2009

[22] Legaz M.E., Vicente C., Ascaso C., Pereira E.C., Filho L.X. Pigment analysis of sun and shade populations of Cladonia verticillaris. *Biochemical Systematics and Ecology* ;**14**:575-82;1986

[23] Culberson C.F. Improved conditions and new data for identification of lichen products by standardized thin-layer chromatographic method. *Journal of Chromatography A* ;**72**:113-25;1972

[24] Jian Z.Y., Woollard A.C., Wolff S.P. Lipid hydroperoxide measurement by oxidation of Fe2+ in the presence of xylenol orang: comparison with TBA assay and an iodometric method. *Lipids* ;**26**:853-6;1991

[25] Michael G. Fiftyfold amplification of the Lowry protein assay. *Analytical Biochemistry* ;**163**:476-81;1987

[26] Draper H.H., Hadley M. Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol* ;**186**:421-31;1990

[27] Misawa M., Yanaura S. Continuous determination of tracheobronchial secretory activity in dogs. *Jpn J Pharmacol* ;**30**:221-9;1990

[28] Hosoe H., Kaise T., Ohmori K., Isohama Y., Kai H., Takahama K. et al. Mucolytic and antitussive effects of erdosteine. *J Pharm Pharmacol* ;**51**:959-66;1999

[29] Lesniewska V., Laerke H.N., Hedemann M.S., Hojsgaard S., Pierzynowski S.G., Jensen B.B. The effect of change of the diet and feeding regimen at weaning on duodenal myoelectrical activity in piglets. *Anim Sci* ;**71**:443-51;2000

[30] Stewart D., Waxman K. Management of postoperative ileus. *Am J Ther* ;**14**:561-6;2012

[31] Ritter C., Andrades M.E., Reinke A., Menna-Barreto S., Moreira J.C., Dal-Pizzol F. Treatment with N-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis. *Crit Care Med* ;**32**:342-9;2004

