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ANNA ELIZA MACIEL DE FARIA MOTA OLIVEIRA

OBTENÇÃO DE PRODUTOS NANOESTRUTURADOS BIOATIVOS A PARTIR DOS FRUTOS DE SUCUPIRA-BRANCA (*Pterodon emarginatus* Vogel)

Macapá
2016

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OBTENÇÃO DE PRODUTOS NANOESTRUTURADOS BIOATIVOS A PARTIR DOS FRUTOS DE SUCUPIRA-BRANCA (*Pterodon emarginatus* Vogel)

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Inovação Farmacêutica da Universidade Federal do Amapá para obtenção do Título de Doutor em Inovação Farmacêutica.

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LISTA DE TABELAS

ATCI	Iodeto de Acetiltiocolina (do inglês: Acetylthiocholine iodide)
CEMIB/SP	Centro Multidisciplinar para investigação Biológica na Área de Ciência em Animais Universidade de Campinas - SP
CEP UNIFAP	Comitê de Ética e Pesquisa - Universidade Federal do Amapá
cm	Centimetro (s)
D	Densidade celular anterior a adição da nanoemulsão
D_0	Densidade celular após cada dia específico
d(0)	Tamanho médio de gotícula após a preparação da nanoemulsão
d(1)	Tamanho médio de gotícula após um dia de preparação da nanoemulsão
DLS	Espalhamento dinâmico da luz (do inglês: Dynamic light scattering)
DP400	Dioleato de polietilenoglicol 400
DP600	Dioleato de polietilenoglicol 600
DTNB	Ácido 5,5-ditiobis-2-nitrobenzóico (do inglês: 5,5-dithiobis-2-nitrobenzoic acid)
eV	Eletron volt
FID	Detector de inonização de chama (do inglês: flame ionization detector)
g	Grama (s)
GC	Cromatógrafo gasoso (do inglês: gas-chromatograph)
GC-FID	Cromatógrafo gasoso acoplado a detector de ionização de chama (do inglês: gas-chromatograph coupled to flame ionization detector)
GC-MS	Cromatógrafo gasoso acoplado a detector de ionização de espectrometria de massas (do inglês: gas-chromatograph coupled to mass spectrometer detector)
g/kg	Grama por quilograma
g/ml	Grama por mililitro
h	Hora(s)
HLB	Equilíbrio Hidrófilo-Lipófilo (do inglês: Hydrophile Lipophile Balance)

HLBm	Equilíbrio Hidrófilo-Lipófilo da mistura
HLB_{sm}	Equilíbrio Hidrófilo-Lipófilo do monooleato de sorbitano
HLB_{p80}	Equilíbrio Hidrófilo-Lipófilo do polisorbato 80
HPLC-DAD	Cromatógrafo líquido de alta eficiência com detector de arranjo de fotodiodos (do inglês: high performance liquid chromatography couple to diodo array detector
LC50	Concentração letal capaz de matar 50% dos organismos expostos (do inglês: Lethal concentration that kills 50% of the exposed organism)
LC90	Concentração letal capaz de matar 90% dos organismos expostos (do inglês: Lethal concentration that kills 90% of the exposed organism)
m	Metro (s)
m_{p80}	Massa de polisorbato 80
m_{sm}	Massa de monooleato de sorbitano
mm	Milimetro (s)
mg/kg	Miligrama por quilograma
MHV	6α,7β-dihydroxyvouacapan-17-β-oato de metila (do inglês: 6α,7β-dihydroxyvouacapan-17-β-oate)
min	Minuto (s)
mL	Mililitro (s)
mL/min	Mililitro por minuto
ml/kg	Mililitro por quilo
MP400	Monooleato de polietilenoglicol 400
MP600	Monooleato de polietilenoglicol 600
MS	Mass spectrometry
mV	Milivolt
m/z	Razão massa/carga
nm	Nanometro (s)
NPK	Nitrogênio, Fósforo e Potássio
OECD	Guidelines for the testing of chemicals
O/W	Óleo em água (do inglês: Oil in water)
PBS	Tampão fosfato-salino (do inglês: Phosphate buffered saline)
PDI	Índice de polidispersão (do inglês: Polydispersity index)
PEG-40	Polietileno Glicol hidrogenado

PEG-40H	Polietileno Glicol 40 hidrogenado
PG	Tamanho de partícula (do inglês: Particle growth)
PIC	Phase inversion composition method
PIT	Phase inversion temperature method
ppm	Partes por milhão
rHLB	Required Hydrophile Lipophile Balance
rpm	Rotação por minuto
scan/s	Número de scans por segundo
S80	Monooleato de sorbitano 80
SOR	surfactant to oil ratio
T20	Polissorbato 20
T80	Polisorbato 80
TS	Trioleato de Sorbitano
UV	Ultravioleta
UV-Vis	Ultravioleta visível
v/v	Volume/volume
w/w	Massa/massa (do inglês: Weight/weight)
WHO	Organização Mundial da Saúde (do inglês: World Health Organization)
ZS	Zeta Size
µL	Microlitro
µm	Micrômetro
%VC	Porcentagem de células Viáveis
ºC	Grau (s) Celsius

RESUMO

Introdução: *Pterodon emarginatus* Vogel (Fabaceae) é uma espécie nativa do Brasil conhecida popularmente como sucupira-branca. Seus frutos são fonte de um oleorresina rico em diterpenos e óleo essencial, ambos com diversas atividades biológicas conhecidas, incluindo ação larvicida frente à *Aedes aegypti* (Diptera:Culicidae). No entanto, a baixa solubilidade em água dos derivados dos seus frutos torna o desenvolvimento de produtos aquosos estáveis extremamente complexo. A utilização da nanotecnologia permite uma melhor disponibilização em água de substâncias de baixa polaridade, além de outras vantagens, como possibilidade de incremento da estabilidade química e física e liberação modificada das substâncias bioativas.

Objetivo: O objetivo deste trabalho foi obter produtos nanoestruturados bioativos a partir do oleorresina, óleo essencial e diterpeno obtidos de frutos de *P. emarginatus*.

Metodologia: O oleorresina foi obtido por prensagem e o óleo essencial obtido por hidrodestilação utilizando aparato do tipo Clevenger, ambos extraídos dos frutos. Foi realizada cromatografia em coluna utilizando-se sílica gel como fase estacionária para obtenção do diterpeno voucapânico. As nanoemulsões do tipo óleo em água foram obtidas por métodos de baixo aporte de energia, empregando-se técnicas de inversão de fases. A nanodispersão contendo o diterpeno foi preparada através do método de deslocamento do solvente. A avaliação da atividade larvicida frente à *A. aegypti* e *Culex quinquefasciatus* (Diptera:Culicidae) foi testada para verificação da disponibilização em água das substâncias bioativas presentes nos produtos nanoestruturados.

Resultados e discussão: Os produtos nanoestruturados obtidos apresentaram tamanho médio de gotícula diminuto e baixo índice de polidispersão, além de valores negativos de potencial zeta. Foram observados indicativos de estabilidade física para as nanoemulsões preparadas com oleorresina e óleo essencial. Elas apresentaram potente atividade larvicida e não foram observados indicativos de toxicidade ambiental. A nanodispersão preparada com o diterpeno apresentou atividade larvicida residual, sugerindo uma liberação modificada da substância ativa.

Conclusões: Este estudo permitiu a obtenção de diversos produtos nanotecnológicos a base de frutos de *P. emarginatus* utilizando-se técnicas de baixo aporte de energia e baixo custo. Portanto, valoriza uma espécie nativa e abre perspectivas para o seu uso sustentável mediante geração de produtos passíveis de serem preparados dentro do contexto tecnológico nacional.

Palavras-Chave: *Pterodon emarginatus, frutos, nanodispersões, nanoemulsões*, a*tividade larvicida.*

Agradecimentos: CNPq, FAPEAP.

ABSTRACT

Introduction: *Pterodon emarginatus* Vogel (Fabaceae) is a species native to Brazil and that is popularly known as sucupira-branca. Its fruits are source of an diterpene-rich oleoresin and essential oil, both with several biological activities, including larvicidal action against *Aedes aegypti* (Diptera:Culicidae). However, poor water solubility of these raw materials makes development of aqueous stable products a complex procedure. Utilization of nanotechnology allows better availability in water of low polar substances and other advantages, such as possible increment of chemical and physical stability and even modified release of bioactive substances.

Objectives: The aim of the present study was to obtain bioactive nanostructured products using oleoresin, essential oil and diterpene from fruits of *P. emarginatus*.

Methodology: The oleoresin was obtained by pressing and essential oil was obtained by hydrodistillation using Clevenger-type apparatus. Column chromatography with silica gel as stationary phase was used for isolation of vouacapan diterpene. Oil in water nanoemulsions were obtained by low energy method using phase inversion techniques. The nanodispersion containing the diterpene was prepared by solvent displacement method. Evaluation of larvicidal activity against *A. aegypti* and *Culex quinquefasciatus* (Diptera:Culicidae) was performed in order to verify availability in water of bioactive substances present in the nanostructured products.

Results and discussion: Obtained nanostructured products presented small mean droplet size and polydispersity index, in addition to negative zeta potential values. Indicative of physical stability were observed for nanoemulsions prepared with oleoresin and essential oil. They presented potent larvicidal activity and no signals of environmental toxicity were observed. The nanodispersion prepared with the diterpene presented residual larvicidal activity, suggesting modified release of active substance.

Conclusions: This study allowed obtainment of nanotechnology products using fruits of *P. emarginatus* by low cost and low energy methods. Thus, it valorizes a native species and open perspectives for its sustainable use by generating products that can be prepared in our national technological context

Keywords: *Pterodon emarginatus, fruits, nanodispersions, nanoemulsions*, larvicidal activity.

Acknowledgements: CNPq, FAPEAP.

1. INTRODUÇÃO

A espécie vegetal *Pterodon emarginatus* Vogel (Fabaceae) é uma árvore popularmente conhecida como "sucupira-branca" ou "faveira". É uma leguminosa de porte médio, geralmente com 10 metros de altura e seus frutos, bem como de outras espécies do gênero, são reconhecidos por diversas atividades biológicas.

Preparações populares à base dos frutos de *P. emarginatus* têm demonstrado eficácia significativa e vêm sendo utilizados pela população sob a forma de macerados hidroalcoólicos para o tratamento de afecções da laringe e ainda como estimulante do apetite e fortificante, principalmente em crianças. Além disso, essa espécie possui comprovada ação antiinflamatória, parasiticida, analgésica, antitumoral e larvicida frente à *Aedes aegypti* (Diptera:Culicidae).

Dos frutos da sucupira-branca extrai-se um oleorresina bastante viscoso, de coloração castanho-clara. Diversos estudos já demostraram que suas substâncias, principalmente terpenóides, são os responsáveis pelas atividades biológicas relatadas. Os marcadores químicos do oleorresina de sucupira-branca são diterpenos de esqueleto vouacapânico e também se pode obter um óleo essencial rico em sesquiterpenos. No entanto, a intrínseca baixa solubilidade dos constituintes desses óleos e a sua imiscibilidade em água tornam o desenvolvimento de um produto aquoso um verdadeiro desafio tecnológico.

A nanotecnologia é uma área em franca expansão com caráter extremamente multidisciplinar, permitindo a geração de produtos inovadores para diversos segmentos, como indústria de alimentos, medicamentos, cosméticos e pesticidas. Dentre os diversos produtos nanoestruturados que podem ser preparados, destacam-se as nanodispersões aquosas, que permitem uma melhor disponibilização de substâncias pouco solúveis nesse meio. Caso o produto disperso seja um líquido imiscível, como um óleo, denominamos a nanodispersão aquosa de nanoemulsão do tipo óleo em água. A classificação quanto ao tamanho médio da gotícula ou partícula pode variar segundo diferentes autores, contudo, assume-se que deve ser inferior a 1 micrômetro e apresentar propriedades diferenciadas do material de origem. Além da vantagem relacionada ao incremento da solubilidade,

observa-se que as nanodispersões podem aumentar a estabilidade física do sistema, tornar o produto mais atraente ao consumidor devido a maior fluidez, transparência e aspecto fino, controlar a liberação das substâncias ativas, aumentar a estabilidade química, entre outras. Atualmente, estudos nanobiotecnológicos com produtos naturais de origem vegetal estão na vanguarda do conhecimento científico e considerados muito promissores. Neste contexto, o presente trabalho apresenta o desenvolvimento e preparação de diversas nanoemulsões do tipo óleo em água com o oleorresina e óleo essencial de frutos de *P. emarginatus*, além de nanodispersões com o diterpeno 6α,7β, dihidroxivouacapanoato de metila, isolado do extrato hexânico.

O mosquito *A. aegypti* é o principal agente transmissor de doenças virais infecciosas como Dengue, Chikungunya e Zika. Já a espécie *Culex quinquefasciatus* (Diptera:Culicidae) transmite parasitas causadores da Filariose Linfática ou Elefantíase. Este é um grave problema de saúde pública, especialmente em países tropicais e subtropicais, onde a incidência é muito alta. A maior preocupação quanto à transmissão dessas doenças é de que o desenvolvimento dos agentes transmissores ocorre em ambientes urbanos, com uma elevada incidência de focos das larvas em água parada, principalmente nos ambientes residenciais. Levando em consideração que muitos pesticidas de origem natural são pouco solúveis em água, o desenvolvimento de nanoformulações seria uma alternativa viável para aumentar a atividade inseticida dessas substâncias, além de induzirem um menor impacto sobre o meio ambiente devido ao fato de serem biodegradáveis. A atividade larvicida frente de óleos e diterpenos de sucupira-branca são conhecidas. Portanto, foi utilizado esse modelo para verificar a bioatividade das nanoformulações obtidas, evidenciando o êxito em se disponibilizar em água os produtos à base da espécie mediante preparação de nanoemulsões e nanodispersões. A produção de produtos nanoestruturados usando o óleo de sucupira-branca, espécie nativa da flora brasileira, também pode se tornar uma alternativa viável para práticas integradas de controle de *A. aegypti* e *C.quinquefasciatus,* contribuindo para o desenvolvimento de produtos frente a doenças tropicais negligenciadas e emergentes.

O presente trabalho encontra-se apresentado sob formato de artigos conforme as normas do Programa de Pós Graduação em Inovação Farmacêutica. Os capítulos que irão compô-lo são:

Capítulo 1: Aspectos químicos, biológicos e fitofarmacêuticos do gênero *Pterodon*: uma fonte de compostos bioativos;

Capítulo 2: Utilization of natural products for novel nano-based insecticidal agents;

Capítulo 3: Development of a larvicidal nanoemulsion with *Pterodon emarginatus* Vogel oil;

Capítulo 4: *Pterodon emarginatus* oleoresin-based nanoemulsion as a promising tool for *Culex quinquefasciatus* (Diptera: Culicidade) control;

Capítulo 5: Utilization of dynamic light scattering to evaluate *Pterodon emarginatus* oleoresin based nanoemulsion formation and stabilization by non-heating and solvent-free method;

Capítulo 6: Essential oil from *Pterodon emarginatus* as a promising natural raw material for larvicidal nanoemulsions against a tropical disease vector;

Capítulo 7: Investigation of potential residual larvicidal activity of a novel aqueous nanodispersion prepared with vouacapan diterpene isolated from *Pterodon emarginatus* oleoresin.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Obter produtos nanoestruturados a partir de óleos oriundos dos frutos de *Pterodon emarginatus* e seus derivados e avaliar o seu potencial biológico.

2.2 OBJETIVOS ESPECÍFICOS

DEVELOPMENT OF A LARVICIDAL NANOEMULSION WITH *Pterodon emarginatus* Vogel OIL

- Determinar o equilíbrio hidrófilo-lipófilo do oleorresina de *P. emarginatus;*
- Obter nanoemulsões contendo oleorresina de *P. emarginatus*;
- Caracterizar as nanoemulsões obtidas;
- Avaliar a atividade larvicida de uma nanoemulsão obtida contra *A. aegypti*;
- Avaliar a influência da nanoemulsão larvicida sobre a atividade da acetilcolinesterase das larvas de *A. aegypti*;
- Avaliar a toxicidade aguda da nanoemulsão larvicida em camundongos Swiss.

Pterodon emarginatus OLEORESIN-BASED NANOEMULSION AS A PROMISING TOOL FOR *CULEX QUINQUEFASCIATUS* (DIPTERA: CULICIDADE) CONTROL

- Investigar a influência de diferentes pares de tensoativos na formação da nanoemulsão contendo oleorresina de *P. emarginatus;*
- Avaliar a atividade larvicida de uma das nanoemulsões obtidas contra *C. quinquefaciatus*;
- Avaliar a influência da nanoemulsão larvicida sobre a atividade da acetilcolinesterase das larvas de *C. quinquefaciatus*.

- ❖ Avaliar a ecotoxicidade da nanoemulsão contra um organismo não-alvo, a microalga *Chlorella vulgaris.*

UTILIZATION OF DYNAMIC LIGHT SCATTERING TO EVALUATE *Pterodon emarginatus* OLEORESIN BASED NANOEMULSION FORMATION AND STABILIZATION BY NON-HEATING AND SOLVENT-FREE METHOD

- ❖ Preparar nanoemulsões contendo oleorresina de *P. emarginatus* utilizando polysorbato 85 ou monooleato de polietilenoglicol 400;
- ❖ Caracterizar as nanoemulsões obtidas utilizando-se diferentes diluições;
- ❖ Avaliar a influência da temperatura sobre o tamanho de partícula, índice de polidispersão e potencial zeta das nanoemulsões obtidas.

ESSENTIAL OIL FROM *Pterodon emarginatus* AS A PROMISING NATURAL RAW MATERIAL FOR LARVICIDAL NANOEMULSIONS AGAINST A TROPICAL DISEASE VECTOR

- ❖ Determinar o equilíbrio hidrófilo-lipófilo do óleo essencial de *P. emarginatus;*
- ❖ Obter nanoemulsões com óleo essencial de *P. emarginatus*;
- ❖ Caracterizar as nanoemulsões obtidas;
- ❖ Avaliar a atividade larvicida de uma nanoemulsão obtida contra *A. aegypti*;
- ❖ Avaliar a influência da nanoemulsão sobre a atividade da acetilcolinesterase das larvas de *A. aegypti*.

INVESTIGATION OF POTENCIAL RESIDUAL ACTIVITY OF A NOVEL AQUEOUS NANODISPERSION PREPARED WITH VOUACAPAN DITERPEN ISOLATED FROM *Pterodon emarginatus* OLEORESIN

- ❖ Isolar um diterpeno de uma mistura pré-purificada obtida do oleorresina de *P. emarginatus*;
- ❖ Propor rota de fragmentação para o diterpeno isolado;
- ❖ Obter uma nanodispersão com o diterpeno isolado;
- ❖ Caracterizar a nanodispersão obtida;
- ❖ Avaliar a ação larvicida residual da nanodispersão obtida.

ASPECTOS QUÍMICOS, BIOLÓGICOS E FITOFARMACÊUTICOS DO GÊNERO *Pterodon*: UMA FONTE DE COMPOSTOS BIOATIVOS

Artigo de revisão.

ASPECTOS QUÍMICOS, BIOLÓGICOS E FITOFARMACÊUTICOS DO GÊNERO *Pterdon*: UMA FONTE DE COMPOSTOS BIOATIVOS

O Brasil possui uma diversificada flora, rica em compostos bioativos de origem vegetal. Durante séculos as plantas desempenharam um papel fundamental, fornecendo uma fonte de agentes bioativos potencialmente ativos para o homem (HOSTETTMAN; QUEIROZ; VIEIRA, 2003). Originalmente, o profundo conhecimento do arsenal químico da natureza por povos ancestrais e antigas civilizações pode ser considerado fator fundamental para o descobrimento dessas substâncias (VIEGAS JR; BOLZANI; BARREIRO, 2006). Outras estratégias envolvem a seleção randômica da espécie vegetal ou uma abordagem quimiosistemática (MACIEL; PINTO; VEIGA JR, 2002).

O gênero *Pterodon* Vogel (1837) pertence à família Fabaceae Lindl., uma das mais representativas das Angiospermas. Compreende quatro espécies de árvores aromáticas nativas do Brasil, distribuídas amplamente em vários domínios fitogeográficos, que são: *Pterodon abruptus* (Moric.) Benth, *Pterodon appariciοi* Pedersoli, *Pterodon emarginatus* Vogel e *Pterodon pubescens* (Benth.) Benth (LIMA, LIMA, 2015). Este pequeno gênero, é conhecido pelas propriedades terapêuticas atribuídas a suas espécies, que são bastante utilizadas na medicina popular (VIEIRA et al., 2008). Os frutos e folhas do gênero são geralmente utilizados em infusões e ingeridos em pequenas doses e períodos regulares para o tratamento de doenças reumáticas, doenças respiratórias, como anti-inflamatório, depurativo e tônico (CARVALHO et al., 1998; COELHO et al, 2005; HOSCHEID; CARDOSO, 2015).

As diversas atividades biológicas verificadas podem ser atribuídas à variedade de substâncias identificadas no gênero. Análises fitoquímicas demostraram a presença de alcalóides (TORRENEGRA; BAUEREIB; ACHENBACH, 1989) e terpenóides (MORAES et al., 2012) na casca (Tabela 1), isoflavonas e terpenóides na madeira do tronco (BRAZ FILHO; GOTTLIEB; ASSUMPÇÃO, 1971; GALINA; GOTTLIEB, 1974; MARQUES et al, 1998, BUSTAMANTE et al., 2010) (Tabela 2), isoflavonas e terpenóides no alburno e cerne (MARQUES et al., 1998) (Tabela 3), sesquiterpenos, esteróides, flavonóides, taninos catequínicos, flavonas e xantonas nas folhas (CAMPOS; CRAVEIRO; TEIXEIRA, 1990; SANTOS et al., 2010; MIRANDA et al., 2014; ARRAIS-SILVA et al., 2014) (Tabela 4). No oleorresina obtido a partir de frutos, também foi relatada uma grande variedade de substâncias (Tabela

5), como terpenóides do tipo furanoditerpenos, sesquiterpenos, diterpenos de esqueleto vouacapânico (NETO, 1976; NUNAN et al, 1982; ARRIAGA et al, 2000; SPINDOLA et al, 2009; CABRAL et al., 2012; HOSCHEID et al., 2013; NUCCI-MARTINS et al., 2015), constituintes fenólicos e flavonóides (DUTRA; LEITE; BARBOSA, 2008).

Tabela 1: Algumas substâncias isoladas nas cascas do caule de *Pterodon* sp.

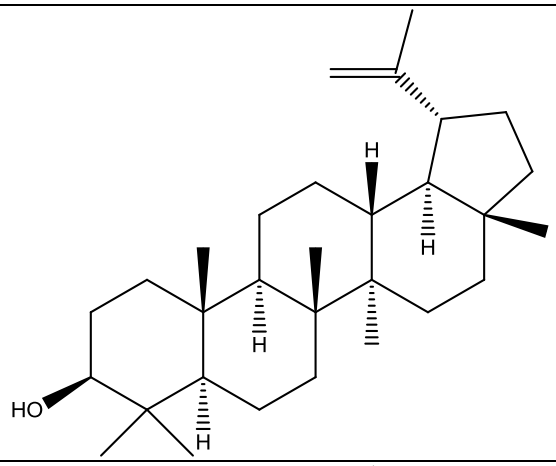
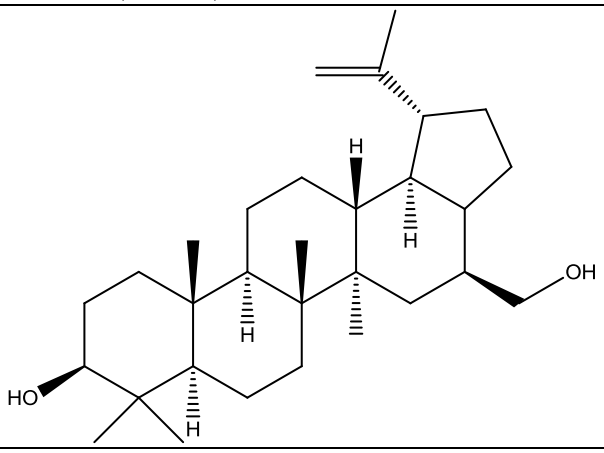
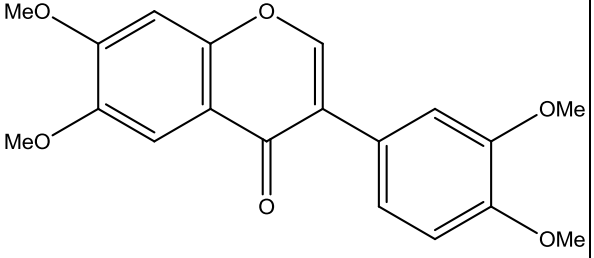
Classe	Substâncias	Estrutura	Referências
Triterpenos	lupeol		MORAES et al., 2012
	betulina		

Tabela 2: Algumas substâncias isoladas na madeira do tronco de *Pterodon* sp.

Classe	Substâncias	Estrutura	Referências
Isoflavonas	3',4',6,7-tetrametoxiisoflavona		BRAZ FILHO; GOTTLIEB; ASSUMPÇÃO, 1971

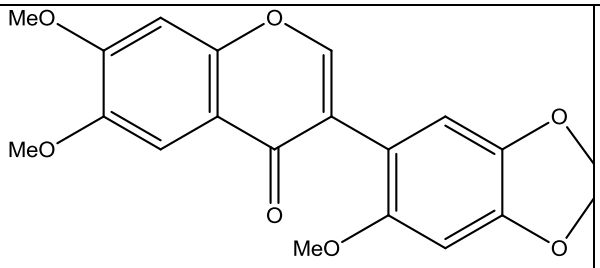
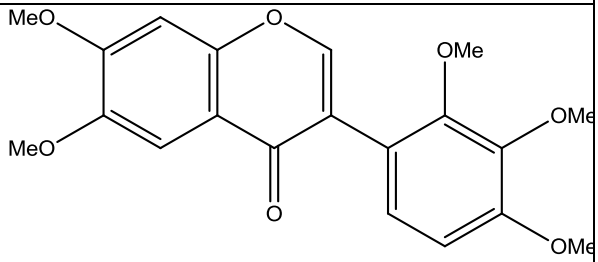
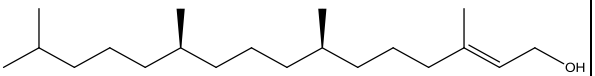
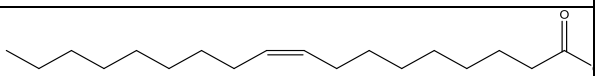
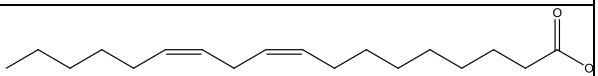
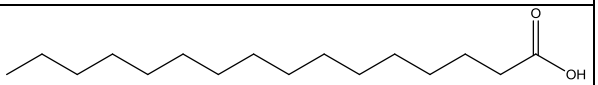
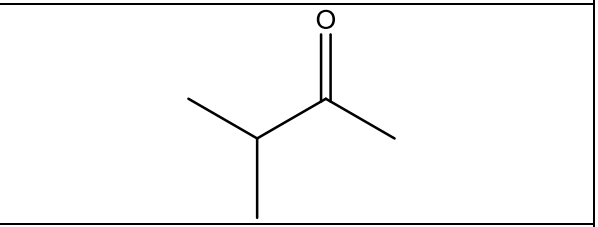
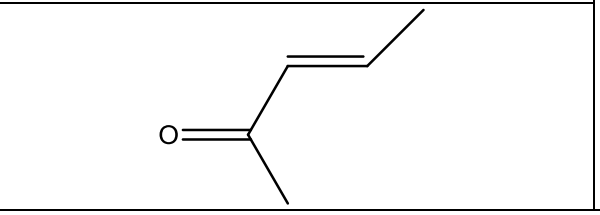
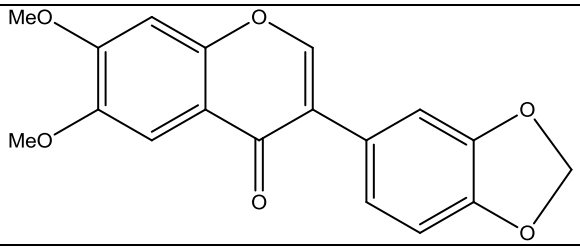
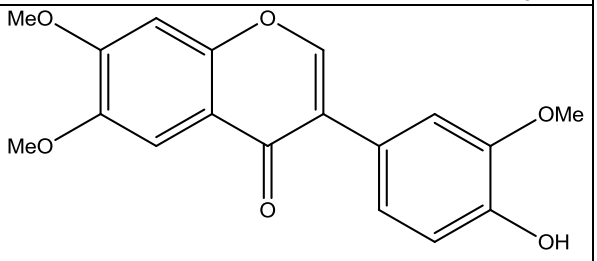
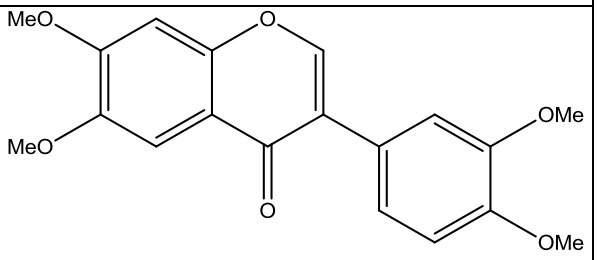
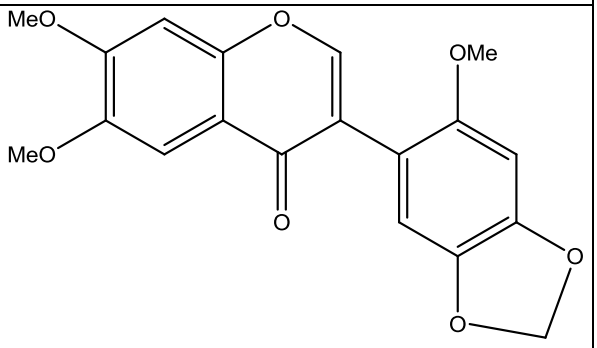
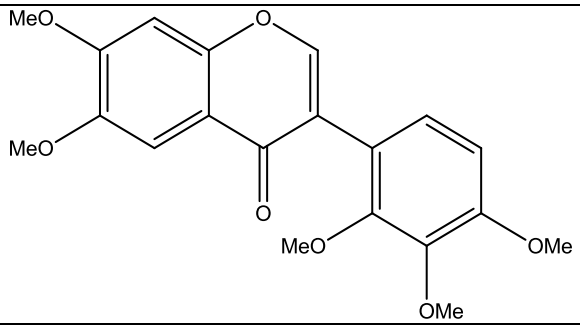
Isoflavonas	2’,6,7-trimetoxi-4’,5’-metilenodioxiisoflavona		BRAZ FILHO; GOTTLIEB; ASSUMPÇÃO, 1971
	2’,3’,4’,6,7penta-metoxiisoflavona		
Terpe-nóide	fitol		HERNÁNDEZ TERRONES et al., 2007
Ácidos graxos	ácido oléico		
	linoleiladato de metila		
	ácido palmítico		
Outros	Isopropenil-metilcetona		
	3-penten-2-ona		

Tabela 3: Algumas substâncias isoladas no alburno e cerne de *Pterodon* sp.

Classe	Substâncias	Estrutura	Referências
Isoflavonas	6,7-dimetoxi-3',4'-metilenodioxiisoflavona		MARQUES et al., 1998
	4'-hidroxi-3',6,7-trimetoxiisoflavona		
	3´,4´,6,7-tetrametoxi-isoflavona		
	2',6,7-trimetoxi-3',4'-metilenodioxiisoflavona		
	2',3',4',7,7-pentametoxiisoflavona		

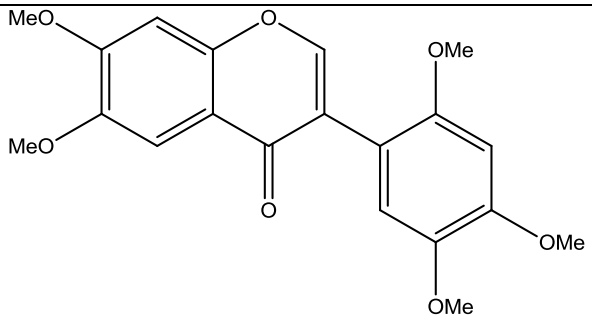
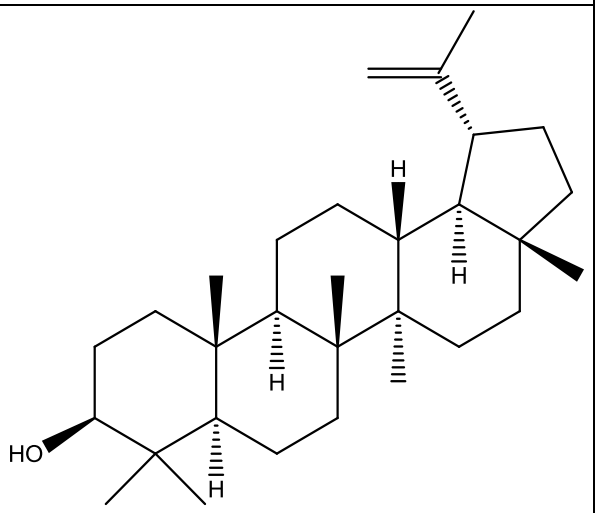
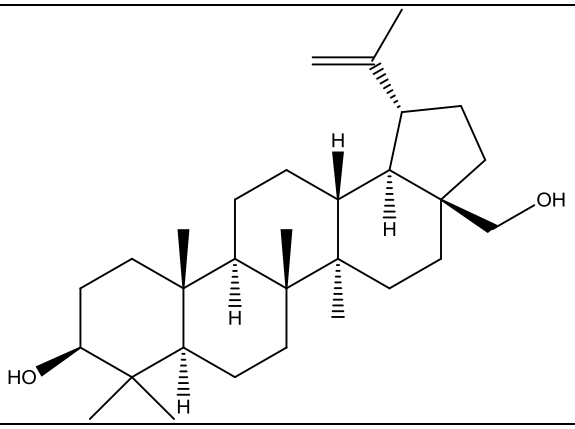
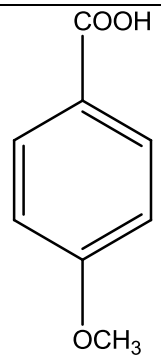
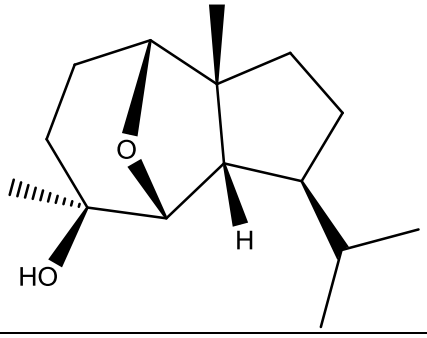
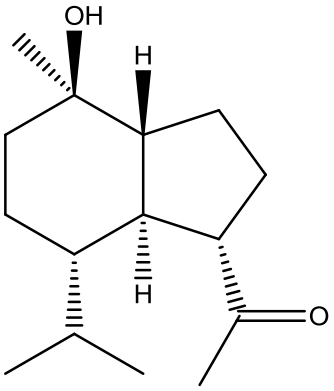
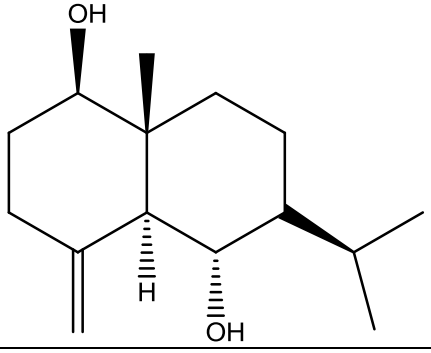
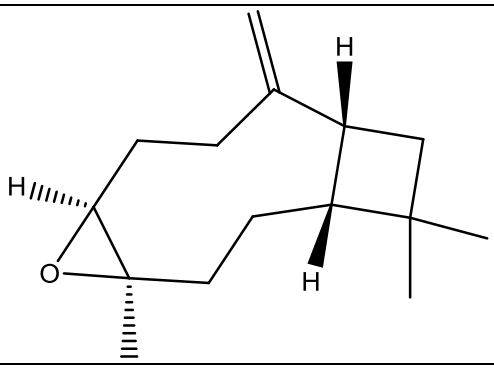
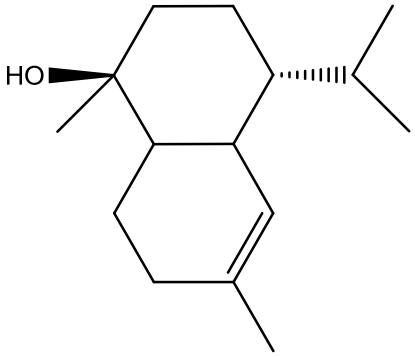
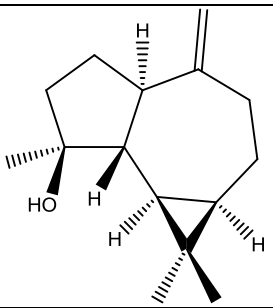
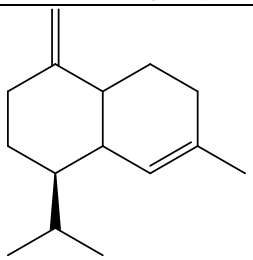
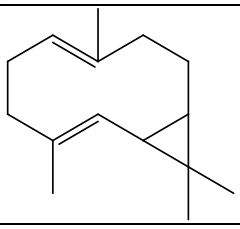
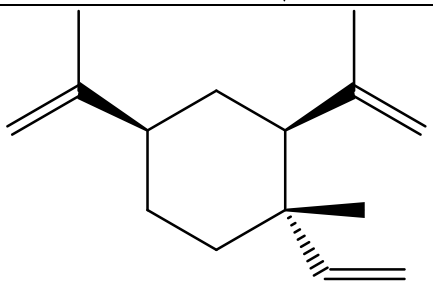
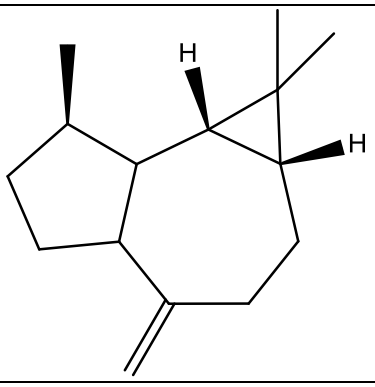
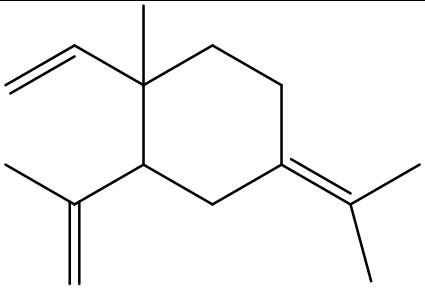
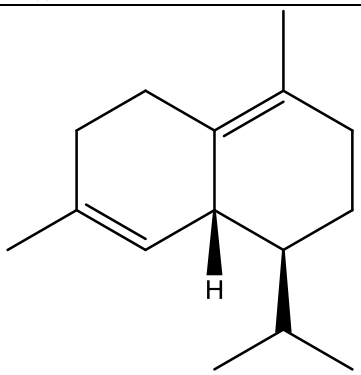
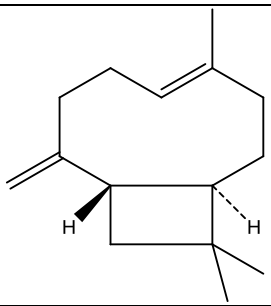
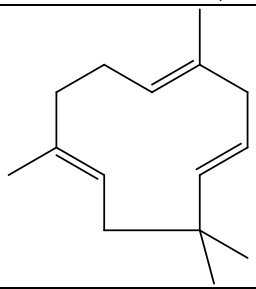
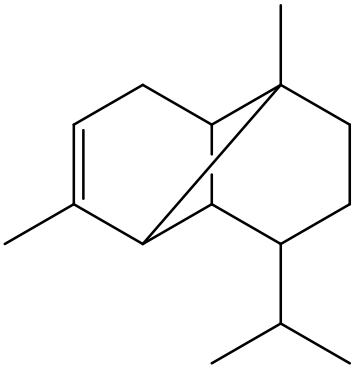
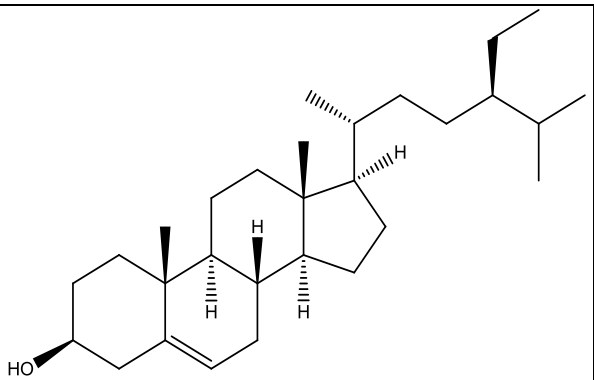
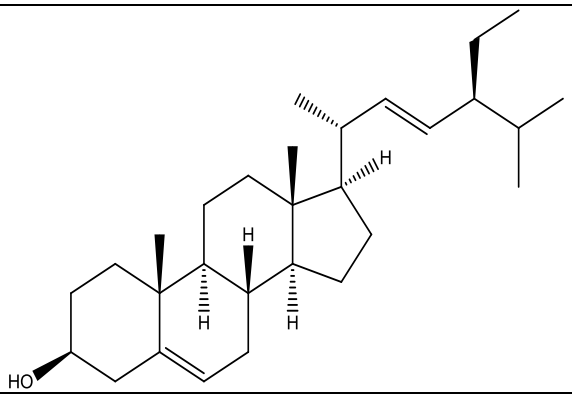
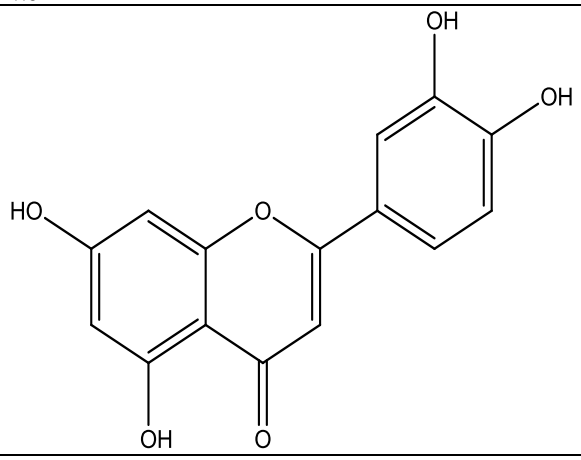
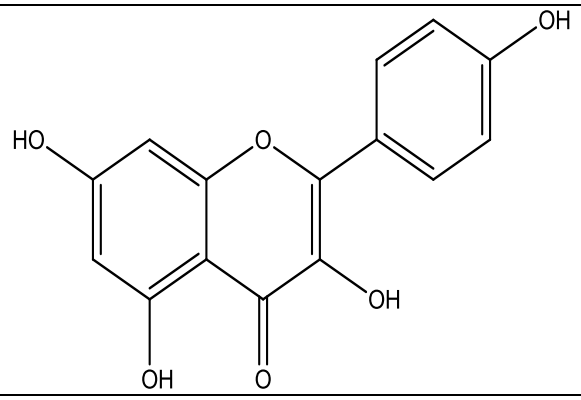
	2’,4’,5,6,7-pentametoxiisoflavona		MARQUES et al., 1998
Terpenos	lupeol		
	betulina		
Outros	ácido 4-metoxibenzóico		

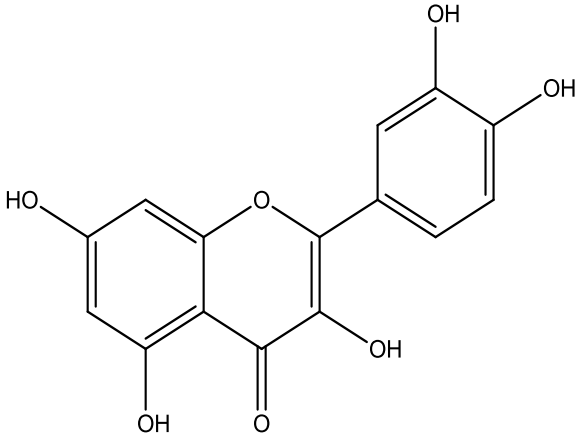
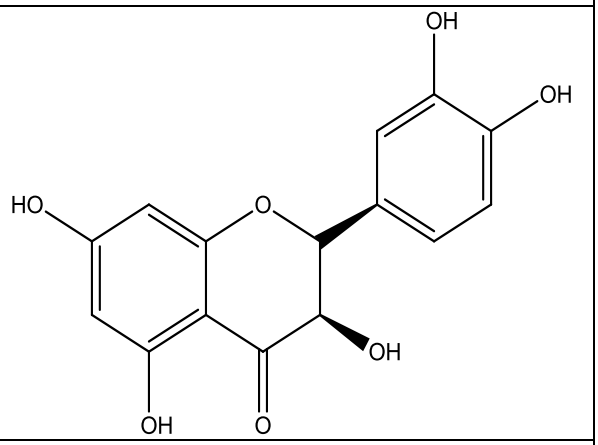
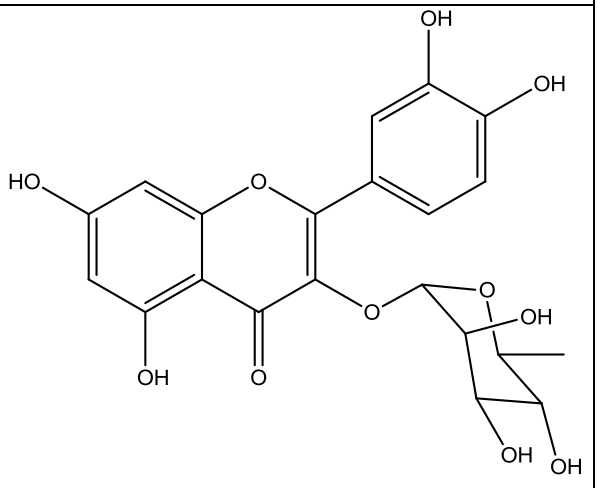
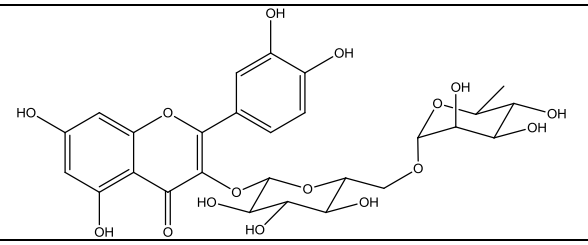
Tabela 4: Algumas substâncias isoladas nas folhas de *Pterodon* sp.

