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AVALIAÇÃO DO POTENCIAL BIOLÓGICO DE ÓLEOS ESSENCIAIS E EXTRATOS ORGÂNICOS DE FOLHAS DE *Indigofera suffruticosa.*

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Tese de Doutorado apresentada ao programa de Pós–Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco como parte dos requisitos para obtenção do título de Doutora em Bioquímica.

Orientadora: Prof. Dra. Vera Lúcia de Menezes Lima

Co-orientador: Prof. Dr. Cláudio Augusto Gomes da Câmara

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"A mente que se abre a uma nova ideia
jamais voltará ao seu tamanho original"

Albert Einstein.

RESUMO

Indigofera suffruticosa Mill (Fabaceae), é um arbusto conhecido como anil. É uma planta com distribuição mundial, e é utilizada na medicina popular contra diversos problemas de saúde. No presente estudo, folhas de *I. suffruticosa* de duas localidades do Estado de Pernambuco, Municípios de São Caetano e Cabo de Santo Agostinho, foram coletadas para extração de óleos essenciais por hidrodestilação e extração com solventes para obtenção de extratos orgânicos (folhas coletadas no Município de São Caetano). O óleo essencial hidrodestilado foi caracterizado de acordo com suas propriedades físico-químicas e biológicas. A análise de CG e CG-EM dos constituintes químicos do óleo essencial do Município de São Caetano revelou a presença de fenilpropanóides (89,9%), sesquiterpenos (6,4%), e monoterpeno (1,4%). Este estudo avaliou a ação acaricida do óleo essencial por fumigação sobre o *Tetranychus urticae*, e exibiu uma LC_{50} de 0.90 μL L^{-1} de ar, bem como, a sua atividade antimicrobiana e potencial citotóxico contra cinco linhagens de células cancerígenas humanas. Além disso, foram também obtidos dados de atividade antimicrobiana de extratos orgânicos e sua ação sinérgica combinada com eritromicina em *Staphylococcus aureus* resistentes a antibióticos. O extrato clorofórmico apresentou melhor efeito sinérgico (FIC = 0,2). A atividade antimicrobiana foi determinada pelo método de difusão em disco e pelo método de microdiluição, contra bactérias Gram-(+), Gram-(-) e fungos e a citotoxicidade foi analizadas pelo ensaio de MTT. Os óleos essenciais investigados mostraram fraca atividade contra bactérias gram-negativas, mas, revelou de moderada a alta atividade contra bactérias gram-positivas (CIM de 64,5 μg/mL a 1000 μg/mL). Os óleos também revelaram atividade citotóxica nas linhagens de células cancerígenas: HL-60 (leucemia pró-mielocítica humano), NCI-H292 (carcinoma do pulmão humano), HEP-2 (carcinoma da laringe humano) e HT29 (carcinoma do cólon humano), MCF-7 (carcinoma da mama). As células tumorais HL-60 e HEP-2 exibiram alta susceptibilidade aos óleos essenciais das duas localidades (IC_{50} de 2,0 μg/mL a 5,4 μg/mL). Neste estudo, também foi demonstrado que os extratos orgânicos de folhas de *I. suffruticosa* tem atividade antimicrobiana. Os óleos essenciais e extratos orgânicos de folhas de *I. suffruticosa* investigados neste trabalho exibiram significantes atividade, indicando que esta planta possui potencial biológico.

Palavras-chave: Fenilpropanóides. Atividade acaricida. Atividade antitumoral. Sinergismo, *Staphylococcus aureus*.

ABSTRACT

Indigofera suffruticosa Mill (Fabaceae) is a shrub popularly known as anil. Is a plant distributed worldwidely, and is utilized in the popular medicine against diverse problem of health. In this study, the leaves of *I. suffruticosa* of two locations from state of Pernambuco (São Caetano and Cabo de Santo Agostinho) were collected for extraction of essential oils by hydrodistillation and solvent extraction to obtain organic extracts (leaves collected in São Caetano). The essential oil Hydrodistilled was characterized for their properties physicochemical and biological. The GC and GC-MS analysis of the chemical constituents of the essential oil of the Municipality of São Caetano revealed the presence of phenylpropanoids, monoterpenes and sesquiterpenes. This study evaluated the acaricidal action of the essential oil on Tetranychus urticae and exhibited a LC_{50} de 0.90 μL L^{-1} de ar, as well as their antimicrobial activity and cytotoxic potential against five human cancer cell lines. In addition, data were also obtained from the antimicrobial activity of organic extracts and their synergistic action combined with erythromycin in *Staphylococcus aureus* resistant to antibiotics (FIC = 0,2). The antimicrobial activity was determined by disk diffusion and the microdilution method against Gram (+) bacteria, Gram (-) and fungi and the cytotoxicity was analyzed by MTT assay. The essential oils investigated showed weak activity against gram-negative bacteria, but revealed of moderate to high activity against gram-positive bacteria (MIC of 64.5 μg/mL to 1000 μg/mL). The oils also showed cytotoxic activity in human cancer cell lines: HL-60 (human promyelocytic leukemia), NCI-H292 (human lung carcinoma), Hep-2 (human larynx carcinoma) and HT29 (human colon carcinoma), MCF-7 (breast carcinoma). The tumor cells HL-60 and HEP-2 exhibited high susceptibility to essential oils of the two localities (IC_{50} of 2,0 μg/mL to 5,4 μg/mL). In this study, also was demonstrated that the organic extracts of leaves of *I. suffruticosa* has antimicrobial activity. The essential oils and organic extracts of leaves of *I. suffruticosa* investigated in this study exhibited significant activity, indicating that this plant has biological potential.

Keywords: Phenylpropanoids. Acaricidal activity. Antitumor activity. Synergism. *Staphylococcus aureus.*

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LISTA DE ABREVIATURAS

UFPE – Universidade Federal de Pernambuco

UFRPE – Universidade Federal Rural de Pernambuco

% – Percentagem

mg – miligramas

mL – Mililitro

° C – Graus Celsius

SD – Desvio padrão

FAL – Fenilalanina

IPP – Isopentanila difosfato

DMAPP – Dimetilalil difosfato

MEP – Metileritritol fosfato

GPP – Geranila difosfato

FPP – Farnesila difosfato

GGPP – Geranilgeranil difosfato

CG – Cromatografia gasosa

CG/EM - Cromatografia gasosa acoplada à espectrometria de massa

CMI / MIC – Concentração mínima inibitória

CMB – Concentração mínima bacterida

FIC – Concentração fração inibitória

DNA – Ácido desoxirribonucleico

RNA – Ácido ribonucleico

MRSA – *Staphylococcus aureus* resistante à meticilina

NaOH – Hidróxido de sódio

HCl – Ácido clorídrico

pH – Potencial de hidrogênio

DMSO – Dimetilsulfóxido

DIZ – Diâmetro de zona de inibição

µl – Microlitro

OEIsSC – Óleo essencial de *Indigofera suffruticosa* de São Caetano

OEIsC – Óleo essencial de *Indigofera suffruticosa* do Cabo de Santo Agostinho

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1. Introdução

1 INTRODUÇÃO

Uma das características dos seres vivos é a presença de atividade metabólica. Do metabolismo se obtém uma grande variedade de substâncias através de reações que ocorrem no interior das células. As substâncias produzidas pelos vegetais são provenintes do metabolismo primário ou secundário. Entende-se por metabolismo primário o conjunto de processos metabólicos responsáveis pelo desenvolvimento e manutenção celular. Os compostos envolvidos no metabolismo primário possuem uma distribuição universal nas plantas. As macromoléculas como carboidratos, proteínas, lipídios, originadas a partir de rota metabólica primária, realizam as principais funções vitais da planta (ALVES, 2001; SANTOS, 2004), tais como o armazenamento de energia, fitormônio, crescimento e reprodução celular. Estas macromoléculas originam o segundo grupo de compostos químicos, denominados metabólitos secundários. Destes resultam substâncias de baixo peso molecular, às vezes produzida em pequenas quantidades e com características químicas variadas, geralmente complexas e com marcante atividade biológica (AGOSTINI-COSTA et al., 2012).

O metabolismo secundário origina compostos que não possuem uma distribuição universal, pois nem sempre são necessários para que uma planta complete seu ciclo de vida. Os produtos resultantes deste metabolismo são comuns entre certos grupos taxonômicos, ou exclusivos para determinada espécie, e oferecem vantagens para a manutenção e desenvolvimento das plantas que os sintetizam (CROTEAU, KUTCHAN, LEWIS, 2000; VERPOORTE, 2000). Eles desempenham um papel importante na interação das plantas com o meio ambiente. Um dos principais componentes do meio externo, cuja interação é mediada por compostos do metabolismo secundário, são os fatores bióticos. Desse modo, produtos secundários possuem um papel contra a herbivoria, ataque de patógenos, competição entre plantas e atração de organismos benéficos como polinizadores, dispersores de semente e microorganismos simbiontes. Além disso, produtos secundários também possuem ação protetora em relação a estresses abióticos, como aqueles associados com mudanças de temperatura, conteúdo de água, níveis de luz, exposição à UV e deficiência de nutrientes minerais. E os metabólitos secundários dividem-se em três grandes grupos: alcaloide, terpenos e compostos fenólicos (CROTEAU, KUTCHAN, LEWIS, 2000).

A descoberta dos compostos secundários tornou possível o uso dos vegetais como fonte de princípios bioativos para o tratamento de uma infinidade de patologias que acometem os seres vivos. São muitas as espécies utilizadas como planta medicinal e uma série de

investigação científica tem destacado a importância e a contribuição de tantas famílias de plantas para o desenvolvimento de novos agentes terapêuticos. As espécies do gênero *Indigofera* da família Fabaceae têm se mostrado promissoras na busca de substâncias bioativa. Entre elas encontra-se a espécie *Indigofera suffruticosa* com grande potencial biológico.

Neste estudo foi investigado as propriedades biológicas de metabólitos secundários da planta *I. suffruticosa* a partir da análise da composição química e atividade acaricida do óleo essencial de *I. suffruticosa* contra *Tetranychus urticae* Koch (capítulo I); da análise da atividade citotóxica e antimicrobiana de óleo essencial de *I. suffruticosa* (capítulo II) e da análise do efeito sinérgico de extratos orgânicos de folhas de *I. suffruticosa* (capítulo III).

2. Revisão de Literatura

2 REVISÃO DE LITERATURA

2.1 Generalidades sobre Plantas Medicinais.

O Brasil, detentor de uma vasta biodiversidade vegetal, possui em seus biomas uma expressiva diversidade de espécies medicinais que constitui uma das mais relevantes fontes de princípios ativos. Essa diversidade vegetal é responsável pelo fornecimento de uma rica fonte de substâncias cujas propriedades químicas são importantes à vida humana, uma vez que fornecem alimentos, corantes e produtos de potencial aplicação biotecnológica, visando sua utilização como fonte de recursos terapêuticos ou ainda como ingredientes para cosméticos e agroquímicos, assim como o desenvolvimento de fitofármacos (MARQUES, SOUZA, 2012). Nessa perspectiva, considera-se a possibilidade de obtenção de novos produtos naturais com atividade biológica, um fator de grande incentivo ao estudo com plantas.

Estudos com plantas medicinais têm avançado nos últimos anos com o objetivo de encontrar novos compostos bioativos em extratos vegetais. A etnofarmacologia é uma abordagem promissora, norteada pelo uso popular da flora medicinal, que aumenta as chances de descoberta de novos princípios ativos (CORDELL, COLVARD, 2005). Espécies de diversas famílias de plantas medicinais têm sido usadas por milhares de anos para o tratamento de muitas doenças.

A espécie *Protium spp* (almécega) da família Burseraceae, tem amplo uso popular para o tratamento de feridas, de úlceras, como agente anti-inflamatório e como repelente de insetos (CORRÊA, 1987). As flores de *Muntingia calabura* (pau de seda) da família Muntingiacea, foram utilizados como antissépticos, anti-espasmódico, calmante, e para o tratamento da dor de cabeça, enquanto as raízes são empregadas como abortivos (CORRÊA, 1987), e a infusão dos frutos de *Xylopia sericea* (pindahiba) da família Annonaceae, é usada popularmente no tratamento de perturbações gástricas (CORRÊA, 1987), enquanto a infusão de folhas e casca do caule de *Anacardium humile* (Anacardiaceae), conhecida popularmente como cajuzinho-do-cerrado, é indicada contra diarreia e como expectorante e a infusão da inflorescência são empregadas contra tosse e também para glicemia em diabéticos (ALMEIDA et al., 1998). A espécie *Plectranthus amboinicus* da família Lamiaceae, vulgarmente conhecida como hortelã da folha grossa é frequentemente usada na medicina popular contra inflamações e infecções respiratórias (CASTILLO, GONZÁLEZ, 1999).

O gênero *Croton* (Euphorbiaceae) amplamente distribuído na região do Nordeste do Brasil têm muitas espécies utilizadas na medicina popular para o tratamento de inflamações, infecções, ferida, hipertensão, úlceras, cancro, reumatismo e malária (AGRA, FRANÇA, BARBOSA-FILHO, 2007; AGRA et al., 2008). Chá de folhas e flores de *Camellia sinensis*, planta conhecida como chá, da família Theaceae, tem sido utilizado na China como medicamentos tradicionais para desodorização, cuidados da pele, supressor da tosse e expectorante (YOSHIKAWA et al., 2008). *Lippia gracillis* (Verbenaceae), popularmente conhecida como "alecrim-de-tabuleiro, é amplamente utilizada no Nordeste do Brasil para cortes na pele, picadas de insetos e garganta dolorida (BOTELHO et al., 2008).

Além disso, o óleo-resina de copaíba, obtido a partir do tronco de espécies de *Copaifera* (Fabaceae) é usado extensivamente na medicina popular como anti-inflamatório, antitumoral, antitetânica, antiblenorrágico, como antisséptico urinário, no tratamento de bronquite, sífilis, doenças cutâneas, úlceras, bem como para cura de feridas (PAIVA et al,. 2004).

Deve-se destacar ainda *Indigofera suffruticosa*, Fabaceae, cujas investigações prévias de atividades biológicas com extrato aquoso têm revelado, entre outras, atividade anti-inflamatória inibindo edema de pata de camundongo (LEITE et al., 2003), atividade citotóxica em células embrionárias de ratos (LEITE et al., 2004), atividade antimicrobiana contra a bactéria Gram-positiva *Staphylococcus aureus* (ATCC-6538) e os fungos *Trichophyton rubrum* (N-09, LM-13) e *Microsporum canis* (EM-828), (LEITE et al., 2006), atividade antitumoral sobre o Carcinoma Ehrlich (SILVA, 2008), atividade gastroprotetora (LUIZ-FERREIRA et al., 2011), atividade repelente contra o *Aedes aegypti* (VIEIRA et al,. 2012) e forte propriedade anti-inflamatória que diminui a expressão de mediadores pró-inflamatórios (CHEN et al., 2013).

Partindo da necessidade de investigar plantas encontradas no Brasil, em especial da região Nordeste, o presente trabalho objetivou estudar a espécie *I. suffruticosa* da família Fabaceae, visto que, de acordo com a literatura, essa família botânica é muito utilizada na medicina popular, com grandes possibilidades de ter potencial farmacológico.

2.2 Família Fabaceae e o Gênero *Indigofera*

A família Fabaceae, também conhecida como Leguminosae (leguminosas), é a terceira maior família botânica. Possui cerca de 727 gêneros com 19.327 espécies cosmopolitas (LEWIS et al., 2005), subordinadas a três subfamílias: Caesalpinioideae, Mimosoideae,

Papilionoideae (POLHILL, RAVEN, 1981; LEWIS et al., 2005). Muitas delas são economicamente importante como fonte de produtos alimentares como soja, ervilha, feijão, alfafa e também como produtos medicinais, ornamentais e madeireiros (TUCKER, 2003).

No Brasil a família é um táxon bem representado no semiárido nordestino e apresenta um grande número de gêneros dentre os quais se destaca *Indigofera*, com importância medicinal (ALCOFORADO-FILHO, 1993; FERRAZ et al.,1998).

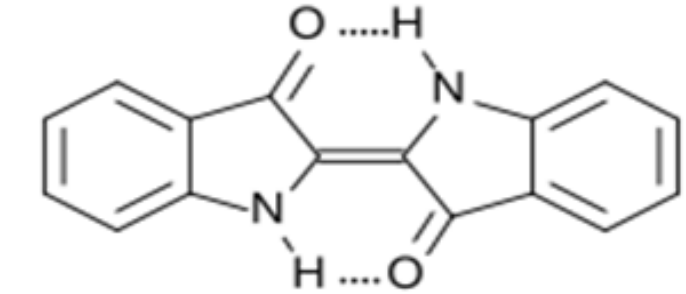
Este gênero compreende aproximadamente 700 espécies dicotiledôneas herbáceas e arbustivas fitogeograficamente distribuídas nas regiões tropicais e subtropicais, encontradas nas Américas e também na África, Ásia e Austrália (HASSEN et al., 2007; MOREIRA, AZEVEDO-TOZZI, 1997).

São plantas que crescem espontaneamente em todos os solos agrícolas, principalmente nas imediações de cidades e vilas (PESAVENTO, 2005). Espécies desse gênero são ricas em metabólitos secundários como: flavonoides, alcaloides, terpenos e saponinas (HARBONE, WILLIAMS, 2000), os quais apresentam significativas atividades farmacológicas e terapêuticas.

A origem do nome *Indigofera* provém da palavra alemã índigo, que significa produção de pigmento azul (Indigo Blue); que pode ser extraído de *I. suffruticosa* Mill (espécie foco de estudo desta pesquisa) e *Indigofera truxilensis* (PESAVENTO, 2005). Portanto, o uso mais conhecido e antigo do gênero *Indigofera* é a produção deste pigmento azul. O índigo foi detectado, sobretudo nas sementes da *I. suffruticosa* (LEITE, 2003), apresentando-se como uma molécula com forte fluorescência, o que, de certa forma, confirma este táxon da planta.

O índigo (Figura 1) foi um dos primeiros corantes naturais a ser obtido de modo sintético e contribuiu para suprir a indústria têxtil. O emprego mais famoso deste corante foi sem dúvida o Jeans. Com o desenvolvimento industrial do setor têxtil a produção sintética de anilina substituiu o pigmento natural. As comunidades do interior do Brasil ainda usam de forma artesanal o pigmento natural para colorir roupas de lã e algodão (PESAVENTO, 2005).

Figura 1- Fórmula estrutural do indigo.



http://pt.wikipedia.org/wiki/Anil_(corante)

As espécies do gênero *Indigofera* têm alto teor em proteínas, característica típica das leguminosas; tem habilidade para tolerar seca; inundações e elevadas salinidade tornando-as assim agronomicamente muito desejáveis (SHERMAN, 1982). Esta combinação de peculiaridades torna-a apta a se desenvolver na Região do Semiárido. No Brasil, a espécie *I. hirsuta* é usada como adubo verde e forragem e tem sido recomendada como potencialmente controladora de nematoides (ALLEN, RODRIGUES-KABANA, 1981; AYLWARD et al., 1987). Na cultura popular essas espécies são utilizadas no tratamento de diversos problemas de saúde (HASTINGS, 1990).

Recentemente, estudos têm sido reportados com diversas atividades biológicas de espécies de *Indigofera*, tais como atividade antioxidante, antimicrobiana e efeito de citotoxicidade em linhagem de células cancerígenas de *I. tinctoria* (RENUKADEVI, SULTANA, 2011); atividade mutagênica de *I. truxillensis* e *I. suffruticosa* (CALVO et al., 2011); atividade antimicrobiana da espécie e *I. trita* e *I. lupatana* (VINOT et al., 2011; NGOCI et al., 2012); atividade antimicrobiana e antioxidante em *I. linnaei* (SANDHYAVALI et al., 2012) entre outras atividades.

A *I. suffruticosa* (Figura 2), espécie selecionada para este estudo, é vulgarmente conhecida como anil, é uma planta arbustiva, medindo de 1-2 m de altura, com ramos pubescentes, propagando-se por sementes, folhas pinadas, com 7-15 folíolos oblongos ou ovais, glabros na face e no verso. Apresenta flores miúdas, numerosas, albo-rósea ou amarelada, e possui pequena vagem falciforme com 6-10 sementes com aparência de feijão (BRAGA, 1976). Ela é nativa das Antilhas (ALMEIDA, 1993) com ampla distribuição nas Américas tropical e subtropical. No Brasil há registro da espécie em todas as regiões e em todos os Estados entre eles São Paulo, Sergipe, Bahia, Rio de Janeiro, Minas Gerais, Mato

Grosso, Maranhão, Alagoas, Paraíba, Ceará, Rio Grande do Norte, Pará e Pernambuco (MOREIRA, AZEVEDO-TOZI, 1997; MIOTTO, IGANCI, 2014).