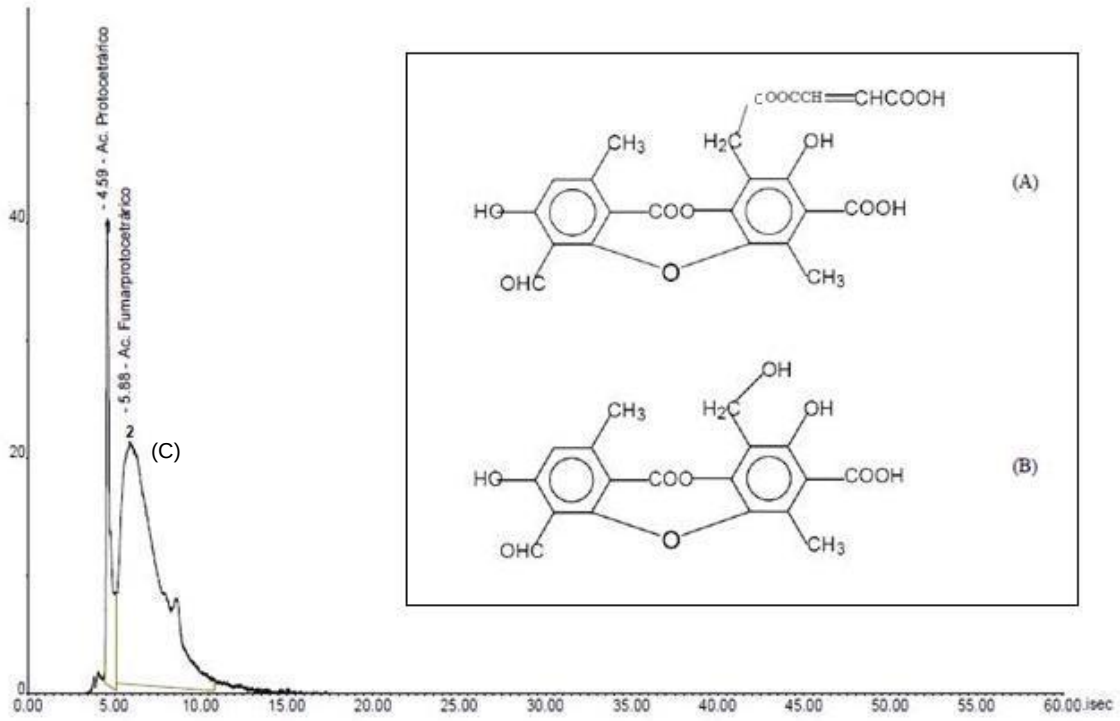


Figure 1. The insert figures show the chemical structure of fumarprotocetraric (A) and protocetraric (B) acids. On the left bottom, chromatogram of acetone extract of *Cladonia verticillaris*.(C)

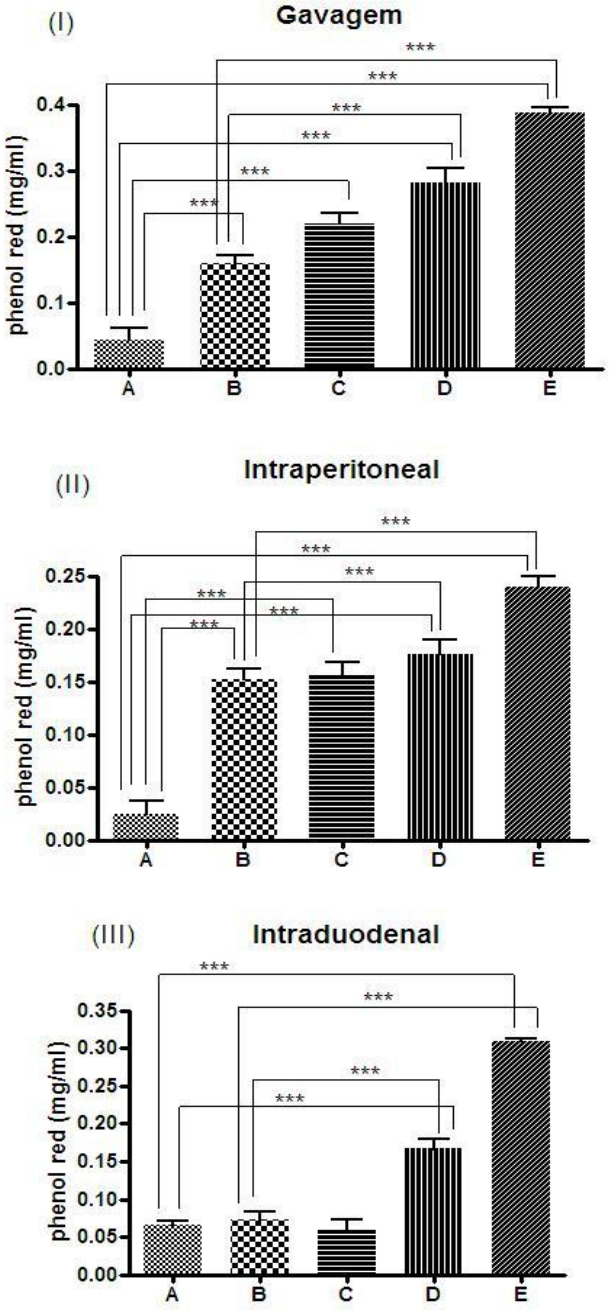


Figure 2. Evaluation of phenol red (mg / mL), in bronchialveolar lavage (BAL), after FUM administration. (I) gavagem (groups: A - saline 7.5 mg / kg, B - ambroxol 1mg/kg, C - FUM 25 mg / kg, D - FUM 50 mg / kg, and E - FUM 100 mg / kg), (II) intraperitoneal (groups: A -saline 7.5 mg / kg, B - ambroxol 1mg/kg, C-FUM 12.5 mg / kg, D - FUM 25 mg / kg, and E - FUM 50 mg / kg) and (III) intraduodenal (groups: A – saline 7.5 mg / kg, B - ambroxol 1mg/kg, C – FUM 25 mg / kg, D - FUM 50 mg / kg and E - FUM 100 mg / kg). Values are expressed as mean and standard error (n = 6 animals for each group). * Significant difference compared with control group (Kruskal-Wallis test followed by Dunn `s post-test, $p < 0.05$).

