Universidade Federal de Pernambuco
Centro de Ciências Biológicas
Mestrado em Bioquímica

# Investigação da ação expectorante do extrato acetônico e do ácido fumarprotocetrárico da *Cladonia verticillaris* (líquen) em camundongos

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Recife
2007

Cynthia Karinne Wessen Pereira Lima

# Investigação da ação expectorante do extrato acetônico e do ácido fumarprotocetrárico da *Cladonia verticillaris* (líquen) em camundongos

Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de mestre em bioquímica pela Universidade Federal de Pernambuco.

**Wessen, Cynthia K.**

**Investigação da ação expectorante do extrato acetônico e do ácido fumarprotocetrárico de C*ladonia Verticillaris* (Líquen) em camundongos/ Cynthia K. Wessen – Recife: O Autor, 2007.**

**55 folhas : il., fig., tab.**

Dissertação (mestrado) – Universidade Federal de Pernambuco. CCB. Bioquímica, 2007.

**Inclui bibliografia e anexo.**

1. Líquens 2. Cladonia Verticillaris 3. Ácido Fumarprotocetrarico I. Título.

| | | |
|---|---|---|
| **577.1** | **CDU (2.ed.)** | **UFPE** |
| **572** | **CDD (22.ed.)** | **CCB – 2007- 038** |

Ata da defesa de dissertação da Mestranda **Cynthia Karine Wessen Pereira Lima,** realizada em 15 de fevereiro de 2007, como requisito final para obtenção do título de Mestre em Bioquímica.

Às 09:10 minutos do dia 15 de fevereiro de 2007, foi aberto, no Auditório Prof. Marcionilo Lins/Depto. de Bioquímica, o ato de defesa de dissertação da mestranda **Cynthia Karine Wessen Pereira**, aluna do Curso de Mestrado em Bioquímica. Iniciando os trabalhos a Profa. Dra. Patrícia Maria Guedes Paiva, Vice-Coordenadora do Curso supra citado, fez a apresentação da aluna, de seu orientador, Prof. Dr. Nicácio Henrique da Silva, de suas co-orientadoras, Profa. Dra. Eugênia Cristina Gonçalves Pereira, do Depto. de Geografia/UFPE e Profa. Dra. Maria Teresa Jansem Catanho, do Depto. de Biofísica/UFPE, e da Banca Examinadora composta pelos professores doutores: Nicácio Henrique da Silva, na qualidade de Presidente, Maria da Paz Carvalho da Silva, ambos do Depto. de Bioquímica/UFPE, Armele de Fátima Dornelas de Andrade, do Depto. de Fisioterapia/UFPE e Maria Bernadete de Souza Maia, do Depto. de Fisiologia dee Farmacologia/UFPE. Após as apresentações, o Sr. Presidente convidou a aluna para a apresentação de sua dissertação intitulada: **"Investigação da ação expectorante do extrato acetônico e do ácido fumarprotocetrárico de *Cladonia verticillaris* (LÍQUEN) em camundongos",** e informou que de acordo com o Regimento Interno do Curso, o candidato disporia de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluno para responder às perguntas seria de 30 (trinta) minutos. A aluna procedeu a explanação e comentários acerca do tema em 30 (trinta) minutos. Em seguida, o Sr. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, a Profa. Dra. Armele de Andrade, em seguida para a Profa. Dra. Bernadete Maia, e finalmente para a Dra. Maria da Paz Carvalho Silva, os quais agradeceram o convite, fizeram alguns comentários, deram sugestões e iniciaram suas respectivas argüições. Ao final das mesmas, os referidos professores deram-se por satisfeitos. Em seguida, o Sr. Presidente usou da palavra para tecer alguns comentários, agradecer à Banca Examinadora e parabenizar a candidata. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Mestrado, na presença da Vice-Coordenadora do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 15 de fevereiro de 2007.

Em Tempo:
na linha 03, onde se lê: CYNTHIA KARINE WESSEN PEREIRA
leia-se: CYNTHIA KARINE WESSEN PEREIRA LIMA.
Recife, 15/02/2007

COMISSÃO EXAMINADORA

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Prof. Dr. Nicácio Henrique da Silva
**Orientador (Presidente)**

Profa. Dra. Maria da Paz Carvalho da Silva
**1° examinador**

Profa. Dra. Armele de Fátima Dornelas de Andrade
**2° examinador**

Profa. Dra. Maria Bernadete de Souza Maia
**3° examinador**

Recife, 2007

À minha família pelo amor
incondicional em todas as horas.

"A alegria está na luta, na tentativa,
no sofrimento envolvido.
Não na vitória propriamente dita".
*(Mahatma Gandhi)*

SUMÁRIO

# AGRADECIMENTOS

Ao Deus do meu coração, cuja infinita bondade, me faz todos os dias fechar os olhos e abrir o coração para enxergar as belezas da vida.

Aos meus orientadores Prof. Dr. Nicácio Henrique que sempre esteve presente dedicando-se carinhosamente com paciência e humildade fazendo-me sorrir nas horas mais difíceis, à Profa. Dra. Eugênia Pereira que praticou o verdadeiro sentido da palavra orientar indo muito além dos ensinamentos científicos, à Profa. Dra. Maria Teresa Jansem Catanho pela atenção e competência.

Ao Departamento de Bioquímica da Universidade Federal de Pernambuco, por tornar possível à realização deste projeto.

Ao Laboratório de Produtos Naturais do Departamento de bioquímica da Universidade Federal de Pernambuco, por ter concedido-me suas instalações e todo o apoio para realização deste trabalho.

À Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) pela concessão de bolsa, utilizada em parte do curso.

Aos técnicos de laboratório do Departamento de Bioquímica/UFPE, especialmente ao Sr João Virgínio.

Aos colegas do Curso de Pós Graduação em Bioquímica/UFRPE, especialmente Alba Serafim, Jenayce Vasconcelos, Flávia Pereira e Paula Hirakawa, pela amizade e companheirismo.

A Ivomar Guimarães, por ser a estrela onde brilha a minha alma.

# LISTA DE ABREVIATURAS

ATR – Atranorina

BL – Bronchial lavage

COPD – Chronic obstructive pulmonary disease

DPOC – Doença pulmonar obstrutiva crônica

FUM – Ácido fumarprotocetrárico

HPLC – High-performance liquid chromatography

$LD_{50}$ – Lethal dose 50%

PRO – Ácido Protocetrárico

TLC – Thin-layer chromatography

RESUMO

Os líquens tem sido utilizados na medicina popular desde a antiguidade para tratar vários tipos de doenças do trato respiratório como irritação da garganta, tosse, tuberculose, e asma. Este estudo tem como objetivo avaliar a atividade expectorante do extrato acetônico e do ácido fumarprotocetrárico (FUM) de *Cladonia verticillaris* em camundongos. Para este propósito, foram usados 60 camundongos albinos suíços, fêmeas, separados em cinco grupos, aos quais foi administrado vermelho de fenol, diluído em salina 0,9%, intraperitonealmente (200 mg/kg em 10 mL/kg) e após isto, foi administrada a droga oralmente. Os animais foram sacrificados 30 minutos e 1 hora após a administração das drogas e tiveram a traquéia dissecada e canulada com uma agulha, através da qual foram realizadas lavagens pulmonares com solução salina. Os fluidos coletados foram centrifugados e ao sobrenadante adicionado NaOH (0,01 N). Em seguida, a leitura do material foi feita em espectrofotômetro a 546 nm. Os resultados foram analisados através do teste não paramétrico de Mann-Whitney ($p \leq 0,05$). Os grupos tratados com ambroxol (75 mg/kg) e com o extrato acetônico (80 mg/kg) apresentaram aumento estatisticamente significante da excreção do vermelho de fenol na secreção traqueobrônquica dos animais. Estes resultados sugerem que, na forma oral, o extrato acetônico, na dose de 80 mg/kg, mostrou-se tão eficaz quanto o ambroxol como expectorante.