A espécie é usada contra diversos problemas de saúde e sua utilização na medicina popular está baseada em infusões e decocções de diferentes partes deste vegetal (MATOS, 1999). São atribuídas a esta planta propriedades febrífuga, anti-espasmódica, diurética, abortiva, analgésica, contra problemas estomacais e urinários, icterícia, úlceras, purgativa, sedativa e inseticida (HASTINGS, 1990). Estudos farmacológicos mostraram que extratos de *I. suffruticosa* apresentaram atividade antiepiléptica (ROIG, MESA, 1974), anticonvulsivante (ALEJO, MIRANDA, RODRIGUES, 1996) e antigenotóxica (BADELL et al., 1998).

As primeiras investigações a respeito da composição química da espécie *I. suffruticosa*, utilizando extratos de sementes, detectaram uma rica fonte de aminoácidos com prováveis ações tóxicas (MILLER, SMITH, 1973). Através da identificação, caracterização e quantificação de seis rotenóides de diferentes órgãos como raiz, caule, semente e folhas dessa planta foram verificadas que a mesma tem bioeficácia contra larvas do mosquito causador da malária (*Anopheles*) e pragas de grãos armazenados a exemplo do *Callosobruchus chinensis* adultos (KAMAL, MANGLA, 1993).

LEITE, (2003), em um estudo fitoquímico preliminar das folhas, caule e sementes desta eapécie, demonstrou abundante presença de metabólitos secundários como alcaloides, polifenóis (cumarina e ácido clorogênico) e flavonoides (nas folhas); triterpenoides e/ou esteroides (abundantes nas folhas e, em menor extensão, no caule e sementes), e também metabólitos primários como oses redutoras (em todas as partes estudadas do vegetal). Outros estudos fitoquímicos de partes aéreas (galhos, folhas e frutos) demonstraram a presença de flavonoides derivados da quercetina (como a rutina), o ácido gálico e alcaloides (CALVO, 2007).

Figura 2- *Indigofera suffruticosa*. **A:** Vista parcial da planta adulta; **B:** folha e inflorescência**;** **C:** ramos com folhas e sementes**; D:** ramos com flores, inflorescência e folhas.

Fonte: A AUTORA (2013)

2.3 Óleos Essenciais

Os óleos essenciais são substâncias naturais de variável poder aromatizante, originados principalmente de matéria prima vegetal por meio de processo físico como hidrodestilação. De uma forma geral, são misturas complexas de substâncias voláteis, lipofílicas, geralmente odoríferas e líquidas (SIMÕES, SPITZER, 2003).

Também podem ser chamados de óleos voláteis, óleos etéreos ou essências. A designação de "óleo" deriva de algumas de suas características físico-químicas como, por exemplo, a de serem geralmente líquidos de aparência oleosa à temperatura ambiente. Entretanto, por ter como característica principal a volatilidade, difere dos óleos fixos, que são misturas de substâncias lipídicas, as quais apresentam sua composição glicerídica quimicamente diferente da composição lipofílica dos óleos essenciais. Outra característica importante é o aroma, geralmente agradável e intenso na maioria dos óleos voláteis, sendo por isso, também chamados de essências. Eles também são solúveis em solventes orgânicos apolares, como éter, recebendo por isso a denominação de óleos etéreos ou em latim *aetheroleum* (RADÜNZ, 2004).

Tem também como característica, apresentar sabor geralmente acre (ácido) e picante; sua coloração quando recentemente extraídos é incolor ou ligeiramente amarelado; são instáveis e sujeitos à degradação na presença de luz, calor, oxigênio atmosférico, umidade e metais (SIMÕES, SPITZER, 2004). Os óleos voláteis podem ser produzidos em diferentes partes das plantas, como folhas, flores, cascas, tronco, galhos, raízes, rizomas, frutos e sementes e são armazenadas em estruturas secretoras especializadas tais como tricomas glandulares, células parenquimáticas diferenciadas ou canais oleíferos (BURT, 2004; BAKKALI et al., 2008; ANWAR et al., 2009). Apesar de vários órgãos de uma planta poder acumular óleos voláteis, numerosos fatores determinam a sua composição química e rendimento. Em alguns casos estes fatores são interdependentes e se influenciam mutuamente. Estas variáveis podem incluir variação sazonal e maturidade, origem geográfica e variação genética (HARTMANN, 2007; HUSSAIN et al., 2008).

Variação sazonal e maturidade: Esses dois elementos estão interligados, devido à fase ontogênica específica de crescimento diferir conforme o andamento da temporada. As mudanças climáticas, a idade da planta e a fase fenológica também interferem com o metabolismo das plantas, sendo que a sazonalidade implica em mudanças de parâmetros como temperatura e pluviosidade, podendo estimular a produção de certos compostos em

detrimento de outros, o que favorece a síntese de determinadas classes de metabólitos secundários (HUSSAIN et al.,2008).

Origem geográfica: As diferentes características geográficas e edafoclimáticas interferem diretamente sobre o modo como as plantas adaptam-se e desenvolvem-se, influenciando assim na produção dos metabólitos secundários produzidos de acordo com a sua necessidade (HARTMANN, 2007). Estas diferenças podem estar ligadas às texturas variadas de solo e possíveis respostas de adaptação de diferentes populações, resultando em diferentes produtos químicos a ser formado, sem diferenças morfológicas observadas nas plantas (HUSSAIN et al.,2008). Altitude parece ser outro importante fator ambiental.

Variação genética: Composição genética da planta é um dos mais importantes contribuintes para a composição de óleo essencial. A evolução das plantas em resposta às condições ambientais sob as quais viveram ao longo do tempo resultou em grande variabilidade genética, que promove diferenças químicas intraespecíficas nos óleos voláteis de plantas da mesma espécie, morfologicamente idênticas e até mesmo cultivadas nas mesmas condições ambientais (HUSSAIN et al., 2008).

Outros fatores que afetam o crescimento das plantas, conduzindo a variações no rendimento e composição de óleo essencial, incluem estágios de crescimento, parte da planta utilizada, secagem pós-colheita, armazenamento, temperatura, disponibilidade de água, entre outros (HUSSAIN et al., 2008).

Todas as plantas aromáticas contêm óleos essenciais. A capacidade da planta de acumular óleos essenciais é bastante elevada em angiospermas dicotiledôneas, em menor frequência em angiospermas monocotiledôneas e raramente são encontradas em gimnospermas. A matéria prima a partir da qual os óleos essenciais são obtidos pode ser fresca, parcialmente desidratada ou seca (HUSSAIN et al., 2008; ANWAR et al., 2009).

Após serem extraídos, os óleos essenciais devem ser analisados para identificação e quantificação de seus componentes, através de técnicas cromatográficas, como a cromatografia gasosa (CG) e a cromatografia gasosa acoplada à espectrometria de massa (CG/EM).

Na análise através da CG, a amostra que é injetada no cromatógrafo volatiliza, permitindo a separação e a identificação dos compostos individuais através do tempo de retenção relativo da amostra quando comparados com padrões (SIMÕES, SPITZER, 1999; ARAÚJO, 1995). A cromatografia gasosa acoplada à espectrometria de massa irá indicar a massa molecular e o padrão de fragmentação (ARAÚJO, 1995).

Do ponto de vista químico, os óleos essenciais de plantas são constituídos de vários compostos naturais com alta complexidade em concentrações muito diferentes. Eles são caracterizados por dois ou três componentes principais em concentrações bastante elevadas (20-70%) em relação aos outros componentes presentes em quantidades muito baixa. Geralmente, os componentes principais refletem as características físicas e determinam as propriedades biológicas dos óleos essenciais (BURT, 2004).

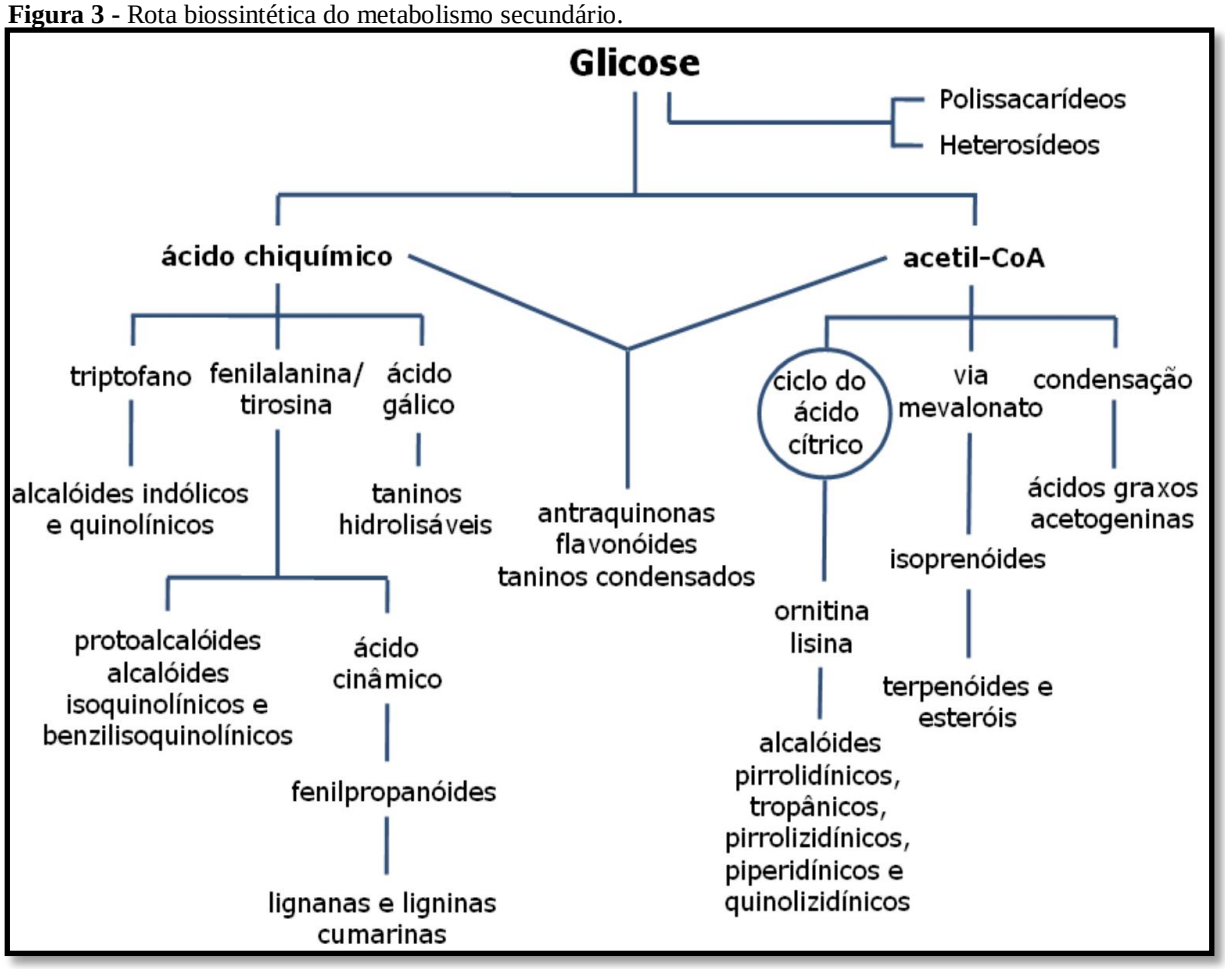
Ao longo do período evolutivo, os vegetais desenvolveram uma complexa variedade de moléculas, a fim de possibilitar sua sobrevivência, como forma de proteção e resistência às intempéries do clima, predadores e poluição (MONTANARI, BOLZANI, 2001; VIEGAS-JR, BOLZANI, BARREIRO, 2006).

A tendência para designar os metabólitos secundários é a utilização do termo "produtos naturais". Considera-se que uma das principais funções do metabolismo secundário nas plantas seja a biossíntese de compostos complexos como alcaloides, terpenóides e derivados de fenilpropanóides. Eles são os princípios ativos que conferem propriedades terapêuticas às plantas. Estes compostos estão envolvidos na defesa do vegetal contra herbívoros, insetos, micro-organismos patógenos, favorecem a atração de polinizadores, atuam no controle da germinação de sementes e na competição com outros vegetais (ALVES, 2001).

Uma característica do metabolismo secundário é a grande plasticidade genética, que garante adaptações das plantas às demandas da pressão seletiva ambiental como produção e acúmulo de metabólitos secundários e defesa anti-herbivorismo (HARTMANM, 2007). Assim, os metabólitos secundários, são parte integrante das interações de espécies em comunidades vegetais e animais e da adaptação das plantas ao seu ambiente (BREITLING, et al., 2013). Além disso, em diversas situações de estresses bióticos e abióticos, novas rotas biossintéticas são iniciadas a partir de metabólitos primários, desencadeando a produção de substâncias químicas com grande variabilidade estrutural (ALVES, 2001).

Os metabólitos secundários ou produtos naturais são formados por caminhos biossintéticos distintos, que produzem moléculas dotadas de grande diversidade de esqueletos e grupamentos funcionais, como entre outros, os fenilpropanóides, compostos aromáticos formados pela via do ácido chiquímico (Figura 3) e os terpenóides, formados pela via do ácido mevalônico-acetato (SANGWAN et al. 2001; OLIVEIRA, GODOY, COSTA, 2003).

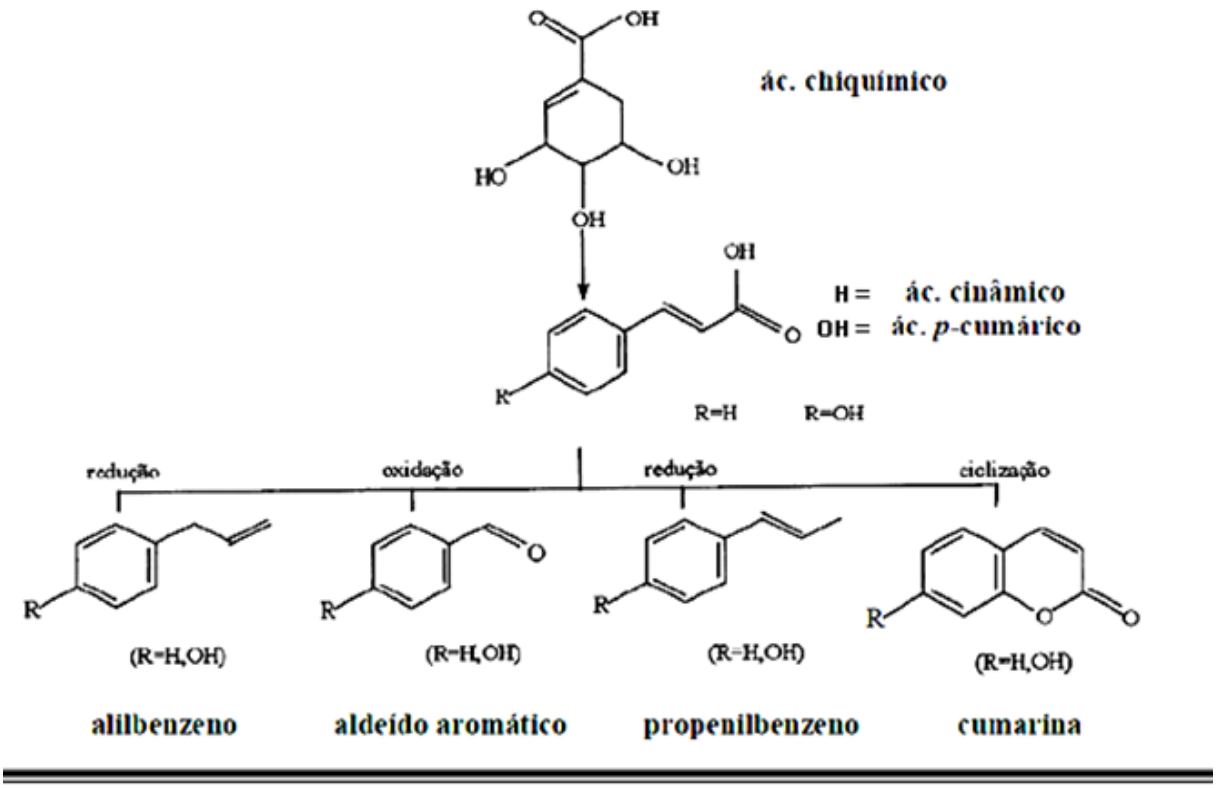
Figura 3 - Rota biossintética do metabolismo secundário.



Fonte: SANTOS, 2004.

Os fenilpropanóides são substâncias naturais amplamente distribuídas nos vegetais e constituídas por compostos formados por um esqueleto carbônico com um anel aromático unido a uma cadeia de três átomos de carbonos derivados de aminoácidos aromáticos (CROTEAU, KUTCHAN, LEWIS, 2000), oriundos biossinteticamente da via do ácido chiquímico, a qual produz o aminoácido aromático fenilalanina, que por ação da enzima fenilalanina amonialiase (FAL), origina o ácido cinâmico e o ácido *p*-cumárico (SANTOS, 2004; SIMÕES, SPITZER, 2004), que por meio de reduções enzimáticas da cadeia lateral destes ácidos leva à formação de alilbenzenos e propenilbenzenos, esqueletos carbônicos dos fenilpropanóides (Figura 4). Os principais fenilpropanóides conhecidos são eugenol, metil eugenol, miristicina, elemicina, chavicol, metil chavicol, dilapiol, anetol, estragol, apiol (SANGWAN et al., 2001).

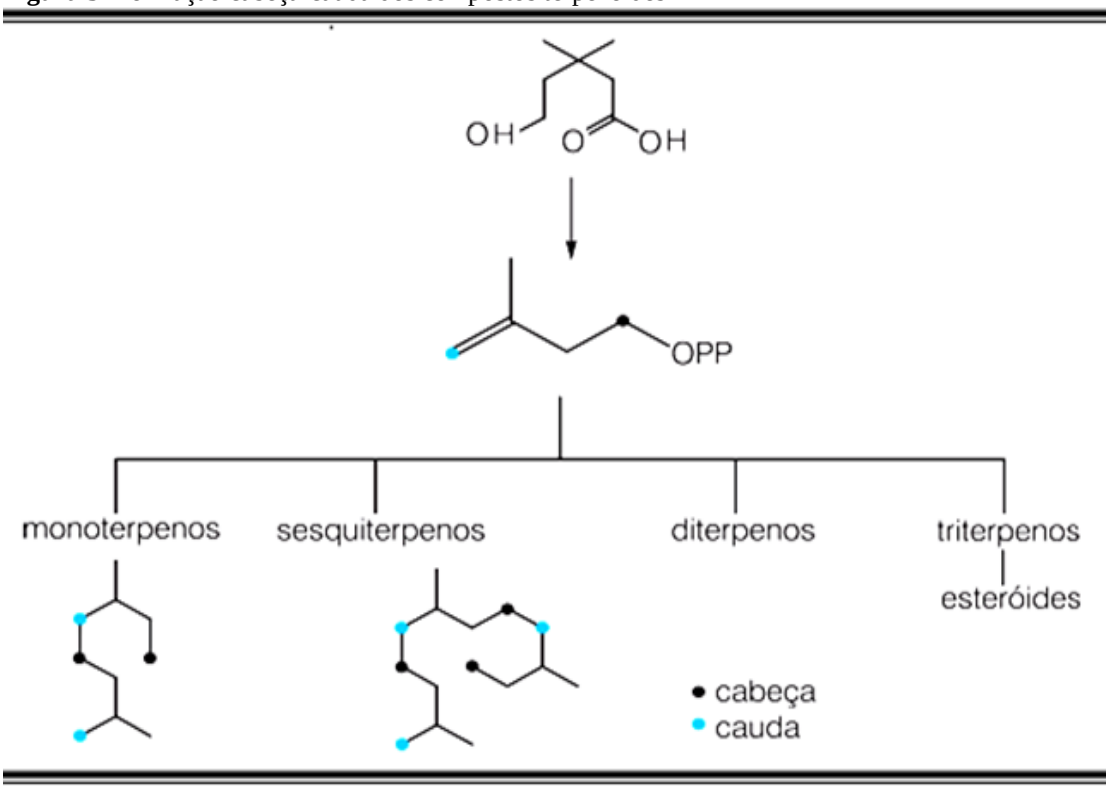
Figura 4- Formação dos compostos fenilpropanoides



Fonte: ALVES, 2001

Os terpenóides, também chamados isoprenoides, são compostos que ocorrem em todas as plantas e compreendem a classe funcional e estruturalmente mais variada de metabólitos secundários (VERPOORTE, 2000). O termo terpenoide foi dado em virtude do primeiro composto dessa classe ter sido isolado da terebentina (BRAMLEY, 1997). Ele também é empregado para indicar todas as substâncias de origem biossintética derivadas de unidades do isopreno (Figura 5) de cinco átomos de carbono (ALVES, 2001).