Classe	Substâncias	Estrutura	Referências
Sesquiterpenos	(rel)-2β,6β-epóxi-5β-hidróxi-isodaucano		MIRANDA et al., 2014
	oplopanona		
	1β,6αdi-hidróxi-4(15)-eudesmeno		
	óxido de cariofileno		

Sesquiterpenos	α-cadinol		MIRANDA et al., 2014
	Spatulenol		
	γ-muuroleno		SANTOS et al., 2010
	biciclogermacreno		
	β-elemeno		
	allo-aromadendreno		

Sesquiterpenos	γ-elemeno		CAMPOS; CRAVEIRO; TEIXEIRA, 1990
	δ- cadineno		CAMPOS; CRAVEIRO; TEIXEIRA, 1990; SANTOS et al., 2010
	β-cariofileno		
	α-humuleno		
	α-copaeno		

Esteróides	β-sitosterol		SANTOS et al., 2010; MIRANDA et al., 2014
	estigmasterol		
Flavonóides	Luteolina		MIRANDA et al., 2014
	kaempferol		

Flavonóides	quercetina		MIRANDA et al., 2014
	(+)-catequina		
	quercetina-3-O-α-L-rhamnopiranosídeo		
	rutina		

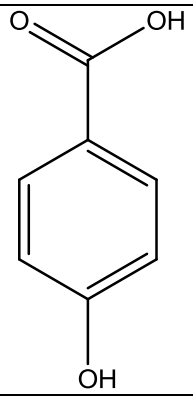
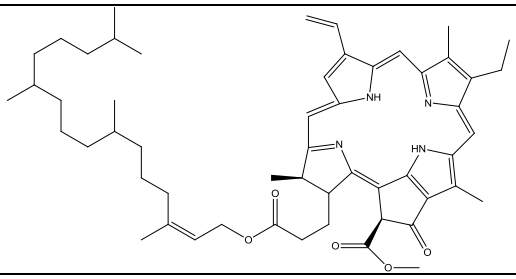
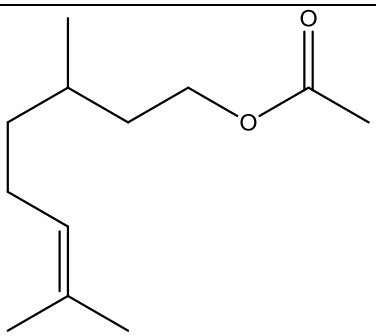
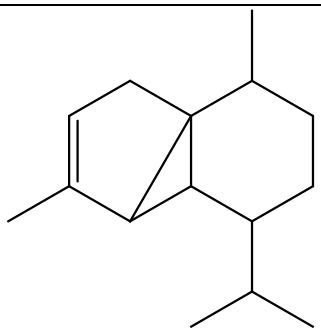
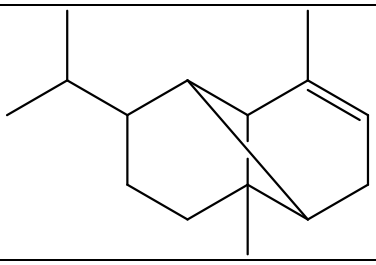
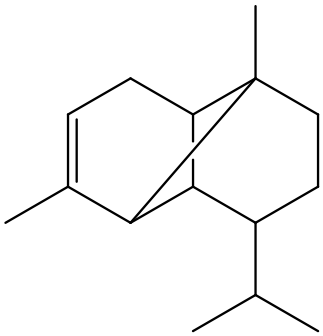
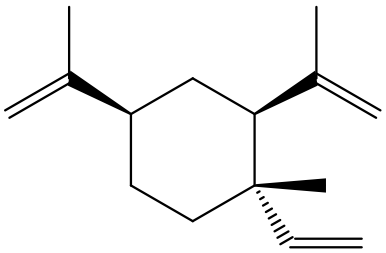
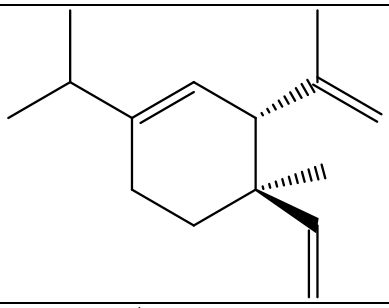
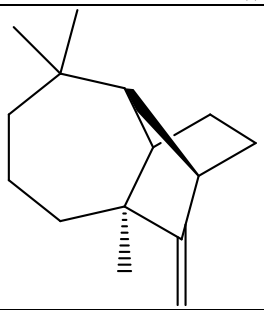
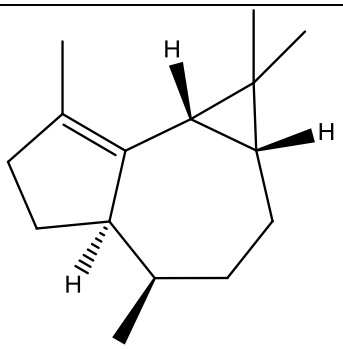
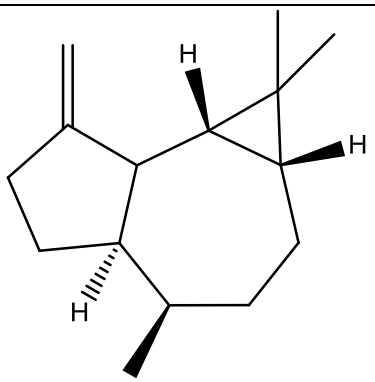
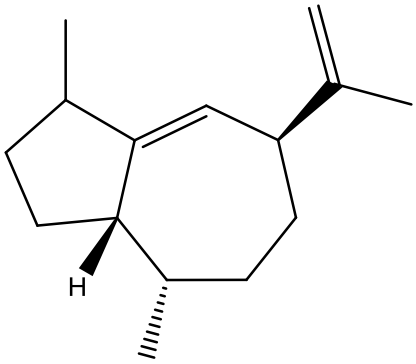
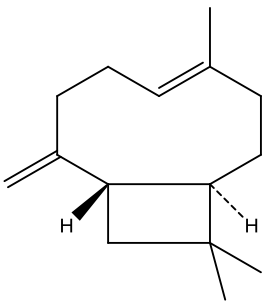
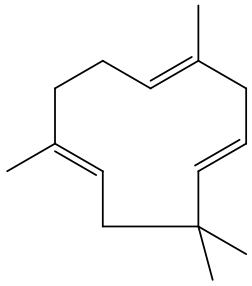
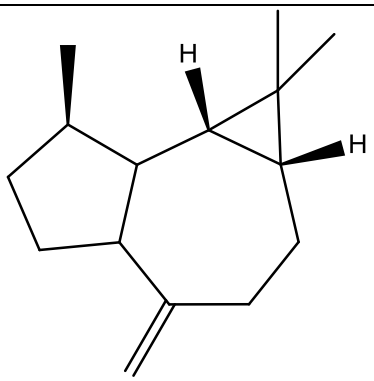
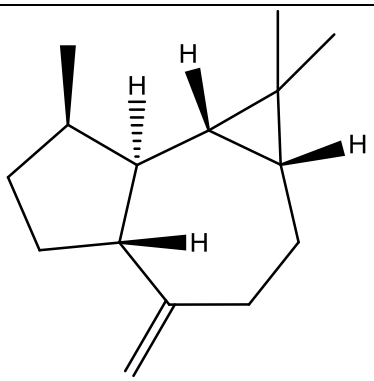
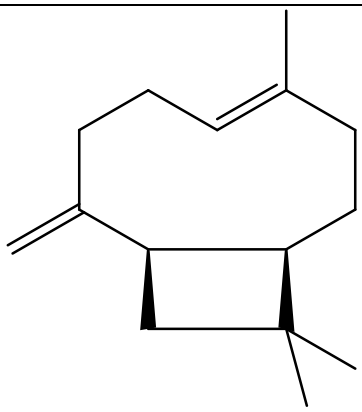
Outros	ácido p-hidroxibenzoico		MIRANDA et al., 2014
	feofitina A		

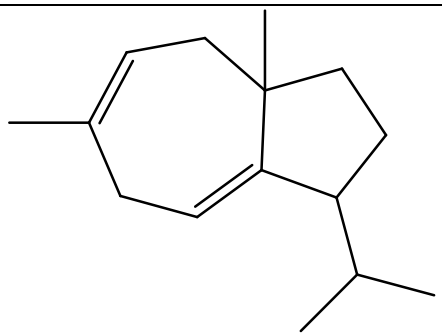
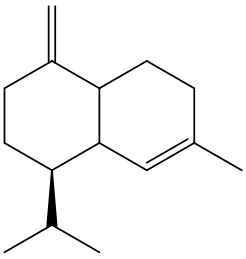
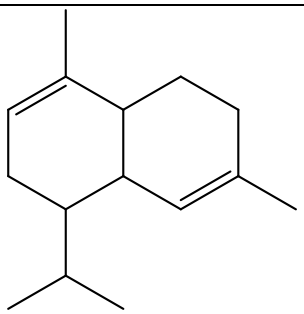
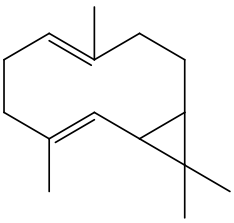
Tabela 5: Algumas substâncias isoladas dos frutos e sementes de *Pterodon* sp.

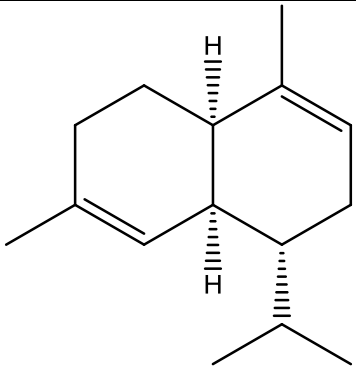
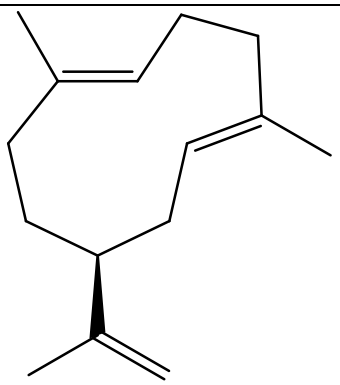
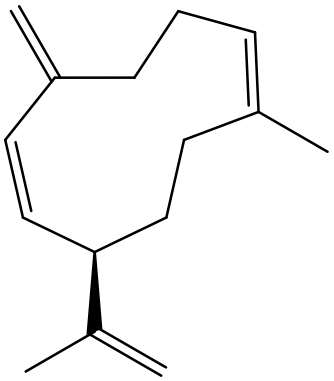
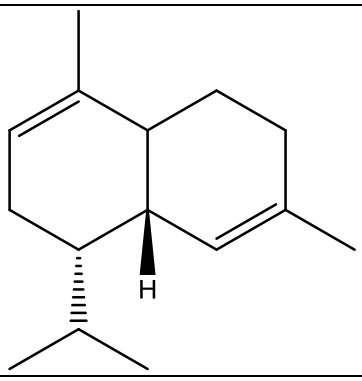
Classe	Substâncias	Estrutura	Referência
Monoterpenos	Acetate de citronelila		ALVES et al., 2013
Sesquiterpenos	α- cubebeno		EVANGELISTA et al., 2007; ALVES et al., 2013; 2014
	α-ylangeno		ALVES et al., 2013

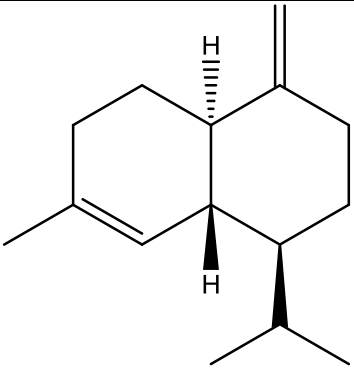
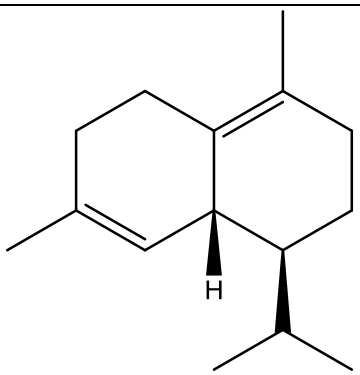
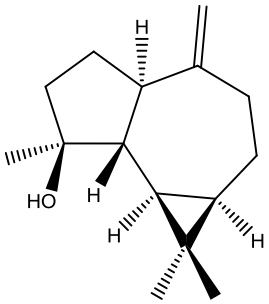
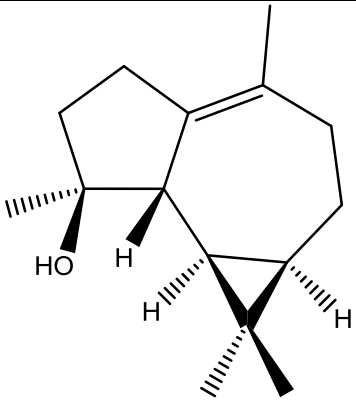
Sesquiterpenos	α-copaeno		EVANGELISTA et al., 2007; NUCCI et al., 2012; PINTO et al., 2013; VELOZO et al, 2013; ALVES et al., 2013; 2014; NUCCI-MARTINS et al., 2015
	β -elemeno		EVANGELISTA et al., 2007; DUTRA et al., 2009b; NUCCI et al., 2012; DUTRA et al., 2012; PINTO et al., 2013; VELOZO et al, 2013; ALVES et al., 2013; 2014; NUCCI-MARTINS et al., 2015
	δ -elemeno		EVANGELISTA et al., 2007; DUTRA et al., 2009b; DUTRA et al., 2012; NUCCI-MARTINS et al., 2015.
	longifoleno		ALVES et al., 2013.

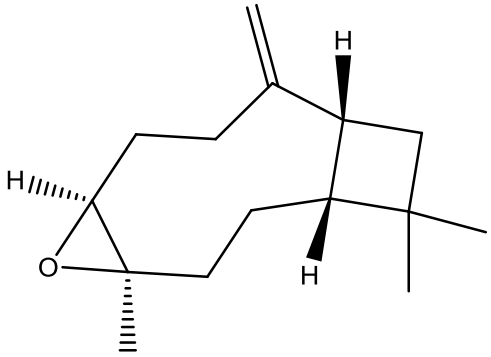
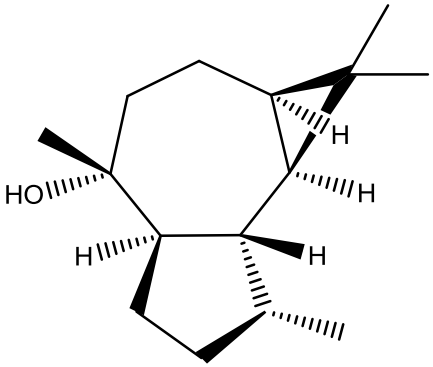
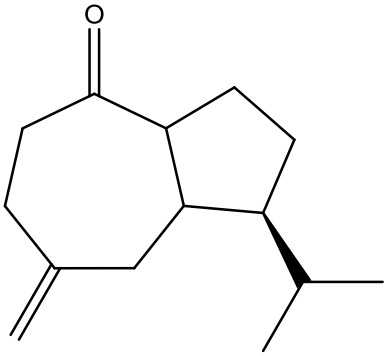
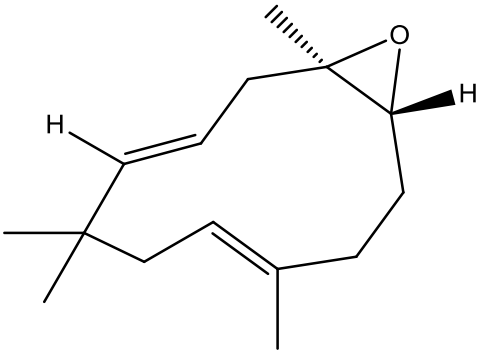
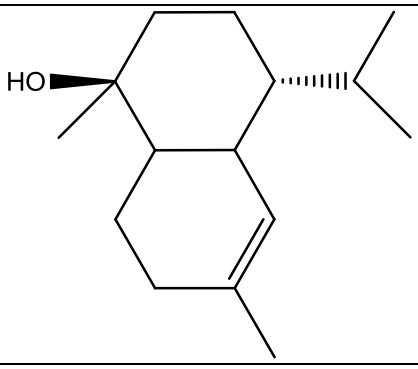
Sesquiterpenos	α -gurjuneno		EVANGELISTA et al., 2007; ALVES et al., 2013.
	β-gurjuneno		EVANGELISTA et al., 2007; DUTRA et al., 2009b; DUTRA et al., 2012.
	γ-gurjuneno		ALVES et al., 2013.
	β-cariofileno		EVANGELISTA et al., 2007; DUTRA et al., 2009b; NUCCI et al., 2012; DUTRA et al., 2012; PINTO et al., 2013; VELOZO et al, 2013; ALVES et al., 2013; 2014; NUCCI-MARTINS et al., 2015; OLIVEIRA et al., 2016.

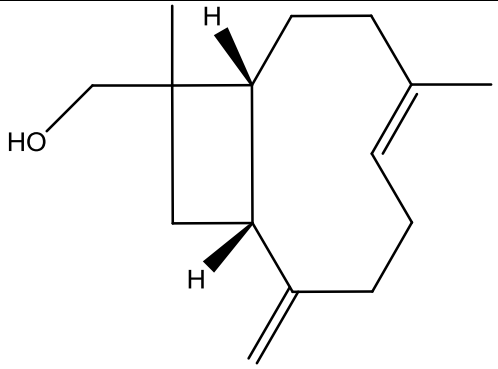
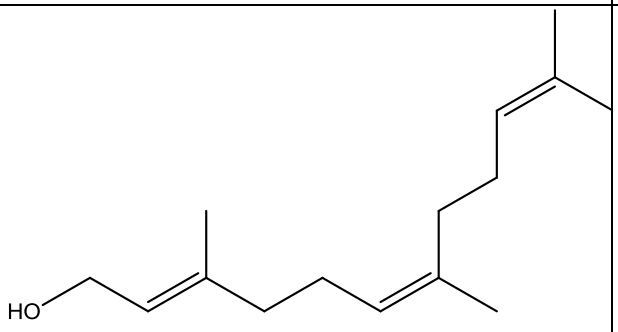
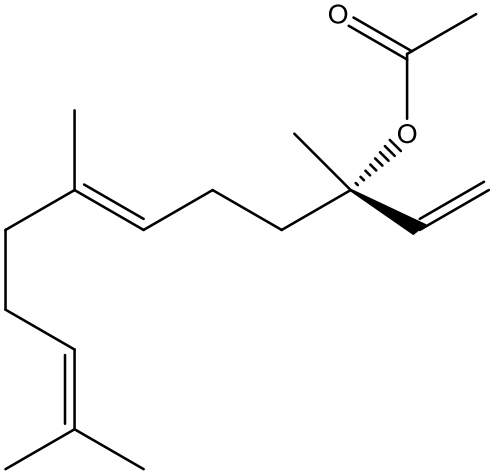
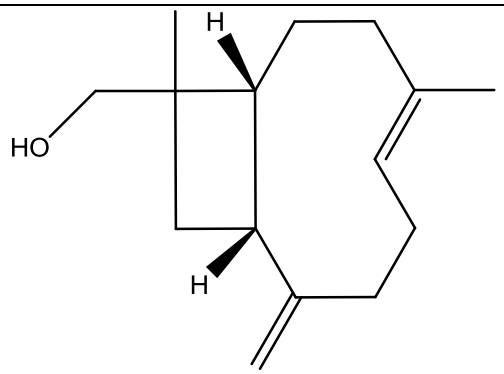
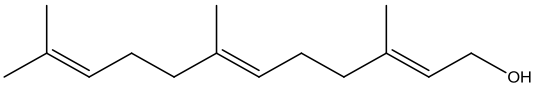
Sesquiterpenos	α-humuleno		EVANGELISTA et al., 2007; DUTRA et al., 2009b; NUCCI et al., 2012; DUTRA et al., 2012; PINTO et al., 2013; VELOZO et al, 2013; ALVES et al., 2013; 2014; NUCCI-MARTINS et al., 2015.
	Allo-aromadendreno		EVANGELISTA et al., 2007; NUCCI et al., 2012; VELOZO et al, 2013; ALVES et al., 2013; NUCCI-MARTINS et al., 2015.
	Aromadendreno		EVANGELISTA et al., 2007.
	9-*epi-(E)*-cariofileno		ALVES et al., 2013; 2014.

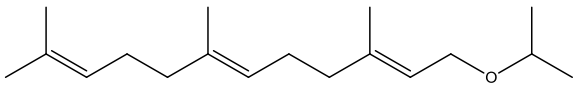
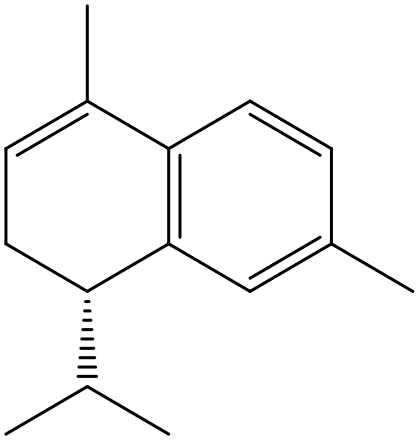
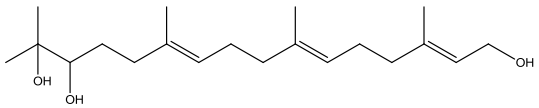
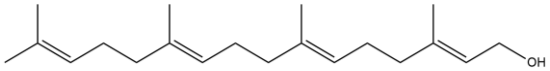
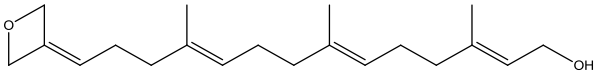
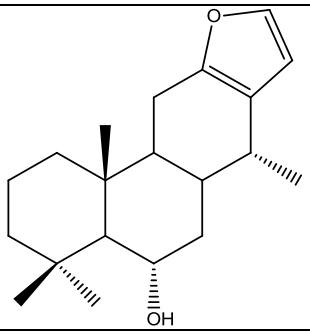
Sesquiterpenos	Dauca-5,8-dieno		ALVES et al., 2013; 2014.
	γ-muuroleno		EVANGELISTA et al., 2007; DUTRA et al., 2009b; NUCCI et al., 2012; DUTRA et al., 2012; ALVES et al., 2013; 2014; NUCCI-MARTINS et al., 2015.
	α -amorpheno		ALVES et al., 2013.
	Biciclogermacreno		EVANGELISTA et al., 2007; DUTRA et al., 2009b; NUCCI et al., 2012; DUTRA et al., 2012; VELOZO et al, 2013; ALVES et al., 2013; 2014; NUCCI-MARTINS et al., 2015.

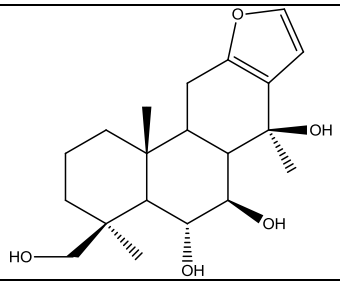
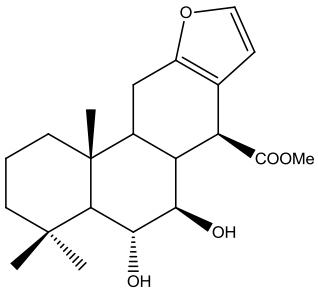
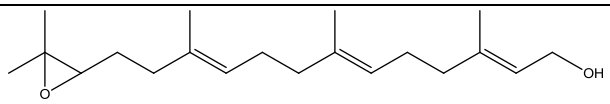
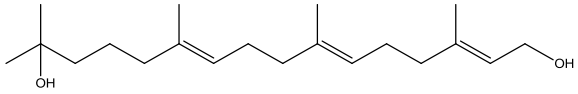
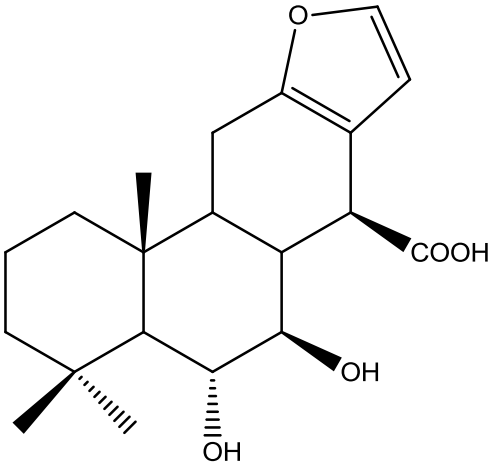
Sesquiterpenos	α -muuroleno		ALVES et al., 2013; 2014.
	Germacreno-A		PINTO et al., 2013; ALVES et al., 2013; VELOZO et al, 2013.
	Germacreno-D		EVANGELISTA et al., 2007; DUTRA et al., 2009b; DUTRA et al., 2012; VELOZO et al, 2013; PINTO et al., 2013.
	δ- amorpheno		ALVES et al., 2013.

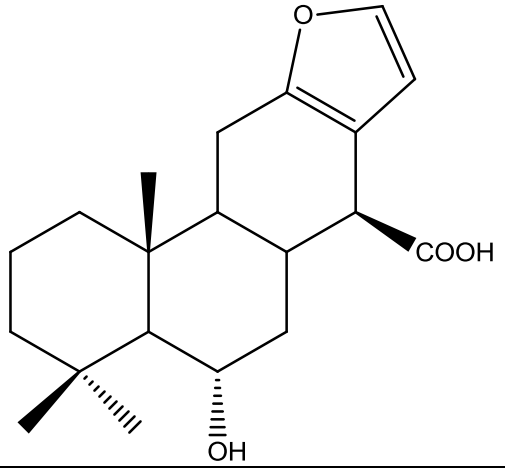
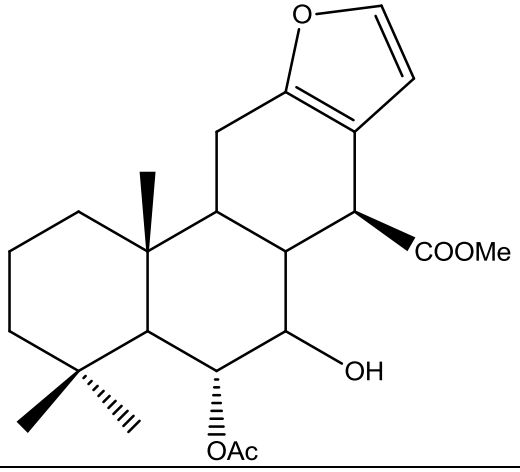
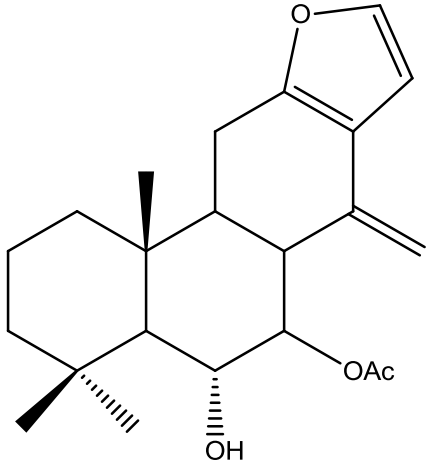
Sesquiterpenos	γ-cadineno		EVANGELISTA et al., 2007; ALVES et al., 2013; 2014.
	δ- cadineno		EVANGELISTA et al., 2007; NUCCI et al., 2012; ALVES et al., 2013; 2014; NUCCI-MARTINS et al., 2015.
	Spatulenol		EVANGELISTA et al., 2007; DUTRA et al., 2009b; DUTRA et al., 2012; PINTO et al., 2013; VELOZO et al, 2013; ALVES et al., 2013; 2014.
	Iso-spatulenol		PINTO et al., 2013.

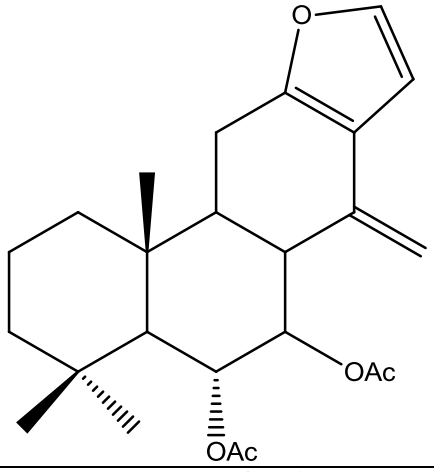
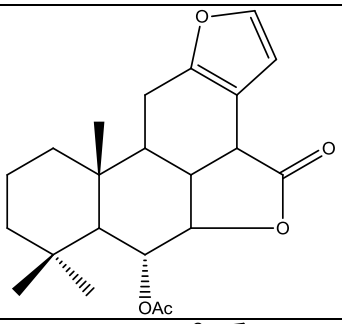
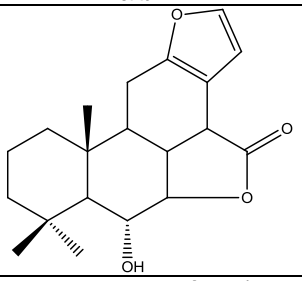
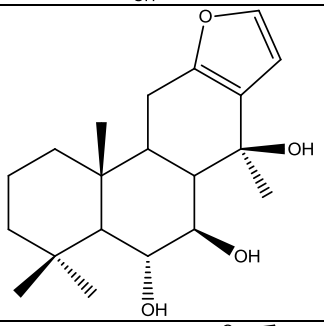
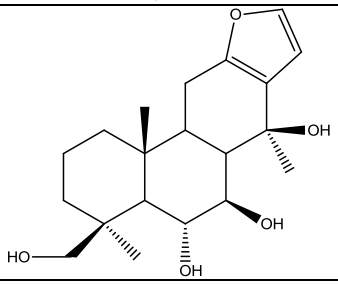
sesquiterpenos	Óxido de cariofileno		DUTRA et al., 2009b; DUTRA et al., 2012; PINTO et al., 2013; VELOZO et al, 2013; ALVES et al., 2013; 2014.
	globulol		ALVES et al., 2013; 2014.
	salvial-4(14)-en-1-ona		ALVES et al., 2013.
	Epóxido de humuleno II		ALVES et al., 2013; 2014.
	α-cadinol		ALVES et al., 2013; 2014.

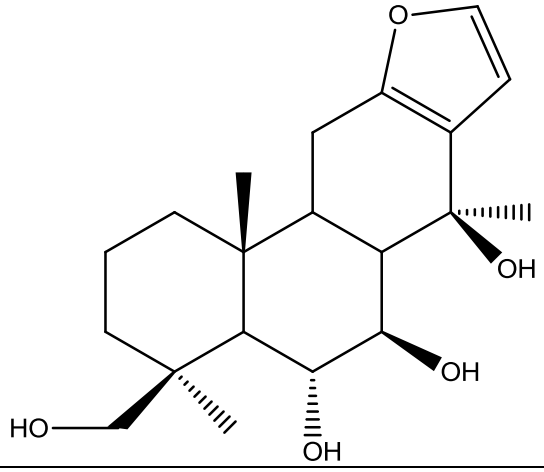
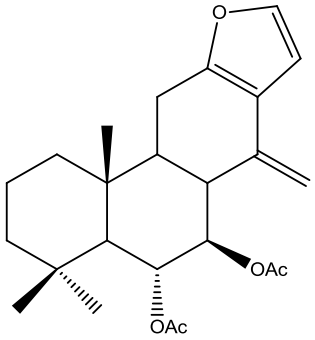
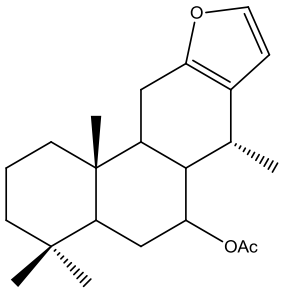
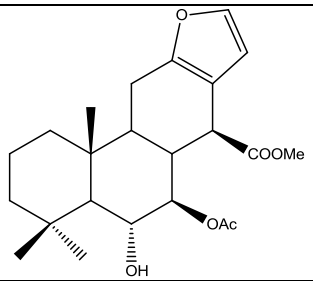
sesquiterpenos	14-hidroxi (E)-cariofileno		ALVES et al., 2013.
	(2E,6Z)-farnesol		ALVES et al., 2013.
	Acetate de (E)-nerolidila		ALVES et al., 2013.
	14-hidroxi-9-Epi-E-cariofileno		ALVES et al., 2014.
	Farnesol		PINTO et al., 2013; VELOZO et al, 2013; ALVES et al., 2014.

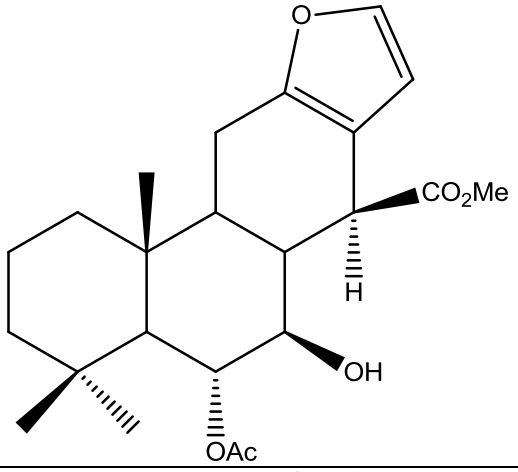
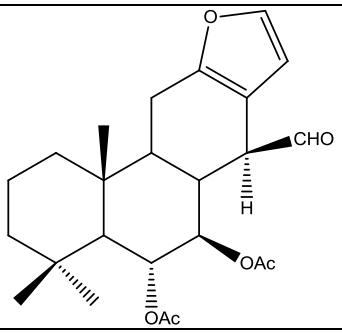
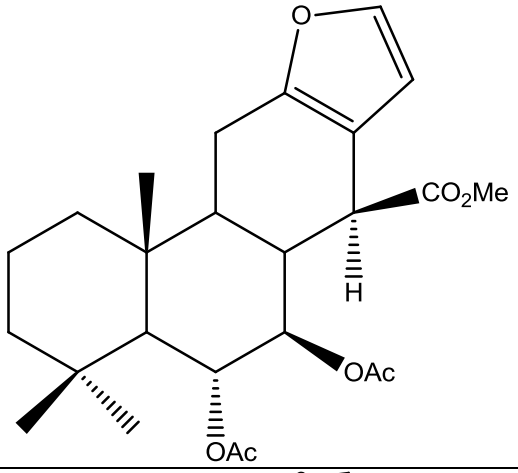
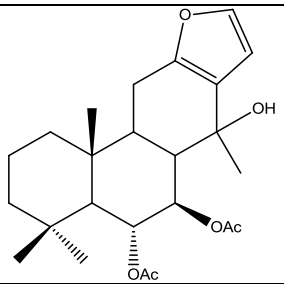
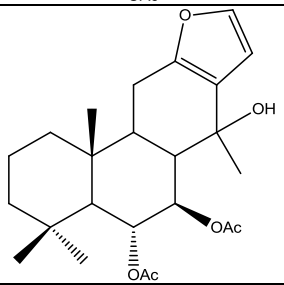
sesquiterpenos	Acetato de cis-farnesila		DUTRA et al., 2009b; DUTRA et al., 2012, PINTO et al., 2013; VELOZO et al, 2013.
	α - calacoreno		EVANGELISTA et al., 2007.
Diterpenos	14,15 dihidroxi-14,15-dihidrogeranilgeraniol		MORS et al., 1967; MAHAJAN; MONTEIRO, 1970, FASCIO et al, 1976
	Geranilgeraniol		MORS et al., 1967; SPINDOLA et al., 2010; OLIVEIRA et al., 2016
	16,17-Epoxigeranilgeraniol		FASCIO et al., 1970.
	6 α-hidroxivouacapano		ARRIAGA et al., 2000; PIMENTA et al., 2006.

