CÍNTIA ALVARENGA SANTOS FRAGA DE MIRANDA

POTENCIAIS BIOLÓGICOS DOS ÓLEOS ESSENCIAIS DE PLANTAS DANINHAS

LAVRAS – MG

2013

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agroquímica, área de concentração em Agroquímica e Agrobioquímica, para a obtenção do título de Doutor.

Orientadora

Dra. Maria das Graças Cardoso

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APROVADA em 05 de setembro de 2013.

Dra. Maria Laene Moreira de Carvalho	UFLA
Dra. Silvana Marcussi	UFLA
Dr. Paulo Sérgio Castilho Preté	UFLA
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Dra. Tânia Toledo de Oliveira	UFV

Dra. Maria das Graças Cardoso

Orientadora

LAVRAS – MG

2013

A Deus, por me amar, perdoar, guiar e fortalecer em todos os momentos da minha caminhada.

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RESUMO

Os óleos essenciais são metabólitos secundários obtidos exclusivamente de plantas e apresentam um conjunto de constituintes químicos que se relacionam diretamente às suas atividades biológicas e farmacológicas. Entre as espécies produtoras de óleos essenciais, destacam-se as plantas daninhas, que embora sejam um problema substancial para a agricultura, possuem metabólitos secundários que podem apresentar aplicabilidades promissoras. O aproveitamento dos óleos essenciais dessas espécies invasoras pode agregar importância a essas plantas, possibilitando descobrir novos compostos bioativos, o que consiste em uma alternativa sustentável e viável econômica e ecologicamente. Os objetivos neste trabalho foram caracterizar quimicamente os óleos essenciais extraídos de folhas frescas de *Parthenium hysterophorus*, *Ambrosia polystachya*, *Baccharis dracunculifolia*, *Conyza bonariensis*, *Tithonia diversifolia* e *Hedychium coronarium* e dos rizomas frescos de *Hedychium coronarium*, espécies selecionadas entre dezesseis plantas invasoras, além de avaliar seus potenciais alelopáticos, antioxidantes, antibacterianos e as habilidades inibidoras da coagulação e fibrinogenólise induzidas por diferentes peçonhas de serpentes. Os constituntes majoritários dos óleos extraídos das folhas de *C. bonariensis* foram o limoneno, trans-β-ocimeno e o cis-verbenol, de *P. hysterophorus*, o germacreno-D, trans-β-ocimeno e o β-mirceno, do *T. diversifolia*, o β-pineno, α-pineno e o limoneno, de *A. polystachya*, o germacreno-D, trans-β-ocimeno e o β-cariofileno, de *H. coronarium*, o β-pineno, α-pineno, e o β-cariofileno, de *B. dracunculifolia* o limoneno, trans-nerolidol e o β-pineno e dos rizomas de *H. coronarium* o β-pineno, 1,8-cineol e o α-pineno. Em relação ao efeito alelopático sobre a germinação de sementes e vigor de plântulas de alface, os óleos essenciais com maiores teores de monoterpenos foram os que apresentaram maior eficiência. Todos os óleos avaliados não demonstraram eficácia antioxidante; entretanto, apresentaram efeito inibitório sobre bactérias Gram positivas. Os óleos essenciais com maiores teores de sesquiterpenos apresentaram maior potencial em inibir a coagulação sanguínea e a fibrinogenólise induzidas por diferentes peçonhas de serpentes.

Palavras-chave: Óleos voláteis. Espécies invasoras. Alelopatia. Antibacteriano. Antipeçonhas.

ABSTRACT

Essential oils are composed of secondary metabolites produced exclusively by plants and present a set of chemical constituents that directly relate to their biological and pharmacological activities. Weeds stand out among the essential-oil-producing species. Although they represent a substantial problem for agriculture, they contain secondary metabolites that may have promising applicability. The use of the essential oils from these invasive species can increase the importance of these plants, enabling the discovery of new bioactive compounds, which consists of a sustainable economically and ecologically viable alternative. The objectives of this study were to chemically characterize the essential oils extracted from fresh leaves of *Parthenium hysterophorus Ambrosia polystachya, Baccharis dracunculifolia, Conyza bonariensis, Tithonia diversifolia* and *Hedychium coronarium* and from the fresh rhizomes of *Hedychium coronarium*. These species were selected among sixteen invasive plants, and the allelopathic, antioxidant, and antibacterial potentials, as well as the capacity to inhibit coagulation and fibrinogenolysis induced by various snake venoms, were evaluated. Limonene, *trans*-β-ocimene and *cis*-verbenol were the principal components in the oil extracted from the leaves of *C. bonariensis*; germacrene-D, *trans*-β-ocimene and β-myrcene from *P. hysterophorus*; β-pinene, α-pinene and limonene from *T. diversifolia*; germacrene-D, β-*trans*-ocimene and β-caryophyllene from *A. polystachya*; β-pinene, α-pinene, and β-caryophyllene from *H. coronarium*; limonene, nerolidol and *trans*-β-pinene from *B. dracunculifolia*; and β-pinene, 1,8-cineole and α-pinene from the rhizomes of *H. coronarium*. Regarding the allelopathic effect on seed germination and seedling vigor of lettuce, the essential oils with the highest levels of monoterpenes were those with the highest efficiency. None of oils presented antioxidant efficacy, although they had inhibitory effect against Gram positive bacteria. Essential oils with the higher concentrations of sesquiterpenes presented a greater potential for inhibiting blood clotting and fibrinogenolysis induced by different snake venoms.

Keywords: Volatile oils. Invasive species. Allelopathy. Antibacterial. Anti-snake venom.

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LISTA DE ABREVIATURAS E SIGLAS

APTT	Activated partial thromboplastin time
BHA	Butil-hidroxi-anisol
BHT	2,6-di-tert-butil-4-hidroxitolueno
CGL	Cromatografia Gás-Líquido
CGL/EM	Cromatografia Gás-Líquido acoplada à espectrometria de massas
CI_{50}	Concentração Inibitória de 50%
CL	Cloranfenicol
CMI	Concentração Mínima Inibitória
CoA	Coenzima A
COEP	Comitê de Ética em Pesquisas com Seres Humanos da Universidade Federal de Lavras
CT	Clotting time
DMAPP	Dimetilalil pirofosfato
DMSO	Dimetilsulfóxido
DPPH	2,2-difenil-1-picrilhidrazila
DXP	1-deoxi-D-xilose-5-fosfato
FPP	Farnesil pirofosfato
GGPP	Geranilgeranil pirofosfato
GPP	Geranil pirofosfato
IPP	Isopentenil pirofosfato
IR	Índice de retenção
IVG	Índice de velocidade de germinação
ISO	International Standard Organization
LMP	Low melting point
MEP	Metilritrinol fosfato
NCCLS	National Committe for clinical Laboratory Standards

NI	Não apresentou inibição na faixa das concentrações avaliadas
NMP	Normal Melting Point
PAGE	Polyacrylamide gel electrophoresis
PCA	Análise de Componentes Principais
PBS	Phosphate Buffered Saline
PG	Propyl galate
SDS	Sodium dodecyl sulphate
TAP	Time for prothrombin activation
TBHQ	Tert-butil-hidroquinona
t	traces
TR	Tempo de retenção
TS	Bleeding time
TSA	Tryptic Soy Agar
UFC	Unidade Formadora de Colônia
UV	Ultravioleta
UV-Vis	Ultravioleta-visível
v	vestigial

LISTA DE SÍMBOLOS

ºC	Unidade de temperatura: Graus Celsius
%	Unidade: Porcentagem
L	Unidade de volume: Litro
mL	Unidade de volume: Mililitro
µL	Unidade de volume: Microlitro
g	Unidade de massa: Grama
v/p	Razão: volume/peso
v/v	Razão: volume/volume
m	Unidade de medida: Metro
mm	Unidade de medida: Milimetro
µm	Unidade de medida: Micrômetro
mL min^{-1}	Unidade de vazão ou fluxo: Mililitros/minuto
mg mL^{-1}	Unidade de concentração: Miligrama/mililitro
ºC min^{-1}	Razão: Graus Celsius/minuto
cm s^{-1}	Razão: Centímetros/segundo
UFC mL^{-1}	Razão: Unidade Formadora de Colônias/ mililitro
cm	Unidade de medida: Centímetro
mg	Unidade de massa: Miligrama
nm	Unidade de medida: Nanômetro
min	Unidade de tempo: Minuto
h	Unidade de tempo: Hora
w/w	Razão: massa/massa

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Os óleos essenciais, conhecidos como óleos voláteis, são metabólitos secundários presentes em diferentes estruturas específicas das plantas, possuem odor cacterístico, apresentam-se na forma líquida e são instáveis em diversas condições. São constituídos, geralmente, por dezenas de moléculas químicas, que se agrupam de maneira característica para cada óleo essencial. Nas plantas, são responsáveis por diversas atividades biológicas, podendo atuar como aleloquímicos, protetores contra certos patógenos, como bactérias, fungos e vírus, além de possuírem ações antioxidativas.

Além desses efeitos biológicos, os óleos voláteis apresentam diversas aplicações farmacológicas, que se relacionam diretamente à sua constituição química. Essa combinação particular de compostos químicos relaciona-se às características genéticas de cada espécie vegetal e, dentro de uma mesma espécie, divergem-se em relação aos quimiotipos e fatores edafoclimáticos. Os constituintes químicos dos óleos essenciais atuam isoladamente ou em conjunto, em sinergismo ou em antagonismo, conferindo, assim, as distintas propriedades bioativas dos óleos essenciais. Os óleos essenciais podem ser extraídos de diferentes espécies agrícolas, mas pouco se sabe sobre o seu conteúdo, em se tratando de plantas daninhas.

As plantas podem ser consideradas daninhas quando nascem espontaneamente em locais e momentos indesejados, apresentando crescimento vegetativo rápido, excelente adaptação climática, ciclo de vida perene, além das diversas estruturas morfológicas responsáveis por sua dispersão, longevidade e produção vegetativa contínua. Essas espécies invasoras são consideradas grandes competidoras de sistemas agrícolas, causando prejuízos na

produtividade das lavouras, uma vez que competem por espaço, luz, água e nutrientes. Entretanto, as potencialidades dos óleos essenciais extraídos dessas espécies invasoras podem ser exploradas. Algumas podem apresentar considerável rendimento de óleo essencial, despertando o interesse para pesquisas nas quais os empreguem como substitutos ou em associação a herbicidas, antioxidantes sintéticos, antibióticos e terapias antiofídicas tradicionais, pois podem apresentar-se eficientes do ponto de vista funcional, adequados econômica e ecologicamente, além de possuírem grande aceitabilidade por parte dos consumidores.

Este trabalho foi desenvolvido com o objetivo de prospectar espécies daninhas com potenciais para a produção de óleos essenciais, como melão-de-são-caetano (*Momordica charantia*), cordão-de-frade (*Leonotis nepetaefolia*), buva (*Conysa bonariensis*), losna-branca (*Parthenium hysterophorus*), ambrosia (*Ambrosia polystachya*), fedegoso (*Senna occidentalis*), cambará (*Lantana camara*), margaridão (*Tithonia diversifolia*), alecrim-do-campo (*Baccharis dracunculifolia*), assa-peixe (*Vernonia polyanthes*), lírio-do-brejo (*Hedychium coronarium*), mostarda-cheirosa (*Sinabis arvensis*), caruru-de-porco (*Amaranthus* spp.), estrelinha (*Melamphodium divaricatum*), capim-gordura (*Melinis minutiflora*) e mata-pasto (*Eupatorium maximilianii*) (LORENZI, 2000) presentes no câmpus da Universidade Federal de Lavras. Diversas partes de todas essas espécies botânicas foram avaliadas quanto ao potencial produtor de óleos essenciais e todos os óleos essenciais obtidos nessa prospecção foram empregados nos experimentos.

Entre as espécies avaliadas, seis plantas daninhas apresentaram capacidade produtora de óleos essenciais, sendo esses, em sua maioria, pertencentes à família Asteraceae, que é o maior grupo sistemático das Angiospermas (VERDI; BRIGHENTE; PIZZOLANTE, 2005). Esses óleos essenciais foram extraídos das folhas frescas das espécies *Parthenium*

hysterophorus, *Ambrosia polystachya*, *Baccharis dracunculifolia*, *Conyza bonariensis*, *Tithonia diversifolia* (Figura 1). Outra espécie que apresentou potencial produtor de óleos essenciais foi o *Hedychium coronarium*, monocotiledônea exótica e aquática componente da família Zingiberaceae; os óleos essenciais foram extraídos separadamente das folhas e dos rizomas (ZENNI; ZILLER, 2011).

Adicionalmente, objetivou-se caracterizar quimicamente todos esses óleos essenciais e avaliar seus potenciais alelopáticos, antioxidantes, antibacterianos, além das habilidades inibidoras da coagulação e fibrinogenólise induzidas por diferentes peçonhas de serpentes.

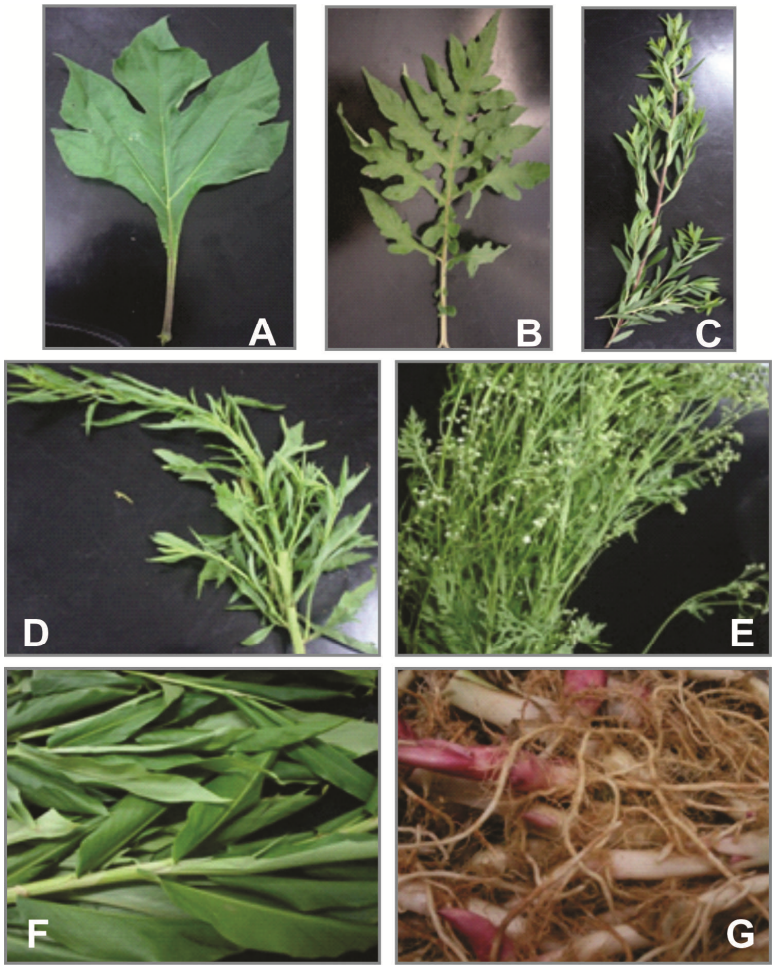

Figura 1 Aspecto geral das espécies selecionadas: A- *Tithonia diversifolia* B- *Ambrosia polystachya*, C- *Baccharis dracunculifolia*, D- *Conyza bonariensis*, E- *Parthenium hysterophorus*, F e G- *Hedychium coronarium*

2 REFERENCIAL TEÓRICO

2.1 Plantas daninhas como fontes de produtos naturais bioativos

A presença das plantas daninhas em áreas agrícolas tornou-se uma preocupação mundial, em razão dos enormes prejuízos causados, com redução da qualidade e quantidade dos produtos agrícolas. O controle dessas espécies invasoras geralmente é feito por métodos químicos, com a utilização de herbicidas sintéticos ou métodos físicos, como a realização de queimadas e capina.

Segundo Bachega et al. (2013), essas espécies daninhas competem com as culturas por luz, nutrientes, água e espaço, e o efeito dessa competição é dependente de fatores relacionados à cultura, à comunidade infestante, ao manejo adotado e ao período de convivência entre cultura e comunidade infestante, sendo todos esses fatores condicionados às condições edafoclimáticas do ambiente.

O uso de herbicidas no controle de plantas daninhas está sendo amplamente adotado em países em desenvolvimento que necessitam elevar a produtividade das lavouras e enfrentam a escassez de mão de obra para capina. Esse cenário pode ser observado no Brasil, onde a migração dos trabalhadores rurais do campo para as cidades têm ocorrido de forma intensa, com redução da população rural de 64% em 1950 para 32% em 1980 e para 16% em 2010. A aplicação desses defensivos químicos aumentou a produção agrícola mundial e essa maior utilização desses herbicidas tem sido acompanhada pelo crescimento de seu valor de mercado, que expandiu 39% entre 2002 e 2011 e está projetado para aumentar mais 11% até o ano de 2016 (GIANESSI, 2013).

Apesar da eficiência na aplicação, elevada produtividade e da fitotoxidez demonstrada por esses herbicidas sintéticos, Al-Sherif et al. (2013) destacam

inconvenientes nessa aplicação, como o fato de sua seletividade ainda ser muito limitada, podendo atuar indististamente nas espécies daninhas e nas espécies vegetais cultivadas nas lavouras. Esses aleloquímicos sintéticos alteram a germinação de sementes e crescimento de plântulas por diversos mecanismos, podendo estar envolvidos na síntese hormonal, divisão celular, mudança na estrutura microscópica, permeabilidade da membrana e/ou na síntese de proteínas de todas as espécies vegetais envolvidas. Adicionalmente, o uso contínuo desses herbicidas sintéticos faz com que as espécies daninhas desenvolvam resistência a esses produtos, o que induz a necessidade de aumentar as dosagens dos produtos já empregados ou até mesmo substituí-los por herbicidas mais potentes e de maior poder alelopático, para que um controle eficaz seja alcançado (BACHEGA et al., 2013).

Assim, torna-se complexo precisar as consequências atuais e principalmente futuras dessas alterações morfológicas e fisiológicas das culturas onde foram aplicados esses herbicidas sintéticos e sobre as espécies animais que as consomem ou habitam a área em que são aplicados. Somam-se ao exposto, o fato de que geralmente esses herbicidas são formados por moléculas hidrossolúveis, o que pode ocasionar a contaminação de lençóis freáticos próximos a essas lavouras, além de serem tóxicos para trabalhadores que os manipulam e os aplicam. Dessa forma, o uso continuado de herbicidas sintéticos constitui uma ameaça à produção agrícola sustentável, podendo resultar em um grave problema ecológico, além de causar riscos à saúde de trabalhadores e consumidores (DE ALMEIDA et al., 2010).

Esse real problema do uso exacerbado dos herbicidas sintéticos tem conduzido diversos estudos que propõe estratégias alternativas para eliminação dessas plantas daninhas. Entre elas, destacam-se o desenvolvimento de compostos biodegradáveis e não tóxicos para se associarem ou substituírem

esses defensivos ou, até mesmo, a exploração do potencial biológico dos metabólitos secundários extraídos dessas plantas invasoras.

Segundo Gianessi (2013), quando a eliminação dessas espécies invasoras é realizada por capina manual em momentos ideais, a produtividade da lavoura não é reduzida, em relação às lavouras onde esse controle é feito por aplicação de herbicidas. Assim, a capina dessas espécies invasoras e posterior aproveitamento dos metabólitos bioativos provenientes das plantas daninhas constituem uma alternativa viável ecológica e economicamente, visto que dariam um destino mais nobre às espécies daninhas que causam prejuízos à agricultura, além de constituírem uma renda alternativa ao agricultor.

Entre os metabólitos secundários que podem ser extraídos das plantas daninhas, destacam-se os óleos essenciais, cujas aplicações biológicas e farmacológicas têm sido pouco pesquisadas, constituindo, assim, uma importante linha de pesquisa a ser estudada.

2.2 Óleos essenciais

O nascimento, crescimento, reprodução, envelhecimento, doenças e morte de um organismo são controlados por reações químicas. Essas reações originam dois tipos de metabólitos; os primários e os secundários. Os primeiros são compostos comuns a todos os organismos e são encontrados em todas as espécies, sendo eles, proteínas, lipídios, ácidos nucleicos, carboidratos, entre outros. Já os metabólitos secundários são característicos e específicos para cada indivíduo e, apesar de estarem presentes em pequenas quantidades, proporcionam-lhes adaptação em seu habitat. Como exemplos desses últimos, podem ser citados os fenilpropanoides e terpenoides constituintes dos óleos essenciais, alcaloides, flavonoides, taninos, cumarinas, saponinas, entre outros (BRAZ FILHO, 2010).

Os óleos essenciais, segundo a "International Organization for Standardization (ISO)", são compostos obtidos de diferentes partes de plantas por hidrodestilação ou destilação por arraste com vapor d'água, bem como os produtos obtidos por expressão dos pericarpos de frutos cítricos. Apresentam-se como líquidos à temperatura ambiente, geralmente sem coloração ou ligeiramente amarelados, lipossolúveis, solúveis em solventes orgânicos e com densidade normalmente menor que a água. São misturas voláteis constituídas por numerosos componentes químicos, característicos de cada espécie vegetal, em que predominam dois ou três compostos majoritários. Possuem odor forte e característico, além de serem extremamente instáveis em diversas condições, como presença de luz, calor, oxigênio e substâncias oxidantes (SIMÕES et al., 2007).

São metabólitos secundários obtidos exclusivamente de plantas e distribuem-se diferentemente em diversos órgãos vegetais, como folhas, flores, caules, rizomas, cascas e frutos, podendo ser encontrados e armazenados em estruturas específicas, como tricomas glandulares, canais oleíferos, bolsas lisígenas ou esquisolisígenas e células parenquimáticas diferenciadas (SILVA et al., 2009).

Assim como os demais metabólitos secundários, os óleos essenciais desempenham diversas atividades biológicas no vegetal, aumentando a sua probabilidade de sobrevivência. Eles protegem o vegetal contra o ataque de patógenos, como bactérias, fungos, vírus e insetos herbívoros, atuam como fitoalexinas, apresentando atividades antigerminativas ou tóxicas para outras plantas. Contribuem também para que as espécies vegetais possam ter uma boa interação com os diferentes ecossistemas, desempenhando papel adicional de atrair animais polinizadores e dispersores de sementes (FUMAGALI et al., 2008).

Essas atividades biológicas específicas de cada óleo essencial estimulam as pesquisas que envolvem suas potencialidades farmacológicas e o possível emprego tanto dos óleos essenciais, como de seus constituintes, isoladamente nas indústrias de medicamentos, cosméticos, alimentos e na agroindústria, substituindo ou associando-se aos compostos utilizados tradicionalmente.

A composição característica do óleo essencial de cada espécie vegetal e a concentração de cada princípio ativo são influenciadas por fatores genéticos e ambientais. Os genéticos são específicos para cada espécie e os ambientais podem variar consideravelmente, dependendo da temperatura, altitude, herbivoria e ataque de patógenos, disponibilidade de água e nutrientes, composição atmosférica, radiação UV, ritmo circadiano, índice pluviométrico, sazonalidade e altitude. Além desses fatores edafoclimáticos, podem ser influenciadas pela idade da planta, o horário e condições de colheita, estabilização, tempo de secagem e estocagem, além dos quimiotipos. Diante disso, infere-se que, em uma mesma espécie botânica, a constância na constituição química e/ou na concentração de metabólitos secundários é incomum, o que torna a identificação e a quantificação dos constituintes desses óleos essenciais necessárias para a abordagem de suas propriedades farmacológicas (GOBBO-NETO; LOPES, 2007).

2.3 Biossíntese dos constituintes dos óleos essenciais

Os óleos essenciais são compostos por uma mistura de numerosos componentes, em que geralmente predominam os terpenoides. Esses constituintes apresentam como precursor comum o isopentenil-pirofosfato, prevalecendo em sua maioria os monoterpenos, sesquiterpenos e seus derivados oxigenados, como álcoois, cetonas, aldeídos, ésteres, fenóis e óxidos. Menos numerosos, nos óleos essenciais estão os fenilpropanoides, que são derivados do

ácido chiquímico e apresentam como característica estrutural um anel benzênico com uma cadeia lateral composta de três carbonos, que apresentam uma dupla ligação (SILVA, A. et al., 2012). As principais vias envolvidas no metabolismo secundário encontram-se sumarizadas na Figura 2.

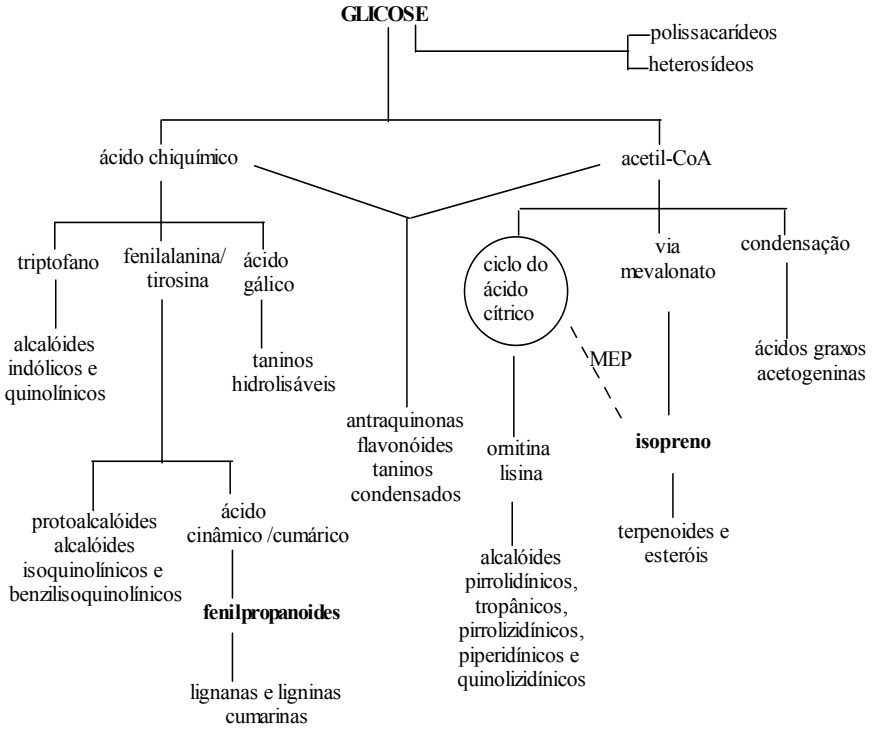

Figura 2 Ciclo biossintético dos metabólitos secundários
Fonte: Adaptado de Simões et al. (2007)

Segundo Baldwin (2010), a origem biossintética dos terpenos deriva de unidades isoprênicas fosforiladas, que são moléculas pentacarbonadas que podem ser originadas por duas vias. A primeira delas ocorre no citoplasma e nas mitocôndrias e é dependente do mevalonato, enquanto a segunda ocorre nos

cloroplastos e plastídios e é independente do mevalonato, sendo conhecida também como via do metileritritol fosfato (MEP) ou rota da 1-deoxi-D-xilulose (DXP).

Na rota do ácido mevalônico, ocorre a condensação aldólica de três moléculas de acetil-CoA, a partir de uma série de etapas, para formar o ácido mevalônico. Esse intermediário de seis carbonos é, então, pirofosforilado, descarboxilado e desidratado, para produzir o isopentenil pirofosfato (IPP), que é a unidade básica dos terpenos (Figura 3). Essa unidade pentacarbonada, por meio de reações de isomerização, é convertida em dimetilalil pirofosfato (DMAPP) (DEWICK, 2009).

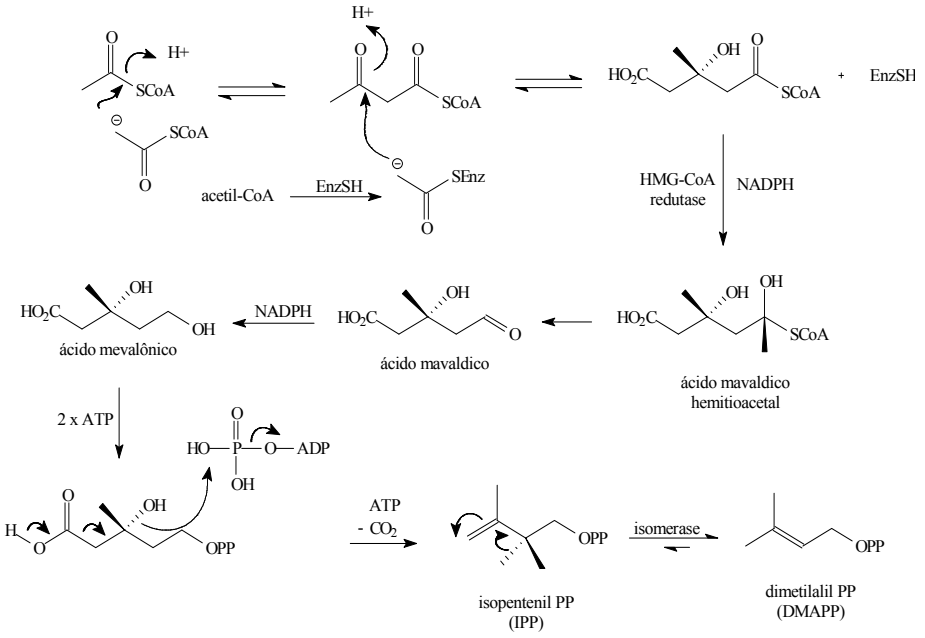

Figura 3 Mecanismo de formação das unidades isoprênicas ativas, dimetilalil difosfato (DMAPP) e isopentenil difosfato (IPP) pela via do mevalonato

Fonte: Adaptado de Dewick (2009)

O IPP também pode ser formado a partir da rota do MEP, onde o gliceraldeído-3-fosfato e dois átomos de carbono derivados do piruvato se combinam, passando por sucessivas reações para produzir o 2-C-metil-D-eritritol-2,4-ciclodifosfato, que é convertido a IPP (Figura 4). As moléculas de IPP e seu isômero DMAPP sintetizadas em ambas as vias unem-se para formar terpenos maiores, por meio do modelo "cabeça-cauda". Essa condensação de Claisen de unidades de IPP e DMAPP, pela ação da enzima prenil-transferase, forma o intermediário pirofosfato de geranila (GPP, C10), que condensa com outra unidade IPP, fornecendo o pirofosfato de farnesila (FPP, C15). Por último, a junção de FPP com outra unidade de IPP conduz à produção de pirofosfato de geranilgeranila (GGPP, C20), precursor dos diterpenos. As ciclizações do GGPP, por meio da formação de diferentes carbocátions e rearranjos, levam a variações estruturais de diterpenos. A dimerização de FPP e GGPP forma os triterpenos e os tetraterpenos, com 30 e 40 átomos de carbono, respectivamente (Figura 4). Provenientes de um precursor comum, as estruturas terpênicas sintetizadas por essas duas rotas podem ser modificadas por reduções, oxidações e ciclizações, formando numerosos e distintos compostos terpênicos, que constituem, assim, um dos maiores e, talvez estruturalmente, o mais diverso grupo de metabólitos secundários derivados dos vegetais (Figura 5) (DEWICK, 2009).