Figura 5- Formação cabeça-cauda dos compostos terpenóides



ALVES, 2001

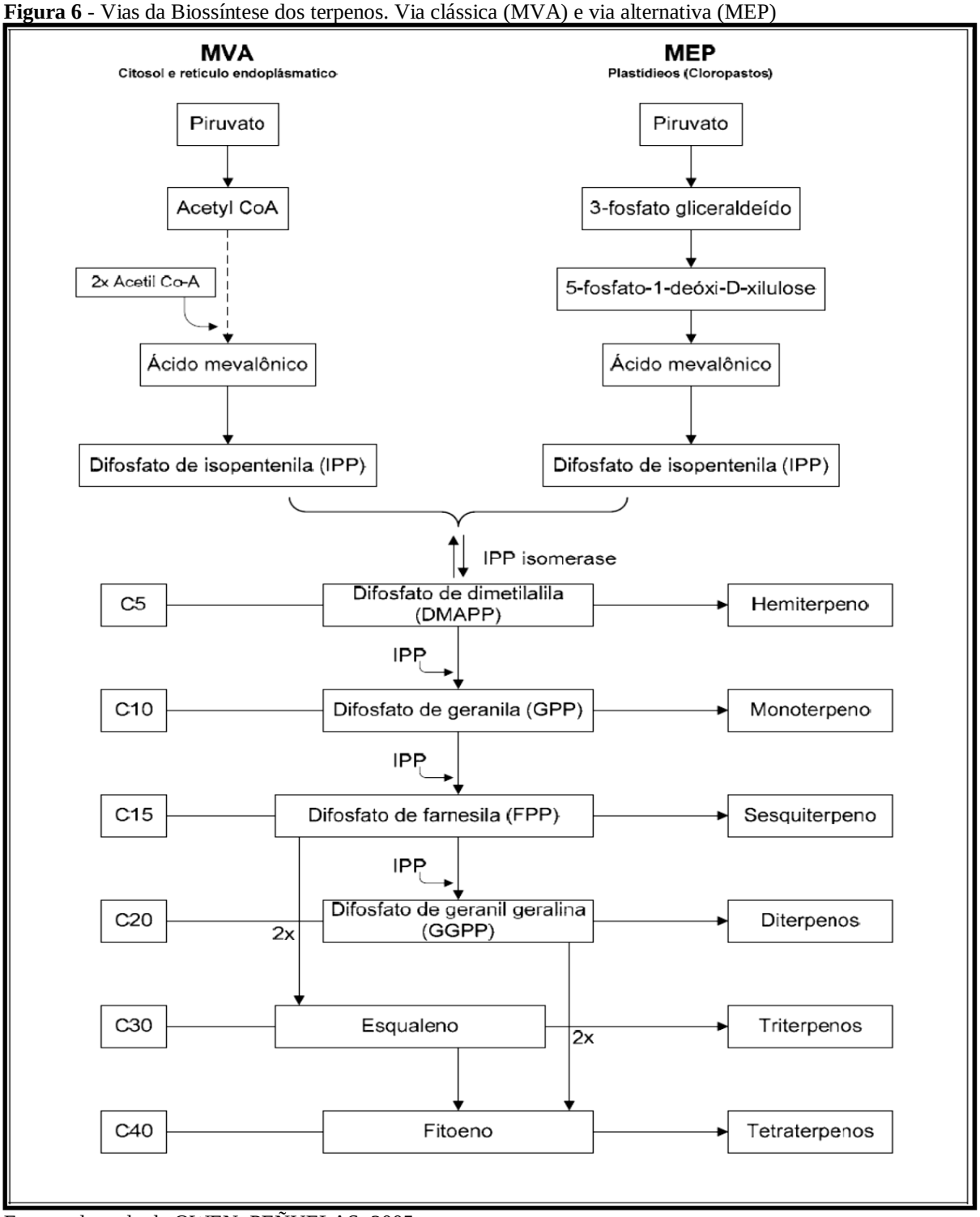
A partir da condensação de unidades pentacarbonadas, o isopentenil difosfato (IPP), e seu isômero dimetilalil difosfato (DMAPP), dá-se início a formação dos terpenos (VERPOORTE, 2000). A formação do IPP pode ocorrer por duas vias biossintéticas: 1) a via do mevalonato ou via clássica, responsável pela formação dos sesquiterpenos (C_{15}) e triterpenos (C_{30}) que ocorre no citosol e cujos precursores são piruvato e acetilcoenzima A e 2) a via alternativa conhecida como via do metileritritol fosfato (MEP), que origina os monoterpenos (C_{10}, uma unidade isoprênica); diterpenos (C_{20}, duas unidades isoprênicas) e tetraterpenos (C_{40}, quatro unidades isoprênicas), ocorrem nos plastídios e tem como precursores piruvato e gliceraldeído-3-fosfato (Figura 6) (CROTEAU, KUTCHAN, LEWIS, 2000; VERPOORTE, 2000; AHARONI et al., 2006).

Para a formação dos monoterpenos (C_{10}), uma unidade de IPP é adicionada a uma de DMAPP, formando o geranil difosfato (GPP), precursor de terpenos com dez carbonos. A junção de uma unidade de IPP ao GPP forma o farnesil difosfato (FPP), com 15 átomos de carbonos, a partir do qual se formam os sesquiterpenos. A adição de uma unidade de IPP ao FPP, por sua vez, forma o de geranilgeranil difosfato (GGPP), com 20 átomos de carbonos, precursor dos diterpenos. Estas estruturas são posteriormente modificadas por enzimas (hidroxilases, desidrogenases, redutases e glicosil, metil e acil transferases), que juntas geram

uma série de compostos diferentes e dão origem aos óleos essenciais, terebentinas e resinas (BOHLMANN, MEYER-GAUEN, 1998; AHARONI et al., 2006). Os triterpenos, com 30 átomos de carbonos, são formados pela união de duas unidades FPP e os tetraterpenos, com 40 átomos de carbonos, pela junção de duas unidades GGPP (CROTEAU, KUTCHAN, LEWIS, 2000; VERPOORTE, 2000).

Os diversos terpenos apresentam funções variadas nos vegetais. Os monoterpenos e sesquiterpenos são constituintes dos óleos voláteis, sendo que os primeiros atuam na defesa química da planta contra a ação de predadores (CASTRO et al., 2007), e na atração de polinizadores. Os sesquiterpenos, em geral, apresentam funções protetoras contra fungos e bactérias, enquanto muitos diterpenóides dão origem aos hormônios de crescimento vegetal. Os triterpenóides e seus derivados, os esteroides, apresentam função protetora contra herbívoros; alguns são antimitóticos e outros atuam na germinação das sementes e na inibição do crescimento da raiz (VICKERY, VICKERY, 1981)

Figura 6 - Vias da Biossíntese dos terpenos. Via clássica (MVA) e via alternativa (MEP)



Fonte: adaptado de OWEN, PEÑUELAS, 2005.

2.3.1 Importância dos Óleos Essenciais

A diversidade de substâncias ativas provenientes do metabolismo vegetal tem motivado o desenvolvimento de pesquisas envolvendo extratos e óleos essenciais de plantas medicinais,

tendo em vista suas diversificadas atividades biológicas, e seu importante papel no processo de desenvolvimento de novos agentes terapêuticos.

Os óleos essenciais obtidos a partir de várias espécies de plantas têm uma ampla e variada aplicação em muitas indústrias na preservação de alimentos (HYLDGAARD, MYGIND, MEYER, 2012), como cosméticos, perfumes, aromatização de alimentos, bebidas, sorvetes e produtos de utilidade doméstica, como detergentes, sabões, repelentes e aromatizantes de ambiente, etc. (BURT, 2004). Recentemente estão ganhando muito interesse por causa de seus múltiplos usos e do seu potencial biológico devido às suas propriedades antioxidante (BURT, 2004), efeitos anti-inflamatórios, antinociceptivo e antiespasmódico (DANIEL et al., 2009; ADORJAN, BUCHBAUER, 2010; PINHO et al., 2012), atividades antitumoral (WANG et al., 2012; QUASSINTI et al., 2013), antibacteriana e antifúngica (KNAAK, FIUZA, 2010).

Diversos estudos também foram realizados por vários pesquisadores para avaliar o potencial de óleos essenciais contra insetos (ISMAN, 2006; AUTRAN et al., 2009; LIU, CHU, JIANG, 2011; CHU et al., 2012; SANTOS et al., 2012; REGNAULT, VICENT, ARNASON, 2012) e ácaros (CHOI, et al., 2004; SERTKAYA, KAYA, SOYLU, 2010; PASAY, et al., 2010; MOTAZEDIAN, RAVAN, BANDANI, 2012), dentre eles, tem sido relatada atividade acaricida contra *Tetranychus urticae* com óleos essenciais de plantas medicinais (MIRESMAILLI, BRADBURY, ISMAN, 2006; CAVALCANTI et al., 2010; PONTES et al., 2010; HAN et al., 2011; ATTIA et al., 2011; ATTIA et al., 2012; ARAÚJO et al., 2012; LABORDA et al., 2013).

2.4 *Tetranychus urticae*

O ácaro *T. urticae* Koch, (Acari: Tetranychidae), é uma praga de grande interesse agrícola, vulgarmente conhecido como ácaro-rajado, um minúsculo artrópode, de coloração amarelo-esverdeada. As fêmeas são grandes, medindo menos de um milímetro de comprimento e geralmente com manchas dorsais escuras, Na língua inglesa possui dois nomes comuns, “two-spotted spider mite” ou “red mite”, referentes à variação de cores apresentada pelas fêmeas em diferentes condições ambientais (MORAES, FLECHTMANN, 2008).

Figura 7- *Tetranychus urticae*

Fonte: http://www.promip.agr.br/pragas_interna.ph

Ácaros de importância agrícola são pragas que provocam grandes prejuízos para os pequenos, médios e grandes agricultores. O ácaro-rajado, *T. urticae* Koch, é considerado uma das principais pragas da agricultura por atacar diversas culturas economicamente importantes, de valor nutritivo ou ornamental em todo o mundo, como culturas de algodão, feijão, milho, soja, mamão, macieira, videira, alface, batata, berinjela, melancia, melão, morangueiro, pepino, tomateiro, além de flores de plantas ornamentais (MORAES, FLECHTMANN, 2008; AFIFY, ALY, TURKY, 2012). Essa espécie cosmopolita de ocorrência em regiões temperadas e tropicais pode ocorrer em casa de vegetação ou no campo (GALLO et al., 2002).

No Brasil, esta praga já foi registrada em diversos Estados de Norte a Sul. Em Pernambuco, seu primeiro registro de ocorrência foi em 1985, após o início dos cultivos irrigados no município de Petrolina (MORAES, 2001). Desde então esse ácaro tem causado grandes prejuízos aos agricultores, atacando diferentes culturas.

O controle do ácaro rajado tem sido realizado, principalmente, por método químico através de inseticidas orgânicos sintéticos, como organoclorados, organofosforados e carbamatos (THACKER, 2002), os quais são frequentemente associados com os resíduos perigosos para o consumo, podendo ainda ser responsável por problemas relacionados à contaminação do meio ambiente, desenvolvimento de resistência de diversas pragas e eliminação de inimigos naturais (POTENZA, TAKEMATSU, BENEDICTO, 1999; HERNANDEZ et al., 2012). Além disso, a alta toxicidade destes produtos afeta também espécies benéficas, inclusive os mamíferos (GONÇALVES et al., 2001).

O desenvolvimento de resistência em insetos e ácaros a produtos químicos sintéticos tem sido um dos maiores problemas no controle de pragas (MORAES, FLECHTMANN, 2008). A resistência de *T. urticae* a pesticidas tem sido documentada em diversos países, incluindo o Brasil (DARP, 2012). Trabalhos científicos têm indicado que diversas populações deste ácaro

já se mostraram resistentes a alguns acaricidas como dimetoato, abamectin, a fenpyroximate e propargite (SATO et al., 2000; SATO et al., 2004; SATO et al., 2005; SATO, et al., 2009). Em videira o ácaro rajado mostrou ser resistente ao dimetoato (SOUZA-FILHO, SUPLICY-FILHO, SATO, 1994); em pessegueiros, a dimetoato, mevinfós, naled e cihexatina (SATO et al., 2000), em morangueiro, a fenpyroximate (SATO et al., 2004) e em plantas ornamentais, como crisântemo, a clorfenapir (SATO et al., 2007).

A busca por novos produtos com potencial acaricida/inseticida a partir de plantas medicinais tem sido amplamente investigada visando minimizar os problemas inerentes ao uso indiscriminado de inseticidas convencionais, que apresentem baixos custos e que sejam inofensivos para os seres humanos e outros animais (TANKIWICZ, FENIK, BIZIUK, 2011). Uma alternativa viável a estes acaricidas/inseticidas convencionais é o uso de produtos naturais obtidos de diferentes partes do vegetal na forma de extratos, pós, óleos fixos e voláteis (GONÇALVES et al., 2001). Esses produtos, usados para o controle de pragas são denominados de inseticidas botânicos. Óleos essenciais de plantas podem ser considerados como fontes promissoras para o controle de pragas, uma vez que constituem uma fonte rica de compostos químicos bioativos e são biodegradáveis (ISMAN, 2006).

2.5 Agentes Antimicrobianos

Óleos essenciais de diversas plantas também tem demonstrado potencial como agentes antimicrobianos (FARIA et al.,2006; OROOJALIAN et al., 2010; MIHAILOVIĆ et al., 2011; ABDELHADY, ALY, 2012; BITU et al., 2012; BNOUHAM et al., 2012; CHAUDHRY et al., 2012; MAKHLOUFI, MOUSSAOUI, LAZOUNI, 2012; NADIR et al., 2013).

Substâncias antimicrobianas ou antibióticas constituem um grupo especial de agentes terapêuticos, geralmente produzidos e obtidos a partir de organismos vivos. São substâncias que, em pequenas concentrações, devem possuir atividade letal ou inibitória contra muitas espécies microbianas (COWAN, 1999).

Os micro-organismos patogênicos vêm se apresentando cada vez mais resistentes aos antibióticos atualmente disponíveis. A busca por substâncias de estrutura química diversa de origem vegetal sob a forma de extratos ou óleos essenciais, que atuem como novos agentes antimicrobianos tem sido objeto de inúmeras pesquisas (SUTCLIFFE, 2003). Os produtos naturais de origem vegetal por apresentarem uma diversidade de estruturas moleculares como compostos alcaloides, flavonoides, polifenóis e terpenóides têm se mostrado eficientes contra as mais variadas espécies microbianas (YUNES, CECHINEL-FILHO, 2001; LIMA, 2001).

Antimicrobianos naturais tem sido usados na conservação de alimentos (SOUZA et al., 2005), no combate a micro-organismos causadores de doenças de pele (WECKESSER, 2007**;** CRUZ et al., 2007), de úlcera gástrica (STEGE, 2006), etc**.** Um dos principais interesses, no entanto, é no combate a micro-organismos resistentes (ARIAS, et al., 2004; NASCIMENTO, 2000).

Para avaliar a atividade antimicrobiana de materiais obtidos de fontes vegetais, diferentes métodos padrão são frequentemente utilizados, tais como: ensaio de difusão em disco (método utilizado neste estudo), bioautografia e a macro ou microdiluição (método também utilizado neste estudo), para a determinação da concentração inibitória mínima (CMI) (BURT, 2004; BAKKALI et al., 2008).

A triagem de óleos essenciais e extratos para a atividade antibacteriana é muitas vezes feito pelo ensaio de difusão em disco, no qual um disco de papel embebido com uma concentração conhecida do material a ser avaliado é colocado em contato com um meio de cultura sólido em que foi inoculado um determinado microrganismo. Esse método geralmente é usado para verificação preliminar da atividade antibacteriana antes de estudos mais detalhados (BAUER, KYRBY, 1966).

A bioautografia é um estudo qualitativo (inibição ou não do crescimento microbiano). O princípio da técnica baseia-se na imersão de uma placa de cromatografia em camada fina, devidamente preparada com a substância desejada, colocada em placa de petri sobre a qual se aplica o meio de cultura e posteriormente sobre o meio, aplica-se uma suspensão de bactéria. (CUNICO et al., 2007).

A eficiência da atividade antimicrobiana pode ser determinada pela diluição da amostra analisada em ágar ou em caldo (PINTORE et al, 2002). O método da diluição em caldo relaciona o crescimento microbiano em um meio de cultura líquido, verificado pela turbidez do meio, com a concentração do material ensaiado, utilizando também um padrão de referência como controle positivo, bem como controles negativos nesta comparação. Permite avaliações quantitativas, possibilitando a verificação da concentração inibitória mínima (CIM), ou seja, o teor mínimo do material ensaiado que inibe o crescimento microbiano, representado pela ausência de turvação no meio. O método mais citado e importante no desempenho antimicrobiano é a medição da concentração inibitória mínima (CIM), que informa os resultados precisos, exato e reprodutível (CLSI, 2009). Em alguns casos, a concentração mínima bactericida (CMB) ou a concentração bacteriostática é indicada, ambos os termos concordando em estreita colaboração com a CIM (HUSSAIN. 2009).

2.6. Câncer

Relevantes atividades citotóxicas de óleos essenciais contra várias linhagens de células cancerígenas também têm sido descritas (FAYED, 2009; SHARMA et al., 2009; PATHARAKORN et al., 2010; JAGANATHAN, SUPRIYANTO, 2012; JAYAPRAKASHA et al., 2013).

Câncer é um conjunto de doenças, que atualmente no mundo, é a causa de inúmeras mortes (Figura 8) (SEUNG *et al*., 2004). Também é denominado de neoplasia maligna ou tumor maligno, corresponde a um grupo de várias doenças que têm em comum a proliferação descontrolada de células anormais e que pode ocorrer em qualquer local do organismo. Ele se caracteriza pela perda do controle da divisão celular e pela capacidade de invadir outras estruturas orgânicas. Assim sendo, as células se dividem de forma rápida, agressiva e incontrolável, espalhando-se para outras regiões do corpo, acarretando transtornos funcionais (INSTITUTO NACIONAL DE CÂNCER, 2011).

Figura 8 - O câncer no mundo

As diferentes formas de câncer correspondem aos vários tipos de células do corpo. Câncer derivado de tecidos epiteliais como pele ou mucosas é denominado carcinoma e os derivados em tecidos conjuntivos como osso, músculo ou cartilagem é chamado de sarcoma**.** Outras características que diferenciam os diversos tipos de câncer entre si é a metástases**,** isto é, a velocidade de multiplicação das células e a capacidade de invadir tecidos e órgãos vizinhos ou distantes (TAVARES, SEGÓVIA, PAULA, 2007).

Vários fatores podem estar envolvidos no aumento da incidência do câncer, como por exemplo, o envelhecimento da população, sedentarismo, hábitos alimentares, fatores

inflamatórios e principalmente predisposição genética e condições ambientais. Atualmente, sabe-se que existe susceptibilidade diferencial à carcinogênese, em parte por diferenças genéticas no metabolismo do carcinógeno e/ou por alteração na capacidade de reparo do DNA (ANDRADE, PEREIRA, 2007).

Estudos com linhagens de células cancerígenas identificaram uma variedade de marcadores moleculares e mutações específicas que podem estar envolvidas na patogênese do câncer. Essas variações incluem a hiperexpressão de oncogenes, deleção de genes supressores tumorais ou perda da expressão de genes supressores tumorais, entre outros (CROCE, SOZZI, HUEBNERK, 1999). Os marcadores tumorais, substâncias utilizadas como indicadores de malignidade, são componentes celulares, estruturais e bioquímicos, que podem definir alterações celulares e moleculares. Na maioria dos casos, são produtos normais do metabolismo celular que apresentam aumento de produção devido à transformação maligna (ALMEIDA, et al., 2007).

A incidência crescente de câncer em todo mundo tem estimulado a busca de pesquisas e terapias, mais seguras e eficazes, para prevenção e combate do mesmo. Ainda que vários progressos venham sendo realizados no estudo das neoplasias, de acordo com Oliveira, Pinheiro e Valadades (2005), o sucesso no tratamento de tumores tem se mostrado discreto, devido ao grau de agressividade da doença, os mecanismos de escape das células neoplásicas, além dos efeitos colaterais causados pelos agentes antineoplásicos.

Assim, continuam abertos novas invesigações de pesquisas para novos compostos antineoplásicos e sua avaliação em vários sistemas tumorais e cultura de tecidos, com a finalidade de selecionar compostos mais efetivos (FLORÊNCIO et al., 2007). Com o desenvolvimento de técnicas cada vez mais avançadas e o elevado interesse nessa área, muito ainda está para ser descoberto (APOLINARIO et al., 1997; WRIGHT, GRUIDL, 2000; YATABE et al., 2000).