Palavras- chaves: *Cladonia verticillaris*; Ácido fumarprotocetrárico; Atividade mucolítica

ABSTRACT

Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiinflammatory, analgesic and antipyretic effects. Throughout the ages, lichens have been used for various purposes in folk medicine for treatment of affections such as throat irritation and cough, tuberculosis and asthma. This study was aimed at evaluating the expectorant activity of acetonic extract from *Cladonia verticillaris* and fumarprotocetraric acid (FUM) in mice. Female Swiss mice (n = 60) were separated into five groups. Phenol red, suspended in saline, was injected intraperitoneally (200 mg/kg in 100 mL/kg) and after this the drugs were administered orally. The mice were sacrificed and their tracheas were dissected and cannulated with a blunt. Through this blunt lung lavages were carried out with saline and the fluids collected were then centrifuged. A portion was taken and mixed with NaOH (0,01 N) and measured at 546 nm. The Mann-Whitney test and a probability level of $p \leq 0.05$ were chosen as the criterion for statistical significance. The groups treated with ambroxol (75 mg/kg) and acetonic extract (80 mg/kg) showed statistical significance with increasing of phenol red in tracheobronchial sputum. These results suggest that acetonic extract (80 mg/kg) administered orally is as an efficient mucolytic agent as ambroxol.

Keywords: Lichen; *Cladonia verticillaris*; Fumarprotocetraric acid; Mucolytic activity

# 1. INTRODUÇÃO

As doenças respiratórias constituem importante causa de morbidade e mortalidade em adultos e crianças no mundo. Segundo dados da Organização Mundial de Saúde (OMS), em 2002, estima-se que as infecções respiratórias representaram 6,9% do total de mortos e que a doença pulmonar obstrutiva crônicas (DPOC) e a asma somaram juntas 6,5% (OMS, 2004). A OMS estima ainda que entre os anos de 2000 e 2003, as infecções respiratórias agudas representaram 19% do total de mortes entre crianças menores de 5 anos (OMS, 2005).

No Brasil, as doenças respiratórias agudas e crônicas também ocupam lugar de destaque nas estatísticas. Segundo o Ministério da Saúde, estas foram responsáveis por 14,91% dos internamentos no Sistema Único de Saúde (SUS) em 2005 e, por 11,24% do total de óbitos em 2003 (BRASIL, 2005).

Nas doenças respiratórias, a inflamação persistente leva a uma excessiva produção de muco com alta viscoelasticidade e adesividade, dificultando, desta forma, a mobilização desta secreção na via aérea e sua expectoração pela tosse. O muco acumulado na via aérea pode levar a obstrução, colonização por bactérias e infecções recorrentes (DAVISKAS & ANDERSON, 2006).

Mucolíticos e outras drogas similares são usados desde a antiguidade. Embora sejam largamente empregados e prescritos, sua eficácia permanece em dúvida (YUTA & BARANIUK, 2005). Em virtude de supostamente apresentarem menos ou nenhum efeito adverso, os medicamentos fitoterápicos são amplamente utilizados como expectorantes. Na Europa, o líquen *Cetraria islandica* é empregado como base em diversos produtos, dentre eles xaropes expectorantes e pastilhas para afecções respiratórias.

O líquen *Cladonia verticillaris*, comum no nordeste brasileiro, possui composição química semelhante a *C. islandica,* ambos contém o ácido fumarprotocetrárico como principal metabólito, que demonstrou ação antitumoral (SANTOS *et al.*, 1997), antiinflamatória aguda e crônica, antinociceptiva e antipirética (SANTOS, 2003).

Visto a efetividade deste composto e sua ocorrência em quantidades suficientes para estudo em *C. verticillaris*, além de ser esta espécie abundante na costa do nordeste brasileiro, justifica-se um estudo que vislumbre mais uma de suas propriedades farmacológicas.

# 2. REVISÃO DA LITERATURA

Os líquens são originados da associação simbiótica entre uma ou mais algas e um fungo, resultando na formação de um talo de estrutura específica, com características morfológicas peculiares que os distinguem das formas que lhes deram origem (HALE, 1983; HARKSWORTH & HILL, 1984; NASH III, 1996). Produzem uma variedade de metabólitos secundários característicos, alguns dos quais exibem uma alta taxa de usos potenciais em atividades biológicas (YAMAMOTO, 1991).

As substâncias químicas produzidas pelos líquens são agrupadas, de acordo com a localização do talo, em produtos intra e extracelulares. O talo liquênico é uma estrutura composta sendo alguns produtos sintetizados pelo fungo e outros pela alga (HALE, 1983). Os produtos intracelulares (carboidratos, carotenóides e vitaminas, aminoácidos e proteínas) estão ligados à parede celular e ao protoplasto. São freqüentemente solúveis em água e, podem ser extraídos a quente. Esses compostos ocorrem não somente em líquens, mas em fungos e algas de vida livre e em plantas superiores (HALE, 1983). Os produtos extracelulares, freqüentemente chamados metabólitos secundários, são encontrados na medula ou no córtex, raramente em ambas as camadas (NASH III, 1996). São ácidos alifáticos, meta e para-depsídeos, depsidonas, ésteres benzílicos, dibenzofuranos, xantonas, antraquinonas, ácidos úsnicos, terpenos e derivados do ácido pulvínico. Embora alguns desses compostos também sejam produzidos por fungos de vida livre e por plantas superiores, a maior parte é considerada exclusiva de líquens (ELIX, 1996).

O uso tópico de extratos liquênicos tem origem nos tempos do Egito antigo (VARTIA, 1973). A espécie *Lecanora esculenta,* comum no deserto, é relatada como sendo o maná bíblico (TREASE & EVANS, 1978). Os líquens eram usados na medicina popular de acordo com a sua semelhança às enfermidades; como exemplo a *Lobaria pulmonaria,* por sua superfície reticulada, era utilizada em problemas pulmonares (ABRAHAN & FLOREY, 1949).

Através dos anos, os líquens têm sido utilizados com vários propósitos, como corantes, perfumes e remédios na medicina popular (MÜLLER, 2001).

Como certos compostos fenólicos produzidos pelos líquens absorvem fortemente raios UVB, estes agentes têm sido usados como fotoprotetores (FERNÁNDEZ *et al*.

1996), e sua capacidade antioxidante (HIDALGO *et al*., 1994; GÜLÇIN *et al*., 2002) justifica o uso destes em cremes cosméticos. Testes com o extrato aquoso da *Lobaria pulmonaria* mostraram forte atividade anti-ulcerogênica em camundongos (SÜLEYMAN *et al*., 2003). O extrato metanólico do líquen *Umbilicaria esculenta* mostrou significante ação antitrombótica *in vivo* e *in vitro* (KIM & LEE, 2006). Testes com líquens comprovaram a ocorrência freqüente de metabólitos com propriedades antibióticas, antimicobacteriana, antiviral, antitumoral, analgésica e antipirética (MÜLLER, 2001).