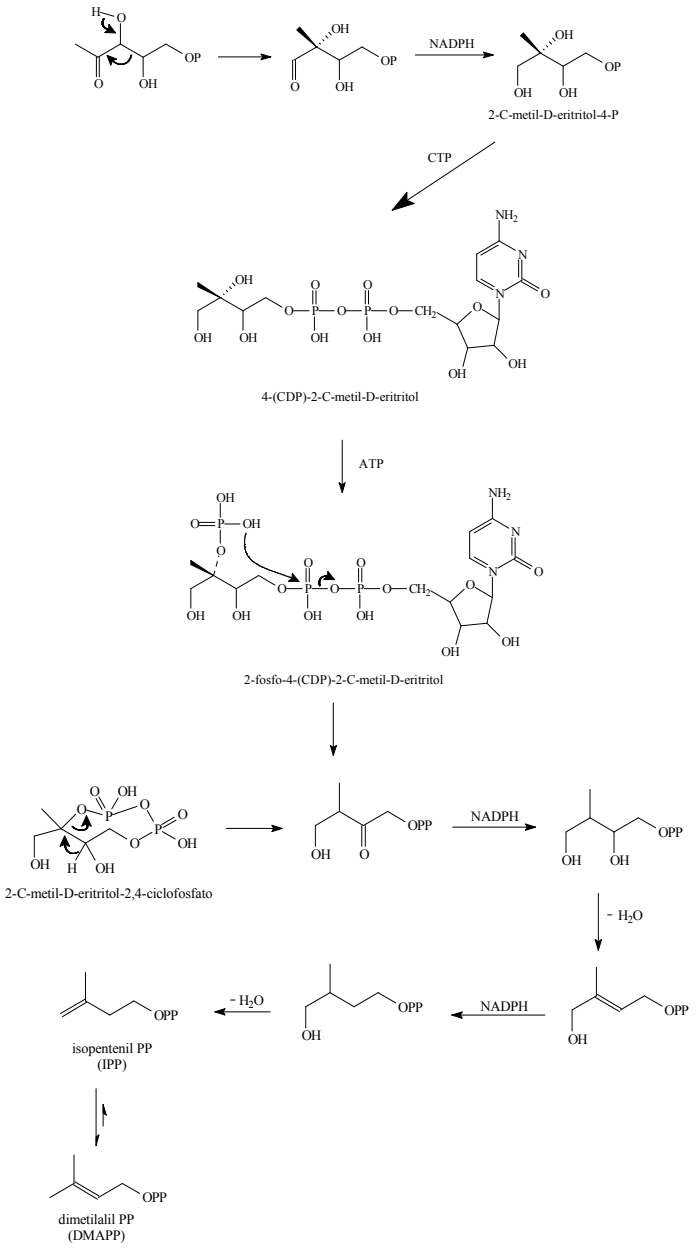

Figura 4 Mecanismo de formação das unidades isoprênica ativas, dimetilalil difosfato (DMAPP) e isopentenil difosfato (IPP), pela via da 1-deoxi-D-xilulose -5- P mevalonato

Fonte: Adaptado de Dewick (2009)

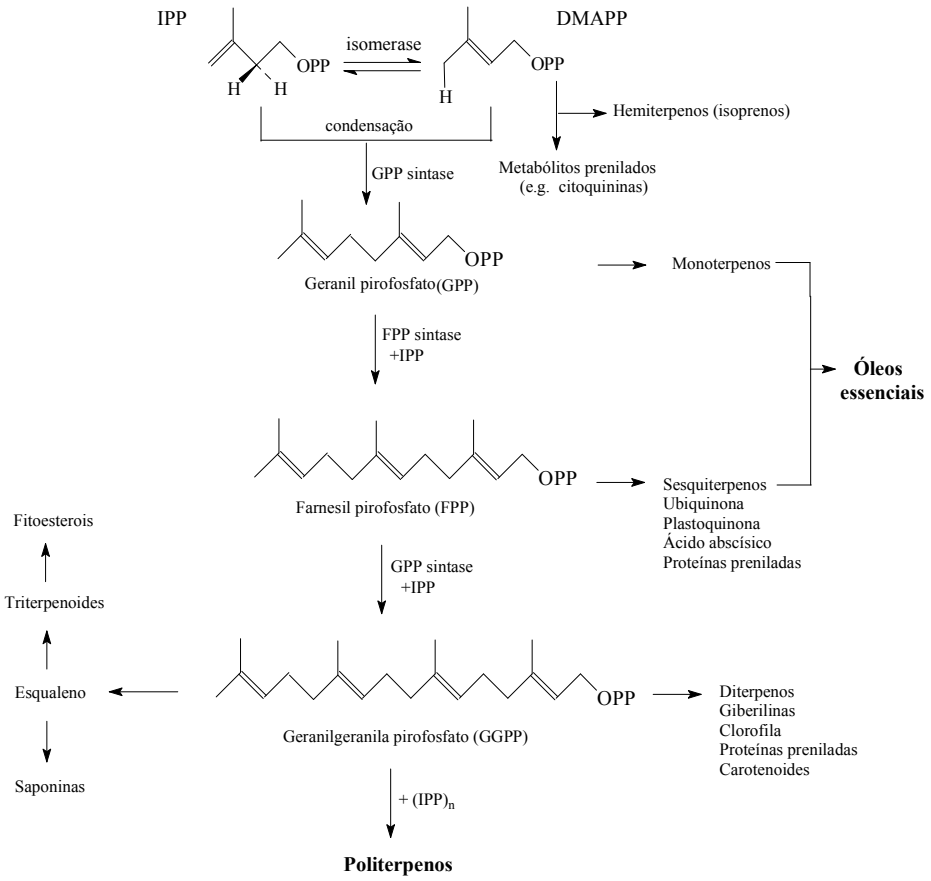

Figura 5 Esquema das vias de biossíntese responsáveis pela produção de terpenoides, a partir da formação dos blocos de isoprenoides isopentenil pirofosfato (IPP) e dimetilalil pirofosfato (DMAPP)
Fonte: Adaptado de Dewick (2009)

Os fenilpropanoides formam-se a partir do ácido chiquímico, que origina inicialmente os aminoácidos fenilalanina e tirosina. Esses sofrem a ação enzimática da fenilalanina amonialiase (PAL), que retira uma molécula de amônia das moléculas desses aminoácidos, gerando os ácidos cinâmico e *p*-

cumárico, respectivamente. Esses últimos, por sua vez, sofrem diferentes tipos de reações, como oxidações, reduções e ciclizações, originando os fenilpropanoides (Figura 6) (SIMÕES et al., 2007).

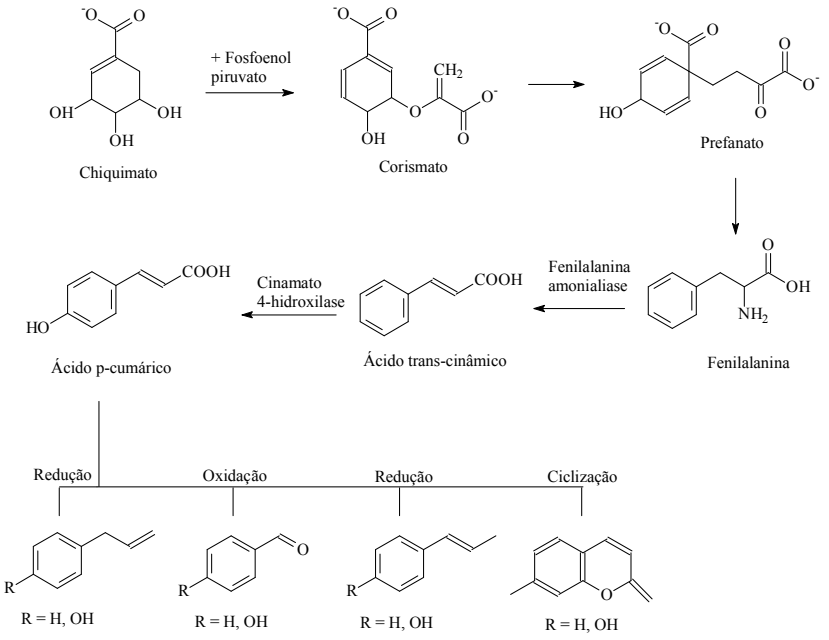

Figura 6 Mecanismo de formação da estrutura básica dos fenilpropanoides a partir do ácido chiquímico
Fonte: Simões et al. (2007)

2.4 Potenciais biológicos de óleos essenciais extraídos de espécies invasoras

O aproveitamento do potencial biológico e farmacológico dos óleos essenciais extraídos de espécies invasoras tem motivado pesquisas nas quais se abordam as mais diversas aplicabilidades desses óleos. Entretanto, essas potencialidades e até mesmo a caracterização química desses óleos ainda são pouco exploradas, o que faz desses estudos uma promissora linha de pesquisa.

A composição química dos óleos essenciais das raízes de duas espécies de *Echinops* (Asteraceae), *E. bannaticus* Rochel ex Schrad. e *E. sphaerocephalus* L., plantas daninhas comumente encontradas na flora da Sérvia, foi estudada pela primeira vez por Radulović e Denić (2013). Os autores encontraram entre 106 e 81 constituintes, respectivamente, na composição desses óleos. A Análise de Componentes Principais (PCA) e agrupamento hierárquico aglomerativo revelaram um agrupamento de *E. bannaticus* e *E. sphaerocephalus* e sua estreita relação com outra espécie do mesmo gênero descrita anteriormente, a *E. grijsii*, o que sugere a inserção dessa espécie ao gênero *Echinops*.

Peebles et al. (2011) avaliaram o potencial contra pragas agrícolas do óleo essencial das folhas frescas da espécie *Thelechitonia trilobata*, considerada uma planta daninha infestante. O óleo essencial apresentou resultados satisfatórios como anticarrapaticidas, sobre espécimes de *Ripicephalus ervertsi* encontrados em ovelhas e nos bioensaios que avaliaram a repelência, fumigação e o contato com gorgulho do milho (*Sitophilus zeamais*).

A espécie *Ligularia virgaurea* é uma erva daninha amplamente distribuída nas pastagens de leste da China, cujos efeitos alelopáticos de seu óleo essencial foram avaliados por Ma et al. (2005). Os autores verificaram que o óleo essencial reduziu a velocidade da germinação das cinco espécies de

gramíneas avaliadas e inferiram que a volatilização é uma das maneiras pelas quais o óleo de *L. virgaurea* propaga seus aleloquímicos.

Estudos que abordam as potencialidades do óleo essencial da espécie invasora exótica *Hedychium coronarium* têm utilizado principalmente o óleo essencial extraído de seus rizomas. Em trabalhos de Joy, Rajan e Abraham (2007), Salubal et al. (2007), Joshi et al. (2008), Prakash et al. (2010) e Ho (2011) apresentaram as atividades antimicrobianas desse óleo em diversas cepas bacterianas e fúngicas, e obtiveram resultados satisfatórios. Entretanto, quando suas propriedades larvicidas contra mosquitos e antioxidantes foram estudadas por Joshi et al. (2008) e Ho (2011), os autores verificaram potenciais moderados dessas atividades. Apenas a caracterização química do óleo essencial extraído das folhas dessa espécie foi estudada por Santos et al. (2010), que encontraram β-cariofileno, óxido de cariofileno e o β-pineno como contituintes principais. Estudos abrangendo as propriedades biológicas do óleo essencial extraído das folhas dessa espécie não foram relatados anteriormente.

O óleo essencial das folhas da espécie invasora *Tithonia diversifolia*, amplamente distribuída em lavouras, foi caracterizado quimicamente por Moronkola et al. (2007). Esses autores encontraram como constituintes principais desse óleo o α-pineno, β-cariofileno e o germacreno-D. Entretanto, estudos sobre as potencialidades biológicas do referido óleo essencial não foram descritas anteriormente na literatura.

A *Conyza bonariensis* é uma espécie invasora com amplo potencial alelopático e grande capacidade de propagação, e segundo Cerdeira et al. (2011), apresenta resistência a diversos herbicidas sintéticos comumente utilizados em culturas agrícolas, como o glifosato, sendo considerada uma das plantas daninhas de controle mais difícil. Apenas o potencial anti-inflamatório do óleo essencial extraído das folhas dessa espécie foi avaliado nas pesquisas realizadas

por Souza et al. (2003), que observaram resultados anti-inflamatórios promissores.

A espécie *Baccharis dracunculifolia* é uma planta utilizada popularmente no tratamento de distúrbios gástricos, cansaço físico, inapetência, afecções febris e debilidade orgânica, apresentando características invasoras, sendo encontrada comumente em campos agricultu ráveis (CANTON; ONOFRE, 2010). As aplicações farmacológicas do óleo essencial extraído das folhas dessa espécie têm sido exploradas em diversos estudos. Ferronato et al. (2007) avaliaram a capacidade antibacteriana desse óleo em cepas de *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 e *Pseudomonas aeruginosa* ATCC 27853 e obtiveram resultados satisfatórios. A capacidade antiulcerogênica do referido óleo foi avaliada por Kloppel et al. (2007) e Massignani et al. (2009), enquanto a anti-inflamatória foi abordada por Florao et al. (2012), e todos observaram resultados promissores em suas avaliações. Parreira et al. (2010) avaliaram os potenciais antileishmanial, antiplasmodial, antiesquistossomicidal e antimicrobiano em espécies de *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, *Staphylococcus aureus* meticilina resistentes ATCC 43300 e *Mycobacterium intracellulare* ATCC 23068 do óleo essencial de *B. dracunculifolia* e verificaram que o óleo apresentou eficiência apenas contra formas promastigotas de *Leishmania donovani* e em ensaios antiesquistossomicidas. A capacidade antibacteriana desse óleo em cepas de *Escherichia coli* e de *Staphylococcus aureus* foi avaliada por Silva, N. et al. (2012), que não verificam essa potencialidade, ao contrário dos estudos realizados por Galvão et al. (2012), que constataram eficácia desse óleo sobre cepas de *Streptococcus mutans*.

Pesquisas realizadas por Chowdhury (2002) sobre a constituição química do óleo essencial extraído das folhas da espécie invasora *P.*

hysterophorus, coletadas na Índia, identificaram 78% dos constituintes desse óleo essencial, totalizando 63 compostos químicos, entre os quais predominaram o acetato de bornila e o geraniol. As aplicabilidades biológicas desse óleo não foram avaliadas anteriormente.

Estudos sobre a composição química e as atividades biológicas do óleo essencial extraído das folhas da espécie *A. polystachya* não foram descritos anteriormente na literatura.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 - CHEMICAL COMPOSITION AND ALLELOPATHIC ACTIVITY OF *PARTHENIUM HYSTEROPHORUS* AND *AMBROSIA POLYSTACHYA* WEEDS ESSENTIAL OILS

Artigo submetido ao Conselho Editorial do periódico Ciência e Agrotecnologia e formatado conforme normas do referido periódico.

Chemical composition and allelopathic activity of *Parthenium hysterophorus* and *Ambrosia polystachya* weeds essential oils

Composição química e atividade alelopática dos óleos essenciais das plantas daninhas *Parthenium hysterophorus* e *Ambrosia polystachya*

ABSTRACT

Some weeds may be potential producers of essential oils that can be studied for use as substitutes or in combination with synthetic herbicides because they exhibit allelopathic activity, are suitable economically and ecologically, and are generally accepted by consumers. The volatile constituents of the essential oils from the weed plants, *Parthenium hysterophorus* and *Ambrosia polystachya*, were identified and quantified by GLC-MS and GLC. Allelopathic activities were determined by methods that evaluate the effects of contact with the volatile components and direct application of the oil to the seeds on the seed germination and seedling vigor of lettuce. We identified 27 compounds in the essential oil from *P. hysterophorus*, and the main constituents were germacrene-D (35.9%), trans-β-ocimene (8.5%) and β-myrcene (7.6%). In

the essential oil from *A. polystachya*, 40 constituents were identified and the principal compounds were germacrene-D (29.3%), *trans*-β-ocimene (13.6%) and β-caryophyllene (9.8%). In both methods, the essential oil from *A. polystachya* presented a greater potential for reducing seed germination and seedling vigor in lettuce than the essential oil from *P. hysterophorus*. This activity might be attributed to its higher content of monoterpenes.

Index terms: volatile oils, allelopathy, Asteraceae.

INTRODUCTION

Weeds are harmful to agriculture because they decrease the productivity and quality of agricultural products. In recent decades, invasive plants have mostly been eliminated by applying synthetic herbicides, which may cause environmental damage with appreciable toxic effects to living organisms, including human beings (Singh et al., 2005). The abuse of these synthetic herbicides has increased the resistance of invasive herbs. This fact makes the use of bioherbicides a promising, economically and environmentally sustainable alternative (Batish et al., 2008).

These allelochemicals of natural origin can be produced by all types of plant tissues. They act by different mechanisms such as volatilization, root exudation and decomposition of residues (Weston & Duke, 2003). The weeds are noted for their allelopathic potential. They compete for resources with adjacent plants, with consequent inhibition of growth and development of cultures (Kong et al., 2007).

Among these weeds, some species of Asteraceae, such as *Parthenium hysterophorus* and *Ambrosia polystachya*, stand out for their high allelopathic potential for crops. *P. hysterophorus*, commonly known as ragweed, is a plant native to the Americas and is one of the ten worst invasive species for

agriculture, causing huge losses in many parts of world (Jayanti et al., 2005). The Ambrosia genus includes over thirty weed species found mainly in the Americas. These species invade cultivated fields and reduce seed production. *A. polystachya*, known as ambrosia or cravorona americana, is one of these species (Kong et al., 2007). Given the potential phytotoxicity of these invasive species, extracts and volatile compounds of *P. hysterophorus* and Ambrosia species have had their allelopathic potentials demonstrated recentemente (Jayanti et al., 2005; Kong et al., 2007; Wakjira et al., 2009; Chen et al., 2011; Patracchini et al., 2011; Khan et al., 2012).

Some weeds can produce essential oils, which are complex mixtures of different secondary metabolites derived from various plant tissues (leaves, flowers, seeds, rhizomes, bark), among which we highlight terpenoids that may have biological functions, such as allelopathy, that are essential for survival and adaptation of the plant to the environment (Silva et al., 2009). Given the fact that herbicides present substantial problems for agriculture, human health, and the environment, and the fact that weeds stand out as a species with allelopathic activity, as well as a potential source of essential oils, the aim of this work was to chemically characterize the essential oils extracted from fresh leaves of *Parthenium hysterophorus* and *Ambrosia polystachya* and to determine their potential allelopathic activities by assessing the effect of direct contact and contact with the volatile components on seed germination and the seedling vigor of lettuce (*Lactuca sativa* L.) by in vitro screening assays.

MATERIAL AND METHODS

Collection of Plant Material

The young leaves (rib and limb) of adult *Parthenium hysterophorus* and *Ambrosia polystachya* plants in the flowering stage were collected on the campus of the Universidade Federal de Lavras (UFLA), in Lavras, MG, Brazil

(21 º 14 'S, longitude 45 º 00' W Gr and 918 m altitude). The species were collected in the early morning of days without precipitation in February 2012, and the species identification were kindly performed by Doctor Mariana Esteves Mansanares, Department of Biology of UFLA and exsiccates were deposited in the ESAL-UFLA Herbarium under records numbering 26944 and 26948, respectively.

Extraction of the essential oils

The essential oils from fresh leaves were extracted by hydrodistillation using a modified Clevenger apparatus adapted to a round bottom flask with a capacity of 4 L over a period of 2 h (Farmacopeia Brasileira, 2000). The hidrolact was centrifuged for 5 min at 965.36*g*, and the oils were separated with a Pasteur pipette, transferred to amber glass bottles and stored at 4 °C.

Determination of moisture and yields of the extractions

The determination of the moisture content of the fresh leaves was performed in parallel with the extraction of the essential oils (Pimentel et al., 2006). In a 250-mL, round-bottom flask coupled to a Dean-Stark trap, was placed 5 g of chopped fresh leaves in 70 ml of cyclohexane. The flask was heated on a heating mantle for 2 h at a temperature of 81±1 °C, and the quantity of water distilled was measured. The yields of the essential oils were expressed on a dry weight basis (DWB).

Identification of essential oil constituents

The gas-liquid chromatography-mass spectrometric (GLC-MS) analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph equipped with a fused-silica DB-1 column (30 m x 0.25 mm ID; film thickness, 0.25 m; J & W Scientific Inc.) coupled to a Perkin Elmer Turbomass mass spectrometer (software version 4.1). The oven temperature was programmed from 45 to 175 ˚C at 3 ˚C/min and then at 15 ˚C/min to 300 ˚C, where the temperature was maintained for 10 min. The temperature of the transfer line was

280 ˚C; the temperature of the ionization chamber was 220 ˚C; the carrier gas was helium, adjusted to a linear velocity of 30 cm/s; and the split ratio was 1:40. The identity of the compounds was determined by comparison of their retention indices relative to C9-C21 n-alkanes, along with the comparison of the mass spectra with those of standard commercial and reference compounds present in oils existing in the laboratory and with a library of mass spectra developed in the laboratory of the Centre for Plant Biotechnology, Faculty of Science, University of Lisbon, Lisbon, Portugal (Mendes et al., 2011).

Quantification of constituents of the essential oils

The essential oils were analyzed by gas-liquid chromatography (GLC) on a Perkin Elmer 8700 gas chromatograph equipped with two flame ionization detectors (FID), a system for data processing and an injector. The chromatograph possessed two columns of different polarity, with the following characteristics: a fused silica capillary column containing the DB-1 methylsilicone stationary phase (30 m x 0.25 mm ID; film thickness, 0.25 m; J & W Scientific Inc.) and a fused silica capillary column containing the DB-17HT phenylmethylsilicone stationary phase (30 m x 0.25 mm id). The oven temperature was programmed from 45 °C to 175 °C at 3 °C/min, followed by an increase at 15 °C/min to 300 °C, where the temperature was held for 10 min. The temperatures of the injector and detector were 290 °C and 280 °C, respectively. Hydrogen was used as the carrier gas with a flow rate of 30 cm/s, and the split ratio was 1:50. The percentage composition of the oils was determined by integration of peak areas without using correction factors. The values given represent the average of two injections.

Allelopathic activity of the essential oils

Two bioassays were conducted to evaluate the allelopathic potential of the essential oils: the first assessed the effect of the volatile components and the second assessed the direct contact of the oil with the seeds on the germination of

lettuce seeds (Regina SF 3500 cultivar). The 1% stock solutions were prepared with 0.5 mL of each essential oil emulsified in Tween 80 in a 1:1 (v/v) ratio and dissolved in distilled water. The remaining concentrations (0.1 and 0 01% v/v) were prepared by dilution. A solution of 1.0% v/v Tween 80 in water was used as a control (Silva et al., 2009). The solutions of the essential oils were added at the beginning of the bioassay. During the experiments, only distilled water was added when necessary (Souza Filho et al., 2009). In both bioassays, seeds were packed in gerbox-type acrylic boxes (dimensions 11x11x4 cm). Two sheets of sterilized blotting paper were employed as substrates, where 50 seeds were distributed, totaling 200 seeds per treatment. The seeds were maintained in a growth chamber (BOD) at a temperature of 20 ± 1 °C and a photoperiod of 12 h. In the direct contact method, the blotting paper was soaked with equivalent amounts of solutions of the different concentrations of the essential oil up to 2.5 times its dry weight (BRASIL, 2009). The evaluation of the effect of the volatile components was determined by adding distilled water to the paper substrate in amounts equivalent to 2.5 times its dry weight (BRASIL, 2009). Three-milliliter portions of the solutions of essential oils were placed on two sheets of filter paper affixed to the incubator lid, thereby avoiding direct contact of the solutions with the seeds (Souza Filho et al., 2009).

Germination was monitored for seven days, starting from the implementation of the test, with daily counts of lettuce seedlings, and the results were expressed in percentage of seedlings normais (BRASIL, 2009). Seed vigor was determined from the following variables: First count of germination, which consisted of the evaluation of normal seedlings on the fourth day after sowing (BRASIL, 2009); Germination Speed Index (GSI) (Maguire, 1962); average lengths of root and seedling shoots on the seventh day, which were with the aid of a millimeter-scale rule and the means were expressed in centimeters(BRASIL, 2009); determination of the dry mass of seedlings in an

incubator at 60 °C in Kraft paper bags to constant weight. After this period, the weighing and determination of the means by repetition were preformed, the results being expressed in grams (Krzyzanowski et al., 1999).

Statistical Analysis

The experimental design for both methods was completely randomized in a 4x2 factorial scheme (four concentrations and two essential oils), with four replications. The essential oils were compared, because these species were of the same botanical family, the Asteraceae. The different methods were not compared because they are known to have different chemical mechanisms. Significant factors from the F test ($p < 0.05$) were submitted to the Scott-Knott test of means (5%) for the determination of the models. The data were analyzed using the Statistical Analysis System Variance for Balanced Data – Sisvar (Ferreira, 2011). The results were plotted in bar graphs with the values of the variables versus the concentrations analyzed using the GraphPad Prism software version 5.0.

RESULTS AND DISCUSSION

The yields of essential oils obtained by hydrodistillation of the leaves from *P. hysterophorus* and *A. polystachya* were 0.04 and 0.33% (w/w), respectively. In the essential oil extracted from *P. hysterophorus*, 31 components were identified, amounting to 76% of its constitution, and 58 compounds (97%, Table 1) were identified in the essential oil extracted from *A. polystachya*. The identified components and their relative proportions are listed in Table 1 in order of elution on a DB-1 column.

Table 1. Percentage composition of the essential oils obtained from the leaves of *P. hysterophorus* and *A. polystachya* harvested during flowering.

Components	RI	*Parthenium hysterophorus* (%)	*Ambrosia polystachya* (%)
Tricyclene	921	-	t
α-Tujene	924	t	t
α-Pinene	930	0.4	1.7
Camphene	938	0.8	0.3
Sabinene	958	0.2	1.7
1-Octen-3-ol	961	1.6	t
β-Pinene	963	0.4	6.6
β-Myrcene	975	7.6	0.3
α-Phelandrene	995	-	t
p-Cymene	1003	t	0.2
1,8-Cineol	1005	-	0.6
β-Phelandreno	1005	0.2	t
Limonene	1009	0.7	8.0
cis-β-Ocimene	1017	t	t
trans-β-Ocimene	1027	8.5	13.6
γ-Terpinene	1035	-	t
trans-Sabinene hydrate	1037	-	t
Terpinolene	1064	-	0.2
Linalool	1074	-	0.5
Octen-3-ol acetate	1086	-	t
α-Campholenal	1098	-	0.1
cis-Verbenol	1110	-	0.2
δ-Terpineol	1134	-	t
Terpinen-4-ol	1148	-	t
α-Terpineol	1159	-	t
Thymol methyl ether	1210	-	0.1
Borneol acetate	1265	1.3	t
trans-α-Necrodol acetate	1265	-	t
δ-Elemene	1332	t	t
α-Cubebene	1345	-	0.1
α-Copaene	1375	-	0.6
β-Bourbonene	1379	-	t
β-Cubebene	1385	0.4	0.8
β-Elemene	1388	0.2	0.2
β-Caryophylene	1414	3.1	9.8
β-Copaene	1426	0.2	0.1
trans-α-Bergamotene	1434	t	t

Components	RI	*Parthenium hysterophorus* (%)	*Ambrosia polystachya* (%)
α-Humulene	1447	1.0	1.0
trans-β-Farnesene	1455	0.1	-
allo-Aromadendrene	1456	-	0.2
γ-Muurolene	1469	-	t
Germacrene D	1474	35.9	29.3
β-Selineno	1476	-	0.7
Bicyclogermacrene	1487	1.5	4.2
α-Muurolene	1494	-	0.2
(*trans,trans*)-α-Farnesene	1500	3.3	-
γ-Cadinene	1500	-	0.2
δ-Cadinene	1505	0.1	1.4
β-Sesquiphelandrene	1508	0.3	-
trans-Nerolidol	1549	-	0.1
Spathulenol	1551	-	2.2
Carota-5,8-diene*	1551	1.5	-
β-Caryophylene oxide	1561	-	2.6
Globulol	1566	-	4.6
Viridiflorol	1569	-	0.8
Carotol	1573	4.8	-
Ledol	1580	-	0.2
3,4-Dehydroglobulol*	1587	-	2.4
T-Cadinol	1616	-	0.3
δ-Cadinol	1618	-	0.5
α-Cadinol	1626	0.7	0.1
Intermedeol	1626	-	0.1
α-Bisabolol	1656	0.6	-
Phytol acetate	2047	0.6	0.4
% Identified		75.7	97.3
Grouped components			
Monoterpene hydrocarbons		18.8	32.7
Oxygen-bearing monoterpenes		1.3	1.5
Sesquiterpene hydrocarbons		47.4	48.8
Oxygen-bearing sesquiterpenes		6.1	13.9
Oxygen-bearing diterpenes		0.6	0.4
Outros		1.6	v

RI: Retention Indices relative to a series of n-alkanes C_9-C_{21} on a DB-1 column. t: trace (<0,05%). *Compounds identified only on the basis of the mass spectra.