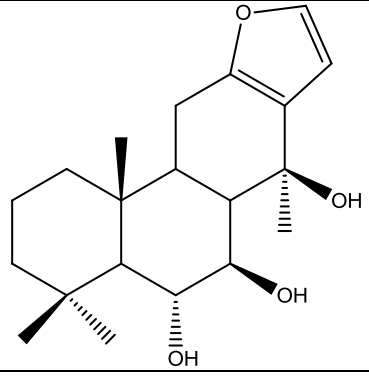
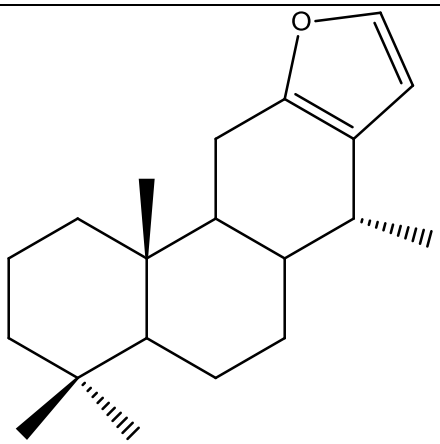
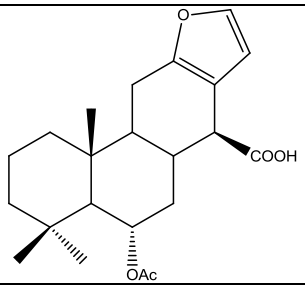
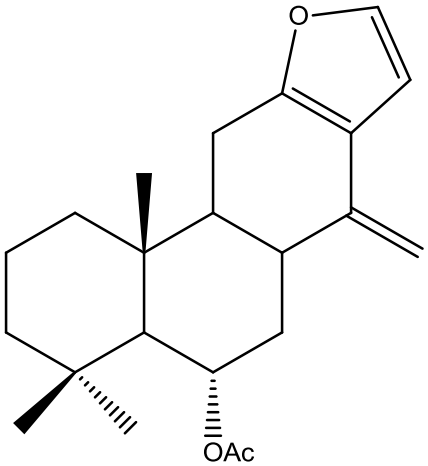
Diterpenos	6α,7β,14β,19-tetraidroxivouacapano		ARRIAGA et al., 2000.
	6α,7β-diidroxivouacapan-17β-oato de metila		MAHAJAN; MONTEIRO, 1973; FASCIO et al., 1976; ARRIAGA et al., 2000; COELHO et al., 2005; OMENA et al., 2006; SPINDOLA et al., 2009; 2010.PINTO et al., 2013; OLIVEIRA et al., 2016
	14, 15-epoxigeranilgeraniol		MORS et al., 1967; CABRAL et al., 2012.
	15-diidroxigeranilgeraniol		CABRAL et al., 2012.
	ácido 6α,7β-diidroxivouacapan-17-β oico		MAHAJAN; MONTEIRO, 1973; FASCIO et al., 1976; CAMPOS et al., 1994; DEMUNER; BARBOSA, 1996; DUARTE et al., 1996; BELINELO et al., 2001; OMENA et al., 2006; EUZEBIO et al., 2009; 2010; GALCERAN et al., 2011

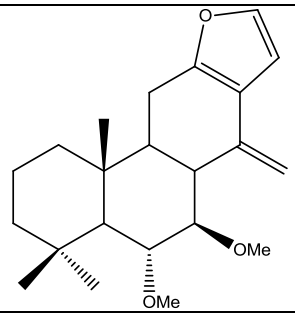
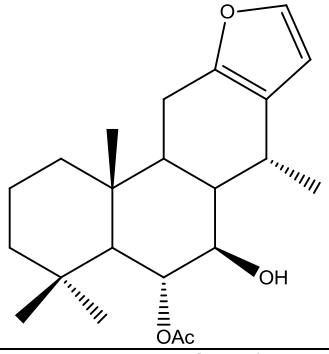
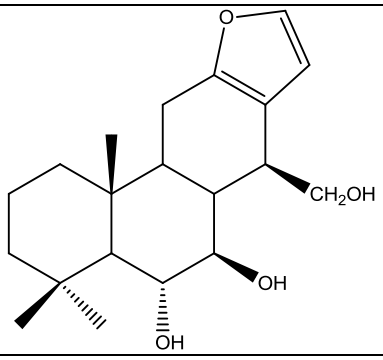
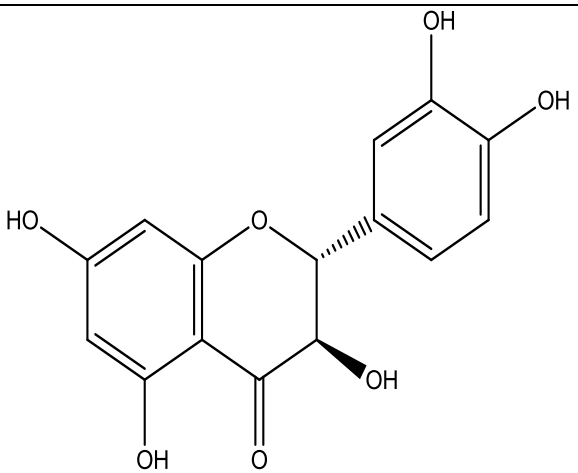
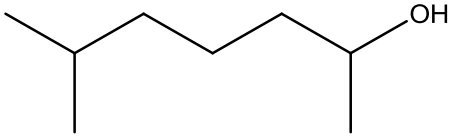
Diterpenos	ácido 6α, hidroxivouacapan-17-β oico		NUCCI et al., 2012; NUCCI-MARTINS et al., 2015
	6α-acetoxi-7β-hidroxivouacapan-17 β-oato de metila		FASCIO et al., 1976; CAMPOS et al., 1994; COELHO et al., 2005; PINTO et al., 2013; SERVAT et al., 2012
	6α-hidroxi-7β-acetoxivouacap-14(17)-eno		CAMPOS et al., 1994; COELHO et al., 2005; PINTO et al., 2013

Diterpenos	6α,7β-diacetoxi-vouacap-14 (17) eno		MAHAJAN; MONTEIRO, 1973
	6 α - acetoxivouacapano-17 β, 7 β – lactona		MAHAJAN; MONTEIRO, 1973; DI MASCIO et al., 1996
	6 α-hidroxivouacapano-7β-17 β-lactona		DEMUNER; BARBOSA, 1996; DI MASCIO et al., 1996; OMENA et al., 2006; NUCCI et al., 2012
	vouacapano-6α,7β-,17 β -triol		DEMUNER; BARBOSA, 1996
	vouacapano-6α,7β-,17β, 19-tetraol		DEMUNER; BARBOSA, 1996

Diterpenos	vouacapano-6α,7β-,14β,19-tetraol		ARRIAGA et al., 2000; VIEIRA et al., 2008
	6α,7β diacetoxivouacapano		MAHAJAN; MONTEIRO, 1973; FASCIO et al., 1976; SPINDOLA et al.; 2009; NUCCI et al., 2012; PINTO et al., 2013; NUCCI-MARTINS et al., 2015
	7β-acetoxivouacapano		MAHAJAN; MONTEIRO, 1973; FASCIO et al., 1976; COELHO et al., 2005; SPINDOLA et al.; 2009
	7 β -acetoxi-6 α -hidroxivouacapan-l7 β-oato de metila		MAHAJAN; MONTEIRO, 1973; SERVAT et al., 2012; PINTO et al., 2013

Diterpenos	6 α -acetoxi-7β-hidroxivouacapan-17β-oato		FASCIO et al., 1976
	6 α, 7β-diacetoxi-vouacapan-14 β -al		FASCIO et al., 1976
	6 α, 7β -diacetoxivouacapan-14 β -oato		FASCIO et al., 1976
	6α,7β -diacetoxi-12,16-diidro-12,14-diidro-16-oxovinhaticoato de metila		FASCIO et al., 1976
	6α,7β –diacetoxi-14-hidroxivinhaticoato de metila		FASCIO et al., 1976

Diterpenos	vouacapan-6 α, 7β, 14 β -triol		MAHAJAN; MONTEIRO, 1973; FASCIO et al., 1976
	vouacapano		PIMENTA et al., 2006; NUCCI et al., 2012; NUCCI-MARTINS et al., 2015
	6α-acetoxivouacapano		PIMENTA et al., 2006; NUCCI et al., 2012; NUCCI-MARTINS et al., 2015
	6α-acetoxivouacapano-17-eno		NUCCI et al., 2012; NUCCI-MARTINS et al., 2015

Diterpenos	6 α, 7β-dimetoxivouacapan-17-eno		NUCCI et al., 2012
	6 α –acetoxi-7β-hidroxivouacapano		SPINDOLA et al.; 2009; NUCCI et al., 2012; PINTO et al., 2013; NUCCI-MARTINS et al., 2015
	6α,7β-diidroxivouacapano17β-metileno-ol		SPINDOLA et al., 2009
Flavonóides	taxifolina		ARRIAGA et al., 2000
Outros	6-metil-2 heptanol		ALVES et al., 2013
	n-hexadecanol		ALVES et al., 2013

O primeiro estudo descrito com o gênero *Pterodon* demonstrou a atividade frente à *Schistossoma mansoni* de um óleo castanho viscoso extraído dos frutos de *P. pubescens* (MORS, PELLEGRINO, SANTOS FILHO; 1966). Em seguida, foi verificada a ação quimioprofilática contra este parasito, que causa a esquistossomose, através da inibição da penetração de cercárias na pele. Essa atividade foi atribuída ao diterpeno linear 14,15-epoxigeranilgeraniol, isolado a partir deste óleo dos frutos (MORS et al., 1967). Outros estudos posteriores comprovaram seus efeitos tóxicos e mutagênicos contra cercárias (PELLEGRINO, 1967; DOS SANTOS FILHO et al., 1972; FASCIO, et al.,1976; KATZ et al., 1993). O geranilgeraniol, isolado do extrato etanólico das sementes, apresentou ação, *in vitro,* contra formas tripomastigotas sanguíneas e amastigotas intracelulares de *Trypanosoma cruzi* em concentrações que não se mostraram tóxicas para o hospedeiro. Sugeriu-se que essa substância teria como principal alvo a mitocôndria celular do protozoário, levando-a ao autofagismo e morte do parasito (MENNA-BARRETO et al., 2008). O oleoresina dos frutos desta espécie não produziu genotoxicidade em células de mamíferos nos testes *in vivo* e *in vitro*, corroborando seu potencial como cercaricida natural (DIAS et al., 1995).

Desde então, os efeitos biológicos dos diferentes extratos, frações e substâncias isoladas de espécies do gênero *Pterodon* foram investigados em vários modelos experimentais *in vivo* e *in vitro*. Foi verificado o efeito frente à artrite reumatóide em um modelo de indução por colágeno em ratos, quando administradas pequenas doses diárias por via oral do extrato hidroalcoólico de sementes de *Pterodon sp.* (SABINO et al, 1999a). Sabino et al. (1999b) verificou que uma fração oleosa extraída das sementes de espécies do gênero não demonstrou sinais de citotoxicidade, mutagenicidade e toxicidade aguda, quando administradas em ratos saudáveis. Em outro estudo, o extrato hidroalcóolico de uma espécie deste gênero foi capaz de reduzir o índice de artrite sem qualquer alteração concomitante em seu exame hematológico e em vários parâmetros bioquímicos. Não foram detectados sinais de toxicidade subaguda em animais, sugerindo seu efeito na prevenção e tratamento da artrite reumatóide (COELHO et al., 2001; COELHO, SABINO, DALMAU; 2004).

Outro estudo demonstrou que o a subfração terpênica do extrato etanólico de sementes do gênero induziu, *in vitro*, a apoptose em células de leucemia mielóide crônica, se apresentando como um potente agente antitumoral (PEREIRA et al.,

2012). Já o extrato oleoso obtido a partir sementes apresentou atividade anti-edematogênica aguda, quando testado no edema de pata induzido por carragenina, e tópica, em ensaios de edema de orelha induzido pelo óleo de cróton. Esses modelos são comumente utilizados para avaliar a atividade anti-inflamatória de fármacos. As atividades observadas foram atribuídas aos diterpenos vouacapânicos, ácido 6α,7β-diidroxivouacapano-17β-óico, 6α-hidroxivouacapano-7β-17β-lactona e 6α,7β-dihidroxivouacapano-17β-oato de metila, além do geranilgeraniol e farnesol, também presentes no extrato (SILVA et al., 2004). A estabilidade acelerada de formulações a base de produtos oriundos de sementes de *Pterodon sp.* microencapsulados por "spray-drying" foi avaliada, comprovando que a atividade antinociceptiva do extrato bruto de sementes de *Pterodon* e da fração constituída pela mistura de isômeros 6α-hidroxi-7β-acetoxi-vouacapano-17β-oato de metila e 6α-acetoxi-7β-hidroxi-vouacapano-17β-oato de metila foram mantidas, mesmo após a microencapsulação (SERVAT et al., 2012). Essa seria uma alternativa útil para aumentar o tempo de prateleira da formulação (OLIVEIRA et al., 2012).

O efeito antinociceptivo e anti-inflamatório do gênero também foram avaliados, além da supressão da resposta de linfócitos B e T e produção de óxido nítrico. Resultados promissores foram observados, sugerindo seu potencial terapêutico no controle da resposta imune celular e humoral exacerbada em doenças autoimunes e processos inflamatórios crônicos (COELHO et al, 2005; CARDOSO et al., 2008). Em outro estudo, foi atribuída a atividade antinociceptiva ao geranilgeraniol e ao diterpeno 6α,7β-diidroxivouacapan-17 β-oato de metila, isolados do extrato bruto de sementes (SPINDOLA et al, 2011). A análise do extrato hidroetanólico também demostrou atividade antinociceptiva em modelos de dor agudo e crônico (NUCCI et al, 2012).

Algumas outras atividades avaliadas mostram a importância terapêutica do gênero *Pterodon*, tais como: ação antiproliferativa do extrato das sementes contra células de melanoma humano (VIEIRA et al, 2008) e efeito antitumoral *in vitro* usando modelo de desenvolvimento de células leucêmicas (PEREIRA et al., 2012); efeito imunomodulatório e tripanocida do extrato oleoso de sementes contra formas amastigotas e tripomastigotas do parasito (CARDOSO et al., 2008; MENNA-BARRETO et al, 2008), ação anti-inflamatória crônica da fração hexânica do extrato etanólico dos frutos reduzindo a migração de células pro-inflamatórias ao mesmo tempo que diminuiu os níveis de glicose, triglicerídeos e colesterol (HOSCHEID et

al., 2013), atividade antimicrobiana in vitro do óleo das sementes (NETO, 1976) e atividade anti-leishmania do extrato etanólico das folhas ao controlar a carga parasitária do protozoário nos macrófagos infectados de maneira dose-dependente (ARRAIS-SILVA et al., 2014).

Formulações inovadoras também já foram desenvolvidas a partir do gênero *Pterodon*. Nanoemulsões preparadas com óleo de frutos e PEG-40H apresentaram tamanho de partícula inferior a 200 nm e indícios de estabilidade cinética. Essa formulação pode ser potencialmente útil para diversas aplicações biológicas associadas a essas espécies (HOSCHEID et al., 2015).

3.1 *PTERODON EMARGINATUS* VOGEL

A espécie *Pterodon emarginatus* Vogel (Figura 1) (sinonímias: *Acosmium inornatum* (Mohlenbr.) Yakovlev, *Commilobium polygalaeflorus* Benth., *Commilobium pubescens* Benth., *Pterodon appariciοi* Pedersoli, *Pterodon polygalaeflorus* (Benth.) Benth., *Pterodon pubescens* (Benth.) Benth. e *Sweetia inornata* Mohlenbr. (THE PLANTLIST, 2016), conhecida popularmente como “sucupira”, “sucupira-branca” ou “faveira” é uma espécie arbórea, aromática, nativa da flora brasileira com distribuição geográfica que abrange as regiões Norte (Rondônia e Tocantins); Nordeste (Bahia, Ceará, Maranhão, Piauí); Centro-oeste (Distrito Federal, Goiás, Mato Grosso do Sul, Mato Grosso) e Sudeste (Minas Gerais, São Paulo) (LIMA; DE LIMA, 2016). Esta leguminosa, normalmente de porte médio, possui árvores que geralmente medem de 5-10 metros, podendo chegar a 20 metros de altura. Possui tronco ereto de 40-50 centímetros de diâmetro e são comuns tanto nas fitofisionomias do cerrado brasileiro como na Mata Atlântica (zonas de transição) (LORENZI, MATOS, 2002; MASCARO, TEIXEIRA, GILBERT, 2004).

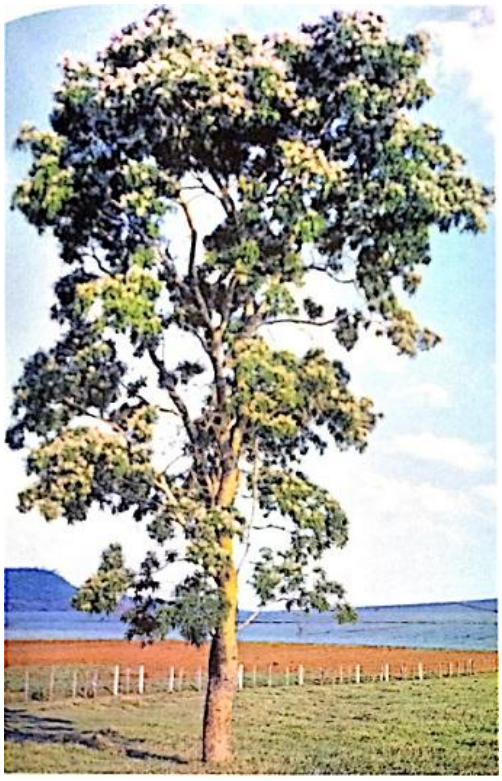


Figura 1: *P. emarginatus* aspecto geral da planta adulta
Fonte: LORENZI, 2014

As árvores de *P. emarginatus* podem frutificar em diferentes épocas do ano (dependendo da região de ocorrência), mas em geral florescem entre abril e maio, frutificam entre maio e junho e liberam os frutos entre junho e agosto (MASCARO, TEIXEIRA, GILBERT, 2004). Possui importância em reflorestamentos mistos, projetados para recuperação de vastas áreas, mesmo com o crescimento lento de suas árvores em solos de baixa fertilidade, pois têm tolerância à luz direta (OLIVEIRA, PAIVA, 2005; HANSEN, HARAGUCHI, ALONSO, 2010; DANIEL et al., 2013). Árvores de sucupira são consideradas promissoras em programas de pecuária bovina sustentável. Sua matéria-orgânica permite uma melhoria do solo e aumenta o valor nutritivo do capim-braquiária utilizado na alimentação do gado (PEZZONI et al., 2011). Suas folhas são opostas pinadas com 5-7 folículos ovalados, glabros de 6-12 centimetros de comprimento por 3-4 centímetros de largura e as inflorescências com panículas terminais e axilares amplas possuem flores amarelo-ouro (LORENZI, 2014) (Figura 2).

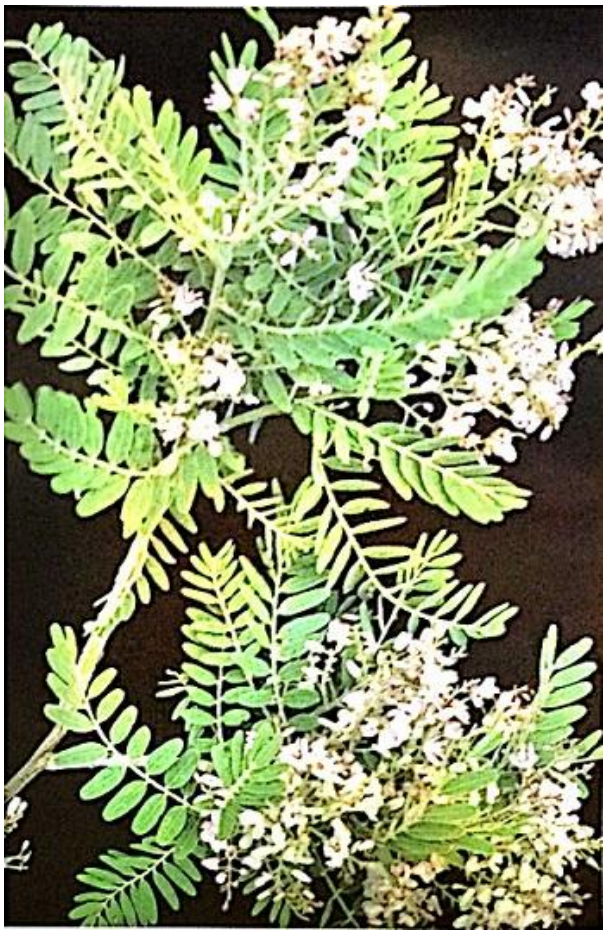


Figura 2: Detalhe da folha e inflorescência de *P. emarginatus* Vogel
Fonte: LORENZI, 2014.

O nome *Pterodon emarginatus* vem do grego-latino e significa “sementes com asas que circundam sua periferia”. Os frutos da espécie, tipo vagens indeiscentes, caem espontaneamente e se dispersam por longas distâncias devido ao seu formato circular que permite um vôo estável com ventos unidirecionais (MASCARO, TEIXEIRA, GILBERT, 2004) (Figura 3 e Figura 4). Cada fruto possui uma ou mais raramente, duas sementes imaturas, onde se observa o acúmulo de compostos fenólicos em pequenos vacúolos na exotesta das células. A elevada quantidade desses compostos fenólicos conferem as sementes alta dureza, baixa permeabilidade a água e proteção ao ataque de patógenos. As sementes alcançam suas dimensões finais em tamanho real de cerca de 4 - 5 cm, onde se observam embriões com cotilédones carnosos, ricos em lipídios e proteínas acumuladas e grãos de amido raros (OLIVEIRA, PAIVA, 2005; HANSEN et al., 2010, LORENZI, 2014).

Figura 3: Frutos de sucupira após queda espontânea mostrando formato circular, o que facilita a sua propagação.
Fonte: Próprio autor

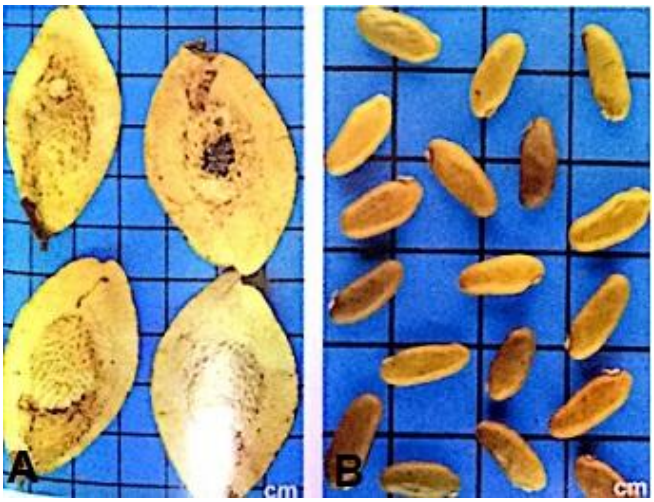


Figura 4: Frutos (A) e Sementes (B) de *P. emarginatus*.
Fonte: LORENZI, 2014

Sua madeira, moderadamente pesada, é resistente e possui textura média, grã-irregular sendo resistente ao ataque de organismos xilófagos (Figura 5) (LORENZI, 2014). A madeira da casca é utilizada em infusões, enquanto as raízes e sementes são comercializadas nos mercados populares devido à ampla atividade farmacológica (SABINO et al, 1999a; EVANGELISTA et al., 2007). Estudos etnobotânicos mostram que as preparações dos frutos da espécie vegetal *P. emarginatus* têm demonstrado eficácia significativa quanto ao seu uso em medicina popular. Eles são usados pela população em macerações hidroalcoólicas para o tratamento de afecções da laringe, disfunções respiratória, como estimulante do

apetite e fortificante, principalmente em crianças. Além disso, são bastante utilizados para o tratamento de reumatismo, problemas ósseos, e até mesmo para fins depurativos (MASCARO, TEIXEIRA, GILBERT, 2004; DUTRA et al., 2009b).

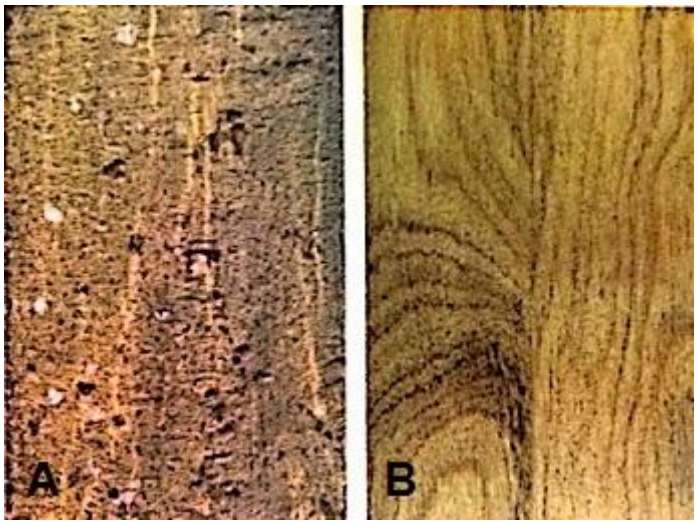


Figura 5: Detalhe do tronco (A) e madeira (B) de *P. emarginatus*.
Fonte: LORENZI, 2014.

3.1.1 Fitoquímica

O oleorresina viscoso de coloração âmbar obtido dos frutos é o principal derivado de *P. emarginatus* (Figura 6). Ele é rico em terpenóides, destacando-se o beta-cariofileno, geranilgeraniol e diterpenos com esqueleto vouacapânico. Mahajan e Monteiro (1973) identificaram o 6α,7β-diacetoxivouacapano, 7β-acetoxivouacapano, , ácido 6α,7β-dihidroxivouacapan-17β-óico, 6α,7β-dihidroxivouacapan-l7β-oato de metila, 7β-acetoxi-6α-hidroxivouacapan-l7β-oato de metila, 6α-acetoxivouacapano-17β,7β-lactona, vouacapano-6a,7β,14β-triol e 6α,7β-diacetoxi-vouacapano. Também foram identificados os diterpenos 6α-hidroxivouacapano (ARRIAGA et al., 2000), 6α,7β-acetoxivouacapano-14(17)-eno (CAMPOS et al., 1994) e os furanoditerpenos vouacapano-6α,7β,17β-triol e vouacapano-6α,7β,17β,19-tetraol (DEMUNER, BARBOSA, 1996) (Tabela 5).

Figura 6: Óleorresina retirado dos frutos de *Pterodon emarginatus* Vogel.
Fonte: Próprio autor

Estudos fitoquímicos realizados com o óleo essencial dos frutos indicaram a presença dos sesquiterpenos β-cariofileno, γ–muuroleno, espatulenol e α-humuleno como substâncias majoritárias. Alem desses, foram identificados outros terpenos aromáticos voláteis e fenilpropanóides (ALVES et al., 2013; DUTRA et al., 2012; ALBERTI et al., 2014).

Estudos fitoquímicos utilizando outras partes de *P. emarginatus* também foram realizados com o intuito de identificar seus constituintes. Nas cascas, os testes fitoquímicos realizados evidenciaram a presença de flavonóides, heterosídeos saponínicos, resinas e traços de esteróides e triterpenóides (BUSTAMANTE et al. 2010). A composição química do óleo essencial de suas folhas mostrou a presença de hidrocarbonetos sesquiterpênicos, sendo os majoritários o γ-muuroleno e o biciclogermacreno (SANTOS et al., 2010). O estudo fitoquímico do extrato etanólico das folhas demonstrou a presença de uma mistura dos fitoesteróides β-sitosterol e estigmasterol, que possuem ampla ocorrência nos vegetais (SANTOS et al., 2010), e também foi sugerida a presença das flavonas *di*-*C*-glicosilflavona, C,*O*-glicosilflavonas e luteina-7-*O*-rutinosideo como constituintes principais do extrato hidroetanólico, além de saponinas, sendo a composição bastante diferente da

demonstrada nos óleos e extratos de sementes e frutos (NEGRI, MATTEI, MENDES, 2014).

3.1.2 Atividade biológica

No Brasil, dentre as diversas plantas com potencial terapêutico, a espécie *P. emarginatus* destaca-se por sua versatilidade de atividades biológicas. Seu uso é bastante difundido na medicina tradicional e uma das principais indicações populares para essa espécie é a utilização da infusão dos seus frutos para tratamento de processos inflamatórios. Diversos estudos foram realizados a fim de comprovar esse efeito. O extrato bruto hexânico dos seus frutos foi usado para avaliação do efeito anti-inflamatório em modelos animais, comprovando sua atividade e sugerindo que o edema gerado está relacionado à liberação de prostaglandinas e de outros mediadores do sistema de cininas (CARVALHO et al., 1998; PINTO et al., 2013). Os diterpenos 6α-hidroxivouacapano-7β-17β-lactona e 6α,7β-diiidroxivouacapano-17β-oato metil ester, presentes no oleorresina de sementes foram previamente descritos como substâncias associadas às atividades anti-inflamatórias desta espécies (NUNAN et al., 1982). Ao ácido 6α,7β-diiidroxivouacapano-17β-óico, um diterpeno de esqueleto vouacapânico presente no extrato hexânico dos frutos, foi atribuída a atividade anti-inflamatória e efeitos analgésicos periféricos (DUARTE, FERREIRA-ALVES, NAKAMURA-KRAIG, 1992; DUARTE et al., 1996; GALCERAN et al., 2011), além de ação broncodilatadora (LEAL et al., 2000). À ação antioxidante do óleo essencial das sementes, sugeriu-se que sesquiterpenos, como biciclogermacreno, *trans*-cariofileno e α-humuleno são os possíveis responsáveis por essa atividade (DUTRA et al., 2009a; DONATI et al, 2014). Um estudo realizado em ratos demonstrou atividades antiulcerogênica e anti-inflamatória acentuadas do óleo essencial de sementes *P. emarginatus.* Elas foram consideradas, ao menos parcialmente, uma conseqüência da modulação de óxido nítrico e de interleucina-1, sugerindo que o *P. emarginatus* ou seus componentes podem representar uma nova opção terapêutica para o tratamento de úlceras gástricas e de doenças inflamatórias (DUTRA et al., 2009b). Foi relatada a ação protetora do extrato bruto hexânico dos frutos contra o estresse oxidativo e nitrosativo induzida pelo exercício agudo em ratos, sugerindo um efeito antioxidante, associado também a processos inflamatórios (PAULA et al., 2005). A atividade

cicatrizante na pele de coelhos foi observada após o uso tópico de cremes contendo óleo essencial em diferentes concentrações (5% e 10%) e fração hexânica a 10%, ambos obtidos das sementes de *P. emarginatus.* Houve significante diminuição no número de células inflamatórias, aumento no número de fibroblastos e vasos sanguíneos, demonstrando a eficácia do fitoterápico no processo de reparação tecidual (DUTRA et al., 2009c). Microemulsões obtida a partir do oleorresina de frutos de sucupira, tensoativos e água também foram avaliadas em teste de edema de orelha em camundongos induzido pelo óleo de cróton. O efeito anti-inflamatório do óleo foi mantido após a incorporação do mesmo nesse sistema, sendo considerada uma alternativa otimizada e mais estável para o uso como anti-inflamatório (PASCOA et al., 2015).

Durante a avaliação do potencial angiogênico do óleo das favas de *P. emarginatus* na concentração de 1g/ml, utilizando-se um ensaio de membrana corioalantóica de embrião de aves, foi demonstrada a estimulação do crescimento de novos vasos sanguíneos, explicada provavelmente pela ativação da resposta inflamatória. A neovascularização está geralmente associada a condições em que ocorrem as diferentes fases da infiltração de células inflamatórias e é um fator importante numa variedade de processos fisiológicos e patológicos (ARAUJO et al., 2015). Em outro estudo com aves, o oleorresina de *P. emarginatus* adicionado na dieta de frangos, aumentou o teor de proteína bruta e reduziu o teor de lipídios totais na carne dos animais, além de apresentar um efeito pró-oxidante (LIMA et al., 2015).

O óleo essencial extraído dos frutos *P. emarginatus* apresentou atividade farmacológica contra bronquite, amigdalites e outras disfunções respiratórias (ARRIAGA et al., 2000). Também reduziu a atividade anti-inflamatória aguda ao diminuir a migração leucocitária, a concentração de proteínas no exsudato e a vasodilatação *in vivo* (VELOZO et al., 2013). Em ensaios biológicos, o óleo essencial das sementes de *P. emarginatus* causou inibição no crescimento de *Staphylococcus aureus*, demonstrando atividade bactericida (DUTRA et al., 2009a). No mesmo estudo, foi observada a atividade leishmanicida do óleo essencial frente às formas promastigotas de *Leishmania.* A administraçao de óleo essencial obtido de sementes de *P. emarginatus*, por via oral (100 mg/kg) em camundongos, mostrou-se potencialmente útil para atenuar os efeitos de encefaloielite autoimune. Portanto, foi considerada promissora para o tratamento de condições imuno-inflamatórias (ALBERTI et al., 2014). Além da atividade citotóxica observada contra linhagens de

células tumorais e células mononucleares de sangue periférico humano (DUTRA et al., 2012)

Estudos realizados com as folhas da espécie mostraram atividades biológicas associadas a esse órgão vegetal. O óleo essencial de folhas apresentou atividade frente a bactérias Gram-positivas, mas mostrou-se inativo frente isolados clínicos de *Candida* (SANTOS et al., 2010). O extrato hidroetanólico demostrou atividade antinociceptiva em ensaios *in vivo*, sugerindo que o efeito estava relacionado à presença de flavonas e saponinas e não de terpenóides, como sugerido anteriormente em testes com óleo e extrato de sementes (NEGRI, MATTEI, MENDES et al., 2013).

O extrato etanólico da casca de *P. emarginatus* demostrou atividades analgésica e anti-inflamatória em testes com camundongos (MORAES et al., 2009). Foram isolados os triterpenos lupeol e betulina desse extrato, sugerindo que estes são ao menos parcialmente responsáveis pelas atividades supracitadas (MORAES et al., 2012), além de apresentar atividade antimicrobiana contra bactérias Gram-positivas, Gram-negativas e *Candida* (BUSTAMANTE et al., 2010). O extrato metanólico do caule da espécie demonstrou atividade alelopática sobre o desenvolvimento da raiz, parte aérea, assim como sobre a germinação do capim-colonião (*Panicum maximum*) (HERNÁNDEZ-TERRONES et al., 2007).

Extratos e frações de baixa polaridade com constituição similar ao oleorresina, além do óleo essencial e diterpenos isolados de *P. emarginatus* foram eficazes agentes larvicidas frente a *Aedes aegypti* (OMENA et al., 2006; PIMENTA et al., 2006). Nanoemulsões desenvolvidas a partir do óleo fixo da espécie também mostraram, em baixas concentrações, alta mortalidade frente a larvas do mosquito. Nas mesmas concentrações, quando administradas por via oral em camundongos, não se mostraram tóxicas, sendo portando uma boa alternativa para o combate de focos domésticos de desenvolvimento de *Aedes* (OLIVEIRA et al., 2016). Esses dados corroboram o potencial dessa espécie para compor práticas integradas de controle de mosquitos que são vetores de doenças tropicais negligenciadas tais como Dengue, Chikungunya e Zika.

Apesar do uso difundido de plantas para fins terapêuticos, são necessários estudos para avaliar o risco à saúde humana. Alguns metabólitos de plantas são potencialmente tóxicos, podendo ser citotóxicos ou genotóxicos. O consumo de alta quantidade de folhas verdes de *P. emarginatus* por bovinos e ovinos (6g/kg e 20g/kg

respectivamente) mostrou sinais de intoxicação e alta hepatotoxicidade nos animais (CRUZ et al., 2012). Entretanto , quando Assunção et al.(2015) avaliaram as atividades genotóxicas, citotóxicas e antimutagênicas *in vivo* do óleo fixo da semente de *P. emarginatus*, foi demostrado que nas concentrações testadas, o óleo não causou efeito clastogênico aos animais e nem danos sobre eritrócitos na medula óssea de camundongos. Portanto, foram corroborados os resultados de outros estudos que envolveram o gênero *Pterodon,* indicando que doses do óleo não resultaram alterações macroscópicas teciduais em animais (SABINO et al, 1999b; COELHO et al, 2004; CARDOSO et al., 2008) e que a análise da metástase de células de medula óssea de ratos não resultou em aumento significativo de alterações cromossômicas (DIAS et al., 1995). Os possíveis efeitos tóxicos da sucupira branca também foram testados ao avaliar alterações hematológicas em ratos e hemostáticas em coelhos. O tempo de protrombina, tempo de tromboplastina parcial ativada e a agregação plaquetária foram analisados, após os animais receberem a dose de 0,5ml/kg do extrato oleoso das sementes durante 30 dias consecutivos. Findo o período de tempo, não foram observadas alterações nos parâmetros analisados (PEDRAZZI et al., 1999). Além disso, foi relatado o efeito anticarcinogênico, atribuído a substâncias presentes no extrato alcoólico dos frutos, como derivados lactonas e ácido 6α,7β-diidroxivouacapano-17β-óico (EUZEBIO et al., 2009; 2010).

Formulações inovadoras também já foram desenvolvidas a partir de *P. emarginatus*. O nanocompósito obtido do oleorresina de sementes da espécie mostrou indícios de possuir propriedades semicondutoras (SILVEIRA et al., 2012). Microcápsulas preparadas com óleo essencial de frutos *P. emarginatus* foram capazes de prevenir a perda de substâncias voláteis, incluindo o sesquiterpeno *beta*-cariofileno. Essa pode ser uma estratégia propícia para evitar a degradação e/ou evaporação das substâncias bioativas presente nesse derivado de droga vegetal (ALVES et al., 2014). Seu óleo em diferentes concentrações foi capaz de gerar meso-estruturas de sílica com diferentes diâmetros de poro, além de possibilitar variados tipos de estruturas, como hexagonal, cúbica ou lamelar. Portanto, foi considerado promissor como agente de baixa toxidade ambiental para indústria química (BATISTA et al., 2012).

REFERÊNCIAS

ALBERTI, T. B.; MARCON R.; BICCA, M. A.; RAPOSO, N. R. B.; CALIXTO, J. B.; DUTRA, R. C. Essential oil from *Pterodon emarginatus* seeds ameliorates experimental autoimmune encephalomyelitis by modulating Th1/Treg cell balance. **Journal of Ethnopharmacology,** v. 155, p. 485-494, 2014.

ALVES, S. F.; BORGES, L. L.; DE PAULA, J. A. M.; VIEIRA, R. F.; FERRI, P. H; COUTO, R. O.; PAULA, J. R.; BARA, M . T. F. Chemical variability of the essential oils from fruits of *Pterodon emarginatus* in the Brazilian Cerrado. **Brazilian Journal of Pharmacognosy,** v. 23, p. 224-229, 2013.

ALVES, S.F.; BORGES, L.L.; SANTOS, T.O.; PAULA, J.R.; CONCEICAO, E.C.; BARA, M.T.F. Microencapsulation of Essential Oil from Fruits of *Pterodon emarginatus* Using Gum Arabic and Maltodextrin as Wall Materials: Composition and Stability. **Drying Technology**, v. 32, p. 96–105, 2014.

ARAUJO, L. A.; ASSUNÇÃO, L. A.; SILVA-JUNIOR, N. J.; LEMES, S. R.; MELO-REIS, P. R. Angiogenic activity of sucupira (*Pterodon emarginatus*) oil. **Scientia Medica** v., 25 ID20351, DOI: http://dx.doi.org/10.15448/1980-6108.2015.2.20351 , 2015.

ARRAIS-SILVA, W.W.; NUNES, P.S.G.; CARVALHO, J.D.; BRUNE, M.W.; ARRAIS-LIMA, C.; BATALINI C. 2014. Preliminary phytochemical and antileishmanial studies of the ethanolic extracts of *Pterodon pudescens*. **Revista Brasileira de Plantas Medicinais**, v. 16, p. 561-565, 2014.

ARRIAGA, A.M.C.; CASTRO, M.A.B.; SILVEIRA, E.R.; BRAZ-FILHO, R. Further diterpenoids isolated from *Pterodon polygalaeflorus*. **Journal of Brazilian Chemical Society**, v.11, p. 187–190, 2000.

ASSUNÇÃO, L. A.; LEMES, S. R.; ARAÚJO, L. A.; COSTA, C. R.; MAGALHÃES, L. G.; MOURA, K. K.; MELO-REIS, P.R. Assessment of the cytotoxic, genotoxic,and antigenotoxic activities of sucupira oil (*Pterodon emarginatus*). **Genetics and molecular research,** v. 14, p. 6323-6329, 2015.

BATISTA, J. N. M.; DE FARIA, E. H.; CALEFI, P. S.; CIUFFI, K. J.; NASSAR, E. J.; CUNHA, W. R.; CAIUT, J. M. A.; ROCLA, L. A. Controlling silicate meso-structures using sucupira oil as a new swelling agent. **Applied Surface Science**; v. 258, n.12, p. 5111-5116, 2012.

BELINELO, V. J.; PILÓ-VELOSO,D.; BORGES, E. E. L.; ALVES, D. L. F.; REIS,G. T. Síntese e atividade fitotóxica de amidas derivadas do ácido 6alfa,7beta-di-hidroxivouacapan-17beta-óico. **Eclética Química**, *26*, 25-39, 2001. https://dx.doi.org/10.1590/S0100-46702001000100002

BRAZ FILHO, R.; GOTTLIEB, O.R.; ASSUMPÇÃO, R.M.V. The isoflavones of *Pterodon pubescens*. **Phytochemistry**, v. 10, p. 2835-2836, 1971.

BUSTAMANTE, K.G.L.; LIMA, A.D.F.; SOARES, M.L.; FIUZA, T.S.; TRESVENZOL, L.M.F.; BARA, M.T.F.; PIMENTA, F.C.; PAULA, J.R. Avaliação da atividade antimicrobiana do extrato etanólico bruto da casca da sucupira branca (Pterodon emarginatus Vogel) – Fabaceae. **Revista Brasileira de Plantas Medicinais**, v.12, 341-345, 2010.

CABRAL, E. C.; SEVART, L.; SPINDOLA, H. M.; COELHO, M. B.; SOUSA, I. M. O.; QUEIROZ, N. C. A.; FOGLIO, M. A.; EBERLIN, M. N.; RIVEROS, J. M. *Pterodon pubescens* Oil: Characterisation, Certification of Origin and Quality Control via Mass Spectrometry Fingerprinting Analysis. **Phytochemical Analysis**, v. 24, p. 184–192, 2012.

CAMPOS, A. M.; CRAVEIRO, A. A.; TEIXEIRA, T. C. Óleo essencial das sementes de Pterodon polygalaeflorus. In: **Resumos da Reunião da Sociedade Brasileira de Química**, PN-004, 1990.

CAMPOS, A.M.; SILVEIRA, E.R.; BRAZ-FILHO, R.; TEIXEIRA, T.C. Diterpenoids from *Pterodon polygalaeflorus*. **Phytochemistry**, v. 36, p. 403-406, 1994.

CARDOSO, C. C.; PINTO, A. C.; MARQUES, P. R.; GAYER, C. R. M.; AFEL, M. I. R.; COELHO, M. G. P.; SABINO, K. C. C. Suppression of T and B Cell Responses by *Pterodon pubescenses* Seeds Ethanolic Extract. **Pakistan Journal of Biological Sciences**, v.11, p.2308-2313, 2008.