A *Cetraria islandica* (Isla moss), é muito conhecida na medicina popular européia, sendo usada no tratamento de doenças como hemorróidas, disenteria, bronquite, tuberculose (DÜLGER *et al.*, 1998), resfriados comuns, asma (HUOVINEN *et al.*, 1986), tosse, irritação na garganta e gastrite (KARTNIG, 1987). Esta espécie também tem sido utilizada como droga hemostática (BAYTOP, 1999) e comprovou-se a presença de compostos neste líquen capazes de inibir, *in vitro,* o crescimento do *Helicobacter pylori,* justificando seu uso no alívio dos sintomas da gastrite e da úlcera duodenal (INGÓLFSDÓTTIR *et al.,* 1997). Pastilhas preparadas a partir de extratos da *C. islandica*, de nomes comerciais de "Isla-Moos ®" e "Isla-Mint ®" (Figura 1), usadas para doenças do trato respiratório superior, tiveram suas tolerabilidades testadas em 3.143 crianças e resultados satisfatórios favoreceram o seu uso (HECKER & VOLP, 2004). Kempe *et al.* (1997) estudaram 61 pacientes que haviam se submetido à cirurgia recente de desvio de septo nasal e, apresentavam secura e inflamação da garganta devido à respiração predominantemente bucal na fase pós-cirúrgica. Verificaram que a pastilha "Isla moos®" foi capaz de causar mudanças diretas nos quadros clínicos observados sem, contudo, causar efeitos adversos no tratamento da inflamação da mucosa oral. Desta forma, o uso desta pastilha na fase pós-operatória de cirurgia nasal, após intubação ou em infecções simples na garganta tem se mostrado eficiente.

Além das pastilhas Isla-Moos ® e Isla-Mint ®, são encontrados na Europa, sobretudo na Alemanha, outros medicamentos e produtos obtidos a partir da *C. islandica* como o xarope expectorante de nome comercial Pulmobronquiol Plus ® (Figura 2), o xampu de nome comercial Natural Shower ® (Figura 3) e o bom-bom de nome Em-Herbal ® (Figura 4).

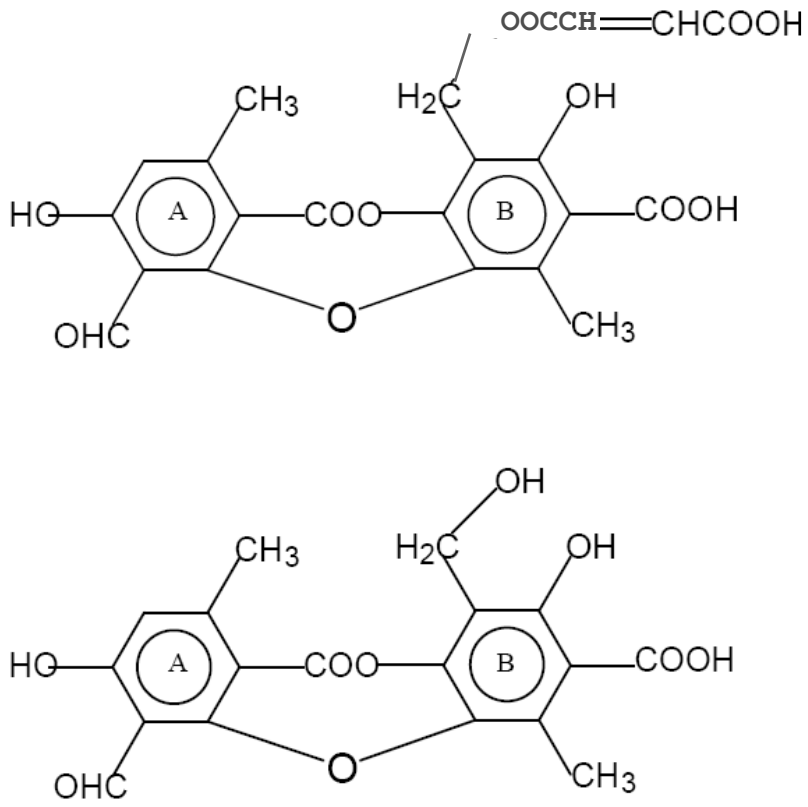

Figura 1. Pastilhas à base de *C. islandica* de nomes comerciais Isla-Moos ® e Isla-Mint ®.


Figura 2. Xarope à base de *C. islandica* de nome comercial Pulmobronquiol Plus ®.


Figura 3. Xampu à base de *C. islandica* de nome comercial Natural Shower ®.


Figura 4. bala à base de *C. islandica* de nome comercial Em-herbal ®.

O ácido fumarprotocetrárico (FUM), ácido protoliquesterínico, α-metileno-γ-lactona, e o β-orcinol são considerados os metabólitos secundários com maior atividade biológica da *C. islandica* (ÖGMUNDSDÓTTIR *et al.*, 1998). O FUM (Figura 5A), produzido apenas por líquens, é classificado como depsidona. Este composto liquênico possui dois anéis aromáticos e um heterocíclo resultante de uma ligação éter e éster. O FUM possui no anel B uma molécula de ácido fumárico adicionada por esterificação direta do grupo $–CH_2OH$ deste anel, quando ainda na forma de ácido protocetrárico (PRO) (Figura 5B) (HONDA & VILEGAS, 1998).




(A)

(B)

Figura 5. Modelo estrutural do FUM (A) e do PRO (B). Fonte: Pereira, 1998.

O líquen *Cladonia verticillaris* (Raddi) Fr. (Figura 6) é considerado por Ahti *et al.* (1993) como espécie endêmica da costa leste do Brasil, encontrada do Rio Grande do Sul à Paraíba.

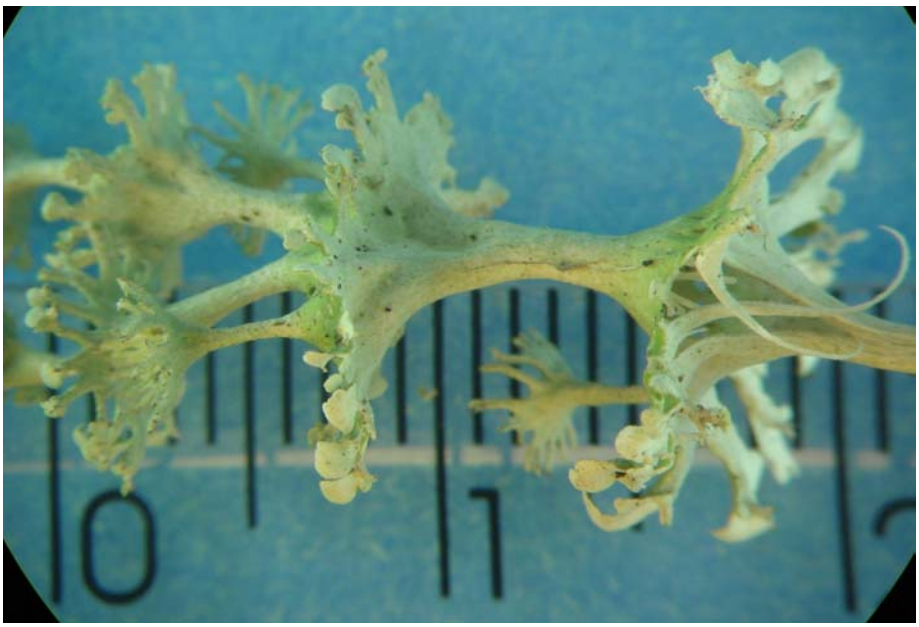


Figura 6. *Cladonia verticillaris* (Raddi) Fr. Ocorrente sobre solos arenosos de tabuleiros. Escala em centímetros. Fonte: Freitas, 2006.