Despite the large differences in the chemical compositions of the essential oils isolated from the two species, the sesquiterpene hydrocarbon fraction was the principal fraction in both cases, reaching 47% of the essential oil from *P. hysterophorus* and 49% of that from *A. polystachya*. Similarly, the monoterpene hydrocarbon fraction was the second most important fraction in these essential oils (19% of the essential oil from *P. hysterophorus* and 33% of that from *A. polystachya*). Germacrene (36%), *trans*-β-ocimene (9%) and β-myrcene (8%) were the dominant components isolated from the essential oil of *P. hysterophorus*. Germacrene (29%) and *trans*-β-ocimene (14%), followed by β-caryophyllene (10%), were also the principal compounds in the essential oil from *A. polystachya*.

Few studies on the chemical composition of these essential oils have previously been described in the literature. Chowdhury et al. (2002) identified bornyl acetate (9.15%), geraniol (7.53%) and phenylacetonitrile as the major constituents of the essential oil extracted from the leaves of *P. hysterophorus* collected in India. These authors identified 78% of the constituents of the essential oil, a total of 63 chemical compounds. With respect to *A. polystachya*, Bolzani & Gotta (1970) evaluated the essential oil from the flowers of this species and determined that it is probably produced in the secretory ducts of the male and female flowers. They identified 14 chemical compounds. According to Gobbo-Neto & Lopes (2007), the variation in the chemical compositions of the essential oils from different botanical species and the essential oils extracted from the same botanical species from different countries is related to the collection site, the vegetative cycle and edaphoclimatic factors.

The effect of volatile components of the essential oils from *P. hysterophorus* and *A. polystachya* on seed germination and seedling vigor of lettuce was evaluated. An increase in the concentration of both essential oils did not significantly affect the first germination count and the total germination of

the lettuce, which had mean values of 88.5% and 95% for the essential oil from *A. polystachya* and 94% and 96.5% for the oil from *P. hysterophorus*, respectively. The concentration of the essential oil from *P. hysterophorus* did not influence the IVG, the dry weight and the lengths of the aerial parts and roots. These variables had values of 75.6, 0.0370 g, 17.05 mm and 38.13 mm, respectively. The volatile components of the essential oil from *P. hysterophorus* did not have any allelopathic effect, unlike the essential oil from *A. polystachya*, which presented dose-dependent effects for the IVG, the dry matter and the measurements of the aerial parts and roots of lettuce seedlings. A 1% concentration of the essential oil from *A. polystachya* produced the largest reductions in the variables, with mean values of 47.4 for the IVG; 0.0328 g of dry matter, 12.48 mm for the length of the shoots and 30.85 mm for the root. The control presented values of 75.7, 0.0363 g, 17.88 mm and 38.43 mm, respectively, for the same variables. The responses of the IVG and lengths of the aerial parts and roots of seedlings to essential oil concentrations of 0.01% and 0.1% did not differ statistically. There was no statistical difference between the concentrations with regard to dry weight, although the weights were different from that of the control (Figure 1).

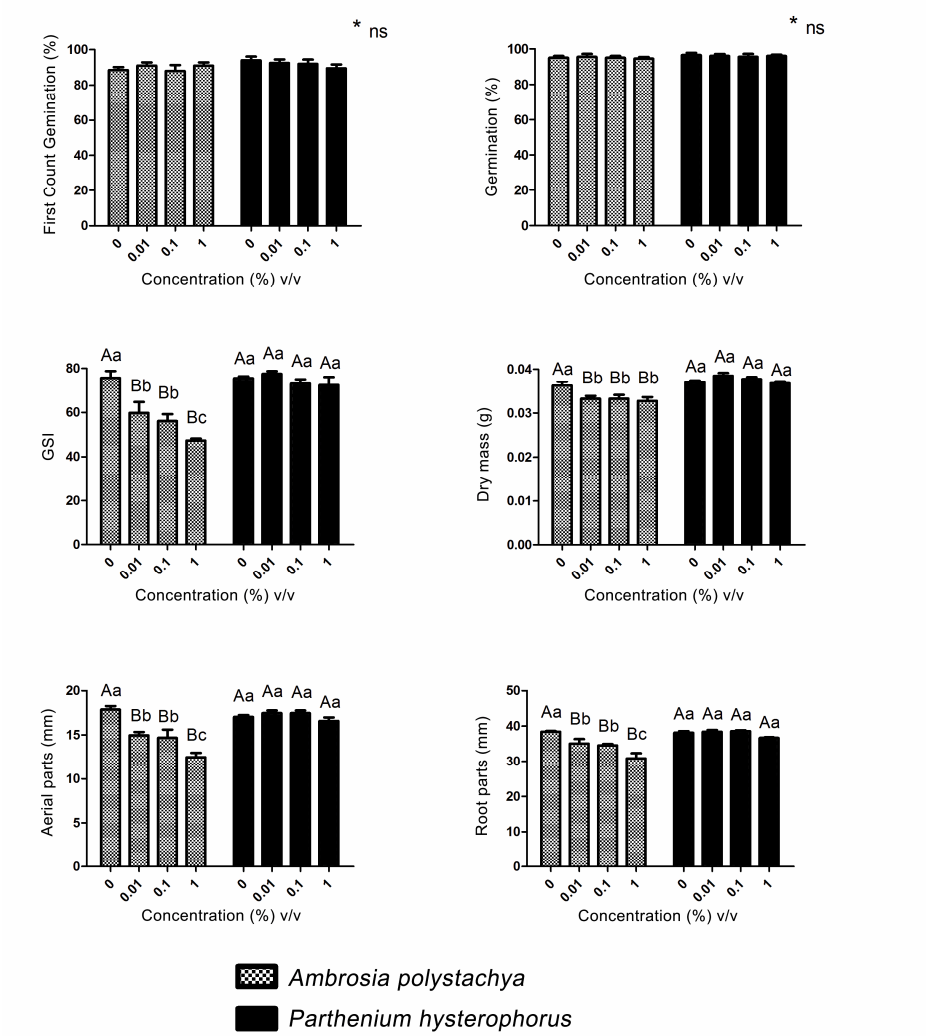

Figure 1. Mean values for the first germination count, total germination, IVG, seedling dry mass, and lengths of shoots and roots as a function of the concentrations of the essential oils from *A. polystachya* and *P. hysterophorus*, evaluated according to the effect of the volatile components. Means followed by the same uppercase letter for the comparison between the essential oils analyzed and the same lowercase letter for the comparison between concentrations of each essential oil do not differ significantly at 5% probability by the Scott-Knott test.

The essential oils from *A. polystachya* and *P. hysterophorus* in direct contact with the lettuce seeds did not affect the first count (fourth day) and total germination (seventh day) responses at the concentrations of 0.01% and 0.1%, with mean responses of 94% and 90.5% for *A. polystachya* and 92.5% and 94.5% for *P. hysterophorus*. Only the 1% concentration differed statistically from the other concentrations, with significantly lower mean values for these variables. The use of the essential oil from *A. polystachya* reduced them to 85% and 89%, while that of *P. hysterophorus* caused a reduction of 82.5% and 75.5%, respectively. This same response pattern was observed when the influence of direct contact with the essential oil from *P. hysterophorus* was analyzed, wherein application of the 1% concentration also caused a reduction in the responses of the IVG variable from 76.51 to 42.76, the dry matter was reduced from 0.0375 g to 0.0265 g, the length of the shoots from 15.55 mm to 11.58 mm and that of the root from 42.95 mm to 21.85 mm. The direct contact with the essential oil from *A. polystachya* also caused allelopathic effects at concentrations lower than that of the essential oil from *P. hysterophorus*. There were no significant differences from the control variables for IVG, dry matter and length of the aerial parts and roots of lettuce seedlings. The application of the 1% concentrations of the essential oil from *A. polystachya* significantly decreased the mean values of the GSI from 76.28 to 49.64, the dry weight from 0.0338 g to 0.0373 g, the length of the shoots from 15.98 mm to 10.28 mm and the length of the roots from 42, 35 mm to 31.6 mm (Figure 2).

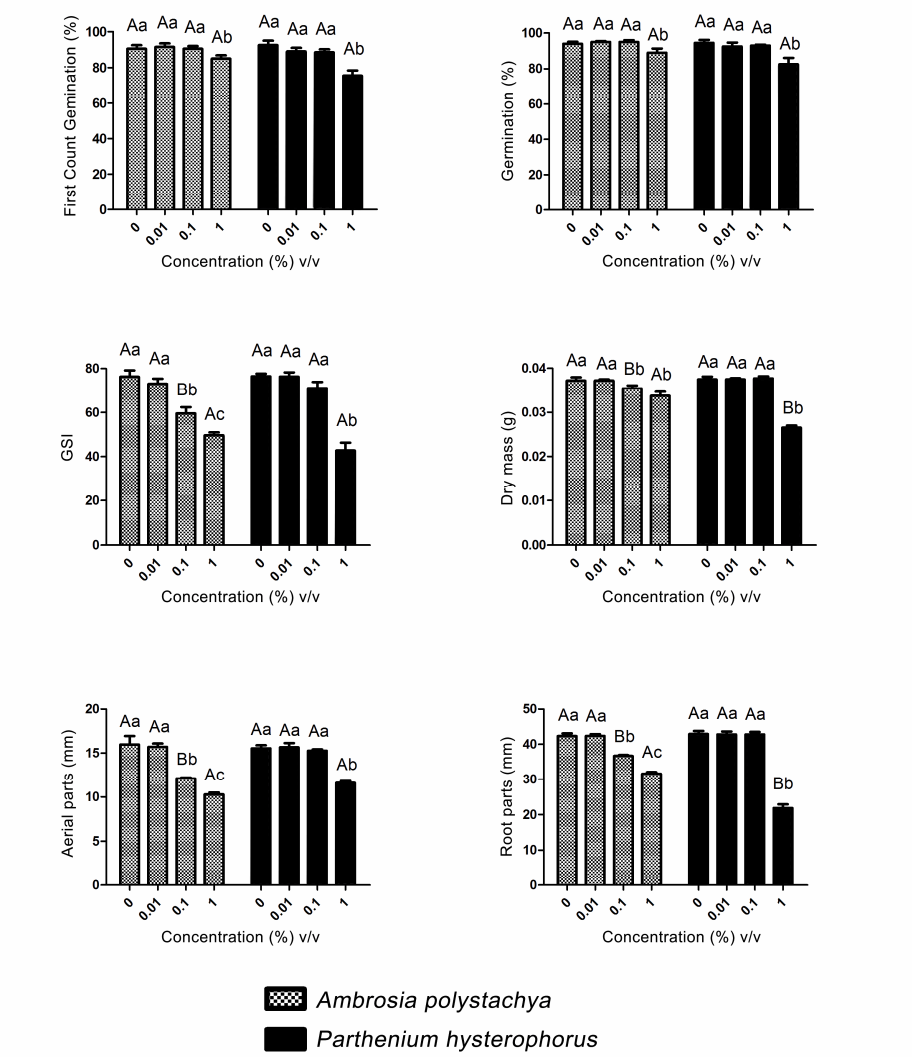

Figure 2. Mean values for the first germination count, total germination, IVG, seedling dry mass, length of shoot and length of root versus the concentrations of the essential oils from *A. polystachya* and *P. hysterophorus*, evaluated by the direct contact method. Means followed by the same uppercase letter for comparison between the essential oils analyzed and the same lowercase letter for comparison between concentrations of each essential oil do not differ significantly at 5% probability by the Scott-Knott test.

The similar response profiles observed for the essential oil from *A. polystachya* in both methods can be explained by the fact that it delayed the initial germination of lettuce seeds (up to the third day of the experiment), which explains the constant values for the first germination and final germination and the decrease in the responses of the other variables so that higher concentrations of this oil were required. When the volatile components of the essential oil from *P. hysterophorus* were evaluated, no phytotoxicity was observed. Direct contact of this oil with the substrate affected the germination and seedling vigor only at the highest concentration tested (1%).

The effects of the volatile compounds from fresh flowers and leaves of *P. hysterophorus* on the growth of the *Echinochloa crus-galli* (L.) Beauv. and *Digitaria sanguinalis* L. grass species in transparent boxes were evaluated by Chen et al. (2011). The volatiles were extracted from the headspace and the constituents were identified by GC-MS. The principal volatile constituents identified from the flowers were the monoterpenes myrcene (56.67%) and ocimene (26.28%), while those from the leaves were myrcene (28.07%) and α-pinene (14.52%). The volatile components from the flowers inhibited the growth of *E. crus-galli* and *D. sanguinalis* seedlings, while those from the leaves only affected the development of *D. sanguinalis* seedlings. The inhibition of growth of these species was justified by the higher content of volatile monoterpenes in the oil from the flowers.

The allelopathic activity of the essential oil from basil (*Ocimum basilicum*) on seed germination and the initial growth of lettuce, tomato and lemon balm seedlings was evaluated by Rosado et al. (2009). The major compounds found in this oil were the monoterpenes linalool and geraniol. The phytotoxic effects of the essential oil from basil were attributed to the monoterpene content, a fact that corroborated the findings of our experimento. This ability of monoterpenes to affect the germination and growth of plants can

be explained by the ability of these compounds to cause morphological and physiological changes in plants, including the inhibition of the respiratory chain in the isolated mitochondria and mitosis, alteration of the integrity of cell membranes, deterioration of cuticular waxes, increased persperation, and lipid peroxidative damage to microtubes (Yoshimura et al., 1911).

The essential oils were extracted from invasive species belonging to the same botanical family, the Asteraceae, both oils contained germacrene-D as the major constituent (29.3% and 35.9% for *A. polystachya* and *P. hysterophorus*, respectively) and had different profiles for allelopathic activities. The allelopathic activity is rarely a result of a single constituent, generally being associated with a group of compounds so the potential difference between allelopathic essential oils of these weeds can be associated with the presence of monoterpenes, a group of chemical compounds that stand out for their allelopathic potential by influencing seed germination and the vigor of seedlings (Silva et al., 2009). This fact explains the different patterns of phytotoxic responses to the essential oils in the present study because, in both methods, the essential oil from *A. polystachya* produced more significant allelopathic effects than those of the essential oil from *P. hysterophorus*. This difference can probably be explained by the higher concentration of monoterpenes present in the essential oil from *A. polystachya* (34.2%) than in the oil from *P. hysterophorus* (20.1%).

CONCLUSIONS

The principal constituents found in the essential oils from *A. polystachya* were germacrene D (29.3%), *trans*-β-ocimene (13.6%) and β-caryophyllene (9.8%), whereas germacrene-D (35.9%), *trans*-β-ocimene (8.5%) and β-myrcene (7.6%) were found in the oil from *P. hysterophorus*. In both the methods used in evaluating the allelopathic activity of the essential oils, that from *A.*

polystachya presented a greater potential for reducing seed germination and seedling vigor of lettuce than the essential oil from *P. hysterophorus*. This difference can possibly be attributed to the higher content of monoterpenes in the essential oil from *A. polystachya*.

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ARTIGO 2 - EVALUATION OF THE CHEMICAL COMPOSITION AND ALLELOPATHIC POTENTIAL OF ESSENTIAL OILS FROM THREE SPECIES OF ASTERECEAE AGAINST SEED GERMINATION AND SEEDLING VIGOR OF LETTUCE

Artigo submetido ao Conselho Editorial do periódico African Journal of Agricultural Research e formatado conforme normas do referido periódico.

Evaluation of the chemical composition and allelopathic potential of essential oils from three species of Astereceae against seed germination and seedling vigor of lettuce

ABSTRACT

The allelopathic properties of the essential oils have been exploited because they are biodegradable natural compounds that can be used in combination or as prototypes for new bioherbicides**.** The objectives of this study were to chemically characterize the essential oils from *B. dracunculifolia*, *C. bonariesis* and *T. diversifolia* and to evaluate their allelopathic potential. The essential oils were extracted by hydrodistillation and their chemical compositions were determined by GC-MS. Allelopathic activities were determined by methods that evaluate the effects of volatile compounds and direct contact of those compounds on the seed germination and seedling vigor of lettuce. The principal constituents of the essential oil from *B. dracunculifolia* were limonene (30.9%), *trans*-nerolidol (22.4%) and β-pinene (14.5%); those in the oil from *C. bonariesis* were limonene (56.7%), *trans*-β-ocimene (26.3%) and *cis*-verbenol (4.4%); and those in the oil from *T. diversifolia* were β-pinene (38.3%), α-pinene (28.6%) and limonene (8.8%). Minor differences in the germination and vigor of lettuce seedlings were observed when they were exposed to the volatile essential oils from the leaves of the three species of the Asteraceae family. However,

upon direct contact with these oils, those of *C. bonariesis* presented the greatest allelopathic potential, which was attributed to its higher content of oxygenated monoterpenes.

Key words: volatile oils, allelopathy, *Baccharis dracunculifolia*, *Conyza bonariesis*, *Tithonia diversifolia***.**

INTRODUCTION

Essential oils are secondary metabolites produced exclusively by plant and are composed of complex mixtures of many compounds. The constituents may be grouped into phenylpropanoids and terpenoids, and, of the latter class, the monoterpene compounds are found in the highest concentration in essential oils (Silva et al., 2009).

According to De Almeida et al. (2010), terpenoid compounds are considered to be important bioactive compounds involved in the defense of plant organisms, which is a reason why essential oils stand out for their allelopathic properties. In addition to this natural property, these volatile compounds do not persist in the soil and contaminate the groundwater and present little or no toxicity to mammals (Singh et al., 2005), which makes these metabolites an alternative to new synthetic herbicides. Vokou et al. (2003) highlighted the importance of evaluating the allelopathic activity of each essential oil individually because terpenes present in the oils can act independently, synergistically or antagonistically, and this type of interaction between the constituents is not predictable.

Among the plants that produce essential oils, the Asteraceae family is the most numerous systematic group of the Angiosperms, and their chemical compositions and biological activities have been widely explored. Species are very varied in appearance, being composed mainly of small herbs or shrubs

comprising about 1100 genera and 25,000 species found mainly in the tropical mountain regions of South America (Verdi et al., 2005).

This study sought to evaluate the chemical composition of the essential oils extracted from fresh leaves of three species of the Asteraceae family -- *Baccharis dracunculifolia*, *Conyza bonariesis* and *Tithonia diversifolia* -- and compare their allelopathic potential using bioassays that assess the effect of direct contact with the oil and the effect of contact with the volatile components on seed germination and seedling vigor of lettuce (*Lactuca sativa* L.), in the laboratory.

MATERIAL AND METHODS

Collection of the plant material

The Asteraceae plants were collected in the morning, at approximately 8:00 a.m. within the same week, without rainfall, in the month of February 2012. Species identification was kindly performed by Doctor Mariana Esteves Mansanares, Department of Biology of UFLA and deposited in the ESAL Herbarium of the Federal University of Lavras (UFLA) and registered under the numbers 26946, 26947 and 26945, respectively. The young leaves (rib and limb) of adult plants of the *Baccharis dracunculifolia*, *Conyza bonariesis* and *Tithonia diversifolia* species were collected on the UFLA campus in Lavras, MG, Brazil (21° 14‘S, longitude 45° 00 ‘W Gr. and 918 m altitude).

Extraction of the Essential Oils

The essential oils from the fresh leaves of the test species were extracted by steam distillation during a period of two hours using a modified Clevenger apparatus adapted to a round bottom flask with a capacity of four liters, in accordance with the method described by the Farmacopeia Brasileira (2000).

The hydrolate was centrifuged at 965,36 g for five minutes, and the essential oils were separated and stored in amber glass bottles in the refrigerator.

Analysis of moisture and yield of the extracted oils

Five grams of fresh chopped leaves from these plants and 70 ml of cyclohexane were added to a 250 mL round bottom flask attached to a Dean Stark collector with a condenser; the mixture was heated at 81 ± 1 °C for 2 hours and the volume of water that distilled was measured. The yield of oil extracted from the plant was calculated on a moisture-free basis (Pimentel et al., 2006).

Identification of the essential oil constituents

The GC-MS analyses was performed using a Perkin Elmer Autosystem XL gas chromatograph equipped with a fused silica DB-1 column (30 m x 0.25 mm ID, film thickness 0.25 m; J & W Scientific Inc.) coupled to a Perkin Elmer Turbomass mass spectrometer (software version 4.1). The oven temperature was programmed from 45 to 175˚C at 3˚C/min and, subsequently, at 15˚C/min to 300˚C, where it was held for 10 min. The temperature of the transfer line was 280˚C; the temperature of the ionization chamber was 220˚C; the flow rate of the helium carrier gas was 30 cm/s; and the split ratio was 1:40.

The identity of the compounds was determined by comparison of their retention indices and mass spectra with those of commercial standards and reference compounds present in oils existing in the laboratory and by comparing the spectra with a library of mass spectra developed in the laboratory of the Centre for Plant Biotechnology of the Faculty of Sciences, University of Lisbon (Mendes et al., 2011).

Quantification of the constituents of the essential oils

The essential oils were analyzed by gas-liquid chromatography on a Perkin Elmer model 8700 gas chromatograph equipped with two flame ionization detectors (FID), a system for data processing and an automatic injector. Two columns of different polarity were installed with the following characteristics: DB-1 fused silica methylsilicone immobilized phase (30 m x 0.25 mm ID, film thickness 0.25 m; J & W Scientific Inc.); DB-17HT phenylmethylsilicone immobilized phase (30 m x 0.25 mm id). The oven temperature was programmed from 45 °C to 175 °C at 3 °C/min and, subsequently, at 15 °C/min to 300 °C, where the temperature was maintained for 10 min. The temperatures of the injector and detector were 290 °C and 280 °C, respectively. The flow rate of the hydrogen carrier gas was 30 cm/s, and the split ratio was 1:50. The percentage composition of the oils was determined by integration of peak areas without using correction factors. The values given represent the average of two injections.

Allelopathic activity of essential oils

Two bioassays were conducted to evaluate the allelopathic potential of the essential oils. The first test evaluated the effect of the volatile components, and the second assessed the effect of the direct contact of the seeds with the oils on seed germination and seedling vigor of lettuce (cultivar: Regina SF 3500). A 1% stock solution of each essential oil was prepared by emulsifying the oil with 0.5 ml Tween 80 in a 1:1 (v/v) ratio and dissolving it in a distilled water emulsion. The 0.1 and 0.01% v/v concentrations were prepared by dilution of the stock solution. A 1.0% v/v solution of Tween 80 in water was used as the control (Silva et al., 2009). The solutions were added at the start of the bioassays, and then only distilled water was added, if necessary (Souza Filho et al., 2009). In both bioassays, seeds were packed in an acrylic box (dimensions 11x11x4 cm)

with two sheets of sterilized blotting paper as the substrate. Fifty seeds were distributed in each box, totaling 200 seeds per treatment. The seeds were kept in a growth chamber at 20 ± 1 °C with a photoperiod of 12 hours. In the direct contact method, the blotter paper was soaked with solutions of different concentrations of essential oils in quantities equivalent to 2.5 times its dry weight (BRASIL, 2009). To evaluate the effect of the volatile compounds, distilled water was added to the paper substrate in quantities equivalent to 2.5 times the dry weight (BRASIL, 2009), and 3 mL of the solutions of the essential oils were placed on two sheets of filter paper affixed to the gerbox cover, thereby avoiding direct contact of the solution with the seeds (Souza Filho et al., 2009). Germination was monitored for seven days after the implementation of the test, with daily counts of lettuce seedlings, and the results were expressed as the percentage of normal seedlings (BRASIL, 2009).

Seed vigor was determined by using the following variables: First Count Germination, which evaluated the germination of normal seedlings on the fourth day after sowing (BRASIL, 2009); Germination Speed Index (GSI), calculated according to the index proposed by Maguire (1962); Medium Root Length and Length of Seedling Shoots on the Seventh Day, conducted with the aid of a millimeter ruler, and the average results were expressed in centimeters (BRASIL, 2009); Determination of Dry Matter of Seedlings, performed in an oven at 60 °C in Kraft paper bags to constant weights. Finally, the seedlings were weighed, and the averages per repetition were determined (Krzyzanowski et al., 1999).

Statistical Analysis

The experimental design for both methods was completely randomized in a 4x3 factorial scheme (concentrations x essential oils), with four replications. The essential oils were compared to one another, because the species are of the

same botanical family, as opposed to comparing the different methods, which were not compared because they are known to involve different chemical mechanisms. Significant factors from the F test ($p < 0.05$) were submitted to the Scott-Knott mean test (5%) for the determination of the models. The data were analyzed using the Analysis of Variance for Balanced Data statistical program - Sisvar, according to Ferreira (2011).

RESULTS AND DISCUSSION

The yields of essential oil obtained from the leaves of *Baccharis dracunculifolia*, *Conyza bonariesis* and *Tithonia diversifolia* were 0.8, 0.4 and 0.2%, respectively. The chemical constituents of these essential oils, their percentages and retention indices are presented in Table 1.

Table 1. Percentages and retention indices (RI) of the components identified in the essential oils of *Baccharis dracunculifolia*, *Conyza bonariesis* and *Tithonia diversifolia*.

Components	**RI**	***B. dracunculifolia***	***C. bonariesis***	***T. diversifolia***
Tricyclene	921	-	-	t
α-Tujene	924	0.9	t	t
α-Pinene	930	7.1	0.4	28.6
Canfene	938	t	0.2	1.0
Sabinene	958	t	0.4	2.1
β-Pinene	963	14.5	0.2	38.3
1.8-Dehydrocineol	973	-	t	-
β-Mircene	975	3.2	1.6	-
δ-3-Carene	1000	0.3	-	-
o-Cimene	1000	t	-	-
α-Terpinene	1002	-	0.1	-
p-Cimene	1003	0.1	t	t
1.8-Cineol	1005	-	0.4	-

β-Phelandrene	1005	t	0.4	0.4
Limonene	1009	30.9	56.7	8.8
cis-β-Ocimene	1017	t	0.3	7.0
Acetofenone	1017	t	-	-
trans-β-Ocimene	1027	0.5	26.3	t
γ-Terpinene	1035	0.4	0.2	t
trans-Sabinene hydrate	1037	-	t	t
Terpinolene	1064	0.4	t	t
Nonanal	1073	-	-	t
Linalol	1074	0.3	t	-
Isopentyl isovalerate	1084	-	-	t
trans-*p*-2-Menten-1-ol	1099	-	t	-
cis-Miroxide		-	0.2	-
cis-Verbenol	1110	-	4.4	-
trans-Miroxide		-	t	-
allo-Ocimene	1110	-	-	t
trans-Limonene oxide	1112	-	0.2	-
trans-Verbenol	1114	-	-	t
Pinocarvone	1121	-	-	t
Borneol	1134	-	0.2	0.2
Criptone	1143	-	-	t
Terpinen-4-ol	1148	0.1	0.2	t
Mirtenal	1153	-	-	t
α-Terpineol	1159	t	0.2	-
Mirtenol	1168	-	-	t
Carvone	1210	-	t	-
Bornyl acetate	1265	-	t	-
Thymol	1275	-	-	t
Geranyl formate	1285	-	-	t
Carvacrol	1286	-	t	t
δ-Elemene	1332	t	t	t
Silfinene		-	t	-
7-*epi*-Silfipertol-5-ene		-	0.1	-
Modheo-2-ene		-	t	-
α-Copaene	1375	-	-	t

Daucene	1379	-	t	-
Isodaucene		-	t	-
β-Elemeno	1388	0.1	t	t
1.4-Dimethyl azulene		-	-	t
β-Caryophyllene	1414	1.1	0.3	3.7
cis-α-Bergamotene		-	0.5	-
Aromandrene	1428	0.3	-	-
α-Humulene	1447	t	0.2	t
trans-β-Farnesene	1455	-	0.1	-
allo-Aromadendrene	1456	-	-	t
trans-β-Ionone	1456	-	-	t
Cabreuva A oxide	1455	0.3	-	-
Cabreuva B oxide	1463	0.7	-	-
Cabreuva C oxide		0.0	-	-
Cabreuva D oxide		0.3	-	-
γ-Muurolene	1469	0.3	0.2	-
Zierene	1473	-	-	t
Germacrene-D	1474	1.9	0.2	0.2
β-Selinene	1476	-	-	t
Bicyclogermacrene	1487	2.3	1.8	1.7
α-Muurolene	1494	0.4	-	-
β-Bisabolene	1500	-	-	t
γ-Cadinene	1500	1.4	0.1	-
δ-Cadinene	1505	1.3	0.2	0.1
Kessane	1517	-	-	0.1
Isocaryophylene	1528	-	-	t
Germacrene-B	1533	-	t	-
cis-3-Hexenyl benzoate	1533	-	-	t
trans-Nerolidol	1549	22.4	0.3	t
Espatulenol	1551	1.6	0.3	3.9
Germacreno-D-4-ol	1557	-	0.3	-
β-Caryophylene	1561	0.9	0.1	2.9
Carotol		-	t	-
Humulene II epoxide		-	-	t
Viridiflorol	1569	0.1	-	-

Ledol	1580	0.8	-	-
T-Cadinol	1616	-	0.1	0.8
epi-α-Cadinol	1616	v	-	-
α-Muurolol	1618	-	0.1	-
α-Cadinol	1626	0.4	0.4	-
Phytol acetate	2047	-	-	t
Diterpene NI		-	1.2	-
% Identification		**95.2**	**97.3**	**99.7**
Grouped components				
Monoterpene hydrocarbons		58.3	86.6	86.6
Oxygen-containing monoterpenes		0.3	5.6	0.2
Sesquiterpene hydrocarbons		9.1	3.4	5.8
Oxygen-containing sesquiterpenes		27.5	1.7	7.6

RI, retention index; t, trace (<0.05%); NI, not identified.