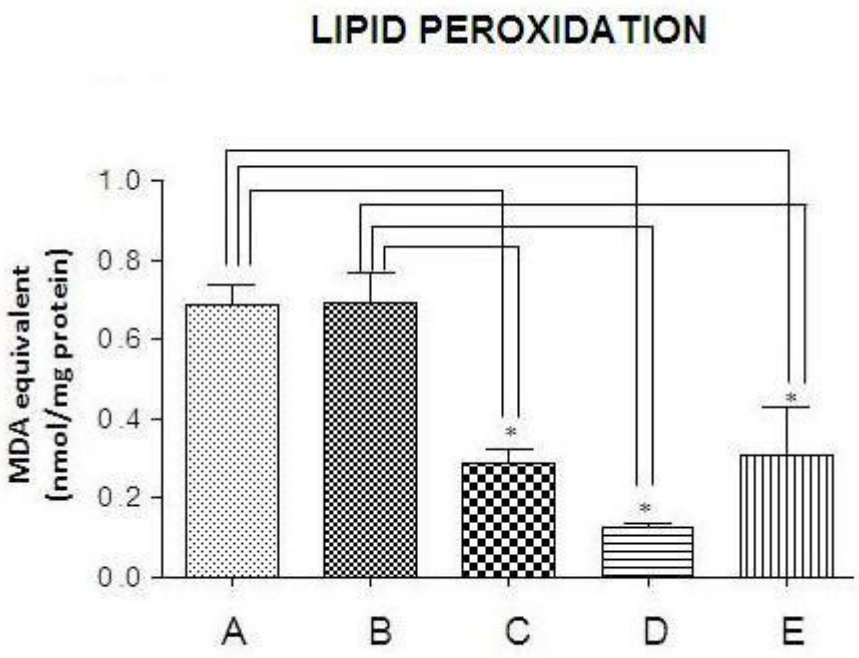


Figure 3. Evaluation of lipid peroxidation, oral administration (MDA equivalents nmol / mg protein) of FUM in lung tissue of mice (groups: A – saline 7.5 mg / kg, B, - N-acetyl-cysteine, C- FUM 25 mg / kg, D - FUM 50 mg / kg, and E- FUM 100 mg / kg, administered orally). Values are expressed as mean and standard error (n = 6 animals for each group). * Significant difference compared with control group (Kruskal-Wallis test followed by Dunn `s post-test, $p < 0.05$).

6 CONCLUSÃO

a) a acetona quando utilizada para purificação do extrato acetônico de *C. Verticillaris* apresentou-se eficaz, uma vez que ao final do processo o grau de purificação do produto obtido foi verificado por CLAE;
b) podemos inferir que o FUM é um, bioproduto que apresenta atividade expectorante dose-dependente sendo esta melhor exarcerbada quando administrado por via oral;
c) no entanto, isso não recorre que seu mecanismo de ação esteja exclusivamente relacionado ao reflexo vagal, pois observamos atividade expectorante quando o FUM foi administrado por via intraperitoneal ou intraduodenal. Provavelmente estes decorrem da característica físico-química do produto, como carater ácido e pouco reativa, fatores que favorecem a rápida absorção e biodistribuição no organismo;
d) além da atividade expectorante do FUM verificamos que ele apresenta atividade antioxidante, no entanto, o provável mecanismo de ação deverá ser investigado futuramente.

ANEXO A – Trabalhos apresentados em Congressos no decorrer do curso

Referência: X Encuentro del Grupo Latinoamericano de Líquenólogos GLAL X. Bogotá - Colombia -- "Effect of fumarprotocetraric acid isolated the Lichen *Cladonia verticillaris* on tracheobronchial phenol red excretion in mice ".