A *C. verticillaris* tem como principal componente químico o FUM e, em menores concentrações, o PRO e a atranorina (ATR) (AHTI *et al*., 1993). Outros compostos podem ocorrer em mínimas concentrações, sobretudo sob influência micro climática, como as substâncias $Cph_1$ e $Cph_2$ (LEGAZ *et al.*, 1986), ou produtos intermediários da biossíntese do FUM como o ácido hipoprotocetrárico e seu aldeído (PEREIRA *et al.*, 1999).

A atranorina, que é um para-depsídeo, é formada de duas unidades aromáticas substituídas. A substância possui, no anel A, duas hidroxilas fenólicas, um grupo metila, e uma função aldeídica; no anel B possui dois grupos metila, uma hidroxila fenólica e uma função éster (Figura 7) (ASAHINA & SHIBATA, 1954).

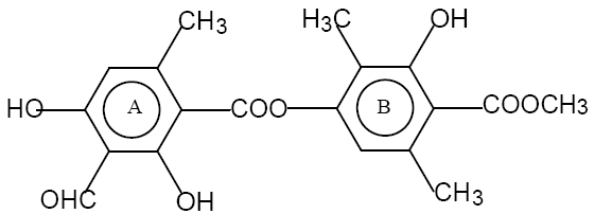



Figura 7. Modelo estrutural da atranorina. Fonte: Pereira, 1998

As depsidonas mostraram-se efetivas como fotoprotetores (FERNÁNDEZ *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 leucócitos 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). O FUM extraído da *Cladonia verticillaris* mostrou ação antitumoral (SANTOS *et al.*, 1997), ação antiinflamatória aguda e crônica, antinociceptiva e antipirética (SANTOS, 2003).

Uma das conseqüências da inalação de cerca de 10.000 litros de ar todos os dias é a de que, junto com esse ar, penetram também no aparelho respiratório partículas em suspensão, gases e microrganismos que, dependendo de sua natureza, concentração e forma de apresentação, têm maior ou menor potencial de provocar danos ao organismo. Para defender-se dessas agressões em potencial, o aparelho respiratório possui um sistema de defesa altamente eficiente e integrado, dos quais o mais bem conhecido e estudado é o da depuração mucociliar, que depende basicamente da integração entre o movimento dos cílios das células do epitélio de revestimento da mucosa respiratória e, o muco produzido pelas glândulas mucosas e pelas células caliciformes (HOSOE *et al*., 1998; SILVA, 2006).

Em várias doenças do sistema respiratório, a exemplo a bronquite crônica, fibrose cística e asma, o sistema de depuração mucociliar está prejudicado pela diminuição dos batimentos ciliares das células epiteliais, ou por uma mudança na produção de muco, ou ambos (HOSOE *et al*., 1998).

Há séculos o homem busca substâncias capazes de facilitar a retirada do excesso de secreção brônquica; entretanto, é importante que isso ocorra como conseqüência e, antes de decidir sobre qual droga mucoativa utilizar, o paciente deve ser avaliado quanto à patologia primária e ao tratamento específico iniciado. Droga mucoativa é definida como um agente que possui, como ação primária, a capacidade de modificar a produção e secreção de muco, sua natureza e composição e/ou sua interação com o epitélio. Freqüentemente são listadas sob uma série de termos como expectorantes, fluidificantes, demulcentes, dentre outros sinônimos (SILVA, 2006).

O ambroxol (trans - 4 - [(2 – amino - 3,5 – dibromofenil - metil) amino] ciclohexanol) (Figura 8), um agente muco regulador, estimula a síntese e secreção do

surfactante, normalizando a produção de muco e, facilitando a expectoração (NOWAK *et al.*, 1994). Exibe atividade antioxidante (GILLISSEN *et al.*, 1997; SUZUKI *et al.*, 1998) e antiinflamatória com redução de citocinas dos macrófagos, monócitos e granulócitos broncoalveolares (PFEIFER *et al.*, 1997; GIBBS *et al.*, 1999).

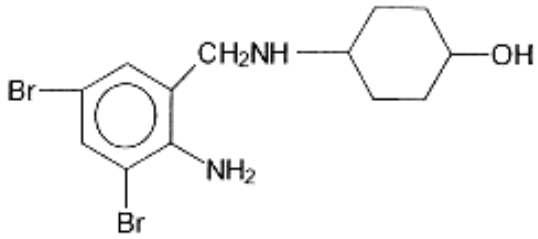



Figura 8. Modelo estrutural do ambroxol. Fonte: Nowak *et al.*, 1994.

Por isso, estudos direcionados à descoberta de novos produtos que minimizem o problema e apresente baixa ou nenhuma toxicidade, são promissores neste ramo da ciência.

Os líquens nordestinos, sobretudo os da família Cladoniaceae, são encontrados em quantidades suficientes para testes, além de produzirem metabólitos secundários bioativos, a exemplo do ácido fumarprotocetrárico.

Diante do fato da *C. islandica* ser um líquen muito usado na medicina popular e da efetividade do FUM, seu principal metabólito secundário, como adjuvante quimioterápico e antiinflamatório, o estudo do FUM como agente mucolítico possibilitará contribuir para o conhecimento das propriedades das substâncias liquênicas ao nível regional.

# 3. OBJETIVOS

## 3.1 Geral

Avaliar o potencial do extrato acetônico e do FUM isolado e purificado de *Cladonia verticillaris* como mucolítico no trato respiratório de camundongos.

## 3.2 Específicos

- Obter extrato acetônico de *C. verticillaris*, isolar, purificar e quantificar o FUM nele contido.
- Determinar a dose de FUM ativa como agente mucolítico em camundongos.
- Realizar testes *in vivo* da atividade mucolítica do extrato acetônico e do FUM de *C. verticillaris* em modelo experimental.

## 4. ARTIGO A SER PUBLICADO

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A ser submetido à revista:

JOURNAL OF ETHNOPHARMACOLOGY:

Impacto: : 1.554

http://www.ethnopharmacology.org

# Investigation of expectorant action of acetonic extract and fumarprotocetraric acid from *Cladonia verticillaris* (lichen) in mice.

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**Abstract**

Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiinflammatory, analgesic and antipyretic effects. Throughout the ages, lichens have been used for various purposes in folk medicine for treatment of affections such as throat irritation and cough, tuberculosis and asthma. This study was aimed at evaluating the expectorant activity of acetonic extract from *Cladonia verticillaris* and fumarprotocetraric acid (FUM) in mice. Female Swiss mice (n = 60) were separated into five groups. Phenol red, suspended in saline, was injected intraperitoneally (200 mg/kg in 100 mL/kg) and after this the drugs were administered orally. The mice were sacrificed and their tracheas were dissected and cannulated with a blunt. Through this blunt lung lavages were carried out with saline and the fluids collected were then centrifuged. A portion was taken and mixed with NaOH (0,01 N) and measured at 546 nm. The Mann-Whitney test and a probability level of $p \leq 0.05$ were chosen as the criterion for statistical significance. The groups treated with ambroxol (75 mg/kg) and acetonic extract (80 mg/kg) showed statistical significance with increasing of phenol red in tracheobronchial sputum. These results suggest that acetonic extract (80 mg/kg) administered orally is as an efficient mucolytic agent as ambroxol.