CARVALHO, J.C.T.; SERTIE, J.A.A.; BARBOSA, M.V.J.; PATRÍCIO, K.C.M.; CAPUTO, L.R.G.; SARTI, S.J.; FERREIRA, L.P.; BASTOS, J.K. Anti-inflammatory activity of the crude extract from the fruits of *Pterodon emarginatus* Vog. **Journal of Ethnopharmacology**, v.64, p.127–133, 1998.

COELHO, M. G. P.; MARQUES, P. R.; GAYER, C. R. M.; VAZ, L. C. A., NOGUEIRA NETO J. F.; SABINO, K. C. C. Subacute toxicity evaluation of a hydroalcoholic extract of *Pterodon pubescens* seeds in mice with collagen-induced arthritis. **Journal of Ethnopharmacology,** v. 77 p. 159–164, 2001.

COELHO, M. G. P.; SABINO, K. C. C.; DALMAU, S. R. Immunomodulatory effects of sucupira (*Pterodon pubescens*) seed infusion oncollagen-induced arthritis. **Clinical and Experimental Rheumatology**, v.22, p. 213–218, 2004.

COELHO, L.P.; REIS, P.A.; DE CASTRO, F.L.; GAYER, C.R.M.; LOPES, S.C.; SILVA, M. C.S.; SABINO, K.C.C.; TODESCHINI, A. R.; COELHO, M.G.P. Antinociceptive properties of ethanolic extract and fractions of *Pterodon pubescens* Benth. Seeds. **Journal of Ethnopharmacology**, v. 98, p. 109–116, 2005.

CRUZ, R. A. S. ; DE OLIVEIRA, L. P. ; CALDEIRA, F. H. B. ; MENDONÇA, F. S. ; BACHA, F. B. ; POTT, A. ; LEMOS, R. A. A. ; COLODEL, E. M. Intoxicação espontânea e experimental por Pterodon emarginatus (Fabaceae Faboideae) em bovinos e experimental em ovinos. **Pesquisa Veterinária Brasileira,** v.32, p.1087-1094, 2012.

DANIEL, O.; PEZZONI, T.; NOGUEIRA, I. M. B.; CREMON, T.; VITORINO, A. C. T. Allometric relations on *Pterodon emarginatus* vogel trees in a silvopastoral system. **Cerne**, v.19, p. 141-149, 2013.

DEMUNER, A.J., BARBOSA, L.C.A. Structure and Plant Growth Regulatory Activity of New Diterpenes from Pterodon polygalaeflorus. **Journal of Natural Products,** v. 59, p. 770-772, 1996.

DIAS, F. L.; TAKANASHI, C. S.; SAKAMOTO-HOJO, E. T.; VICHNEWSKI, W.; SARTI, S. J.Genotoxicity of the natural cercaricides “sucupira” oil and eremanthine in mammalian cells in vitro and in vivo. **Environmental and Molecular Mutagenesis**, v.26, p.338-344, 1995.

DI MASCIO, P., A.; MEDEIROS M., H., G.; SIES., H.; BERTOLOTTI, S.; BRASLAVSKY, S. E.; VELOSO D., P.; SALES, H., L., N., B.; MAGALHÃES, E.; BRAZ-FILHO, R.; BECHARA, E. J. H. Quenching of singlet molecular oxygen by natural furan diterpenes. **Journal of Photochemistry and Phetobiology B: Biology,** v. 38, p. 169-173, 1996.

DONATI, M.; MONDIN, A.; CHEN, Z.; MIRANDA, F. M.; NASCIMENTO JUNIOR, B.B.; SCHIRATO, G.; PASTORE, P.; FROLDI, G. Radical scavenging and antimicrobial activities of Croton zehntner, Pterodon emarginatus and Schinopsis brasiliensis essential oils and their major constituents: estragole, trans-anethole, β-caryophyllene and myrcene. **Natural Product Research: Formerly Natural Product Letters**, DOI: 10.1080/14786419.2014.964709, 2014.

DOS SANTOS-FILHO, D.; VICHNEWSKI, W.; BAKER, P. M.; GILBERT, B. Prophilaxys of Schistosomiasis: Diterpenes from *Pterodon pubescences* Benth. **Anais da Academia Brasileira de Ciências,** v. 44, p. 45-49, 1972.

DUARTE, I. D.G; FERREIRA-ALVES, D.L.; NAKAMURA-KRAIG, M. Possible participation of endogenous opioid peptides on the mechanism involved in analgesia induced by vouacapan. **Life Sciences**, v. 50, p. 891-897, 1992.

DUARTE, I.D.G.; ALVES, D. L. F., VELOSO, D.P., NAKAMURA-CRAIG, M. Evidence of the involvement of biogenic amines the antinociceptive effect of a vouacapan extracted from Pterodon polygalaeflorus Benth. **Journal of Ethnopharmacology**, v. 55, p. 13-18, 1996.

DUTRA, R.C.; LEITE, M.N.; BARBOSA, N.R. Quantification of Phenolic Constituents and Antioxidant Activity of *Pterodon emarginatus* Vogel Seeds. **International Journal of Molecular Science***,* v. 9*,* p. 606-614, 2008.

DUTRA, R.C.; BRAGA, F.G.; COIMBRA, E.S.; SILVA, A.D.; BARBOSA, N.R. Atividades antimicrobiana e leishmanicida das sementes de *Pterodon emarginatus* Vogel. **Brazilian Journal of Pharmacognosy**, v. 19, p. 429- 435, 2009a.

DUTRA, R.C.; FAVA, M.B.; ALVES, C.C.S.; FERREIRA, A.P.; BARBOSA, N.R. Antiulcerogenic and antiinflammatory activities of the essential oil from *Pterodon emarginatus* seeds. **Journal of Pharmacy And Pharmacology**, v. 61, p. 243-250, 2009b.

DUTRA, R.C.; PITTELLA, F.; FERREIRA, A. S.; LARCHER, P.; Rogério E. FARIAS, R. E.; BARBOSA, N.R. Efeito Cicatrizante das Sementes de *Pterodon emarginatus* Vogel em Modelos de Úlceras Dérmicas Experimentais em Coelhos. **Latin American Journal of Pharmacy**, v. 28, p. 375-82, 2009c.

DUTRA, R. C.; PITTELLA, F.; DITTZ, D.; MARCON, R.; PIMENTA, D. S.; LOPES, M. T. P.; RAPOSO, N. R. B. Chemical composition and cytotoxicity activity of the essential oil of *Pterodon emarginatus*. **Brazilian Journal of Pharmacognosy**, v. 22, p. 971-978, 2012.

EUZEBIO, F. P. G.; SANTOS, F. J. L.; PILO-VELOSO, D.; RUIZ, A. L. T. G.; CARVALHO, J. E.; FERREIRA-ALVES, D. L.; DE FATIMA, A. Effect of 6α,7β-dihydroxyvouacapan-17β-oic acid and its lactone derivatives on the growth of human cancer cells. **Bioorganic Chemistry,** v. 37, p. 96-100, 2009.

EUZÉBIO, F. P. G.; SANTOS, F. J. L.; PILÓ-VELOSO, D.; ALCÂNTARA, A. F. C.; RUIZ, A. L. T. G.; CARVALHO, J. E.; FOGLIO, M. A.; FERREIRA-ALVES, D. L.; FATIMA, A. Synthesis, antiproliferative activity in cancer cells and theoretical studies of novel 6a,7b-dihydroxyvouacapan-17b-oic acid Mannich base derivatives. **Bioorganic & Medicinal Chemistry**, v. 18, p. 8171-8177, 2010.

EVANGELISTA, G. L.; COELHO-DE-SOUZA, A. N.; SANTOS, C. F.; LEAL-CARDOSO, J. H.; LOPES, E. A. B.; DOS SANTOS, M.V.; LAHLOU, S.; MAGALHÃES, P. J. C. Essential oil of *Pterodon polygalaeflorus* inhibits electromechanical coupling on rat isolated trachea. **Journal of Ethnopharmacology**, v. 109, p. 515-522, 2007.

FASCIO, M.; GILBERT, B.; MORS, W.B.; NISHIDA, T. Two new diterpenes from Pterodon pubescens. **Anais da Academia Brasileira de Ciências,** v.42, p.97-101, 1970.

FASCIO, M.; MORS, H.B.; GILBERT, B.; MAHAJAN, J.R.; MONTEIRO, M.B. Diterpenoid furans from *Pterodon* species. **Phytochemistry**, v. 15, p. 201-203, 1976.

GALCERAN, C. B.; SERTIE, J. A. A.; LIMA, C. S.; CARVALHO, J. C. T. Anti-inflammatory and analgesic effects of 6a,7b–dihydroxy-vouacapan-17b-oic acid isolated from *Pterodon emarginatus* Vog. Fruits**. Inflammopharmacology**, v. 19, p. 139–143, 2011.

GALINA, E.; GOTTLIEB, O.R. Chemistry of Brazilian Leguminosae. XLVI Isoflavones from Pterodon appariciori. **Phytochemistry**, v.13, n.11, p.2593-2595, 1974.

HANSEN, D.; HARAGUCHI, M.; ALONSO, A. Pharmaceutical properties of 'sucupira' (*Pterodon* spp.). **Brazilian Journal of Pharmaceutical Sciences**, v. 46, 607-616, 2010.

HERNÁNDEZ-TERRONES,M.G.; MORAIS,S.A.L.; FERREIRA,S.; SANTOS,D.Q.; NASCIMENTO, E.A.; CHANG,R. Phytochemistry and Allelophatic Studyof *Pterodon emarginatus* Stem Extract. **Planta Daninha**, v.25, n.4, p.755-762, 2007.

HOSCHEID, J.; BERSANI-AMADO, C.A.; ROCHA, B.A.; OUTUKI, P.M.; DA SILVA, M.A.C.P.; FROEHLICH, D.L.; CARDOSO, M.L.C. Inhibitory Effect of the Hexane Fraction of the Ethanolic Extract of the Fruits of *Pterodon pubescens* Benth in Acute and Chronic Inflammation. **Journal Evidence-Based Complementary and Alternative Medicine**, Article ID 272795,7 p. http://dx.doi.org/10.1155/2013/272795, 2013.

HOSCHEID, J.; CARDOSO, M. L. C. Sucupira as a Potential Plant for Arthritis Treatment and Other Diseases. **Hindawi Publishing Corporation Arthritis**, Article ID 379459, 12 p., http://dx.doi.org/10.1155/2015/379459, 2015.

HOSCHEID, J.; OUTUKI, P.M.; KLEINUBING, S. A.; SILVA, M.F.; BRUSCHI, M.L. CARDOSO, M. L.C. Development and characterization of *Pterodon pubescens* oil nanoemulsions as a possible delivery system for the treatment of rheumatoid arthritis. **Colloids and surfaces. A, Physicochemical and engineering aspects,** v. 484, p.19-27, 2015.

HOSTETTMAN, K.; QUEIROZ, E. F.; VIEIRA, P. C. **Princípios Ativos de Plantas Superiores**. São Carlos: EdUFSCar, p. 59-100, 2003.

KATZ, N.; SANTOS FILHO, D.; SARTI, S.J.; MENDES, N.M.; ROCHA FILHO, P.A.; ARAUJO, N. Atividade quimioprofilática na esquistossomose mansoni de sabonetes contendo óleo essencial dos frutos de *Pterodon pubescens*. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 35, p. 183-191, 1993.

LEAL, L. K. A. M.; FERREIRA, A. A. G.; BEZERRA, G. A.; MATOS, F. J. A.; VIANA, G. S. B. Antinociceptive, anti-inflammatory and bronchodilator activities of Brazilian medicinal plants containing coumarin: a comparative study. **Journal of Ethnopharmacology**, v. 70, p. 151-159, 2000.

LIMA, C. B.; RACANICCI, A. M. C.; OLIVEIRA, G. R.; MIGOTTO, D. L.; AMADOR, S. A.; SOUZA, T. C.; TANURE, C. B. G. S.; VIEIRA, A. Effects of the Dietary Supplementation of Sucupira (Pterodon Emarginatus Vog.) and Copaiba (Copaifera Langsdorffii) Resinoils on Chicken Breast and Thigh Meat Quality and Oxidative Stability. Brazilian Journal of Poultry Science, p.47-56, 2015.

LIMA, H.C. DE; LIMA, I.B. *Pterodon* in **Lista de Espécies da Flora do Brasil**. Jardim Botânico do Rio de Janeiro, 2015. Disponível em: <http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB29842>. Acesso em: 03 Jan. 2016

LORENZI, H.; MATOS, F.J.A. **Plantas Medicinais no Brasil: Nativas e Exóticas**. Nova Odessa, Brazil: Instituto Plantarum, 369 p., 2002.

LORENZI, H. **Árvores Brasileiras – Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil**. Nova Odessa, Brazil: Instituto Plantarum, 6ª Ed., v. 01, 2014.

MACIEL, M. A. M.; PINTO, A., C.; VEIGA JR, V. F. Quim. Nova, Vol. 25, No. 3, 429-438, 2002. Plantas medicinais: a necessidade de estudos multidisciplinares. **Química Nova**, v. 29, n. 2, p. 326-337, 2006

MAHAJAN, J.R.; MONTEIRO, M.B. New diterpenoids from Pterodon emarginatus. Anais da Academia Brasileira de Ciências, v.42, p.103-107, 1970.

MAHAJAN, J.R.; MONTEIRO, M.B. New diterpenoids from Pterodon emarginatus. **Anais da Academia Brasileira de Ciências**, v.44, p.51-53, 1973.

MARQUES D. D., MACHADO, M. I. L., CARVALHO, M. G., MELEIRA, L. A. C., BRAZ-FILHO, R. Isoflavonoids and Triterpenoids Isolated from Pterodon polygalaeflorus. **Journal of th. Brazilian Chemical Society**., v. 9, n. 3, p. 295-301, 1998.

MASCARO, U.C.P.; TEIXEIRA, D.F.; GILBERT, B. Avaliação da sustentabilidade da coleta de frutos de sucupira branca (*Pterodon emarginatus* Vog.) após queda espontânea. **Revista Brasileira de Plantas Medicinais**, v. 7, p. 131-138, 2004.

MENNA-BARRETO, R.F.S., LARANJA, G.A.T., SILVA, M.C.C., COELHO, M.G.P., PAES, M.C., OLIVEIRA, M.M., DE CASTRO, S.L. Anti-*Trypanosoma cruzi* activity of *Pterodon pubescens* seed oil: geranylgeraniol as the major bioactive component. **Parasitology Research**, v. 103, p.111–117, 2008.

MIRANDA, M.L.D.; GARCEZ, F.R.; ABOT, A.R.; GARCEZ, W.S. Sesquiterpenos e outros constituintes das folhas de *Pterodon pubescens* Benth (Leguminosae). **Quimica Nova***, v.* 37, p. 473-476, 2014.

MORAES, W. F.; MATOS, L. G.; NASCIMENTO, M. V. M.; PAULA, J. R.; BARA, M. T. F.; CUNHA, L. C.; VALADARES, M. C.; COSTA, E. A. Anti-inflammatory and anti-nociceptive effects of *Pterodon emarginatus* stem bark alcohol extract*.* **Pharmaceutical Biology**, v. 47, p. 146-150, 2009.

MORAES, W. F.; GALDINO, P. M.; NASCIMENTO, M. V. M.; VANDERLINDE, F. A.; BARA, M. T. F.; COSTA, E. A.; PAULA, J. R. Triterpenes involved in the anti-inflammatory effect of ethanolic extract of Pterodon emarginatus Vogel stem bark. **Journal of Natural Medicines** v. 66, p. 202–207, 2012.

MORS, W. B.; PELLEGRINO, J.; SANTOS FILHO, M. F. Ação profilática do óleo de frutos da sucupira branca, *Pterodon pubestens* Benth., contra a infecção pelo *Schistosoma munsoni*. **Anais da Academia Brasileira de Ciências,** v. 38, p. 325-330, 1966.

MORS, W.B.; SANTOS FILHO, M.F.; MONTEIRO, H.J.; GILBERT, B.; PELLEGRINO, J. Chemoprophylactic agent in schistosomiasis: 14,15-epoxygeranylgeraniol. **Science**, v. 157, p. 950-951, 1967.

NEGRI, G., MATTEI, R., MENDES, F.R. Antinociceptive activity of th HPLC- and MS-standardized hydroethanolic extract of *Pterodon emarginatus* Vogel leaves. **Phytomedicine, v.**21, p. 1062-1069, 2014.

NETO, J.J. Contribuição ao Estudo Farmacognóstico dos frutos de Pterodon pubescens Bentham. Parte III- Identificação cromatográfica em fase gasosa, química e ação microbiológica do óleo do fruto. **Revista da Faculdade de Farmácia e Odontologia de Araraquara**, v.10, n.2, p.317-327, 1976.

NUCCI, C.; MAZZARDO-MARTINS, L.; STRAMOSK, J.; BRETHANHA, L.C.; PIZZOLATTI, M.G.; SANTOS, A.R.S.; MARTINS, D.F. Oleaginous extract from the fruits Pterodon pubescens Benth induces antinociception in animal models of acute and chronic pain. **Journal of Ethnopharmacology**, v. 143, p. 170-178, 2012.

NUCCI-MARTINS, C.; MARTINS, D.F.; NASCIMENTO, L.F.; VENZKE, D.; OLIVEIRA, A.S.; FREDERICO, M.J.S.; SILVA, F.R.M.B.; BRIGHENTE, I.M.C.; PIZZOLATTI, M.G.; SANTOS, A.R.S. Ameliorative potential of standardized fruit extract of Pterodon pubescens Benth on neuropathic pain in mice: Evidence for the mechanisms of action. **Journal of Ethnopharmacology,** v. 175, p. 273-286, 2015.

NUNAN, E. A.; CARVALHO, M. G.; PILOÂ-VELOSO, D.; TURCHETTI-MAIA, R. M. M.; FERREIRA A. D. L. Furane diterpenes with anti and pro-inflammatory activity. **Brazilian Journal of Medical and Biological Research**, v. 15, p. 450-451, 1982.

OLIVEIRA, D. M. T.; PAIVA, E. A. S. Anatomy and ontogeny of *Pterodon emarginatus* (Fabaceae: Faboideae) seed. **Brazilian Journal of Biology**, v. 65, p. 483-494, 2005.

OLIVEIRA, P. C.; CONCEIÇÃO, E. C.; OLIVEIRA, P. A.; NASCIMENTO, M. V.; COSTA, E. A., PAULA, J. R.; BARA, M. T. F. Obtaining a dry extract of Pterodon emarginatus (Fabaceae) fruits by spray drying. **Journal of Pharmacy Research**,v.5, n.1, p.641-645, 2012.

OLIVEIRA, A. E. M. F. M.; DUARTE, J. L.; AMADO, J. R. R.; CRUZ, R. A. S.; ROCHA, C. F.; SOUTO, R. N. P.; FERREIRA, R. M.A.; SANTOS, K.; CONCEIÇÃO, E. C.; OLIVEIRA, L. A. R.; KELECOM, A.; FERNANDES, C. P.; CARVALHO, J. C. T. Development of a Larvicidal Nanoemulsion with Pterodon emarginatusVogel Oil. **PLoS ONE** 11(1): e0145835. doi:10.1371/journal.pone.0145835. 2016.

OMENA, M.C; BENTO, E.S.; DE PAULA, J.E.; SANT'ANA, A.E.G. Larvicidal Diterpenes from *Pterodon polygalaeflorus*. **Vector-Borne and Zoonotic Diseases**, v. 6, p. 216-222, 2006.

PASCOA, H., DINIZ, D. G. A., FLORENTINO, I. F., COSTA, E. A., BARA, M. T. F. Microemulsion based on Pterodon emarginatus oil and its anti-inflammatory potential. **Brazilian Journal of Pharmaceutical Sciences**, v. 51, p. 117-126, 2015.

PAULA, F. B. A., GOUVÊA, C. M. C. P., ALFREDO, P. P., SALGADO, I. Protective action of a hexane crude extract of *Pterodon emarginatus* fruits against oxidative and nitrosative stress induced by acute exercise in rats. **BMC Complementary and Alternative Medicine**, DOI:10.1186/1472- 6882-5-17, 2005.

PEDRAZZI, A.H.P.; RODRIGUES, E.R.; ZANARDO FILHO, A.; FRANCO, J.J.; RIBEIRO, W.O. Preclinical (hematologic and hemostatic) evaluation of the possible toxic side effects of a *pterodon emarginatus* vog extract. **Acta Horticulturae**, DOI : 10.17660/ActaHortic.1999.501.7, 1999.

PELLEGRINO, J. Protection Against Human Schistosome Cercariae. **Experimental Parasitology**, v. 21, p. 112-131, 1967.

PEREIRA, M. F.; MARTINO, T.; DALMAU, S. R.; PAES, M. C.; BARJA-FIDALGO, C.; ALBANO, R. M.; COELHO, M. G. P.; SABINO, K. C. C. Terpenic fraction of *Pterodon pubescens* inhibits factor kappa B and extracelular signal-regulated protein kinase ½ activation and deregulates gene expression in leukemia cells. **Complementary & Alternative Medicine**, DOI: 10.1186/1472-6882-12-231, 2012.

PEZZONI, T.; VITORINO, A. C. T.; DANIEL, O.; LEMPP, B. Influência de Pterodon emarginatus Vogel sobre atributos físicos e químicos do solo e valor nutritivo de Brachiaria decumbens Stapf em sistema silvopastoril. **Cerne,** v. 18, n. 2, p. 293-301, 2011.

PIMENTA, T.A.A.; SANTIAGO, G.M.P.; ARRIAGA, A.M.C.; MENEZES, G.H.A.; BEZERRA, S.B. Estudo fitoquímico e atividade larvicida de *Pterodon polygalaeflorus* Benth (Leguminosae) sobre *Aedes aegypti*. **Revista Brasileira de Farmacognosia**, v. 16, p.501-505, 2006.

PINTO, F.A.; VIGLIANO, M.V.; LEAL, N.R.F.; SILVA, G.P.; MARQUES, P.R.; VELOZO, L.S.M.; SABINO, K.C.C.; COELHO, M.G.P. Phytochemical and pharmacological analysis of *Pterodon polygalaeflorus* extracts. **Pharmacology online,** *v.* 3, p. 56-70, 2013.

SANTOS, A. P.; ZATTA, D. T.; MORAES, W. F.; BARA, M. T. F.; FERRI, P. H.; SILVA, M. R. R.; PAULA, J. R. Composição química, atividade antimicrobiana do óleo essencial e ocorrência de esteróides nas folhas de *Pterodon emarginatus* Vogel, Fabaceae. **Brazilian Journal of Pharmacognosy**, v. 20, p. 891-896, 2010.

SABINO, K. C. C.; CASTRO, F. A.; OLIVEIRA, J. C. R.; DALMAU, S. R. A.; COELHO, M. G. P. Successful Treatment of Collagen-induced Arthritis in Mice with a Hydroalcohol Extract of Seeds of Pterodon pubescens. **Phytotherapy Research***, v.* 13, p. 613-615, 1999a.

SABINO, K. C. C.; GAYER, C. R. M.; VAZ, L. C. A.; SANTOS, L. R. L.; FELZENSZWAL, B. I.; COELHO, M. G. P. In vitro and in vivo toxicological study of the *Pterodon pubescens* seed oil. **Toxicology Letters**, v.108, p.27–35, 1999b.

SERVAT, L.; SPINDOLA, H. M.; RODRIGUES, R. A. F.; SOUZA, I. M. O.; RUIZ, A. L. T. G.; CARVALHO, J. E.; FOGLIO, M. A. *Pterodon pubescens* Benth: Stability Study of Microencapsulated Extract and Isolated Compounds Monitored by Antinociceptive Assays. **Journal of the Brazilian Chemical Society**, v. 23, p. 1244-1253, 2012.

SILVA, M. C. C.; GAYER, C. R. M.; LOPES, C. S.; CALIXTO, N. O.; REIS, P. A.; PASSAES, C. P. B.; PAES, M. C.; DALMAU, S. R.; SABINO, K. C. C.; TEDESCHINI, A. R.; COELHO, M. G. P. Acute and topic antiedematogenic fractions isolated from the seeds of *Pterodon pubescens*. **Journal of Pharmacy and Pharmacology**, v. 55, p. 135-141, 2004.

SILVEIRA, L. B.; MARTINS, Q. S.; MAIA, J. C.; SANTOS, J. G. Preparation of Nanocomposites Resin from Seed Pterodon emarginatus Doped Maghemite Nanoparticles. **Journal of Nanoscience and Nanotechnology**, v.12, 4832–4835, 2012.

SPINDOLA, H.M.; CARVALHO, J.E.; RUIZ, A.L.T.G.; RODRIGUES, R.A.F.; DENNY, C.; SOUZA, I.M.O.; TAMASHIRO, J.Y.; FOGLIO, M.A. Furanoditerpenes from *Pterodon pubescens* Benth with Selective in vitro Anticancer Activity for Prostate Cell Line**. Journal of the Brazilian Chemical Society**, v. 20, p. 569-575, 2009.

SPINDOLA, H.M.; SERVAT, L.; DENNY, C.; RODRIGUES, R.A.F.; EBERLIN, M.N.; CABRAL, E.; SOUZA, I.M.O.; TAMASHIRO, J.Y.; CARVALHO, J.E.; FOGLIO, M.A. Antinociceptive effect of geranylgeraniol and 6a,7b-dihydroxyvouacapan-17b-oate methyl ester isolated from *Pterodon pubescens* Benth. **BMC Pharmacology***, v.*10, article 1, DOI: 10.1186/1471-2210-10-1 2010.

SPINDOLA, H. M.; SERVAT, L.; RODRIGUES, R. A. F.; SOUSA, I. M. O.; CARVALHO, J. E.; FOGLIO, M. A. Geranylgeraniol and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester isolated from *Pterodon pubescens* Benth.: Further investigation on the antinociceptive mechanisms of action. **European Journal of Pharmacology**, v. 656, p. 45-51, 2011.

THE PLANTLIST , 2013. Disponível em: http://www.theplantlist.org/tpl1.1/record/ild-33212. Acesso em: 11 jun 2016.

TORRENEGRA, R., BAUEREIB, P., ACHENBACH, H. Homoormosanine-type alkaloids from *Bowdichia virgilioides*. **Phytochemistry**, v. 28, p. 2219–2221, 1989.

VELOZO, L. S. M.; MARTINO, T.; VIGLIANO, M. V.; PINTO, F. A.; SILVA, G. P.; JUSTO, M. G. A.; SABINO, K. C. C.; COELHO, M. G. P. *Pterodon polygalaeflorus* Essential Oil Modulates Acute Inflammation and B and T Lymphocyte Activation. **The American Journal of Chinese Medicine**, v.41, p. 545-563, 2013.

VIEGAS JR, C.; BOLZANI, V.S.; BARREIRO, E. J. Os Produtos naturais e a Química Medicinal Moderna. **Química Nova**, v. 25, n. 3, p. 429-438, 2002.

VIEIRA, C.R.; MARQUES, M.F.; SOARES, P.R.; MATUDA, L.; DE OLIVEIRA, C.M.A.; KATO, L.; DA SILVA, C.C.; GUILLO, L.A. Antiproliferative activity of *Pterodon pubescens* Benth. seed oil and its active principle on human melanoma cells. **Phytomedicine: International Journal of Phytotherapy and Phytopharmacology**, v. 15, p. 528-532, 2008.

UTILIZATION OF NATURAL PRODUCTS FOR NOVEL NANO-BASED INSECTICIDAL AGENTS

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Utilization of natural products for novel nano-based insecticidal agents

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INTRODUCTION

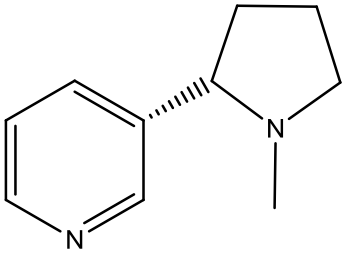
Insects can be benefit for human being and are used since ancient times, such as in case of bee for honey production or silk for clothing. New approaches relies on utilization of these organisms as source of bioactive compounds, since many of them have folk medicine use [1,2]. Moreover, they are useful as pollinators and therefore are very important for farming [3]. However, when they interact with our society, causing problems with impairment to agriculture or public health, they are considered pests [4]. Major problems associated to utilization of pesticides relies in the fact that they can be harmful for environment and non-targets organism, including humans. Thus, integrated control programs using natural products are very promising for ecofriendly pest agents, including those using nanotechnology approach [5,6].

INSECTICIDES FROM PLANT ORIGIN

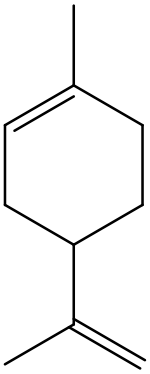
Natural products were used as effective insecticides since ancient times. Latter, with development of synthetic pesticides, a decline in the use of natural compounds was observed. However, due to undesired effects of synthetic compounds, growing interest is observed again for natural products, especially those from plant origin [7]. Co-evolution of organisms that share the same ecosystem make some of them able to create a chemical defense against their enemies [3]. On this context, it is well know that plants are able to produce their own defenses, being the secondary metabolites a chemical interface between the plants and their environment [8]. Thus, it is worth mentioning that secondary metabolites from plant origin develop a main role in the development of potential insecticides. A large number of natural products, from different classes (eg. alkaloids, terpenoids) (Figure 1) act as insecticides by a

wide range of mechanism of action. They may interfere in the cholinergic, GABA, mitochondrial, octopamenergic system or others [9]

The natural products can be fumigated or be in contact to the crop pests [10], act as repellent to crop pests [11], larvicidal or repellent agents against vectors of tropical diseases [12]. For example, natural oils as repellent agents against disease vectors are increasingly popular due to reduced toxicity and customer approval [13]. In this case, synergistic effect among components of a complex matrix is associated to improved bioactivity when compared to isolated substances. Moreover, novel delivery systems to improved repellent activity of the natural compounds are considered very promising [14]. Interesting approach in this subject include application of microencapsulated essential oils to cotton textile [15]. Considering the great concern worldwide related to effective ways to prevent bites from mosquitoes that are disease vectors and, nanotechnology was considered very interesting to address the problem of residual control [16].



Nicotine (alkaloid)



Limonene (terpenoid)

Figure 1. Chemical structures of secondary metabolites from plant original with insecticidal activity.

NANOTECHNOLOGY

Nanotechnology involves manipulation and organization of structures in a sub-micron level. It is widespread in several areas of knowledge, including pest control. Novel products with enhanced properties can be prepared using nanotechnology and predictions suggest that, in the next decades, they will change our understanding and will create new developing opportunities [17]. Growing interest has been observed for nanotechnology-based pest agents. The nanosize products offer a great advantage regarding improvement of insecticide agent bioavailability and even controlled release [18]. The two main targets of nanopesticides are related to integrated control strategies against crop pests [19,20,21] or disease vectors [22].

Despite some authors define nanoformulations using a upper size limit of 100 nm for mean diameter, this definition may fail in the case of nanopesticides. Thus, more appropriate definition should indicate that nanopesticides should have particles up to 1000 nm and exhibit novel properties due to size reduction, when compared to bulk material [23]. Nowadays, combination of natural products with nanotechnology is in the spotlight research for novel ecofriendly pesticide agents.

NATURAL PRODUCT-BASED NANOFORMULATIONS AGAINST INSECTS

Development of nanoformulations containing natural products have been considered a very promising strategy to enhance the insecticidal activity of these compounds. Moreover, they may induce lower impact on the environment and non-target organism, better solubilization in water of the insecticide (eg. larvicides) agents, improved efficacy due to higher surface area and induction of systemic activity due to smaller particle size [24]. Encapsulation of substances may induce a controlled release, which also offer several advantages [25]. Most of

literature data regarding natural-product based nanoformulations are related to nanoemulsions, metallic nanoparticles or polymeric nanoparticles.

Nanoemulsions are kinetically disperse systems constituted by two immiscible liquids. They are mainly prepared using two different approaches, name high energy methods or low energy methods. High energy methods use disruptive forces to reduce droplet size and main equipment used are high-shear stirrers, high-pressure homogenizers and ultrasound generators. Low energy methods use the internal energy of the system and often involve phase inversion (PIT or PIC) or spontaneous emulsification [26]. Polymeric nanoparticles are mainly sub-divided into two groups, name nanospheres or nanocapsules. Nanospheres are basically a dense polymeric matrix in which the bioactive compounds are dispersed. Nanocapsules are consituted by a cavity surrounded by a polymeric coating layer, being the bioactive compounds entrapped or distributed in the membrane [27]. Metallic nanoparticles are usually prepared by adding metal salt into aqueous solution, stirring and monitoring by UV-Vis in order to access color change and nanoparticle formation [28,29]. Most recent papers regarding development of natural-product based nanoformulations are presented in this chapter.

INSECTICIDE NANOEMULSIONS CONTAINING NATURAL PRODUCTS AS BIOACTIVE COUMPOUNDS

Nanoemulsions prepared by high-energy sonication method with neem (*Azadirachta indica*) oil were able to induce high mortality levels in *Culex quinquefasciatus*(Diptera: Culicidae) larvae after 24 h of exposure. At a constant oil concentration, nanoemulsions with different surfactant (polysorbate 20) induced different mortality levels, while the surfactant alone was

not toxic to larvae. This fact was associated to different mean droplet size, since as the mean droplet size decreased, the mortality level increased [30].

Ultrasonication was performed to obtain basil (*Ocimum basilicumi*) nanoemulsions. Intense disruptive forces allowed achievement of mean droplet size around 30 nm. Different fold dilutions of the nanoemulsion were prepared revealed a dose and time dependent mortality of *Aedes aegypti* larvae [31]. On another study with this oil, spontaneous emulsification allowed achievement of low size nanoemulsion with great physical stability. This nanoproduct was effective against *C. quinquefasciatus* larvae, inducing a dose-dependent mortality that was increased as function of exposure time. Moreover alteration on epithelial and peritrophic membranes were observed [32].

Rosemary (*Rosmarinus officinalis*) essential oil was used as oil phase to prepare a nanoemulsion by low energy method using polysorbate 80 (oil to surfactant ratio of 1:1) and 90% of water (w/w). It presented mean droplet size below 200 nm and its larvicidal potential agent against *A. aegypti* was investigated. The diluted nanoemulsion containing 250 ppm of essential oil at the experiments induced around 80% of mortality after 24 h and around 90% of mortality after 48 h [33].

A study carried out with guracica (*Manilkara subsericea*) fruits extracts revealed that hexane-soluble fraction, as well as it pentacyclic triterpenes, presented high activity against *Dysdercus peruvianus* and *Oncopeltus fasciatus* [5]. However, aiming to develop an ecofriendly insecticide with this non-water soluble natural, avoiding use of organic solvents, a nanoemulsion was prepared with hexane fraction obtained from guracica fruits. Dynamic light scattering analysis of the nanoemulsion prepared with 5% (w/w) of octyldodecil myristate, 2 % (w/w) of sorbitan monooleate, 3% (w/w) of polysorbate 80, 5% (w/w) of guracica fraction and 85% (w/w) of water suggested mean droplet size of 155.2 ± 3.8 nm and polydispersity index of 0.270 ± 0.006. Topical application of this nanoemulsion containing insecticide

triterpenes (Figure 2) induced mortality in *D. peruvianus*, which causes severe damage to cotton crops. Moreover, no acute toxic effects were observed in mice and the nanoemulsion did not inhibit the acetylcholinesterase activity, a enzyme that develop a main role in cases of toxicity to non-target organism [6].

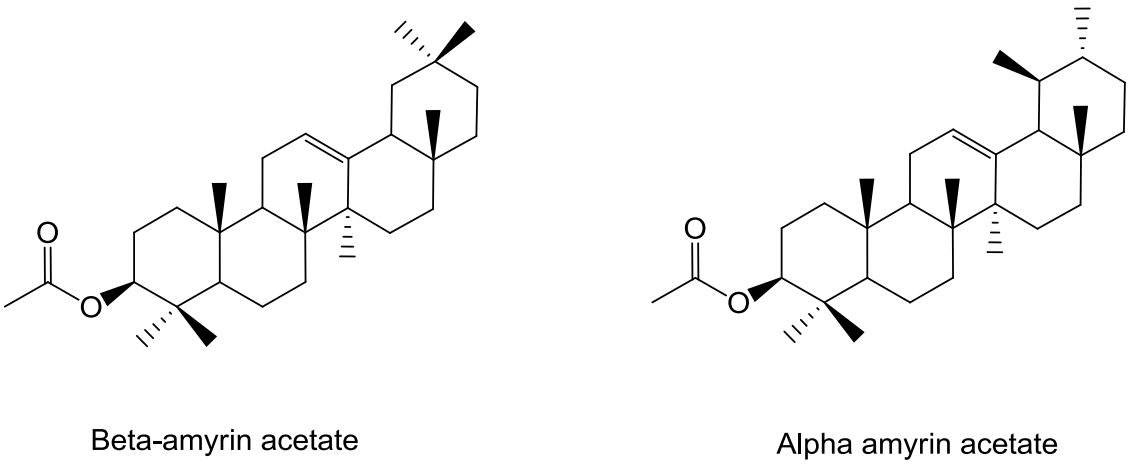


Figure 2. Chemical structures of pentacyclic triterpenes with insecticidal action present in the nanoemulsion prepared with *Manilkara subsericea* extract

Essential oils from three Asteraceae species (*Ageratum conyzoides, Achillea fragrantissima* and *Tagetes minuta*) were used to prepared nanoemulsions. High energy method using high pressure homogenization was performed. The bioactivity of the oils against cowpea bettle (*Callosobruchus maculatus*) was highly increased when they were available as the nanoemulsions, being toxic to eggs and adults [34].

The nanoemulsions prepared with citronella (*Cymbopogum nardus*), hairy basil (*Ocimum americanum*) and vetiver (*Vetiveria zizanioides*) oils that were prepared by high-pressure homogenization method presented repellent activity against *A. aegypti*. Reduction of droplet size increased physical stability and improved repellent activity. Improved mosquito

protection time was achieved using the optimized nanoemulsion constituted by 10% (w/w) of citronella oil, 5% (w/w) of hairy basil oil and 5% (w/w) of vetiver oil. Citotoxic test using normal human foreskin fibroblast suggest low toxic effects, highlighting potential of these essential oil-based nanoemulsions [35]. Utilization of glicerol at high amounts proved to be a good strategy to achieve slow release of citronella oil from nanoemulsions, providing a controlled release and better mosquito repellent activity [36].

Oleoresin from *Copaifera duckei* was used for preparation of several oil in water nanoemulsions. Optimized nanoemulsion was able to induce high mortality level (approximately 90%) at 200 ppm (expressed as oleoresin content) after 48 h and was considered a promising larvicidal agent against *A. aegypti* [37]. *Eucalyptus globulus* oil nanoemulsions were prepared by ultrasonication and presented improved larvicidal activity against *C. quinquefasciatus*, when compared to bulk emulsion. Analysis of larvae homogenate revealed that decrease in total protein content and decrease in the acetylcholinesterase activity were observed after treatment with the nanoemulsion, in addition to reduction of acid and alkaline phosphatase levels [38].

Sucupira-branca (*Pterodon emarginatus*) oleoresin containing the diterpenes methyl 6α,7β-dihydroxyvouacapan-17-β-oate, geranylgeraniol the sesquiterpene β-caryophyllene was used as natural raw material for a larvicidal nanoemulsion against *A. aegypti* larvae. The optimized nanoemulsion obtained by low energy titration method induced high mortality levels and low LC_{50} value 34.75 (7.31–51.86) ppm after 48 h. Moreover, it was considered potentially safe for mammals and mechanism of insecticidal action may envolve inhibition of acetylcholinesterase [39].

Aqueous filtrates of karanja (*Pongamia glabra*) and jatropha (*Jatropha curcas*) oil-free cakes were used as external phased for preparation of *Eucalyptus globulus* oil nanoemulsions. This

strategy was considered promising for food grain storage due to insecticidal activity against *Tribolium castaneum* [40].

INSECTICIDE NANOPARTICLES CONTAINING NATURAL PRODUCTS AS BIOACTIVE COUMPOUNDS

Mettalic nanoparticles

Nickel nanoparticles prepared aqueous leaf extract obtained from *Aegle marmelos* showed stronger larvicidal against *A. aegypti*, *Anopheles stephensi* and *C. quinquefasciatus* than the pure aqueous, ethyl acetate and methanol extracts. Moreover, it presented lower LC50 values when compared to this extracts, being comparable to positive control, the known chemical larvicidal temephos [28]. Evaluation of larvicidal and pupal toxicity effects of silver nanoparticles prepared with floral extract from *Chrysanthemum indicum* showed that this nanoproduct was more potent than the aqueous extract used for its fabrication. LC50 and LC90 of this nanoparticles against first, second, third, fourth instar and pupa were 5.07/29.18, 10.35/47.35, 14.19/65.53, 22.81/87.96 and 35.05/115.05 ppm, respectively [29]. Mangrove plant (*Rhizophora mucronata*) aqueous extract from leaves was subjected to silver nanopartice fabrication, which presented potential larvicidal activity against *A. aegypti* and *C. quinquefasciatus* [41].

The aqueous extract obtained from leaves from *Anisomeles indica* was used for preparation of silver nanoparticles that were active against *Anopheles subpictus* (LC50 = 31.56 ppm), *Aedes albopictus* (35.21 ppm) and *Culex tritaeniorhynchus* (38.08 ppm) [42].

The silver nanoparticles prepared with aqueous extract from *Toddalia asiatica* were able to induce mortality levels in *C. quinquefasciatus* larvae. They were more active than pure aqueous extract and potency of the larvicidal action decreased as function of insect growth,

ranging from 16.48 (I instar) to 31.83 (pupae) ppm. Moreover, it was considered an ecofriendly agent, since it did not induce major alteration in predation rates of guppy fish, a natural enemy of the mosquito larvae (*Poecilia reticulata*) [43]. *Bruguiera cilindrica* aqueous extract from leaves was used as raw material for preparation of silver nanoparticles and LC50 values also decreased as function of *A. aegypti* development stage, ranging from 9.93 (instar I) to 30.69 (pupa). Non-target effect on *Carassius auratus* was also evaluated and sub-lethal doses of the phytofabricated nanoparticles did affect the predation of the goldfish [44].