We identified 95.2, 97.3 and 99.7% of the chemical compounds in the essential oils from fresh leaves of *B. dracunculifolia*, *C. bonariesis* and *T. diversifolia*, respectively. Monoterpenes (58.6, 92.2 and 86.8%, respectively) were the most abundant in all the oils, while sesquiterpenoids were found in concentrations of 36.6, 5.1 and 13.4%, respectively.

The principal constituents found in the oil from *B. dracunculifolia* were limonene (30.9%), *trans*-nerolidol (22.4%) and β-pinene (14.5%). However, Massignani et al. (2009) identified nerolidol (23.58%), germacrene-D (21.54%) and bicyclogermacrene (19.24%) as the principal components of the essential oil from the leaves of *B. dracunculifolia* collected in the city of Franca, SP, Brazil. Some constituents were found in larger quantities in the oil from *C. bonariesis*: limonene (56.7%), *trans*-β-ocimene (26.3%) and *cis*-verbenol (4.4%). Barbosa et al. (2005) identified limonene (29.6%), manool (13.8%) and *trans*-α-bergamotene (10.3%) as major components of the leaf oil from *C. bonariensis* collected in Viçosa, MG, Brazil. The essential oil from *T. diversifolia* contained

β-pinene (38.3%), α-pinene (28.6%) and limonene (8.8%) as the principal constituents, while the main compounds found in essential oils extracted from the same species collected in Nigeria and studied by Moronkola et al. (2007) were α-pinene (32.9%), β-caryophyllene (20.8%) and germacrene-D (12.6%). The differences in chemical compositions of the essential oils of the same botanical species are possibly due to the fact that they were collected at different sites and, consequently, were submitted to different soil and climatic conditions (Gobbo-Neto and Lopes, 2007).

When the volatile allelopathic effects of the components of the essential oils from the three species of the Asteraceae family were evaluated (Table 2), it was found that an increase in the concentration did not affect the first count germinated seedlings or the final germination, which had mean values of 89 and 94.3% respectively. There was no statistically significant difference in the effects of these variables on the GSI of the oils, the percentage of dry matter or the measurements of the aerial parts of the plants. However, the phytotoxic effect of the concentrations of each oil showed to be dose-dependent. In relation to the root length of the seedlings, the effect of different concentrations of the essential oil from *C. bonariesis* did not differ from that of the control, with a mean length of 38.3 mm. However, when the interference of the essential oils from *B. dracunculifolia* and *T. diversifolia* on this variable was evaluated, it was found that the concentration of 1% caused a decrease in the length to 32.6 and 33.9 mm, respectively.

Table 2. Allelopathic volatile effects of components of the essential oils from fresh leaves of the *B. dracunculifolia*, *C. bonariesis* and *T. diversifolia* species on seed germination and seedling vigor of lettuce.

EO (%) v/v	1st cont. (%)	Germ. (%)	GSI	Dry M.(g)	P. aerial (mm)	P. root (mm)
B.dracunculifolia						
0	88Aa	94Aa	75.8Aa	0.0370Aa	17.6Aa	38.4Aa
0.01	90.5Aa	93.5Aa	69.5Aa	0.0360Aa	14.4Aa	36.8Aa
0.1	93Aa	97Aa	69.2Ab	0.0293Aa	14.9Ab	36.3Aa
1	88.5Aa	93.5Aa	56.0Ac	0.0290Ab	13.7Ab	32.6Bb
C. bonariesis						
0	88.5Aa	95Aa	75.2Aa	0.0370Aa	17.1Aa	38.3Aa
0.01	85.5Aa	91.5Aa	74.0Aa	0.0370Aa	17.1Aa	38.3Aa
0.1	90.5Aa	94Aa	73.3Ab	0.0365Aa	16.4Ab	38.0Aa
1	90Aa	95.5Aa	64.2Ac	0.0346Ab	15.0Ab	36.4Aa
T. diversifolia						
0	90.5Aa	94Aa	75.5Aa	0.0375Aa	17.0Aa	39.9Aa
0.01	91Aa	93.5Aa	74.1Aa	0.0378Aa	17.1Aa	38.7Aa
0.1	93Aa	95Aa	64.6Ab	0.0345Aa	14.2Ab	36.7Ab
1	89.5Aa	93.5Aa	60.4Ac	0.0340Ab	12.9Ab	33.9Bc

EO, concentration of solutions of essential oils; 1st cont., Percentage of seeds germinated on the fourth day of the experiment; Germ., Percentage of seeds germinated on the seventh day of the experiment; GSI, germination speed index; Dry M., average seedling dry mass; P. aerial, average length of the aerial parts of the plants; P. root; average length of seedling roots. For each variable, means followed by the same letter, uppercase for comparison between the essential oils analyzed and lowercase for comparison between concentrations in each essential oil, do not differ significantly at 5% probability by the Scott-Knott test.

According to the data described in Table 3, the direct contact with different concentrations of the essential oil from *B. dracunculifolia* did not affect the first germination count or the final number of seedlings germinated. However, the other variables were affected by concentrations of 0.1 and 1% of oil, which caused significant reductions in the GSI, percentage of dry matter and aerial and root lengths of the seedlings. This behavior can be explained by the delay in the germination of the seeds, especially in the first two days of the

experiment, caused by higher concentrations of the oil from *B. dracunculifolia*. This fact justifies the constant value of the variables for the first count and total germination and the dose-dependent responses of other variables. Unlike the oil from *B. dracunculifolia*, the oils from *C. bonariesis* and *T. diversifolia* presented dose-dependent allelopathic responses for all the variables. This fact shows that these oils reduced seed speed germination and seedling vigor throughout the experimental period.

Table 3. Effects of direct contact of the essential oils from fresh leaves of the *B. dracunculifolia*, *C. bonariesis* and *T. diversifolia* species on seed germination and seedling vigor of lettuce.

EO (%) v/v	1st cont. (%)	Germ. (%)	GSI	Dry M.(g)	P. aerial (mm)	P. root (mm)
B.dracunculifolia						
0	91Aa	96Aa	76.4Aa	0.0378Aa	15.9Aa	42.6Aa
0.01	91.5Aa	94.5Aa	72.9Aa	0.0375Aa	15.8Aa	41.3Aa
0.1	93Aa	96Aa	44.8Ab	0.0300Ab	10.5Ab	31.5Ab
1	87.5Aa	91.5Aa	42.9Ac	0.0295Ab	10.2Ab	29.6Bc
C. bonariesis						
0	87Aa	92.5Aa	76.5Aa	0.0373Aa	15.2Aa	42.6Aa
0.01	82Ba	89Ba	49.2Cb	0.0338Bb	11.0Bb	32.1Cb
0.1	76.5Cb	90.5Ba	39.7Bc	0.0303Ac	9.6Ac	26.2Bc
1	69.5Bc	74Bb	26.4Bd	0.0218Bd	4.2Bd	13.9Cd
T. diversifolia						
0	94Aa	95.5Aa	76.4Aa	0.0370Aa	16.4Aa	41.3Aa
0.01	89Aa	95.5Aa	63.2Bb	0.0360Aa	12.1Bb	38.4Bb
0.1	83.5Bb	89.5Bb	47.9Ac	0.0293Ab	10.6Ac	32.1Ac
1	85Ab	90.5Ab	44.0Ad	0.0290Ab	10.4Ac	32.1Ac

EO, concentration of solutions of essential oils; 1st cont., Percentage of seeds germinated on the fourth day of the experiment; Germ., Percentage of seeds germinated on the seventh day of the experiment; GSI, germination speed index; Dry M., average seedling dry mass; P. aerial, average length of the aerial parts of the plants; P. root; average length of seedling roots. For each variable, means followed by the same letter, uppercase for comparison between the essential oils analyzed and lowercase for comparison between concentrations in each essential oil, do not differ significantly at 5% probability by the Scott-Knott test.

A dose-dependent phytotoxicity was observed upon direct contact of the essential oils from Asteraceae plants with the seeds or seedlings, with more pronounced allelopathic effects caused by the use of a 1% concentration of the essential oils. The oil from *C. bonariesis* had a higher allelopathic efficiency than the essential oils from *B. dracunculifolia* and *T.diversifolia* with regard to all the variables. It caused significant reductions in the first count from 87 to 69.5%, the total germination from 92.5 to 74%, the GSI from 76.5 to 26.4, the dry mass from 0.0373 to 0.0218, the length of the aerial parts from 15.2 to 4.2 mm and the length of the roots from 42.6 to 13.9 mm.

The greater allelopathic potential presented by the oil from *C. bonariesis* may be associated with the higher content of oxygenated monoterpenes, because the oil contained 5.6% of these compounds, whereas the oils from *B. dracunculifolia* and *T. diversifolia* contained 0.3 and 0.2%, respectively. These results corroborate those found by Vokou et al. (2003), who studied the allelopathic activity of 47 hydrocarbons and oxygenated monoterpenes alone and in pairs on seed germination and seedling vigor of lettuce. The authors found that oxygenated monoterpenes have the greatest phytotoxicity. The allelopathic efficiency of the essential oil from *Artemisia scopariada*, another species of the Asteraceae family, was studied by Kaur et al. (2010). The authors evaluated the influence of the essential oil by direct contact bioassay on the emergence and growth of the roots and shoots of five weed species: *Achyranthes aspera, Cassia occidentalis, Parthenium hysterophorus, Echinochloa crus-galli* and *Ageratum conyzoides*. The authors found a high content of monoterpenes (55.18%), of which those with oxygen totaled 4.99%, similar to the essential oil from *C. bonariesis* in the present study which contains 5.1% oxygenated monoterpenes. The oil from *A. scopariada* presented bioherbicidal properties in concentrations of 2%, 4% and 6% v/v, causing changes to the photosynthetic, respiratory metabolism, affecting the growth and physiological processes of

some weed species, like the oil from *C. bonariesis* that caused reduction in seed germination and the seedling vigor of lettuce in a concentration of 1%.

Given the above results, the need to individually analyze the chemical composition and allelopathic potential of essential oils from different species of the same botanical family was confirmed, because essential oils have a unique combination of chemical compounds that directly influence their phytotoxicity. Additionally, the need to examine the allelopathic activity by different bioassays should be emphasized, because the application of the volatile components of the oils caused similar allelopathic effects, unlike the effect of direct contact, which generated different profiles for each individual essential oil that were proportional to the content of oxygenated monoterpenes.

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ARTIGO 3 - CARACTERIZAÇÃO QUÍMICA E ATIVIDADE ALELOPÁTICA DOS ÓLEOS ESSENCIAIS DE FOLHAS E RIZOMAS DE *HEDYCHIUM CORINARIUM*.

Artigo submetido ao Conselho Editorial do periódico Planta Daninha e formatado conforme normas do referido periódico.

Caracterização química e atividade alelopática de óleos essenciais de folhas e rizomas de *Hedychium coronarium*.

RESUMO

Os óleos essenciais apresentam potencial para serem empregados como bioerbicidas possuindo vantagens em função da sua biodegradabilidade, elevada diversidade estrutural e reduzida resistência natural às plantas daninhas. A espécie *Hedychium coronarium* é uma planta invasora exótica adaptada a diferentes regiões do Brasil e possui óleos essenciais em diferentes órgãos vegetativos. Os óleos essenciais das folhas e dos rizomas de *H. coronarium* foram extraídos por hidrodestilação e suas composições químicas foram determinadas por Cromatografia Gás-Líquido/Espectrometria de Massas. As atividades alelopáticas foram determinadas pelas metodologias que avaliam os efeitos voláteis e o contato direto dos mesmos sobre a germinação de sementes e vigor de plântulas de alface. Os constituintes majoritários do óleo das folhas foram o β-pineno (46,9%), o α-pineno (19,2%) e o β-cariofileno (13,2%) e o dos rizomas, o β-pineno (41,5%), o 1,8-cineol (23,6%) e o α-pineno (13,1%). O óleo essencial extraído dos rizomas apresentou maior potencial alelopático que o óleo essencial das folhas da mesma espécie, o que possivelmente deve-se aos seus maiores conteúdos monoterpênicos, principalmente do 1,8-cineol.

Palavras-chave: óleos voláteis, rizomas, folhas, *Hedychium coronarium*, alelopatia.

Chemical characterization and allelopathic activity of essential oils from leaves and rhizomes of the invasive exotic plant *Hedychium coronarium*.

ABSTRACT

Essential oils have potential to be used as bioherbicides possessing several advantages due to their biodegradability, high structural diversity and reduced natural resistance to weeds. The species *Hedychium coronarium* is an invasive exotic plant adapted to different Brazil regions and possesses essential oils in different plant parts. The essential oils of leaves and rhizomes of *H. coronarium* were extracted by hydrodistillation and their chemical composition were determined by Chromatography Gas-Liquid/Mass spectrometry. The allelopathic activities were determined by methodologies which assess the volatile effects and direct contact on the germination of seeds and seedling vigor of lettuce. The major constituents of the oil extracted from leaves were β-pinene (46.9%), α-pinene (19.2%) and β-caryophyllene (13.2%) and from rhizomes, β-pinene (41, 5%), 1,8-cineole (23.6%) and α-pinene (13,1%). The oil essential extracted from rhizomes presented greater allelopathic potential compared to oils extracted from leaves on the same species, possibly may be due to their largest monoterpenic contents, mainly 1.8-cineol.

Keywords: volatile oils, rhizomes, leaves, *Hedychium coronarium*, allelopathy.

INTRODUÇÃO

O controle de plantas daninhas em áreas agricultáveis tem sido realizado principalmente com o uso de herbicidas sintéticos, que embora eficientes do ponto de vista funcional, provocam prejuízos ambientais, além de aumentar a resistência das espécies (Souza Filho et al., 2006).

De acordo com Duke et al. (2000) o controle químico de plantas daninhas precisa ser dinâmico e inovador e o uso de produtos naturais é uma alternativa viável econômica e ecologicamente. Dentre essas substâncias de origem natural os óleos essenciais podem ser empregados como bioerbicidas, que embora estruturalmente mais complexos que os herbicidas sintéticos, são substâncias provenientes exclusivamente do metabolismo secundário de espécies vegetais, além de serem biodegradáveis, possuírem uma elevada diversidade estrutural e consequentemente, induzirem reduzida ou nenhuma resistência natural por parte das plantas daninhas.

Os óleos essenciais são misturas complexas de compostos voláteis, que podem ser produzidos em diversos órgãos dos vegetais, tais como flores, folhas, sementes, rizomas, cascas, entre outras. São constituídas por fenilpropanoides e terpenoides. Entre os terpenoides, Silva et al. (2009) destacam principalmente os monoterpenos, que por serem a classe de substâncias presentes em maior quantidade nos óleos essenciais, possuem diversas funções nos vegetais como fitoalexinas, hormônios, repelentes de inseto e micro-organismos, agentes de atração polínica e de defesa contra herbivoria, além de aleloquímicos.

Entre as espécies invasoras produtoras de óleos essenciais destaca-se o *Hedychium coronarium*, que é uma monocotiledônea macrófita aquática da família Zingiberaceae, originária da Ásia Tropical e adaptada em toda América. Por ser comumente encontrada em regiões de brejo é conhecida popularmente no Brasil por “lírio-do-brejo”, “gengibre-branco” ou “lírio-borboleta” (Martins et al, 2010). Devido ao rápido crescimento e dispersão, é considerada planta

daninha exótica, encontrada em diversas regiões do Brasil, principalmente no Cerrado, Florestas Costeiras da Serra do Mar, Matas Ciliares de Araucária, Florestas costeiras e do interior da Bahia, Caatinga, Mata Atlântica do Alto Paraná, substituindo a vegetação original e causando prejuízos (Zenni and Ziller, 2011).

Espécies invasoras são conhecidas por seu potencial alelopático, por promovem interações bioquímicas deletérias entre as plantas superiores. Pesquisas de Barbosa et al. (2007) indicam que diversas substâncias naturais são conhecidas como aleloquímicas, incluindo muitos compostos presentes nos óleos essenciais, que podem inibir a germinação das sementes e/ou afetar o vigor das plantas. Esses efeitos negativos sobre as espécies nativas tornam essas plantas daninhas um real problema nos locais onde proliferam (Santos et al., 2005).

No presente trabalho foi objetivo caracterizar quimicamente e avaliar o potencial alelopático dos óleos essenciais de folhas e rizomas frescos de *H. coronarium*, pelas metodologias que avaliam os efeitos voláteis e o contato direto dos mesmos sobre a germinação de sementes e vigor de plântulas de alface.

MATERIAL E MÉTODOS

Coleta do Material Vegetal

A espécie *Hedychium coronarium* foi coletada no câmpus da Universidade Federal de Lavras (UFLA), no município de Lavras/MG, por volta de 8:00 horas da manhã em 25 de fevereiro de 2012. A cidade de Lavras localiza-se no sul do estado de Minas Gerais (Brasil), 21º14’ S, longitude 45º00’ W Gr. e 918 m de altitude. Foram colhidas folhas jovens (nervura e limbo) e rizomas de plantas adultas, no estágio de floração plena. A identificação da espécie foi realizada pela Doutora Mariana Esteves Mansanares, do

Departamento de Biologia da UFLA e a excicata foi depositada no Herbário ESAL na UFLA, sob o registro de numeração 26942.

Extração do óleo essencial

Os óleos essenciais das folhas e rizomas da espécie foram extraídos por hidrodestilação, conforme metodologia descrita pela Farmacopeia Brasileira (2000), utilizando um aparelho de Clevenger modificado adaptado a um balão de fundo redondo com capacidade de 4 litros, por um período de 2 horas, obtendo-se o hidrolato. Esse foi centrifugado a 965,36 g por 5 minutos, em seguida os óleos essenciais foram separados com pipeta de Pasteur e acondicionados em frascos de vidro âmbar e armazenados a 4°C.

Determinação da umidade e rendimento das extrações

Em um balão de fundo redondo com capacidade de 250 mL, acoplado a um coletor de vidro tipo Dean Stark, foram colocados 5 g de folhas ou rizomas frescos picados em 70 mL de cicloexano. O balão foi colocado em manta aquecedora por 2 horas, à temperatura de 81 ± 1°C, medindo-se o volume de água. Após determinação do teor de umidade, o rendimento dos óleos essenciais foram expressos em base vegetal livre de umidade (BLU) (Pimentel et al., 2006).

Identificação dos constituintes dos óleos essenciais

Nas análises de Cromatografia Gás-Líquido/Espectrometria de Massas (CGL/EM) foi utilizado um cromatógrafo Perkin Elmer Autosystem XL, equipado com uma coluna de sílica fundida DB-1(30m x 0,25mm d.i., espessura de filme 0,25µm; J & W Scientific Inc.) acoplado a um espectrômetro de massas Perkin Elmer Turbomass (versão do software 4.1). A temperatura do forno foi programada de 45 a 175˚C, aumentando 3˚C/min, e subsequentemente 15˚C/min até 300˚C. Atingidos 300˚C, a temperatura foi mantida constante durante 10min; temperatura da linha de transferência, 280˚C; temperatura da

câmara de ionização, 220˚C; gás de arraste, hélio, ajustado para uma velocidade linear de 30 cm/s e relação de repartição de fluxo, 1:40.

A identidade dos compostos foi determinada por comparação dos seus índices de retenção, em relação aos dos n-alcanos C_9-C_{21} e espectros de massas, com os padrões comerciais e compostos de referência presentes em óleos existentes no laboratório e por comparação com uma biblioteca de espectros de massas desenvolvida no Laboratório do Centro de Biotecnologia Vegetal da Faculdade de Ciências da Universidade de Lisboa (Mendes et al., 2011).

Quantificação dos constituintes dos óleos essenciais

Os óleos essenciais foram analisados por Cromatografia Gás-Líquido (CGL), num cromatógrafo Perkin Elmer 8700 equipado com dois detectores de ionização de chama (DIC), um sistema de tratamento de dados e um injetor, no qual foram instaladas duas colunas de polaridade diferente com as seguintes características: DB-1 de sílica fundida, de fase imobilizada em metilsilicone (30m x 0,25mm d.i., espessura de filme 0,25µm; J & W Scientific Inc.); DB-17HT de fase imobilizada em fenilmetilsilicone (30m x 0,25mm d.i.). A temperatura do forno foi programada de 45°C a 175°C, aumentando 3°C/min, e subsequentemente 15°C/min até 300°C. Atingidos 300°C a temperatura foi mantida constante durante 10 min. A temperatura do injetor e dos detectores foram de 290°C e 280°C, respectivamente. Utilizou-se hidrogênio como gás de arraste, ajustado para uma velocidade linear de 30 cm/s. e a relação de repartição de fluxo foi de 1:50.

A composição percentual dos óleos foi determinada pela integração das áreas dos picos sem utilização de fatores de correção. Os valores apresentados correspondem ao valor médio de duas injeções.

Atividade alelopática dos óleos essenciais

O potencial alelopático dos óleos essenciais foi avaliado por dois bioensaios, o primeiro avaliando o efeito volátil e, o segundo, o contato direto

desses sobre a germinação das sementes de alface (cultivar: Regina SF 3.500). As soluções foram preparadas com 0,5 mL de cada óleo essencial emulsionado com Tween 80, na proporção 1:1(v/v) e dissolvido em água destilada, obtendo-se a solução estoque na concentração de 1%. As demais concentrações (0,1 e 0,01% em v/v) foram preparadas por diluição. Como controles foram utilizados uma solução de Tween 80 a 1,0% v/v e água (Silva et al., 2009). As soluções testadas foram adicionadas apenas no início dos bioensaios, e posteriormente, quando necessário, apenas água destilada foi acrescentada (Souza Filho et al., 2009). Em ambos os bioensaios as sementes foram acondicionadas em caixa acrílica tipo gerbox (dimensões 11x11x4 cm), tendo como substrato duas folhas de papel mata-borrão esterilizadas. Em cada gerbox, foram distribuídas 50 sementes, totalizando 200 sementes por tratamento e esses foram mantidos em câmara de crescimento (BOD) à temperatura de 20 ± 1°C e fotoperíodo de 12 horas. Na metodologia do contato direto, o papel mata-borrão foi embebido, com quantidades equivalentes a 2,5 vezes o seu peso seco, com soluções dos óleos essenciais em diferentes concentrações (Brasil, 2009). Para avaliação do efeito volátil, água destilada foi adicionada ao substrato de papel em quantidades equivalentes a 2,5 vezes o seu peso seco (Brasil, 2009) e 3 mL das soluções foram colocados em duas folhas de papel de filtro afixadas na tampa do gerbox, evitando contato direto da solução testada com as sementes (Souza Filho et al., 2009).

A germinação foi monitorada por sete dias, contados a partir da implantação do ensaio, com contagens diárias das plântulas de alface, sendo os resultados expressos em porcentagem de plântulas normais (Brasil, 2009).

O vigor das sementes foi determinado pelas seguintes variáveis: primeira contagem de germinação, avaliação da germinação de plântulas normais no quarto dia após a semeadura (Brasil, 2009); Índice de Velocidade de Germinação (IVG), calculado segundo Maguire (1962); comprimentos médios

de raiz e de parte aérea das plântulas ao sétimo dia, realizado com auxilio de régua milimetrada sendo os resultados médios expressos em centímetros (Brasil, 2009); determinação da massa seca de plântulas realizada em estufa a 60°C, em embalagens de papel Kraft, até obtenção de pesos constantes. Após esse período, efetuou-se a pesagem e determinação das médias por repetição, sendo os resultados expressos em gramas (Krzyzanowski et al., 1999).

Análise Estatística

O delineamento experimental utilizado para ambas as metodologias foi inteiramente casualizado em esquema fatorial 4x2 (4 concentrações x 2 óleos essenciais), com quatro repetições. Os óleos essenciais foram comparados entre si, por se tratarem de óleos extraídos de diferentes partes de uma mesma espécie botânica, ao contrário das diferentes metodologias, que não foram comparados por se tratarem de mecanismos químicos sabidamente diferentes. Os fatores significativos pelo teste de F ($p<0,05$) foram submetidos ao teste de média (Scott-Knott 5%) para determinação dos modelos. Os dados foram analisados pelo programa estatístico Sistema de Análise de Variância para Dados Balanceados – Sisvar, segundo Ferreira (2011). Os resultados foram plotados em gráficos de barras com os valores das variáveis em relação às concentrações analisadas empregando o software GraphPad Prism versão 5.0.

RESULTADOS E DISCUSSÃO

As extrações dos óleos essenciais de diferentes partes de *H. coronarium*, apresentaram rendimentos de 0,30% para as folhas e 1,02% para os óleos dos rizomas. Foram identificados 99,3% dos constituintes químicos do óleo essencial das folhas e 99,2% dos rizomas, sendo esses apresentados na Tabela 1.

Tabela 1. Componentes dos óleos essenciais das folhas e rizomas de *H. coronarium*, porcentagens e índices de retenção.

Componentes	**IR**	**% Constituintes**	
		Folhas	**Rizomas**
Tricicleno	921		v
α-Tujeno	924	0,1	v
α-Pineno	930	19,2	13,1
Canfeno	938	0,1	1,2
Sabineno	958	4,3	4,1
β-Pineno	963	46,9	41,5
β-Mirceno	975	1,1	1,2
α-Felandreno	995	0,1	1,8
δ-3-Careno	1000		1,8
α-Terpineno	1002	0,2	0,5
p-Cimeno	1003	0,1	0,3
1,8-Cineol	1005	7,8	23,6
Limoneno	1009	2,8	3,5
cis-β-Ocimeno	1017	v	
trans-β-Ocimeno	1027	0,2	v
γ-Terpineno	1035	0,6	0,9
Hidrato de *trans*-Sabineno	1037	v	v
Terpinoleno	1064	0,1	0,3
Linalol	1074		v
Borneol	1134	0,1	0,6
Terpinen-4-ol	1148	0,4	1,3
α-Terpineol	1159	0,2	1,7
Acetato de bornila	1265	v	0,6
δ-Elemeno	1332	0,3	
Acetato de α-Terpenila	1334		0,3
β-Elemeno	1388	0,1	
β-Cariofileno	1414	13,2	0,5
Guaia-6,9-dieno	1447	v	
α-Humuleno	1447	0,5	
trans-β-Farneseno	1455	v	0,1

γ-Muuroleno	1469	v	
γ-Cadineno	1500		v
(*trans,trans*)-α-Farneseno	1500	v	0,3
δ-Cadineno	1500	v	0,1
Isocariofileno	1528	v	
Germacreno-B	1533	0,1	
trans-Nerolidol	1549	0,1	
Óxido de β-cariofileno	1561	0,6	
Viridiflorol	1569	v	
α-Cadinol	1626		v
Acetato de Fitol	2047	v	

IR, índice de retenção; v, vestigial (<0,05%).

Os constituintes majoritários no óleo das folhas foram os monoterpenos β-pineno (46,9%), o α-pineno (19,2%) e o sesquiterpeno β-cariofileno (13,2%) e nos rizomas, os monoterpenos β-pineno (41,5%), o 1,8-cineol (23,6%) e o α-pineno (13,1%). Santos et al. (2010) estudaram os óleos essenciais de folhas e rizomas frescos de *H. coronarium* coletados na Mata Atlântica do Sudeste do Brasil e obtiveram um rendimento de 0,68% e 0,20%, respectivamente. Como componentes principais do óleo das folhas encontraram o β-cariofileno (43,0%), o óxido de cariofileno (12,1%) e o β-pineno (11,6%,), enquanto no dos rizomas, o 1,8-cineol (34,8%), o β-pineno (16,7%) e o α-terpineol (13,1%). Ao avaliar a composição química do óleo essencial dos rizomas dessa mesma espécie botânica, coletados na Índia, Prakash et al. (2010) identificaram 98,7% dos constituintes químicos, e como majoritários encontraram o linalol (29,3%), o limoneno (20,3%), o trans-meta-menta-2,8-dieno (12,9 %) e o γ-terpineno (8,9%). Essa variabilidade do rendimento e da composição química observada nos óleos essenciais de *H. coronarium* possivelmente está relacionada com localidade e época de coleta da espécie vegetal, ciclo vegetativo e fatores edafoclimáticos (Gobbo-Neto e Lopes, 2007).