Keywords: Lichen; *Cladonia verticillaris*; Fumarprotocetraric acid; Mucolytic activity

## 1. Introducion

The respiratory diseases are essential cause of morbidity and mortality in adults and children around the world. According to World Health Organization (WHO), in 2002 respiratory infections represented 6.9% of the total of deaths. The chronic obstructive pulmonary disease (COPD) as well asthma were responsible for 6.5% of deaths (WHO, 2004).

Respiratory diseases have taken a position of great importance in Brazil. Patients entered public health centers all over the country, only in 2005, victims of such diseases, according to the Health Minister, corresponds to 14.91% of people suffering from the problem. In 2003, 11.24% of the people died as a result of the problem (Brasil, 2005).

In respiratory diseases, persistent inflammation leads to excessive production of mucus, with high viscoelasticity and adhesivity, which is not easily transported by cilia or cough interactions. Accumulated mucus in the airways can lead to airway obstruction, bacterial colonisation, and recurrent infections, resulting in poor quality of life and increased morbidity and mortality (Daviskas & Anderson, 2006).

Mucolytic and related agents have been in use since prehistoric times. Although widely prescribed and used extensively in over-the-counter preparations, their efficacy and mechanisms of action remain unexplained (Yuta & Baraniuk, 2005).

Lichens are symbiotic association between one or more algae and one fungi, resulting in a form of thallus with morphological differences of the original form (Hale, 1983; Harksworth & Hill, 1984; Nash III, 1996). They produce a large variety of secondary metabolites, some of them having potential biological activites (Yamamoto, 1991). Throughout the ages, lichens have been used for various purposes, particulary as dye, perfumes and medicines in folk medicine (Müller, 2001). An example is the lichen

*Cetraria islandica* (L.) Ach., a very common lichen in Turkey, that is extensively used in folk medicine for treatment of diseases such as hemorroids, bronchitis, dysentery and tuberculosis (Dülger et al., 1998). Protolichesterinic acid, α-methylene-γ-lactone, fumarprotocetraric (FUM) acid and β-orcinol depsidone are considered to be the major biologically active secondary metabolites in the lichen *C. islandica (O*gmundsdóttir et al., 1998). There are a lot of manufactured products made from *C. islandica* in Europe such lozenges for treatment of diseases of upper respiratory tract, syrup with expectorant action, shampoo and many others products.

*Cladonia verticillaris* (Raddi) Fr., a very common lichen in the northeast of Brazil, has a similar chemical composition to *C. islandica*, and has as essential biologically active secondary metabolites the FUM and protocetraric acid (PRO) (Figure 1).

The lung is a tissue that is in direct contact with the environment which contains several pollutants that need to be expelled from the airway. These pollutants are removed by a system called mucociliary clearance (Hosoe et al., 1998). Throughout the centuries men research substances which may be to facilitate the excess bronchial sputum removal (Silva, 2006).

Due to the positive activity of FUM as antitumoral, antiinflammatory and antimicrobial agent, studies of mucolytic action from this compound will enlarge the knowledge of lichen substances properties.

## 2. Materials and methods

### *2.1. Lichen collection*

*C. verticillaris* was collected from sandy soils of tableland in Alhandra-Paraíba, in the northeast of Brazil. A sufficient quantity was collected in order to identify it and to take chemical and biological tests. Samples were identified according to their morphological characterisitics. Thalli was dried in air and stored at room temperature (28 ± 3º C). The lichen was identified and deposited in UFP herbarium at the Botanic Departament of the Universidade Federal de Pernambuco, Brazil, register nº 361638.

### *2.2. Preparation of extracts*

Samples of *C. verticillaris* were Soxhlet extracted with ether (250 mL) and after with acetone (250 mL) and then concentrated by vacum.

### *2.3. Isolation and purification of fumarprotodetraric acid*

Fumarprotocetraric acid was isolated and purified after repeated recrystallisation as described by Asahina & Shibata (1954) and modified by Pereira *et al.* (1999). Samples were analysed by thin-layer chromatography (TLC), according to Culberson (1972), and by high-performance liquid chromatography (HPLC), according to Legaz & Vicente (1983).

### *2.4. Animals*

Female Swiss mice (24 -51 g) were obtained from the Aggeu Magalhães Research Center (Pernambuco, Brazil). All recommendations by the Brazilian National Law (no. 6.638, 05 November 1979) for management of animals were respected. The animals had free access to a commercial pellet diet and drinking water before experiments.

*2.5. Estudy of mucolytic activity from C. verticillaris*

The bronchial lavage (BL) was done according to Coppi & Gatti (1989). The animals (n= 60) were fed overnight and divided into five groups. Phenol red, suspended in saline 0.9% (200 mg/kg in 100 mL/kg) was administered intraperitonealy (0.4 mL). FUM or acetonic extract, diluted in saline, and Ambroxol were administered orally with a gavage needle five minutes before the phenol red administration. The FUM and the extract were tested in two different doses (65 mg/Kg and 80 mg/Kg, both of them) following the $LD_{50}$ tested by Santos et al. (1997). The mice were sacrificed thirty minutes and one hour after the dye injection; their tracheas were dissected and cannulated with a blunt hipodermic needle of 1.5 cm. The needle was connected to a 1mL syringe through which six lung washings were done with 0.5 mL of saline. The liquid was collected after each washing. Samples were then centrifuged at 1600 xg for 10 minutes in order to separete the red cells. Two mL of supernadant was mixed with 1 mL of NaOH 0.01 N and measured the absorbance at 546 nm. Then, the total of phenol red eliminated in the tracheobronchial secretion was calculated.

*2.6. Statistical analysis*

Experimental results were expressed as the mean ± S.D., and the Mann-Whitney test was used to determine the significance of the differences between the control and experimental groups ($P \leq 0.05$) considered statistically significant.

## 3. Results and discussion

The extraction with ether was used so that impurities and pigments were removed from the lichen. Afterwards, the same procedure was done with acetone. The ether extract wasn't used in *in vivo* experiments, because in this extract there wasn't FUM, like observed in TLC. However, TLC showed the presence of the FUM and PRO in acetonic extract confirmed by HPLC (Figure 2).

According to HPLC, acetonic extract reached peaks such retention time (RT) were 4.11 min and 4.30 min, that corresponding to FUM and PRO, respectively. The shortest peaks indicated on the chromatogram corresponds to methanol (Figure 3).

Protolichesterinic acid, α-methylene-γ-lactone, fumarprotocetraric (FUM) acid and β-orcinol depsidone are considered to be the major biologically active secondary metabolites in *C. islandica* (Ogmundsdóttir et al., 1998). *C. verticillaris* has FUM, PRO and ATR as its main chemical components (Ahti et al., 1993). FUM represents the metabolite with higher concentration in both species (Ahti et al., 1993; Ingólfsdóttir & Gudjónsdóttir, 1997). Due to FUM be present at both species and being an antiinflammatory agent, its expectorant action was investigated.

Phenol red excretion in BL from all groups of animals being treated with the drugs tested, in different doses was higher compareted to that of the control group. This increase showed a direct relation with the drug doses (Table 1 ).