Preparation of silver nanoparticles increased larvicidal potential of aqueous leaf extract from *Cassia roxburghii* against three disease vector mosquitoes. LC50 for pure aqueous extract and herbal-based nanoparticles were 26.35 / 133.69 ppm for *A. stephensi*, 28.67 / 149.68 ppm for *A. aegypti* and 31.27 / 163.48 ppm for *C. quinquefasciatus* [45].

Larvicidal bioassy revealed that silver nanoparticles prepared with aqueous extract from *Eclipta prostata* presented potential action against *Culex quiquefasciatus* and *A. subpictus*. LC50 or LC90 values are respectively, 4.56 mg/L or LC90 = 13.14 mg/L for *C. quinquefasciatus* and 5.14 or LC90 25.68 ppm for *A. subpictus*. These activities were more pronounced then larvicidal effect induced by pure aqueous extract, which exhibited LC_{50} = 27.49; LC90 = 70.38 ppm), and against the larvae of *A. subpictus* (LC50 = 27.85 mg/L; LC90 = 71.45 ppm) [46].

The aqueous extract from fresh or dry leaves and green berries from *Solanum nigrum* encapsulated in silver nanoparticles showed potent larvicidal activity against *C. quinquefasciatus* and *A. stephensi*. Third instar of the larvae were more susceptible than second instar for both species and LC50 values below 3.0 ppm were observed in all groups [47].

Silver nanoparticles prepared with aqueous leaf extract from the seaweed *Hypnea musciformis* were evaluated against tropical disease vector (*A. aegypti*) and agricultural pest (*Plutella xylostella*). They presented mean droplet size around 40-65 nm and were considered more active in earlier-instar. LC50 for *A. aegypti* and *P. xylostella* were, respectively, 18.14 and 24.51 (I instar), 20.54 and 26.47 (II intar), 26.61 and 28.35 (III instar), 27.99 and 32.55 (IV instar) and 30.79 and 38.23 (pupae). They also reduced longetivity and fecundity of both insects [48].

Mimusops elengi aqueous leaf extract was subjected to preparation of silver nanoparticles. Mean values of LC50 and LC90 (ppm) against *A. stephensi* were 12.53 and 31.25 (I instar), 14.67 and 38.93 (II instar), 16.90 and 45.29 (III instar), 19.13 and 55.18 (IV instar), 23.55 and 65.26 (pupa). Mean values of LC50 and LC90 (ppm) against *Aedes albopictus* were 11.72 and 30.07 (I instar), 13.55 and 37.59 (II instar), 16.05 and 43.25 (III instar), 17.77 and 51.85 (IV instar), 21.46 and 62.49 (pupa). The nanoparticles were also evaluated against adults of both insects. Adulticidal activity revealed that silver nanoparticles were more active than pure aqueous extract agains *A. stephensi* and *A. albopictus* [49].

Enhanced larvicidal activity against *A. aegypti* was observed for silver nanoparticles prepared with aqueous extract from leaves of *Leucas aspera*, when compared to aqueous and organic solvent-based extracts. LC50 and LC90 values were, respectively, as follows: 8.56 and 21.6 ppm (nanoparticles), 10.0 and 93.0 ppm (methanol), 14.5 and 39.6 (petroleum ether), 13.5 and 42.2 ppm (chloroform), 17.4 and 31.3 ppm (ethyl acetate), 27.5 and 53.3 ppm (aqueous extract) [50].

The aqueous extract from seeds of *Moringa oleifera* was used for preparation of silver nanoparticles. Spherical shape nanoparticles with mean diameter around 100 nm presented promising larvicidal activity against *A. aegypti* at different larval stages. LC50 ranged from

10.24 ppm (I instar larvae) to 21.17 ppm (pupae), while LC90 ranged from 23.05 ppm (I instar larvae) to 42.59 ppm (pupae) [51].

Silver nanoparticles (diameter around 25 nm) prepared with *Hybanthus enneaspermus* were active against *A. subpictus* (LC50 = 17.24 ppm) and *C. quinquefasciatus* (LC50 = 13.12 ppm). This results suggst that silver nanoparticles are more potent than pure extract, which presented higher LC50 values for *A. subpictus* (117.83 ppm) and *C. quinquefasciatus* (126.59 ppm) [52]. Leaves of *Feronia elephantum* were extracted with water and silver nanoparticles were prepared. Larvicidal potential of this nanoproduct was observed against *A. stephensi* (LC50 = 11.56 ppm and LC90 = 20.56 ppm), *A. aegypti* (LC50 = 13.13 ppm and LC90 = 23.12 ppm) and *C. quinquefasciatus* (LC50 = 14.19 ppm and LC90 = 29.30 ppm). These nanoparticles were potentially more active than aqueous leaf extract [53].

Polymeric nanoparticles

The essential oil from *Zanthoxylum rhoifolium* were encapsulated into polymeric nanospheres using biopolymer (PCL) and non-ionic biodegradable surfactants (Span 60/Tween 80). Main constituents of the essential oil were β-elemene (31.26 %), β-caryophyllene (12.09 %) and D-germacrene (18.16 %). Considerable reduction in the number of eggs and nymphs, as high as 95%, was observed against *Bemisia tabaci*. Considering the effects induced by pure essential oil, both products were considered promising against this insect [54].

CONCLUDING REMARKS

Natural products, especially from plant origin, are historicaly considered alternatives as potential insecticides. However, poor water solubility of some compounds, high volatility and

in some cases, chemical instability, make development of viable novel products a technological challenge. Nanotechnology emerged as a promising area to solve this main problems, in addition to another advantages. Recent investigations in the field shows increasing interest and suggest that several natural-product based nanoformulations may be available for consumers.

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ABREVIATIONS

PIT – phase inversion temperature method

PIC – phase inversion composition method

LC50 – lethal concentration that kills 50% of the exposed insects

LC90 – lethal concentration that kills 90% of exposed insects

REFERENCES

[1] N. A. Ratcliffe, C. B. Mello, E. S. Garcia, T. M. Butt, P. Azambuja, Insect Biochemistry and Molecular Biology 41 (2001) 747-769.

[2] N. Ratcliffe, P. Azambuja, C. B. Mello, Evidence-Based Complementary and Alternative Medicine 2014 (2014) 21p.

[3] J. B. Harborne, Natural Products Reports 18 (2001) 361–379.

[4] Gullan P. J. & Cranston P. S. Os Insetos. Um Resumo de Entomologia. 3º Ed. Department of Entomology, Univercidade da California, Davis, EUA. Roca, 2008.

[5] C. P. Fernandes , F. B. Almeida, A. N. Silveira, M. S. Gonzalez, C. B. Mello, D. Feder, R. Apolinário, M. G. Santos, J. C. T. Carvalho, L. A. C. Tietbohl, L. Rocha, D. Q. Falcão, Journal of Nanobiotechnology (2014) 12 – 22.

[6] C. P. Fernandes, A. X., J. P. F. Pacheco, M. G. Santos, R. M., N. A Ratcliffe, M. S. G., C. B. M., L. Rocha, D. Feder, Pest Management Science 69 (2013) 292–301.

[7] M. B. Isman, Pest Management Science 64 (2008) 8–11.

[8] L. Gobbo-Neto, N. P. Lopes, Química Nova 30 (2007) 374-381.

[9] R. S. Rattan. Crop Protection 29 (2010) 913-920.

[10] P. K. Bett, A. L. Deng, J. O. Ogendo, S. T. Kariuki, M. Kamatenesi-Mugisha, J. M. Mihale, B. Torto, Industrial Crops and Products 82 (2016) 51–62.

[11] W. Du, X. Han, Y. Wang, Y. Qin, Scientific Reports 6 (2015) DOI: 10.1038/srep22140.

[12] B. Conti, G. Benelli, G. Flamini, P. L. Cioni & R. Profeti, L. Ceccarini, M. Macchia, A. Parasitology Research 110 (2012) 2013–2021.

[13] T. M. Katz, J. H. Miller, A. A. Hebert, Journal of the American Academy of Dermatology 58 (2008) 865-871.

[14] L. S. Nerio, J. Olivero-Verbel, E. Stashenko, Bioresource Technology 101 (2010) 372–378.

[15] M. M. Miró Specos, J. J. García, J. Tornesello, P. Marino, M. Della Vecchia, M. V. Defain Tesoriero, L.G. Hermida, Transactions of the Royal Society Tropical Medicine & Hygiene 104 (2010) 653-658.

[16] J. U. Rehman, A. Ali, I. A. Khan, Fitoterapia 95 (2014), 65–74.

[17] S. Huang, L. Wang, L. Liu, Y. Hou, L. Li, Agronomy for Sustainable Development, 35 (2015) 369–400.

[18] A. L. Boehm, I. Martinon, R. Zerrouk, E. Rump, H. Fessi, Journal Microencapsulation 20 (2003) 433–441.

[19] T. Stadler, M. Butelerb, D. K. Weaver, Pest Management Science 66 (2010) 577–579.

[20] L. R. Khot, S. Sankaran, J. M. Maja, R. Ehsani, E. W. Schuster, Crop Protection 35 (2012) 64–70.

[21] M. Rai, A. Ingle, Applied Microbiology and Biotechnology 94 (2012) 287–293.

[22] C.H. Anjali, S. S. Khan ,K. Margulis-Goshen, S. Magdassi, A Mukherjee, N Chandrasekaran Ecotoxicology and Environmental Safety 73 (2012) 1932-1936.

[23] M. Kah, T. Hofmann, Environment International 63 (2013) 224–235.

[24] J. L. de Oliveira, E. V. R. Campos, M. Bakshi', P.C. Abhilash, L. F. Fraceto, Biotechnology Advances 32 (2014) 1550–1561.

[25] B. Solomon, F. F. Sahle, T. Gebre-Mariam, K. Asres, R. H. H. Neubert, European Journal of Pharmaceutics and Biopharmaceutics 80 (2012) 61–66.

[26] C. Solans, I Solé, Current Opinion in Colloid & Interface Science 17 (2012) 246–254.

[27] J. M. Irache, I. Esparza, C. Gamazo, M. Agüeros, S. Espuelas, Veterinary Parasitology 180 (2011) 47– 71.

[28] G. Angajala, R. Ramya, R. Subashini, Acta Tropica 135 (2014) 19–26.

[29] S. Arokiyaraj, V. D. Kumar, V. Elakya, T. Kamala, S. K. Park, M. Ragam, M. Saravanan, M. Bououdina, M. V. Arasu, K. Kovendan, S. Vincent, Environmental Science and Pollution Research 22 (2015) 9759–9765.

[30] C.H. Anjali, Y. Sharma, A. Mukherjee, N. Chandrasekaran, Pest Management Science 68 (2012) 158–163.

[31] V. Ghosh, A. Mukherjee, N. Chandrasekaran, Asian Journal of Chemistry 25 (2013), 321-323.

[32] V. Ghosh, A. Mukherjee, N. Chandrasekaran, Bio Nano Science.4 (2014) 157–165.

[33] J. L. Duarte, J. R. R. Amado, A. E. M. F. M. Oliveira, R. A. S. Cruz, A. M. Ferreira, R. N. P Souto,. D. Q. Falcão, J. C. T. Carvalho, C. P. Fernandes, Revista Brasileira de Farmacognosia, 25 (2015) 189-192.

[34] G. E. Nenaah, S I.A. Ibrahim, B. A. Al-Assiuty, Journal of Stored Products Research 61 (2015) 9–16.

[35] O. Nuchuchua, U. Sakulku, N. Uawongyart, S. Puttipipatkhachorn, A. Soottitantawat, U. Ruktanonchai, AAPS PharmSciTech 10 (2009) 1234-1242.

[36] U. Sakulku, O. Nuchuchua, N. Uawongyart, S. Puttipipatkhachorn, A. Soottitantawat, U. Ruktanonchai, International Journal of Pharmaceutics 372 (2009) 105–111.

[37] E. C. R. Rodrigues, A. M. Ferreira, J. C. E. Vilhena, F. B. Almeida, R. A. S. Cruz, A. C. Florentino, R. N. P. Souto, J. C. T. Carvalho, C. P. Fernandes, Revista Brasileira de Farmacognosia 24(2014) 699-705.

[38] S. Sugumar, S. K. Clarke, M. J. Nirmala, B. K. Tyagi, A. Mukherjee, N. Chandrasekaran, Bulletin of Entomological Research 104 (2014) 393–402.

[39] A. E. M. F. M. Oliveira, J. L. Duarte, J. R. R. Amado, R. A. S. Cruz, C. F. Rocha, R. N. P. Souto, R. M. A. Ferreira, K. S., E. C. Conceição,L. A. R. Oliveira, A. K., C. P. Fernandes, J. C. T. Carvalho, PlosOne 11 (2016) doi:10.1371/journal.pone.0145835

[40] M. Pant, S. Dubey, P. K. Patanjali, S. N. Naik, S. Sharma, International Biodeterioration & Biodegradation 91 (2014), 119–127.

[41] M. Gnanadesigan, M Anand, S Ravikumar, M. Maruthupandy, V Vijayakumar, S Selvam, M. Dhineshkumar, A.K. Kumaraguru, Asian Pacific Journal of Tropical Medicine (2011)799-803.

[42] M. Govindarajan, M. Rajeswary, K. Veerakumar, U. Muthukumaran, S.L. Hoti, G. Benelli, Experimental Parasitology 161(2016) 40–47

[43] K. Murugan, J. S. E. Venus, C. Panneerselvam, S. Bedini, B. Conti, M. Nicoletti, S. K. Sarkar, J. S. Hwang, J. Subramaniam, P. Madhiyazhagan, P. M. Kumar, D. Dinesh, U. Suresh, G. Benelli, Environmental Science and Pollution Research 22 (2015b) 17053–17064.

[44] K. Murugan, D. Dinesh, M. Paulpandi, A. D. M. Althbyani, J. Subramaniam, P. Madhiyazhagan, L. Wang, U. Suresh, P. M. Kumar, J. Mohan, R. Rajaganesh,, H. Wei, K. Kalimuthu, M. N. Parajulee, H. Mehlhorn, G. Benelli, Parasitology Research 114 (2015) 4349–4361.

[45] U. Muthukumaran, M, Govindarajan, M, Rajeswary, Parasitology Research 114 (2015) 4385–4395.

[46] G. Rajakumar, A.A. Rahuman, Acta Tropica 118 (2011) 196–203.

[47] A. Rawani, A. Ghosh, G. Chandra, Acta Tropica 128 (2013) 613– 622.

[48] M. Roni, K. Murugan, C. Panneerselvam, J. Subramaniam, M. Nicoletti, P. Madhiyazhagan, D. Dinesh, U. Suresh, H. F. Khater, H. Wei, A. Canale, A. A. Alarfaj, M. A. Munusamy, A. Higuchi, G. Benelli, Ecotoxicology and Environmental Safety 121 (2015) 31–38.

[49] J. Subramaniam, K. Murugan, C. Panneerselvam, K. Kovendan, P. Madhiyazhagan, P. M. Kumar, D. Dinesh, B. Chandramohan, U. Suresh, M. Nicoletti, A. Higuchi, J. Hwang, S. Kumar, A. A. Alarfaj, M, A. Munusamy, R. H. Messing, G. Benelli, Environmental Science and Pollution Research 22 (2015) 20067–20083.

[50] G. Suganya, S. Karthi, M. S. Shivakumar, Parasitology Research 113 (2014) 1673–1679.

[51] V. Sujitha, K. Murugan, M. Paulpandi, C. Panneerselvam, U. Suresh, M. Roni, M. Nicoletti, A. Higuchi, P. Madhiyazhagan, J. Subramaniam, D. Dinesh, C. Vadivalagan, B. Chandramohan, A. A. Alarfaj, M. A. Munusamy, D. R. Barnard, G. Benelli, Parasitology Research 114 (2015) 3315–3325.

[52] T. Y. Suman, S. R. R. Rajasree, C. Jayaseelan, R. R. Mary, S. Gayathri, L. Aranganathan, R. R. Environmental Science and Pollution Research 23 (2016) 2705–2714.

[53] K. Veerakumar, M. Govindarajan, M. Rajeswary, U. Muthukumaran, Parasitology Research 113 (2014) 1775–1785.

[54] M. Christofoli, E. C. C. Costa, K. U. Bicalho, V. C. Domingues, M. F, Peixoto, C. C. F. Alves, W. L. Araújo, C. M. Cazal, Industrial Crops and Products 70 (2015) 301-308.

ABSTRACT

Plants produce a wide range of substances from secondary metabolism in response to environment. They are biosynthesized for many purposes, including protection against insect attack. Since ancient times, empirical knowledge guided utilization of several plant species as insecticidal agents. Latter, laboratorial investigation allowed identification of many of these bioactive natural products, such as essential oil constituents, diterpenes, triterpenes, alkaloids and others. However, several insecticidal agents from plant origin has poor water solubility in water, being more soluble in organic solvents, potentially toxic to non-targets organisms, including humans, and to the environment. Thus, this fact is considered one of most important technological challenge to develop viable products. Nanotechnology emerged as a promising area to solve this main problem. It comprehends manipulation or obtainment of structures in the nano scale, allowing achievement of several distinctive properties, including enhancement of water solubility, improved chemical and physical stability and even controlled release of substances. On this context, the aim of this review is to show state of art in utilization of natural products to obtain nano-base insecticidal agents.

Key-words: metallic nanoparticles, nanoemulsions, nanopesticides, polymeric nanoparticles, secondary metabolites.

DEVELOPMENT OF A LARVICIDAL NANOEMULSION WITH *Pterodon emarginatus* Vogel OIL

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RESEARCH ARTICLE

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Development of a Larvicidal Nanoemulsion with *Pterodon emarginatus* Vogel oil

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Abstract

Pterodon emarginatus Vogel is a Brazilian species that belongs to the family Fabaceae, popularly known as sucupira. Its oil has several biological activities, including potent larvicidal property against *Aedes aegypti*. This insect is the vector of dengue, a tropical disease that has been considered a critical health problem in developing countries, such as Brazil. Most of dengue control methods involve larvicidal agents suspended or diluted in water and making active lipophilic natural products available is therefore considered a technological challenge. In this context, nanoemulsions appear as viable alternatives to solve this major problem. The present study describes the development of a novel nanoemulsion with larvicidal activity against *A. aegypti* along with the required Hydrophile Lipophile Balance determination of this oil. It was suggested that the mechanism of action might involve reversible inhibition of acetylcholinesterase and our results also suggest that the *P. emarginatus* nanoemulsion is not toxic for mammals. Thus, it contributes significantly to alternative integrative practices of dengue control, as well as to develop sucupira based nanoproducts for application in aqueous media.

Keywords: Hydrophile Lipophile Balance, Nanoemulsion, *Pterodon emarginatus*, Sucupira.

Introduction

Pterodon emarginatus Vogel is a Brazilian species that belongs to the family Fabaceae. It is widely distributed in some regions of this country, including the states of Goiás, São Paulo and Minas Gerais [1]. *P. emarginatus* is commonly known as 'sucupira' and 'sucupira-branca' [2], being recognized as an important plant used in folk medicine. It was described by naturalists from 19th century [3] and has been used to treat inflammation and influenza [3,4]. Essential oils mainly obtained from fruits and seeds of this species have been extensively studied. Biological activities attributed to these volatile mixtures of substances include antimicrobial [5,6], anti-ulcerogenic, anti-inflammatory [7], and cytotoxic [8] properties, besides the control of autoimmune encephalomyelitis [9]. Seeds *of P. emarginatus* are also used as raw material to obtain an amber-colored viscous oil that has been considered a rich source of diterpenes from the vouacapane type [10,11,12]. These terpenes are important bioactive substances, responsible for anti-inflammatory and analgesic properties [11]. In addition to the anti-inflammatory activity [2], the seed oil also present protection against oxidative stress [13] and leishmanicidal activity against promastigotes of *Leishmania amazonensis* [5]. Finally, bioguided fractionation indicated that seed oil and vouacapane diterpenes are promising natural biocontrol agents against the mosquito *Aedes aegypti* [12].

A. aegypti is the main vector of a disease called dengue, which is considered a public health problem in tropical countries. Brazil had more than 4 million reported cases of dengue between 2010-2014. Therefore, it is considered one of most important public health problems in the country [14]. Natural products from plant origin have been considered very promising for integrative practices to control vectors of tropical diseases [15,16]. Nowadays, growing interest has been devoted to the development of nano-size products for this purpose. This innovative kind of products emerged as an alternative for vector control and has potential

application in different stages of insect development [17]. Merging natural products and nanotechnology opens a very promising field for integrated control programs. For example, nanoformulations may act as adulticidal [18], repellent [19] and larvicidal agents [20]. The later approach is one of most effective alternatives to prevent tropical diseases, eliminating vector's larvae. Several natural product-based nanoformulations were reported as potential larvicidal agents [21], including nanoemulsions [20]. One major advantage of nano-size products as larvicidal products rely on the fact that they turn possible highly homogeneous dispersion of low water soluble substances in aqueous media, including herbal oils [22,23]. Moreover, natural product-based nanoformulations have being considered potential environmental low toxic agents and are thus recognized as ecofriendly products [20,21,24].

Nanoemulsions are dispersed systems constituted by two immiscible liquids often stabilized by one or more surfactants. They have small droplet size, often comprised between 20-200 nm, which allows translucent or transparent appearance [25]. Moreover, fine small droplets obtained even at relatively low surfactant concentrations generate kinetic stable systems thus preventing unstable behavior during storage of nanoemulsions, such as gravitational separation or particle aggregation [26,27]. This is a great advantage of nanoemulsions over microemulsions, which are thermodynamically stable systems that require large amounts of surfactant(s) [28]. Methods for nanoemulsion preparation are basically classified into two main groups, named high energy methods and low energy methods. High energy methods involve specific equipment, including high-shear stirring, high-pressure homogenizers and ultrasound generators. Low energy methods use physicochemical properties of the systems and often involve phase inversion [29]. It is well known that surfactants play a crucial role in nanoemulsion formation, especially if low energy methods are employed [30]. In this context, development stage for nanoemulsion preparation is a critical step in order to obtain stable fine systems. Determination of required Hydrophile

Lipophile Balance (rHLB) of the oil has been recognized as an important parameter that should be investigated. This approach relies on the fact that small droplets are formed when the rHLB of oil coincides with HLB of surfactant(s) [31,32].

Despite its great biological potential, the oil of *P. emarginatus*, remains to our knowledge unexplored regarding the development of an innovative nanoformulation. Hence, the present study aims to obtain an optimized larvicidal nanoemulsion using *P. emarginatus* as the bioactive constituent.

Materials and Methods

Chemicals

Sorbitan monooleate and polysorbate 80 were purchased from Praid Produtos Químicos Ltda (SP, Brazil). Acetylthiocholine iodide (ATCI) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich (St Louis, MO).

Plant material

Fruits from *Pterodon emarginatus* Vogel (Fabaceae) were obtained at the Central Market of Goiânia – GO (Brazil). Identification of plant material was performed by Dr. José Realino de Paula and a voucher specimen was deposited at the Herbarium of Goiás Federal University (GO, Brazil) under the register number 41714.

Pterodon emarginatus oil

Oil from *P. emarginatus* fruits was obtained by cold pressing, using a mini mechanical press (MPE-40 ECIRTEC). It was then weighed and hermetically stored in amber glass flask and kept at −20 °C until utilization. Extraction yield (in %) was calculated according to the ratio between obtained oil and starting fruits masses.

Identification of *P. emarginatus* metabolites

Identification of secondary metabolites from *P. emarginatus* oil was performed by comparison of the ultraviolet (UV) spectra and retention times (unpublished data) with those of external standards: β-caryophyllene (purity ≥ 98.5% Sigma – Aldrich®), geranylgeraniol (purity ≥ 85% Sigma – Aldrich®) and the vouacapan diterpenes 6α,7β-dihydroxyvouacapan-17-β-oic acid, methyl 6α,7β-di-hydroxyvouacapan-17-β-oate and 6α,hydroxyvouacapan-7β,17β-lactone (kindly provided by Dra. Dorila Piló Veloso.

Emulsification method

Emulsification was performed using a modification of the low energy method of Ostertag et al. [27]. Emulsions were constituted as follows: 5% (w/w) of *P. emarginatus* oil, 5% (w/w) of surfactants and 90% (w/w) of water. Oily phase was constituted by *P. emarginatus* oil and surfactants, being pooled together and submitted to magnetic stirring (400 rpm) for 30 min under controlled temperature (80 ± 5 ºC) in a water bath. After this, the aqueous phase (distilled water) was added to the oily phase under continuous magnetic stirring (400 rpm) for 1 hour, allowing the temperature to decrease gradually to room temperature (approximately 30 min). Final step consisted in the addition of deionized water,

under magnetic stirring (400 rpm, 10 min) in order to restore the original mass (10 g) of emulsions.

Required Hydrophile-Lipophile Balance (rHLB) Determination

Determination of rHLB of *P. emarginatus* was performed by blending sorbitan monoleate (HLB – 4.3) and polysorbate 80 (HLB – 15) at different ratios [33], in order to obtain several emulsions at a wide range of HLB (4.3-15). Composition and preparation of emulsions was according to emulsification method presented above.

Optimized *P. emarginatus* nanoemulsion

An optimized *P. emarginatus* nanoemulsion was prepared by diluting emulsion at rHLB immediately after preparation with distilled water (1:20); final oil concentration was 2500 ppm. This nanoemulsion was immediately used for *in vivo* and *in vitro* biological assay. It was stored, protected from light, at room temperature for further analyses.

Nanoemulsion characterization

Droplet size, polydispersity index and zeta potential of the nanoemulsion were determined by photon correlation spectroscopy (Zetasizer ZS, Malvern, UK). Emulsions from HLB determination were diluted with water (1:25, v/v), for injection [32]. Optimized *P. emarginatus* nanoemulsion was monitored immediately after preparation and after 1, 2, 7, 14, 21, 30 and 60 days of preparation, being diluted with distilled and deionized water (1:10, v/v). Droplet measurements were performed in triplicate and average droplet size was expressed as the mean diameter ± standard deviation.

Larvicidal assay

Aedes aegypti larvae were obtained from the Arthropoda Laboratory (Universidade Federal do Amapá, Brazil). Biological assay was performed under controlled conditions, using fourth-instar larvae kept at 25±2 ºC, under relative humidity of 75±5% and a 12h light:dark cycle. Experimental protocol was performed according to WHO (2005) [34]. All experiments were performed in triplicate with 10 forth-instar larvae in each replicate (n = 30) Optimized *P. emarginatus* nanoemulsion was diluted in distilled water at 250, 100, 75, 50, 25, 12.5 ppm (relative to *P. emarginatus* oil). Control group was treated with deionized water. Mortality levels were recorded after 24 and 48 hours of exposure.

Preparation of whole body homogenate

Whole body homogenate was prepared according to Sugumar et al. (2014) [23]. Larvae from treated (250 ppm) and control groups were collected and water was gently removed using tissue paper. Then each group was separately homogenized using 3.0 mL phosphate buffered saline (PBS) 0.1 M (pH = 7.5). This step was performed using a T25 Ultra-Turrax homogenizer (Ika-Werke, Staufen, Germany) running at 12000 rpm for 1 min. Then, the homogenates were centrifuged for 30 min (5000 rpm) under controlled temperature (10 ºC). Whole body homogenate supernatants were collected and immediately used for enzymatic assays.

Enzymatic assays

Anticholinesterase activity was performed grossly according to the method described by Ellman et al. (1961) [35].

Acetylcholinesterase activity in whole body homogenate

Briefly, 0.25 mL of whole body homogenate supernatant from treated group (250 ppm) and 0.5 mL of DTNB were added to 2.0 mL of PBS. This solution was incubated for 10 min (25 ± 1 ºC). After this, 0.25 mL of ATCI was added and the absorbance measured at 400 nm using a UV-Mini spectrophotometer (Shimadzu). Blank was performed using whole body homogenate supernatant from control group and assays were performed in triplicate.

Anticholinesterase activity induced by optimized *P. emarginatus* nanoemulsion

Activity of acetylcholinesterase from control group whole body homogenate, after exposure to optimized *P. emarginatus* nanoemulsion (A_1), was determined as follows. Solution of 0.25 mL of this nanoemulsion, 0.25 mL of whole body homogenate supernatant (control group) and 0.5 mL of DTNB to 1.75 mL of phosphate buffer was incubated for 10 min (25 ± 1 ºC). Then 0.25 mL of ATCI were added and the absorbance measured at 410 nm using a UV-Mini spectrophotometer (Shimadzu). Blank was obtained by replacing the ATCI by a same amount of PBS. Maximum acetylcholinesterase activity (A_2) was achieved by replacing the amount of *P. emarginatus* nanoemulsion by PBS. Assays were performed in triplicate. The percentage of inhibition was calculated as follows:

$$\% \text{ Inhibition} = 100 - [(A_1 \times 100)/ A_2]$$

Acute toxicity of optimized *P. emarginatus* nanoemulsion on non-target species

Animals

This study was approved by the Animal Ethics Committee of Universidade Federal do Amapá (CEP – UNIFAP – 0018/2014). All procedures were performed according to the International Committee for animal care in accordance with established national regulations for animal experimentation. The experiments were performed using adult female Swiss albino mice (*Mus musculus*), 8 weeks age, provided by the Multidisciplinar Center for Biological Investigation in Experimental Animals Science Area from (CEMIB/SP) Campinas University. Each experimental group was composed of 3 animals. They were kept in polyethylene cages on a temperature-controlled rack (23°C ± 2°C), under a 12-hour light-dark cycle. Food and water where furnished *ad libido*, except for the 12 hours before the experiments, when food was suppressed and they had access only to water.

Experimental protocol

Acute toxicity studies were performed using female mice according to a modified OECD 423 protocol (2001) [36,37,38]. Treated groups had free access to optimized *P. emarginatus* nanoemulsion diluted in water at 250, 125 and 50 ppm (expressed as *P. emarginatus* oil content in aqueous media, see above) daily for fourteen days.

Behavioral analysis was performed at 0.5, 1, 2, 4, 8, 12 and 24 h after the oral treatment and then daily for fourteen days. Behavioral changes (hyperactivity, convulsions, vocal fremitus, irritation, stereotyped movements, touch response, salivation, tremors, writhing, body

distension, ptose, sleepiness, defecation, diarrhea, piloerection), weight, food and water intake, clinical signs of toxicity and mortality were recorded daily. At the end of fourteen days, they were sacrificed by cervical dislocation and taken to autopsy for macroscopic observation of the organs (heart, lung, liver, kidney and spleen).

Statistical Analysis

Analysis of variance (ANOVA) followed by Tukey´s test was conducted using the Software GraphPad Prism v.5.03 (San Diego, California, USA). Differences were considered significant when $P \leq 0.05$. Probit analysis was performed with 95% confidence interval for LC50 determination.

Results and Discussion

Extraction of *P. emarginatus* fruits yielded 30% (w/w) of a viscous yellow oil. Diterpenes methyl 6α,7β-dihydroxyvouacapan-17-β-oate (MHV), geranylgeraniol and sesquiterpene β-caryophyllene were detected on *P. emarginatus* oil by comparison to external standards. Vouacapan diterpenes have been considered potential bioactive substances from fruits of *P. emarginatus* [11]. Moreover, literature data indicates that some of them, including methyl 6α,7β-dihydroxyvouacapan-17-β-oate, presented potential larvicidal activity [12]. Fig 1 shows the UV spectrum of the diterpene MHV. The UV spectra recorded for other substances present in chromatogram of *P. emarginatus* oil that may be associated to additional vouacapan skeleton diterpenes in this sample are presented in Fig 2.

Fig 1: UV spectra obtained by HPLC-DAD of diterpene MHV.
Fig 2: UV **s**pectra from non-identified compounds from *P. emarginatus* oil suggesting presence of other vouacapan diterpenes.

Natural products display a wide range of biological activities that make them promising candidates for a number of applications. Among the various classes of natural

products, terpenoids and more specifically diterpenes are potentially useful as insecticide agents [39]. However, these substances often possess poor water solubility, being usually much more soluble in toxic organic solvents. Alternatives to enhance their water solubility are desirable looking for viable eco-friendly active products [40]. Classical chemical reactions may be used, giving rise to water soluble compounds such as forming terpenoid salts [41]. Another strategy relies on the development of stable nanosystems, which can improve water solubility of these substances by entrapping then into micelles with nano-size diameter [42]. Nanobiotechnology is an emerging area with great potential for innovative products, including eco-friendly pesticides. Thus, we decided to develop *P. emarginatus* oil in water nanoemulsions, using oil as the internal phase in order to enhance its water solubility and verify its potential as a vector control.

Emulsions at HLB 4.3, 5, 6, 7, 8 and 9 presented some signals of instability after preparation, including presence of sedimentation and phase separation. Emulsions at HLB 10, 11, 12, 13, 14 and 15 presented slight yellow appearance and homogeneous aspect. After one day of storage, emulsions at HLB 15 and 14 presented highest mean droplet size (HLB 15: 459.2 ± 6.8 nm; HLB 14: 262.1 ± 4.3 nm) and polydispersity index (HLB 15: 0.393 ± 0.006; HLB 14: 0.192 ± 0.008). Emulsions at HLB 10 and 13 presented mean droplet size below 200 nm after 1 day of preparation. However, after 7 days, values increased above this upper limit. Smallest droplets size was observed for emulsions at HLB 12 (126.7 ± 0.9 nm) and HLB 11 (135.8 ± 0.2 nm), analyzed after 1 day.

After 7 days, smallest mean droplet size was observed for formulation at HLB 11. Moreover, this emulsion presented less variation of both mean droplet size and polydispersity index parameters, when compared to emulsion at HLB 12. Characterization of nanoemulsions must involve determination of droplet size, since these nanodispersed system are classified according to droplet diameter. Dynamic light scattering (DLS) is one of most used technique

for this purpose, giving average size and size distribution of droplets, also offering some advantages, such as fast and easy analysis. However, complementary techniques may be used to corroborate data obtained by DLS, such as transmission electron microscopy, which measures mean diameter of individual droplets and also provides important information about surface and morphology of droplets [43]. Zeta potential is also considered an important parameter regarding stability of nanoemulsions. It is associated to surface potential of droplets that constitute these dispersed systems. Zeta potential values obtained in the present study were in accordance with stable behavior [44]. Mean droplet size, polydispersity index and zeta potential of emulsions at HLB 11 and 12 are presented in table 1.

Hydrophile-Lipophile Balance was formerly described as an useful scale for selecting emulsifiers. Low HLB values were attributed to more lipophilic agents and high HLB values were attributed to more hydrophilic agents [45]. It is well established that most stable emulsion, and therefore smaller droplets, are achieved when HLB of surfactant(s) coincides with rHLB of the oil [46]. In this context, it is desirable that rHLB of oily phase constituents, including herbal oils, should be measured. Basically, the procedure aiming this purpose has been performed by blending pairs of surfactants at different ratios and monitoring most stable emulsion as a valuable tool to determine rHLB [32,47]. Thus, our results suggest that oil from fruits of *P. emarginatus* has HLB of 11. Some of our recent studies reported generation of nanoemulsions with good physical properties using this approach [33,48,49,50], as it could be achieved in the present study. However, in some cases it could be observed that it was not possible to obtain nanoemulsions during rHLB determination [46,47]. To overcome this problem, some studies with important Brazilian herbal oils were carried out with organic solvent-based methods, high energy methods or merging both techniques. Addition of aqueous phase to an oily phase containing copaiba (*Copaifera multijuga*) oil was unsuccessful to induce formation of nanoemulsions, being required an additional step using a high pressure

homogenizer to generate intense disruptive forces and achieve droplets with mean diameter around 160-300 nm. In this same study, spontaneous generation of nanoemulsions was only achieved by solubilizing oily phase with organic solvents, resulting in mean droplet size around 160-315 nm [51]. In another study carried out with andiroba (*Carapa guianensis*) and aroeira (*Schinus molle*) oils, solubilization of oily phase with organic solvent allowed only to obtain coarse emulsions; nanoemulsions (respectively with mean droplet around 240 and 130 nm) could finally be produced by a further high pressure homogenization step [52]. Besides the aforementioned disadvantage of high energy methods due to increasing costs of the process, utilization of organic solvents should be avoided if development of an eco-friendly product is desired. It is well known that a supplementary evaporation step using a rotary evaporator should be performed to remove the organic solvent from internal or external phases of the nanoemulsion. However, literature data suggests that residual levels of the organic solvent may remain entrapped and gradually released [53].

Due to their small size and potential kinetic stability, even at relatively low surfactant concentrations [25], nanoemulsions have been considered potential innovative products for a wide range of applications. Transparent or translucent aspect of nanoemulsions is also an attractive, being this type of nanoformulation very useful to incorporate lipophilic agents, such as herbal oils, into aqueous products [54]. Special attention is given for natural-products-based nanoemulsions as ecofriendly pesticides, considering their potential advantages, including low risk for non-target organisms and the fact that they are often more easily biodegraded [20,23,42]. Optimized *P. emarginatus* nanoemulsion was prepared by diluting nanoemulsion at HLB 11 and presented fine appearance and translucent aspect (Fig 3). It was also characterized as an O/W system, being able to be dispersed in aqueous media. Almost no variation was observed for droplet size and polydispersity index during storage (60 days), suggesting that the system has a great tendency to achieve kinetic stability. Narrow size

distribution with monomodal aspect was observed, being in accordance with high homogeneity and low polydispersity nanodispersed systems [55,56]. Particle size distribution during storage of optimized *P. emarginatus* nanoemulsion is presented in Fig 4.

Fig 3. Optimized *P. emarginatus* nanoemulsion used for in vitro and in vivo biological assays.

Fig 4. Mean droplet size - Day 0: 125.1 ± 0.5 nm; Day 1: 124.4 ± 0.4 nm; Day 2: 125.6 ± 0.5 nm; Day 7: 124.8 ± 0.3 nm; Day 14: 127.5 ± 0.2 nm; Day 21: 131.0 ± 0.5 nm; Day 30: 134.3 ± 0.8 nm; Day 60: 129.2 ± 1.0 nm. Polydispersity index - Day 0: 0.175 ± 0.014; Day 1: 0.185 ± 0.012; Day 2: 0.196 ± 0.006; Day 7: 0.174 ± 0.003; Day 14: 0.194 ± 0.013; Day 21: 0.192 ± 0.007; Day 30: 0.210 ± 0.006; Day 60: 0.193 ± 0.001.Zeta potential - Day 0: -30.9 ± 0.4 mV; Day 1: -29.6 ± 1.4 mV; Day 2: -29.6 ± 1.4 mV; Day 7: -33.1 ± 3.3 mV; Day 14: -35.4 ± 2.8 mV; Day 21: -35.4 ± 1.2 mV; Day 30: -47.1 ± 1.5 mV; Day 60: -41.0 ± 6.34 mV.

As part of our ongoing studies on the development of larvicidal nanoemulsions with Brazilian herbal oils, we decided to investigate the effects of *P. emarginatus* nanoemulsion on *Aedes aegypti* larvae. Groups of larvae treated with optimized *P. emarginatus* nanoemulsion at 250 ppm (expressed as *P. emarginatus* oil content in aqueous media) presented mortality level of 100 %. According to literature data, a natural product is considered a promising larvicidal agent when mortality of treated group at 250 ppm reaches mortality levels higher than 75 % [57]. A similar effect was observed for a larvicidal nanoemulsion against *Culex quinquefasciatus*, which induce 98% of mortality at 250 ppm after 24 h of treatment [23]. No mortality was observed in the *A. aegypti* control groups. ANOVA test indicated statistical differences in mortality between tested concentrations after 24 h (F value = 27.88; p = 0.0000)

and 48 h (F value = 31.10; p = 0.0000). It was also observed that mortality rate was dependent on time for treated groups at 12.5, 25, 50 and 75 ppm. This observation is in accordance with previous data of larvicidal nanoemulsions, in which mortality levels increased with time exposure [58]. Mortality levels induced by nanoemulsion prepared with *P. emarginatus* oil are presented in table 2. LC50 after 48 h was estimated as 34.75 (7.31 – 51.86) ppm (expressed as *P. emarginatus* oil content in aqueous media).

Most studies carried out with larvicidal nanoemulsions do not present LC50 values [20,23,48,50]. Anjali et al. (2012) [58], in turn, observed that neem nanoemulsion with mean droplet size of 93.0 nm presented LC50 of 25.99 ppm against *C. quinquefasciatus*. Regarding nanoparticles, a study aiming the investigation of larvicidal potential of phyto-based nickel nanoparticles indicated a LC50 of 534.83 ppm against *A. aegypti*, while temephos showed a LC50 of 507.62 ppm [59]. Considering that bioaccumulation is considered a major problem regarding environmental toxicity [60], safety of this metal-based nanoparticles should be deeply investigated. In this respect, our results suggest that optimized *P. emarginatus* nanoemulsion presented a larvicidal activity in accordance with literature data for classical herbal derived nanoemulsions. It also may be considered potentially more effective than other nanoformulations and classical chemicals used for vector control.

Despite aforementioned advantages of oil in water nanoemulsion as larvicidal agents, a possible increase of biological activity should also be mentioned. Study carried out with nanoformulations containing herbal oil and geraniol indicated a positive correlation between larvicidal and small size droplets [22]. Anjali et al. (2012) [58] also reported that neem nanoemulsions with smaller mean droplet size presented smaller LC50 values against *C. quinquefasciatus*. Hence, development of a nanoemulsion can be considered a good approach to develop viable innovative products for insect control, even enhancing biological activity of natural compounds. Classical bioactive Brazilian herbal oils, such as *Carapa guianensis* and

Copaifera sp presented LC50 around 50 and 150 ppm, respectively [61]. On the other hand, previous study carried out with vouacapan diterpenes from *P. emarginatus* indicated that MHV presented LC50 of 21.76 ppm [12]. In the present study, the observed LC50 is closer to those expected for isolated diterpenes and indicated that this nanoformulation is more active than some non-formulated herbal oils of great interest.

Table 2. Mortality levels (%) of *Aedes aegypti* larvae after treatment with optimized *P. emarginatus* nanoemulsion (expressed as *P. emarginatus* oil content in aqueous media).