Efeito do ácido fumarprotocetrárico isolado de líquen *Cladonia verticillaris* sobre a excreção traqueobrônquica de vermelho fenol em camundongos Swiss

Alves, G.M.B.[1] ; Franco, E.S.[2]; Melo, R.G.[2]; Cordeiro D.P.[2];
Pereira, E.C.G.[1]; Silva, N.H.[1]; Maia, M.B.S.[2]
[1] Laboratório de Produtos Naturais, Depto de Bioquímica, Centro de Ciências Biológicas /UFPE
[2] Laboratório de Farmacologia de Produtos Bioativos, Depto de Fisiologia e Farmacologia, Centro de Ciências Biológicas /UFPE

INTRODUÇÃO

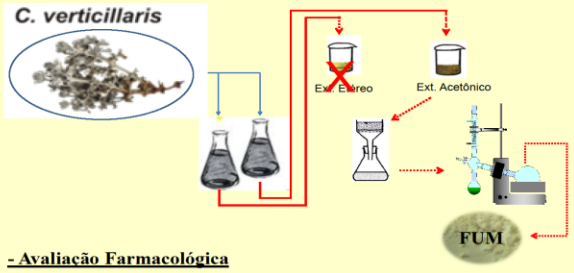
O líquen *Cladonia verticillaris* é muito comum no nordeste do Brasil. Tem como metabólitos secundários o ácido fumarprotocetrarico (FUM) e ácido protocetrárico, os quais também são encontrados no líquen *Cetraria islandica* o qual é amplamente usado na medicina popular turca para o tratamento de bronquite e tuberculose.

OBJETIVO

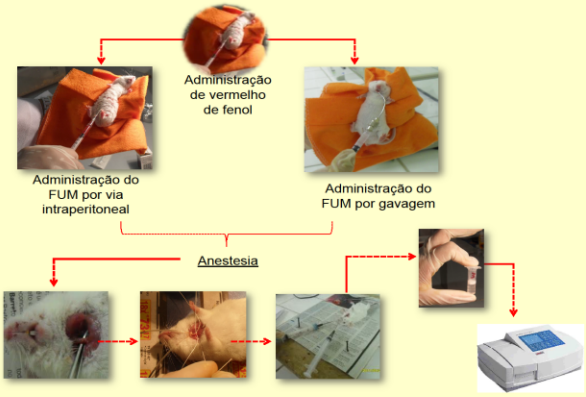
Este tem como objetivo avaliar o efeito do FUM isolado de *Cladonia verticillaris* sobre a excreção traqueobrônquica de vermelho fenol em camundongos Swiss.

METODOLOGIA

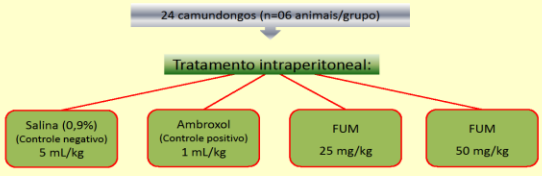
- Obtenção do FUM

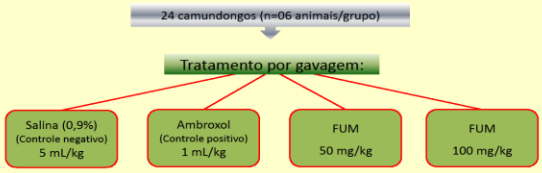


- Avaliação Farmacológica



- Grupos Experimentais





RESULTADOS

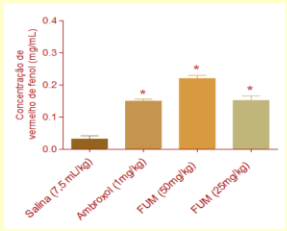


Figura 1 - Concentração de vermelho de fenol no lavado traqueobrônquico dos grupos tratados com FUM (25 ou 50 mg/kg; i.p.) ou Ambroxol (1 mg/kg; i.p.) ou salina (7,5ml/kg; i.p.). *(p<0,05).