Métodos *in vitro* que medem o potencial citotóxico de drogas continuam sendo desenvolvidos para uma avaliação mais sensível da concentração que danifica componentes, estruturas ou vias bioquímicas celulares, utilizando corantes fluorescentes ou coloração específica de célula incluindo o método colorimétrico MTT ou 3-(4,5 dimethyl thiazole-2yl)-2,5 diphenyl tetrazolium bromide (SUN et al , 2005; YOO et al., 2005), teste de exclusão com Trypan Blue (HORVATHOVA et al., 2006), método de vermelho neutro (CHUNG et al , 2007), entre outros. MTT tem sido amplamente relatado devido à sua simplicidade e confiabilidade para medir a viabilidade celular para o rastreamento de agentes antiproliferativos (MANOSROIA, DHUMTANOMA, MANOSROIA, 2006; JIE et al, 2007).

Várias plantas medicinais são interessantes para ser investigadas e desenvolvidas como agentes anticancerígenos, uma vez que na atualidade, estão aumentando as buscas sobre os mecanismos envolvidos com atividades farmacológicas (OLIVEIRA, PINHEIRO, VALADARES, 2005).

3. Objetivos

3 OBJETIVOS

3.1 Objetivo Geral

❖ Investigar o potencial biológico do óleo esencial e extratos orgânicos obtidos de folhas de *Indigofera suffruticosa* Mill.

3.2 Objetivos Específicos

- Obter o óleo essencial da folha de *I. suffruticosa.* e identificar os seus constituintes químicos por CG/EM.
- Avaliar a atividade acaricida do óleo essencial da folha de *I. suffruticosa.*
- Avaliar a atividade antitumoral de óleos essenciais da folha de *I. suffruticosa.*
- Avaliar a atividade antimicrobiana de óleos essenciais e extratos orgânicos da folha de *I. suffruticosa*.
- Investigar o potencial sinérgico de eritromicina com extratos orgânicos da folha de *I. suffruticosa* sobre *Staphylococcus aureus.*

4. Referências

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5. Capítulo I

5. CAPÍTULO I – Artigo 1

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Abstract: The essential oil obtained from leaves of Indigofera suffruticosa was characterized by GC and GC-MS. Chemical analysis allowed the identification of 17 constituents, representing 97.7% of the total chemical composition with a high percentage of phenylpropanoids (89.9%), of which Eugenol (45.6%), (E)-asarone (17.4%) and Dill apiole (10.6%) were identified as the principal agents. The oil revealed strong fumigant activity (LC50 = 0.90 μL L-1). Laboratory trials of the fumigant action of the oil and its constituents, eugenol, (E)-asarone, dill apiole, (Z)-asarone, thymol, nerolidol, and safrole, and blends of these selected constituents were evaluated for activity against Tetranychus urticae. Ovicidal and fertility tests revealed that the vapors of I. suffruticosa as well as its main compounds interfered with the viability and number of eggs produced by the T. urticae females. Eugenol, Thymol and the complete blend showed the same level of toxicity and were about 225 times more potent than the oil. The results suggest that the essential oil from leaves of I. suffruticosa and some of its selected components seems to be a promising agent for future studies towards the preparation of a product formulated against T. urticae in greenhouse.

Highlights

- Essential oil analyses of *Indigofera suffruticosa* showed 89.9% of phenylpropanoids.
- Seventeen constituents were identified, in essential oil of *I. suffruticosa* leaves.
- Eugenol, (E) asarone and dill apiole were major constituents of *I. suffruticosa* oil.
- The essential oil revealed strong fumigant activity against *Tetranychus urticae.*
- Eugenol, thymol and complete blend showed the same level of toxicity on *T. urticae*.

Chemical composition and acaricidal activity of essential oil from *Indigofera suffruticosa* Mill against *Tetranychus urticae* Koch.

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ABSTRACT

The essential oil obtained from leaves of *Indigofera suffruticosa* was characterized by GC and GC-MS. Chemical analysis allowed the identification of 17 constituents, representing 97.7% of the total chemical composition with a high percentage of phenylpropanoids (89.9%), of which Eugenol (45.6%), (E)-asarone (17.4%) and Dill apiole (10.6%) were identified as the principal agents. The oil revealed strong fumigant activity (LC_{50} = 0.90 µL L^{-1}). The fumigant action of the oil and its constituents, eugenol, (E)-asarone, dill apiole, (Z)-asarone, thymol, nerolidol, and safrole, and blends of these selected constituents were evaluated for activity against *Tetranychus urticae*. Ovicidal and fertility tests revealed that the vapors of *I. suffruticosa* as well as its main compounds interfered with the viability and number of eggs produced by the *T. urticae* females. Eugenol, Thymol and the complete blend showed the same level of toxicity and were about 225 times more potent than the oil. The results suggest that the essential oil from leaves of *I. suffruticosa* and some of its selected components seems to be a promising agent for future studies towards the preparation of a product formulated against *T. urticae* in greenhouse.

KEY-WORDS: Spider mites; Leaf essential oil; Fumigant activity; Toxicity; Blend.

1. Introduction

Tetranychus urticae Koch (Acari: Tetranychidae), commonly known as the two-spotted spider mite occurs both in the greenhouse and in the field and has a wide distribution in temperate and tropical regions (Flamini, 2006). It is considered a major agricultural pest for attacking crops of economic importance (Afify et al., 2012). In Pernambuco, in irrigated systems in the city of Petrolina, this pest has attacked various crops of ornamental and agricultural interest as culture of bean, raspberry, peach, tomato and cotton (Sato et al. 2009).

The use of conventional insecticides in Brazil has grown dramatically and has caused great concern among various segments of society, which have demanded ecological alternatives, i.e. the use of products with low toxicity to mammals and low persistence in the soil. The resistance of *T. urticae* to miticide abamectin and fenpyroximate in commercial fields of several crops was described in the literature (Sato et al 2009). Natural Products are a good alternative to control the dissemination of this insect pest. Considering the already proven biological activity, including against arthropods (Isman, 2006), added to its volatility, biodegradability and, in general, due to low or no toxicity to mammals, essential oils, obtained from different parts of plants, are strong candidates in the search for a leading product for the formulation of alternative products to synthetic pesticides to be used in pest control. In recent years, plant essential oils and their constituents have been investigated for several biological activities (Interaminense et al., 2013; Pinho et al., 2012). A significant number of studies aimed at assessing the miticide potential of essential oils from medicinal plants has been carried out by several researchers (Laborda et al., 2013; Araújo et al., 2012; Sertkaya et al., 2010; Attia et al., 2011; Pontes et al., 2007; Miresmailli et al., 2006).

Among the species with widespread use in folk medicine, the members of the genus *Indigofera* in the legume family Fabaceae, subfamily Papilionoideae, stand out. This genus has approximately 700 species distributed in Africa, Asia, Australia and the Americas (Hassen et al., 2007). Among the species of this genus, *Indigofera suffruticosa* Mill is noted for its biological properties and use in folk medicine. Known commonly as indigo, it is a plant native to the West Indies (Almeida, 1993) with a distribution in tropical and subtropical America. In Brazil, it has been introduced and cultivated on a large scale for natural indigo dye extraction to supply the textile industry. Being easily found in the northeastern region, infusions and decoctions of different parts of the plant are used in folk medicine as a febrifuge, purgative, sedative, and insecticide (Hastings, 1990). Previous investigations of the biological potential of aqueous extracts of the leaves of this plant have revealed mammal

embryotoxic (Leite et al., 2004) and antimicrobial effects (Leite et al., 2006), as well as mutagenic activity and anticonvulsant from methanolic extracts (Calvo et al., 2011; Almeida et al., 2013). However, no investigation of chemical composition and acaricidal potential has been reported with essential oil of *I. suffruticosa* leaves.

In order to contribute to the phytochemical knowledge and potential biological application of plants that occur in Brazil, this study aimed to identify the chemical composition and evaluate the fumigant, ovicidal and oviposition deterrence effect on *T. urticae* of essential oils from the leaves of *I. suffruticosa*. Furthermore, it was also evaluated the miticide effect of the main components and of its blends prepared in the same proportion as identified in nature.

2. Material and methods

2.1. Plant material

Fresh leaves of *I. suffruticosa* were collected in the morning in the municipality of São Caetano, Pernambuco, Brazil. The plant was identified by Dra. Marlene Carvalho Alencar Barbosa from the Department of Botany, Federal University of Pernambuco (UFPE), Brazil. A voucher specimen was deposited with the identification number 45.217 at the UFP Geraldo Mariz Herbarium at UFPE.

2.2. Essential oil extraction

Fresh leaves (100 g) were submitted to hydrodistillation for 2 h in a Clevenger-type apparatus. The oil layers obtained were separated and dried over anhydrous sodium sulfate, weighed, stored in hermetically sealed glass containers and kept at -20 ^{0}C until further analysis. The oil yields (w/w) were calculated from the weight of fresh material. All procedures were performed in triplicate.

2.3. Chemicals

Thymol, nerolidol, eugenol, safrole, dill apiole, (E)- and (Z)-asarone standard compounds used for the identifications of volatile components and in bioassays were purchased from Sigma-Aldrich.

2.4. Gas chromatography and gas chromatography-mass spectrometry

2.4.1. Gas chromatography

Quantitative GC analyses were performed on a Hewlett-Packard 5890 Series II GC apparatus equipped with a flame ionization detector (FID) and using a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness) (J & W Scientific) . The oven temperature was programmed from 50 °C to 250 °C at 3 °C/min during the integration purposes. Injector and detector temperatures were at 250 °C. Hydrogen was used as the carrier gas at a flow rate of 1 L/min and 30 p.s.i. inlet pressure in split mode (1:30). The injection volume was 0.5 μL containing diluted oil (1/100) v/v with n-hexane. The amount of each compound was calculated from GC peak areas in the order of DB-5 column elution and expressed as a relative percentage of the total area of the chromatograms. Analyses were conducted in triplicate.

2.4.2. Gas chromatography-mass spectrometry

The qualitative GC/MS analyses was carried out using Hewlett-Packard GC/MS (CG: 5890 SERIES II/CG-MS: MSD 5971) , system operating in the EI mode at 70 eV fitted with the same column and temperature program as described for the GC experiments, with the following parameters: carrier gas was helium; flow rate: 1 mL/min; split mode (1:30); injected volume: 1 μL of diluted solution (1/100) of oil in n-hexane.

2.5. Identification of essential oil constituents

Identification of the individual components of the essential oil was based on GC retention indices (RI) with reference to a homologous series of n-alkanes (C_7-C_{30}) calculated according to the equation of Van den Dool and Kratz (1963) and by computer matching against the mass

spectral library of the GC/MS data system (NIST 98 and WILEY) and co-injection with authentic standards, as well as other published mass spectra (Adams, 2007). Area percentages were obtained electronically from the GC-FID response without the use of an internal standard or correction factors.

2.6. Optical Rotation

Measurements of the optical rotation of the *Indigofera* oil was performed with a digital polarimeter (A. Krüss model Px800, West Germany) at 589 nm and 26°C as a solution in dichloromethane.

2.7. Acaricidal assay

2.7.1. Biological material

The mite *T. urticae* was identified by the acarologist Dr. Manoel Guedes C. Gondim-Júnior from Agronomy Departament of Federal Rural University of Pernambuco. The mite *T. urticae* used for the bioassay were obtained from established laboratory colonies maintained for more than 10 years without any pesticide exposure, as describe in a previous work (Araujo et al., 2012). The spider mites were reared on beans plants *Canavalia ensiformes* in the greenhouse, at temperature of 25 ± 1 ºC, relative humidity of 65 ± 5% and 12:12 h photoregime.

2.7.2. Fumigant assay

The method to evaluate the fumigant activity of the oil was the same as that used by Pontes et al. (2007). Glass recipients with a capacity of 2.5 L were used as test arenas. Adult female spider mites placed on *C. ensiformes* leaf disks (2.5 cm diameter) were exposed to *I. suffruticosa* volatile oils. A fine haired brush was used to transfer the mites onto the leaf disks. To maintain the turgor of the disks and avoid the escape of mites, the leaf disks were placed onto filter paper disks saturated with water in Petri dishes (9 cm). The experiments were performed in three replicates. Each replicate consisted of 30 specimens of *T. urticae* placed on 3 leaf disks (10 mites per disk) in a Petri dish. The amounts of oil applied on a strip

of filter paper (5 x 3 cm) attached to the underside of the recipient lid by an automatic pipette, were of 0.02 to 12 µL, corresponding of 0.08 to 4.8 µL L^{-1} air. Control glass recipients contained no essential oil or other products. Eugenol was used as a positive control and its concentration ranged from 6.4 x 10^{-5} to 1.2 µL L^{-1} air. Mortality was determined after 24 h. Following exposure, the Petri dishes with spider mites were then removed from the recipients arenas and the mites were touched slightly with a brush in order to determine mortality, and those with no sign of movement were considered dead, as reported in previous work (Pontes et al., 2007; Cavalcanti et al., 2010). The % mortality data for *I. suffruticosa* oil was submitted to analysis of variance, with mean values compared by Tukey's test ($P \leq 0.05$) using the SAS software (SAS Institute, 2002). The same data were also analyzed with the Probit model using the POLO-PC program for the determination of LC_{50} values, with 95% confidence levels set for all experiments (LeOra Software, 1987).

2.7.3. Effects of *Indigofera suffruticosa* oil on the fecundity of two-spotted spider mite

The methodology used for the fertility experiment was as reported by Pontes et al. (2007) with modifications. Glass containers with a capacity of 1.0 L were used as fumigation chambers. Five leaf discs from bean-Pig (1.5 cm) were placed equidistant in a Petri dish (10 cm) which contained a disc of filter paper saturated with water in order to prevent the escape of mites and to maintain the turgor of the leaves. In each leaf disc was placed only one adult female mite, then each Petri dish was placed into the fumigation chamber. The essential oils and their major constituents were applied with the aid of automatic pipette into strips of filter paper (10 x 2 cm) attached to the inner surface of the fumigation chamber lid. The concentration of the essential oil of *I. suffruticosa* (0.08 µL L^{-1} air), and its major compounds (0.0002 µL L^{-1} to 8 µL L^{-1} air), and also of eugenol, as the positive control (0.000064 µL L^{-1} air) was the same that reduced the mite oviposition, but differ significantly from that of the control found in the bioassay that evaluated the toxicity by fumigation assay. For each concentration were used 10 replicates. Fertility was assessed after 24 h of exposure, by counting the number of eggs. The experimental design was randomized and the data obtained after meet the tests of normality and homogeneity of variance were analyzed by Tukey's test at 5% probability by SAS (SAS Institute 2002).

2.7.4. Ovicidal assay

The methodology used for the ovicidal activity was that of Pontes et al. (2007) with modifications. Glass containers with a capacity of 1.0 L were used as fumigation chambers. Ten adult female spider mites were transferred to leaf discs from bean-Pig (2.5 cm) for obtaining the eggs. After a period of 12 hours, all females were removed from the leaves and their eggs counted, and only 30 eggs per leaf disc were left. The leaves were kept onto filter paper discs saturated with distilled water and placed in Petri dishes (8 cm in diameter and 1.5 cm height), as a manner to maintain the turgescence of the material. The experiments were performed in triplicate; a replicate consisted of 90 eggs of *T. urticae* placed on three leaf discs (30 eggs per disk) in a Petri dish, thus a total of 270 eggs per treatment were used. The essential oils and their major constituents were applied with the aid of automatic pipette into strips of filter paper (10 x 2 cm) attached to the inner surface of the fumigation chamber lid. The concentration of the essential oil of *I. suffruticosa* and its major compounds, as well as that of the positive control was 4.8 μL L^{-1} air, in relation to the concentration of oil responsible for 95% mortality of the adults in the bioassay of toxicity. The Petri dishes containing the eggs were exposed to vapors of oils, to the major constituents and to a positive control, for 24 h; then, the Petri dishes were removed from the fumigation chambers, and after 96 h the percentage of viable eggs and hatch were evaluated. Three repetitions were performed. Data of viability, after meet the tests of normality and homogeneity of variance, were subjected to analysis of variance and means were compared by Tukey test at 5% probability by SAS (SAS Institute 2002).

2.8. Comparative toxicity of compounds

The protocol described above for the fumigant assay was used in order to investigate the potential contribution of each constituent to the toxicity of the oil blend. Seven compounds of the oil, included the principal constituents, were selected and their fumigant activities were evaluated individually and in the form of a complete blend. A blend was also prepared with all seven constituents as well as blends removing one constituent at a time, based on the natural composition of the oil indicated by GC-MS and tested at the concentration at which the pure oil caused ≥ 95% mortality. The toxicity of the complete and incomplete blends was statistically compared with that of *I. suffruticosa* oil.

3. Results and Discussion

3.1 Chemical composition of *I. suffruticosa* leaves

Hydrodistillation from the leaves of *I. suffruticosa* provided a viscous, yellow oil with a strong odor and a yield of 0.04% w/w. Seventeen compounds, representing 97.7% of the total oil were identified by GC-MS. The constituents identified and their retention indices are listed in Table 1, according to their order of elution in a DB-5 capillary column. Phenylpropanoids (89.9 ± 0.5) was the predominant oil chemistry class in the oil of *I. suffruticosa*, followed by sesquiterpenes (6.4 ± 0.1) and monoterpenes (1.4 ± 0.1). The principal component of the oil was eugenol (45.6 ± 0.4), followed by (E)-asarone (17.4 ± 0.2) and Dill apiole (10.6 ± 0.2). Unlike the oil composition of *I. suffruticosa*, in the essential oil of another species in the genus *Indigofera* (*I. microcarpa*) the presence of phenylpropanoids and monoterpenes was not detected, however large amounts of sesquiterpenes (83.7%) were observed, among these β-caryophyllene (56.0%) and α-humulene (25.1%) were the principal constituents (Arriaga et al., 2008).

3.2 Acaricidal activity of *Indigofera suffruticosa* essential oil

Table 2 presents the toxicities of *I. suffruticosa* oil, selected constituents and their blend. Vapors of the essential oil from leaves of *I. suffruticosa* were toxic to adult *T. urticae*, promoting 96.6% mortality at concentration of 4.8 µl L^{-1} of air, and by linear regression analysis to obtain LC_{50} of *I. suffruticosa* oil revealed that by fumigation the average lethal concentration (LC_{50}) of the leaves oil was estimated at 0.90 µl L^{-1} of air. The positive control (eugenol) was about 225 times more toxic than the observed toxicity of *I. suffruticosa* oil. Because of the method used, the toxicity observed with oil can be attributed to vapor penetration of its chemical constituents through the respiratory system of the mite. Out of the seven chemical constituents selected from the oil of *I. suffruticosa*, just the (E)- and (Z)-asarone (LC_{50} = 6.64 µl L^{-1} of air and 8.51 µl L^{-1} of air, respectively) showed toxicity lower than that displayed by the essential oil. On the other hand, eugenol (LC_{50} = 0.004 µl L^{-1} of air) and thymol (LC_{50} = 0.002 µl L^{-1} of air), with the same level of toxicity, had the greatest fumigant action, followed by nerolidol (LC_{50} = 0.04 µl L^{-1} of air), dill apiole (LC_{50} = 0.28 µl L^{-1} of air) and safrole (LC_{50} = 0.36 µl L^{-1} of air), in comparison with the essential oil.

Several studies have shown positive results for fumigation activity of essential oils and their constituents against mites and other arthropods. The essential oils of *Mentha longifolia, Salvia officialis, Myrtus communis* (Motazedian et al., 2012), *Lippia siloides* and its component thymol (Cavalcanti et al., 2010) exhibited potent acaricidal activity by fumigation against *T. urticae*. Moraes et al (2012) have demonstrated that *T. urticae* mites were more susceptible to *Eugenia langsdorffii* leaf oils by fumigation than by residual contact. Furthermore, other work also have demonstrated that in experiments done with closed containers the mites *T. urticae* were more susceptible to vapors of *Piper aduncum* oil and its components dilapiol e (E)-nerolidol in fumigation tests, than to direct use in contact tests, in the latter test the mortality of mites was reduced by around 50% (Araújo et al., 2012). Previous studies have demonstrated that eugenol exhibit high toxicity against the mites *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* and *Tyrofagus putrescentiae*, which are etiological agents of allergy, and that the toxicic effect of this phenylpropanoid was more effective in closed containers than in open containers, indicating that the the effect of this compound was largely due to the action of the volatile phase (Kim et al., 2003a, Kim et al., 2003b). It has been demonstrated that eugenol presents high toxicity against the mite *Sarcoptes scabiei* (Pasay et al., 2010), and other studies have shown that eugenol also has insecticidal activity by fumigation against the insects *Rhyzopertha dominica, Callosobruchus chinensis* and *Oryzaephilus surinamensis* (Ogendo et al., 2008), and also against eggs and adult females of *Pediculus capiti* in closed containers (Yang et al., 2003). Furthermore, volatile phase effects of essential oils were also found to be more effective than contact phase effect against fungal growth (Soylu et al, 2006).

Therefore, our results suggest that eugenol and thymol, constituents of the essential oil, are those which contribute the most to the fumigant action observed for the oil of *I. suffruticosa*.