Several natural products from plant origin were reported as potential insecticides, including diterpenes [62]. One of the possible mechanisms involved is inhibition of acetylcholinesterase of the insects [39,63]. Comparison between acetycholinesterase activity of *A. aegypti* larval homogenates (control and tested group) indicated no statistically significant difference ($P>0.05$) regarding acetylcholinesterase activity. However, it was observed that *P. emarginatus* oil-based nanoemulsion added to whole body homogenate from control group of *A. aegypti* larvae presented anticholinesterase activity, being able to inhibit the enzyme activity around 50.22%. Previous study performed with terpenoids indicated that some of them induced a reversible inhibition of acetylcholinesterase, verified by recovery of enzyme activity after 15 min of incubation [63]. Thus, it could be suggested that acetylcholinesterase inhibitors from *P. emarginatus* detached from the enzyme, explaining similar acetylcholinesterase activity on *A. aegypti* whole body larval homogenates (control and treated groups).

A. aegypti preference for clearwater and its ability to grow and develop in human-made receptacles, such as discarded pots, tires and water storage containers is well known [64]. Moreover, this vector became fully adapted to human environment [65]. Vector control in drinking water sources and containers using larvicidal chemicals, such as temephos, is

recognized as an effective strategy [66]. However, utilization of home pesticides may generate intoxication of human [16] and/or domestic animals, such as dogs and cats [67]. Thus, low toxicity for both humans and other non-target organisms is a main characteristic that should be considered for development of novel larvicidal products [68].

In the present study, it was decided to investigate the action of optimized *P. emarginatus* nanoemulsion on non-target species using adult female Swiss albino mice (*Mus musculus*) as a model for mammals. No behavioral alteration, death or macroscopical changes in organs (heart, lung, liver, kidney and spleen) could be observed (Data not shown). Moreover, the absence of any significant change in body weight, food and water intake (Fig 5) also suggests that optimized *P. emarginatus* nanoemulsion may be not toxic for mammals.

Fig 5. Analysis of body weight (A), water (B) and food intake (C) variation in mice treated with optimized *P. emarginatus* nanoemulsion.

Conclusions

Oil from seeds of *P. emarginatus* is recognized as a plant derived product with great biological potential, including for larval control of dengue vectors. However, it remained unexplored until this moment regarding development of a nanobiotechnology product. The present study allowed development of a novel nanoemulsion with larvicidal activity against *A. aegypti* along with determination of rHLB of this oil. Moreover, it could be suggested that the mechanism of action may involve reversible inhibition of acetylcholinesterase and that the nanoemulsion may be safe for mammals.

It is worth mentioning that our optimized *P. emarginatus* nanoemusion was prepared by a low energy and solvent-free method, reducing costs of the process and being in

agreement with eco-friendly products requirements. Absence of coating which is necessary for nanoparticle preparation with no impairment to physical stability and larvicidal activity can be considered an advantage in terms of process costs. Moreover, utilization of eco-friendly surfactants is also an advantage, avoiding bioaccumulation which is associated to other type of nanoformulations. Thus, it contributes significantly to alternative integrative practices of dengue control, as well as developing of sucupira based nanoproducts for application on aqueous media.

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REFERENCES

1. Lima HC, Lima IB. *Pterodon* in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. Avaiable at: <http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB29842>. Acessed in 17 Jun. 2015.
2. Carvalho JC, Sertié JA, Barbosa MV, Patrício KC, Caputo LR, Sarti SJ, et al. Anti-inflammatory activity of the crude extract from the fruits of *Pterodon emarginatus* Vog. J Ethnopharmacol. 1999; 64: 127-33.
3. Fagg CW, Lughadha EN, Milliken W, Hind DJN, Brandão MGL. Useful Brazilian plants listed in the manuscripts and publications of the Scottish medic and naturalist George Gardner (1812–1849). J Ethnopharmacol. 2015; 161: 18-29.
4. Medeiros PM, Pinto BLS, Nascimento VT. Can organoleptic properties explain the differential use of medicinal plants? Evidence from Northeastern Brazil. J Ethnopharmacol. 2015; 159: 43-48.
5. Dutra RC, Braga FG, Coimbra ES, Silva AD, Barbosa NR. Atividades antimicrobiana e leishmanicida das sementes de *Pterodon emarginatus* Vogel. Braz J Pharmacog. 2009; 19: 429-435.
6. Ferreira SB, Dantas IC, Catão RMR. Avaliação da atividade antimicrobiana do óleo essencial de sucupira (Pterodon emarginatus Vogel). Rev Bras Pl Med. 2014; 16: 225-230.

7. Dutra RC, Fava MB, Alves CCS, Ferreira AP, Barbosa NR. Antiulcerogenic and anti-inflammatory activities of the essential oil from Pterodon emarginatus seeds. J Pharm Pharmacol. 2009, 61; 243-250.
8. Dutra RC, Pittella F, Dittz D, Marcon R, Pimenta DS, Lopes MTP, et al. Chemical composition and cytotoxicity activity of the essential oil of Pterodon emarginatus. Braz J Pharmacog. 2012; 22: 971-978.
9. Alberti TB, Marcon R, Bicca MA, Raposo NRB, Calixto JB, Dutra RC. Essential oil from Pterodon emarginatus seeds ameliorates experimental autoimmune encephalomyelitis by modulating Th1/Treg cell balance. J Ethnopharmacol. 2014; 155: 485-494.
10. Mahajan JR, Monteiro MB. New Diterpenoids from Pterodon emarginatus Vog. J Chem Soc Perkin 1.1973; 5: 520-525.
11. Galceran CB, Sertie JAA, Lima CS, Carvalho JCT. Anti-inflammatory and analgesic effects of 6a,7b–dihydroxy-vouacapan-17b-oic acid isolated from Pterodon emarginatus Vog. Fruits. Inflammopharmacol. 2011; 19:139–143
12. Omena MC, Bento ES, De Paula JE, Sant'ana AEG. Larvicidal Diterpenes from Pterodon polygalaeflorus. Vector Borne Zoonotic Dis. 2006; 6: 216-222.
13. Paula FBA, Gouvêa CMCP, Alfredo PP, Salgado I. Protective action of a hexane crude extract of *Pterodon emarginatus* fruits against oxidative and nitrosative stress induced by acute exercise in rats. BMC Complementary and Alternative Medicine. 2005 Aug 17. Doi: 10.1186/1472-6882-5-17.
14. Brasil. Casos de Dengue. Brasil, Grandes Regiões e Unidades Federadas. 1990 a 2014. Portal da Saúde – Ministério da Saúde. 13 Jul 2015. Available at: <http://portalsaude.saude.gov.br/images/pdf/2015/julho/29/Dengue-at---2014.pdf>. Accessed in 29 Sep. 2015.

15. Rehman JU, Ali A, Ikhlas A. Khan. Plant based products: use and development as repellents against mosquitoes: a review. Fitoterapia. 2014; 95: 65-74.
16. Mann RS, Kaufman PE. Natural Product Pesticides: Their Development, Delivery and Use Against Insect Vectors. Mini-Rev Org Chem. 2012; 9: 185-202.
17. Barik TP, Kamaraju R, Gowswami A. Silica nanoparticle: a potential new insecticide for mosquito vector control. Parasitol Res. 2012; 111: 1075–1083.
18. Veerakumar K, Govindarajan M. Adulticidal properties of synthesized silver nanoparticles using leaf extracts of Feronia elephantum (Rutaceae) against filariasis, malaria, and dengue vector mosquitoes. Parasitol Res. 2014; 113:4085–4096.
19. Nuchuchua O, Sakulku U, Uawongyart N, Puttipipatkhachorn S, Soottitantawat A, Ruktanonchai U. In Vitro Characterization and Mosquito (Aedes aegypti) Repellent Activity of Essential-Oils-Loaded Nanoemulsions. AAPS PharmSciTech. 2009; 10: 1234-1242.
20. Ghosh V, Mukherjee A, Chandrasekaran N. Formulation and Characterization of Plant Essential Oil Based Nanoemulsion: Evaluation of its Larvicidal Activity Against Aedes aegypti. Asian J Chem. 2013; 25: 321-323.
21. Angajala G, Ramya R, Subashini R. In-vitro anti-inflammatory and mosquito larvicidal efficacy of nickel nanoparticles phytofabricated from aqueous leaf extracts of *Aegle marmelos* Correa. Acta Trop. 2014; 135: 19-26.
22. Montefuscoli AR, González JOW, Palma SD, Ferrero AA, Band BF. Design and development of aqueous nanoformulations for mosquito control. Parasitol Res. 2014; 113: 793–800.
23. Sugumar SKS, Clarke MJ, Nirmala BK, Tyagi AM, Chandrasekaran N. Nanoemulsion of eucalyptus oil and its larvicidal activity against Culex quinquefasciatus. Bull Entomol Res. 2014; 104: 393–402.

24. Soni N, Prakash S. Green Nanoparticles for Mosquito Control. The Scientific World Journal. 2014 Aug 27. Article ID496362, 6 pages. doi: 10.1155/2014/496362.

24. Solè I, Solans C, Maestro A, González C, Gutiérrez JM. Study of nanoemulsion formation by dilution of microemulsions. J Colloid Interface Sci. 2012 376: 133-139.

25. Tadros T, Izquierdo P, Esquena J, Solans C. Formation and stability of nanoemulsions. Adv Colloid Interface Sci. 2004; 108: 303–318.

26. Ostertag F, Weiss J, McClements DJ. Low-energy formation of edible nanoemulsions: Factors influencing droplet size produced by emulsion phase inversion J Colloid Interface Sci. 2012; 388: 95–102.

27. Forgiarini A, Esquena J, González C, Solans C. Studies of the relation between phase behavior and emulsification methods with nanoemulsion formation. Progr Colloid Polym Sci. 2000; 115: 36-39.

28. Solans C, Izquierdo P, Nolla J, Azemar N, Garcia-Celma MJ. Curr Opin Colloid Interface Sci. 2005; 10: 102-110.

29. Wang L, Dong J, Chen J, Eastoe J, Li X. Design and optimization of a new self-nanoemulsifying drug delivery system. J Colloid Interface Sci. 2009; 330: 443–448.

30. Sevcikova P, Kasparkova V, Vltavska P, Krejci J. On the preparation and characterization of nanoemulsions produced by phase inversion emulsification. Colloids and Surfaces A: Physicochem Eng Aspects. 2012; 410: 130-135.

31. Fernandes CP, Mascarenhas MP, Zibetti FM, Lima BG, Oliveira RPRF, Rocha L, et al. HLB value, an important parameter for the development of essential oil phytopharmaceuticals. Braz J Pharmacog. 2013; 23: 108-114.

32. Costa IC, Rodrigues RF, Almeida FB, Favacho HAS, Falcão DQ, Ferreira AM, et al. Development of Jojoba Oil (Simmondsia chinensis (Link) C.K. Schneid.) Based Nanoemulsions. Lat Am J Pharm. 2014; 33:459-463.

33. WHO. World Health Organization 2005. Guidelines for laboratory and field testing of mosquito larvicides. World health organization Communicable disease control, prevention and eradication Who pesticide evaluation scheme. Geneva, Swizerland.
34. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmaco.1961; 7: 88-95.
35. OECD (Organization for economic cooperation and development) 2001. *Guideline for Testing of Chemicals: Acute Oral Toxicity-Acute Toxic Class Method.* Guideline: 423.
36. Hamdaoui M, Hedhili A, Doghri T, Yacoub M, Nagati K, Tritar B. Effect of tea decoction and the mixture: Lemon juice and tea decoction on iron bioavailability in rats. Toxicol Lett. 1995; 78: 38-38.
37. Niwattisaiwong N, Luo XX, Coville PF, Wanwimolruk S. Effects of Chinese, Japanese and Western Tea on Hepatic P450 Enzyme Activities in Rats. Drug Metabol Drug Interact. 2004; 20: 43–56.
38. Rattan RS. Mechanism of action of insecticidal secondary metabolites of plant origin. Crop Protection. 2010; 29: 913-920.
39. Kanis LA, Prophiro JS, Vieira, ES, Nascimento MP, Zepon KM, Kulkamp-Guerreiro IC, et al. Larvicidal activity of Copaifera sp. (Leguminosae) oleoresin microcapsules against Aedes aegypti (Diptera: Culicidae) larvae. Parasitol Res. 2012; 110: 1173–1178.
40. Martins MCB, Silva MC, Silva LRS, Lima VLM, Pereira EC, Falcão EPS, et al. Usnic Acid Potassium Salt: An Alternative for the Control of *Biomphalaria glabrata* (Say, 1818). PLoS ONE. 2014 Nov 6. 9(11): e111102. doi:10.1371/journal.pone.0111102
41. Fernandes CP, Almeida FB, Silveira AN, Gonzalez MS, Mello CB, Feder D, et al. Development of an insecticidal nanoemulsion with *Manilkara subsericea* (Sapotaceae) extract. J Nanobiotechnology. 2014 May 18; 12-22. doi:10.1186/1477-3155-12-22.

42. Grapentin C, Barnert S, Schubert R. Monitoring the Stability of Perfluorocarbon Nanoemulsions by Cryo-TEM Image Analysis and Dynamic Light Scattering. PLoS ONE. 2015 Jun 22. e0130674. doi:10.1371/journal.pone.0130674
43. Bruxel F, Laux M, Wild LB, Fraga M, Koester LS, Teixeira HF. Nanoemulsões como sistemas de liberação parenteral de fármacos.Quim Nova. 2012;35: 1827-1840.
44. Griffin WC. Classification of surface-active agents by HLB. J Soc Cosmet Chem. 1949; 1: 311-326.
45. Rodríguez-Rojo S, Varona S, Núnez M, Cocero MJ. Characterization of rosemary essential oil for biodegradable emulsions. Ind Crop Prod 2012; 37: 137-140.
46. Orafidiya LO, Oladimeji FA. Determination of the required HLB values of some essential oils. Int J Pharm. 2002; 237: 241-249.
47. Rodrigues ECR, Ferreira AM, Vilhena JCE, Almeida FB, Cruz RAS, Florentino AC, et al. Development of a larvicidal nanoemulsion with Copaiba (*Copaifera duckei*) oleoresin. BrazJPharmacog. 2014; 24: 99-705.
48. Rodrigues ECR, Ferreira AM, Vilhena JCE, Almeida FB, Cruz RAS, Amado JRR, et al. Development of Babassu Oil Based Nanoemulsions. Lat Am J Pharm. 2015; 34: 338-343.
49. Duarte JL, Amado JRR, Oliveira AEMFM, Cruz RAS, Ferreira AM, Souto RNP, et al. Evaluation of larvicidal activity of a nanoemulsion of *Rosmarinus officinalis* essential oil. Bras JPharmacog. 2015; 25: 189-192.
50. Dias DO, Colombo M, Kelmann RG, Kaiser S, Lucca LG, Teixeira HF, et al. Optimization of Copaiba oil-based nanoemulsions obtained by different preparation methods. Ind Crop Prod. 2014; 59: 154–162.
51. Baldissera MD, Silva AS, Oliveira CB, Zimmermann CEP, Vaucher RA, Santos RCV, et al. Trypanocidal activity of the essential oils in their conventional and nanoemulsion forms: In vitro tests. Exp Parasitol. 2013; 134: 356–361.

52. Silva HD, Cerqueira MA, Souza BWS, Ribeiro C, Avides MC, Quintas MAC, et al. Nanoemulsions of β-carotene using a high-energy emulsification–evaporation Technique. J Food Eng. 2011; 102: 130–135.

53. Chang Y, McClements DJ. Optimization of Orange Oil Nanoemulsion Formation by Isothermal Low-Energy Methods: Influence of the Oil Phase, Surfactant, and Temperature. J Agric Food Chem. 2014; 62:2306−2312.

54. Leong WF, Lai OM, Long K, Man YBC, Misran M, Tan CP. Preparation and characterization of water-soluble phytosterol nanodispersions. Food Chemistry. 2011; 129: 77-83.

55. Cheong JN, Tan CP, Man YBC, Misran M. α-Tocopherol nanodispersions: Preparation, characterization and stability evaluation. Journal of Food Engineering. 2008; 89: 204–209.

56. Montenegro LHM, Oliveira PES, Conserva LM, Rocha EMM, Brito AC, Araújo RM, et al. Terpenóides e avaliação do potencial antimalárico, larvicida, anti-radicalar e anticolinesterásico de *Pouteria venosa* (Sapotaceae). Braz J Pharmacog. 2006; 16: 611–617.

57. Anjali CH, Sharma Y, Mukherjee A , Chandrasekaran N. Neem oil (*Azadirachta indica*) nanoemulsion - a potent larvicidal agent against *Culex quinquefasciatus*. Pest Manag Sci. 2012; 68: 158–163.

58. Angajala G, Ramya R, Subashini R In-vitro anti-inflammatory and mosquito larvicidal efficacy of nickel nanoparticles phytofabricated from aqueous leaf extracts of *Aegle marmelos* Correa. Acta Trop. 2014; 135: 19–26

59. Hovda LR, Hooser SB. Toxicology of newer pesticides for use in dogs and cats. Vet Clin North_Am Small Anim Pract. 2002; 32:455-67.

60. Prophiro JS, Silva MAN, Kanis LA, Rocha LCBP, Duque-Luna JE., Silva OS. First report on susceptibility of wild *Aedes aegypti* (Diptera: Culicidae) using *Carapa guianensis* (Meliaceae) and Copaifera sp. (Leguminosae). Parasitol Res. 2012; 110: 699-705.

61. Júnior CV. Terpenos com atividade inseticida: uma alternativa para o controle químico de insetos. Quim Nova. 2003; 26: 390-400.

62. López MD, Pascual-Villalobos MJ. Mode of inhibition of acetylcholinesterase by monoterpenoids and implications for pest control. Ind Crop Prod. 2010; 31: 284-288.

63. Seng CM, Setha T, Nealon J, Socheat D, Chantha N, Nathan MB. Community-based use of the larvivorous fish Poecilia reticulata to control the dengue vector Aedes aegypti in domestic water storage containers in rural Cambodia. J Vector Ecol. 2008; 33:139-44.

64. Gubler DJ. The Global Emergence/Resurgence of Arboviral Diseases As Public Health Problems. Archives of Medical Research. 2002; 33: 330–342.

65. WHO. World Health Organization 2011. Guidelines for drinking-water quality. Geneva, Swizerland.

66. Caloni F, Cortinovis C, Rivolta M, Davanzo F. Suspected poisoning of domestic animals by pesticides. Sci Total Environ. 2016; 539: 331–336.

67. Morrison AC, Zielinski-Gutierrez E, Scott TW, Rosenberg R. Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti*. PLoS Med. 2008 Mar 18. e68. doi:10.1371/journal.pmed.0050068.

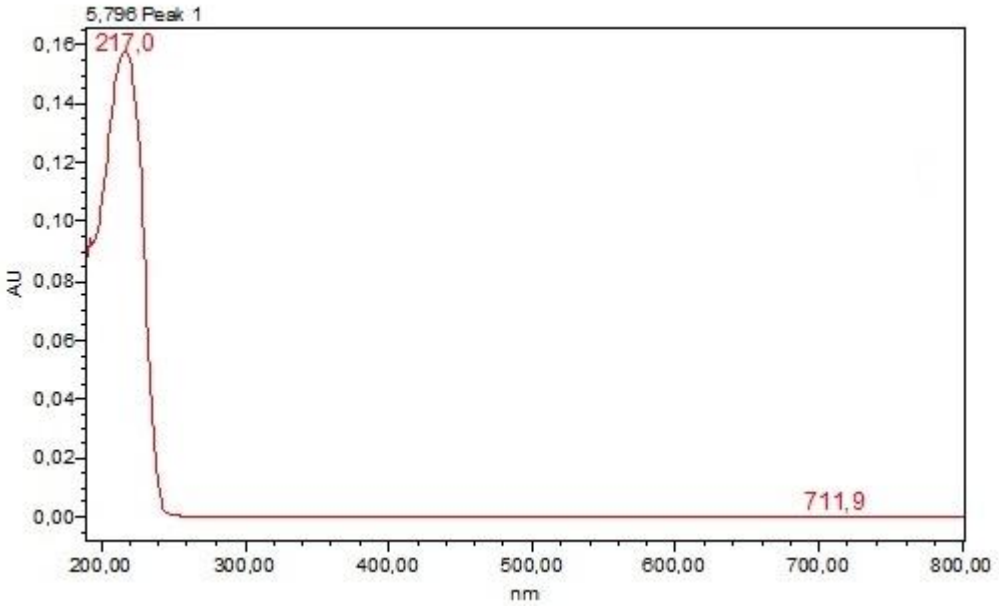

Figure 1:UV spectra obtained by HPLC-DAD for diterpene MHV.

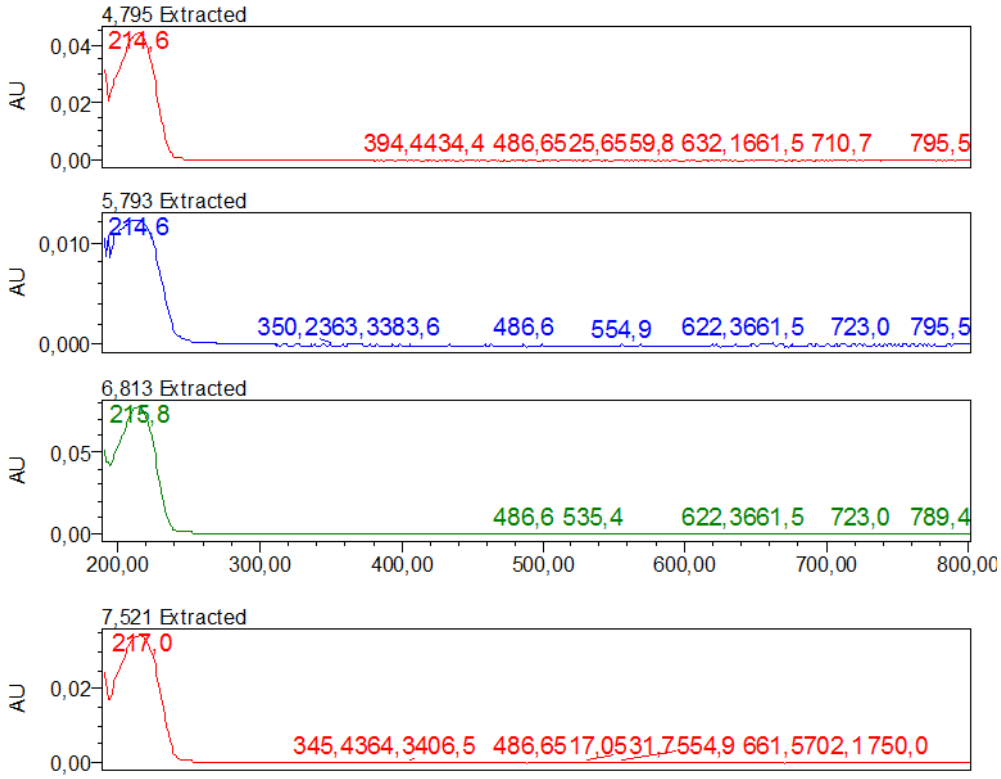

Figure 2: UV spectra from non-identified compound from *P. emarginatus* oil suggesting the presence of other vouacapan diterpenes.

Figure 3. Optimized *P. emarginatus* nanoemulsionused for in vitro and in vivo biological assays.

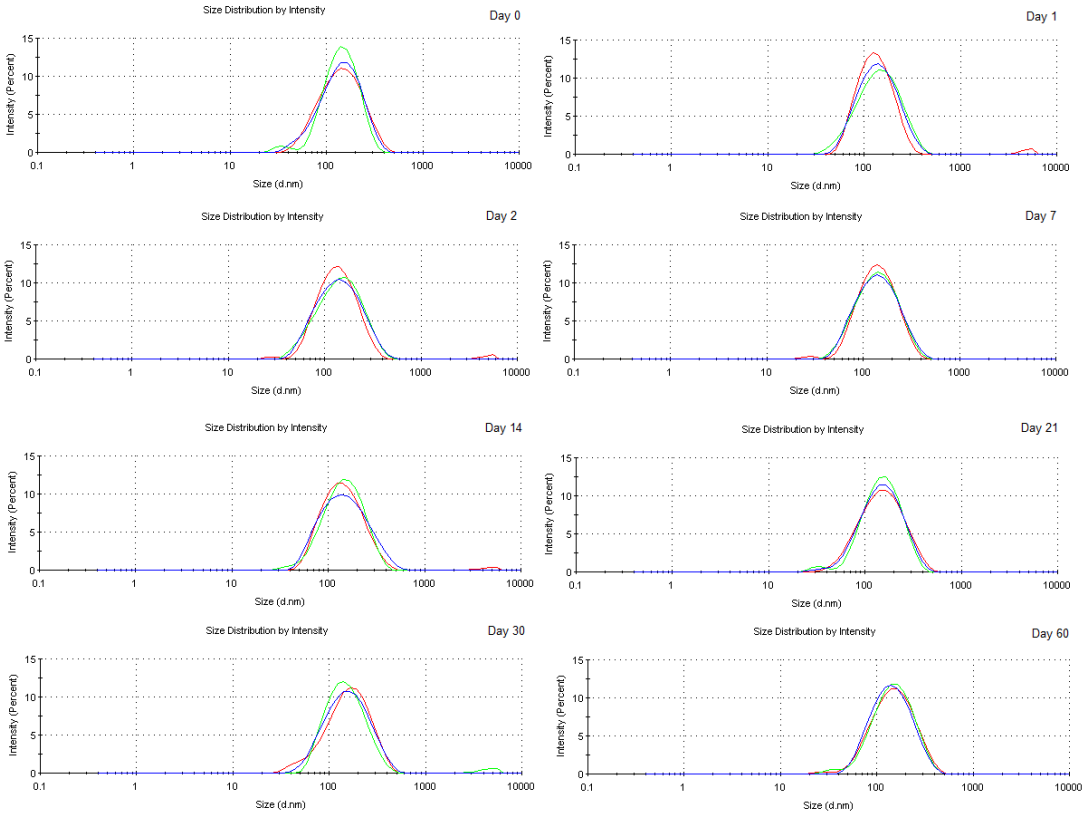

Figure 4. Mean droplet size -Day 0: 125.1 ± 0.5 nm; Day 1: 124.4 ± 0.4 nm; Day 2: 125.6 ± 0.5 nm; Day 7: 124.8 ± 0.3 nm; Day 14: 127.5 ± 0.2 nm; Day 21: 131.0 ± 0.5 nm; Day 30: 134.3 ± 0.8 nm; Day 60:129.2 ± 1.0 nm.Polydispersity index - Day 0: 0.175 ± 0.014; Day 1: 0.185 ± 0.012; Day 2: 0.196 ± 0.006; Day 7: 0.174 ± 0.003; Day 14: 0.194 ± 0.013; Day 21: 0.192 ± 0.007; Day 30: 0.210 ± 0.006; Day 60:0.193 ± 0.001.Zeta potential - Day 0: -30.9 ± 0.4 mV; Day 1: -29.6 ± 1.4 mV; Day 2: -29.6 ± 1.4 mV; Day 7: -33.1 ± 3.3 mV; Day 14: -35.4 ± 2.8 mV; Day 21: -35.4 ± 1.2 mV; Day 30: -47.1 ± 1.5 mV; Day 60:-41.0 ± 6.34 mV.

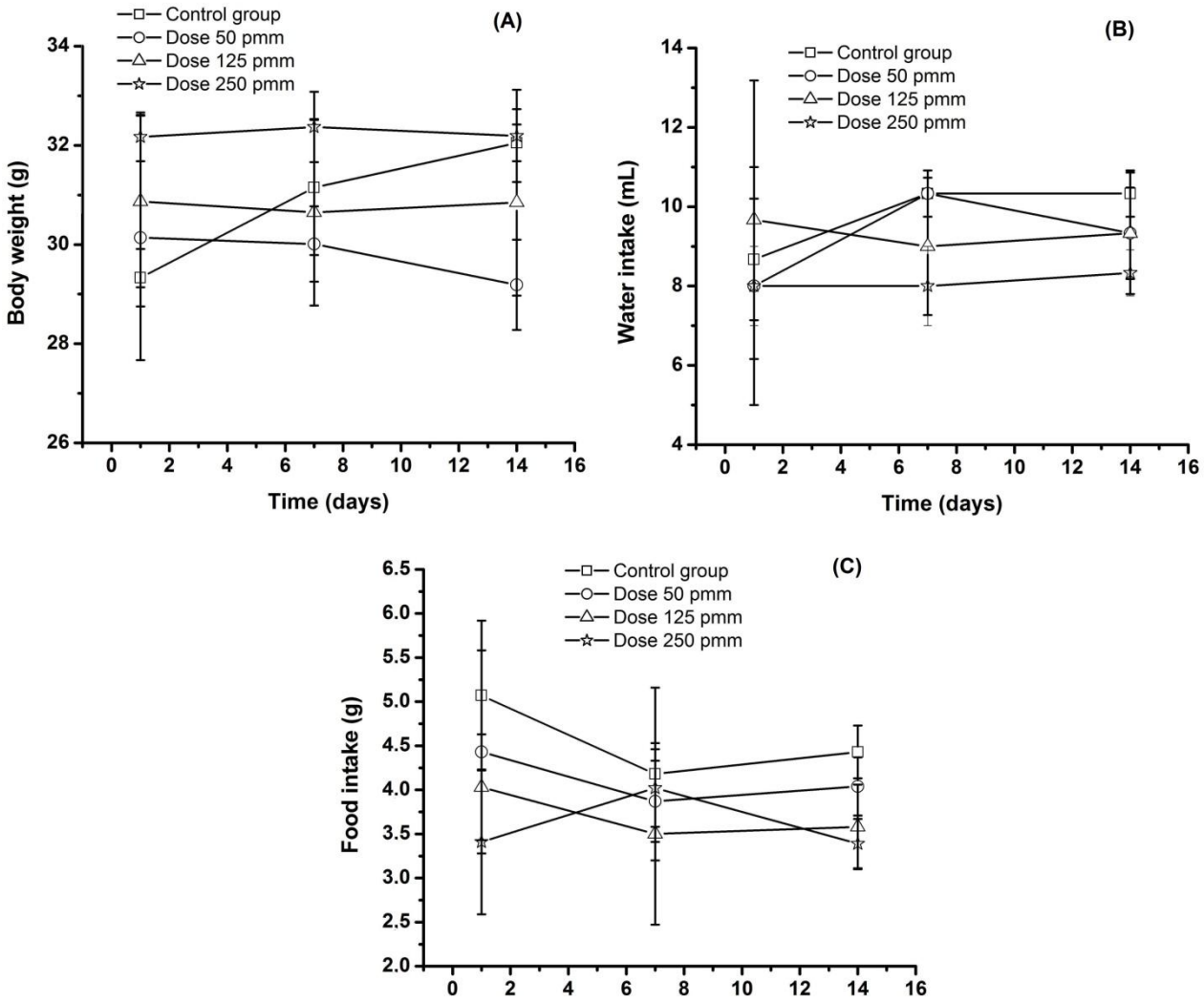

Figure 5. Analysis of body weight (A), water (B) and food intake (C) variation in mice treated with optimized *P. emarginatus* nanoemulsion.

Table 1. Mean droplet size, polydispersity index and zeta potential during of emulsions prepared during rHLB determination of *P. emarginatus* oil.

HLB	1 Day			7 Days		
	Mean droplet size (nm)	Polydispersity index	Zeta potential (mV)	Mean droplet size (nm)	Polydispersity index	Zeta potential (mV)
11	135.8 ± 0.2	0.173 ± 0.002	-27.2 ± 0.6	160.3± 1.4	0.188± 0.02	-24.3 ± 0.5
12	126.7 ± 0.9	0.096 ± 0.019	-25.1 ± 1.3	182.0± 0.7	0.140± 0.005	-25.6 ± 1.1

Table 2. Mortality levels (%) of *Aedes aegypti* larvae after treatment with optimized *P. emarginatus* nanoemulsion (expressed as *P. emarginatus* oil content in aqueous media).

Exposure time (h)	Control	Tested concentrations					
		12.5 ppm*	25 ppm*	50 ppm*	75 ppm*	100 ppm	250 ppm
24	0	0	0	40 ± 17.32^{a}	50 ± 20.0^{a}	90 ± 0.0^{c}	100 ± 0.0
48	0	23.33 ± 5.77^{a}	50 ± 17.32^{b}	63.33 ± 5.77^{b}	83.33 ± 5.77^{c}	93.33 ± 5.77^{c}	100 ± 0.0

Data is expressed as mean ± standard deviation

Analysis of variance (ANOVA) followed by Tukey´s test was performed to evaluate statistical significance of the results (n = 30). Experiment was performed in triplicate and each replicate was composed by 10 larvae.

* Statistical significant increase in mortality level as function of exposure period

Means in the same line with different superscript mean statistical significant difference ($P < 0.05$)

Pterodon emarginatus OLEORESIN-BASED NANOEMULSION AS A PROMISING TOOL FOR *CULEX QUINQUEFASCIATUS* (DIPTERA: CULICIDADE) CONTROL

Artigo submetido ao periódico Nanobiotecnology*:
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***Pterodon emarginatus* oleoresin-based nanoemulsion as a promising tool for *Culex quinquefaciatus* (Diptera: Culicidae) control**

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Abstract

Background: Preparation of nanoformulations using natural products as bioactive substances is considered very promising for innovative larvicidal agents. On this context, oil in water nanoemulsions develop a main role, since they satisfactorily disperse poor-water soluble substances, such as herbal oils, in aqueous media. *Pterodon emarginatus*, popularly known as sucupira, has a promising bioactive oleoresin. However, to our knowledge, no previous studies were carried out to evaluate its potential against *Culex quinquefasciatus*, the main vector of the tropical neglected disease called lymphatic filariasis or elephantiasis. Thus, we aimed to investigate influence of different pairs of surfactants in nanoemulsion formation and investigate if a sucupira oleoresin-based nanoemulsion has promising larvicidal activity against this *C. quinquefasciatus*. We also evaluated possible mechanism of insecticidal action and ecotoxicicity of the nanoemulsion against a non-target organism. **Results:** Among the different pairs of surfactants that were tested, nanoemulsions obtained with polysorbate 80/sorbitan monooleate and polysorbate 80/sorbitan trioleate presented smallest mean droplet size just afterwards preparation, respectively 151.0 ± 2.252 nm and 160.7 ± 1.493 nm. They presented high negative zeta potential values, low polydispersity index (<0.300) and did not present great alteration in mean droplet size and polydispersity index after 1 day of preparation. Overall, nanoemulsion prepared with polysorbate 80/sorbitan monooleate was considered more stable and was chosen for biological assays. It presented low LC_{50} value against larvae (34.75; 7.31–51.86 mg/L) after 48 h of treatment and did not inhibit acetylcholinesterase. It was not toxic to green algae *Chlorella vulgaris* at low concentration (25 mg/L). **Conclusions:** Our results suggest that optimal nanoemulsions may be prepared with different surfactants using a low cost and low energy simple method. Moreover, this

prototype proved to be effective against *C. quinquefasciatus*, being considered an ecofriendly novel nanoproduct that can be useful in integrated control programs of vector control.

Keywords: Larvicidal, nanoemulsion, oleoresin, sucupira.

Background

Culex quinquefasciatus (Diptera: Culicidae) is a nocturnal domestic mosquito with high occurrence on tropical and subtropical regions [1]. Often, its population density is associated to deforestation and urbanization process [2,3,4]. *C. quinquefasciatus* deposits its eggs and develops on standing water with high concentration of organic material. Thus, it is associated to substandard housing, absence of basic sanitation, treated water and others [5,6]. Moreover, hematophagic-feeding habits favors *C. quinquefasciatus* proliferation close to human population [7]. This species is highly anthropophilic and responsible by transmission of filarial nematodes, which cause several diseases in humans [8].

Lymphatic filariasis is caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* [9]. In Brazil, etiological agent of this disease is *Wuchereria bancrofti* [2]. The cycle of the disease begins during blood repast, when the infected female of the vector transmit *Wuchereria brancofti* larvae to human host. These larvae migrate to lymphatic system and develop to adult stage, causing dilatation of vessels [1,10]. This disease is also known as elephantiasis and it is recognized as a neglected disease associated to poverty [11,12] with high prevalence in tropical and sub-tropical countries [13]. According to World Health Organization (WHO), it is estimated that around 120 million of people worldwide has lymphatic filariasis [14]. Various form of manifestation of this disease include asymptomatic behavior and physical incapacity [15], mainly due to cronical hydrocele and

elephantiasis of the legs or arms [16]. More than 40 million of people around the world were marginalized until 2012 [14]. On this context, WHO launched a global program for eradication of lymphatic filariasis until the year 2020 [9]. The main strategy involves treatment of population on endemic areas, control of morbidity and prevent incapacity that is associated to the disease [14]. However, another potential alternative involves environmental control, aiming to interrupt transmission by the vector.

Growing interest is observed worldwide for new integrative practices for vector control. Several of them involve utilization of natural products as bioactive agents. These compounds are biodegradable and may be used as potent ecofriendly insecticides [17]. A new approach relies on utilization of these plant-derived insecticides to prepare nanosize products [18]. The nano-scale allows achievement of optimized properties regarding biological activities, chemical and physical stability, making them versatile innovative products [19]. On this context, several nanoformulations can be prepared, including nanoemulsions. They are dispersed systems with sub-micrometer size droplets, often ranging from 20 to upper limits between 100-500, according to different author criteria [20]. Nanoemulsions have been considered very promising to enhance solubility of poor water-soluble substances [21]. On this context, development of herbal bioactive oil-based nanoemulsions has great potential for mosquito larvae control [22,23,24,25]. Moreover, several effective nanoemulsions containing natural oils were considered effective larvicidal agents against *C. quinquefasciatus* [26,27,28].

Pterodon emarginatus Vogel is a traditional plant species with a wide range of folk use in Brazil, being popularly known as “sucupira” or “sucupira-branca” [29]. Terpenoids from seeds of sucupira, especially vouacapan diterpenes, develop a main role as bioactive

compounds of this plant [30,31], being major constituents of the ambar coloured oleoresin obtained from its seeds. This oily material was subjected to some studies aiming to develop emulsions with submicrometer droplets [29,32]. Considering the larvicidal potential of sucupira oleoresin and its terpenoids against *Aedes aegypti* [33,34], a promising larvicidal nanoemulsion against this vector larvae was prepared using this raw material [25]. However, to our knowledge, no studies were carried out for another pest and/or vector insects. Thus, as part of our ongoing studies with larvicidal natural product-based nanoemulsions, the present study aim to evaluate insecticidal activity of sucupira-based ecofriendly nanoemulsion against *C. quinquefasciatus*.

Results and Discussion

Table 1 shows droplet size, particle size distribution (polydispersity index) and zeta potential of formulations prepared with sucupira oleoresin. All of them presented high negative zeta potential values. Adsorption of hydroxyl groups and/or conjugated bases of secondary metabolites, which naturally occur on some natural oils, at the surface of micelles has been associated to this phenomenon [35]. Thus, dissociation of some substances from sucupira oleoresin, such as fatty acids and others (eg. vouacapan diterpene acids) may be responsible by this observation. Most of them presented high mean droplet size (> 200 nm) and high polydispersity index (>0.500), in addition to large amount of precipitate. Nanoemulsions obtained with polysorbate 80/sorbitan monooleate and polysorbate 80/sorbitan trioleate presented smallest mean droplet size just afterwards preparation, respectively 151.0 ± 2.252 nm and 160.7 ± 1.493 nm. They also presented fine appearance, translucent aspect and bluish reflect, which are in accordance with the concept of nanoemulsions [20]. Influence of surfactant type, as well as required hydrophile-lypophile

balance (rHLB) value of the oil, is a major factor of influence on nanoemulsion formation. Regarding literature data, it can be observed that utilization of different pairs of sorbitan alkanoates/ethoxylated sorbitan alkanoates at rHLB of different oils (interval between 11-12) also successfully generated nanoemulsions with mean droplet size below 200 nm [36]. Thus, considering surfactant pairs employed in the present study, our results suggest that polysorbate 80/sorbitan monooleate and polysorbate 80/sorbitan trioleate may be considered the best pairs for preparation of sucupira oil based nanoemulsions.

Table 1. Droplet size, polydispersity index and zeta potential of nanoemulsions prepared with sucupira oleoresin and different pairs of surfactants (rHLB = 11). Results are expressed as mean ± standard deviation.

		Size (nm)	PDI	Zeta potential (mV)	Size (nm)	PDI	Zeta potential (mV)
		Day 0			Day 1		
T80	S80	151.0 ± 2.252	0.221 ± 0.006	-32.5 ± 1.07	146.3 ± 1.450	0.219 ± 0.005	-30.5 ± 0.819
T20	S80	2540.0 ± 996.7	1.000 ± 0.000	-52.6 ± 4.14	1461.0 ± 470.8	1.000 ± 0.000	-43.8 ± 0.603
T80	TS	160.7 ± 1.493	0.252 ± 0.012	-29.2 ± 0.346	159.8 ± 3.46	0.277 ± 0.027	-31.4 ± 0.404
T20	TS	650.0 ± 663.9	0.756 ± 0.200	-31.3 ± 1.08	526.2 ± 397.2	0.601 ± 0.194	-34.3 ± 1.11
MP400		223.7 ± 28.69	0.391 ± 0.034	-43.2 ± 0.82	352.3 ± 198.8	0.536 ± 0.078	-40.6 ± 1.10
MP600	DP600	507.1 ± 171.6	0.576 ± 0.109	-35.8 ± 1.00	345.1 ± 161.1	0.568 ± 0.025	-30.3 ± 0.34
MP600	DP400	464.9 ± 176.1	0.569 ± 0.085	-44.0 ± 0.351	345.7 ± 83.2	0.476 ± 0.078	-39.3 ± 0.569
T20	DP600	750.8 ± 238.2	0.763 ± 0.063	-40.7 ± 0.451	322.6 ± 49.73	0.579 ± 0.092	-35.5 ± 0.200
T20	DP400	460.2 ± 134.6	0.608 ± 0.125	-52.2 ± 1.38	484.1 ± 59.68	0.576 ± 0.103	-51.6 ± 1.71
T80	DP600	1055 ± 65.11	0.779 ± 0.026	-14.3 ± 0.289	898.2 ± 141.1	0.921 ± 0.136	-37.3 ± 0.208
T80	DP400	248.3 ± 30.51	0.605 ± 0.157	-49.7 ± 1.16	214.5 ± 18.6	0.504 ± 0.060	-44.0 ± 3.67

T80 = polysorbate 80. S80 = sorbitan monooleate. T20 = polysorbate 20. TS = sorbitan trioleate. DP400 = polyethyleneglycol 400 dioleate. DP600 = polyethyleneglycol 600 dioleate. MP400 = polyethyleneglycol 400 monooleate. MP600 = polyethyleneglycol 600 monooleate.