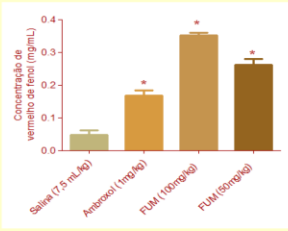


Figura 2 - Concentração de vermelho de fenol no lavado traqueobrônquico dos grupos tratados com FUM (50 ou 100 mg/kg; v.o.) ou Ambroxol (1 mg/kg; v.o.) ou salina (7,5ml/kg; v.o.). *(p<0,05).

CONCLUSÃO

Os resultados sugerem que ação expectorante do FUM não é mediada por um reflexo vagal iniciado pela estimulação da mucosa gástrica após a administração oral.

Congresso Luso-Brasileiro de patologia experimental. “Evaluation of the expectorant activity of acid fumarprotocetrárico isolated from the lichen Cladonia verticillaris in swiss mice”.

Evaluation of the expectorant activity of acid fumarprotocetraric isolated from the lichen *Cladonia verticillaris* in swiss mice

Alves, G.M.B.[1] ; Franco, E.S.[2]; Oliveira, A.P.[2]; Cunha,H.P..[2]; Melo, R.G.[2]; Cordeiro D.P.[2]; Pereira, E.C.G.[1]; Silva, N.H.[1]; Maia, M.B.S.[2]

1-Laboratório de Produtos Naturais/UFPE
2-Laboratório de Farmacologia de Produtos Bioativos/ UFPE

INTRODUCTION

The lichens have been used in folk medicine since ancient times to treat many kinds of respiratory diseases such as: throat irritation, cough, tuberculosis and asthma.

OBJECTIVE

To evaluate the expectorant activity fumarprotocetraric acid (FUM) from *Cladonia verticillaris* in swiss mice.

METHODOLOGY

We used 30 swiss mice, males (30-35g), which received phenol red (200mg/kg; i.p) (fig. 1) diluted in 0.9% NaCl. After thirty minutes , the animals were separed into five groups (n=6), and treated by intraduodenally with: FUM (25, 50 or 100mg/kg), ambroxol (1 mg/kg) or NaCl 0.9%, (0.5ml/kg) (control group). Thirty minutes after treatment the animals where anesthetized, tracheostomized and cannulated (fig.2), and we carried out a cycle of lung lavage (2ml/animal) with NaCl 0.9% solution (fig. 3). The fluid obtained from lavage where centrifuged and read using a spectrophotometer at 535 nm (fig. 4).

Figure 1-Phenol Red

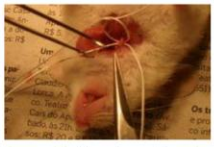


Figure 2- Tracheostomized

Figure 3- Cycle of lung lavage

Figure 4-Fluid lung

RESULTS

The results where expressed as mean standard error. And these were subjected to one way ANOVA and *Bonferroni post-test* ($p<0,05$). The groups treated with the FUM (50 or 100mg/kg) showed significant increase ($p<0,05$) excretion of phenol red, measured in tracheobronchial lavage, compared with positiv controls and saline groups. The results showed that FUM (50 or 100mg/kg) produced an expectorant higher (108 ± 1.2% and 270 ± 0.3%, respectively) that obtained in the group treated with ambroxol, both intaduodenal administered.

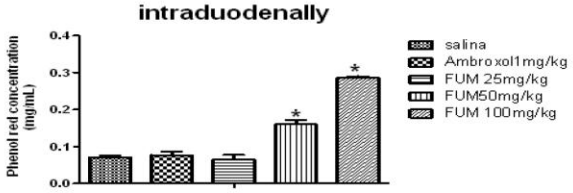


Figure 1. Phenol red concentration in the lavage fluid following intraperitoneal administration (i.p.) of ambroxol or FUM and intraduodenally administration of saline (control group). (*significantly different from other groups, $p < 0.05$ by ANOVA Turkey). Data is presented as mean ± SD.

CONCLUSION

Considering the results we conclude that the FUM (50 and 100mg/kg) showed expectorant activity in mice.