Ao analisar os efeitos voláteis dos óleos essenciais das diferentes partes de *H.coronarium* observou-se que o aumento das concentrações não alterou a primeira contagem de plântulas germinadas e germinação total, apresentando porcentagens médias, respectivamente, de 91,5 e 95%, para o óleo das folhas e 91 e 94%, para o óleo dos rizomas. Ao avaliar as variáveis IVG e comprimento das partes radiculares das plântulas de alface, os óleos essenciais não apresentaram diferenças significativas entre si em todas as concentrações analisadas e em cada óleo, somente a maior concentração diferiu-se do controle. O tratamento com 1% (v/v) do óleo das folhas reduziu o IVG de 75,9 para 74,9, e o comprimento radicular das plântulas de 38,3 para 37,3 mm, enquanto o óleo dos rizomas, de 75,9 para 65,8 o IVG, de 38,5 para 35,7 mm o tamanho das radículas (Figura 1).

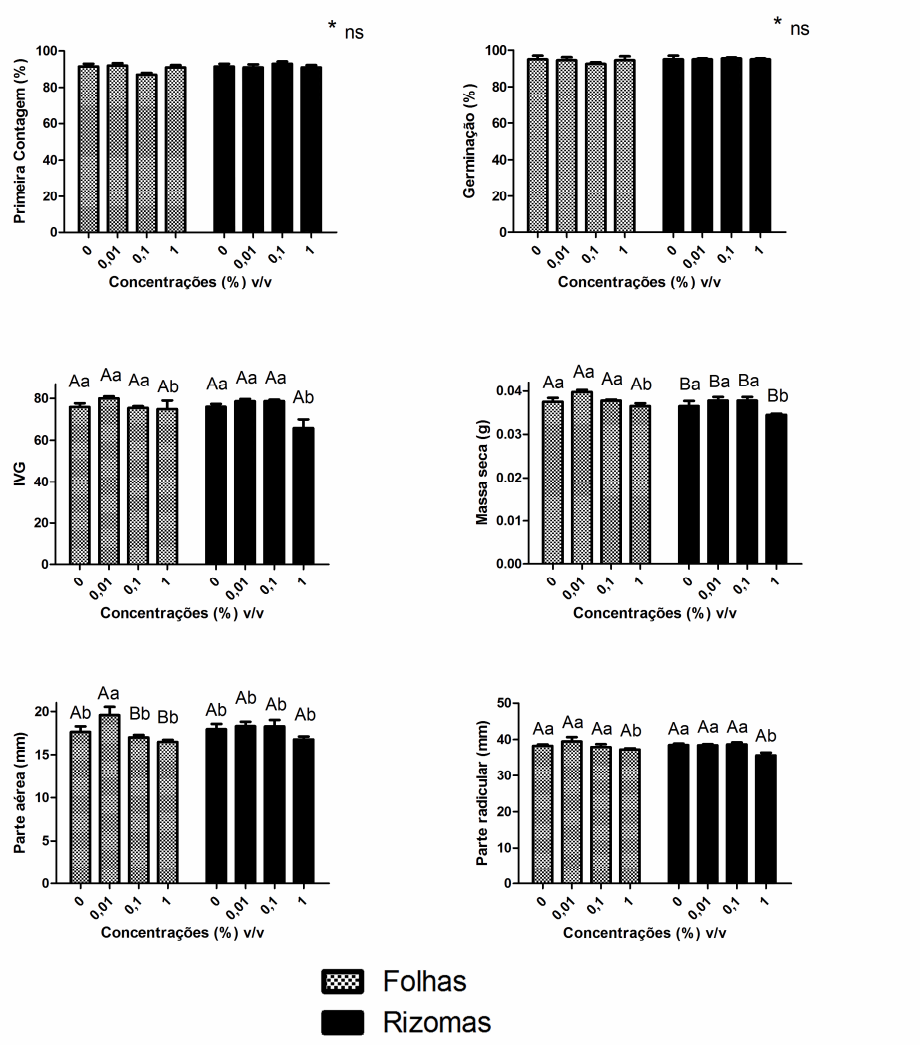

Figura 1. Valores médios das variáveis primeira contagem de germinação, germinação total, IVG, massa de plântulas secas, comprimentos de parte aérea e de raiz em função das concentrações dos óleos essenciais extraídos de folhas e rizomas de *H. coronarium*, avaliadas pela metodologia do efeito volátil.
Médias seguidas da mesma letra, maiúsculas para comparação entre os óleos essenciais analisados e minúsculas para comparação entre as concentrações dentro de cada óleo essencial, não diferem significativamente entre si a 5% de probabilidade, pelo teste de Scott-Knott.

Em relação à matéria seca de plântulas, o óleo dos rizomas provocou reduções estatisticamente superiores ao óleo das folhas, entretanto ao avaliar cada óleo separadamente, verificou-se que somente a maior concentração de cada óleo diferiu-se dos controles, com reduções da matéria seca de 0,0375 para 0,0365 g, para o óleo das folhas, e, de 0,0365 para 0,0345 g para o óleo dos rizomas. A concentração de 0,01% de ambos os óleos provocou estímulo do crescimento das partes aéreas das plântulas de alface, com comprimentos médios de 19,6 mm para o óleo das folhas e 18,3 mm para os rizomas, ao contrário das outras concentrações, que não diferiram estatisticamente do controle, com comprimentos médios de 17,6 mm e 18,0 mm, respectivamente. De acordo com Souza Filho et al. (2009), os aleloquímicos possuem atributos inibitórios e estimulatórios, sendo que em baixas concentrações, podem apresentar efeitos estimulatórios em determinados casos e não inibitório para dada espécie receptora, o que pode justificar esse aumento do comprimento da parte aérea, provocado pela concentração inicial avaliada dos óleos essenciais.

Santana et al. (2006) afirmam que os aleloquímicos não podem alterar significativamente a germinação de sementes e afetar a velocidade e sincronia da germinação destas, o que pode justificar o fato dos efeitos voláteis desses óleos essenciais avaliados não influenciarem as variáveis primeira contagem de plântulas germinadas e a germinação total de plântulas e alterarem as variáveis IVG, massa seca e comprimento de partes radiculares de plântulas.

Ao avaliar o efeito do contato direto dos óleos essenciais na germinação e vigor de plântulas de alface, observou-se que o óleo dos rizomas provocou reduções estatisticamente superiores ao óleo das folhas nas variáveis primeira contagem de plântulas germinada, IVG, massa seca de plântula e comprimentos de partes aéreas e radiculares de plântulas (Figura 2). Os óleos essenciais não demonstraram diferenças significativas somente na variável germinação final, uma vez que o óleo dos rizomas provocou atraso na geminação inicial das

sementes, principalmente nos três primeiros dias de observação, não afetando a germinação final.

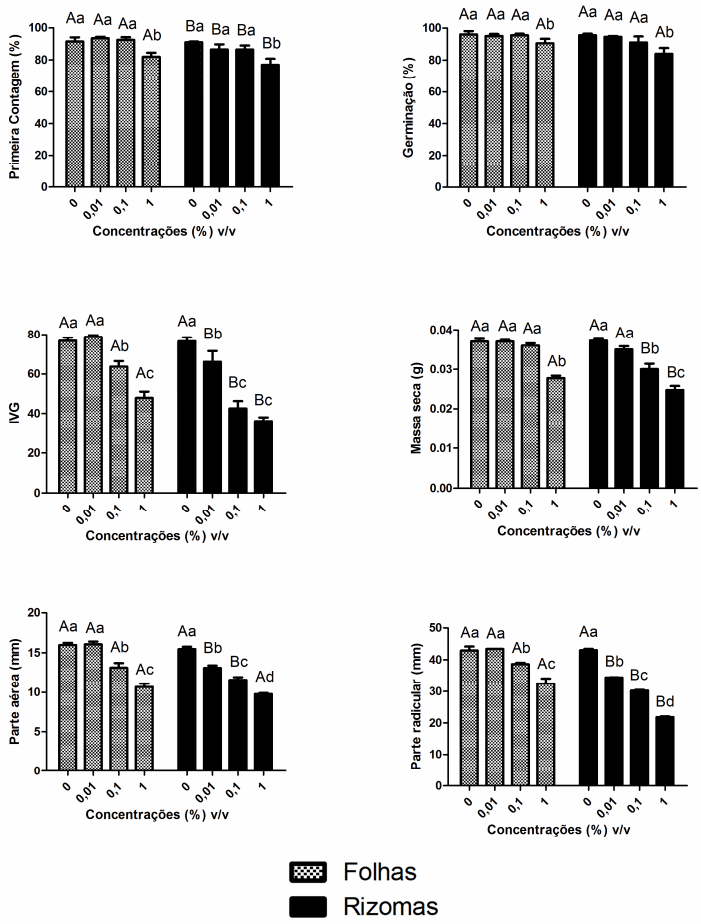

Figura 2. Valores médios das variáveis primeira contagem de germinação, germinação total, IVG, massa de plântulas secas, comprimentos de parte aérea e de raiz em função das concentrações dos óleos essenciais de folhas e rizomas de *H. coronarium*, avaliadas pela metodologia do contato direto.
Médias seguidas da mesma letra, maiúsculas para comparação entre os óleos essenciais analisados e minúsculas para comparação entre as concentrações dentro de cada óleo essencial, não diferem significativamente entre si a 5% de probabilidade, pelo teste de Scott-Knott.

Na análise da germinação inicial e final para ambos os óleos, somente a concentração de 1%, diferiu-se estatisticamente das demais concentrações, sendo que essa concentração do óleo das folhas reduziu a primeira contagem de germinação de 91,5 para 82% e a germinação final de 96 para 90,5%, enquanto que o óleo dos rizomas reduziu de 91 para 77% a geminação inicial e de 95,5 para 84%, a germinação final das sementes. Essa mesma tendência de resposta foi verificada apenas quando a influência do óleo essencial das folhas sobre a massa seca de plântulas foi avaliada, com diminuição de 0,0370 para 0,0278 g, diferentemente do óleo dos rizomas, que somente na concentração de 0,01% não diferiu estatisticamente do controle, que apresentou uma massa seca de 0,0373 g ao passo que a concentração de 1% reduziu essa variável para 0,0248 g. Ambos os óleos essenciais provocaram reduções nas variáveis IVG e comprimentos de partes aéreas e radiculares das plântulas dependentes das concentrações, entretanto em todas essas variáveis o óleo dos rizomas provocou reduções significativamente superiores, sendo que o óleo das folhas na concentração de 1% apresentou IVG de 47,9, comprimento de parte aérea de 10,7 mm e radicular de 32,5 mm, enquanto que a mesma concentração do óleo dos rizomas, apresentou valores de 36,07; 9,78 mm e 21,8 mm, respectivamente, para as mesmas variáveis.

De acordo com Souza Filho et al. (2009) os efeitos dos óleos essenciais na germinação e vigor de plântulas, podem ser explicados de forma individualizada considerando os principais constituintes, corroborando com as observações feitas no presente estudo em que houve a influência da composição dos óleos essenciais extraídos de diferentes partes da espécie *H. coronarium* nas diferentes atividades alelopáticas apresentadas por esses óleos. O óleo dos rizomas apresentou 98,2% de monoterpenos e 1% de sesquiterpenos, enquanto que o das folhas, 84,3% de monoterpenos e 14,9% de sesquiterpenos. Adicionalmente, verificou-se que o óleo essencial dos rizomas apresentou

potencial alelopático superior ao extraído das folhas, o que pode ser explicado por seu maior conteúdo de monoterpenos, que segundo Rosado et al. (2009), compõem o grupo de compostos identificados com maior potencialidade inibitória, por provocarem alterações na estrutura e função das membranas, impedindo o crescimento e atividade das células. Em seus estudos Duke et al. (2000), afirmam que entre esses compostos monoterpênicos, o 1,8-cineol demonstrou eficiência em suprimir o crescimento de plantas daninhas, o que corrobora com os dados encontrados no presente trabalho, em que entre os compostos químicos dos óleos em estudo, o 1,8-cineol foi encontrado como um dos constituintes majoritários apenas do óleo essencial dos rizomas, o que pode justificar seu maior potencial alelopático.

Diante dos dados analisados, pode-se verificar que o óleo essencial extraído dos rizomas demonstrou maior eficiência alelopática que o óleo essencial das folhas da mesma espécie, nas condições analisadas, o que possivelmente deve-se a maior quantidade de compostos monoterpênicos, principalmente o 1,8-cineol.

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ARTIGO 4 - ÓLEOS ESSENCIAIS DE FOLHAS DE DIVERSAS ESPÉCIES: PROPRIEDADES ANTIOXIDANTES E ANTIBACTERIANAS.

Artigo submetido ao Conselho Editorial do periódico Ciência Agronômica e formatado conforme normas do referido periódico.

Óleos essenciais de folhas de diversas espécies: propriedades antioxidantes e antibacterianas.

Essential oils of the leaves of various species: antioxidant and antibacterial properties.

RESUMO - Os óleos essenciais apresentam possibilidade de serem empregados nas indústrias de alimentos, bebidas, produtos de higiene pessoal e cosméticos para evitar ou reduzir a deterioração lipídica e a contaminação por micro-organismos. Neste trabalho foram objetivos avaliar as propriedades funcionais antimicrobianas e antioxidantes de óleos essenciais de folhas frescas de *Coniza bonariensis, Parthenium hysterophorus, Tithonia diversifolia, Ambrosia polystachya*, *Hedychium coronarium* e *Baccharis dracunculifolia*, extraídos por hidrodestilação. O potencial antioxidante foi avaliado pelas metodologias do consumo do radical DPPH e da inibição da oxidação do sistema β-caroteno/ácido linoleico. A sensibilidade das bactérias *Salmonella* Cholerasuis, *Listeria monocytogenes*, *Staphylococcus aureus* e *Escherichia coli* frente aos óleos essenciais foi determinada pela utilização do método de difusão em cavidade ágar. Os óleos essenciais destacaram-se pelo elevado conteúdo de terpenoides. Todos os óleos essenciais avaliados pela metodologia do sequestro do radical DPPH não apresentaram CI_{50} significativos. Pela metodologia do β-caroteno/ácido linoleico, os óleos essenciais de *T. diversifolia* e *H. coronarium*

não apresentaram atividades significativas e os de *C. bonariensis, P. hysterophorus, A. polystachya*, e *B. dracunculifolia* apresentaram CI_{50} superiores a maior concentração avaliada. Os óleos essenciais das espécies *C. bonariensis*, *T. diversifolia*, *H. coronarium* e de *B. dracunculifolia* apresentaram atividade antibacteriana para bactérias Gram-negativas e Gram-positivas, com exceção do óleo essencial de *P. hysterophorus*, que não impediu o crescimento de nenhuma das cepas bacterianas testadas. O óleo essencial de *A. polystachya* apresentou potencial antibacteriano apenas nas cepas de *S. aureus*.
Palavras-chave: Óleos voláteis. Micro-organismos. Oxidação.

ABSTRACT - Essential oils have the possibility of being employed in industry of food, drinks, toiletries and cosmetics to prevent or reduce lipid deterioration and contamination by microorganisms. In this work, we have aimed to evaluate the antimicrobial and antioxidants properties of essential oils from fresh leaves of *Coniza bonariensis, Parthenium hysterophorus, Tithonia diversifolia, Ambrosia polystachya*, *Hedychium coronarium* and *Baccharis dracunculifolia,* extracted by hydrodestillation. The antioxidant capacity was evaluated by the methods of DPPH consumption and inhibition of oxidation of the β-carotene/linoleic acid system. The sensitivity of bacteria *Salmonella* Cholerasuis, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* in the presence of essential oils was determined using agar diffusion method. Essential oils highlighted by the high content of terpenoids. All of the essential oils tested by the radical scavenging DPPH method presented no significant CI_{50}. Using the β-carotene/linoleic acid system, the essential oils from *T. diversifolia* and *H. coronarium* showed no significant activity and the *C. bonariensis, P. hysterophorus, A. polystachya*, and *B. dracunculifolia* showed CI_{50} greater than the highest concentration evaluated. The essential oils of species of *C. bonariensis*, *T. diversifolia*, *H. coronarium* and *B. dracunculifolia*

showed antibacterial activity for Gram-negative and Gram-positive, with the exception of the volatile oil from *P. hysterophorus*, which did not show inhibition on the growth of any of the bacterial strains tested. The *A. polystachya* essential oil showed antimicrobial potential only in strains of *S.aureus*.
Key words: Volatile oils. Microorganisms. Oxidation.

INTRODUÇÃO

O crescente interesse dos consumidores em ingredientes funcionais a partir de fontes naturais está permitindo a aplicação dos óleos essenciais nas indústrias de alimentos, bebidas, produtos de higiene pessoal e cosméticos, com o objetivo de evitar a deterioração lipídica, oxidação e a contaminação por micro-organismos. Aliado ao interesse do consumidor destaca-se o fato desses produtos de degradação gerarem enormes prejuízos para a indústria pelo desenvolvimento de compostos tóxicos, que afetam gravemente a vida de prateleira de muitos produtos. Somam-se ainda a esses fatos o desenvolvimento crescente de resistência bacteriana aos antimicrobianos utilizados atualmente, além da possibilidade dos antioxidantes sintéticos mais empregados como o butil-hidroxi-anisol (BHA), 2,6-di-tert-butil-4-hidroxitolueno (BHT), tercbutil-hidroquinona (TBHQ) e galato de propila (PG) apresentarem efeito carcinogênico em experimentos com animais. Desta forma os óleos essenciais demonstram ser uma possibilidade viável para substituir ou associar-se a antioxidantes sintéticos e antimicrobianos convencionais, com a finalidade de diminuir a quantidade dessas substâncias nos alimentos (ANDRADE *et al.*, 2012; LANG; BUCHBAUER, 2012; SACCHETTI *et al.*, 2004).

Os óleos essenciais são metabólitos secundários extraídos de diversas partes de plantas, possuem composição química complexa e garantem aos vegetais vantagens adaptativas no meio em que estão inseridos (OUSSALAH *et al.*, 2007). A composição química dos óleos essenciais varia entre as espécies e

entre as partes de um mesmo vegetal. De acordo com Gobbo-Neto e Lopes (2007), em uma mesma espécie botânica, essa constituição pode ser afetada pelo local de cultivo, condições de coleta, estabilização e estocagem, além dos fatores edafoclimáticos. Os constituintes dos óleos essenciais são principalmente os derivados terpênicos, como os mono, sesquiterpenos e os fenilpropanoides (SOLÓRZANO-SANTOS; MIRANDA-NOVALES, 2012).

Segundo Gutierrez, Barry-Ryan e Bourke (2008), essas diversas combinações de constituintes químicos apresentadas pelos óleos essenciais, podem controlar a oxidação de alimentos e a proliferação de bactérias que apresentam resistência consistentemente elevada a agentes antimicrobianos, tais como *Salmonella* Cholerasuis, *Listeria monocytogenes*, *Staphylococcus aureus* e *Escherichia coli*.

Diante da possibilidade de utilização dos óleos essenciais para aumentar a conservação, prolongar a validade de alimentos e diminuir o uso de antioxidantes sintéticos e agentes antimicrobianos, numerosos estudos têm se dedicado a demonstrar os potenciais promissores desses compostos no combate às bactérias patogênicas e aos radicais livres, tornando o emprego desses metabólitos uma alternativa viável ecológica e economicamente (ANDRADE *et al*,. 2012; BURT, 2004; EBRAHIMABADI *et al.*, 2011; GUIMARÃES *et al.*, 2008; GUTIERREZ; BARRY-RYAN; BOURKE, 2008; JOSHI *et al.*, 2008; KULISIC *et al.*, 2004).

Embora os óleos essenciais representem uma possibilidade interessante para a preservação de alimentos, é necessário conhecer suas propriedades antibacterianas e antioxidantes, por meio da determinação da Concentração Mínima Inibitória (CMI) e da Concentração de 50% de Inibição (CI_{50}) desses metabólitos, podendo estabelecer assim uma correlação entre esses potenciais e a composição química dos mesmos (HYLDGAARD; MYGIND; MEYER, 2012). No presente experimento, relatam-se os resultados de um estudo

destinado a comparar, as propriedades funcionais antimicrobianas e antioxidantes de óleos essenciais de folhas frescas das espécies botânicas *Coniza bonariensis, Parthenium hysterophorus, Tithonia diversifolia, Ambrosia polystachya*, *Hedychium coronarium* e *Baccharis dracunculifolia*.

MATERIAL E MÉTODOS

Material Vegetal

As partes das folhas jovens (nervura e limbo) das espécies *C. bonariensis, P. hysterophorus, T. diversifolia, A. polystachya*, *H. coronarium* e *B. dracunculifolia* foram coletadas em plantas adultas no estágio de floração plena no câmpus da Universidade Federal de Lavras (UFLA), no município de Lavras/MG. A cidade de Lavras localiza-se no sul do estado de Minas Gerais (Brasil), 21º14' S, longitude 45º00' W Gr. e 918 m de altitude. As espécies foram coletadas no início da manhã de dias sem precipitação, no mês de fevereiro de 2012, identificadas pela Doutora Mariana Esteves Mansanares, do Departamento de Biologia da UFLA e as excicatas foram depositadas no Herbário ESAL na UFLA, sob os registros de numeração 26945, 26944, 26947, 28948, 26942 e 26946, respectivamente.

Os óleos essenciais foram extraídos por hidrodestilação, utilizando um aparelho de Clevenger modificado adaptado a um balão de fundo redondo com capacidade de 4 litros, por um período de 2 horas conforme metodologia descrita pela Farmacopeia Brasileira (2000). Para purificação do óleo essencial, o hidrolato foi centrifugado a 965,36 g por 5 minutos, e, os óleos foram acondicionados em frascos de vidro âmbar e armazenados sob refrigeração.

Identificação e quantificação dos constituintes dos óleos essenciais

Nas análises de Cromatografia Gás-Líquido/Espectrometria de Massas (CGL/EM) foi utilizado um cromatógrafo Perkin Elmer Autosystem XL,

equipado com uma coluna de sílica fundida DB-1(30m x 0,25mm d.i., espessura de filme 0,25µm; J & W Scientific Inc.) acoplado a um espectrômetro de massas Perkin Elmer Turbomass (versão do software 4.1). A temperatura do forno foi programada de 45 a 175˚C, aumentando 3˚C/min, e subsequentemente 15˚C/min até 300˚C. Atingidos 300˚C, a temperatura foi mantida constante durante 10min; temperatura da linha de transferência, 280˚C; temperatura da câmara de ionização, 220˚C; gás de arraste, hélio, ajustado para uma velocidade linear de 30 cm/s e relação de repartição de fluxo, 1:40. A identidade dos compostos foi determinada por comparação dos seus índices de retenção, em relação aos dos n-alcanos C_9-C_{21} e espectros de massas, com os padrões comerciais e compostos de referência presentes em óleos existentes no laboratório e por comparação com uma biblioteca de espectros de massas desenvolvida no Laboratório do Centro de Biotecnologia Vegetal da Faculdade de Ciências da Universidade de Lisboa (MENDES *et al*., 2011).

Os óleos essenciais foram analisados por Cromatografia Gás-Líquido (CGL), num cromatógrafo Perkin Elmer 8700 equipado com dois detectores de ionização de chama (DIC), um sistema de tratamento de dados e um injetor, no qual foram instaladas duas colunas de polaridade diferente com as seguintes características: DB-1 de sílica fundida, de fase imobilizada em metilsilicone (30m x 0,25mm d.i., espessura de filme 0,25µm; J & W Scientific Inc.); DB-17HT de fase imobilizada em fenilmetilsilicone (30m x 0,25mm d.i.). A temperatura do forno foi programada de 45°C a 175°C, aumentando 3°C/min, e subsequentemente 15°C/min até 300°C. Atingidos 300°C a temperatura foi mantida constante durante 10 min. A temperatura do injetor e dos detectores foram de 290°C e 280°C, respectivamente. Utilizou-se hidrogênio como gás de arraste, ajustado para uma velocidade linear de 30 cm/s. e a relação de repartição de fluxo foi de 1:50.

A composição percentual dos óleos foi determinada pela integração das áreas dos picos sem utilização de fatores de correção. Os valores apresentados correspondem ao valor médio de duas injeções.

Ensaios de atividade antioxidante

O potencial antioxidante dos óleos essenciais foi avaliado pelas metodologias do consumo do radical DPPH e da inibição da oxidação do sistema β-caroteno/ácido linoleico. Para fins de comparação foram empregados os padrões timol e ácido ascórbico, por possuírem atividade antioxidante reconhecida e o antioxidante sintético BHT. Todas as análises foram realizadas em triplicata, sendo calculada a média dos resultados.

Análise quantitativa da atividade antioxidante pelo consumo do radical livre DPPH

Este ensaio foi realizado de acordo com metodologia descrita por Sousa *et al.* (2007), com algumas modificações. Preparou-se uma solução estoque de DPPH em metanol na concentração de 40 μg mL^{-1}. As amostras foram diluídas em metanol nas concentrações de 250, 200, 150, 100, 50 e 25 μg mL^{-1}. As medidas das absorbâncias das misturas reacionais (0,3 mL da solução das amostras e 2,7 mL da solução de DPPH) foram realizadas no tempo de 60 minutos em espectrofotômetro UV-Vis (SHIMATZU UV-160 1PC), no comprimento de onda 515 nm. Como branco, foram utilizados 2,7 mL de metanol e 0,3 mL da solução metanólica mais concentrada de cada amostra e, como controle, 2,7 mL de solução estoque de DPPH e 0,3 mL de metanol. Os valores de absorbância foram convertidos em porcentagem de atividade antioxidante. Para determinação da CI_{50}, construiu-se uma curva, plotando-se na abscissa as concentrações das amostras (μg mL^{-1}) e, na ordenada, a porcentagem de atividade antioxidante.

Análise quantitativa da atividade antioxidante pelo sistema β-caroteno/ácido linoleico

A determinação da atividade antioxidante no sistema ácido linoleico/β-caroteno foi realizada conforme metodologia descrita por Lopes-Lutz *et al.* (2008), com algumas modificações. A uma solução contendo β-caroteno (2 mg) e clorofórmio (10 mL), foram adicionados 20 mg de ácido linoleico e 200 mg Tween 20. O clorofórmio foi totalmente evaporado em evaporador rotatório. Posteriormente, foram adicionados 50 mL de água destilada, previamente saturada com oxigênio por 30 minutos (emulsão A). Alíquotas (200 μL) de cada amostra foram dissolvidas em metanol, nas concentrações de 250, 200, 150, 100, 50 e 25 μg mL^{-1} e, em seguida, foram adicionadas a 2,5 mL de emulsão de ácido linoleico/β-caroteno. As amostras foram submetidas à oxidação, incubando-as a 50°C por 60 minutos. Como branco, utilizou-se a emulsão A sem o β-caroteno (2,5 mL) e 200 μL da solução metanólica mais concentrada de cada amostra e, como controle, empregaram-se 2,5 mL da emulsão de β-caroteno e 200 μL de metanol. A absorbância foi lida a 470 nm e a atividade antioxidante relativa foi determinada. Foram plotados gráficos com os percentuais da degradação do β-caroteno *versus* as concentrações analisadas, para a obtenção da CI_{50}.

Ensaios biológicos

As bactérias empregadas foram cepas padrões ATCC, previamente purificadas e mantidas em eppendorfs contendo meio de congelamento, de *Salmonella* cholerasuis ATCC 6539, *Listeria monocytogenes* ATCC 19117, *Staphylococcus aureus* ATCC 13565 e *Escherichia coli* ATCC 11229. Para a ativação das culturas, as cepas foram colocadas em caldo BHI, com posterior incubação em BOD à 37°C, por 24 horas para a obtenção do inóculo.

A sensibilidade das bactérias frente aos óleos essenciais foi determinada pelo método de difusão em cavidade ágar. Após ativação das bactérias, alíquotas desse meio foram transferidas para um tubo com 5 mL de caldo de soja tríptica

(TSB). Os tubos foram incubados a 37ºC, até alcançar a turbidez de uma solução-padrão McFarland de 0,5, o que resultou em uma suspensão contendo 10^8 UFC mL^{-1}. As leituras de turbidez foram realizadas utilizando espectrofotômetro (Shimadzu UV-160 1 PC), no comprimento de onda de 625 nm (NCCLS, 2003).

A concentração do inóculo obtida pela solução-padrão McFarland de 0,5 foi diluída em TSB até a concentração de 10^6 UFC mL^{-1}, sendo, posteriormente transferida para o meio de cultura TSA, para a espécie *L. monocytogenes*, e para as demais espécies, foi utilizado o Ágar Mueller-Hinton. Nos meios de cultura foram colocadas pérolas de vidro, para a formação dos poços de deposição, os quais foram preenchidos com 10 µL do óleo essencial diluído em dimetilsulfóxido (DMSO), nas concentrações de 500; 250; 125; 62,5; 31,25; 15,62; 7,81 e 3,90 µL mL^{-1}. Para o controle positivo 10 µL de cloranfenicol (100 µg mL^{-1}) foram depositados no poço. Como controle negativo foi utilizado a mesma quantidade de dimetilsulfóxido (DMSO). As placas foram incubadas em BOD à 37°C por 24 horas. Decorrido esse tempo, foram medidas os diâmetros dos halos formados, com três repetições para cada tratamento. A CMI foi definida como a menor concentração do óleo essencial em que foi observada a formação do halo de inibição.