Sucupira oil based nanoemulsions prepared with polysorbate 80/sorbitan monooleate and polysorbate 80/sorbitan trioleate did not present great alteration in mean droplet size and polydispersity index after 1 day of preparation (Table 1). Moreover, we observed high homogeneity of particle size and almost monomodal distribution even after 7 days of storage (Figure 1). Regarding physical stability of these nanoemulsions, we observed that the

one prepared with polysorbate 80/sorbitan trioleate presented slight few precipitate after 7 days. Sucupira oil has several bioactive substances with low water solubility, such as diterpenes. These terpenoid substances are often found as white powders, as free diterpenes or even as acids or esters. Despite our result suggests the potential of polysorbate 80/sorbitan trioleate to form sucupira nanoemulsions, the surfactant to oil ratio (1:1) used in the present study probably was not sufficient to entrap and stabilize all these substances. Considering that surfactant to oil ratio is considered one of most important parameters that affect stability of nanoemulsions, especially for low energy methods [37], further studies may be performed to access its influence on sucupira oil based nanoemulsions formation and stability.

INSERT FIGURE 1 HERE

Preparation of nanoemulsion by low-energy methods, in contrast to high-energy methods, should be encouraged. These methods make use of chemical energy released due to a dilution process (self-emulsification methods) or make use of chemical energy released by phase transitions or change in surfactant curvature during the emulsification process, being able to induce formation of small droplets. Phase transitions can be induced by changing the temperature (PIT - phase inversion temperature method) or composition (PIC – phase inversion composition method) [20]. A great advantage of methods that involve low aport of energy is associated to reduction of process costs. We proved that sucupira nanoemulsions with good indicatives of physical stability could be obtained using this approach. Considering our aforementioned results, we opted to use the nanoemulsion

prepared with polysorbate 80/sorbitan monooleate for biological investigation. Table 2 shows mortality levels induced by sucupira nanoemulsion (expressed as sucupira oleoresin content). After 24 h, treatment with nanoemulsion at 25 mg/L was not considered statistically different from control group ($p>0.05$). Group treated at 100 mg/L reached 26.67 ± 9.43 % of mortality, which is significantly different from control group ($p<0.0001$), treated groups at 25 mg/L ($p<0.01$) and 200 mg/L ($p<0.0001$). Higher mortality level was observed for group treated at 200 mg/L, which reached 86.67 ± 4.71 % after 24 h and was considered significantly different from control and treated groups ($p<0.0001$). After 48 h of treatment, statistically significant difference in mortality was observed for treated groups at 25 mg/L ($p<0.05$), 100 mg/L ($p<0.0001$) and 200 mg/L ($p<0.0001$), when compared to control group. Lowest mortality level was observed for group treated at 25 mg/L ($p<0.0001$), which reached 20 ± 0 %. No statistically significant difference was observed in mortality levels induced by groups treated at 100 and 200 mg/L ($p>0.05$), which reached 93.33 ± 4.71 % and 100 ± 0 %, respectively. Increased mortality levels were observed for groups treated at 25 mg/L ($p<0.1$), 100 mg/L ($p<0.0001$) and 200 mg/L ($p<0.01$) as function of exposure time. *P. emarginatus* oil nanoemulsion presented median-lethal concentration (LC_{50}) of 56.70 (30.12-94.97) mg/L after 48 h, in the larvicidal assay against *C. quinquefasciatus*. Other studies aimed to generate larvicidal herbal nanoemulsions against *C. quinquefasticatus*. The oil in water nanoemulsion prepared with neem oil decreased as function of droplet size as follows: 11.75 mg/L (mean droplet size around 31.03 nm), 25.99 mg/L (mean droplet size around 93.0 nm) and 62.89 mg/L (mean droplet size around 251.43 nm) [26]. After 24 h, it was observed that a mortality level below 40% was achieved with eucalyptus oil-based nanoemulsion at an experimental concentration of 50 mg/L [28]. Thus, our results are in accordance with mortality levels in this range of mean droplet diameter for classical larvicidal natural oils.

Table 2. Mortality levels of *Culex quinquefasciatus* after exposure to different concentrations of sucupira oil based nanoemulsion.

Exposure Time	Control	25 mg/L	100 mg/L	200 mg/L
24	0^{a}	10 ± 0^{a}	26.67 ± 9.43^{b}	86.67 ± 4.71^{c}
48	6.67 ± 4.71^{a}	20 ± 0^{b}	93.33 ± 4.71^{c}	100 ± 0^{c}

Data is expressed as mean ± standard deviation

Means in the same line with different superscript indicates statistical significant difference ($P < 0.05$)

Natural products have been recognized a valuable resource of potential larvicidal agents against disease vectors. Different criteria were proposed as standard for promising agents. Overall, satisfactory results are associated to samples that induce mortality levels higher than 75% [38] at 250 mg/L or have LC_{50} values below 100 mg/L [33], which are in accordance with our results. Optimized sucupira nanoemulsion, similar to the larvicidal nanoproduct that was used in the present study, was recently described as a promising larvicidal agent against *A. aegypti* larvae. It presented LC_{50} of 34.75 (7.31–51.86) mg/L [25]. Some plant extracts, including some obtained from species associated to diterpenoid-rich genus, revealed lower LC_{50} and LC_{90} values against *A. aegypti* larvae, when compared to *C. quinquefasciatus* larvae under same experimental conditions [39]. This data is in accordance with our results, which suggested that *C. quinquefasciatus* larvae are less susceptible to sucupira nanoemulsion than *A. aegypti* larvae.

Several mechanism of action have been proposed for insecticidal natural products, including inhibition of acetylcholinesterase [17]. No statistically significant difference was observed between acetylcholinesterase activity in the presence and absence of *P.*

emarginatus nanoemulsion ($p>0.05$) (Figure 2). A larvicidal nanoemulsion prepared with eucalyptus oil was able to reduce about 80 % of acetylcholinesterase activity of the enzyme from *C. quinquefasciatus* and this inhibitory activity may be, at least partially, attributed to 1.8-cineole (eucalyptol) [28]. Sucupira oleoresin is mostly constituted by the sesquiterpene β-caryophyllene (three isoprene units) and diterpenes, such as geranylgeraniol and vouacapan compounds [25]. Eucalyptol is a monoterpene that is well recognized as a potent insecticidal agent and acetylcholinesterase inhibitor. However, geraniol, which also have two isoprene units and play a main role in diterpenes formation [40], has weak inhibitory activity against acetylcholinesterase, despite it has strong insecticidal activity [41]. In addition to the fact that bioactive substances may present significant differences in acetylcholinesterase inhibitory activities, differences attributed to insect enzymes inbibitory sites may be attributed to absence of anticholinesterase activity found in the present study. This hypothesis should also be considered since a similar nanoemulsion prepared with this natural raw material was able to inhibit acetylcholinesterase from the *A. aegypti* larvae [25]. Further studies to investigate another possible mechanism of action, in addition to quantification of levels of secondary metabolites released from sucupira nanoemulsion during acetylcholinesterase assay should be carried out to support these findings.

INSERT FIGURE 2 HERE

Environmental toxicology assay was carried out using the green algae *Chlorella vulgaris* subjected to different sucupira nanoemulsion concentrations (expressed as sucupira oleoresin content) (Table 3). We observed formation of a precipitate and loss of typical green color of the algae dispersion just afterwards addition of nanoemulsion at 1000 mg/L, while

no change in macroscopical appearance was observed for groups containing nanoemulsion at 500, 100 and 25 mg/L. After 1 day of treatment, 50% of reduction in cell density was observed for the group containing nanoemulsion at 500 mg/L, while no viable cell was observed on the group containing nanoemulsion at 1000 mg/L. Significantly decrease in cell viability was observed after additional period of 24 h ($p<0.0001$), reaching 12% of viable cells after 2 days of treatment. No viable cell was found for the group treated with nanoemulsion at 500 mg/L after 3 days. No statistically significant difference was observed for the group treated with nanoemulsion after 3 days of treatment ($p>0.05$). However, significantly decrease in cell density was observed after 7, 14, 21 and 28 days ($p<0.0001$), reaching 80.0 ± 0.0%, 62.7 ± 3.4 %, 47.7 ± 4.0 % and 13.3 ± 18.9 % of viable cells, respectively. During 14 days, no significant difference ($p>0.05$) in cell density was for the group containing nanoemulsion at 25 mg/L. Low decrease in percentage of viable cells was observed after 21 and 28 days ($p<0.0001$). Cell count in control group revealed 16% of increase in cell density from day 3 to day 7 ($p<0.001$), which was kept constant until a total of 14 days of treatment ($p>0.05$). This result was statistically different when compared to group treated with nanoemulsion at 25 mg/L in the same period ($p<0.0001$). This fact is probably associated to a spontaneous growth that was suppressed by constituents of sucupira nanoemulsion. However, we can conclude that no significant difference was observed after the end of the experiment between control and group tested at 25 mg/L ($p>0.05$). *C. vulgaris* is a green microalgae that develop a main role in the aquatic ecosystem, being in the first level of the trophic chain. Moreover, it has been considered a promising agent for bioremediation due to its ability to degrade oil [42] and other contamintants, such as nonylphenol [43]. This organism has been considered valuable as a biondicator for ecotoxicological studies. It has short life cycle and is easily cultured in laboratory, being also sensitive to toxicants, among

other advantages [44,45]. Nano-size may enhance toxicological effects, when compared to bulk material. Thus, evaluation of ecotoxicological impact of nanostructures should be encouraged, including aquatical toxicology using *C. vulgaris* as biological indicator [46]. Complexes of carbon nanotubes-diuron increased toxicity of the herbicide against *C. vulgaris* [47]. Another study observed ecotoxicological effects of cellulose nanofibers in *C. vulgaris* and suggested impact of carbon nanotubes on this algae [45]. Aqueous extract of soil containing zinc oxide nanoparticles did not induce any toxicological effect on this aquatic organism [48]. To our knowledge, this is the first report of evaluation of ecotoxicological assay for a proposed larvicidal natural product-based nanoemulsion against *C. vulgaris*. Our previous data suggests that sucupira oleoresin-based nanoemulsion is potentially safe mammals, considering a non-target toxicological assay performed with mice. Thus, it presented potential application at domestic environment [25]. However, a major problem for utilization of pesticides is the possibility of they being leached by water and reach the environment, such rivers, estuaries and ocean [49]. It is worth mentioning that this situation involves dilution of the pesticide agent. Thus, in addition to biodegradable nature of natural products, concentration of nanoemulsion (expressed as sucupira oleoresin content) in the environment will probably be not toxic for green algae, considering our results using this non-target model.

Table 3. Percentage of viable cells of the green algae *Chlorella vulgaris* subjected to different sucupira nanoemulsion concentrations (expressed as sucupira oleoresin content). Results are expressed as mean ± stander deviation.

Day	Concentration (mg/L)				
	25	100	500	1000	control
1	100 ± 0^{a}	100 ± 0^{a}	50 ± 0^{a}	0 ± 0^{a}	100 ± 0^{a}
2	100 ± 0^{a}	100 ± 0^{a}	12 ± 0^{b}	0 ± 0^{a}	100 ± 0^{a}
3	100 ± 0^{a}	100 ± 0^{a}	0 ± 0^{c}	0 ± 0^{a}	100 ± 0^{a}
7	100 ± 0^{a}	80 ± 0^{b}	0 ± 0^{c}	0 ± 0^{a}	116 ± 0^{b}
14	100 ± 0^{a}	62.7 ± 3.4^{c}	0 ± 0^{c}	0 ± 0^{a}	116 ± 0^{b}
21	80 ± 0^{b}	47.7 ± 4.0^{d}	0 ± 0^{c}	0 ± 0^{a}	80 ± 0^{c}
28	70 ± 0^{c}	13.3 ± 18.9^{e}	0 ± 0^{c}	0 ± 0^{a}	70 ± 0^{d}

Means in the same column with different superscripts are significantly different ($p<0.05$).

Conclusions

Novel nanobiotechnology larvicidal agents using natural products from plant origin are very promising for vector control. *Culex quinquefasciatus* is responsible for transmission of filariasis, a neglected tropical disease. Our results suggest that optimal nanoemulsions may be prepared with different surfactants using a low cost, organic solvent-free and low energy simple method. Moreover, this prototype proved to be effective against *C. quinquefasciatus* and probably has low toxic effects to environment. Thus, it can be concluded that sucupira oleoresin-nanoemulsion is potentially an ecofriendly novel nanoproduct that can be useful in integrated control programs of vector control.

Materials and Methods

Chemicals

Sorbitan trioleate, sorbitan monooleate, polyethyleneglycol 400 dioleate, polyethyleneglycol 600 dioleate, polyethyleneglycol 400 monooleate, polyethyleneglycol 600 monooleate, polysorbate 80 and polysorbate 20 were purchased from Praid Produtos Químicos Ltda (SP, Brazil). Acetylthiocholine iodide (ATCI) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich (St Louis, MO). Distilled water was used for general procedures.

Obtainment of *P. emarginatus* oleoresin

Fruits from *Pterodon emarginatus* Vogel (Fabaceae) were obtained from Central Market of Goiânia – GO (Brazil). Identification of plant material was performed by Dr. José Realino de Paula and a voucher specimen was deposited at the Herbarium of Goiás Federal University (GO, Brazil) under the register number 41714. Oleoresin from *P. emarginatus* fruits was obtained by cold pressing using a mini mechanical press (MPE-40 ECIRTEC), weighed and hermetically stored in amber glass flask and kept at −20 °C until utilization.

Emulsification method

Emulsification method was performed using low energy method [37] with some modifications [25]. Emulsions were prepared with sucupira oleoresin and surfactant (s) to oil ratio was 1:1. Final concentration of sucupira oleoresin or surfactant (s) were on the emulsions were 2500 mg/mL. Oily phase was constituted by *P. emarginatus* oleoresin and different pairs of surfactants at rHLB of *P. emarginatus* oil (rHLB = 11) (Table 4). Surfactants

and oil were mixed using magnetic stirring (400 rpm) for 30 minutes under controlled temperature using a water bath (80 ± 5 ° C). Aqueous phase was added through oily phase under constant magnetic stirring rate (400 rpm) and temperature gradually decreased to room temperature in approximately 30 min. System was stirred for 1 hour and after this period, an additional amount of water was added to restore final mass (50 g).

Table 4: Composition of oily phase of *P. emarginatus* nanoemulsions.

Formulation	Surfactants	Concentration (mg/L)
1	T80/S80	1560/940
2	T20/S80	1360/1140
3	T80/TS	1740/760
4	T20/TS	1540/960
5	MP600/DP600	840/1660
6	MP600/DP400	1380/1120
7	MP400	2500
8	T20/DP600	380/2120
9	T20/DP400	760/1740
10	T80/DP600	500/2000
11	T80/DP400	1540/960

P. emarginatus oil concentration was 2500 mg/L. Surfactant mixture final concentration was 2500 mg/L (rHLB = 11). Final mass of each formulation was 50 g. T80 = polysorbate 80. S80 = sorbitan monooleate. T20 = polysorbate 20. TS = sorbitan trioleate. DP400 = polyethyleneglycol 400 dioleate. DP600 = polyethyleneglycol 600 dioleate. MP400 = polyethyleneglycol 400 monooleate. MP600 = polyethyleneglycol 600 monooleate.

Nanoemulsion characterization

Droplet size, polydispersity index and zeta potential of the nanoemulsions were determined using a Zetasizer ZS (Malvern, UK). Each sample was diluted with distilled water (1:20) for analysis. Measurements were performed in triplicate and results were expressed as the mean diameter ± standard deviation.

Larvicidal assay

Culex quinquefasciatus female were collected at Macapá (Universidade Federal do Amapá, Brazil), identified in the Laboratory of Arthropoda of Amapá Federal University and its eggs were used for the reared colony. Biological assay was performed under controlled conditions, being fourth-instar larvae kept at 25±2 ºC, relative humidity of 75±5% and a 12h light : dark cycle. Experimental protocol was performed according to WHO protocol [50] with some modifications. All experiments were performed in triplicate with 10 forth-instar larvae in each sample. Nanoemulsion was diluted in distilled water at 200, 100, 25 mg/L (expressed as sucupira oleoresin content on aqueous media). Control group was constituted by deionized water. Mortality levels were recorded after 24 and 48 hours of exposure. If mortality level of the control was between 5 – 20 %, correction of mortality levels of treated groups should was performed using Abbott´s formula as follows: Mortality (%) = 100 (X – Y) / X, where X = percentage survival in the untreated control and Y = percentage survival in treated sample.

Enzymatic assays

Whole body homogenate was prepared according to previously established method [28]. Larvae from control group was collected and water was gently removed using tissue paper. Then, they were separately homogenized with 3.0 mL phosphate buffered saline (PBS) 0.1 M (pH = 7.5). This step was performed using a T25 Ultra-Turrax homogenizer (Ika-Werke, Staufen, Germany) running at 12000 rpm for 1 min. The homogenate was centrifuged for 30 min (5000 rpm) under controlled temperature (10 ºC). Whole body homogenate supernatants were collected and immediately used for enzymatic assay.

Anticholinesterase activity was performed according to the well-established method described by Ellman et al. (1961) [51] with some modifications. Activity of acetylcholinesterase from whole body homogenate, after exposure to optimized *P. emarginatus* nanoemulsion, was determined as follows: Aliquot of 0.25 mL of this nanoemulsion, 0.25 mL of whole body homogenate supernatant and 0.5 mL of DTNB were added to 1.75 mL of phosphate buffer. The mixture was incubated for 10 min (25 ± 1°C). Then 0.25 mL of ATCI was added and the absorbance was measured at 410 nm using a UV-Mini spectrophotometer (Shimadzu). Maximum acetylcholinesterase activity was achieved by replacing the amount of *P. emarginatus* nanoemulsion by PBS. Blank was obtained by replacing the ATCI by a same amount of PBS. Assays were performed in triplicate and results were considered significant when ($P<0.05$).

Environmental toxicology assay

The green algae *Chlorella vulgaris* was isolated from water samples obtained from Lagoa dos Índios, situated on the municipality of Macapá (latitude 0.031368 and longitude

51.102559). Serial dilution was carried out in order to isolate the colony and cells were inoculated into NPK media. Algae counting was carried out using a Neubauer chamber [52]. This organism was used as a non-target model for environmental toxicology assay. Aliquot of 10 ml of *C. vulgaris* inoculum was cultivated in nitrogen/phosphorus/potassium (NPK, 08:08:08) aqueous solution. Initial cell density was $1x10^6$ cel⁄ml for all tested groups. Nanoemulsion was tested at different concentrations (25, 100, 500 and 1000 mg/L, expressed as oleoresin content). Control group was constituted by *C. vulgaris* dispersion ($1x10^6$ cel⁄ml) and NPK aqueous solution. Cell count was performed after 1, 2, 3, 7, 14, 21 and 28days. Percentage of viable cells (%VC) was calculated as follows: $\%VC = (D/D_0) \times 100$, where: D is cell density before nanoemulsion addition, D_0 is cell density after at each specific day.

Statistical Analysis

Analysis of variance (Two-way ANOVA) followed by Tukey´s test or Bonferroni´s test was conducted using the Software GraphPad Prism 6.0 (San Diego, California, USA). Differences were considered significant when $p \leq 0.05$. Probit analysis was performed with 95% confidence interval for LC_{50} determination.

Competing interests

All authors declare no conflict of interests.

Authors' contributions

AEMFMO contributed in this paper running the laboratory work, analysis of the data and drafted the paper, which is part of her doctorate thesis. JLD contributed in preparation of nanoemulsions. RNPS and RMAF contributed in insect bioassay. ECC LARO contributed in preparation of natural product raw material. RASC contributed in AChE bioassay. SMMF contributed in non-target organism toxicological assay. ACF contributed in statistical analysis of data. JCTC and CPF designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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REFERENCES

1. Fonseca, D.M., Smith, J.L.; Wilkerson, R.C., Fleischer, R.C.: Pathways of expansion and multiple introductions illustrated by large genetic differentiation among worldwide populations of the southern house mosquito. Am J Trop Med Hyg **74**, 284–289 (2006).
2. Albuquerque, M.F.P.M.: Urbanization, Slums and Endemics: The Production of Filariasis in Recife, Brazil. Cad Saúde Públ. **9** , 487-497 (1993).
3. Adebote, A.D., Oniye, J.S., Ndams, S.I., Nache, K.M: The breeding of mosquitoes (Diptera: Culicidae) in peridomestic containers and implication in yellow fever transmission in villages around Zaria, Northern Nigeria. J. Entomol **3**, 180-188 (2006).
4. Bonfim, C., Netto, M.J.E., Pedroza, D., Portugal, J.L., Medeiros, Z.A.: Socioenvironmental composite index as a tool for identifying urban areas at risk of lymphatic filariasis. Trop Med Int Health **14**, 877-884 (2009).
5. Barbosa, R.M.R., Souto, A., Eiras, A.E., Regis, L.: Laboratory and field evaluation of an oviposition trap for *Culex quinquefasciatus* (Diptera: Culicidae). Men Inst Oswaldo Cruz **102**, 523-529 (2007).
6. Correia, J.C., Barbosa, R.M.R., Oliveira, C.M.F., Albuquerque, C.M.R.: Residential characteristics aggravating infestation by *Culex quinquefasciatus* in a region of Northeastern Brazil. Rev Saúde Pública **46**, 935-941 (2012).
7. Dibo, M.R., Menezes, R.M.T., Ghirardelli, C.P., Mendonça, A.L., Neto, F.C.: The presence of Culicidae species in medium-sized cities in the state of São Paulo, Brazil and the risk of West Nile fever and other arbovirus infection. Rev Soc Bras Med Trop **44**, 496-503 (2011).
8. Pérez-Arellano, L.J., Carranza-Rodríguez, C., Vieira-Lista, C.; Muro, A.: Nematodosis I: filariosis. Medicine **10**, 3729-38 (2010).

9. Dorkenoo, A., Sodahlon, Y., Dubray, C.: Progress toward elimination of lymphatic filariasis – TOGO, 2000-2009. Morbidity and Mortality Weekly Report **60**, 989-991 (2011).
10. Morais, S.A.; Almeida, F.; Suesdek, L.; Marrelli, M.T. Low genetic diversity in wolbachia-infected *Culex quinquefasciatus* (Diptera: Culicidae) from Brazil and Argentina. Rev Inst Med Trop **54**, 325-329 (2012).
11. Conteh, L., Engels, T., Molyneu, D.H.: Socioeconomic aspects of neglected tropical diseases. Lancet **375**, 239–247 (2010).
12. Resolution Cd49.R19 Elimination Of Neglected Diseases And Other Poverty-Related Infections. Washington, D.C., Usa, 28 September-2 October 2009. Pan American Health Organization World Health Organization.
13. World Health Organization Weekly Epidemiological Record Relevé Épidémiologique Hebdomadaire 19 October 2007, 82nd Year / 19 Octobre 2007, 82e Année No. 42, 2007, 82, 361–380. Geneva.
14. World Health Organization . Global Programme To Eliminate Lymphatic Filariasis. Lymphatic Filariasis: Managing Morbidity And Preventing Disability. 2013
15. BRASIL- MINISTÉRIO DA SAÚDE. Secretaria de Vigilância em Saúde Departamento de Vigilância Epidemiológica . Guia de Vigilância Epidemiológica e Eliminação da Filariose Linfática. Brasília / DF 2009
16. Ramaiah, K.D., Ottesen, E.A.: Progress and Impact of 13 Years of the Global Programme to Eliminate Lymphatic Filariasis on Reducing the Burden of Filarial Disease**.** Plos Neglected Tropical Diseases. **8** e3319. 2014. doi:10.1371/journal.pntd.0003319.
17. Rattan, R.S.: Mechanism of action of insecticidal secondary metabolites of plant origin. Crop Prot **29**, 913-920 (2010).

18. Veerakumar, K., Govindarajan, M.: Adulticidal properties of synthesized silver nanoparticles using leaf extracts of *Feronia elephantum* (Rutaceae) against filariasis, malaria, and dengue vector mosquitoes. Parasitol Res **113**, 4085–4096 (2014).
19. Hamburg, M.A.: FDA's Approach to Regulation of Products of Nanotechnology. Science. **336**, 299-300 (2012).
20. Solans, C., Solé, I.: Nano-emulsions: formation by low-energy methods Curr Opin Colloid Interface Sci **17**: 246-254 (2012).
21. Chen, H., Khemtong, C., Yang, X., Chang, X., Gao, J.: Nanonization strategies for poorly water soluble drugs. Drug Discovery Today **16**, 354-360 (2011).
22. Ghosh V, Mukherjee A, Chandrasekaran N.: Formulation and Characterization of Plant Essential Oil Based Nanoemulsion: Evaluation of its Larvicidal Activity Against Aedes aegypti. Asian J Chem **25,** 321-323 2013.
23. Duarte, J.L., Amado, J.R.R., Oliveira, A.E.M.F.M., Cruz, R.A.S., Ferreira, A.M., Souto, R.N.P., Falcão, D.Q, Carvalho, J.C.T., Fernandes, C.P.: Evaluation of larvicidal activity of a nanoemulsion of *Rosmarinus officinalis* essential oil. Braz J Pharmacog **25**, 189-192(2015).
24. Rodrigues, E.C.R., Ferreira, A.M., Vilhena, J.C.E., Almeida, F.B., Cruz, R.A.S., Florentino, A.C., Souto, R.N.P., Carvalho, J.C.T., Fernandes, C.P.: Development of a larvicidal nanoemulsion with Copaiba (*Copaifera duckei*) oleoresin. Braz J Pharmacog. **24**, 699-705 (2014).
25. Oliveira, A.E.M.F.M., Duarte, J.L., Amado, J.R.R., Cruz, R.A.S., Rocha, C.F., Souto, R.N.P., Ferreira, R.M.A., Santos, K., Conceição, E.C., Oliveira, L.A.R., Kelecom, A., Fernandes, C.P., Carvalho, J.C.T.: Development of a Larvicidal Nanoemulsion with *Pterodon emarginatus* Vogel Oil Plos ONE **11**. e0145835 (2016). doi:10.1371/journal.pone.0145835.

26. Anjali, C.H., Sharma, Y., Mukherjee, A., Chandrasekaran, N.: Neem oil (*Azadirachta indica*) nanoemulsion a potent larvicidal agent against *Culex quinquefasciatus*. Pest Manag Sci **68**, 158-163 (2012).

27. Ghosh, V., Mukherjee, A., Chandrasekaran, N.: Optimization of Process Parameters to Formulate Nanoemulsion by Spontaneous Emulsification: Evaluation of Larvicidal Activity Against *Culex quinquefasciatus* Larva. Bio Nano Sci **4**, 157–165 (2014).

28. Sugumar, S.K.S., Clarke, M.J., Nirmala, B.K., Tyagi, A.M., Chandrasekaran, N.: Nanoemulsion of eucalyptus oil and its larvicidal activity against *Culex quinquefasciatus*. Bull Entomol Res **104**, 393–402 (2014).

29. Hoscheid, J., Cardoso, M.L.C. Sucupira as a Potential Plant for Arthritis Treatment and Other Diseases. Arthritis **Volume 2015**, Article ID 379459, 12 pages (2015).

30. Hansen, D., Haraguchi, M., Alonso, A.: Pharmaceutical properties of 'sucupira' (Pterodon spp.). Braz J Pharm Sci **46**, 607-616 (2010).

31. Dutra, R.C., Campos, M.M., Santos, A.R.S., Calixto, J.B. Medicinal plants in Brazil: Pharmacological studies, drug discovery, challenges and perspectives. Pharmacological Research. doi:10.1016/j.phrs.2016.01.021 (2016).

32. Pascoa, H., Diniz, D.G.A., Florentino, I.F., Costa, E.A., Bara, M.T.F.: Microemulsion based on *Pterodon emarginatus* oil and its anti-inflammatory potential. Braz J Pharm Sci **51**, 117-126 (2015).

33. Pimenta, A.T.A., Santiago, G.M.P., Arriaga, A.M.C., Menezes, G.H.A., Bezerra, S.B.: Estudo fitoquímico e avaliação da atividade larvicida de *Pterodon polygalaeflorus* Benth (Leguminosae) sobre *Aedes aegypti*. Braz J Pharmacog **16,** 501-505 (2006).

34. Omena, M.C., Bento, E.S., De Paula, J.E., Sant'ana, A.E.G.: Larvicidal Diterpenes from *Pterodon polygalaeflorus*. Vector Borne Zoonotic Dis **6**, 216-222 (2006).

35. Dias, D.O., Colombo, M., Kelmann, R.G., Kaiser, S., Lucca, L.G., Teixeira, H.F., Limberger, R.P, Veiga Junior, V.F., Koester, L.S.: Optimization of Copaiba oil-based nanoemulsions obtained by different preparation methods. Ind Crop Prod **59**, 154–162 (2014).
36. Wang, L., Dong, J., Chen, J., Eastoe, J., Li, X.: Design and optimization of a new self-nanoemulsifying drug delivery system. J Colloid Interface Sci 330, 443–448 (2009).
37. Ostertag F, Weiss J, McClements DJ.:Low-energy formation of edible nanoemulsions: Factors influencing droplet size produced by emulsion phase inversion J Colloid Interface Sci 388, 95–102 (2012).
38. Montenegro, L.H.M., Oliveira, P.E.S., Conserva, L.M., Rocha, E.M.M., Brito, A.C., Araújo, R.M., Trevisan, M.T.S., Lemos, R.P.L.: Terpenóides e avaliação do potencial antimalárico, larvicida, anti-radicalar e anticolinesterásico de *Pouteria venosa* (Sapotaceae). Braz J Pharmacogn **16**, 611-617 (2006).
39. Rahuman, A.A.,Gopalakrishnan, G., Venkatesan, P., Geetha, K.: Larvicidal activity of some Euphorbiaceae plant extracts against *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae). Parasitol Res **102**, 867-873 (2008.
40. Dewick, P.M. (2009): Medicinal natural products – A biosynthetic approach. 3a ed, John Wiley & Sons, Ltd. P 187-189.
41. Lópes, M.D., Pascual Villa-Lobos, M.J.: Mode of inhibition of acetylcholinesterase by monoterpenoids and implications for pest control. Ind Crop Prod **31**, 284-288 (2010).
42. El-Sheekh, M.M., Hamouda, R.A., Nizam, A.A.: Biodegradation of crude oil by Scenedesmus obliquus and *Chlorella vulgaris* growing under heterotrophic conditions. Int Biodeter Biodegr **82**, 67-72 (2013).
43. Gao, Q.T., Wong, Y.S., Tam, N.F.: Removal and biodegradation of nonylphenol by immobilized Chlorella vulgaris. Bioresour Technol **102,** 10230–10238 (2011).

44. Silva, A., Figueiredo, S.A., Sales, M.G., Delerue-Matos, C.: Ecotoxicity tests using the green algae *Chlorella vulgaris*—A useful tool in hazardous effluents management. J Hazard Mater **167**, 179–185 (2009).

45. Pereira, M.M., Mouton, L., Yéprémian, C., Couté, A., Lo, J., Marconcini, J.M., Ladeira, L.O., Raposo, N.R.B., Brandão, H.M., Brayner, R. Ecotoxicological effects of carbon nanotubes and cellulose nanofibers in Chlorella vulgaris. J Nanobiotechnol, 12-15 (2014).

46. Polonini, H.C., Brandão, H.M., Raposo, N.R.B., Brandão, M.A.F., Mouton, L., Couté, A., Yéprémian, C., Sivry, Y., Brayner, R.: Size-dependent ecotoxicity of barium titanate particles: the case of Chlorella vulgaris green algae. Ecotoxicology **24**, 938–948 (2015)

47. Schwab, F., Bucheli, T.D., Camenzuli, L., Magrez, A., Knauer, K., Sigg, L., Nowack, B.: Diuron Sorbed to Carbon Nanotubes Exhibits Enhanced Toxicity to Chlorella vulgaris. Environ Sci Technol., **47**, 7012−7019 (2013).

48. García-Gómez, C., Babin, M., Obrador, A., Álvarez, J.M., Fernández, M.D.: Toxicity of ZnO Nanoparticles, ZnO Bulk, and $ZnCl_2$ on Earthworms in a Spiked Natural Soil and Toxicological Effects of Leachates on Aquatic Organisms. Arch Environ Contam Toxicol **67**, 465–473 (2014).

49. Sánchez-Bayo, F.: Insecticides Mode of Action in Relation to Their Toxicity to Non-Target Organisms. J Environment Analytic Toxicol **S:4** (2011).

50. WHO. World Health Organization 2005. Guidelines for laboratory and field testing of mosquito larvicides. World health organization Communicable disease control, prevention and eradication Who pesticide evaluation scheme. Geneva, Swizerland.

51. Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M.: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmaco. **7**, 88-95 (1961).

52. Lourenço. S. O. 2006. Cultivo de Microalgas Marinhas: Princípios e Aplicações. Editora Rima, São Carlos. 606 p. ISBN-85-7656-113-1.

FIGURES

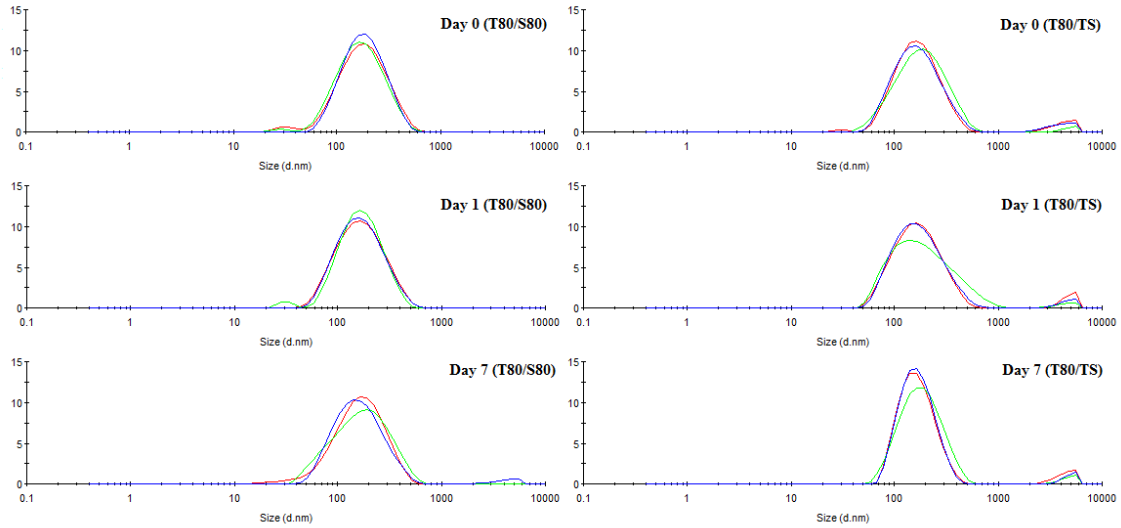

Figure 1. Particle size distribution of *P. emarginatus* nanoemulsions prepared with different surfactant pairs. Mean droplet size S80/T80. Day 0: 151.0±2.252 nm; Day 1: 146.3±1.450 nm; Day 7: 141.6±0.9504 nm. Polydispersity index. Day 0: 0.221±0.006; Day 1: 0.219±0.005; Day 7: 0.245±0.004. Mean droplet size T80/TS. Day 0: 160.7±1.493 nm; Day 1: 159.8±3.460 nm; Day 7: 167.9±1.473 nm. Polydispersity index. Day 0: 0.252±0.012; Day 1: 0.277±0.027; Day 7: 0.231±0.012.

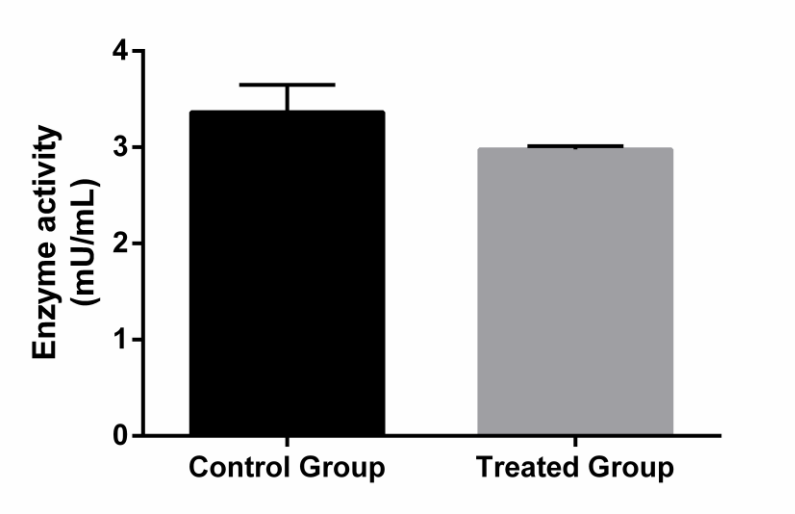

Figure 2. Acetylcholinesterase activity of enzyme from whole body homogenates of *Culex quinquefasciatus* larvae.

UTILIZATION OF DYNAMIC LIGHT SCATTERING TO EVALUATE *Pterodon emarginatus* OLEORESIN-BASED NANOEMULSION FORMATION BY NON-HEATING AND SOLVENT-FREE METHOD

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Utilization of dynamic light scattering to evaluate *Pterodon emarginatus* oleoresin-based nanoemulsion formation by non-heating and solvent-free method

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Abstract: *Pterodon emarginatus* (Fabaceae) is a great source of bioactive compounds. The most known and studied herbal derivative from this species is an ambar-colored oleoresin that contains vouacapane diterpenes and volatile terpenoids, such as β-caryophyllene. Some recent papers aimed to generate nanoemulsions using this oleoresin for biological applications. However, they used high-energy methods that elevate costs of the process or heating procedures, which offer the disadvantage of volatile substances lost. Thus, as part of our ongoing studies with nanobiotechnology of natural products, especially regarding preparation of nanoemulsions with promising plant-based oils by low cost and low energy methods, we decided to evaluate the ability of non-heating and solvent-free method to generate *P. emarginatus* oleoresin-based nanoemulsions. Two non-ionic surfactants were used to generate nanoemulsions by using a simple homogenization method with vortex stirrer. Low mean droplet size (<180 nm) and low polydispersity index (<0.200) were observed even after one day after preparation. Programmed temperature ramp analysis revealed that no major effect on droplet size and polydispersity index were observed, suggesting the robustness of formed nanoemulsions. Thus, we show for the first time formation of sucupira-based nanoemulsions by simple, low cost and ecofriendly method that may be useful to prevent volatile lost. This study open new perspectives for bioactive evaluation of this novel nano-product.

Keywords: Fruits, Low energy method, Sucupira-branca.

Conflict of interest: All authors have none to declare.

Introductory remarks

Pterodon emarginatus Vogel belongs to the family Fabaceae and is widespread in Midwest of Brazil, being also found South-Eastern, Northeast and North regions (Lima & Lima, 2015). Its fruits, as well as other *Pterodon* species, are commonly known as sucupira-branca. From this plant part, it is extracted an oleoresin that have vouacapan diterpenes and sesquiterpenes. This herbal derivative has several biological properties, including analgesic, antiinflammatory, antinoceptive and larvicidal properties (Hoscheid & Cardoso, 2015; Hansen et al., 2010).

Natural products from plant origin have been sujected to several studies aiming to generate novel nanotechnology-based bioactive products. Regarding several types of nano-size formulations, herbal oils (including oleoresins) are very interesting for nanoemulsion preparation (Zorzi et al., 2015). Nanoemulsions are kinetically stable disperse systems constituted by two immiscible liquids, often stabilized by surfactants. Droplet diameter is in a nanometer range, often ranging from 20 nm to different upper limits (eg. 500, 300, 200 and 100) that varies according to author criteria (Solans & Solé, 2012). High-energy methods involved in nanoemulsion formation often make use of high energy devices that elevate costs of the process, such as high pressure homogenisers or ultrasonicators. Spontaneous energy methods involve utilization of volatile organic solvents, that may not be considered ecofriendly, considering toxicity of some of them. Moreover, it is necessary to remove this component of the nanoemulsification process. Low-energy methods make use of intrinsic characteristics of the system, often involving phase inversion (Sugumar et al., 2016). Most common phase inversion methods are associated to phase transitions induced by change in temperature (phase inversion temperature – PIT) or composition (phase inversion composition – PIC) (Solans & Solè, 2012).

Growing interest is observed for nanobiotechnology studies with oleoresin from sucupira-branca olereosin (Hoscheid et al., 2015; Pascoa et al., 2015; Oliveira et al., 2016). The oleoresin obtained from fruits of *P. emarginatus* that was used in the present study was previously submitted to chemical characterization. Chromatographic analysis carried out using HPLC-DAD and comparison to authentic external standards revealed the presence of methyl 6α,7β-dihydroxyvouacapan-17-β-oate, geranylgeraniol and β-caryophyllene, being in accordance with phytochemical profile of the genus (Oliveira et al., 2016). Thus, is worthmentioning that utilization of a phase inversion temperature may lead to some lost of volatiles, even if it is able to generate fne droplets. The present study aim to prepare *P.emarginatus* nanoemulsions using a simple non-heating and solvent-free method that can be considered very promising for development of viable, low cost and ecofriendly nanoproduct with this natural raw material.