Apoio: Capes

ANEXO B – Normas do periódico ao qual o trabalho da dissertação será submetido

PULMONARY PHARMACOLOGY AND THERAPEUTICS

ELSEVIER

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 1094-5539

DESCRIPTION

Pulmonary Pharmacology and Therapeutics (formerly *Pulmonary Pharmacology*) is concerned with lung pharmacology from molecular to clinical aspects. The subject matter encompasses the major diseases of the lung including asthma, cystic fibrosis, pulmonary circulation, ARDS, carcinoma, bronchitis, emphysema and drug delivery. Laboratory and clinical research on man and animals will be considered including studies related to chemotherapy of cancer, tuberculosis and infection. In addition to original research papers the journal will include review articles and book reviews.

Research Areas Include:

- All major diseases of the lung
- Physiology
- Pathology
- Drug delivery
- Metabolism
- Pulmonary Toxicology

Before submitting, please note the "**Rules of 5**" defining immediate rejection criteria of PPT:

1. Out of scope: The paper should report on the use of pharmacological or toxicological studies (positive or negative) that are directly related to *Pulmonary Pharmacology & Therapeutics*.

Immediate rejection criteria are:
a) Testing of extracts or plant parts that are not characterized
b) Case Reports or n=1 studies

2. Too preliminary: A paper must be based on a thorough and extensive study, using proper controls

Immediate rejection criteria are:
a) In vitro studies with a single dose, or very high concentration b) Single dose studies with very few animals, no dose-response studies c) No proper controls. d) Repetition or confirmation of an earlier work

3. Unethical: The study must have been carried under appropriate ethical approval for the study (either animal or human).

4. Inappropriate experiments or lacking essential detail

Immediate rejection criteria are: a) Wrong species or lacking details on the exact number of animals/ subjects used
b) No details of structure if a new drug is used for the first time

5. Lack of novelty: The study must represent a novel approach to the field.

Immediate rejection criteria are:
a) Repetition of well known data
b) No advance of knowledge in the field

Note that only manuscripts written in good English will be considered by the Editors (British usage is preferred).

IMPACT FACTOR

2010: 2.093 © Thomson Reuters Journal Citation Reports 2011

ABSTRACTING AND INDEXING

ADONIS
Biological Abstracts
Current Awareness in Biological Sciences
Current Contents
EMBASE
Excerpta Medica
Research Alert
Scopus

EDITORIAL BOARD

Honorary Editors:

Peter Barnes, London, UK
Clive Page, London, UK

GUIDE FOR AUTHORS

INTRODUCTION

Pulmonary Pharmacology & Therapeutics is concerned with lung pharmacology from molecular to clinical aspects. The subject matter encompasses the major diseases of the lung (including asthma, cystic fibrosis, pulmonary circulation, ARDS, cardnoma, bronchitis, emphysema) and drug delivery. Laboratory and clinical research on man and animals will be considered including studies related to chemotherapy of cancer, tuberculosis and infection. In addition to original research papers the journal will include review articles and book reviews.

For more details on how to write a world class paper, please visit our Pharmacology Author Resources page.

Authors are encouraged to submit video material or animation sequences to support and enhance your scientific research. For more information please see the paragraph on video data below.

Before submitting, please note the "**Rules of 5**" defining immediate rejection criteria of PPT:

1. Out of scope: The paper should report on the use of pharmacological or toxicological studies (positive or negative) that are directly related to *Pulmonary Pharmacology & Therapeutics*.

Immediate rejection criteria are:
a) Testing of extracts or plant parts that are not characterized
b) Case Reports or n=1 studies

2. Too preliminary: A paper must be based on a thorough and extensive study, using proper controls

Immediate rejection criteria are:
a) In vitro studies with a single dose, or very high concentration b) Single dose studies with very few animals, no dose-response studies c) No proper controls. d) Repetition or confirmation of an earlier work

3. Unethical: The study must have been carried under appropriate ethical approval for the study (either animal or human).