RESULTADOS E DISCUSSÃO

Caracterização química e quantificação dos constituintes dos óleos essenciais

Os compostos majoritários do óleo essencial de *C. bonariensis* foram o limoneno (56,7%), o trans-β-ocimeno (26,3%) e o cis-verbenol (4,4%), no óleo de *P. hysterophorus*, o germacreno-D (35,9%), o trans-β-ocimeno (8,5%) e o β-mirceno (7,6%), enquanto no óleo de *T. diversifolia* foram o β-pineno (38,3%), o α-pineno (28,6%) e o limoneno (8,8%). No óleo essencial de *A. polystachya*

foram encontrados como componentes majoritários o germacreno-D (29,3%), o trans-β-ocimeno (13,6%) e o β-cariofileno (9,8%), no de *H. coronarium,* o β-pineno (46,9%), o α-pineno (19,2%) e o β-cariofileno (13,2%), enquanto no de *B. dracunculifolia*, o limoneno (30,9%), o trans-nerolidol (22,4%) e o β-pineno (14,5%).

Tabela 1 - Percentual de constituintes químicos agrupados dos óleos essenciais das folhas de *C. bonariensis* (Cb), *P. hysterophorus* (Ph), *T. diversifolia* (Td), *A. polystachya* (Ap), *H. coronarium* (Hc) e *B. dracunculifolia* (Bd)

Componentes agrupados	%					
	Cb	Ph	Td	Ap	Hc	Bd
Monoterpenos hidrocarbonetos	86,6	18,8	86,1	32,7	75,8	58,2
Monoterpenos oxigenados	5,6	1,3	0,2	1,5	8,5	0,3
Sesquiterpenos hidrocarbonetos	3,4	47,4	5,8	48,8	14,2	9,1
Sesquiterpenos oxigenados	1,7	6,1	7,6	13,9	0,7	27,5
Diterpenos oxigenados	-	0,6	-	0,4	-	-
Outros	-	1,6	-	-	-	-
Total	97,3	75,7	99,7	97,3	99,2	95,2

Em relação à composição desses óleos essenciais, pôde-se destacar o elevado conteúdo de terpenoides, em que os monoterpenoides predominaram nos óleos essenciais de *C. bonariensis*, *T. diversifolia*, *H. coronarium* e *B. dracunculifolia* e os sesquiterpenoides nos óleos essenciais de *P. hysterophorus* e *A. polystachya* (Tabela1). Santos *et al.* (2006) destacaram os monoterpenoides e os sesquiterpenoides como os principais compostos encontrados nos óleos essenciais, corroborando com os dados encontrados.

Atividade antioxidante

Os óleos essenciais são uma mistura complexa de dezenas de compostos com diferentes comportamentos, grupos funcionais e polaridade. Devido a essa complexidade estrutural, Wang *et al*. (2008) afirmam que o efeito antioxidante

de um óleo essencial não pode ser atribuído a um ou alguns de seus constituintes, visto que além dos compostos principais, os constituintes encontrados em concentrações menores podem contribuir de forma significativa para a atividade do óleo. Anteriormente Kulisic *et al*. (2004) salientaram a importância da utilização de diferentes metodologias avaliadoras da atividade antioxidante, para tentar descrever a capacidade de combater os radicais livres apresentada por esses metabólitos. Nas metodologias empregadas no presente estudo, a capacidade de cada óleo essencial de combater os radicais, foi determinada com base nas respectivas CI_{50}. Os valores de CI_{50} para a atividade antioxidante dos óleos essenciais, antioxidantes sintéticos, BHT, ácido ascórbico e padrão timol, pelos métodos do sequestro de radicais DPPH e do β-caroteno/ácido linoleico estão descritos na Tabela 2.

Tabela 2 - Atividade antioxidante dos óleos essenciais de *C. bonariensis, P. hysterophorus, T. diversifolia, A. polystachya*, *H. coronarium* e *B. dracunculifolia*, BHT, timol e ácido ascórbico pelos métodos de sequestro de radicais DPPH (1,1-difenil-2-picrilidrazila) e da inibição da oxidação do sistema β-caroteno/ácido linoleico

Amostras	Metodologias	
	DPPH	β-caroteno/ácido linoleico
	CI_{50} (μg mL^{-1})	CI_{50} (μg mL^{-1})
C. bonariensis	NI	> 250
P. hysterophorus	NI	> 250
T. diversifolia	NI	NI
A. polystachya	NI	> 250
H. coronarium	NI	NI
B. dracunculifolia	NI	> 250
BHT	48,84	< 25
Timol	> 250	105,82
Ácido Ascórbico	44,36	118,15

*CI_{50}: Concentração de inibição de 50%, **NI: não apresentou inibição na faixa das concentrações avaliadas

De acordo com os dados apresentados pôde-se observar que todos nenhum dos óleos essenciais avaliados apresentaram habilidade em capturar radicais estáveis DPPH, não apresentando atividades antioxidantes consideráveis. O timol apresentou CI_{50} superior à maior concentração avaliada, ao contrário dos compostos antioxidantes padrões, ácido ascórbico e BHT, que demonstraram eficiência no combate aos radicais DPPH, reduzindo as absorbâncias das soluções. As baixas atividades apresentadas pelos óleos essenciais, podem ser explicadas pelo fato dos mesmos serem pouco solúveis nas condições do experimento e, segundo Andrade *et al.* (2012), essa metodologia deve ser aplicada, principalmente para compostos hidrofílicos, como o ácido ascórbico, justificando seu elevado desempenho em sequestrar os radicais DPPH.

O ensaio do β-caroteno/ácido linoleico é apropriado para avaliar antioxidantes lipofílicos, como os óleos essenciais que, de acordo com Kulisic *et al.* (2004), explica a atividade reduzida de moléculas polares, como o ácido ascórbico. Entre os óleos essenciais estudados, o de *T. diversifolia* e *H. coronarium* não apresentaram atividades significativas e os óleos das espécies *C. bonariensis, P. hysterophorus, A. polystachya* e *B. dracunculifolia* apresentaram CI_{50} superiores a maior concentração avaliada (250 μg mL^{-1}). Essa baixa atividade pode ser explicada pelo fato de que, os constituintes majoritários dos óleos avaliados no presente experimento, não são compostos que contêm átomos de hidrogênio na posição alílica e/ou posições benzílicas. Segundo Ebrahimabadi *et al.* (2010), os compostos fenólicos são aqueles que mostram melhor atividade nessa metodologia, devido a abstração relativamente fácil de um átomo de hidrogênio a partir desses grupos funcionais, por radicais peróxidos nas circunstâncias do teste.

As atividades antioxidantes dos óleos essenciais extraídos das folhas de *C. bonariensis, P. hysterophorus, T. diversifolia, A. polystachya*, *H. coronarium* e

B. dracunculifolia não foram descritas anteriormente na literatura. Entretanto a composição química e as propriedades antioxidantes do óleo essencial extraído dos rizomas da espécie *H. coronarium*, coletados na Índia, foram estudadas por Joshi *et al.* (2008), que empregaram as metodologias do sequestro de radicais DPPH, da redução do Fe^{3+} e quelação do Fe^{2+}. Os autores encontraram como constituintes majoritários desse óleo, o diterpeno trans-meta-menta-2,8-dieno (25,2%) e os monoterpenos linalol (21,7%) e α-terpineol (10,9%). De acordo com as análises do seu potencial antioxidante, observaram moderados potenciais na redução do Fe^{3+}, na capacidade dose-dependente de quelar Fe^{2+}, através da ferrozina e na habilidade de sequestrar radicais DPPH. Os resultados encontrados pelos referidos autores não corroboraram com os encontrados no presente estudo, por se tratarem de diferentes constituintes químicos e consequentemente potenciais antioxidantes distintos. De acordo com Gobbo-Neto e Lopes (2007), os metabólitos secundários encontrados em diferentes partes de uma mesma espécie botânica podem ser diferentes, o que justifica essas divergências verificadas.

Atividade antibacteriana

O potencial antibacteriano dos óleos essenciais pode ser atribuído, segundo Solórzano-Santos e Miranda-Novales (2012), à hidrofobicidade dos mesmos, o que lhes permite partição com os lípidios da membrana celular e mitocôndrias das bactérias, causando perturbação das estruturas celulares e aumentando a permeabilidade da membrana, provocando o vazamento de moléculas essenciais à sua sobrevivência e levando as bactérias à morte. Os resultados da avaliação antimicrobiana dos óleos essenciais estão sumarizados na Tabela 3, onde os valores de CMI dos óleos essenciais desse estudo estão expressos.

Tabela 3 - Concentração mínima inibitória dos óleos essenciais de *C. bonariensis, P. hysterophorus, T. diversifolia, A. polystachya*, *H. coronarium* e *B. dracunculifolia*, cloranfenicol e dimetilsulfóxido encontrada para as bactérias *S. aureus, L.monocytogenes, E. coli,* e *S.* Cholerasuis

Amostras	CMI (μL mL^{-1})			
	S. aureus	*L.monocytogenes*	*E. coli*	*S.*Choleraesuis
Gram	+	+	-	-
C. bonariensis	15,62	500	NI	3,9
P. hysterophorus	NI	NI	NI	NI
T. diversifolia	15,62	NI	NI	250
A. polystachya	125	NI	NI	NI
H. coronarium	125	250	250	NI
B. dracunculifolia	NI	3,9	NI	500
CL	I	I	I	I
DMSO	NI	NI	NI	NI

*NI: não ocorreu inibição, I: ocorreu 100% de inibição, CL: Cloranfenicol (100 μL mL^{-1}), DMSO: dimetilsulfóxido

A análise dessas CMI permitiu afirmar que os óleos essenciais das espécies avaliadas foram, em sua maioria, mais eficientes para inibir bactérias Gram-positivas, apresentando CMI menores, quando comparadas com os valores apresentados sobre as Gram-negativas. De acordo com Mann, Cox e Markham (2000), esse comportamento pode ser explicado pelo fato das bactérias Gram-negativas possuírem uma membrana externa que impede a penetração de macromoléculas e compostos hidrofóbicos, aumentando sua resistência aos óleos essenciais. Entre as bactérias avaliadas, a *E. coli* mostrou-se mais resistente aos óleos avaliados, sendo inibida somente pelo óleo essencial de *H. coronarium*. Já o óleo essencial de *P. hysterophorus* não inibiu o crescimento de nenhuma das bactérias avaliadas.

Os óleos essenciais com conteúdos maiores de monoterpenos mostraram-se mais eficientes na inibição do crescimento bacteriano. O óleo essencial de *C. bonariensis* mostrou-se efetivo contra as cepas de *S. aureus, L. monocytogenes* e

S. Cholerasuis, o de *T. diversifolia* sobre as cepas de *S. aureus* e *S.* Cholerasuis, o de *H. coronarium* nas cepas de *S. aureus, L. monocytogenes* e *E. coli* e o de *B. dracunculifolia* sobre as cepas de *L. monocytogenes* e *S.* Cholerasuis. Os óleos essenciais ricos em sesquiterpenoides mostraram-se menos ativos na inibição do crescimento das bactérias avaliadas, sendo que o de *P. hysterophorus* não impediu o crescimento de nenhuma das cepas bacterianas testadas nesse experimento e, o de *A. polystachya* apresentou potencial antibacteriano apenas em cepas de *S. aureus*. Essa atividade antibacteriana sobre bactérias Gram-negativas apresentadas por monoterpenoides pode ser justificada, pela capacidade desses compostos de chegarem ao citoplasma dessas bactérias, passando pelos poros de proteínas presentes em sua membrana externa (HELANDER *et al.*, 1998).

Os óleos essenciais extraídos de folhas frescas de *C. bonariensis, P. hysterophorus, T. diversifolia, A. polystachya* e *H. coronarium* não tiveram suas propriedades antibacterianas descritas anteriormente. A atividade antibacteriana do óleo essencial das folhas de *B. dracunculifolia* coletadas no Paraná, sobre as bactérias *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 e *Pseudomonas aeruginosa* ATCC 27853, pelo método da difusão em disco de papel foi avaliada por Ferronatto *et al.* (2007). Esses aplicaram o óleo essencial diretamente nos discos (nas concentrações de 1, 3, 5 e 10 μL/disco) incubando as placas a 36°C por 24 a 48 horas, usando cloranfenicol e amoxicilina como controles. Como resultados os autores verificaram que o óleo apresentou atividade antimicrobiana contra todas as bactérias avaliadas, ao contrário do presente experimento, em que o óleo de *B. dracunculifolia* inibiu o crescimento das bactérias *L.monocytogenes* e *S.* Cholerasuis. Essa divergência de potencial antibacteriano entre os óleos essenciais da mesma espécie botânica coletada em diferentes localidades, possivelmente deve-se as diferenças entre a constituição química desses óleos essenciais, que segundo Gobbo-Neto e Lopes (2007), pode

variar de acordo com a localidade e a época de coleta da espécie vegetal, ciclo vegetativo e fatores edafoclimáticos, corroborando com os dados encontrados.

CONCLUSÕES

1. Os óleos essenciais extraídos das folhas de *C. bonariensis*, *P. hysterophorus*, *T. diversifolia*, *A. polystachya*, *H. coronarium* e de *B. dracunculifolia* destacam-se pelo elevado conteúdo de terpenoides, onde os monoterpenoides predominam nos óleos essenciais de *C. bonariensis*, *T. diversifolia*, *H. coronarium* e *B. dracunculifolia* e os sesquiterpenoides nos óleos de *P. hysterophorus* e *A. polystachya*

2. Nenhum dos óleos essenciais avaliados pela metodologia do sequestro do radical DPPH apresentam CI_{50} significativos. Pela metodologia do β-caroteno/ácido linoleico, os óleos essenciais de *T. diversifolia* e *H. coronarium* não apresentam atividades significativas e os de *C. bonariensis, P. hysterophorus, A. polystachya*, e *B. dracunculifolia* apresentaram CI_{50} superiores a maior concentração avaliada (250 $\mu g\ mL^{-1}$).

3. Os óleos essenciais das espécies *C. bonariensis*, *T. diversifolia*, *H. coronarium* e de *B. dracunculifolia* apresentam atividade antibacteriana para bactérias Gram-negativas e para bactérias Gram-positivas, com exceção do óleo essencial de *P. hysterophorus*, que não impede o crescimento de nenhuma das cepas bacterianas testadas no presente trabalho e o de *A. polystachya* apresenta potencial antibacteriano apenas em cepas de *S. aureus*.

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ARTIGO 5 - ANTIOPHIDIAN PROPERTIES OF ESSENTIAL OILS FROM SPECIES OF THE ASTERACEAE FAMILY: CLOTTING AND FIBRINOGENOLYSIS INDUCED BY *BOTHROPS* AND *LACHESIS* SNAKE VENOMS.

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Antiophidian properties of essential oils from species of the Asteraceae family: clotting and fibrinogenolysis induced by *Bothrops* and *Lachesis* snake venoms

ABSTRACT

Natural inhibitors of toxins and snake venoms have been widely investigated for supplementation or replacement of traditional serum therapy. The essential oils and their pharmacologically active constituents can present inhibitory potential against snakebite and are scientifically unexplored in this context. The essential oils from leaves of species of the Asteraceae family (*Baccharis dracunculifolia*, *Conyza bonariesis*, *Tithonia diversifolia* and *Ambrosia polystachya)* were extracted by hydrodistillation and the inhibition of coagulation and fibrinogenolysis induced by *Lachesis muta*, *Bothrops atrox* and *Bothrops moojeni* venoms was evaluated. All the oils evaluated were able to inhibit the coagulant effect when previously incubated for 15 min at 37°C with the venoms. Clotting times were also expanded in a lesser degree when the essential oils were previously incubated with the plasma, suggesting that the constituents of oils interacted with proteases present in the venoms but also with constituents of blood plasma. The essential oil of *A. polystachya* proved to be effective in inhibiting the fibrinogenolysis induced by *B. moojeni* venom when it was

previously incubated with the venom or with fibrinogen. However, the oil from *B. dracunculifolia* inhibited the cleavage of fibrinogen induced by *L. muta* venom only when it was previously incubated with the venom. Thus, the essential oils are promising as potential adjuvants in the treatment of snakebites, especially considering that in general this type of oils can be applied topically and do not require special pharmaceutical preparations.

1. INTRODUCTION

Snake venoms are complex combinations of several proteins, mostly with enzymatic activity, peptides, nucleotides, free amino acids, biogenic amines, some lipids and several ions capable of inducing physiopathologies characteristic of each species. Venoms generally produce local effects such as hemorrhage, necrosis, edema and intense pain, and systemic effects as bleeding disorders, internal bleeding, cardiovascular shock and acute renal failure [1]. Snake venoms present different actions depending on specific combinations of their components, such as phospholipases A_2, serineproteases, metalloproteinases, hyaluronidases and L-amino acid oxidases [2].

In Brazil, accidents with snakes of the *Bothrops* genus, wide distributed throughout the national territory, stand out in number and severity, being of great relevance due to permanent sequels characterized mainly by the loss of the affected limb [3]. With similar relevance, considering the severity of the local effects induced by their venoms, are the snakes of the *Lachesis* genus, although they have a geographical distribution restricted to the Amazon basin (*Lachesis muta*) and the Atlantic forest, from north of Rio de Janeiro to Paraiba (*Lachesis rhombeata*)[4].

The search for new inhibitors of isolated toxins and snake venoms is essential to complement or even to replace the traditional serum therapy treatment, once it is not very effective in counteracting the local effects observed

after snakebites and it is not very accessible to high-risk areas. The popular use of medicinal plants for the treatment of snakebites has been consecrated, once they have a broad spectrum of effects [5][6][2][7][8][9].This fact stands out the economic and medical importance of ethnopharmacological studies that evaluate the therapeutic properties of plant species. These studies have been exploring the possibility of using plant extracts and isolated active principles for the inhibition of clotting/hemorrhage, inflammation, myotoxicity, edema, cardiotoxicity, among others. The active components derived from plants usually act as enzyme inhibitors, inactivating chemicals or immunomodulation agents capable of interacting with macromolecules present in the venom from different species of snakes or with target molecules belonging to the animal organism [6].Some of the tests used to evaluate the inhibitory potential of plant compounds against snake venoms are the determination of the effects on blood clotting and fibrinogenolysis. Proteases are the major responsible for these activities, corresponding to the main classes involved in necrosis and hemorrhage induction [2][10][7][11][12][8].

Although consisting of a wide variety of molecules with pharmacological properties, many of these scientific studies reporting the antiophidian properties of essential oils are scarce. The inhibitory properties of ethanolic extracts and the essential oil extracted from the leaves of *Nectandra angustifolia* on clotting and the hemolysis induced by the *Bothrops neuwiedi* venom were reported [12].

The chemical composition of essential oils is characteristic to each species, presenting biological function vital to the survival and adaptation of the plants to the environment. Even though they are complex mixtures of chemicals, phenylpropanoids and terpenoids predominate in their compositions, and, within this last class, the monoterpenes are the compounds found in greater proportion in essential oils, followed by sesquiterpenes. The combination of constituents of each essential oil is related to its pharmacological properties [13]. Among the

plants producing essential oils, the Asteraceae family has been widely explored because it is the largest systematic group of Angiosperms, comprising about 1,100 genera and 25,000 species [14].

Considering the wide applicability of secondary metabolites present in essential oils in pharmaceutical, cosmetic and food sciences, and the scarcity of studies on antiophidian properties, a new and promising line of research to be explored is the pharmacological characterization of these oils with a view to the development of products for the treatment of snakebites. Therefore, the aim of this study was to assess the inhibitory properties of essential oils extracted from the leaves of four species of the Asteraceae family (*Baccharis dracunculifolia*, *Conyza bonariesis*, *Tithonia diversifolia* and *Ambrosia polystachya*) on the coagulating and fibrinogenolysis activities induced by *Lachesis muta, Bothrops atrox* and *Bothrops moojeni* snake venoms.

2. MATERIAL AND METHODS

2.1. Plant material and essential oil isolation

The young leaves (rib and limbo) from adult plants species (*Baccharis dracunculifolia*, *Conyza bonariesis*, *Tithonia diversifolia* and *Ambrosia polystachya*) belonging to the Asteraceae family were collected around 08:00 hours on the Campus of the Universidade Federal de Lavras (UFLA) (21° 14‘S, longitude 45° 00 ‘W Gr. and 918 m altitude), on days without precipitation in the month of February 2012. Species identification was kindly performed by Doctor Mariana Esteves Mansanares, Department of Biology of UFLA and exsiccates were deposited in the Herbarium ESAL at UFLA under the registration numbers 26946, 26947, 26945 and 26948, respectively.

The essential oils from fresh leaves were extracted by hydrodistillation using a modified Clevenger apparatus adapted to a 4-L round-bottomed flask for

a period of 2 h [15]. The hydrosols were centrifuged for 5 minutes at 965*g*, and the oils were packaged in amber glass bottles and stored at a temperature of 4ºC.

2.2. Citrated human plasma

Blood samples were obtained from the researchers involved in the work, in good health and with normal tests for BT (Bleeding Time), CT (Clotting Time), TPA (Time for Prothrombin Activation) and APTT (Activated Partial Thromboplastin Time). The samples were collected in BD Vacutainer® tubes containing 0.105 and 0.109 mol buffered sodium citrate (3.2%), at a ratio of nine parts of blood to one part of citrate solution, as recommended by the CLSI (Clinical and Laboratory Standards Institute). This study was approved by the Committee of Ethics in Research with Humans of the UFLA and filed with the number 09978312.8.0000.5148.

2.3. Snake venoms

Desiccated *Lachesis muta, Bothrops atrox* and *Bothrops moojeni* venoms were purchased from Bioagents Serpentarium (Batatais, SP, Brazil) and stored at 4°C until the preparation of solutions for use in the biological tests. The venoms were weighted and then solubilized in phosphate buffered saline (PBS).

2.4. Effects of the essential oils on clotting: induction or inhibition

The coagulant potential of the essential oils was assessed as previously described [16]. Volumes of 200 µl of citrated human plasma were maintained in a water bath at 37°C, and the essential oils were added in different volumes (0.6 and 1.2 µL), following by measurement of time and observation. The samples were gently shaken every 5 minutes for 45 minutes. The incoagulability was defined as the absence of clot after 24 hours of observation. The inhibitory action of the essential oils on the coagulant activity induced by the venoms was also performed [17]. Pilot tests were performed to each venom in order to determine the necessary dose to clot 200 µL of citrated plasma at 37°C in times between 40 and 120 seconds.

Two interacting variables were evaluated: the first predict the possible interaction between essential oil constituents and blood plasma components; the second considered the possible interaction between essential oil constituents and coagulating toxins present in the venoms. In the first test, prior incubation of the plasma (200 µL) with different volumes of the essential oils (0.6 and 1.2 µl) was performed at 37°C for 15 minutes, followed by the addition of 1 µL (10 µg) of each venom (*L. muta*, *B. atrox* and *B. moojeni*) separately and measurement of time. In the second test, different volumes of the essential oils (1.2 and 0.6 µL) were incubated with the snake venoms (1 µL; 10 µg) for 15 minutes at 37°C, followed by the addition of plasma (200 µL), and measurement of time until clot formation.

2.5. The fibrinogenolytic and the antifibrinogenolytic activities of the essential oils

These activities were performed according to Czaikoski et al. (2010) [18]. Essential oils (0.6 and 1.2 µl) were incubated with bovine fibrinogen (60 µg; 6 µl) for 1.5 h at 37°C in a final volume of 25 µl (PBS). The reactions were stopped by adding 10 µL of bromophenol blue solution (Tris-HCl 0.05 mol/L, pH 8.0, containing10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate (SDS) and 0.05% (w/v) bromophenol blue) and heating of samples for 5 minutes at 100°C. The samples were analyzed by polyacrylamide (acrylamide: bis-acrylamide, 19:1) gel electrophoresis at12% with SDS (SDS-PAGE) [19]. A control sample containing only fibrinogen (60 µg in 25 µl of PBS) was submitted to electrophoresis under the same conditions.

The inhibitory potential of the essential oils on the proteolysis induced by *L. muta*, *B. atrox* and *B. moojeni* venoms was assessed by SDS-PAGE. The essential oils (0.6 and 1.2 µl) and snake venoms (30 µg) were pre incubated for 30 minutes at 37°C at first, followed by addition of fibrinogen molecules (60µg) and subsequent incubation for an additional 1.5 h. After that, the essential oils

and fibrinogen molecules were incubated for 30 minutes at 37°C, followed by addition of snake venoms and subsequent incubation for an additional 1.5 h.

The reactions were stopped by adding 10 μL of bromophenol blue solution and heating for 5 minutes at 100°C, and the samples were analyzed by SDS-PAGE [19].

2.6. Statistical Analysis

The experimental designs used for the clotting inhibition tests were completely randomized blocks in 3 x 4 (3 concentrations x 4 essential oils with three repetitions) factorial schemes. Essential oils were compared with each other because they were extracted from species of the same botanical family. The significant factors by the F test ($p < 0.05$) were subjected to the Scott-Knott test of means (5%) for determination of the models. The data were analyzed by the statistical program Sisvar [20].

3. RESULTS AND DISCUSSION

The majority constituents of the essential oils from fresh leaves of *B. dracunculifolia* were limonene (30.9%), *trans*-nerolidol (22.4%) and β-pinene (14.5%), while those in the oil from *C. bonariesis* were limonene (56.7%), *trans*-β-ocimene (26.3%) and *cis*-verbenol (4.4%). The oil from *T. diversifolia* present as majority constituents β-pinene (38.3%), α-pinene (28.6%) and limonene (8.8%), and the main compounds found in oil from *A. polystachya* were germacreme D (29%), *trans*-β-ocimene (14%) and β-caryophyllene (10%) [21].

In the characterization of the components present in the essential oils from species of the Asteraceae family, *B. dracunculifolia*, *C. bonariesis*, *T. diversifolia* and *A. polystachya,* 97.3, 95.2, 99.7 and 97.3% of constituents were identified, respectively. Among these components, the high concentrations of terpenes stand out, with the monoterpenes in the essential oils of *C. bonariensis*,

T. diversifolia and *B. dracunculifolia,* and sesquiterpenes in the essential oil from *A. polystachya*. The essential oils from *B. dracunculifolia*, *C. bonariesis*, *T. diversifolia* and *A. polystachya* presented 58.5%, 92.2%, 86.3% and 34.2% of monoterpenes, and 36.6%, 5.1%, 13.4% and 62.7% of sesquiterpenes, respectively [21].

Although belonging to the same family, the essential oils evaluated presented different chemical compositions that are directly related to their therapeutic properties. The variability in the constitution of essential oils is directly related to the species, and, within the same species, several factors can affect the composition, such as chemotype, location, collection period, vegetative cycle, among others [22]. Additionally, the authors suggested that the biological and pharmacological properties of secondary metabolites are related to the group of compounds characteristic of each species. The essential oils are mixtures of numerous compounds, with one or two major constituents, and their biological activities can be attributed to a synergic action between the molecules [13].

Among the pharmacological potentials, the antiophidian properties of the essential oils from species of the Asteraceae family were partially evaluated in this work. Snakebites represent a public health problem in Brazil, and the search for natural compounds that act effectively in neutralizing especially the local effects induced by toxins are of fundamental importance [23].

The essential oils (0.6 and 1.2 μL) assessed showed no clotting activity over a 24 h period. After previous incubation of venoms and oils, the clotting times (CT) obtained for *L. muta* and *B. moojeni* venoms were significantly increased by all the essential oils (Table1). The use of the essential oil from *C. bonariensis* extended the clotting time of *L. muta* venom from 52.2 to 115.2 seconds and that of *B. moojeni* from 108.3 to 2340.0 seconds. The concentration of the essential oils was significant only for the action of the oil from *T.*

diversifolia against *L. muta* venom and that from *C. bonariensis* against *B. moojeni* venom.

Table 1 - Clotting time of citrated human plasma after the addition of essential oils from *B. dracunculifolia*, *C. bonariensis, T. diversifolia,* and *A. polystachya*previously incubated with *L. muta*, *B. moojeni* and *B. atrox*venoms.

Essential oil (µL)	**Clotting time (sec.)**		
	Venom (10 µg)		
	L. muta	***B. moojeni***	***B. atrox***
B. dracunculifolia			
0	52.2 ± 0.5 Ab	108.3 ± 0.4 Aa	100.8 ± 2.0 Ab
0.6	82.8 ± 2.6 Ca	184.8 ± 2.4 Ba	143.5 ± 1.7 Ca
1.2	87.0 ± 2.0 Ca	234.9 ± 1.9 Ba	154.5 ± 4.5 Da
C. bonariensis			
0	52.2 ± 0.5 Ab	108.3 ± 0.4 Ac	100.8 ± 2.0 Ac
0.6	110.1 ± 1.1 Aa	1977.9 ± 50.6 Ab	191.1 ± 0.6 Bb
1.2	115.2 ± 1.0 Aa	2340.0 ± 52.9 Aa	264.0 ± 2.4 Aa
T. diversifolia			
0	52.2 ± 0.5 Ac	108.3 ± 0.4 Ab	100.8 ± 2.0 Ac
0.6	75.6 ± 1.7 Cb	283.5 ± 3.8 Ba	207.9 ± 1.9 Ab
1.2	91.2 ± 1.8 Ca	315.6 ± 2.6 Ba	227.7 ± 2.7 Ba
A. polystachya			
0	52.2 ± 0.5 Ab	108.3 ± 0.4 Aa	100.8 ± 2.0 Ac
0.6	96.0 ± 1.4 Ba	174.0 ± 1.8 Ba	156.9 ± 0.8 Cb
1.2	99.9 ± 1.8 Ba	188.4 ± 8.3 Ba	180.0 ± 2.1 Ca

For each venom analyzed, the means followed by the same letter (uppercase for comparisons between essential oils and lower case for comparison of the concentrations), do not differ significantly at the 5% probability by Scott-Knott test. *The previous incubations, containing oils and venoms, were performed for 15 minutes at 37^{o}C, followed by the addition of plasma and measurement of time.