3.3 Comparative toxicities of *I. suffruticosa* essential oil and its compounds

To investigate the relative toxicity and the level of interaction of these selected constituents of the *I. suffruticosa* oil, new fumigation experiments were conducted with the mixture of these compounds, using the same proportion at which they were identified in the oil. The results obtained indicated that the toxicity of this mixture (LC_{50} = 0.002 µl L^{-1}) did not differ significantly from that observed with eugenol which was our positive control (LC_{50}

= 0.004 µl L^{-1}), since they are within the same confidence interval, but it was 225 times more toxic than the oil from *I. suffruticosa* (Table 2). Similar result was reported for the mixtures of select compounds from fruits essential oil of the *Eugenia langsdorffii* (Moraes et al., 2012).

The result of this study suggests that the reduced effect seen for the *I. suffruticosa* oil in relation to the mixtures of select compounds may be due to putative synergistic interactions among the selected constituents within the complete mixture. Furthermore, we suppose that the other constituents of the oil, which were unidentified (2.3%) along with those that were not selected for this study (20.6%) may exert antagonistic interactions that result in reducing the toxicity of the the *I. suffruticosa* oil. Jiang et al. (2009) studying the toxicity of essential oil of *Litsea pungens* and *Litsea cubeba* and some of its selected compounds considered that unidentified compounds could be responsible for a sinergic effect since the oil showed an high toxic effect than the mixture on *Trichoplusia ni*.

In order to investigate the level of interaction of the constituents of the complete mixture, in the proportion at which they were identified by CG/EM, fumigation tests were repeated with new mixes prepared by the removal of a component, one at a time, from the complete mixture at the concentration the oil promoted ≥ 95% mortality (4.8 µl L^{-1}).

Between the two, individually tested constituents which showed higher toxicity than observed for *Indigofera* oil (eugenol and thymol), only the experiment performed with the blend from which the eugenol was removed revealed a drastic reduction in mortality of mites (6.66%) (Fig. 1), suggesting that this phenylpropanoid, in the proportion at which it is found, is what contributes most to the toxicity observed for the complete oil mixture. These results indicate that the fumigant action on *T. urticae* from a complex mixture of phenylpropanoids and terpenes, as from the oil of *Indigofera*, is related to the complex, synergistic and/or antagonistic interactions and proportions among the constituents, as well as their individual toxicities.

3.4 Ovicidal activity and effect on fecundity of *I. suffruticosa* oil and main constituents.

Results on the fecundity of mite induced by *I. suffruticosa* oil and some selected chemical constituents are shown in Fig. 2. The essential oil and its constituents were toxic to the mite *T. urticae* showing oviposition deterrent activity by reducing the amount of eggs laid, in comparison with the controls. Recently, Roh et al. (2011) evaluating the activity of essential oil of *Santalum album* and the main compound (santalol) in the fecundity of *T. urticae*

observed that only the main compound showed an oviposition deterrent effect. In addition, Topuz and Erler (2007) demonstrated that the essential oils from *Laurus nobilis, Myrtus communis* e *Artemisia absinthum* were toxic against adults and eggs of *T. Cinnabarinus*, exibiting oviposition deterring activity.

The ovicidal activity was measured by the percentage of non-viable eggs as demonstrated in Fig. 3. The results exhibited significant difference among the treated samples ($F_{8,18}$ = 484.88, P< 0.001). After 24 h of exposure, eugenol and safrol exhibited the highest ovicidal activity and the percentage of non-viable eggs did not differ significantly from each other. Previous work demonstrated that eugenol was also highly effective against eggs of *Pediculus capitis* (Yang et al., 2003). On the other hand, in the present study, the essential oil of *I. suffruticosa* and the selected compounds (E)-nerolidol, (E) and (Z)-asarone showed the lowest ovicidal activity, since the percentage of non-viable eggs promoted by vapors of *I. suffruticosa* did not differ significantly from the percentage obtained for these compounds mentioned. At 4.8 μL L^{-1} air the results obtained with *I. suffruticosa* oil are similar to the ovicidal activity observed with *Citronella java* of essential oil reported by Choi et al. (2004) when using 4.7 μL L^{-1} air, who also investigated the effect of *C. java* oil against *T. urticae*. Furthermore, previous studies suggest that the mode of action of some plant essential oils, such as *Caraway seed*, *Peppermint* and *Spearmint*, on adult and eggs of *T. urticae* are caused by the vapor phase via the respiratory system (Choi et al., 2004). Moreover, our results demonstrated that at relatively low concentrations of essential oil of *I. suffruticosa* and some of its constituents have potential to control the fecundity of *T. urticae* by reducing the egg oviposition and also the number of larvae that emerged after vapor exposition.

4. Conclusion

To the best of our knowledge, this is the first report of the chemical composition of the essential oil from *I. suffruticosa* Mill and evaluation of its miticide activity on *T. urticae*. The results obtained in this work indicate that under laboratory conditions the vapor of the essential oil from the leaves of *I. suffruticosa* is toxic to *T. urticae* and may be a promising candidate to be used against *T. urticae* for the development of a botanical pesticide for the integrated management of *T. urticae*. However, further studies should be conducted to better evaluate the use of this oil for the management of *T. urticae* in greenhouse.

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Table 1

Percentage composition of the essential oil of leaves of *Indigofera suffruticosa.*

Compound	RI[a]	RI[b]	(%) ± SD	Method of Identification
Methyl chavicol	1194	1195	5.2 ± 0.1	RI, MS
Safrole	1288	1285	0.1 ± 0.0	RI, MS, CI
Thymol	1293	1289	1.4 ± 0.1	RI, MS, CI
Eugenol	1352	1356	45.6 ± 0.4	RI, MS, CI
Croweacin	1452	1457	5.4 ± 0.3	RI, MS
α-Patchoulene	1458	1454	0.8 ± 0.0	RI, MS
(E)-*β*-Ioneno	1483	1487	2.3 ± 0.2	RI, MS
cis-Eudesma- 6,11-dieno	1494	1489	0.7 ± 0.0	RI, MS
Elemicin	1552	1555	0.8 ± 0.0	RI, MS
Nerolidol	1557	1561	0,4 ± 0,0	RI, MS, CI
(E)-Isoelemicin	1568	1568	0.3 ± 0.0	RI, MS
(Z)-asarone	1620	1616	1.6 ± 0.1	RI, MS, CI
Dill apiole	1625	1620	10.6 ± 0.2	RI, MS, CI
γ-eudesmol	1635	1630	1.0 ± 0.0	RI, MS
β-eudesmol	1645	1649	1.2 ± 0.1	RI, MS
4,6-dimetoxi-5-vinyl-1,2-benzoioxide	1648	1653	2.9 ± 0.1	RI, MS
(E)-asarone	1675	1675	17.4 ± 0.2	RI, MS, CI
Monoterpenes			1.4 ± 0.1	
Sesquiterpenes			6.4 ± 0.1	
Phenylpropanoids			89.9 ± 0.5	
Total			97.7 ± 0.6	

[a] Retention indices calculated from retention times in relation to those of the series *n*-alkanes on a 30m DB-5 capillary column. [b] Linear retention indices from the literature. SD = Standard Deviation. RI = Retention Index, MS = Mass Spectrum, CI = Co-injection with authentic standards.

Table 2

LC_{50} values (µL $L^{-1}$of air) of the essential oil of leave of *Indigofera suffruticosa*, individual constituents and their mixture against *Tetranychus urticae* in toxicity by fumigation.

Oil/compound/blend	N	df	slope	χ^2	Fumigation LC_{50} (CI 95%)
Indigofera	540	4	1.88	9.34	0.90 (0.61-1.29)[a]
FM	450	3	1.10	5.47	0.002 (0.001-0.004)[b]
Eugenol*	630	5	0.84	2.50	0.004 (0.002-0.008)[b]
Thymol	449	3	1.45	6.35	0.002 (0.001-0.003)[b]
Nerolidol	450	3	1.09	3.57	0.04 (0.02-0.07)[c]
Safrole	442	3	3.81	6.69	0.36 (0.23-0.46)[d]
Dill apiole	702	5	3.24	5.13	0.28 (0.24-0.32)[d]
(Z)-asarone	630	4	5.39	8.38	8.51 (7.14-9.61)[e]
(E)-asarone	540	3	6.93	6.84	6.64 (5.05-7.70)[e]

FM = full mixture of 7 constituents based on the composition of the *Indigofera* oil as indicated by GC-MS analyses. n = number of mites/dose. df = degrees of freedom. χ^2 = chi-squared. CI = confidence interval. * The positive control used in our study was the major constituent of the oil of *I. suffruticosa*. Columns followed by the same letter do not differ significantly based on the confidence interval (P = 0.05).

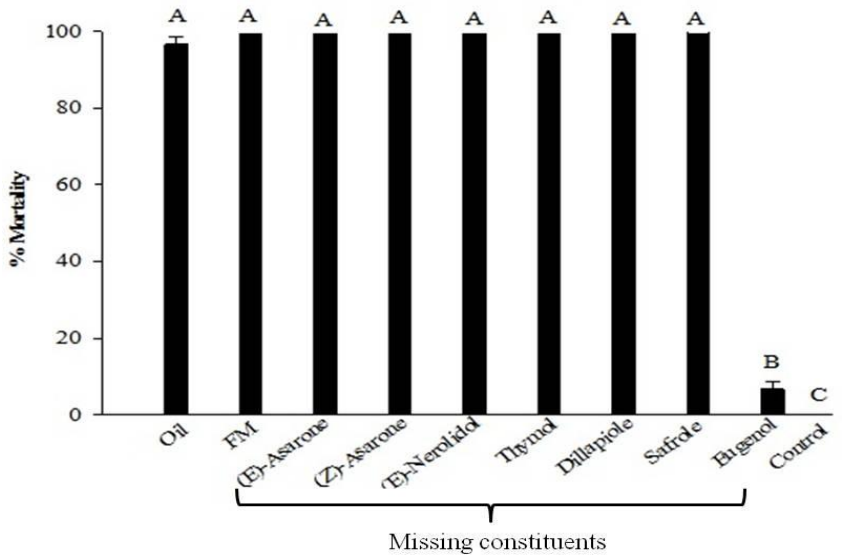

Fig. 1. Mortality caused by the fumigant activity of natural oils, complete blend, and selected blends of constituents of oils from the leaves of *I. suffruticosa* to *Tetranychus urticae* applied at levels equivalent to those found in the ≥ 95% lethal concentration of the natural oil (4.8 µl $L^{-1}$of air). Error bars represent the standard error of the mean of 90 mites. Means corresponding to each treatment with different letters are significantly different from each other according to the Tukey's test ($P \leq 0.05$). FM indicates a blend of 7 constituents, whereas all others indicate full mixture missing the constituent noted.

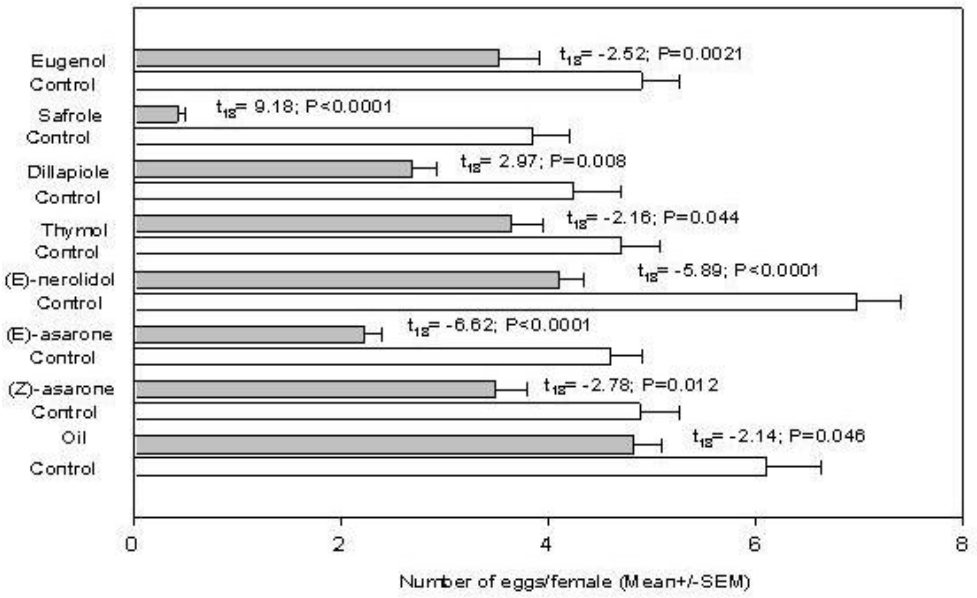

Fig. 2. Mean number of eggs laid per female of *T. urticae* when subjected to oil *I. suffruticosa* and some selected chemical constituents in the lowest concentration used in fumigation experiments that differed significantly from control constituents. Means corresponding to each treatment (Tukey's test).

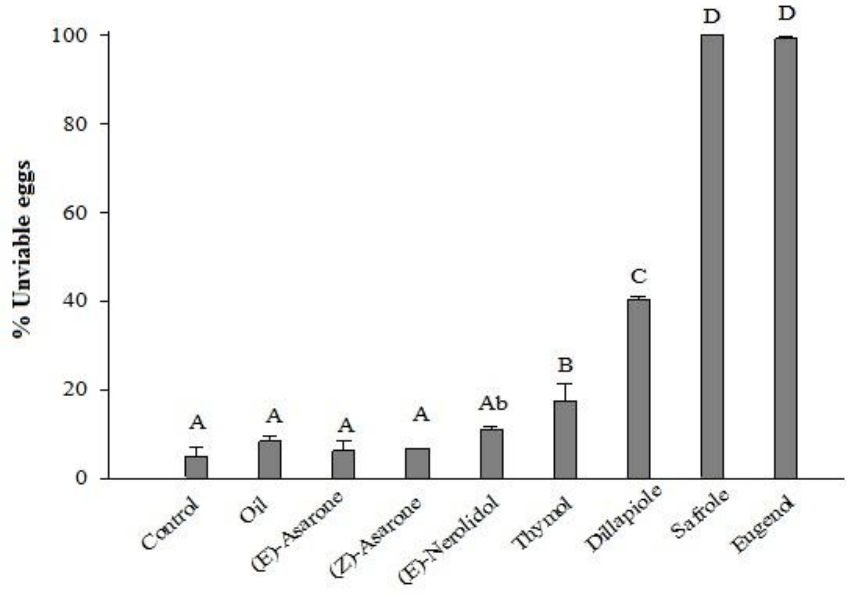

Fig. 3. Percentage of unviable eggs of *T. urticae* when exposed to essential oil of *I. suffruticosa* and selected constituents in concentration 4.8 µL L^{-1} de ar. Means corresponding to each treatment with different letters are significantly different from each other according to the Tukey's test ($P \leq 0.05$).

6. Capítulo II

6. CAPÍTULO II – Artigo 2

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Cytotoxic and antimicrobial activity of essential oil of leaves of *Indigofera suffruticosa* Mill.

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ABSTRACT

Indigofera suffruticosa Mill, commonly known as indigo, is a plant widely in the folk medicine used for infections. In this study, fresh leaves of *I. suffruticosa* were collected in two different cities (São Caetano and Cabo de Santo Agostinho) from Pernambuco (Brazil) and the essential oils were extracted and evaluated for their antimicrobial activity and *in vitro* cytotoxicity using five human cancer cell lines. Essential oils exhibit potent antimicrobial activity against gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), standard *Staphylococcus aureus*, *Bacillus subtilis* and yeast *Candida albicans* with significant result of the minimum inhibitory concentration (MIC) ranging from 64.5 µg/mL to 250 µg/mL. Except for MRSA, the essential oil of Cabo de Santo Agostinho (EOIsC) showed higher MIC than that of São Caetano (EOIsSC), but both oils had low activity against gram-negative bacteria *Escherichia coli* with MIC of 2000 µg/mL. On the other hand, the anticancer activity of EOIsSC indicated that this oil was cytotoxic to all five human cancer cell lines, inhibiting the growth HL-60, NCI-H292, HEP-2, HT29 and MCF-7, and with the average inhibitory concentration (IC_{50}) value ranging from 2.0 µg/mL to 25.4 µg/mL. The better inhibitory effect of EOIsSC was observed to the human cancer cells Hep-2 (IC_{50} 2.0 µg/mL) and HL-60 (IC_{50} 4.8 µg/mL). Nevertheless, when using the EOIsC no cytotoxic effect was observed for HT29 and MCF -7 (IC_{50} > 50 µg/mL), but a potent cytotoxicity was also found for Hep-2 (IC_{50} 5.0 µg/mL) and HL-60 (IC_{50} 5.4 µg/mL). This is the first investigation on anticancer and antimicrobial activity of essential oil from leaves of *I. suffruticosa*. The results suggest that essential oils extracted from fresh leaves of *I. suffruticosa* may be potential candidates as natural anticancer and antimicrobial products for pharmaceutical proposes.

Keywords: Fabaceae. Minimal inhibitory concentration. Pathogenic micro-organisms. Cancer cell lines. HL-60. HEP-2.

Introduction

Cancer and bacteria are nowadays one of the leading causes of morbidity and mortality in humans. Medicinal plants are growing increasingly used in the treatment of various diseases, because they have a rich source of active chemical compounds. The plant *Ocimum gratissimum* (Lamiaceae) is used in Meru Central district, Kenia, to treat pneumonia and malaria (Gakuya et al., 2013). Likewise, the species *Prunus africana* (Rosaceae) has a broad use for treatment of cough, cold and cancer (Gakuya et al.,2013). Moreover, oil-resin of genus *Copaifera* (Caesalpinoideae) has been widely used by populations from north and northeast regions of Brazil for treatment of inflammations, cancer, tetanus, venereal diseases, as urinary antiseptic to treat bronchitis, syphilis, skin diseases, ulcers as well as in wound healing (Paiva et al., 2004).

In this context, scientific investigations have highlighted the importance and contribution of various plant families such as Euphorbiaceae, Lauraceae, Lamiaceae, Leguminosae, used as medicinal plants. Recent progress in the discovery of new drugs from sources of natural products from medicinal plants have resulted in important compounds being developed for the treatment of cancer, resistant bacteria, viruses and immunosuppressive disorder (Bezerra et al., 2009). Many of the properties exhibited by different plant species are associated with volatile substances that make up essential oils. However, genetic, physiological and environmental factors, as well as origin geographical and distances geographical, can play an important role in the chemical composition of the essential oils (Figueiredo, et al., 2008), and can influence their biological activities. The literature has reported the influence on the composition and content of essential oil associated with environmental factors (Hussain et al, 2008), geographical origin (Teles et al, 2013) and geographic distances greater (Vilela et al., 2013).

Among the natural products with growing interest in the research, the essential oils of plants, due to their potential bioactive, stand out (Zu et al, 2010.); beyond their known use for the food additives , flavoring and fragrance industry (Bakkali et al., 2008). A significant number of research with the essential oil of medicinal plants has been carried out by various studious for divers biological properties such as antimicrobial activity (Bitu et al., 2012), antifungal (Nadir et al., 2013), miticide (Laborda et al., 2013) and antitumor (Jayaprakasha et al., 2013).

The genus *Indigofera* belongs to the Fabaceae family with about 700 species distributed across Africa, Australia, Asia and the Americas. Among the species of this genus, the shrubby plant *Indigofera suffruticosa* is commonly known as indigo and stands out due to its biological properties. This species is native to Central America, is widely distributed in tropical and subtropical Americas, being widespread in the Northeast region of Brazil. This plant has popular intensive use as antispasmodic, sedative, febrifuge and purgative (Hasting, 1990). Although, previous investigations on biological properties of aqueous extracts of the leaves of this plant have been reported to inhibit Sarcoma 180 tumor (Vieira et al., 2007), antimicrobial activity (Leite et al., 2006) embryotoxic effect (Leite et al., 2004) and mutagenic activity from methanol extracts of the aerial parts (Calvo et al activity., 2011), its antimicrobial activity and *in vitro* cytotoxic effects of essential oil of *I. suffruticosa* on human tumor cells not were described.

The present study was conducted to investigate the antimicrobial activity of essential oil of *I. suffruticosa* and to evaluate their cytotoxic property against tumor cell lines of human promyelocytic leukemia (HL-60) and human lung carcinoma (NCI-H292), human larynx (HEp-2), human colon (HT29) and breast (MCF-7).

Materials and Methods

Plant material

Fresh leaves of *I. suffruticosa* were collected in the municipality of agreste region in São Caetano and litoral in the Cabo de Santo Agostinho, Pernambuco – Brazil. The plant material of São Caetano was authenticated by Dr. Marlene de Alencar Carvalho Barbosa (Herbário UFP Geraldo Mariz, Universidade federal de Pernambuco), and a voucher specimen was deposited with the identification number 45217. The plant material of Cabo de Santo Agostinho was uthenticated by Dr. Rita Pereira (Herbarium IPA - Empresa Pernambucana de Pesquisa Agropecuária) and a voucher specimen was deposited with the identification number 87813. The basic characteristics and climatic conditions of each region are shown in Table 1.