The animals treated with ambroxol showed an average concentration of 7.64 $\mu g/mL.10^2$ of phenol red in BL. This represented a statistically significant increase ($p=0.0207$) of 46.92% in comparison with the control group (Figures 4 and 5). Silva (2006), mentioned that ambroxol is tolerated by the organism showing rare occurency of pyrosis and diarrhoea. Although it has been widely used as expectorant, its clinical importance remains unclear. Hosoe et al. (1998) reported that ambroxol was enable to

improve the mucociliary clearance in rats. Weiss et al. (1981) observed significant increase in such clearance only in the third part of the lung, after the oral administration of the ambroxol for 4 days.

Acetonic extract (80 mg/kg) raised in 45% the excretion of phenol red in the tracheobronchial secretion in animals. It was possible to obtain 7.54 μg/mL.$10^2$ of phenol red in BL from these animals. This increase was statistically significant with p=0.0291 ( Figures 4 and 5).

In table 1, acetonic extract induced a higher excretion of phenol red in BL than FUM in both doses, 65 mg/kg and 80 mg/kg, but it wasn't statistically significant. One of the possibilities that might explain that is the presence of protocetraric acid in the extract. Huovinen et al. (1986), mentioned that the FUM had been hydrolised during the preparation of teas and lichen infusions in folk medicine, and the loss of the fumarate portion converts FUM in PRO. Another possibility is that the sinergism between the compounds of the extract may intensify its action.

Acetonic extract (80 mg/kg) showed a similar increase to ambroxol on phenol red excretion in BL, without statistical significance (table 1). Santos et al. (1997) established in 668.5 mg/kg the acetonic extract $LD_{50}$. This high lethal dose supported the use of the acetonic extract as a possible mucolytic agent, therefore none of the animals used in this experiment showed any sign of toxicity.

## 4. Conclusion

FUM and acetonic extract proved to be eficcient as mucolytic agent in mice. However, the increase of phenol red excretion in BL in animals treated with FUM hasn't got statistical significance. The acetonic extract (80 mg/kg) developed an expectorant action as efficient as ambroxol. The extract showed an action about three times more active than the FUM, using the same dosage.

Further studies should be done to improve the influence of FUM and PRO, acetonic and aqueous extract on bronchial secretion and their use in aerosol form.

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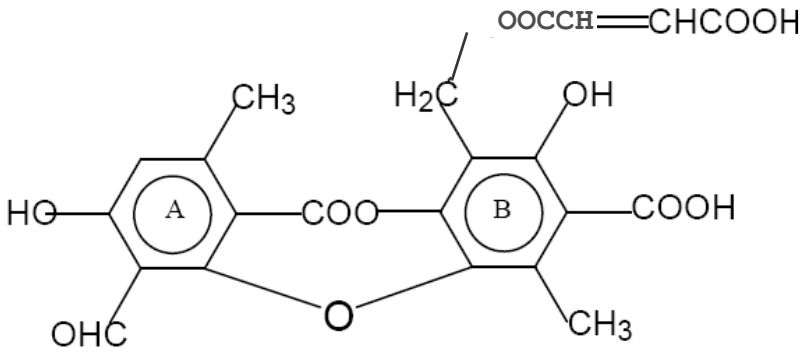
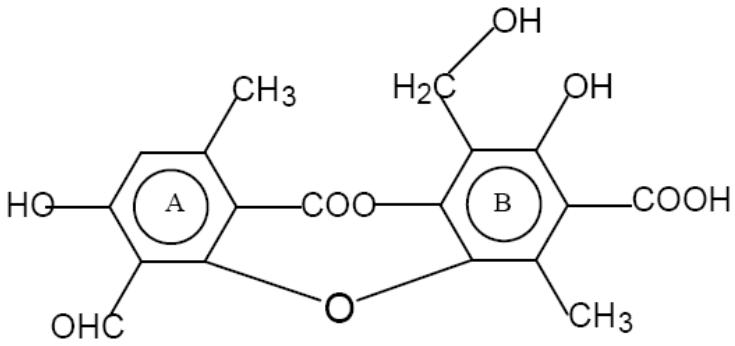



(A)




(B)

Figure 1. Chemical structure of FUM (A) and PRO (B).

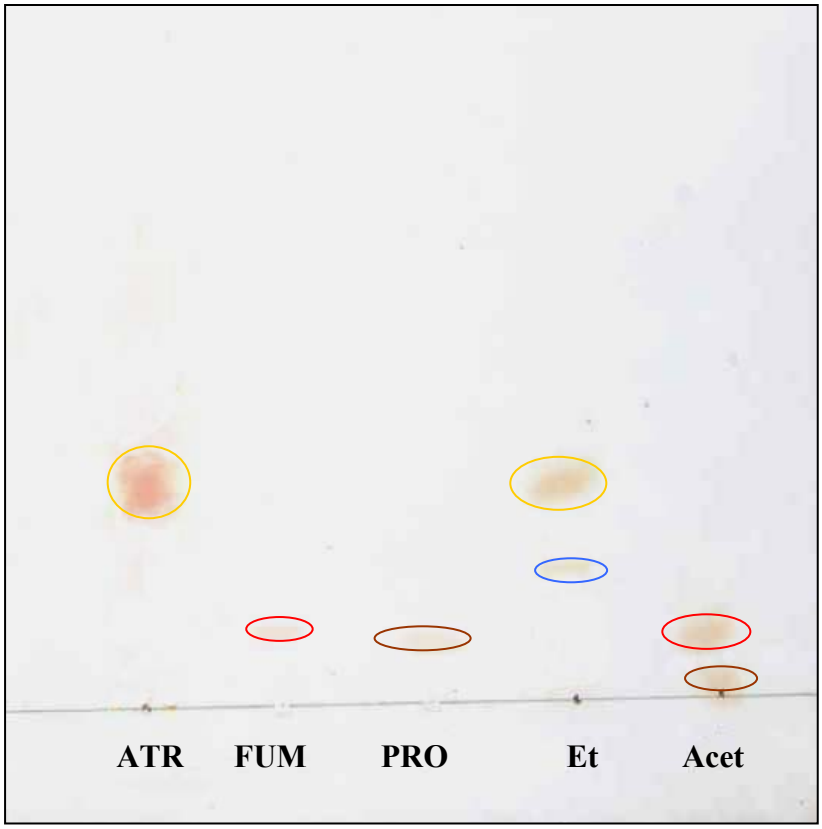



Figure 2. Thin Layer chromatography of organic extracts of *C. verticillaris*. Standards: (ATR) - Atranorin, (FUM) – Fumarprotocetraric acid, (PRO) – Protocetraric acid. Extracts: (Et) – Ether extract, (Acet) – Acetonic extract.