4. Inappropriate experiments or lacking essential detail

Immediate rejection criteria are: a) Wrong species or lacking details on the exact number of animals/ subjects used
b) No details of structure if a new drug is used for the first time

5. Lack of novelty: The study must represent a novel approach to the field.

Immediate rejection criteria are:
a) Repetition of well known data
b) No advance of knowledge in the field

Types of paper

1. *Regular papers*. Original full-length research papers which have not been published previously, except in a preliminary form, may be submitted as regular papers.
2. *Review articles*. Review articles are welcome but should be topical and not just an overview of the literature. Before submission please contact one of the Chief Editors.
3. *Letters to the Editor*. Letters are published from time to time on matters of topical interest. These should be submitted via the online submission system by selecting the article type "Letter to the Editor".
4. *Book reviews*. Book reviews may be submitted to the Editor-in-Chief.

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/ethicalguidelines.

Policy and ethics

The work described in your article must have been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans* http://www.wma.net/en/30publications/10policies/b3/index.html; *EU Directive 2010/63/EU for animal experiments* http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; *Uniform Requirements for manuscripts submitted to Biomedical journals* http://www.icmje.org. This must be stated at an appropriate point in the article.

Also take note of the ARRIVE guidelines on Animal Research: Reporting In Vivo Experiments

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest.

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

Contributors

The corresponding Author of a manuscript for *Pulmonary Pharmacology & Therapeutics* has the duty to ensure that all the named Authors have seen and approved the original and any revised version of the paper and are in agreement with its content before it is submitted to the Editorial Office. Each Author should have participated sufficiently in the work to take public responsibility for the content. The corresponding Author should also ensure that all those who have contributed to the research are acknowledged appropriately either as a co-Author or in the Acknowledgements. In addition, the corresponding Author has the prime responsibility for ensuring the paper is correctly prepared according to the Guide for Authors. Submitted manuscripts not complying with the Guide for Authors may be returned to the Authors for possible revision and resubmission.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Copyright

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

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are

Policy and ethics

The work described in your article must have been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans* http://www.wma.net/en/30publications/10policies/b3/index.html; *EU Directive 2010/63/EU for animal experiments* http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; *Uniform Requirements for manuscripts submitted to Biomedical journals* http://www.icmje.org. This must be stated at an appropriate point in the article.

Also take note of the ARRIVE guidelines on Animal Research: Reporting In Vivo Experiments

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest.

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

Contributors

The corresponding Author of a manuscript for *Pulmonary Pharmacology & Therapeutics* has the duty to ensure that all the named Authors have seen and approved the original and any revised version of the paper and are in agreement with its content before it is submitted to the Editorial Office. Each Author should have participated sufficiently in the work to take public responsibility for the content. The corresponding Author should also ensure that all those who have contributed to the research are acknowledged appropriately either as a co-Author or in the Acknowledgements. In addition, the corresponding Author has the prime responsibility for ensuring the paper is correctly prepared according to the Guide for Authors. Submitted manuscripts not complying with the Guide for Authors may be returned to the Authors for possible revision and resubmission.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Copyright

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

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are

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

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

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

Results
Results should be clear and concise.

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

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

Essential title page information

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

Abstract

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

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

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

Acknowledgements

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

Database linking and Accession numbers

Elsevier aims at connecting online articles with external databases which are useful in their respective research communities. If your article contains relevant unique identifiers or accession numbers (bioinformatics) linking to information on entities (genes, proteins, diseases, etc.) or structures deposited in public databases, then please indicate those entities according to the standard explained below.

Authors should explicitly mention the *database abbreviation (as mentioned below) together with the actual database number*, bearing in mind that an error in a letter or number can result in a dead link in the online version of the article.