The coagulant activity of *B. atrox* venom was inhibited by all the essential oils. The longest CTs were observed after incubation with 1.2 µL of *C. bonariensis* and *T. diversifolia*, with increases in the clotting times from 100.8 seconds to 264.0 and 227.7 seconds, respectively.

The inhibitory effect of plant constituents on the clotting induced by venoms was also observed when the antiophidian activity of the aqueous extract of *Bauhinia forficate* leaves against *B. moojeni* and *Crotalus durissus terrificus* venoms was studied [24]. Previous incubation of the extract with the venom in different proportions (w/w) resulted in partial inhibition of clotting and 100% inhibition at a ratio of 1:200 (w/w). The extract of this plant was considered to be a promising source of natural inhibitors of serine proteases, the main enzymes involved in clotting disorders induced by snake venoms. In the present study, the inhibitory effect was observed for all the essential oils. The partial inhibitory effect observed for the oils can be related to the volumes employed, which were lower than those generally used in the tests involving aqueous extracts because of limitations of the method to the use of compositions of low-polarity.

The venoms from *L. muta*, *B. moojeni* and *B. atrox* have different constitutions and, consequently, possess different action mechanisms. However, all of them act on the blood clotting cascade, inducing clotting in the absence of calcium [25][4]. Only terpenes were identified in all of the essential oils evaluated, with individual variations in the contents of mono-and sesquiterpenes. The results obtained suggest that some constituents of the essential oils interacted with proteins present in the venoms to reduce the rate of clotting. In addition, the clotting induced by the venoms was inhibited largely by the essential oil of *C. bonariensis*, which has the highest content of monoterpenes (92.2%) among the essential oils evaluated.

Tests with previous incubation of the oils with plasma and subsequent addition of venoms were also conducted (Table 2) to determine whether a possible interaction occurred between the constituents of the oils and proteins of the clotting cascade. Unlike the first incubation described, all the essential oils accelerated the clotting time induced by *L. muta* venom. No significant differences in the proclotting effects of *B. dracunculifolia*, *C. bonariensis* and *A.*

polystachya were observed, but these effects were statistically lower than those observed for the oil from *T. diversifolia*.

Table 2 - Clotting times for citrated human plasma previously incubated with different volumes of the essential oils from *B. dracunculifolia*, *C. bonariensis, T. diversifolia*and *A. polystachya*, followed by the addition of *L. muta*, *B. moojeni* and *B. atrox* venoms.

Essential Oil (µL)	**Clotting time (sec.)**		
	Venom (10 µg)		
	L. muta	***B. moojeni***	***B. atrox***
B. dracunculifolia			
0	45.0 ± 1.5 Aa	114.3 ± 2.7 Ab	106.5 ± 0.9 Aa
0.6	39.6 ± 1.5 Bb	117.0 ± 0.6 Aa	106.8 ± 2.4 Ba
1.2	33.0 ± 0.6 Bb	114.6 ± 1.8 Aa	87.6 ± 0.5 Bb
C. bonariensis			
0	45.0 ± 1.5 Aa	114.3 ± 2.7 Ab	106.5 ± 0.9 Aa
0.6	34.2 ± 0.8 Bb	117.9 ± 1.2 Aa	84.3 ± 0.9 Db
1.2	33.6 ± 0.7 Bb	119.7 ± 0.5 Aa	89.4 ± 1.3 Bb
T. diversifolia			
0	45.0 ± 1.5 Aa	114.3 ± 2.7 Ab	106.5 ± 0.9 Ab
0.6	43.2 ± 1.4 Ab	123.6 ± 2.5 Aa	116.4 ± 1.2 Aa
1.2	42.9 ± 1.2 Ab	122.4 ± 1.9 Aa	114.3 ± 1.3 Aa
A. polystachya			
0	45.0 ± 1.5 Aa	114.3 ± 2.7 Ab	106.5 ± 0.9 Aa
0.6	36.3 ± 1.0 Bb	127.8 ± 2.0 Aa	95.1 ± 2.3 Cb
1.2	37.2 ± 0.9 Bb	123.6 ± 1.9 Aa	95.4 ± 2.7 Bb

For each venom analyzed, the mean followed by the same letter (uppercase to comparison between the essential oils tested and lowercase for comparison of concentrations of each essential oil), do not differ significantly at 5% of probability by the Scott-Knott test. *The previous incubations of the essential oils with the plasma were performed for 15 minutes at 37°C, followed by the addition of venom and measurement of clotting time.

The clotting time increased to values statistically insignificant with the addition of *B. moojeni* venom after incubation of the oils (0.6 and 1.2 µl) with plasma. The oil of *T. diversifolia* inhibited the coagulation, extending the plasma

clotting time to statistically significant values (CT: 116.4 ± 1.2 for 0.6 µl of oil; CT: 114.3 ± 1.3 for 1.2 µL of oil), featuring anticlotting effect. However, the oils of *B. dracunculifolia* (CT: 106.8 ± 2.4 and 87.6 ± 0.5 to 0.6 and 1.2 µl, respectively), *C. bonariensis* (CT: 84.3 ± 0.9 and 89.4 ± 1.3) and *A. polystachya* (CT: 95.1 ± 2.3 and 95.4 ± 2.7) were proclotting in the presence of the venom of *B. atrox* (CT: 106.5 ± 0.9) under the same conditions of incubation.

The ethanol extract of leaves from *Nectandra angustifolia* was effective in inhibiting the hemolytic and coagulant activities induced by *Bothrops neuwiedi* venom, while the essential oil from the same plant was only active against the coagulant effect [12]. The authors analyzed the essential oil by GC and GC-MS and found α-pinene, β-pinene and limonene. The same compounds were the predominant molecules in the essential oils evaluated in the present study.

Ten ethanol extracts obtained from species belonging to different families inhibited 100% of the defibrinogenation induced by the *B. asper* venom, when evaluated *in vivo.* They also inhibited the clotting induced by that same venom [26].

The aqueous extract of leaves from *Casearia sylvestris* inhibited the proteases of venoms from various *Bothrops* species, neutralizing the hemorrhagic activity and partially inhibiting the proteolytic activity evaluated on the substrates casein and fibrinogen, and the coagulant effect [27]. Although the aqueous extract of *Mandevilla velutina* has been primarily reported as an inhibitor of phospholipasesA_2, the inhibitory potential on the hemorrhagic, fibrinogenolytic and caseinolytic activities, attributed mainly to proteases, has also been observed [28]. Thus, it's possible to conclude that plant molecules of different polarities interact with the different classes of enzymes present in the venoms, as well as with free molecules and animal cell components to protect

them, resulting in partial or total inhibition of local symptoms of snakebites, especially by *Bothrops* venoms.

A review on medicinal plants reported nine plants species from Asteraceae family (*Baccharis sp.*, *Bidens pilosa*, *Calendula officinalis*, *Clibadium silvestre*, *Cynarasco lymus*, *Eclipat aprostata*, *Lychnophora pinaster*, *Mikania glomerata* and *Solidago chilensis*) with inhibitory properties against snake venoms [29]. The plants are potential inhibitors of toxic and pharmacological effects induced mainly by phospholipases A_2 and proteases present in snake venoms from *Bothrops*, *Crotalus*, *Agkistrodon*, *Echis* and *Calloselasma* genera. No studies of this nature have been performed with the extracts or essential oils from the plant species evaluated in the present work.

Compounds isolated from species of Asteraceae with antiophidian properties have also been reported, such as the diterpeneclerodane (Bt-CD) isolated from *Baccharis trimera*, cynarin from *Cynaras colymus*, wedelolactone, sitosterol, stigmasterol, D-mannitol and dimethylwedelolactone from *Eclipta prostata*, coumarin from *Mikania glomerata,* silymarin from *Silybum marianum*, and caffeic acid and chromogenic acid derivatives from *Vernonia condensata*. Although there are reports of proteins and steroids with antiophidian properties, these properties stand out in phenolic compounds (flavonoids, cumestans, pterocarpans, nitro-compounds, tannic acid and others) and terpenes (mainly diterpenes and triterpenes) [29].

Mendes et al. (2008) [2], assessed the antiophidian potential of the aqueous extract of *Schizolobium parahyba* (Caesalpinoideae) and found that the clotting induced by *B. pauloensis* venom was partially inhibited by the extract at ratios of 1:1 and 1:5 (venom/extract, w/w). The extract prolonged the clotting time around three and eight times, respectively, to the 1:10 and 1:50 w/w proportions. However, very weak inhibition was observed when the clotting was induced with *C. durissus terrificus* venom.

The inhibitory potential of essential oils against *Bothrops* venoms was higher than that observed for *Crotalus* (results not shown), reinforcing the hypothesis of occurrence of a specific interaction between oil compounds and proteins present in the venoms from different snake species.

In general, the essential oils acting on the clotting induced by snake venoms increase or decrease the clotting time after previous incubation with plasma or with venoms. A variety of synergistic interactions between the molecules could be better exploited in future studies using standards corresponding to major compounds, both separately or in mixtures that simulate the composition of the oils. In addition, there are some hypotheses to be evaluated regarding the possible existence of interactions between terpenes and proteins involved in the blood coagulation cascade, such as fibrinogen and thrombin.

Fibrinogen is a dimeric glycoprotein of 340 kDa present in the blood, which consists of polypeptide chains (α, β and γ) with molecular weights of 66.5, 52 and 47 kDa, respectively [30]. Fibrinogen molecules have been widely used as substrates to evaluate the proteolytic effect induced by venoms related to hemorrhagic and/or thrombolytic activities, once isolated proteases may be coagulants, anticoagulants or proclotting [31].

These essential oils from *B. dracunculifolia*, *C. bonariensis, T. diversifolia* and *A. polystachya,* previously incubated with fibrinogen solution, did not induce cleavage or differential migration of the fibrinogen chains (α, β and γ) in the analyzes performed by polyacrylamide gel electrophoresis. However, some molecules of fibrinogen incubated with the essential oils resisted reduction induced by β-mercaptoethanol and heating at 100º for 5 minutes. This result suggests that an interaction between molecules of fibrinogen and constituents of essential oils may have protected the disulfide bridges that

connect those chains. Some examples of the effect observed during the analysis can be viewed in Figure 1A.

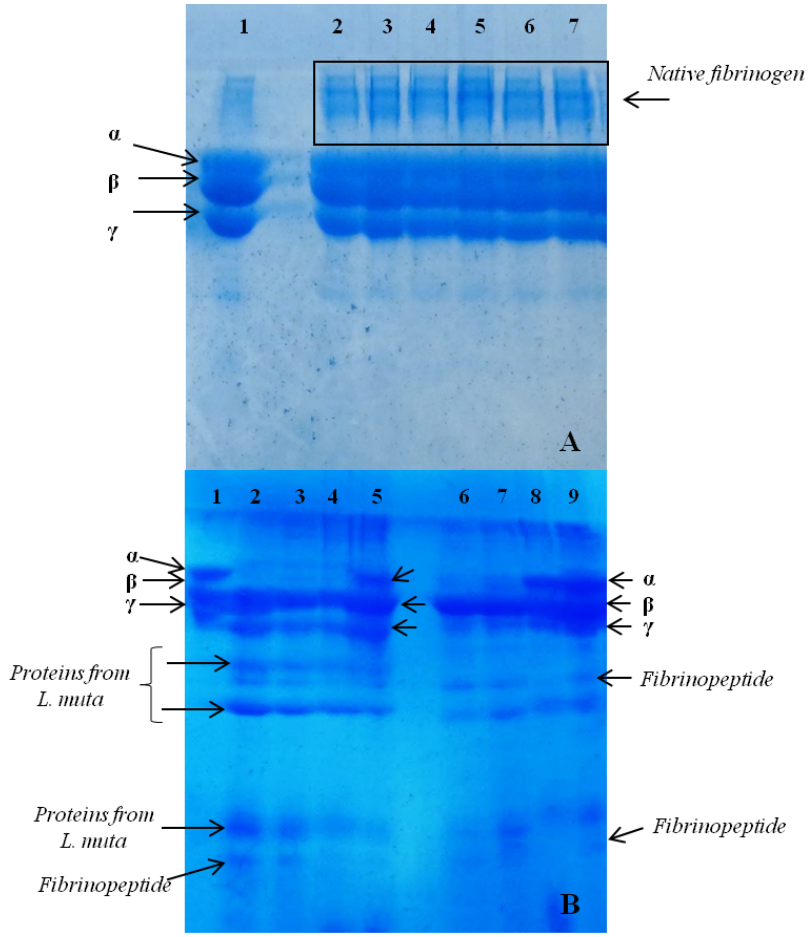

Figure 1. SDS-PAGE.**(A)** Samples: **1-** Fibrinogen (60 µg); **2-** Fibrinogen + EO from *C. bonariensis*(0.6 µl); **3-** Fibrinogen + EO from *T. diversifolia*(0.6 µl); **4-** Fibrinogen + EO from *B. dracunculifolia*(0.6 µl); **5-** Fibrinogen + EO from *B. dracunculifolia*(1.2 µl); **6-** Fibrinogen + EO from *A. polystachya*(0.6 µl); **7-** Fibrinogen + EO from *A. polystachya*(1.2 µl). **(B)** Samples: **1-** Fibrinogen (60 µg); **2-** Fibrinogen + *L. muta*venom (30 µg), 30 min. of incubation; **3-** Fibrinogen + *L. muta,* 60 min. of incubation; **4-***L. muta*venom + EO from *B. dracunculifolia*(1.2 µl) (previously incubated for 30 min.) + fibrinogen (60 min. of incubation); **5-** Fibrinogen + EO from *B. dracunculifolia*(previously incubated for 30 min.) + *L. muta*venom (60 min. of incubation); **6-** Fibrinogen + *B. moojeni*venom, 30 min. of incubation; **7-** Fibrinogen +

*B. moojeni*venom,60 min. of incubation; **8-***B. moojeni*venom + EO from *A. polystachya*(1.2 µl) (previously incubated for 30 min.) + fibrinogen (60 min. of incubation); **9-** Fibrinogen + EO from *A. polystachya*(previously incubated for 30 min.) + *B. moojeni*venom(60 min. of incubation).

L. muta, B. moojeni and *B. atrox* venoms were used as sources of proteolytic agents to induce the cleavage mainly of the α chain of fibrinogen molecules (Figure 1B).The fibrinogenolytic activity of *B. atrox* venom was not inhibited by essential oils from *B. dracunculifolia*, *C. bonariensis, T. diversifolia* or *A. polystachya*. Batroxase, a metalloproteinase with high fibrinogenolytic and thrombolytic activities present in this venom, is probably one of the enzymes responsible for the proteolysis observed, which was not inhibited [3].

Several experiments revealed the existence of interactions between some oils and fibrinogen molecules. Tests using different volumes of oils, incubation times, run times, concentrations of polyacrylamide in the gel and variations of the staining times in the presence of Coomassie Brilliant Blue (results not shown) resulted in the conclusion that the fibrinogen molecules were not degraded and that they could be interacting with constituents of the oils, making it impossible to bind the dye. Coomassie Brilliant Blue interacts with protein macromolecules containing amino acids side chains with basic or aromatic character. Several substances can interfere in these interactions, including polyphenols and polyphenol oxidases [32], which could present binding mechanisms similar to those of the molecules present in the essential oils evaluated in the present work.

The fibrinogenolysis induced by *L. muta* venom was only inhibited when the *B. dracunculifolia* oil was previously incubated with fibrinogen, with subsequent addition of the venom. This fact reinforces the hypothesis that there are interactions between constituents of the oils and the fibrinogen molecules (Figure 1B, sample 5). It is suggested that molecules of the sesquiterpene

fraction of this oil (36.6%) protect the fibrinogen molecules against the action of proteases present in *L. muta* venom.

The essential oil from *A. polystachya* proved to be effective in inhibiting the fibrinogenolytic activity induced by *B. moojeni* venom in trials with previous incubation with fibrinogen or venom (Figure 1B, samples 8 and 9, respectively). The fact that the *B. dracunculifolia* oil inhibited *L. muta* venom, and the oil from *A. polystachya* only inhibited *B. moojeni* venom, indicates the presence of specific and not random interactions, considering the wide composition range and diversity of homologous proteins present in snake venoms [2].

These observations indicate the possibility that the essential oil components from *A. polystachya*, mostly sesquiterpenoids (62.7%), connect efficiently with fibrinogen, thereby reducing the fibrinogenolysis induced by the venom. In addition, incubation of *A. polystachya* with *B. moojeni* venom and evaluation of the results by SDS-PAGE confirmed the interactions of compounds present in the oils with proteins present in the venoms. Bands with molecular weights corresponding to phospholipases A_2 were observed after staining, but not those corresponding to proteases (results not shown).

A 100% inhibition of the fibrinogenolytic activity of *Bothrops* venoms was observed after incubation with aqueous extract of *Shizolobium parahyba,* suggesting that the tannins participate in the inhibitory effect [10]. The interactions that occur between tannins and proteins are influenced by the number of these molecules present in the reaction medium, as well as by the pH and ionic strength of the medium [33]. Thus, future studies evaluating variations in the reaction medium during the incubations of essential oils with snake venoms would make it possible to define more efficiently the mechanisms of these inhibitory effects.

The essential oils from *C. bonariesis* and *T. diversifolia,* withless inhibitory effect on the activities induced by the venoms, are composed

predominantly by monoterpenes (92.2 and 86.3%, respectively), with sesquiterpenoids only comprising 5.1 and 13.4% of the oils. The compositions of these oils were very different from those of the most active essential oils (*B. dracunculifolia* and *A. polystachya*). However, the oils evaluated presented some relevant therapeutic properties since all inhibited the clotting and fibrinogenolysis induced by the venoms.

Considering the low degree of effectiveness of traditional therapy with antibodies in the treatment of the local effects induced by snakebites, the essential oils hold promise for topical use as potential inhibitors of venom toxins. The oils in general do not require specific pharmaceutical preparations and could have direct applications soon after extraction. Many oils with antimicrobial, anti-inflammatory and healing properties are described in the literature, and these actions are of great value in the treatment of snake envenomations. Further studies on the isolation of constituents of these oils and the elucidation of the mechanisms of interaction between secondary metabolites, venom proteins and animal proteins are essential for the development of new antiophidian therapies and to establish the ideal conditions for the application of essential oils.

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ARTIGO 6 - ESSENTIAL OILS OF *HEDYCHIUM CORONARIUM*: INHIBITION OF COAGULATION AND FIBRINOGENOLYSIS INDUCED BY *BOTHROPS* AND *LACHESIS* SNAKE VENOMS.

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Essential oils of *Hedychium coronarium*: inhibition of coagulation and fibrinogenolysis induced by *Bothrops* and *Lachesis* snake venoms

ABSTRACT

The search for new inhibitors of snake venom toxins is essential to complement or even replace traditional antivenom therapy, especially in relation to compounds that neutralize the local effects induced by envenomations. Plant species possess bioactive secondary metabolites such as essential oils, which, besides their possible use as alternative to traditional antivenom therapy, can be extracted from weeds that are considered substantial problems for agriculture, such as *Hedychium coronarium* species. In this context, the essential oils of leaves and rhizomes of *H.coronarium* species were extracted by hydrodistillation, and their potential inhibitory effects on the coagulant and fibrinogenolytic activities induced by the venoms of *Lachesis muta*, *Bothrops atrox* and *Bothrops moojeni* were evaluated. All oils evaluated, when previously incubated with venoms, were able to inhibit the clotting effect, with less inhibition when oils and plasma were preincubated prior to the addition of venoms. The essential oils showed no effectiveness in inhibiting the fibrinogenolysis induced by the venoms evaluated, when previously incubated with venoms or fibrinogen. The results suggest the presence of interactions between the oils and venom proteases, especially serine proteases. Thus, the essential oils can be used as an alternative to complement the serum therapy,

especially considering that these plant metabolites generally do not require specific formulations and may be used topically immediately after extraction.

1. INTRODUCTION

Weeds are a serious threat to the conservation of native plants and cultivated crops, altering the balance of ecosystems, with consequent damage to agriculture. The removal of these weeds has been carried out by physical methods, such as burning and hoeing or mainly by chemical methods such as the use of synthetic herbicides (Gianessi, 2013). Although efficient in the functional point of view, these agrochemicals have low selectivity, acting indiscriminately on invasive and cultivated species and changing them morphologically and physiologically. These alterations make it hard to determine current and especially future changes to the animal species that consume these plants (De Almeida et al. 2010).

According to Gianessi (2013), manual hoeing of weeds at ideal times doesn't reduce crop yields in relation to crops in which this control is accomplished with the use of herbicides. Considering this, it is important to propose an alternative and economically and ecologically sustainable destination for these species, such as the exploration of the biological potential of their secondary metabolites, among which we highlight the essential oils.

Essential oils are complex mixtures of volatile, lipophilic and odoriferous substances from various plant tissues. They are composed primarily of terpenoids, predominantly monoterpenes, sesquiterpenes and their oxygenated derivatives such as alcohols, ketones, aldehydes, esters, phenols and oxides. These metabolites play essential biological functions for survival and adaptation of the plant to the environment, as well as present pharmacological activities of medical-scientific interest (Silva et al., 2012).

Hedychium coronarium stands out among the invasive exotic species, an aquatic monocot belonging to the botanical family Zingiberaceae, widely distributed in Brazil and adapted to the Cerrado fields, coastal forests of Serra do Mar, the Araucaria rainforests, coastal and in land forests of Bahia, Caatinga and Atlantic Forest of High Parana (Zenni and Ziller, 2011).

The biological potential of the essential oils, extracted from rhizomes of this species, has been widely explored and there are reports of antibacterial (Joy et al., 2007; Sabulal et al., 2007; Joshi et al ., 2008; Prakash et al., 2010; Ho, 2011), antifungal (Joy et al., 2007; Salubal et al., 2007; Ho, 2011), larvicide (Ho, 2011) and antioxidant activities (Joshi et al., 2008; Ho, 2011). Researches with essential oils extracted from leaves are restricted to a single work published by Ho (2011), reporting antioxidant, larvicide, antifungal and antibacterial activities. Antiophidian studies to evaluate the properties of oils extracted from the rhizomes and leaves of *H. coronarium* species, exploring their potential pharmacological effects on coagulation and fibrinogenolysis, had not been described in the literature up until the present work.

Snake envenomations are a problem of public health that deserves the attention of health authorities, constituting an occupational and environmental disease that mainly affects rural workers in Brazil (Souza et al., 2012). Snakes of the *Bothrops* genus are responsible for most accidents in Latin America, although the *Lachesis* genus, widely distributed in humid regions as the remote rainforest areas in Central America and South America, is responsible for accidents of equal severity, resulting in prominent local effects, permanent sequels and even death (Sanz et al., 2008).

Snake venoms are mainly composed of proteins with enzymatic activity, belonging to different classes, such as L-amino acid oxidases, phospholipases A_2, serine proteases and metalloproteases, the two latter being the main responsible for effects on hemostasis (Sanz et al., 2008). The combination of

these protein compounds is directly related to the damage caused by these venoms, whose pathophysiology includes local effects (intense pain, edema, hemorrhage and necrosis) and /or systemic effects, such as nausea, coagulopathy, hypotension, cardiovascular shock and kidney disorders (Morais et al., 2012).

Plant extracts have been shown to be a promising alternative for the treatment of snakebites, and in many countries, their use for this purpose is already quite traditional even though often carried out empirically, without scientific evidences of their efficacy (Maiorano et al., 2005). Several studies are wagering on the use of plant extracts as sources of molecular prototypes which can be used in the treatment of snake envenomations, or to complement the traditional antivenoms, which are little effective in minimizing the local damages (Maiorano et al., 2005; Cavalcante et al., 2007; Mendes et al., 2008; Vale et al., 2008; Diego et al., 2009; Torres et al., 2011; Da Silva et al., 2012; Vásquez et al., 2013). However, studies employing essential oils for that purpose are still restricted (Torres et al., 2011).

With that in mind, the present study aimed to evaluate the inhibitory potential of essential oils extracted from leaves and rhizomes of *H. coronarium* species on the coagulant and fibrinogenolytic activities induced by *Lachesis muta*, *Bothrops atrox* and *Bothrops moojeni* snake species.

2. MATERIAL AND METHODS

2.1. Collection and registration of plant material

Hedychium coronarium plant species was collected at the Federal University of Lavras (UFLA), in Lavras / MG (21 º 14 'S, longitude 45 º 00' W Gr and 918 m altitude), around 8:00 am on February 25th, 2012. Young leaves (rib and limb) and rhizomes of mature plants at the stage of full flowering were harvested. Species identification was kindly performed by Doctor Mariana

Esteves Mansanares, Department of Biology of UFLA and exsiccate was deposited in the Herbarium ESAL UFLA under the registration number 26942.

2.2. Essential Oil Extraction

The essential oils from fresh leaves were extracted by steam distillation using a modified Clevenger apparatus, adapted to a round bottom flask with a capacity of 4 liters, for a period of 2 hours, according to the methodology described by the Farmacopeia Brasileira (2000). Resulting samples were centrifuged for 5 minutes at 965.36 g, and the essential oils were separated with a Pasteur pipette, disposed in amber glass bottles and stored at 4°C. The oil volumes used during the analyses were selected from the results obtained in toxicity tests (results not shown).

2.3. Obtaining human citrated plasma

Blood samples were obtained from the researchers involved in the work, which presented good health and normal exams of BT (Bleeding Time), CT (clotting time), PAT (Prothrombin Activity Time), PATT (Partially Activated Thromboplastin Time). The samples were collected in BD Vacutainer ® tubes with buffered sodium citrate 0.109 mol and 0.105 mol (3.2%) in the proportion of nine parts of blood to one part of citrate solution, as recommended by CLSI (Clinical and Laboratory Standards Institute). The original project whose present work is included was approved by Ethics Commit in Human Research of UFLA, protocol number 09978312.8.0000.5148.

2.4. Snake venoms

Crystallized venoms corresponding to a single specimen of each snake species (*Lachesis muta*, *Bothrops atrox* and *Bothrops moojeni*) were purchased from BioAgents Serpentarium, located in Batatais- SP, and stored at 4°C until their use in the biological assays. At this point, venoms were dissolved in phosphate buffered saline (PBS) at different concentrations (g/ml).

2.5. Effects of essential oils on coagulation: induction and/or inhibition of clot formation

The coagulant capacity of essential oils was previously evaluated according to the methodology described by Selistre et al. (1990), with adaptations related to the volumes of samples evaluated. Volumes of 200 µL of citrated human plasma had their temperatures previously stabilized in a water bath at 37°C. Then, essential oils were added separately in tubes at different volumes (0.6 and 1.2 µl) and the reaction mixtures remained under observation at 37°C for 24 hours. The observation was followed by gentle agitation every 5 minutes for a period of 45 minutes, and if no coagulation of the plasma could be observed, this observation was extended for a period of 24 hours, to define plasma uncoagulability.

The inhibitory action of oils on the coagulant activity induced by snake venoms was assessed according to the methodology described by Valentin and Lambeau (2000), with adaptations. Pilot tests were conducted to determine the concentration of each venom able to coagulate 200 µL of citrated plasma in a time interval between 40 and 120 seconds at 37°C.

We evaluated two possible ways of interaction for the oil molecules: firstly, with fibrinogen, and secondly with snake venom proteases. Accordingly, the first assay was made preincubating plasma (200 µl) with essential oils (0.6 and 1.2 µl) at 37°C for 15 minutes, proceeding with the subsequent addition of 1 µl (10 µg) of each venom (*L. muta*, *B. atrox* and *B. moojeni*) separately, measuring the time for the formation of a rigid clot. The second assay was performed with preincubation of essential oils (0.6 and 1.2 µl) with the same venoms (10 µg; 50 µg/mL) for 15 minutes at 37°C, followed by the addiction of plasma (200 µl) and measurement of the clotting time.

2.6. Effects of essential oils on the fibrinogen structure and the fibrinogenolytic activity induced by venoms

The activities were carried out according to the methodology described by Czaikoski et al. (2010), with modifications. The essential oils (0.6 and 1.2 µl) were incubated with bovine fibrinogen (60 µg) for 90 minutes at 37°C in a final volume of 25 µl (PBS). The reactions were stopped by addition of 10 µl of bromophenol blue solution (0.05 M Tris-HCl, pH 8.0, comprising 10% (v/v) glycerol, 10% (v/v) mercaptoethanol, 2% (w/v) sodium dodecyl sulfate (SDS) and 0.05% (w/v) bromophenol blue) and boiling the samples for 5 minutes in a water bath. The samples were analyzed by poliacrilamide gel electrophoresis at 12% (acrylamide: bisacrylamide, 19:1) in the presence of SDS (SDS-PAGE) under denaturing conditions (Laemmli, 1970). A sample control containing only fibrinogen was used for the visualization of the band pattern corresponding to α, β and γ chains of fibrinogen.