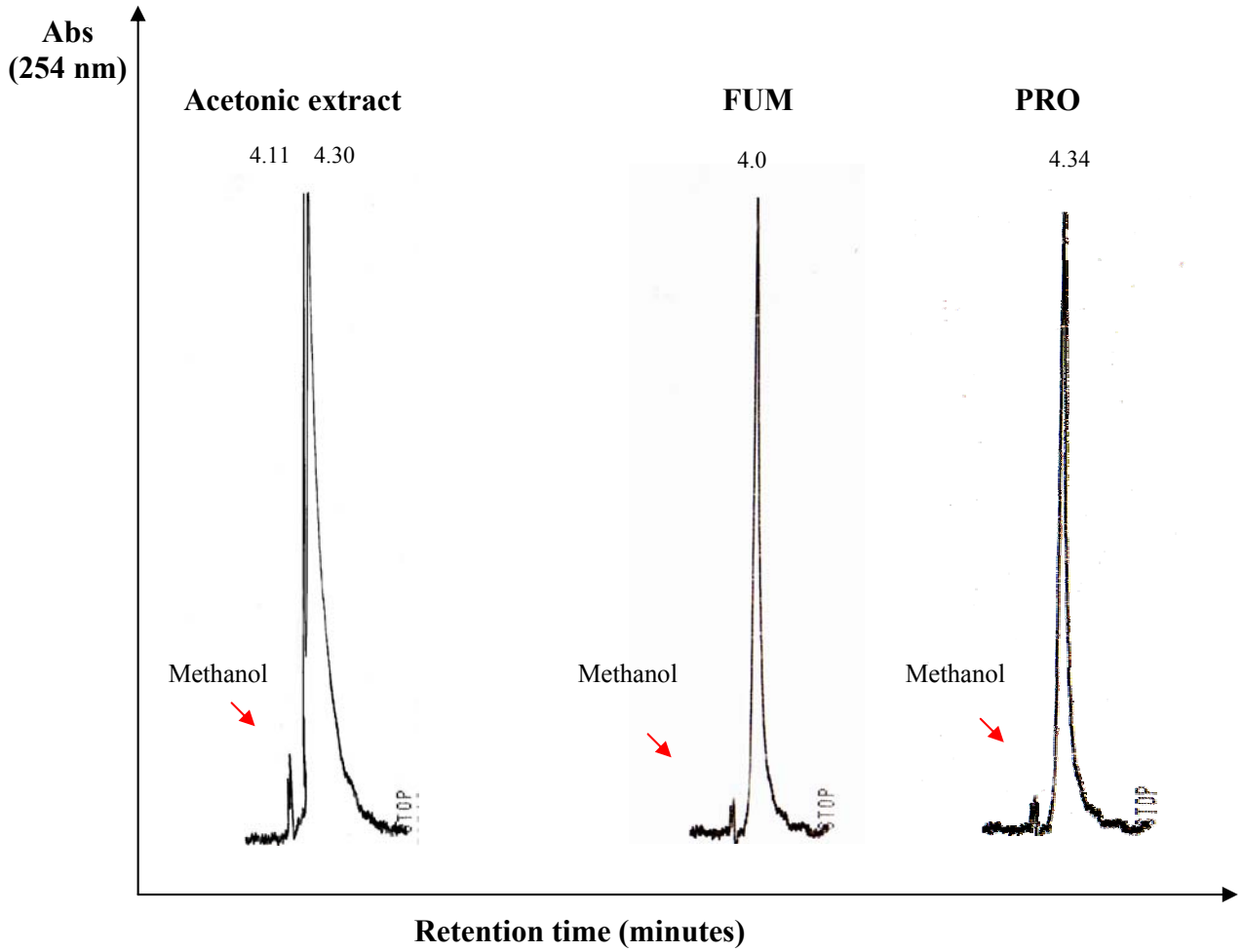



Figure 3. High-performance liquid chromatography of acetonic extract from *C. verticillaris* and standards: PRO e FUM.

**Table 1. Effect of ambroxol, FUM and acetonic extract on the phenol red excretion in bronchial secretion of mice.**

| *Samples* | *Dose (mg/kg)* | *Phenol red concentration in BL (μg/mL.10²)* | *Increase in phenol red excretion in BL (%)* |
|---|---|---|---|
| Control | - | 5.2 ± 1.85 | - |
| Ambroxol | 75 | 7.64 ± 1.14 | 46.92 |
| FUM | 65 | 5.56 ± 1.60 | 6.92 |
| FUM | 80 | 5.58 ± 1.98 | 12.69 |
| Acetonic extract | 65 | 6.94 ± 3.69 | 33.46 |
| Acetonic extract | 80 | 7.54 ± 1.91 | 45.00 |

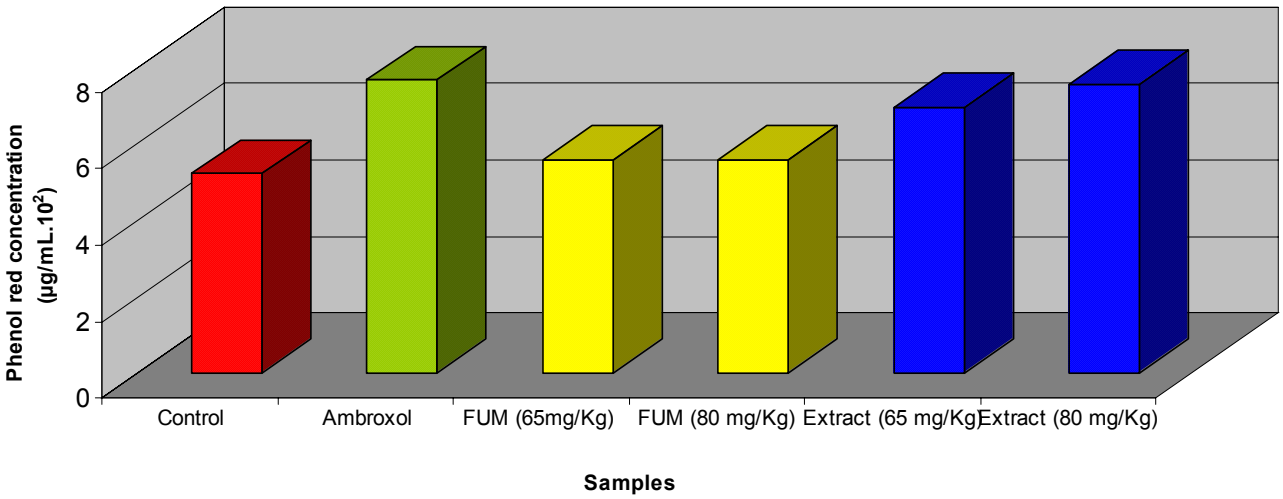



Figure 4. Phenol red concentration in bronchial lavage from animals treated with ambroxol, FUM and acetonic extract.

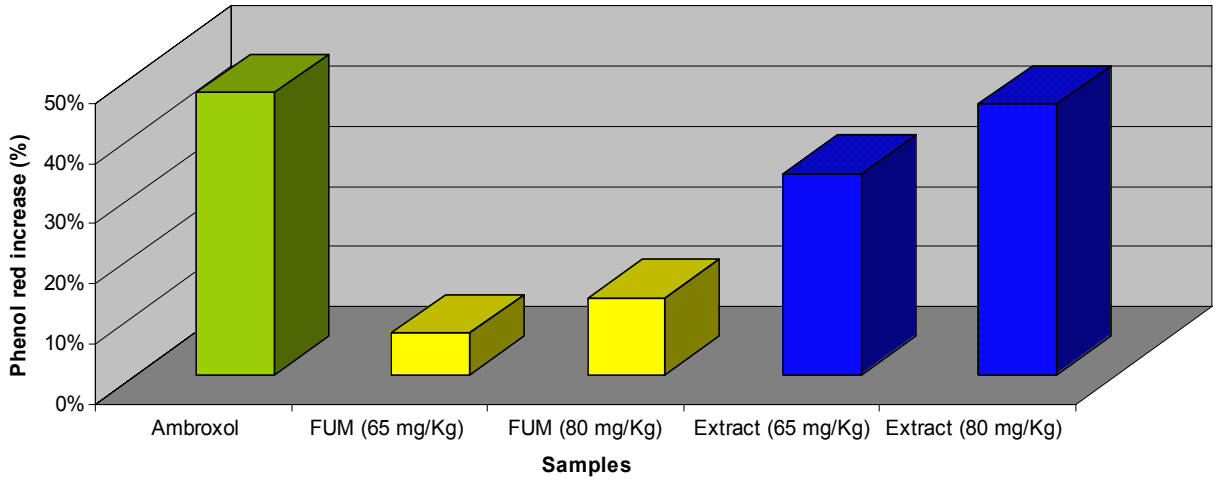



Figure 5. Percentual of increasing phenol red excretion in bronchial lavage from animals treated with ambroxol, FUM and acetonic extract.