Please use the following format: **Database ID: xxxx**

Links can be provided in your online article to the following databases (examples of citations are given in parentheses):

- GenBank: Genetic sequence database at the National Center for Biotechnical Information (NCBI) (GenBank ID: BA123456)
- PDB: Worldwide Protein Data Bank (PDB ID: 1TUP)
- CCDC: Cambridge Crystallographic Data Centre (CCDC ID: AI631510)
- TAIR: The Arabidopsis Information Resource database (TAIR ID: AT1G01020)
- NCT: ClinicalTrials.gov (NCT ID: NCT00222573)
- OMIM: Online Mendelian Inheritance in Man (OMIM ID: 601240)
- MINT: Molecular INTeractions database (MINT ID: 6166710)
- MI: EMBL-EBI OLS Molecular Interaction Ontology (MI ID: 0218)
- UniProt: Universal Protein Resource Knowledgebase (UniProt ID: Q9H0H5)

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

- Make sure you use uniform lettering and sizing of your original artwork.
- Save text in illustrations as 'graphics' or enclose the font.
- Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Produce images near to the desired size of the printed version.
- Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS: Vector drawings. Embed the font or save the text as 'graphics'.
TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.
Please do not:
- Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

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

Tables
Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

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

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

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

Reference management software
This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference style
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:

Reference to a journal publication:
[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9.
Reference to a book:
[2] Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.
Reference to a chapter in an edited book:
[3] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.
Note shortened form for last page number. e.g., 51–9, and that for more than 6 authors the first 6 should be listed followed by 'et al.' For further details you are referred to 'Uniform Requirements for Manuscripts submitted to Biomedical Journals' (J Am Med Assoc 1997;277:927–34) (see also http://www.nlm.nih.gov/bsd/uniform_requirements.html).

Journal abbreviations source

Journal names should be abbreviated according to
Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;
List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;
CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.
Ensure that the following items are present:
One author has been designated as the corresponding author with contact details:
- E-mail address
- Full postal address
- Telephone and fax numbers

All necessary files have been uploaded, and contain:
- Keywords
- All figure captions
- All tables (including title, description, footnotes)

Further considerations
- Manuscript has been 'spell-checked' and 'grammar-checked'
- References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print
• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes
For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

Use of the Digital Object Identifier

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

Proofs

One set of page proofs (as PDF files) will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post) or, a link will be provided in the e-mail so that authors can download the files themselves. Elsevier now provides authors with PDF proofs which can be annotated; for this you will need to download Adobe Reader version 7 (or higher) available free from http://get.adobe.com/reader. Instructions on how to annotate PDF files will accompany the proofs (also given online). The exact system requirements are given at the Adobe site: http://www.adobe.com/products/reader/tech-specs.html.
If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return them to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and return by fax, or scan the pages and e-mail, or by post. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately – please let us have all your corrections within 48 hours. It is important to ensure that all corrections are sent back to us in one communication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.

Offprints

The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail or, alternatively, 25 free paper offprints. If the corresponding author opts for paper offprints, this preference must be indicated via the offprint order form which is sent once the article is accepted for publication. Additional paper offprints can also be ordered via this form for an extra charge. The PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use.

AUTHOR INQUIRIES

For inquiries relating to the submission of articles (including electronic submission) please visit this journal's homepage. Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher. You can track accepted articles at http://www.elsevier.com/trackarticle. You can also check our Author FAQs (http://www.elsevier.com/authorFAQ) and/or contact Customer Support via http://support.elsevier.com.

ANEXO C – Carta de aceite do Comitê de ética ensino e pesquisa animal

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

Av. Prof. Nelson Chaves, s/n
50670-420 / Recife - PE - Brasil
fones: (55 81) 2126 8840 | 2126 8351
fax: (55 81) 2126 8350
www.ccb.ufpe.br

Recife, 12 de agosto de 2011.

Ofício nº 387/11

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Nicácio Henrique da Silva**
Departamento de Bioquímica e Fisiologia
Universidade Federal de Pernambuco
Processo nº 23076.023133/2011-37

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Avaliação da atividade expectorante/mucolítica e anti-oxidante do ácido fumarprotocetrárico isolado de *Cladônia verticillaris* (líquen) em roedores."**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério do LIKA; Animais: Camundongos; Linhagem: Swiss; Sexo: Machos; Idade: 22 dias; Peso: 25-30g; Número de animais previsto no protocolo: 180 animais.

Atenciosamente,

Profa. Maria Teresa Jansem
Presidente do CEEA
UFPE

CCB: Integrar para desenvolver