Essential oil extraction

Fresh leaves (100 g) were submitted to hydrodistillation for 2 h in a Clevenger-type apparatus. The layers of essential oils obtained of São Caetano (EOIsSC) and Cabo de Santo

Agostinho (EOIsC) were separated and dried over anhydrous sodium sulfate, weighed, stored in hermetically sealed glass containers and kept at -20 ^{0}C until further analysis. The oil yields (w/w) were calculated from the weight of fresh material. All procedures were performed in triplicate.

Antimicrobial activity

Microbial Strains

The antimicrobial activity of essential oil of *I. suffruticosa* leaves were evaluated against the following microorganisms: *Staphylococcus aureus* padrão (UFPEDA02), *Staphylococcus aureus* Clínico (UFPEDA705), *Staphylococcus aureus* resistente a meticilina – MRSA (UFPEDA699), *Bacillus subtilis* (UFPEDA16), *Escherichia coli* (UFPEDA224), *Pseudomonas aeruginosa* (UFPEDA416), *Candida albicans* (UFPEDA1007) e *Fusarium oxysporum* (UFPEDA2455). All microorganisms were obtained of the collection held by the Departamento de Antibióticos at the Universidade Federal de Pernambuco (UFPEDA) - Brazil, and maintained in Nutrient Agar (bacterium) and Sabouraud (fungi) and stored at 4 °C.

Agar diffusion assay

The antimicrobial activity of essential oils was determined by the agar diffusion method (Kirby-Bauer, 1966) according to standards of the NCCLS (1997). The bacterial strains were cultured on Mueller-Hinton agar and the fungi on Sabouraud Agar. After growing, the culture were suspended in saline with turbidity of 0.5 McFarland scale. An aliquot of 100 µL of microorganisms was inoculated in Petri dishes containing culture medium specific for each test-microorganism. Paper discs were individually impregnated with 10 µL of essential oil of *Indigofera* and placed on the surface of the plate with the culture medium. The samples were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for fungal strains. After incubation, the diameter of the inhibition zone (DZI) was examined and average values were calculated. The measurements of zones of inhibition were performed in duplicate.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Microdilution susceptibility assay was performed according to the recommendations of NCCLS (1997) for the determination of minimum inhibitory concentration (MIC). The stock solution of essential oil (20 mg/mL) was prepared in 1 mL of dimethyl sulfoxide (DMSO) PA. 100 μL of this solution was transferred to a microplate containing 100 μL of culture medium. Then, serial dilutions of the oil was prepared in culture medium. The suspension inoculum, of each test strain was prepared in sterile saline solution (0.9%) and turbidity of the suspension was adjusted to 0.5 MacFarland scale and 10 μL (approximately 10^6 UFC/mL) solution of microorganism was added to the wells containing Mueller-Hinton agar for bacteria and Sabouraud agar for fungi. In all tests, were included positive and negative control. Oxacillin (4 mg/mL) and streptomycin (10 mg/mL) reference standard antibacterial agent and fluconazole (2 mg/mL) standard antifungal agents, were used as positive controls. The Mueller-Hinton plates were incubated at 37 °C for 24 h and Sabouraud at 30 °C for 48 h. The microbial growth was observed on the bottom of microplate wells. The MIC was defined as the lowest concentration of oil that visibly inhibited the growth of each microorganism. Posteriorly, cultures were seeded under the appropriate conditions for bacteria and fungi to determine the minimum bactericidal concentration (MBC) which corresponds to the minimum concentration of oil that eliminated the microorganisms.

The MBC/MIC ratio was calculated and used to classify *I. suffruticosa* essential oils as bacteriostatic or bactericidal antimicrobial. MBC/MIC ratio > 4 indicates bacteriostatic essential oils and MBC/MIC ratio ≤ 4 indicates bactericidal essential oils (Gatsing et al., 2009).

Cytotoxicity assay

HL-60 (human pro-myelocytic leukemia), NCI-H292 (human lung carcinoma), HEP-2 (human larynx carcinoma) and HT29 (human colon carcinoma), MCF-7 (breast carcinoma) were obtained from Rio de Janeiro Cell Bank (RJ-Brazil). All cancer cells were maintained in DMEM or RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C with 5% CO_2. The cytotoxicity of the two oils was tested using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) (Sigma Aldrich Co., St. Louis, MO/USA) reduction assay. For all

experiments, tumor cells were plated in 96-well plates (10^5 cells/mL for adherent cells or 3×10^5 cells/mL for leukemia). Tested Compounds (0.78; 1.56; 3.12; 6.25; 12.5; 25 and 50 μg/mL) dissolved in DMSO 1% were added to each well and incubated for 72 h. Control groups received the same amount of DMSO. After 69h of treatment 25 μL of MTT (5mg/mL) was added, three hours later, the MTT formazan product was dissolved in 100 μL of DMSO, and absorbance was measured at 595 nm in plate spectrophotometer. The IC_{50} values and their 95% confidence intervals for two different experiments were obtained by nonlinear regression using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California USA).

Results and Discussion

The results of the antimicrobial activity involving the diameter of the inhibition zone (DIZ), the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBCs) of both essential oils are shown in Table 2. The present study showed that the essential oils of *I. suffruticosa* displayed antimicrobial activity against all tested pathogens, with the exception of *P. aeruginosa.*

The EOIsSC and EOIsC showed different levels of antimicrobial activity with inhibition zone of 8-31 mm in the agar diffusion test. The EOIsSC exhibited high activity against the gram-positive pathogens standard *S. aureus, B. subtilis,* MRSA and *S. aureus* clinical with diameters of inhibition zone (DIZs) of 31 mm, 30 mm, 24 mm and 14 mm, respectively and showed lower activity against gram-negative bacteria *E. coli* with DIZ of 8.5 mm and the fungus *F. oxysporum* with DIZ of 8.0 mm. This oil was inactive against *C. albicans* and *P. aeruginosa* by the method mentioned above. The EOIsC sample also was ineffective against *P. aeruginosa* in disc paper assay.

In this context, the inhibitory activity of two essential oils showed MIC ranging from 64.5 μg/mL to 2.000 μg/mL. Both oils showed great activity for methicillin-resistant *S. aureus* (MRSA) with MIC of 64.5 μg/mL, whereas the standard *S. aureus* showed the same activity (64.5 μg/mL) only for EOIsC. For the *B. subtilis* and the yeast *C. albicans,* the oils investigated showed strong antibacterial activity with MICs of 125 μg /mL for EOIsC and 250 μg/mL for EOIsSC. These oils also showed similar and moderate susceptibility against the microorganisms *S. aureus* of clinical isolates (MIC: 1000 μg/mL), *E. coli* (MIC: 2000 μg/mL) and filamentous fungus *F. oxysporum* (MIC: 500 μg/mL). The results obtained from this

method showed that the oils were more effective on gram-positive pathogens than on gram-negative pathogens.

The essential oil of *Phyllanthus amarus* exhibited strong activity against *S. aureus, B. subtilis* and *C. albicans*, and was inactive against *P. aeruginosa* as observed by Ogunlesi et al. (2009). In addition, Oliveira et al. (2012) showed that the hydroalcoholic extract of *Buchenavia tetraphylla* did not show activity against the *E. coli, Enterococcus faecalis* and *Klebsiella pnuemoniae* by agar diffusion technique, but exhibited activity by microdilution method.

The literature has reported antimicrobial activity of essential oils of plants more pronounced against gram-positive bacteria than against Gram-negative bacteria (Ashour et al, 2008; Wang et al, 2012). This may be due to the absence of phospholipids in the outer membrane of gram-positive bacteria, which allow the penetration of bioactive compounds of essential oils and can compromise the integrity of the cell membrane (Delamore et al., 2007).

The essential oils of *I. suffruticosa* of both regions showed similar values for MBC. To evaluate the bactericidal or bacteriostatic effect of the essential oils, the value of the ratio MBC/MIC was calculated. Antimicrobial substances are considered as bacteriostatic agents when the ratio MBC/MIC is > 4 and bactericidal agents when the ratio MBC/MIC is ≤ 4 (Gatsing et al., 2006). Thus, the two essential oils were bactericidal agents for almost all tested pathogens, except for resistant methicillin *S. aureus* (MRSA) and *B. subtilis,* which showed a high bacteriostatic activity. Also EOIsC was bacteriostatic to standard *S. aureus*. Our results revealed that EOIsSC and EOIsC possess potent antibacterial property.

Five tumor cell lines were used: HL-60 (human pro-myelocytic leukemia), NCI-H292 (human lung carcinoma), HEP-2 (human larynx carcinoma) and HT29 (human colon carcinoma), MCF-7 (breast carcinoma). The assay results for the extracts screened were separated into four categories: inactive (TGI > 50 μg/ml), weak activity (15 μg/ml < TGI < 50 μg/ml), moderate activity (6.25 μg/ml < TGI < 15 μg/ml) and potent activity (TGI < 6.25 μg/ml) (Fouche et al., 2008). As shown in Table 3 and Figure 1, the results indicated that both essential oils presented different cytotoxic activities on tumor cells. In general, a dose-dependent manner inhibited growth of the human cancer cells investigated. EOIsSC presented a potent activity with IC_{50} values of 4,8 μg/mL and 2.0 μg/mL for HL60 and HEP, respectively. OEIsSC moderate activity was found on NCI-H29 e HT29, with IC_{50} values of 6.9 μg/mL e 8.2 μg/mL, respectively, while OEIsSC weak activity was obtained for MCF-7 (25.4 μg/mL). OEIsC also presented potent citoxic activity on HL60 and HEP (IC_{50} 5.4

µg/mL and 5,0 µg/mL), respectively, although it presented moderated activity on NCIH292 (IC_{50} 13.8 µg/mL) and no activity on HT29 and MCF-7 (IC_{50} > 50 µg/mL).

Many biological activities such as antimicrobial, antioxidant and cytotoxic activity of *Indigofera* species have been described with extract of *I. tinctoria, I. trita* and *I. linnaei* (Renukadevi e Sultana, 2011; Vinoth et al, 2011; Sandhyavali et al., 2012). The aqueous extract of *I. suffruticosa* leaves obtained by infusion exhibited results against *S. aureus*, *Trichophyton rubrum* and *Microsporum canis* (Leite et al., 2006). The *in vivo* anticancer effect was observed for aqueous extract of *I. suffruticosa* on the tumor sarcoma 180 (Vieira et al., 2007). Although some studies indicated antimicrobial activity and potencial antitumor effects of *I. suffruticosa* extracts, none of them investigated the cytotoxic activity of *I. suffruticosa* essential oil.

Numerous studies have demonstrated anticancer, antibacterial and antifungal activity for the essential oils obtained from plants (Ogunlesi et al., 2009; Patharakorn et al., 2010; Mihailović et al., 2011; Bitu et al., 2012; Wang et al., 2012). In fact, the anticancer potential of essential oils obtained from Brazilian northeastern plants has been studied recently (Ferraz, et al., 2013a, Ferraz, at al., 2013b). Essential oil from *Xylopia frutescens* presented *in vivo* anticancer activity (Ferraz, et al., 2013a). In addition *Lippia gracilis* essential oil induces apoptosis on human liver tumor cells (Ferraz, at al., 2013b). *I. suffruticosa* essential oil presented potent cytotoxic activity, at least for two cancer cells lines, indicating its anticancer potential.

In this study the differences obtained in results of the biological activities of essential oils of *I. suffruticosa* of the distinct regions can be related to environmental factors as climatic conditions and characteristics of each region (Table 1), different geographical origin and geographical distance between the regions (169 km). The literature has reported the influence of environmental factors and physical-chemical variations of essential oils of many plants. It was demonstrated by Hussain et al. (2008), that the growing season affected the chemistry composition , the antimicrobial and antioxidant activity of essential oil of *Ocimum basilicum*. Teles et al. (2013) revealed that the composition and content of leaves essential oil of *Mentha x villosa* Hudson were considerably affected by the geographic region where they were grown. In addition, Vilela and collaborators (2013) demonstrated that essential oils from eight populations of *Eugenia dysenterica*, of the Central Brazilian Cerrado, chemically differ with geographic distance greater than 120 km.

Conclusion

In this study the essential oils of *I. suffruticosa* showed high antibacterial and cytotoxic activity. These oils have strong effect bactericidal, bacteriostatic and cytotoxic. To the best of our knowledge, this is the first report of cytotoxic activities and antibacterial and antifungal properties of essential oils obtained from fresh leaves of *I. suffruticosa.*. The results suggest further investigation into the discovery of new agents with antimicrobial and anticancer potential.

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Table 1

Basic characterization of the regions of collecting the plant material.

	São Caetano	Cabo de Sto Agostinho
Localization	08º 19´ 33" S/ 36 º 04´ 21"W	8° 17´1"S / 35° 02´00"W
Altitude	552 m	29 m
Precipitation (annual average)	491 mm	1309.9 mm
Temperature (annual average)	23 ºC	25 ºC
Climate	BSh'(warm semi-arid climate)	As' (Tropical rainy with dry summer)

Fonte: MINISTÉRIO DE MINAS E ENERGIA, 2005; CONDEPE/FIDEM (2005).

Table 2

Antimicrobial activity of essential oil from leaves of *Indigofera suffruticosa*

microorganism	EOIsSC				EOIsC				Standard
	DZI	MIC	MBC	MBC/MIC	DZI	MIC	MBC	MBC/MIC	MIC
S. aureus	31	250	1000	4	14	64.5	1000	>4	< 0.781[a]
S. aureus (clínical)	14	1000	2000	2	15	1000	2000	2	< 6.25 [a]
MRSA	24	64,5	1000	>4	15.5	64.5	500	>4	12.5 [a]
B. subtillis	30	250	>2000	>4	17	125	>2000	>4	< 0.781[a]
E. coli	8,5	2000	2000	1	0	2000	2000	1	< 16.125[b]
P. aeruginosa	0	NT	NT	----	0	NT	NT	----	NT
C. albicans	0	250	250	1	9.5	125	250	2	12.5 [c]
F. oxysporum	8	500	500	1	9	500	500	1	200 [c]

EOIsSC: Essential oil of *I. suffruticosa* São Caetano
EOIsC: Essential oil of *I. suffruticosa* Cabo de Santo Agostinho.
Standard: antimicrobial agent – [a] oxacillin, [b]streptomycin, [c]Fluconazole
MIC: Minimum inhibitory concentration (expressed in µg/mL)
DIZ: Diameter of inhibition zone (in mm)
NT: not tested

Table 3

Cytotoxic activity of essential oil from leaves of *Indigofera suffruticosa* on human cancer cell lines.

Cell lines	Origin	IC_{50} (ug/mL)		
		EOIsSC	EOIsC	Doxorrubicina
HL – 60	pro-myelocytic Leukemia	4.8 4.3 - 5.3	5.4 4.7 - 6.3	0.02 0.01 - 0.02
MCF – 7	Breast Carcinoma	25.4 21.8 - 30.9	> 50	0.2 0.17 - 0.24
HEP - 2	Larynx Carcinoma	2.0 1.8 - 1.3	5.0 4.3 - 5.9	0.7 0.3 - 1.4
HT 29	Colon Carcinoma	8.2 7.4 - 9.1	> 50	0.4 0.2 - 0.6
NCI H-292	Lung Carcinoma	6.9 6.0 - 7.9	13.8 11.6 - 16.3	0.01 0.004 - 0.3

The data are presented as IC_{50} and 95% confidence interval.
EOIsSC: essential oil of *I. suffruticosa* of São Caetano.
EOIsC: essential oil of *I. suffruticosa* of Cabo de Santo Agostinho.

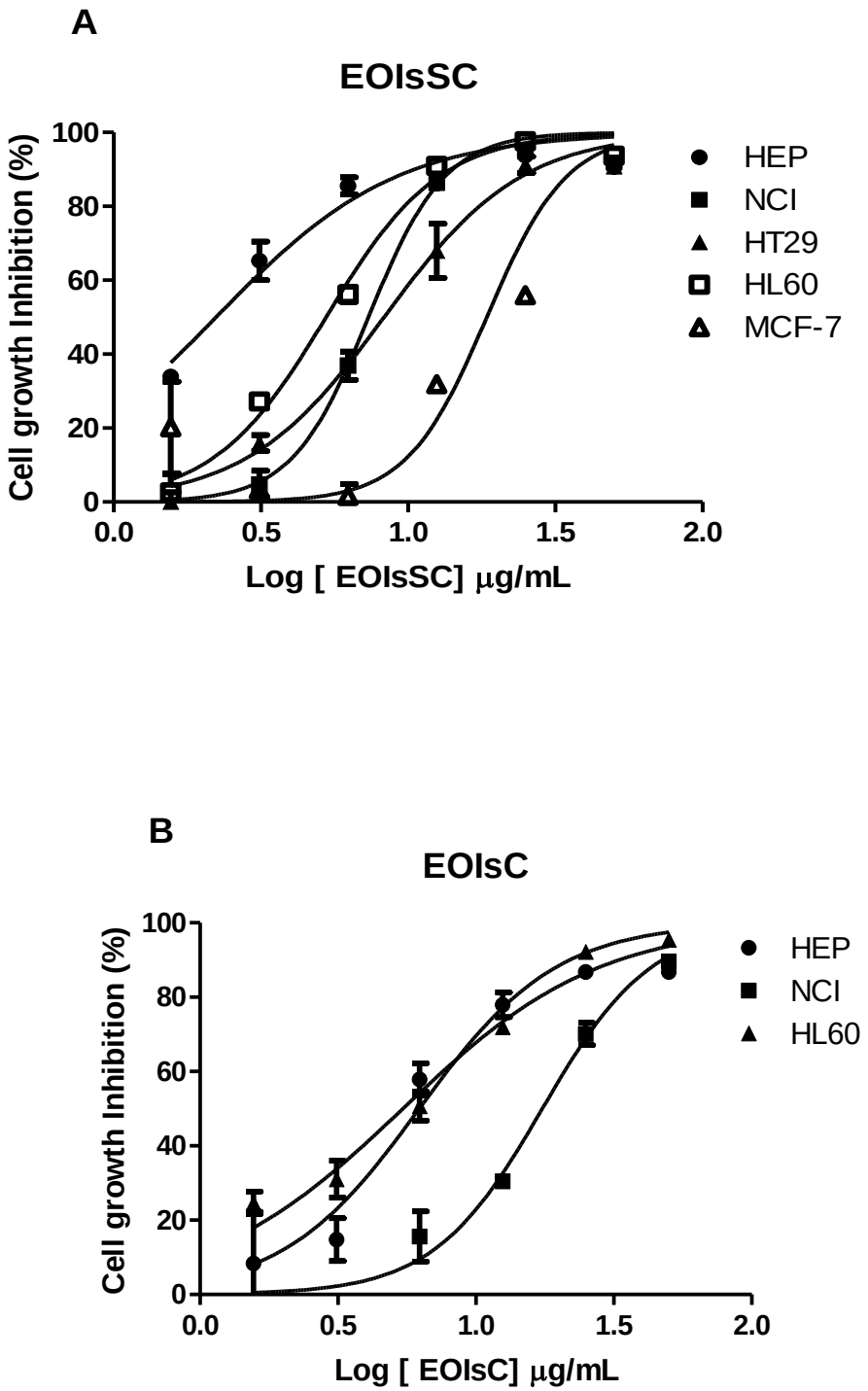

Figure 1. (A) Growth inhibition curves for EOIsSC in Five câncer cell lines. **(B)** Growth inhibition curves for EOIsC in three câncer cell lines.

7. Capítulo III

7. CAPÍTULO III – Artigo 3

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Qualis B1 – Área CAPES: CIÊNCIAS BIOLÓGICAS II.

Synergic effect of organic extracts of leaves of *Indigofera suffruticosa* with erythromycin against *Staphylococcus aureus*

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Abstract

Erythromycin is a macrolide antibiotic which has been prescribed for the treatment of various infections caused by the nosocomial pathogen *Staphylococcus aureu.* Resistance of *S. aureus* to erythromycin has been observed, and one strategy employed to overcome bacterial resistance is the synergic use of the plant extracts and antibiotics. *Indigofera suffruticosa* is a plant popularly used to treat infections. This study aimed to investigate a synergistic effect of the extracts of *I. suffruticosa* leaves with erythromycin against *S. aureus*. *I. suffruticosa* extracts were obtained by fractionated extraction with diethyl ether, chloroform and acetone and the antimicrobial activity was tested against nine clinically isolates of *S. aureus* strains. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by microdilution tests. Fractional inhibitory concentration (FIC) was accessed by checkerboard titer assay. All organic extracts showed antimicrobial activity against the *S. aureus* strains. Acetone extract exhibited the best potential to inhibit *S. aureus* (MIC=0.78, MBC=3.12 mg/mL), and chloroform extract presented also a good MIC=3.12 and MBC=6.25 mg/mL. Furthermore, acetone and chloroform extracts of *I. suffruticosa* were able to enhance the erythromycin activity against *S. aureus*: FIC $\leq$ 0.5. Therefore, the results suggest that organic extracts of leaves of *I. suffruticosa*, alone or erythromycin combined may be promising natural products for the development of new anti-*S. aureus* formulation.