The inhibitory potential of the essential oils on the proteolytic action of *L. muta*, *B. atrox* and *B. moojeni* venoms was assessed by SDS-PAGE after preincubation of samples. We evaluated possible interactions between the essential oils (0.6 and 1.2 µl) and proteases present in the venoms (30 µg), incubating them (final volume of 25 µl; PBS) for 30 minutes at 37°C with subsequent addition of fibrinogen (60 µg) and incubation for 90 minutes at the same temperature. Possible interactions between oil molecules and fibrinogen were considered in another assay, preincubating bovine fibrinogen (60 µg) and essential oils (0.6 and 1.2 µl) for 30 minutes at 37°C with subsequent addition of each venom (*L. muta*, *B. atrox* and *B. moojeni*; 30 µg) and incubation for 90 minutes at the same temperature.

The reactions were stopped by addition of 10 µl of bromophenol blue solution and boiling for 5 min in a water bath, followed by analysis on SDS-PAGE under denaturing conditions, as described by Laemmli (1970).

2.7. Statistical Analysis

The experimental design used for testing the inhibitory action on coagulation were randomized in a 3x2 factorial arrangement (concentration x essential oils), with three replications. The essential oils were compared since they are extracted from different vegetal organs of the same botanical species, *H. coronarium*. Significant factors by F test ($p < 0.05$) were tested for average (Scott-Knott 5%) for the determination of the models. Data were analyzed using the Statistical Analysis System Variance for Balanced Data - Sisvar described by Ferreira (2011).

3. RESULTS AND DISCUSSION

We identified 99.2 and 99.3% of the chemical constituents of the essential oils of rhizomes and leaves of *H. coronarium*, respectively, being the two oils composed entirely by terpenoids. Among these terpene constituents, the oil extracted from rhizomes showed 98.2% monoterpenes and 1% sesquiterpenes, while that from leaves presented 84.3% monoterpenes and 14.9% sesquiterpenes. Among the major compounds of the oil from rhizomes are the monoterpenoids β-pinene (41.5%), 1.8-cineole (23.6%) and α-pinene (13.1%), and for the oil of leaves are the monoterpenoids β-pinene (46.9%) and α-pinene (19.2%), and the sesquiterpene β-caryophyllene (13.2%) (Miranda, 2013). Such variability observed in the chemical compositions of the essential oils of leaves and rhizomes of a botanical species is commonly observed according Gobbo-Neto and Lopes (2007), due to the fact that the location and distribution of secondary metabolites occur in a characteristic manner between different parts of the same vegetal. Additionally, these individual chemical compositions of essential oils directly influence the biological activities and pharmacological effects of these secondary metabolites.

The search for natural products with antiophidian properties has motivated studies of toxic and pharmacological characterization of extracts and essential oils from several plant species, attributing major economic and ethnopharmacological importance to the secondary metabolites. In this context, we highlight the widespread use of popular plant species with antiophidian properties, capable of mainly neutralizing the local effects of venoms, the lack of studies that investigate the antiophidian potential of essential oils and the importance of exploring the pharmacological potential of secondary metabolites extracted from invasive species, making this an innovative and valuable research area of scientific research. Thus, the study of the antiophidian properties of essential oils extracted from rhizomes and leaves of *H. coronarium*, as their inhibitory effects on coagulation and fibrinogenolysis induced by the venoms of *L. muta*, *B.atrox* and *B. moojeni*, evaluated in the present work, may result in information that point to the use of these compounds not only in the treatment of snakebites, but also in the therapy of diseases responsible for hemostatic disorders or inflammatory processes.

The essential oils tested did not cause coagulation of citrated human plasma in the volumes tested (0.6 and 1.2 µl), following observation for a period of 24 hours, thus not showing coagulant potential.

By previously incubating citrated human plasma at 37ºC in the presence of essential oils from rhizomes and leaves of *H. coronarium*, with subsequent addition of the venoms of *L. muta* and *B. atrox*, a decrease in the clotting times induced by the venoms could be observed. For *L. muta* venom (CT = 45.0 ± 1.5 seconds), clotting times of 32,7±1,8 and 36,0±1,0; 35,7±0,7 and 40,5±0,8 (corresponding to oil volumes of 0.6 and 1.2 µl) for oils of rhizomes and leaves, respectively, were observed. For *B. atrox* venom (CT = 106.5 ± 0.9 s), significant changes were detected for the oil from leaves, with clotting times of 83.1±0.6 and 85.2±1.1, corresponding to oil volumes of 0.6 and 1.2 µl, and 78.6

± 1.0 for the rhizome oil volume of 0.6 µl . On the other hand, the oils from the different parts of *H. coronarium* did not statistically change the clotting time induced by the venom of *B. moojeni* (Figure 1).

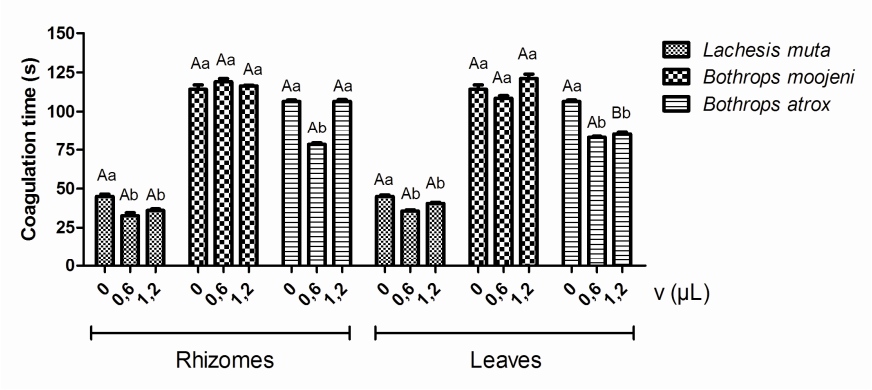

Figure 1. Mean values of the clotting time of citrated human plasma previously incubated at 37°C with different volumes of essential oils extracted from rhizomes and leaves of *H. coronarium*, followed by addition of the venoms of *Lachesis muta*, *B. moojeni* and *B. atrox* (50µg/ml). Means followed by the same letter, in uppercase for comparison between the essential oils analyzed and in lowercase for comparison between the volumes assessed for the same essential oil, do not differ significantly at 5% probability by the Scott-Knott test.

The data presented in Figure 1 show a procoagulant action previously unreported in the literature regarding the effects of antiophidian plant extracts. This is possibly due to the structural variability of molecules, mainly of hydrophobic character, present in essential oils and absent in most aqueous or hydroalcoholic extracts described in the literature. The results suggest that the oils possibly interact with plasma proteins involved in the coagulation cascade, making them more susceptible to the proteolytic action of venoms and consequently accelerating the plasma coagulation. This interaction probably occurs between individual constituents of the essential oils and proteins such as thrombin, fibrinogen or fibrin, which are the major targets of coagulant toxins, thereby acting as procoagulants.

It's important to considerer the limitations of these tests, as the maximum volume of sample in relation to the volume of plasma and limited available quantities of oils for the assays. Considering them, future studies will be performed using variations in incubation times, volumes of plasma, oils and venoms, as well as the analysis of the effects of oils on isolated toxins.

Analyzing the effect of preincubation of snake venoms with the essential oils on the clotting time of citrated plasma, we observed that the oils from rhizomes and leaves of *H. coronarium* were able to extend the clotting time induced by all the venoms evaluated, being potential anticoagulants. Additionally, the results showed that the essential oil from leaves extended plasma coagulation time induced by all three venoms evaluated at higher levels than those obtained for the oil extracted from rhizomes (Figure 2).

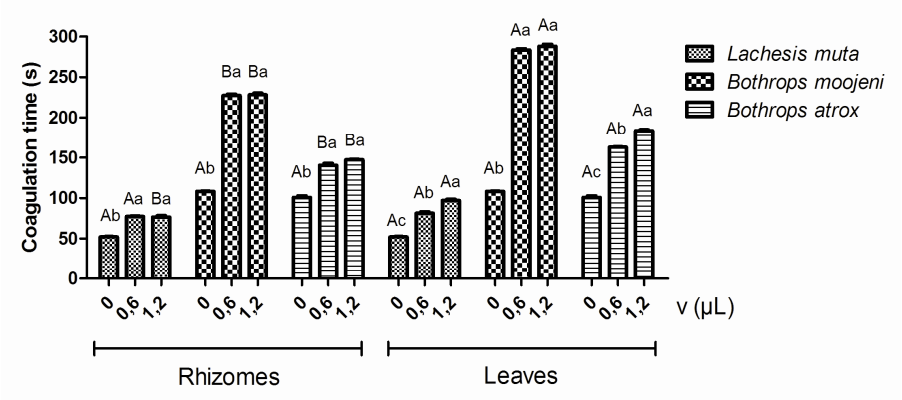

Figure 2. Mean values of the clotting time of citrated human plasma using different volumes of essential oils extracted from rhizomes and leaves of *H. coronarium* previously incubated at 37°C with the venoms of *Lachesis muta*, *B. moojeni* and *B. atrox* (50µg/ml). Means followed by the same letter, in uppercase for comparison between the essential oils analyzed and in lowercase for comparison between the volumes assessed for the same essential oil, do not differ significantly at 5% probability by the Scott-Knott test.

The clotting time of the venom of *L. muta* (52.2 ± 0.5 seconds) was extended by the oil from leaves to 81.3 ± 1.8 and 97.2 ± 1.5, corresponding to volumes of 0.6 and 1.2 µl, respectively. The clotting time of *B. atrox* (100.8 ± 2.0) was extended by the same oil to 162.9 ± 0.8 and 183.9 ± 1.3, respectively.

Statistically significant variations in clotting times were observed with the use of two different volumes (0.6 and 1.2 µl) of the essential oil of leaves, with no significant differences between the results obtained with the same volumes of the oil from rhizomes. The oil obtained from rhizomes, when previously incubated with venoms, prolonged clotting times induced by *L. muta* to 77.4 ± 07 and 76.5 ± 1.8, and by *B. atrox* to 140.7 ± 2.2 and 147.3 ± 0.7, with values corresponding to volumes of 0.6 and 1.2 µl, respectively. Preincubation of oils from rhizomes and leaves with venoms before addition of the plasma resulted in greater inhibition of the clotting effect of the venom of *B. moojeni* in comparison to the tests with preincubation of the oils with the plasma. The clotting time induced by *B. moojeni* venom (108.3 ± 0.4) was prolonged to 227.4 ± 1.6 and 283.5 ± 1.5 for the oil volume of 0.6 µl, and to 228.3 ± 2.1 and 288.0 ± 2.5 for 1.2 µl, corresponding to oils from rhizomes and leaves, respectively (Figure 2).

These findings suggest possible interactions between terpene compounds present in the oils and proteases present in the venoms, which are responsible for their clotting effect. Considering the different clotting times obtained with the different oils and different snake venoms, a pattern of inhibition could not be observed, and therefore, various terpenes could be responsible for specific interactions with different toxins present in the venoms evaluated. Possible mechanisms of inhibition could include ion-cofactors scavenging by the oils, terpenes binding to specific catalytic sites or acting as ligands of enzymatic cofactors, terpenes interactions with hydrophobic amino

acids of the toxins, changing their three dimensional conformation, solubility and hence interfering with their proteolytic activity.

Future trials to evaluate the effects of oils and their major isolated constituents on molecules or classes of venom toxins can provide accurate information about the interactions, predicting specificity and inhibition mechanisms. Silva et al. (2009) stated in their studies that the biological effects of essential oils are rarely associated with a single constituent present in them, relating primarily to a group of secondary metabolites, which agrees with the observations made in this work. This differentiated interaction of essential oils from leaves and rhizomes of *H. coronarium* with the venoms of *L. muta*, *B. moojeni* and *B. atrox*, in which the oil from leaves induced higher inhibition of coagulation, suggests that its higher content of sesquiterpenoids (14.9% in comparison to merely 1% in the oil from rhizomes) possibly accounts for greater antioxidant efficiency and may be associated with the inhibition of toxins, which possess pathophysiological effects that are partly attributed to oxidative actions.

In general, the composition of venoms present minor variations among species of the same genus (e.g. *B. atrox* and *B. moojeni*) and higher among species of different genera (e.g. *B. atrox* and *L. muta*). Nevertheless, the results oppose this observation, suggesting the presence of homologous proteases in the venoms of *B. atrox* and *L. muta*, largely responsible for the induction of coagulation and absent in the venom of *B. moojeni*. Differences in the chemical compositions of venoms are related not only to distinct genera and species, but also to the age of the animal, sex, geographic region of inhabiting, this latter being related to different diet, climate, among other factors (Mendes et al., 2008).

The effect of venoms on blood hemostasis is mainly associated with the action of proteases, which can act on factors of the beginning of the coagulation cascade, as well as on fibrinogen, thrombin, fibrin and on platelet surface

receptors (Bernardes et al., 2008; Gomes et al., 2009; Akao et al., 2010; Patiño et al., 2010; Cintra et al., 2012; Ullah et al., 2013; Madrigal et al., 2012; Menaldo et al., 2012). These various targets for proteases hamper the development of hypotheses on the mechanisms of action of natural inhibitors, such as the essential oils evaluated in the present study.

There are few reports in the literature on the antiophidian properties of essential oils. Torres et al. (2011) evaluated the inhibitory effect of extracts and essential oils from *Nectandra angustifolia* leaves on the hemolysis and coagulation induced by *B. neuwiedi* venom, showing that the essential oil was only effective in inhibiting coagulation. The monoterpenes α-pinene, β-pinene and limonene were the major constituents identified in the essential oils evaluated and were considered by these authors as possible coagulation inhibitors. Additionally, these authors expressed the need for further analysis of the active components of these oils, since there is no conclusive data on mechanisms of specific interactions between plant and venom molecules.

Fibrinogen is a dimeric plasma glycoprotein of 340 kDa, composed of six polypeptide chains (α, β, γ), which participates in the coagulation cascade, being converted by thrombin to fibrin monomers (Monaco et al., 2007). This protein has been extensively used to evaluate the potential fibrinogenolytic effects of snake venoms, and for the verification of potential antiophidian properties of natural compounds, mainly of plant origin (Oliveira et al., 2005; Maiorano et al., 2005; Mendes et al., 2008; Vale et al., 2008; Diogo et al., 2009; Salazar et al., 2011; Torres et al., 2011; Da Silva et al., 2012).

The essential oils from rhizomes and leaves of *H. coronarium* showed no proteolytic effect on fibrinogen. These essential oils were also unable to inhibit the fibrinogenolysis induced by the venoms of *L. muta*, *B. moojeni* and *B. atrox*, since electrophoretic migration profiles observed for samples of fibrinogen incubated with venoms were identical to those obtained with venoms

previously submitted to incubations in the presence of oils (results not shown). These evaluations were performed with preincubation of oils (0.6 and 1.2 µl) with fibrinogen and subsequent addition of venoms, and with previous incubation of oils and venoms and subsequent addition of fibrinogen.

These results suggest that the compounds present in the oils possibly interact with specific non-fibrinogenolytic proteases involved in the coagulation disorders promoted by snake envenomations, thus ruling out a number of metalloproteases described in the literature, mainly isolated from the venoms of *B. atrox* (Batroxase, BaTX-I botroxostatine and atroxlysin-I) and *B. moojeni* (BMMP-III and BthMP) (Gomes et al., 2009; Sánchez et al., 2010; Akao et al., 2010; Cintra et al., 2012; Ullah et al., 2013; Vu et al., 2013) and highlighting the serineproteases mainly responsible by the coagulation disorders (Vu et al., 2013; Madrigal et al., 2012; Menaldo et al., 2012; Costa et al., 2010; Morais-Zani; Tanaka; Tanaka-Azevedo, 2009; Sant'Ana et al., 2008; Oliveira et al., 1999).

Maiorano et al. (2005) evaluated the potential neutralization of different parts (leaves, stems and roots) of fresh and dried *Mikania glomerata* aqueous extracts on the enzymatic and pharmacological effects of *Bothrops* and *Crotalus* snake venoms. All extracts were active in inhibiting the hemolytic, fibrinogenolytic and coagulant activities of the venoms, however the intensity of the inhibitions showed specific to each extract acting on each activity evaluated. The individuality observed in these activities, as those observed in the present work, may be due to the fact that the metabolites are distributed in a particular way in each organ of the same plant, and this characteristic chemical composition of each extract or essential oil is crucial for its pharmacological activity (Gobbo-Neto and Lopes, 2007).

Although the anticoagulant mechanisms of action of the essential oils evaluated are still unknown, they significantly inhibited coagulation induced by

the snake venoms tested, which indicates that they could be used as an alternative to complement the available antivenoms, especially considering that essential oils do not require specific formulations and their topical use may be performed immediately after extraction. In addition, snake venoms have been widely used in studies of normal physiological mechanisms and of development of various diseases, considering that many human enzymes are structurally and functionally similar to molecules present in venoms, indicating the importance of the characterization of new natural compounds capable of interacting with different classes of snake toxins (metalloproteases, serine proteases, phospholipases A_2, L-amino acid oxidases, etc.), thus directing scientific basis for the development of new therapies.

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APÊNDICE

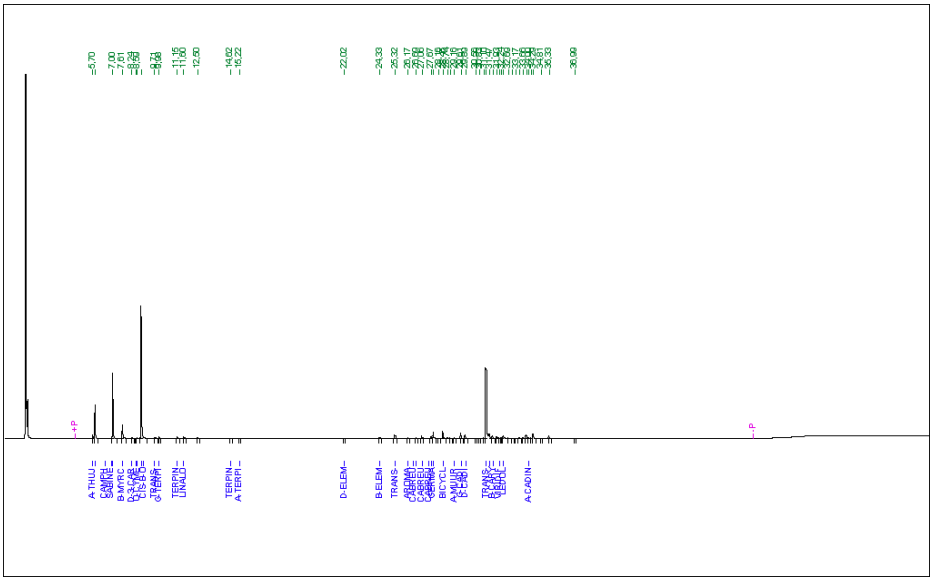

Figura 1 Cromatograma do óleo essencial de folhas frescas de *B. dracunculifolia*

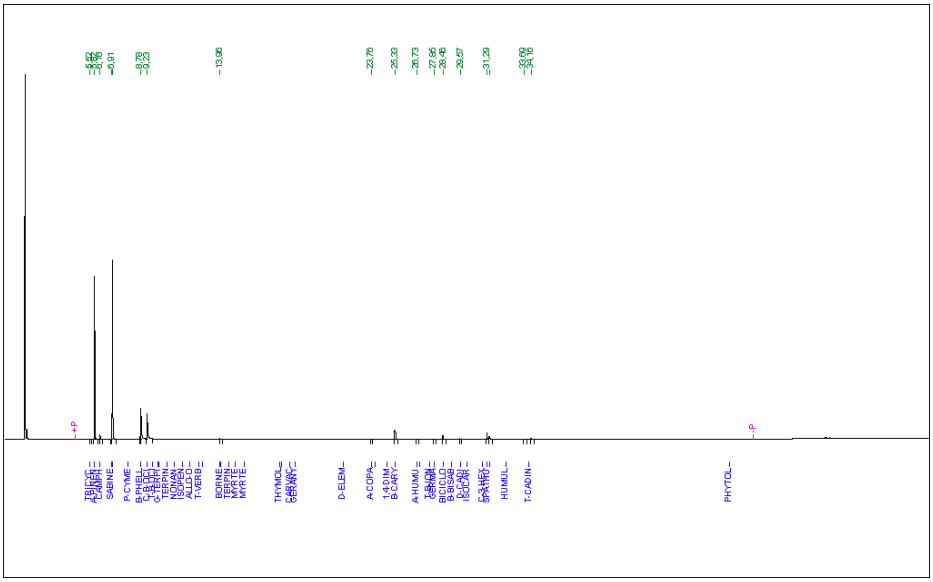

Figura 2 Cromatograma do óleo essencial de folhas frescas de *T. diversifolia*

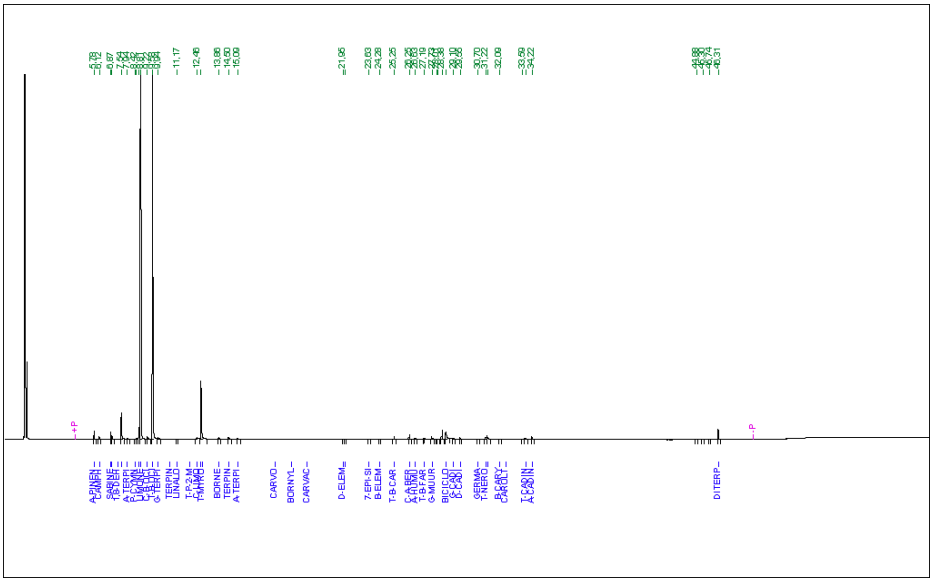

Figura 3 Cromatograma do óleo essencial de folhas frescas de *C. bonariensis*

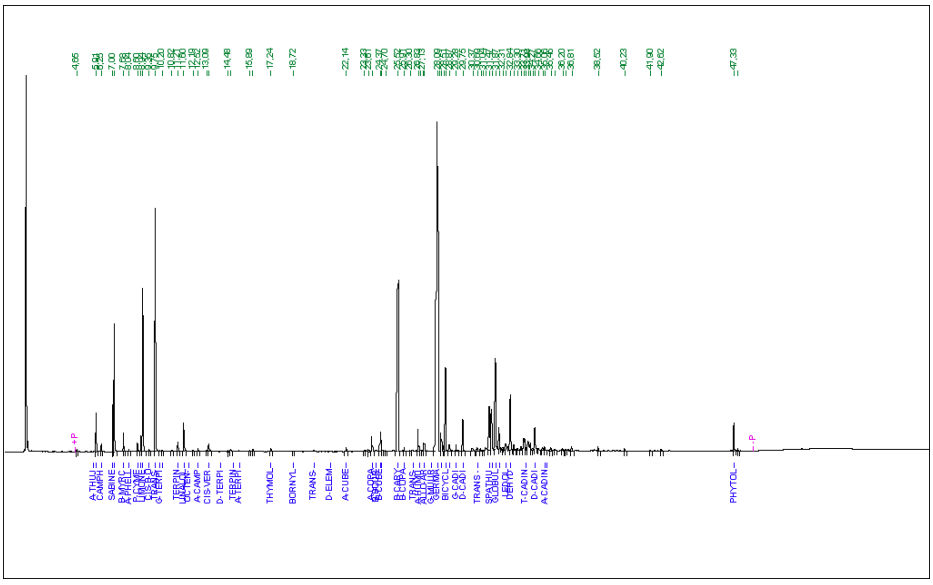

Figura 4 Cromatograma do óleo essencial de folhas frescas de *A. polystachya*

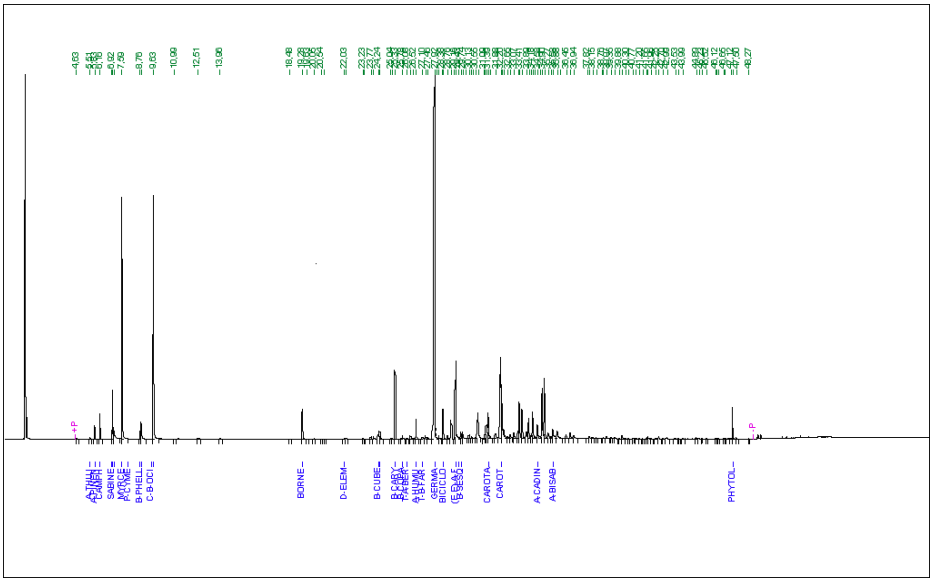

Figura 5 Cromatograma do óleo essencial de folhas frescas de *P. hysterophorus*

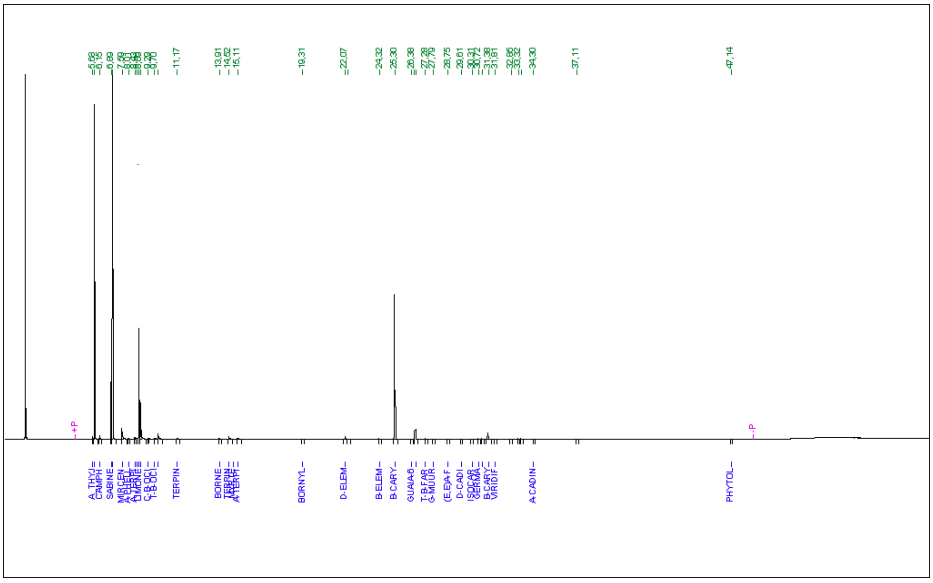

Figura 6 Cromatograma do óleo essencial de folhas frescas de *H. coronarium*

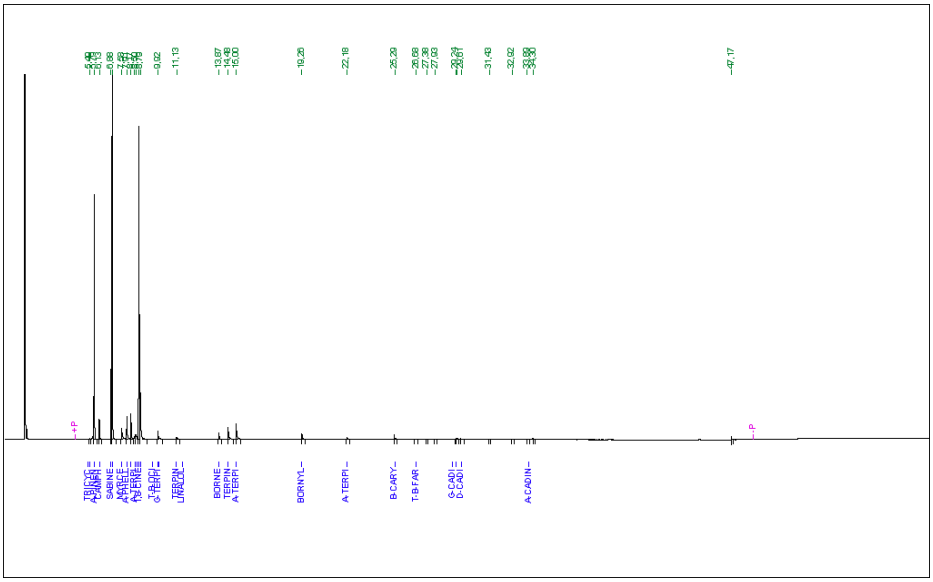

Figura 7 Cromatograma do óleo essencial de rizomas de *H. coronarium*