# 5. CONCLUSÃO

O FUM e o extrato acetônico mostraram-se eficazes como mucolítico em camundongos, embora o aumento da excreção de vermelho fenol no lavado brônquico dos animais tratados com o FUM não tenha sido estatisticamente significante. O extrato acetônico, na dose de 80 mg/kg mostrou-se um agente expectorante tão eficaz quanto o ambroxol e cerca de três vezes mais ativo que o FUM na mesma dose.

Vislumbramos, no futuro, realizar testes *in vivo* e *in vitro* com o extrato acetônico, o FUM e o PRO e também com o extrato aquoso deste líquen a fim de determinar a influência que estes podem ter sobre a secreção brônquica e utilizá-los em novos ensaios administrando estes compostos na forma de aerosol.

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# 7. ANEXOS

## 7.1 Resumo referente ao assunto da dissertação, publicado e apresentado em Congresso no decorrer do curso.

VIII Reunião Regional Nordeste da SBBq / 3rd International Symposium in Biochemistry of Macromolecules and Biotechnology

### MUCOLYTIC ACTION FROM *CLADONIA VERTICILLARIS* EXTRACT AND OF FUMARPROTOCETRARIC ACID IN THE MICE.

Wessen, C.K.[1]; Serafim, A.T.N.[1],Silva, N.H.[1]; Pereira, E.C.[2]; Catanho, M.T.J.A.[3]

[1]Departamento de Bioquímica; [2]Departamento de Ciências Geográficas;
[3]Departamento de Biofísica; Universidade Federal de Pernambuco (UFPE)

Lichens are organisms in symbiotic relationship with fungi and algae. Throughout the ages, lichens have been used for various purposes in folk medicine for treatment of affections such as throat irritation and cough, tuberculosis and asthma. This study was aimed at evaluating the expectorant activity of an extract from *Cladonia verticillaris* and of fumarprotocetraric acid (FUM) in the mice. Sixty (60) female Swiss mice, weighting 25-50g were separated into five groups. Into each group were used four controls animals. Phenol red was injected intraperitoneally, five minutes after, a drug was administered orally: Ambroxol (3mg) and the extract and FUM were used in two different concentration (2.6mg and 3.2mg). The mice were sacrificed thirty minutes after the dye injection; their tracheas were dissected and cannulated with a blunt. Through this blunt six lung lavages were repeated with 0.5 mL saline. The washing fluids collected were then centrifuged at 1600xg for 10 minutes. A portion was taken and brought to 3 mL with NaOH and the read at 546nm. The results showed an increase of 46.15% ($P<0.05$) in phenol red secretion with the use of Ambroxol and an increase in phenol red secretion with acetonic extract (19.23%) and FUM (3.84%) in the group treated with 3.2mg of drug, but it wasn't statistically significant. These results suggest that just Ambroxol enhanced the mucolytic action.

**Key words**: C*ladonia verticillaris, fumarprotocetraric acid, mucolytic.*

# Mucolytic Action from *Cladonia verticillaris* Extract and of Fumarprotocetraric Acid in the Mice

Wessen, C.K.[1]; Silva, N.H.[1]; Serafim, A.T.N.[1]; Pereira, E.C.[2]; Catanho, M.T.J.A[3];
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## Objective

This study has aimed to evaluate the expectorant activity of acetonic extract from *Cladonia verticillaris* and of fumarprotocetraric acid (FUM) in the mice.

## Methodology

Sixty (60) female Swiss mice, weigthing 25-50g were separated into five groups, each one containing twelve animals. Phenol red was injected intraperitoneally, five minutes after, a drug was administred orally. The drugs were administred as follows: Ambroxol (3mg), the extract acetonic and FUM were used in two concentrations (2.6mg and 3.2mg). The mice were sacrificed thirty minutes and one hour after the dye injection; their tracheas were dissected and cannulated with a blunt. Through this blunt six lung lavages were repeated with 0.5 mL saline. The washing fluids collected were then centrifuged at 1600xg for 10 minutes. A portion was taken and brought to 3 mL with NaOH 0.01N and the read at 546nm.

## Results

The results showed an increase of 46.15% ($P<0.05$) in phenol red secretion with the use of Ambroxol. On the other hand, this study showed an increase in phenol red secretion with acetonic extract (19.23%) and FUM (3.84%) in the group treated with 3.2mg of drug, but it wasn´t statistically significant.

Fig.1. Lung lavage.

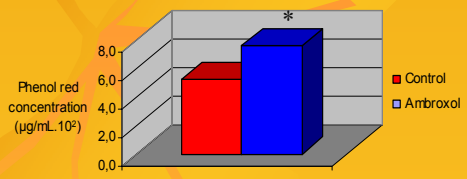
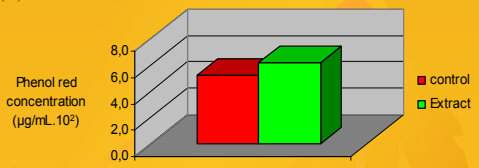



Fig. 2. Effect of Ambroxol in phenol red excretion. *$p<0,05$

(A)




(B)

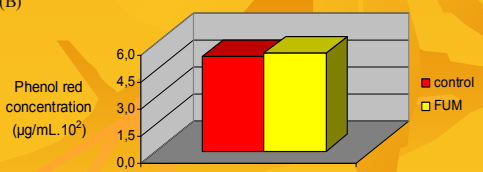



Fig. 3. Effect of acetonic extract (A) and FUM (B) in phenol red excretion. *p<0,05

## Conclusion

The results suggest that just Ambroxol enhaced the mucolytic action. An Improved access to these lichen substances in drug discovery high-troughput screening programs will provide impetus for identifying novel lead-compounds with therapeutic potential and poses new challenges for medicinal chemistry.

## 7.3 Normas do periódico especializado, ao qual o trabalho da dissertação foi submetido.

JOURNAL OF
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An Interdisciplinary Journal Devoted to Indigenous Drugs

The Official Journal of the International Society of Ethnopharmacology

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**Guide for Authors**


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In recent years the preservation of local knowledge, the promotion of indigenous medical systems in primary health care, and the conservation of biodiversity have become even more of a concern to all scientists working at the interface of social and natural sciences but especially to ethnopharmacologists. Recognizing the sovereign rights of States over their natural resources, ethnopharmacologists are particularly concerned with local people's rights to further use and develop their autochthonous resources.
Accordingly, today's Ethnopharmacological research embraces the multidisciplinary effort in the documentation of indigenous medical knowledge, scientific study of indigenous medicines in order to contribute in the long-run to improved health care in the regions of study, as well as search for pharmacologically unique principles from existing indigenous remedies.

The *Journal of Ethnopharmacology* publishes original articles concerned with the observation and experimental investigation of the biological activities of plant and animal substances used in the traditional medicine of past and present cultures. The

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