Keywords: Plant extracts. Antibacterial agent. Macrolide antibiotic. Nosocomial infections.

1. Introduction

The patients who are hospitalized in the intensive care units because of the use of invasive devices and extended hospital stay, are at risk of acquiring nosocomial infections (Streit et al., 2004). Long-term hospitalization may further complicate thepatients' health status following exposure to various antimicrobial agents. The *Staphylococcus* genus is largely distributed in natural environments, being part of the normal skin and soft-tissue microbiota of various animals. Some *Staphylococcus* species are recognized as etiological agents of many human infections (Coutinho et. al., 2009, Deurenberg e Stobberingh, 2008). Erythromycin is a 14-membered ring macrolide antibiotic and has been prescribed for the treatment of various infections caused by *Staphylococcus aureus. Staphylococcus aureus* is an important pathogen associated with nosocomial human infections and this microorganism has successfully involved numerous strategies for resistance to different antibiotics (Coutinho et al., 2009, Chung et al., 2011). In this context, the indiscriminate use of antibiotics in the treatment of bacterial infections has led to the emergence of antibiotic resistant *S. aureus* strains (Adwan, Mhanna, 2008). Resistance of gram-positive bacteria such as staphylococci, enterococci and pneumococci to erythromycin has been observed (Tavares, 2000) Erythromycin is metabolized in the liver, and excretion occurs via bile, through the gut, it is eliminated in the feces. This antibiotic is the 1st choice of specific treatment of pertussis, diphtheria, legionellosis (a form atypical pneumonia) and mycoplasma infections.

Medicinal plants represent an important health and economic component used to many cultures for thousands of years (Silva et al., 2012, Agra et al., 2008). According to the World Health Organization approximately 80% of the global population uses medicinal plants or herbal medicine for primary health care (Pereira et al., 2012). The Brazil comprises one of the highest levels of plant diversity and represents 20% of biodiversity in the world (Ministério do Meio Ambiente, 2010). Some of the Brazilian vegetation consists of species of the family Fabaceae where the subfamily Papilionidae is represented by 188 genus and 2100 native species [13]. *Indigofera suffruticosa* Mill is a member of subfamily Papilionidae, and it is originally from Antilha and América Central. *I. suffruticosa* Mill is popularly known as "anileira" or "anil", the term " *Indigofera*" comes from the German word "indigo", a dye staining blue (Indigo Blue) widely used by the textile industry. This specie occurs in the Northeast countryside of Brazil, and although some toxic effects have been reported this plant has a popular use for the treatment of inflammations, anti-spasmodic, sedative, diuretic, infections, and some effects has been studied (Salvador et al., 2011, Hastings, 1990).

Previously, we demonstrated that aqueous extract of leaves of *I. suffruticosa* obtained by infusion showed strong inhibitory activity against the Gram-positive *S. aureus* and dermatophyte strains (Calvo et al., 2011).

Synergism assessment has become a key tool in phytomedicine research in recent years, and the use of drug combination with herbal products on antimicrobial activity of antibiotics resistant *S. aureus* has been investigated (Vovy et al., 2013, Celenze et al., 2012, Eukeb et al 2010, Wagner e Ulrich-Merzenich, 2009). The secondary metabolites from plant are good sources for synergic therapy and are a wide range of phytochemicals which acts as modifiers of multidrug resistance mechanisms (Hemaiswarya et al., 2008). Pharmacological and clinical studies have described the synergistic multi-target effects of some phytopharmaceuticals. Among the machanisms involved in target antibacterial effects of synergism between drugs and plant extracts are: enzymes, substrates, metabolites, receptors, ion channels, transport proteins, deoxyribonucleic acid and ribonucleic acid (Wagner, 2011)

Thus, this study aimed to evaluate the anti *Staphylococcus aureus* activity of organic extracts of *I. suffruticosa* leaves and their synergistic effect with erythromycin against clinical isolates.

1. Materials and Methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO), erythromycin and 7-hydroxi-3H-phenoxazin-3-one-10-oxide sodium salt (Resazurin) was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO. Mueller-Hinton Agar and Nutrient Agar medium was purchased from HIMEDIA Laboratories®. Diethyl ether, chloroform and acetone were purchased from Merck, Darmstadt, Germany.

2.2. Plant Material and Preparation of Organic Extracts

Leaves of *I. suffruticosa* were collected in São Caetano, Pernambuco, Brazil (latitude: 08º 19’ 33” S; longitude: 36º 04’ 21” W). The samples were collected between 10 and 11 a.m. The plant was identified by Dr. Marlene Carvalho Alencar Barbosa from the Department of

Botany, Federal University of Pernambuco (UFPE), Brazil. A voucher specimen was deposited with the identification number 45.217 at the UFP Geraldo Mariz Herbarium-UFPE.

About 100 g of dried leaves of *I. suffruticosa* were successively extracted (fractionated extraction) with 200 mL of solvents of increasing polarity such as diethyl ether, chloroform and acetone. The same powdered material was homogenized with each solvent for two hours in a mechanical stirrer, kept under overnight refrigeration (4 ºC) and filtrated with Whatman no.1 filter paper. The individual extracts of leaves dried of *I. suffruticosa* were separately concentrated and in a rotary evaporator pressure at 45 ºC. All steps were carried out in dark condition and dried samples were stored in desiccator until used in experimental procedures.

2.3. Clinically Isolated *Staphylococcus aureus* Strains

Nine strain of clinically isolated *S. aureus* strains were used in this study. The strains originally obtained from the vaginal secretion (UFPEDA 660); catheter tip (UFPEDA 663); urine sample (UFPEDA 670); blood sample (UFPEDA 672); prostate secretion (UFPEDA 676); wound secretion (UFPEDA 677 and 679); ocular secretion (UFPEDA 687) and standart strain (UFPEDA 02). All strains were obtained from the Departamento de Antibióticos, Universidade Federal de Pernambuco and maintained in Nutrient Agar (NA) and stored at 4 °C. The antibiotics sensitivity of the all strains has been previously determined (Table 1).

2.4. Effects of Temperature and pH on the activity of the Organic Extracts of Leaves of *I. suffruticosa*

In this study, the effect activity of the organic extracts in different pH and temperature were evaluated on the growth *S. aureus* standard (UFPEDA 02). Thermostability of the organic extracts of leaves of *I. suffruticosa* was determined by storing the samples in sterile tubes and kept at different temperatures (28, 30, 60 and 100 ºC). The effect of pH on the activity of the organic extracts was tested at room temperature by adjusting the pH with 1M NaOH and/or 1M HCl (pH 3, 4, 5, 6, 7, 8, 9 and 10). All organic extracts of *I. suffruticosa* were dissolved in DMSO (1%) and antibacterial activity was tested using the agar diffusion method.

2.5. Determination of Antibacterial Activity Using the Disc Diffusion Method

The antibacterial activity of the organic extracts of *I. suffruticosa* was determined by the disc diffusion method (Oliveira et al., 2012). Briefly, all clinically isolated *S. aureus* strains were grown on Mueller-Hinton Agar (MHA) medium at 37 °C for 18 hours, suspended in distillated water (approximately 1.5×10^8 CFU/mL). An aliquot of 100 µL of bacterial suspension was immediately inoculated in Petri dishes containing MHA medium. Sterile paper discs (6 mm diameter) containing 20 µL of organic extracts of *I. suffruticosa* (100 mg/mL) were applied to agar and the Petri dishes were incubated at 37 °C for an additional 18 hours. After incubation, the diameter of the inhibition zone of growth was examined.

2.6. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and MBC/MIC Ratio

To determine the minimal inhibitory concentration (MIC) we performed microdilution tests (Clinical and Laboratory Standards Institute, 2011). A twofold serial dilution of the organic extracts of *I. suffruticosa* was prepared in Mueller Hinton Broth (MHB) and 10 μL (approximately 1.5×10^8 CFU/mL) of *S. aureus* strains suspension was added. The inoculum bacterial concentration was determined by adjusting their turbiditity (at 625 nm) to 0.5 McFarland scale. The samples were incubated for 24 h at 37◦C. The minimum inhibitory concentration (MIC) was considered as the lowest concentration of organic extracts of *I. suffruticosa* that inhibits visible growth of *S. aureus* strains.

Resazurin (0.01%) was used as an indicator by color change visualization: any color changes from purple to pink were recorded as bacterial growth. The lowest concentration at which no color change occurred was taken as the MIC. Afterwards, cultures were seeded in MHA medium and incubated for 24 h at 37◦C to determine the minimum bactericidal concentration (MBC) which corresponds to the lowest concentration of organic extracts of *I. suffruticosa* at which 99.9% of the *S. aureus* were killed. All experiments were performed in triplicate.

The MBC/MIC ratio was calculated and used to classify antimicrobial *I. suffruticosa* extracts such as bacteriostatic or bactericidal. MBC/MIC ratio > 4 indicates bacteriostatic extracts and MBC/MIC ratio ≤ 4 indicates bactericidal extracts (Gatsin et al., 2009).

2.7. Evaluation of Synergism

Synergism between organic extracts of *I. suffruticosa* and erythromycin was assessed using the checkerboard test. Two-fold serial dilutions of erythromycin prepared in horizontal rows of microtiter plate were subsequently cross-diluted vertically by two-fold serial dilutions of organic extracts of *I. suffruticosa*. Synergistic effect between erythromycin and *I. suffruticosa* was assessed in 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9 proportions (drug:plant extract). The Fractional Inhibitory Concentration (FIC) was calculated according to the equation:

FIC index = FIC of Organic Extracts of *I. suffruticosa* + FIC of Erythromycin

Where, FIC of organic extracts of *I. suffruticosa* = MIC of organic extracts of *I. suffruticosa* in combination with erythromycin / MIC of organic extracts of *I. suffruticosa*; and FIC of Erythromycin = MIC of erythromycin in combination with organic extracts of *I. suffruticosa* / MIC of erythromycin.

The combinatory effect were then determined based on the FICs according to the European Committee on Antimicrobial Susceptibility Testing , (2000) criteria for synergism as follows: FIC ≤ 0.5 = synergy; FIC $> 0.5 - 1$ = additivity; FIC > 1 to < 2 = indifference; and FIC ≥ 2 = antagonism.

2.8. Statistical Analysis

Each experiment was performed in triplicate and results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed by ANOVA. All analyses were carried out using software StatView, version 4.5, Abacus Concept, Inc, Berkeley, CA. The significance of the differences was considered at $P < 0.05$.

3. Results and Discussion

3.1 Antibacterial Activity of Organic Extracts from Leaves of *I. suffruticosa*

The results from the present study showed that all organic extracts of leaves of *I. suffruticosa* presented antimicrobial activity against different *S. aureus* strains (Table 2). The

inhibition zone diameter (DIZ) of *S. aureus* promoted by organic extracts of leaves of *I. suffruticosa* ranged from 25.3 to 36.0. UFPEDA 670 and UFPEDA 02 *S. aureus* strains was isolated from urine and blood samples, respectively, and are as previously characterized with resistant strains to all antibiotics tested: oxacillin, cefoxitin erythromycin and clindamycin. Organic extracts of leaves of *I. suffruticosa* proved as potent antimicrobial agents for these *S. aureus* resistant to antibiotics strains. The DIZ of ether and acetone extracts were higher than 30.0 for both strains, except the chloroform extract of *I. suffruticosa* showed that DIZ = 27.7 ± 2.5 for the *S. aureus* strain isolated from urine sample. In Table 2 we highlight the best inhibitory activities of organic extracts of *I. suffruticosa* against standard strain UFPEDA02.

The analysis of the growth inhibition activity by the disk diffusion method showed that *I. suffruticosa* commonly used by traditional medical practitioners in Northeast of Brazil were active against clinically isolated *S. aureu*s, a Gram-positive bacteria. Omar et al. (2013), reported that extracts of plants exhibited greater inhibition effect on *S. aureus*. These authors mention that gram-positive bacteria present a unique cell membrane, which increases the sensitivity reaction by antibacterial substances. Organic extracts of leaves of *I. suffruticosa* is an effective anti-*S. aureus* natural product and reports have shown that antibacterial products are effective to destroy the gram-positive bacteria cell wall and cytoplasmic membrane, causing a linkage from the cytoplasm (Gao et al., 1999).

Studies have reported the increasing occurrence of nosocomial infections of *S. aureus* resistant to antibiotics (Novy et al., 2013; Zai-Chang et al., 2005). So, strategies have been stimulated to control the infections and the research for new ways with low and active concentration to treat *S. aureus* nosocomial infections (Abu-Shanab et al., 2008). In this context, the most active organic extracts of *I. suffruticosa* was acetone which showed better potential (MIC=1.56 mg/mL) to inhibit the growth of *S. aureus* strains of vaginal secretion, urine samples, prostate secretion and standard strain (Table 3). Chloroform extract of *I. suffruticosa* showed a MIC=3.12 in *S. aureus* strains clinically isolated from prostate secretion, wound secretion and standard strains. Acetone extracts showed this effect only the *S. aureus* standard strain, as shown in the Table 3.

Also in Table 3 it is shown the values of MBC. The acetone extract of *I. suffruticosa* showed the lower values for MBC, in comparison with the chloroform and ether extracts. The MBC/MIC ratio was calculated to determine whether the observed antimicrobial effect of the organic extracts of leaves of *I. suffruticosa* was bactericidal or bacteriostatic. Antimicrobial substances are considered as bacteriostatic agents when the MBC/MIC ratio > 4 and bactericidal agents when the MBC/MIC ratio ≤ 4 (Gatsing et al., 2006). Ether and chloroform

extracts of *I. suffruticosa* were the best bactericidal agents for all clinically isolated *S. aureus* strains tested. Whilst the acetone extract of *I. suffruticosa* was bactericidal for almost all clinically isolated *S. aureus* strains tested, except for the *S. aureus* strains obtained from urine sample and ocular secretion, in which it was observed a bacteriostatic activity. The results of the present study indicate that ether, chloroform and acetone extract exhibit excellent antibacterial property. Several reasons may explain the variance in the antibacterial activity against the clinical *S. aureus* isolates in the presence of the organic extracts which were obtained by increasing the solvent polarity. First, each *S. sureus* strain was clinical isolates from different samples, and they had different resistance profiles to antibiotics, as demonstrated in Table 1. Second, it may be due to presence of secondary metabolites extracted with different affinity to the solvents; hence the activity of the different organic extracts of *I. suffruticosa* was restricted to some *S. aureus* strains. Thus, it is possible that the presences of different secondary metabolites with various mechanism of action are responsible for the variations of the activity of *I. suffruticosa* extracts against *S. aureus.* This hypothesis is supported by a previous study which demonstrated that the fractionation of plant extracts widen the spectrum and increase the potency of plant-derived drugs (Okoli and Iroegbu, 2004).

Therefore, the results of the present study further extend the biological antibacterial potentials of *I. suffruticosa,* a member of the Fabaceae family which has been considered one important medicinal plant.

3.2 Influence of Temperature and pH on the Activity of Organic Extracts from Leaves of *I. suffruticosa*

In this study it was evaluated the effect activity of the organic extracts in different values of pH and temperature on the growth of *S. aureus* standard. The selection of UFPEDA02 strain was based on the results with the disc diffusion, MIC and MBC, because all organic extracts of *I. suffruticosa* leaves exhibited the best anti-*S. aureus* activity against this standard strain. Our results showed that the temperature variation did not affect the antibacterial activity of the organic extracts of *I. suffruticosa* (Figure 1A). In spite of a previous report demonstrated that the temperature may influence the antimicrobial activity of plant extracts (Lu et al., 2005), in this work, we demonstrated that the extracts of *I. suffruticosa* is not influenced by temperature variations up to 100 ^{o}C. These results are in agreement with

Doughari et al. (2006) who also reported that the variation in temperature did not affect the antimicrobial activity of organic extracts from *Tamarindus indica* Linn.

In Figure 1B is shown the effect of pH variation in the antibacterial activity of organic extracts of *I. suffruticosa*. The results show that the anti-*S. aureus* activity of most organic extracts of *I. suffruticosa* was stable at different pH variations. However, we observed a significant increase in activity of the ether extract of *I. suffruticosa* in pH 8.0. The antibacterial activity in pH 8.0 has been reported in a previous study, in which the authors found the best antimicrobial activity with extracts of propolis against *S. aureus* (Lu et al., 2005).

Previous study demonstrated that *S. aureus* grew well between 7 and 47 °C and pH 4 to 9 (Brener et al., 2004). Therefore, these conditions may favor different nosocomial human infections. Temperature and pH might be the key factors in ensuring stability of the extracts of plant and in order to explore further the extracts' potential as antibacterial for future use at different temperatures e pH formulations. In this context, organic extracts of *I. suffruticosa* leaves may be promising natural products for the future development of alternative fitoterapia against *S. aureus* infection.

3.3 Synergistic Effect of Organic Extracts of Leaves of *I. suffruticosa* and Erythromycin

The effect of combinating erythromycin with organic extracts were tested using the checkerboard method (Table 4). Synergistic effect between erythromycin and *I. suffruticosa* was found in the acetone extracts in 9:1, 8:2, 7:3, 5:5, and 3:7 proportions (drug:plant extract); chloroform extract in the proportions 8:2, 6:4, and 3:7 (drug:plant extract). We also highlight that the best synergistic effect between *I. suffruticosa* and erythromycin was with the chloroform extract in the proportion 5:5 (FIC= 0.2), which demonstrated the efficacy of the extract against the UFPEDA 02 standard strain. However, ether extract of leaves of *I. suffruticosa* did not show a synergistic antibacterial effect with erythromycin against clinical *S. aureus* isolates.

Our results showed a clear synergistic effect between choloroform and acetone extracts of *I. suffruticosa* with erythromycin. Synergistic effect of extracts of plants with erythromycin has been found, for example extract of *Euphobia hirta* with erythromycin for *S. aureus*

(Adikwu et al., 2010). Nevertheless, research on synergistic action with *I. suffruticosa* is unpublished.

It appears that it is not easy and nor are feasible that all plants have capacity for synergistic activity with erythromycin against *S. aureus*. In a previous study it was demonstrated that synergistic effect with erythromycin was achieved in association with only half of the several plants tested (Betoni et al., 2006). Therefore, the synergistic ability of plant extracts should be evaluated independent of its antimicrobial activity. Thus, it is possible to infer that *I. suffruticosa* is a plant with potential anti-*S. aureus*, since besides the synergistic effect of the organic extracts of *I. suffruticosa*, it was also found a bacteriostatic and bactericidal activity.

Furthermore, the results of this study demonstrated that organic extracts of leaves of *I. suffruticosa* present a marked antimicrobial activity for clinical *S. aureus* isolates exhibiting a good bacteriostatic and bactericidal effect. Also, the chloroform and acetone extracts of *I. suffruticosa* demonstrated a synergistic activity against *S. aureus* standard strain when associated to erythromycin. Finally, we conclude that organic extracts of leaves of *I. suffruticosa* are promising natural products for the development of new anti-*S. aureus* formulation, thus deserving further studies in order to understand the mechanism of action.

Conflict of Interests

All authors declare that they have no competing interests in the present work.

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Table 1. Susceptibility to antibiotics of *Staphylococcus aureus* strains.

S. aureus Strains	Clinical Source	Susceptibility to Antibiotics			
		Oxacillin	Cefoxitin	Erythromycin	Clindamycin
02	Standart Strain	S	S	S	S
660	Vaginal Secretion	S	S	S	S
663	Catheter Tip	S	S	S	S
670	Urine Sample	R	R	R	R
672	Blood Sample	R	R	R	R
676	Prostate Secretion	S	S	S	S
677	Wound Secretion	S	R	R	S
679	Wound Secretion	S	S	R	S
687	Ocular Secretion	S	S	S	S

R – resistant; S – sensitive.

Table 2. Antimicrobial activity of organic extracts of *I. suffruticosa* in *S. aureus* strains.

S. aureus Strains	Organic Extracts of Leaves of *I. suffruticosa*		
	Ether	Chloroform	Acetone
	DIZ (mm)	DIZ (mm)	DIZ(mm)
02	34.7 ± 0.6	36.0 ± 0.0	35.7 ± 1.1
660	29.0 ± 1.7	28.0 ± 2.0	28.0 ± 2.0
663	28.7 ± 0.6	27.7 ± 0.6	26.7 ± 0.6
670	32.7 ± 1.1	27.7 ± 2.5	30.7 ± 0.6
672	32.6 ± 1.1	32.3 ± 0.6	31.0 ± 3.0
676	27.3 ± 0.6	25.3 ± 0.6	26.3 ± 0.6
677	30.0 ± 1.0	29.0 ± 1.7	29.7 ± 0.6
679	29.0 ± 1.0	26.3 ± 2.3	25.7 ± 2.1
687	26.7 ± 2.3	26.0 ± 2.6	25.3 ± 2.1

DIZ – inhibition zone diameter.

Table 3. Minimum Inhibitory Concentration, Minimum Bactericidal Concentration and MBC/MIC ratio of organic extracts of leaves of *I. suffruticosa* against *S. aureus* strains.

S. aureus Strains	Organic Extracts of Leaves of *I. suffruticosa*								
	Ether			Chloroform			Acetone		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
02	3.12	12.5	4	3.12	12.5	4	1.56	3.12	2
660	6.25	12.5	2	6.25	25.0	4	1.56	6.25	4
663	6.25	25.0	4	6.25	25.0	4	3.12	12.5	4
670	6.25	25.0	4	6.25	25.0	4	1.56	12.5	8
672	6.25	12.5	2	6.25	12.5	2	3.12	6.25	2
676	6.25	12.5	2	3.12	12.5	4	3.12	3.12	1
677	6.25	25.0	4	3.12	6.25	2	3.12	6.25	2
679	6.25	12.5	2	3.12	6.25	2	1.56	6.25	4
687	6.25	12.5	2	6.25	12.5	2	0.78	12.5	16

MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration.
MIC and MBC are expressed in mg/mL

Table 4. Synergistic effect between organic extracts of leaves of *I. suffruticosa* and erythromycin against *S. aureus*.

Erythromycin/Extracts Proportion	FIC Index		
	Organic Extracts of Leaves of *I. suffruticosa*		
	Ether	Chloroform	Acetone
9:1	0.9	0.9	0.4
8:2	0.9	0.4	0.4
7:3	0.7	0.7	0.3
6:4	0.6	0.3	0.6
5:5	0.6	0.2	0.5
4:6	0.8	0.8	0.8
3:7	1.2	0.3	0.3
2:8	0.8	0.8	0.8
1:9	0.8	1.7	1.7

FIC – fractional inhibitory concentrations.

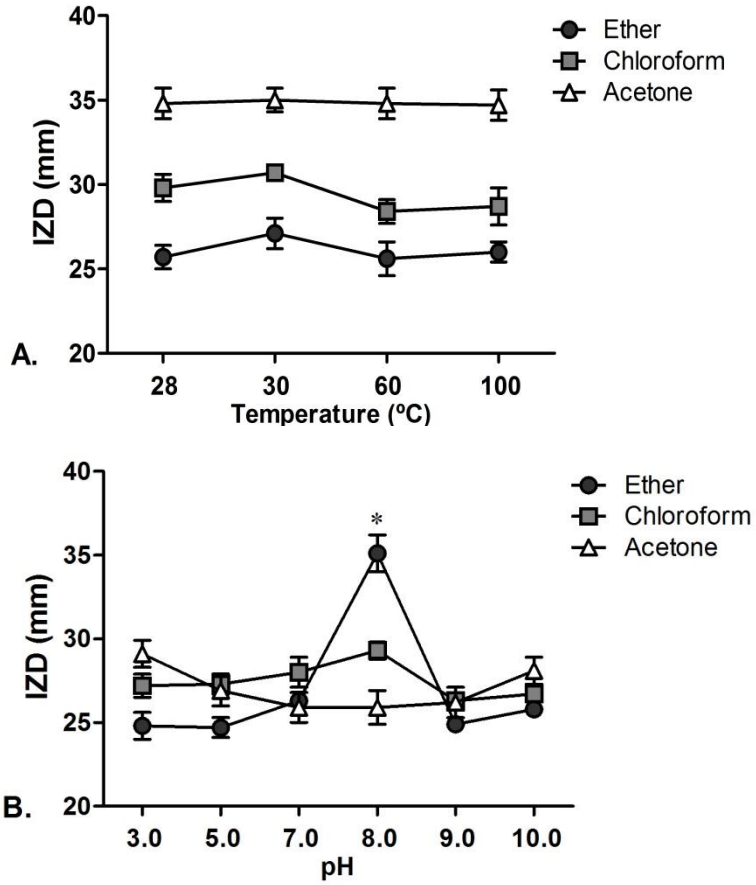


Figure 1. Effect of temperature and pH on the activity of the organic extracts of leaves of *I. suffruticosa*. DIZ – inhibition zone diameter.

8. Conclusões

8. CONCLUSÕES

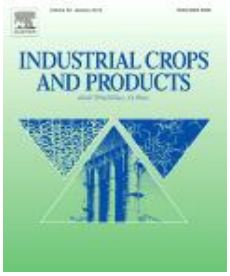
- O óleo essencial de folhas de *I. suffruticosa* é constituído predominantemente por fenilpropanóides, sendo o eugenol, E-asarone e dilapiol os constituintes majoritários.
- O óleo essencial de folhas de *I. suffruticosa* apresenta atividade acaricida contra o *Tetranychus urticae.*
- Óleos essenciais de folhas de *I. suffruticosa* possuem atividade antimicrobiana contra bactérias gram-positivas, sensíveis e resistente ao antibiótico meticilina.
- Óleos essenciais de *I. suffruticosa* apresentaram potente atividade citotóxica contra as linhagens celulares HL-60 (leucemia pró-mielocítica humano) e HEP-2 (carcinoma da laringe humano), moderada atividade citotóxica para NCI-H292 (carcinoma do pulmão humano), HT29 (carcinoma do cólon humano) e fraca citotoxicidade para MCF-7 (carcinoma da mama).
- Os extratos de etér, clorofórmio e acetona de folhas de *I. suffruticosa* apresentara potente atividade bactericida e bacteriostática para o *Staphylococcus aureus.*
- A variação de temperatura e pH não afetaram as propriedades antimicrobiana dos extratos de etér, clorofórmio e acetona de folhas de *I. suffruticosa.*
- O extrato clorofórmico associado à eritromicina apresentou o melhor efeito sinergico contra o *Staphylococcus aureus.*

Anexos

ANEXO

INDUSTRIAL CROPS AND PRODUCTS

An International Journal



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Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples:'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51-59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281-304.

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations: http://www.issn.org/2-22661-LTWA-online.php.

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All necessary files have been uploaded, and contain:

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• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web) • Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print • If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes

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http://dx.doi.org/10.1016/j.physletb.2010.09.059

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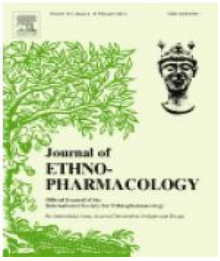

GUIDE FOR AUTHORS

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

In recent years the preservation of local knowledge, the promotion of indigenous medical systems in primary health care, and the conservation of biodiversity have become even more of a concern to all scientists working at the interface of social and natural sciences but especially to ethnopharmacologists. Recognizing the sovereign rights of States over their natural resources, ethnopharmacologists are particularly concerned with local people's rights to further use and develop their autochthonous resources.

Accordingly, today's Ethnopharmacological research embraces the multidisciplinary effort in the documentation of indigenous medical knowledge, scientific study of indigenous medicines in order to contribute in the long-run to improved health care in the regions of study, as well as search for pharmacologically unique principles from existing indigenous remedies.

The *Journal of Ethnopharmacology* publishes original articles concerned with the observation and experimental investigation of the biological activities of plant and animal substances used in the traditional medicine of past and present cultures. The journal will particularly welcome interdisciplinary papers with an **ethnopharmacological**, an **ethnobotanical** or an **ethnochemical** approach to the study of indigenous drugs. Reports of **anthropological** and **ethnobotanical** field studies fall within the journal's scope. Studies involving **pharmacological** and **toxicological** mechanisms of action are especially welcome. **Clinical studies** on efficacy will be considered if contributing to the understanding of specific ethnopharmacological problems.

The journal welcomes review articles in the above mentioned fields especially those highlighting the multi-disciplinary nature of ethnopharmacology. Commentaries are by

invitation only. All reviews and commentaries are fully peer-reviewed. Potential authors are strongly encouraged to contact the Reviews Editor jethnopharmacol@pharmacy.ac.uk prior to writing a review. A one-page outline and a short C.V. of the (senior) author should also be included.

THE "RULES OF 5"

The Editors and Editorial Board have developed the "Rules of 5" for publishing in JEP. We have produced five clear criteria that each author needs to think about before submitting a manuscript and setting the whole process of editing and reviewing at work.

II. Preparation of manuscripts

Authors who want to submit a manuscript should consult and peruse carefully recent issues of the journal for format and style. Authors must include the following contact details on the title page of their submitted manuscript: full postal address; fax; e-mail. All manuscripts submitted are subject to peer review. The minimum requirements for a manuscript to qualify for peer review are that it has been prepared by strictly following the format and style of the journal as mentioned, that it is written in good English, and that it is complete. Manuscripts that have not fulfilled these requirements will be returned to the author(s).

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In the covering letter, the author must also declare that the study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights. See below for further information. The ethnopharmacological importance of the study must also be explained in the cover letter.

Animal and clinical studies - Investigations using experimental animals must state in the Methods section that the research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in for example the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or the US guidelines (NIH publication #85-23, revised in 1985). Investigations with human subjects must state in the Methods section that the research followed guidelines of the Declaration of Helsinki and Tokyo for humans, and was approved by the institutional human experimentation committee or equivalent, and that informed consent was obtained. The Editors will reject papers if there is any doubt about the suitability of the animal or human procedures used.

Biodiversity rights - Each country has its own rights on its biodiversity. Consequently for studying plants one needs to follow the international, national and institutional rules concerning the biodiversity rights.

1.Manuscript types

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1. Original research articles - whose length is not limited and should include Title, Abstract, Methods and Materials, Results, Discussion, Conclusions, Acknowledgements and References. As a guideline, a full length paper normally occupies no more than 10 printed pages of the journal, including tables and illustrations
2. Ethnopharmacological communications (formerly Short Communications) - whose average length is not more than 4 pages in print (approx. 2000-2300 words, including abstract and references). A maximum of 2 illustrations (figures or tables) is allowed. See paragraph below for description and format.
3. Letters to the Editors;
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7. Conference announcements and news.

2. General procedures

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2.1. Title, author(s), address(es)

The title should be no longer than 100 letters, including spaces. Initials or first and middle names followed by last name of the author or authors must be given (**not** last name followed by initials). If there are two or more authors with different addresses, use a superscripted letter (a, b, c etc.), not a number, at the end of the last name of each author to indicate his her corresponding address. The full address of the corresponding author (the way the author

wishes to be contacted) should be provided. The corresponding (usually, the senior) author, to whom correspondence and proofs will be sent, must be indicated by an asterisk and footnoted, and in the footnote, his/her the telephone and fax numbers, and e-mail address must be indicated. Address(es) should be underlined or italicized.

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The abstract should present a summary of the problem, scientific method, major findings and conclusions, in no more than 200 words and in one paragraph and presented at the beginning of the paper. Unsubstantiated speculation should not be included. Footnotes may not be used. References, if cited, must provide complete publication data.

2.3. Text layout

The text of a research paper should be divided into the following headings: Introduction, Methodology (or Materials and Methods), Results, and Discussion and conclusions. Each heading (and subheading) must be numbered using the convention established in the journal. Acknowledgements should come after Discussion and conclusions and before References; Acknowledgements and References are not to be numbered. Headings must be bold-faced and written in an upper-and-lower case style [not in caps], while subheadings should be underlined or italicised. Tables and figures are to be placed at the end of the text, after References. Authors are required to include: (i) the chemical structure, formula and proprietary name of novel or ill-defined compounds; (ii) the w/w yield of prepared extracts in terms of starting crude material; (iii) complete formulation details of all crude drug mixtures; (iv) the voucher herbarium specimen number of the plant(s) studied in case of less well known plants, cited using the collector and collection number (e.g., *Doe 123*), and indicating the name of the herbarium institution where it has been deposited. All plant materials must be fully identified as in the following illustration: *Catharanthus roseus* (L.) G. Don f. *albus* Pich. (Apocynaceae) as authenticated by Dr. John Doe, Department of Botany, University of Connecticut.

2.4. Guidelines for Plant and Animal Names

All scientific names (Latin binomials) must be underlined or italicised throughout the text and in the tables and figures. For plant and animal species, full or complete scientific names, genus-species and the correct authority citation, must be used, *when that name appears for the first time in text*. The authority citation may be dropped in subsequent mention of that name throughout the text. The family name must follow the scientific name in parentheses when the name appears for the first time in the text. Full scientific names and the family name of the subject plants/animals must be used in the Abstract. Synonyms must be indicated in parentheses and preceded by the word "syn." followed by a colon. Authors are advised to consult the International Plant Name Index (IPNI) (http://www.ipni.org and W3Tropicos (http://www.mobot.org) web-based databases to determine the correct spelling of full plant scientific names. Generic names may be abbreviated (e.g., C. *roseus* for *Catharanthus roseus*), provided such practice does not lead to confusion; generic names, however, must not be abbreviated when the name appears for the first time in the text. Specific epithets must never be abbreviated; thus, the use of *Catharanthus r*. is not allowed.

2.5. Keywords

Authors are requested to assign 3-6 keywords to the manuscript, preferably taken from Index Medicus or Excerpta Medica Index, for abstracting and indexing purposes. These keywords

should be typed at the end of the Abstract. Each keyword should start with a capital letter and be separated from each other by a semi-colon.

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Tables should be on separate sheets, one table per sheet, and should bear a short descriptive title. Footnotes in tables should be indicated by consecutive superscript letters, not numbers. **Figures** should be original ink drawings, photographs or computer drawn figures in the original, and of high quality, ready for direct reproduction. Xerox copies are unacceptable as they give unsatisfactory results after final printing. Figures should be drawn in such a way that they can be reduced to **8 cm** in width (i.e., the column width); in exceptional cases a reduction to a width of **17.5** cm will be allowed. All lettering should be such that height of **1.2-1.5mm (minimum)** of numbers and capital letters results after reduction. Numerical scales, scale and curve legends, and all other lettering within the figure itself should be drawn with a lettering guide (stencil) or should be done using stripletters (Letraset, etc). All figures should have captions. Each figure should be identified in the margin or at the back in a corner with the name of the author and the figure number. The figure captions should be on a separate sheet. One set of original drawings is required.

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References should be referred to by name and year (Harvard system) chronologically in the text (e.g.: Brown and Penry, 1973; Stuart, 1979; Ageel et al., 1987) and listed alphabetically at the end of the paper. No ampersand should be used and the words "et al." should not be underlined or italicized. Only papers and books that have been published or in press may be cited.

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Examples:
Journals:
Britton, E.B., 1984. A pointer to a new hallucinogen of insect origin. Journal of Ethnopharmology 12, 331-333.
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Farnsworth, N.R., 1988. Screening plants for new medicines. In: E.O. Wilson and F.M. Peter (Eds.), Biodiversity, National Academy Press, Washington, D.C., pp. 83-97.
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Phytomedicine
International Journal of Phytotherapy and Phytopharmacology

INSTRUCTIONS TO AUTHORS

Aims and Scope

The following areas are covered:

Clinical, pharmacological, moleculargenomic, pharmacokinetic and *bioavailability studies* of standardized plant extracts, fractions, isolated constituents and phytopharmaceuticals thereof having significant bioactivities or could be promising candidates for further thorough pharmacological and clinical studies.

1. Basic and stringent Requirements for consideration of submitted papers:

The standardisation of all above listed plant materials used for the investigations, has to be carried out by means of HPLC, HPLC/MS or HPLC/NMR-fingerprinting inclusive the identification and quantitation of the main bioactive compounds which are or might be responsible for pharmacological activities. The methods have to be described in details: apparatus, columns, solvent systems, gradient, flow rate, detection etc. If the authors do not posess the required analytical equipment or expertise, they are asked to seek cooperation with a phytochemical laboratory. For all plant materials used in investigations stated as derived from cultivated plants or from their natural origin, voucher specimens must be deposited in a specific location with a voucher number. The site (GPS coordinates) and date of collection, with the part(s) used in the study, have to be documented. Without phytochemical standardisation of the plant extracts, the results presented cannot be pharmacologically reproduced and are not acceptable for experimental and clinical studies.

Note: With immediate effect Phytomedicine Will only accept two revisions of a manuscript.

2. The following areas have a restricted scope within Phytomedicine:

- Papers on the isolation and structure elucidation of novel bioactive compounds or the development of new analytical methods do not fall into the scope of Phytomedicine and should be reported elsewhere (e.g. Phytochemistry, Journal of Chromatography or Phytochemical Analysis). Extraordinary pharmacological and clinical studies of these novel natural products, however, are welcome.

Screening results of a large number of plant extracts or plant constituents for antimicrobial or other pharmacological activities will not be considered unless they are focused on those plants or constituents which show extraordinary activities in comparison wit internationally accepted positive (reference) compounds.

- "Dietary Supplements", "Botanicals" or "Functional Food" are not within the scope of Phytomedicine unless they are standardized and pharmacologically investigated analogue to herbal drugs and if the evidences presented are comparable to therapeutic outcomes of a positive control.

Clinical Studies

- Clinical studies must be designed, implemented and analyzed in a manner to meet current standards for clinical trials (GCP = Good Clinical Practice), which are equivalent to those required for synthetic drugs.
- For guidelines and necessary information see the following internet address: www.consortstatement. org with the "Revised Recommendations for Improving the Quality of Reports of Parallel-Group Randomized Trials'' which provides links for downloading the Consort Statement and a checklist as well as explanatory and elaboratory documents. Extensions of the Consort Statement for different types of trials including Herbal Medical Interventions are provided. (The Consort Statement is available in 10 different languages).
- Clinical studies must be approved by na Institutional Ethics Committee or its equivalent and it must be stated in the Method section that the research followed the guidelines of the Declarationof Helsinki and Tokyo for humans.

Pharmacological and molecular biological studies (*in vitro*, *ex vivo* or *in vivo*)

- Investigations with animals must state in the, method section that the research was conducted in accordance with the internationally accepted principles for laboratory animal use and care with stating the guidelines (e.g. European community guidelines/ EEC Directive of 1986 or the US guidelines/ NIH publication) Results have to be based on adequate statistics. Positive controls (reference/standard compounds) and at least three dose responses for conventional pharmacological experiments have to be included.
- Many polyphenolic- and terpenoids containing plant extracts exhibit polyvalent (pleiotropic) activities. Such extracts are of interest for further thorough pharmacological

and therapeutic investigations only if one or two pharmacological activities are dominant and justify the therapeutic application for specified indications.

- Pharmacological studies with herbal drug combinations (e.g. 2–5 plants) will be accepted only if the single herbal extracts are HPLCfinger printed and their major bioactive constituents are quantified before the single extracts are mixed (combined) (see also as an example for the 3D-HPLC-analysis of multidrug combinations Amagaya S. et al., 2001, Phytomedicine 8, 338–342.).
- Two plant extracts or a single constituent of these combined with a synthetic drug or antibiotic which are suggested to exhibit synergistic effects have to be investigated by the "isobol method" according to Berenbaum M. 1989, Pharmacol.Rev. 41: 93-141 (see also Wagner H. and Ulrich-Merzenich G. Synergy research: Approaching a new generation of phyto-Pharmaceuticals Phytomedicine 16: 97- 110 (2009).
- Antimicrobial evaluation of plants are of scientific value only if these plant extracts show extraordinary biological activities in comparison with a synthetic or natural antimicrobial agent standard. It is not useful IF the in vitro activity (MIC) of an extract exceeds 100µg/ml. For the correct determination of MIC values, see Eloff J.N., 2004, Phytomedicine 11: 3701.
- Papers which describe classes of pharmacological activities such as flavonoids with antioxidative activity and isoflavones with estrogenic antiinflammatory activity, will be accepted only if the activities presented exceed those of standard substances and could be promising candidates for further pharmacological and clinical investigations.
- All papers reporting gene expression profiling data
- (microarray experiments) should comply with the Minimum Information about microarray experiments (MIAME) standard: (www.mged.org/Workgroups/ MIAME/miame.html).

At least two **microarrays** should be provided for each experimental condition. Results of selected genes should be validated by a second method (e.g. RT-PCR) or protein data should be provided. In addition functional test (animal experiments/ clinical data) undertaken simultaneously are desirable to allow an appraisement of the biological/clinical relevance of the data. Alternatively, results of in vivo experiments with comparable dosages can be discussed. The presentation of a sole data collection is not acceptable. Biologically relevant information should be presented.

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[a]Department of Biology, The Plant Biocentre, Norwegian University of Science and Technology N-7491 Trondheim, Norway

[b]Institute of Pathobiology, Addis Ababa University P.O. 1176; Addis Abeba, Ethiopia

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An **Abstract** should contain brief information on purpose, methods, results and conclusions in no more than 1000 words.

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Introduction

Materials and Methods

Results

Discussion

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Liu, C.D., Kwan, D., Saxton, R.E., McFadden, D.W., 2000. Hypericin and photodynamic therapy decreases human pancreatic cancer in vitro and in vivo. J. Surgical Res. 93, 137–143.

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