Material and Methods

Pterodon emarginatus Vogel (Fabaceae) fruits were obtained at the Central Market of Goiânia – GO (Brazil) and identified by Dr. José Realino de Paula. Voucher specimen was deposited at the Herbarium of Goiás Federal University (GO, Brazil) under the register number 41714. Oleoresin was previously obtained from fruits by cold pressing, being stored in amber glass flask and kept at −20 °C until utilization (Oliveira et al., 2016). Oil in water nanoemulsions were prepared by blending non-ionic surfactant (polysorbate 85, Sigma-Aldrich, St Louis, MO, or polyethyleneglycol 400 monooleate, Praid, SP, Brazil) and *P. emarginatus* oleoresin at a fixed ratio (9:1). After complete homogenization of the oily phase (surfactant + oleoresin), distilled water was slowly added dropwise to this mixture under vigorous agitation using a vortex stirrer. Each emulsion presented final mass of 10 g and 90 % (w/w) of water. Dynamic light scattering (DLS) analysis was carried out using a Zetasizer

Nano ZS, Malvern, UK). Effect of dilution in mean droplet size, polydispersity index and zeta potential was performed using different dilutions factors (1:10, 1:25, 1:50 and 1:100). Nanoemulsions were also subjected to analysis using a programmed linear ramp of temperature starting from 25 ºC to 80 ºC at a rate of 5ºC / min. Results are expressed as mean ± standard deviation.

Results and Discussion

Pterodon emarginatus oleoresin-based nanoemulsions prepared with polysorbate 85 or polyethyleneglycol 400 monooleate presented a fine aspect with bluish reflect (Figure 1) that is characteristic for this type of system. Several dilutions were performed just afterwards the preparation of *P. emarginatus*-based nanoemulsions with polysorbate 85 or polyethyleneglycol 400 monooleate (Table 1). Higher mean droplet size and polydispersity index (pdi) were observed at the dilution ratio 1:10 for nanoemulsions prepared with both surfactants, which also presented lower zeta potential (in module). On another hand, no major differences were observed for mean droplet size on the remaining dilution ratios (1:25, 1:50 and 1:100), which were around 160-170 nm for both nanoemulsions. Slighly lower polydispersity index was observed for nanoemulsion prepared with polysorbate 85, which was around 0.111-0.122, while pdi of droplets from nanoemulsion prepared with polyethyleneglycol 400 monooleate were around 0.149-1.152. Zeta potential of these nanoemulsions ranged from -21.9±0.5 mV (dilution ratio 1:25 for nanoemulsion prepared with polysorbate 85) to -28.7±0.7 mV (dilution ratio of 1:100 of nanoemulsion prepared with polyethyleneglycol 400 monooleate. Dilution prior to particle size distribution analysis is considered a critical parameter. Optimized dilution factor should be determined, in order to avoid multiple scattering effects. Moreover, dilution of previously prepared nanoemulsions have been considered an important parameter that affects stability and particle growth on

nanoemulsions (Saberi et al., 2013a,b; Saberi et al., 2014). Considering that more concentrated stock nanoemulsion should be preferred for further dilutions in practical applicable products and since no major difference was observed for most of them, we decided to further investgate effect of temperature using the nanoemulsion at 1:25 dilution.

INSERT TABLE 1 HERE

INSERT FIGURE 1 HERE

Figure 2 shows influence of temperature on particle size distribution and zeta potential. Analysis of mean droplet size of nanoemulsion prepared with polysorbate 85 as function of temperature revealed that it remained almost constant from 25 to 35 ºC (around 170 nm). Then, it rapidly decreased, being around 140 nm from 40 to 80 ºC. On another hand, droplet diameter reduction observed for nanoemulsion prepared with polyethyleneglycol monooleate occurred more gradually, also reaching low values in higher temperatures. Probably the coating film that this surfactant form through the droplets is more rigid due to plasticizer character of polyethyleneglycol surfactants. Thus, rearragement of the internal phase may not be so drastically as it was observed for polysorbate 85. Polydispersity index of both nanoemulsions started below 0.200, being the value associated to nanoemulsion prepared with polysorbate 85 slighly lower than value observed for nanoemulsion prepared with polyethylene 400 monooleate. Then they reached a maximum around 0.250 after 35ºC and decreased to minimum values below 0.100 at 80ºC. Zeta potential slight changed below 40 ºC, however, overall gradual increase was observed as function of temperature.

Non-ionic surfactants stabilizes nanoemulsions by reducing interfacial tension and also form a layer that promotes steric repulsion between droplets (Wang et al., 2009). The main mechanism for nanoemulsion destabilization is associated to Ostwald ripening, which

induces formation of larger droplets (Solans & Solè, 2012). Interestingly, increased temperatures induced formation of smaller droplets while no increase above 0.300 on polydispersity index was observed. Moreover, pdi values returned to basal values around 0.100. This parameter reflects homogeneity of particle size distribution and gradual formation of larger droplets from a starting monomodal distribution would results in increased polydispersity index.

Migration of acid compounds from copaiba oleoresin was associated to negative zeta potential values on nanoemulsions prepared with this material (Dias et al., 2014). Vouacapan diterpenes, including some with acid moiety are characteristics for sucupira oleoresin and may explain these starting zeta potential values. Further increase of temperature may have induced growing partition of additional substances that do not easily dissociate, therefore reducing this zeta potential. Moreover, deposition of some substances on the interface may form more compact filmes, and therefore enhancing the stability of the system (Wang et al., 2009). This results may be contributing together to better stabilization of *P. emarginatus* nanoemulsions and reduced particle growth that was observed in the present study.

INSERT FIGURE 2 HERE

The optimized larvicidal nanoemulsion prepared with the same oleoresin that was used in the present study, mixture of polysorbate 80/sorbitan monooleate (HLBmixture = 11) and water presented mean droplet size around 130 nm, polydispersity index around 0.200 and zeta potential around -30 mV. The nanoemulsification method involved homogenization of oily phase (*P. emarginatus* oleoresin and surfactants) at 80 ºC for 30 min (Oliveira et al., 2016). A procedure that also involved heating process was also performed aiming to prepare *P. emarginatus* based nanoemulsions for antiinflammatory evaluation (Pascoa et al., 2015). It is

worth mentioning that volatile substances from the oleoresin may be lost during heating of oily phase. For this reason, utilization of effective nanoemulsification methods that do not involve heating step should be considered an advantage, considering that they would protect the undesired release of volatiles, including terpenoids. *Rosmarinus officinalis* essential oil was formerly used for development of an oil in water nanoemulsion using phase inversion temperature (PIT) method and droplets with mean diameter around 100 nm were obtained (Fernandes et al., 2013). Latter, emulsion phase inversion using a titration process was performed for achievement of bioactive rosemary-based nanoemulsions using the same natural raw material. After seven days of storage, mean droplet size aroung 115.0 nm and low polidispersity index (<0.300) was observed (Duarte et al., 2015).

The nanoemulsions prepared with oleoresin from *Copaifera duckei* were prepared by using phase inversion temperature method. Low mean droplet size (145.2 nm) and relatively broad particle size distribution (pdi = 0.378) were observed for this larvicidal nanoproduct after one day of preparation (Rodrigues et al., 2014). The oleoresin from other copaiba species (*C. multijuga*) was nanoemulsified using a high-pressure homogenizer and mean droplets size around 120-140 nm and zeta potential around -20 mV were obtained (Dias et al., 2012). On another study, nanoemulsions prepared with *C. multijuga* oleoresin were prepared using high-pressure homogenization method or spontaneous emulsification using organic solvents. Most of nanoemulsions prepared by high energy method presented mean droplet size around 160-200 nm and monomodal distribution, being considered more efficient than solvent-based method in this study (Dias et al., 2014). The oleoresin from *P. pubescens* mixed with phospholipon 90G was added through aqueous dispersions of several surfactants and then nanoemulsified using high-speed shear homogenizer. Mean diameter of droplet ranged from 199 to 860 nm and lowest size were observed for nanoemulsions prepared with polyethylene

glycol (PEG-40) castor oil/sorbitan oleate and PEG-40 hydrogenated castor oil/sorbitan oleate (PEG-40H) (Hoscheid et al., 2015).

Conclusion

Due to high biological potential of sucupira oils, recent studies focused on development of nanoemulsions using this natural raw material. However, these studies used high-cost equipments of metodologies that invole heating, probably inducing some lost of volatile components. Additional strategies for nanoemulsions containing oleoresin make use of organic solvents, that also may cause damage to the envinronment. Thus, considering the high potential of studies that make possible to generate nanoemulsions by low cost and ecofriendly methods, the present study aimed and successfully generated *P. emarginatus* nanoemulsions using a non-heating and solvent free method. Homogenization using vortex stirrer allowed achievement of particles with low diameter and low polydispersity index, with no impariment when compared to described methods. Moreover, utilization of this simple method and single low cost surfactants make this nanoproduct very promising for practical applications. We believe that the present study will make possible to disseminate preparation of this nanoemulsions for a wide range of aplications, highligting an important brazilian chemical raw material.

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Authors' contributions

AEMFMO (PhD student), RASC (PhD student) and JLD (undergraduate student) contributed running the laboratory work, analysis of the data and drafted the paper. ECC contributed to oleoresin extraction, characterization and critical reading of the manuscript. JCTC and CPF designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

References

Dias, D.O., Colombo, M., Kelmann, R.G., Souza, T.P., Bassani, V.L., Teixeira, H.F., Veiga Jr., V.F., Limberger, R.P., Koester, L.S., 2012. Optimization of headspace solid-phase microextraction for analysis of β-caryophyllene in a nanoemulsion dosage form prepared with copaiba(Copaifera multijuga Hayne) oil. Anal. Chim. Acta. 721, 79– 84.

Dias, D.O., Colombo, M., Kelmann, R.G., Kaiser, S., Lucca, L.G., Teixeira, H.F., Limberger, R.P., Veiga Jr., V.F., Koester, L.S., 2014. Optimization of Copaiba oil-based nanoemulsions obtained by different preparation methods. Ind. Crop. Prod. 59, 154–162.

Duarte, J.L., Amado, J.R.R., Oliveira, A.E.M.F.M., Cruz, R.A.S., Ferreira, A.M., Souto, R.N.P., Falcão, D.Q., Carvalho, J.C.T., Fernandes, C.P., 2015. Evaluation of larvicidal activity of a nanoemulsion of Rosmarinus officinalis essential oil. Rev. Bras. Farmacogn. 25, 189–192.

Fernandes, C.P., Mascarenhas, M.P., Zibetti, F.M., Lima, B.G., Oliveira, R.P.R.F., Rocha, L., Falcão, D.Q., 2013. HLB value, an important parameter for the development of essential oil phytopharmaceuticals. Rev. Bras. Farmacogn. 23, 108-114.

Lima, H.C. de, Lima, I.B. *Pterodon* in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. Available at: <http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB29842>. Accessed on June, 28, 2016.

Hansen, D., Haraguchi, M., Alonso, A., 2010. Pharmaceutical properties of "sucupira" (*Pterodon spp*.). Braz. J.Pharm. Sci. 46, 607-616.

Hoscheid, J., Cardoso, M.L.C., 2015. Sucupira as a Potential Plant for Arthritis Treatment and Other Diseases. Arthritis. 2015, Article ID 379459, 12 pages.

Hoscheid, J., Outuki, P.P., Kleinubing, S.A., Silva, M.F., Bruschi, M.L., Cardoso, M.L.C., 2015. Development and characterization of *Pterodon pubescens* oil nanoemulsions as a possible delivery system for the treatment of rheumatoid arthritis. Colloids and Surfaces A: Physicochem. Eng. Aspects. 484, 19-27.

Oliveira, A.E.M.F.M., Duarte, J.L., Amado, J.R.R., Cruz, R.A.S., Rocha, C.F., Souto, R.N.P., Ferreira, R.M.A., Santos, K, Conceição, E.C., Oliveira, L.A.R., Kelecom, A., Fernandes, C.P., Carvalho, J.C.T., 2016. Development of a Larvicidal Nanoemulsion with *Pterodon emarginatus* Vogel Oil. PLoS ONE. 11, e0145835.

Pascoa, H., Diniz, D.G.A., Florentino, I.F., Costa, E.A., Bara, M.T.F., 2015. Microemulsion based on *Pterodon emarginatus* oil and its anti-inflammatory potential. Braz. J. Pharm. Sci. 51, 117-126.

Rodrigues, E.C.R., Ferreira, A.M., Vilhena, J.C.E., Almeida, F.B., Cruz, R.A.S., Florentino, A.C., Souto, R.N.P., Carvalho, J.C.T., Fernandes C.P., 2014. Development of a larvicidal nanoemulsion with Copaiba (*Copaifera duckei*) oleoresin. Rev. Bras. Farmacogn. 24: 699-705.

Saberi, A.H., Fang, Y., McClements, D.J., 2013a. Effect of glycerol on formation, stability, and properties of vitamin-E enriched nanoemulsions produced using spontaneous emulsification. J. Colloid Interface Sci. 411, 105–113.

Saberi, A.H., Fang, Y., McClements, D.J., 2013b. Fabrication of vitamin E-enriched nanoemulsions by spontaneous emulsification: Effect of propylene glycol and ethanol on formation, stability, and properties. Food Res. Int. 54, 812–820.

Saberi, A.H., Fang, Y., McClements, D.J., 2014. Stabilization of vitamin E-enriched mini-emulsions: Influence of organic and aqueous phase compositions. Colloids and Surfaces A: Physicochem. Eng. Aspects. 449, 65–73.

Solans, C., Solé, I., 2012. Nano-emulsions: formation by low-energy methods. Curr Opin Colloid Interface Sci. 17, 246-254.

Sugumar, S., Ghosh, V., Mukherjee, A., Chandrasekaran, N., 2016. Essential oil-based nanoemulsion formation by low- and high-energy methods and their application in food preservation against food spoilage microorganisms. In Preedy, V.R. (org.) Essential Oils in Food Preservation, Flavor and Safety. Academic Press, p. 93-100.

Wang, L., Dong, J., Chen, J., Eastoe, J., Li, X., 2009. Design and optimization of a new self-nanoemulsifying drug delivery system. J. Colloid Interface Sci. 330, 443–448.

Zorzi, G.K., Carvalho, E.L.S., von Poser, G.L., Teixeira, H.F., 2015. On the use of nanotechnology-based strategies for association of complex matrices from plant extracts. Rev. Bras. Farmacogn. 25, 426-436.

FIGURES AND TABLES

Table 1. Effect of dilution on droplet size, polydispersity index (pdi) and zeta potential of nanoemulsions prepared with oleoresin extracted from *Pterodon emarginatus* fruits and non-ionic surfactants (polyethyleneglycol monooleate or polysorbate 85). Each measurement represents mean ± standard deviation. Each analysis was performed in triplicate.

Dilution	Polysorbate 85			Polyethyleneglycol 400 monooleate		
	Size (nm)	Pdi	Zeta (mV)	Size (nm)	Pdi	Zeta (mV)
1:10	224.6±0.6	0.265±0.008	-16.1±0.3	176.5±1.8	0.188±0.014	-20.1±0.4
1:25	166.7±1.8	0.113±0.011	-21.9±0.5	167±1.9	0.149±0.005	-25.2±0.8
1:50	164.4±0.5	0.122±0.001	-24.9±0.1	160.8±0.2	0.152±0.021	-23.3±0.3
1:100	163.5±0.2	0.111±0.015	-26.2±1.2	159.2±0.9	0.150±0.006	-28.7±0.7

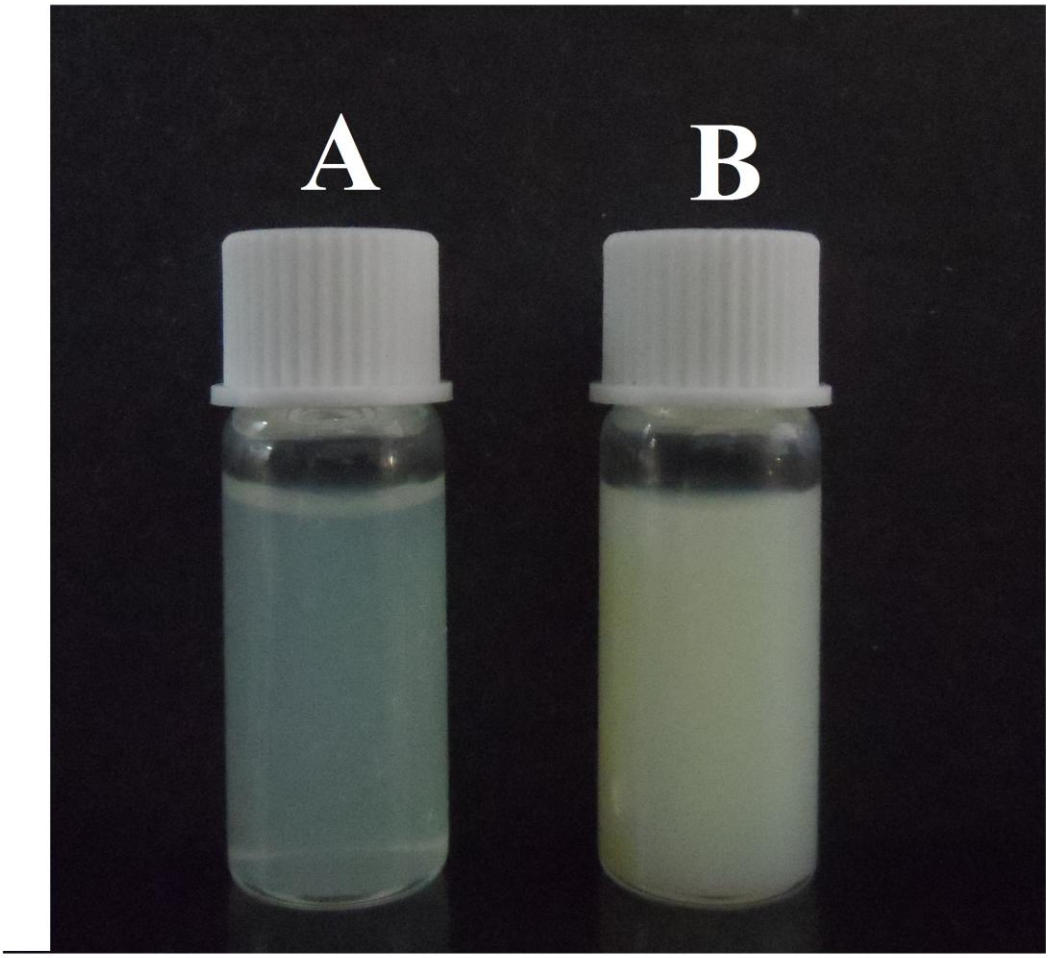
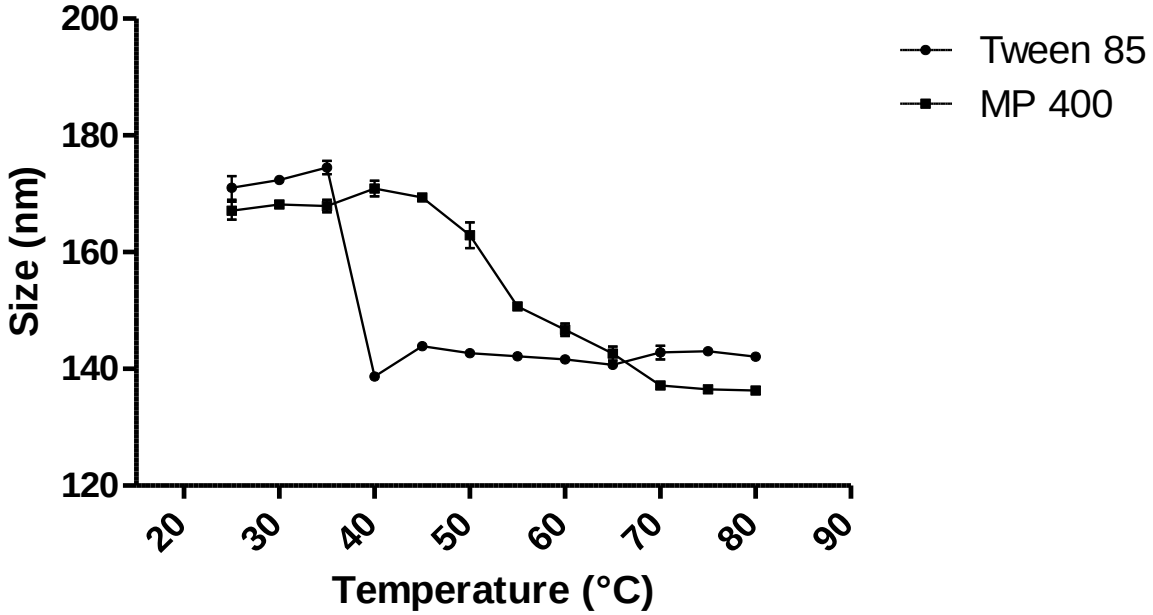


Figure 1. Nanoemulsions prepared with oleoresin extracted from *Pterodon emarginatus* fruits and non-ionic surfactants (A) polyethyleneglycol monooleate or (B) polysorbate 85.


200
180
160
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Size (nm)
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80
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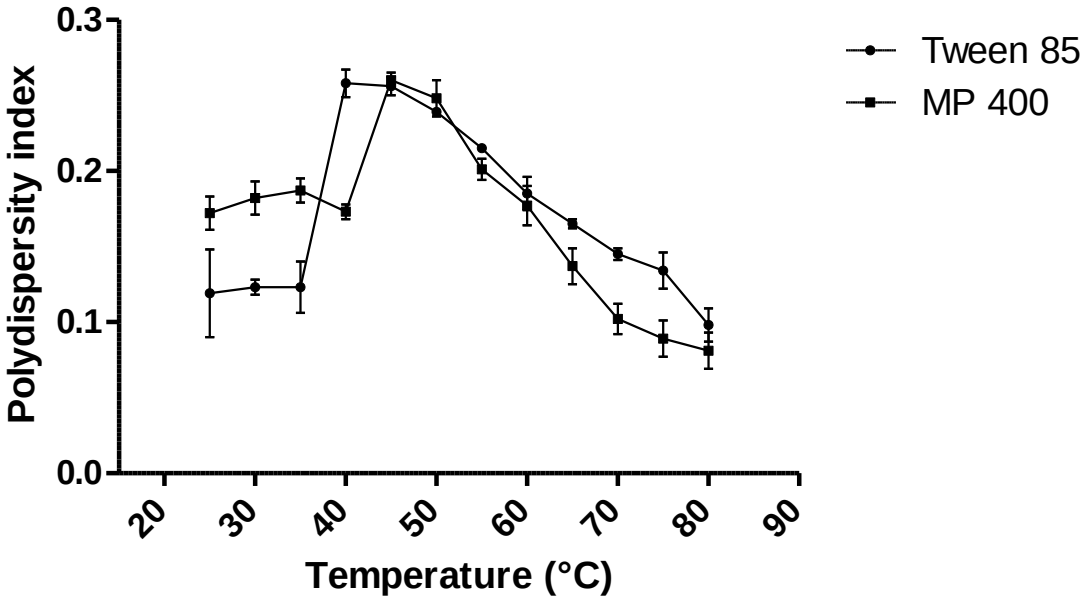
Temperature (°C)
Tween 85
MP 400


0.3
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MP 400

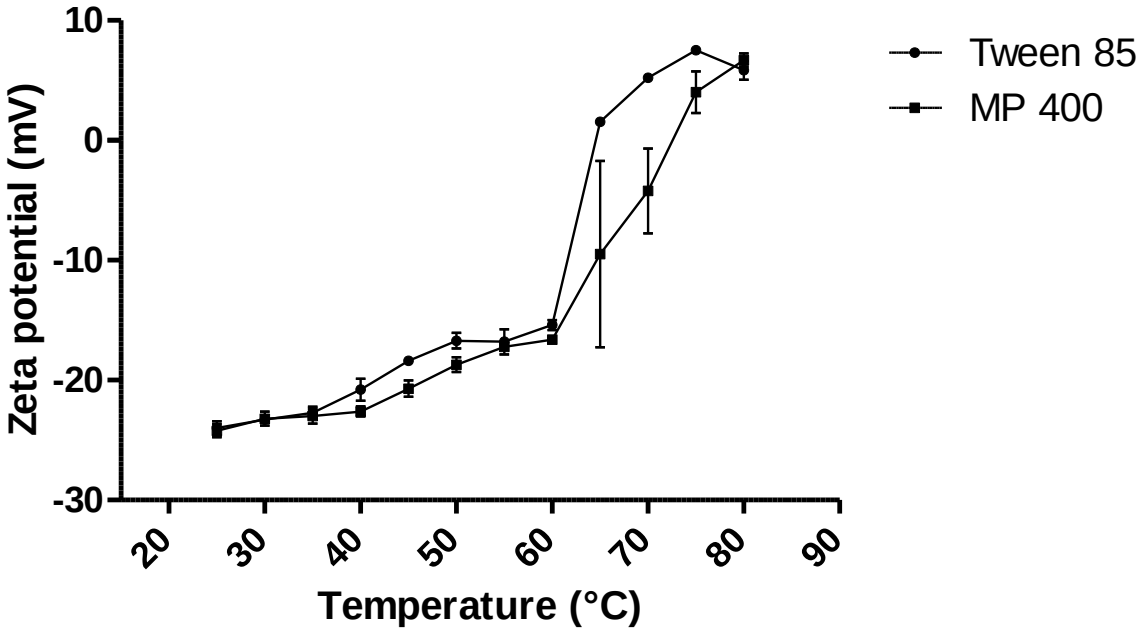

Figure 2. Influence of temperature on droplet size, polydispersity index (pdi) and zeta potential of nanoemulsions prepared with oleoresin extracted from *Pterodon emarginatus* fruits and non-ionic surfactants (polyethyleneglycol monooleate or polysorbate 85). Each measurement represents mean ± standard deviation. Each analysis was performed in triplicate.

ESSENTIAL OIL FROM *Pterodon emarginatus* AS A PROMISING NATURAL RAW MATERIAL FOR LARVICIDAL NANOEMULSIONS AGAINST A TROPICAL DISEASE VECTOR

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* A formatação deste capítulo segue as normas da revista Parasites & Vectors.

Title Page

Essential oil from *Pterodon emarginatus* as a promising natural raw material for larvicidal nanoemulsions against a tropical disease vector

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Abstract

Background: *Pterodon emarginatus* oleoresin obtained from fruits was previously subjected to development of larvicidal nanoemulsions. However, to our knowledge, no studies aiming to develop nanoemulsions with its essential oil were carried out. This study describes preparation and evaluation of larvicidal activity of a novel oil in water nanoemulsion prepared with essential oil from fruits of *P. emarginatus* against *Aedes aegypti* larvae. **Results:** Gas-chromatographic analysis revealed that β-caryophyllene is the major compound, corresponding to 25.8 % of relative percentage of the essential oil. Series of nanoemulsions were obtained and better results were achieved using polysorbate 80, suggesting that required Hydrophile-Lipohile Balance value of the oil is around 15. Mean droplet size decreased from 128.0±6.217 nm to 53.21±0.483 nm, when surfactant to oil ratio was enhanced from 1.0 to 2.0. High mortality levels were observed on 4^{th} instar larvae. **Conclusions:** Ecofriendly nano-based larvicidal products using natural products, especially essential oils are very promising for novel viable larvicidals. This study allowed preparation of nanoemulsions with *P. emarginatus* essential oil for the first time, which proved to be potential larvicidals against *A. aegypti*. Moreover, low cost, solvent-free method was performed, being useful for further applications in integrative practices of vector control.

Keywords: *Aedes aegypti*, dengue, chikungunya fever, hydrodistillation, nanotechnology, sucupira-branca and zika.

Background

Dengue fever is a tropical neglected disease in which more than 2.5 billion of people worldwide are in risk to be infected with dengue vírus. Moreover, approximately 500,000 people require hospitalization, being mostly children, and 2.5% of cases result in death [1]. In Brazil, a total of 170,103 probable cases of dengue were reported in the year 2016. Higher prevalence of virus serotype 1 (DENV1) have been observed in this country. Moreover, growing cases related to other diseases transmitted by *Aedes aegypti* (Diptera: Culicidae), including chikungunya fever and zika, have been reported [2]. The main strategy to prevent diseases transmitted by *A. aegypti* is associated to vector control [1]. These methods involve elimination of water media in which oviposition occurs and mosquito larvae develops, application of larvicidal chemical agents, utilization of biological control and adulticidal agents [3]. Several studies have demonstrated larvicidal activity of plant-based natural products against *A. aegypti*, including essential oils [4,5,6].

Pterodon Vogel is a genus of plants native to Brazil, being widely distributed in several regions and phytogeographic domains, including Amazon and Brazilian savanna (cerrado) [7]. Its species are commonly known as “sucupira” and have fruits that are widely used in folk medicine, mainly due to anti-inflammatory and analgesic properties [8,9]. Most studies about this genus are associated to the species *P. emarginatus* Vogel, mainly using its fruits or seeds. These plant parts have an oleoresin that is highly viscous and considered a source of vouacapan diterpenes [10]. Fruits or seeds of *P. emarginatus* are also used as source of an essential oil with a wide range of biological activities, being able to ameliorate autoimune encephalomyelitis [11], to inhibit contractions in rat isolated trachea [12]. Moreover, it shows antinoceptive [13], antiulcerogenic [14], anti-inflammatory [14, 15], and

antiproliferative [16] effects, antimicrobial activity against *Staphylococcus aureus* ATCC25923 [17] and bactericidal activity against *Mycobacterium bovis*. P*. emarginatus* essential oil also presented larvicidal activity against *A. aegypti* [19]. However, intrinsic low water miscibility of these natural products is a technological challenge for viable products.

Nanoemulsions are disperse systems constituted by two immiscible liquids, usually stabilized by surfactants [20]. They are metastable and have transparent or translucent aspect [21]. Due to small particle size, nanoemulsions have enhanced kinetic stability, when compared to macroemulsions [22,23]. They are obtained by two major groups of methods in terms of energy. Increasing interest have been observed for low energy methods, since they enable obtainment of nanoemulsions with fine droplets using low cost equipment. Spontaneous emulsification or phase inversion composition are the main mechanism of nanoemulsification on low energy methods [24].

Oil in water nanoemulsions have been considered very promising for delivery of poor water soluble larvicidal substances in aqueous media, such as essential oils and other natural products. Thus, it is in the spotlight of research for integrative practices of tropical diseases vector control [25,26,27]. The oleoresin obtained from seeds of *P. emarginatus* was successfully used for the obtention of a novel larvicidal nanoemulsion against *A. aegypti* [28]. However, to our knowledge, no study was carried out in order to generate an oil in water nanoemulsion with the essential oil from this plant. Thus, as part of our ongoing studies with nanobiotechnology of plant-based larvicidals, the aim of the present study was to obtain essential oil-based larvicidal nanoemulsions using fruits of *P. emarginatus*, an important classical Brazilian plant.

Results and Discussion

Hydrodistillation of *P. emarginatus* fruits yielded 2.29 % (w/w) of a transparent essential oil. In this oil, 26 compounds were detected, of which 24 were identified. Beta-caryophyllene (Fig. 1) was the major compound, corresponding to 25.8% of relative composition of the essential oil. Delta-cadinene (9.2%), germacrene D (7.4%), alfa-humulene (6,0%), beta-elemene (5.8%), biciclogermacrene (5.6%) were also representative in this sample. All identified substances are presented in Table 1. Regarding chemical variability, literature data suggests occurrence of chemical polymorphism on essential oils of *P. emarginatus*, highlighted by the presence of two major groups, represented by β-caryophyllene or α-copaene [29]. Thus, ours results are in accordance with previous results obtained for essential oil from fruits of *P. emarginatus*.

INSERT FIGURE 1 HERE

INSERT TABLE 1 HERE

Seven formulations were prepared at different ratios of polysorbate 80 and sorbitan monooleate. All of them presented a bluish reflect, which is characteristic for nanoemulsions [30]. Some of them were opaque (HLB of surfactant mixture from 9-13) and translucent (HLB 14), while a transparent appearance was observed for the formulation prepared solely with polysorbate 80 (HLB 15) (Fig. 2). Analysis of the formulations by dynamic light scattering (DLS) indicated that all of them showed low mean droplet size after preparation (Table 2),

supporting their classification as nanoemulsions. An important criterion for classification of nanoemulsions is related to their droplet size. Usually, they are referred as emulsions that have mean droplet size ranging from 20 to upper limits that may vary (e.g. 500, 300, 200 and 100) [24]. The nanoemulsion prepared solely with polysorbate 80 presented the smallest mean droplet size (128.0±6.2 nm) and almost no variation was observed after one day of storage (133.6±1.8 nm). Low polydispersity index was also observed after preparation (0.250±0.0) and after one day of storage (0.288±0.03).

INSERT FIGURE 2 HERE

INSERT TABLE 2 HERE

Surfactants develop a main role in nanoemulsion formation, since it reduces interfacial tension and enable formation of a film around droplets, enhancing physical stability of the system [31]. They can be classified according to the HLB scale, in which more polar surfactants have higher values, when compared to low polar surfactants. Often, higher HLB values enable formation of oil droplets dispersed in aqueous media (oil in water emulsions), which are especially important for larvicidal agents [32]. Despite a wide range of surfactants can be used in a wide range of HLB, an important strategy rely on utilization of different ratios of a hydrophilic and lipophilic surfactant [31]. Therefore, it is possible to achieve a wide range of formulations with different surfactant HLB values. It is well known that a disperse system with increased stability is formed when the HLB value of the surfactant or surfactant mixture coincides with the required HLB (rHLB) value of the oil [33].

For this reason, determination of rHLB value of important natural oils, including essential oils, have been considered a valuable tool for nanoemulsions preparation [34]. To our knowledge, rHLB of essential oil from fruits of *P. emarginatus* was not previously obtained. Our results suggest that for an essential oil with this composition is around 15, giving valuable information for further studies aiming to generate nanoemulsions with this natural raw material.

Another important parameter for nanoemulsion preparation is related to surfactant amount [35, 36]. We decided to increase surfactant amount to evaluate its influence on nanoemulsion formation. The formulation constituted by *P. emarginatus* essential oil and polysorbate 80, which proved to form most stable nanoemulsion, was prepared at SOR = 2. DLS analysis revealed that mean droplet size decreased (Day 0: 53.21±0.483 nm / Day 1: 57.44±0.280 nm), when compared to SOR = 1. However, it was observed a more polydisperse distribution, highlighted by higher polydispersity indices (Day 0: 0.361±0.008 / Day 1: 0.398±0.003). This parameter reflects homogeneity of particle populations and values below 0.300 are desirable and higher values are associated to polymodal distribution [37]. Increase of SOR and have been associated to lower mean droplet size, as it was observed in the present study. Surfactant adsorption through droplet interface will prevent aggregation and coalescence after droplet collision, if its concentration is enough to cover the droplets that were formed [38]. Smallest mean droplet size was achieved with polysorbate 80 at SOR = 2, being in accordance with literature data for increased surfactant amounts and supporting that this surfactant is able to form optimized *P. emarginatus* essential oil-based nanoemulsions. However, we considered that nanoemulsion prepared with this surfactant at SOR = 1 is suitable for larvicidal assays, since it also presented satisfactory mean droplet size and lower pdi after preparation. Considering that increased surfactant amount also increase

costs of the formulations, it can be considered an additional advantage of this nanoemulsion.

Thus, the nanoemulsion prepared using polysorbate 80 and *P. emarginatus* essential oil at SOR 1 was chosen for larvicidal assay and subjected to DLS characterization for an extended period. Figure 3 shows particle size distribution after different storage periods. After 7 days of storage, increase in polydispersity index was observed. This result is highlighted by increase of droplet population below 100 nm, while a decrease in particle population around 130 nm is observed. Peak of both populations curve coincides after 14 days and then, predominance of droplets with diameter below 100 nm is observed after 21 and 30 days of storage. The main mechanism for nanoemulsions instability is called Ostwald ripening. It happens when the oil has some degree of solubility in the external phase, leading to formation of higher droplets until phase separation occur [22]. However, as discussed earlier, our results showed that increase of polydispersity index was not associated to formation of larger droplets populations. A possible explanation of this fact may be due to gradual release of the volatile substances after reaching the external phase. Spontaneous diffusion and evaporation of volatile substances from internal phase have been considered a mechanism for droplet size reduction [39] and may associated to this results.

INSERT FIGURE 3 HERE

Table 3 shows mortality levels induced by the nanoemulsion prepared with *P. emarginatus* essential oil. Complete dispersibility of the nanoemulsion in the aqueous media confirmed that they are oil in water type. After 24 h of treatment, no mortality was observed

for groups treated with nanoemulsion at 25 ppm (concentrations expressed as function of essential oil) and control group, while higher mortality was observed for group treated at 500 ppm (66.7 ± 12.5 %) ($p<0.0001$). Mortality induced by nanoemulsion prepared with essential oil from fruits of *P. emarginatus* was dependent of exposure time on concentrations of 150 ($p<0.01$) and 250 ($p<0.01$) ppm, while no statistical difference in mortality levels, as function of time, was observed for other tested and control groups ($p>0.05$). After 48 h, significant difference in mortality levels were observed for groups treated at 150 ppm ($p<0.001$), 250 ppm ($p<0.001$) and 500 ppm ($p<0.0001$), when compared to control group. No significant difference was observed between groups treated at 150 and 500 ppm, which reached higher mortality levels, respectively, 73.3 ± 9.4 and 76.7 ± 12.4 . Despite mean mortality level decreased in the group treated at 250 ppm, no statistical difference was observed ($p>0.05$), when compared to groups treated at 150 and 500 ppm. Low percentage of deviance explained by the model (t24 = 59.1353; t48 = 57.2029) and low adjusted percentage (t24 = 31.4399; t48 = 29.038) were observed for the model in the analysis of deviance after 24 h ($p = 0.0035$) and 48 h ($p = 0.0044$) (Fig. 4). Estimated lethal concentrations for this nanoemulsion, expressed as function of essential oil content, were 371.6 (252.5 – 759.7) ppm ($LC_{50\ /\ 24h}$) and 213.7 (71.4 – 410.9) ppm ($LC_{50\ /\ 48h}$).

INSERT TABLE 3 HERE

INSERT FIGURE 4 HERE

Nanoemulsions prepared with neem oil induced various mortality levels in *Culex quinquefasciatus* larvae as function of different mean droplet size, when concentration of the oil was kept constant at 100 ppm. As droplet size increased, mortality level decreased [40]. Dilution of the nanoemulsion has major influence in the stability of the system, which is mainly associated to droplet size increase [41,42,43]. On the larvicidal assay of the present study, this influence of dilutions to achieve the wide range of experimental concentrations may induced slight differences in droplet size and consequently influenced mortality and LC_{50} determination.

Other study also reported the relevance of essential oil-based nanoemulsions as promising larvicidal agents against tropical disease vectors. Oil in water prepared with rosemary essential oil and polysorbate 20 induced around 90% of mortality in *A. aegypti* larvae after 48 h [44]. Neem oil-based nanoemulsion prepared with SOR 1.5 and which presented mean droplet size around 93.0 ppm induced approximately 73 % of mortality in *C. quinquefasciatus* larvae after 24 h [40]. It was observed that increased fold dilution of basil essential oil nanoemulsion affected exposure time necessary to complete loss of *A. aegypti* larval viability [45]. Several studies aiming to generate nano-based larvicidal with natural product active ingredients use metallic nano-products, such as silver nanoparticles [46,47,48,49,50,51,52]. However, it is worth mentioning that eco-safety and bioaccumulation of metals is a major concern for this approach. On this context, natural-product based nanoemulsions prepared with biodegradable surfactants appear as a promising alternative. Moreover, reduced costs of raw materials used for nanoemulsions, such as non-ionic surfactants (eg. polysorbate 80) makes then attractive.

Volatility of essential oils is a great advantage in terms of envinronment non persistency [58]. However, this could be an disadvantage if the concentration of its larvicidal constituents decreases to non-toxic levels to larvae. Thus, products that extend larvicidal activity are considered very relevant for vector control [54]. Oil in water nanoemulsions have been considered of great interest due to several advantages. Enhancement of water miscibility of water-immiscible samples, such as essential oils, is a major advantage. However, formulation of natural products as oil in water nanoemulsions also allows improved chemical and physical stability. Thus, utilization of nanotechnology has been considered a promising tool for ecofriendly pesticides due to its capacity to minimize rapid evaporation of essential oils [60]. Microencapsulation of essential from fruits of *P. emarginatus* was previously performed for this purpose [61]. However, to our knowledge, no nanoemulsions containing this essential oil was previously prepared.

Conclusions

Natural products, especially essential oils, have been considered valuable tools for larvicidal agents. However, poor water solubility of their chemical constituents makes development of viable products a technological challenge. The present study allowed achievement of a series of oil in water nanoemulsions with this important plant-based raw material. Moreover, it was possible to determine its rHLB value and evaluate the influence of some parameters, such as optimal surfactant mixture and influence of surfactant concentration on nanoemulsion formation. The nanoemulsion proved to be active against *A. aegypti*, the main vector of dengue and other diseases that have been considered public health problems. A organic solvent-free and low energy method with reduce costs was

employed for nanoemulsion preparation. Valorization of *P. emarginatus* by preparing a novel, biodegradable and ecofriendly potential larvicidal nanoproduct based on a natural product that can be obtained by sustainable use of biodiversity is also a remarkable contribution of this study.

List of abbreviations

DLS – dynamic light scattering

HLB – hydrophile/lipophile balance

PDI – polydispersity index

PG – particle growth

rHLB – required hydrophile lipophile balance

SOR – surfactant to oil ratio

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article and any of the datasets during the current study may be available from the corresponding author on reasonable request.

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Authors' contributions

AEMFMO contributed in this paper running the laboratory work, analysis of the data and drafted the paper, which is part of her doctorate thesis. DCB and JLD contributed in preparation and characterization of nanoemulsions. RNPS and RMAF contributed in insect bioassay. ECC contributed in preparation of natural product raw material. RASC and JN contributed in essential oil extraction. SL, HRB and PAG contributed to chemical characterization of essential oil. JCTC and CPF designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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Materials and Methods

Chemicals

Surfactants (polysorbate 80 – HLB 15, sorbitan monooleate - HLB 4,3) were obtained from Praid (SP, Brasil).

Essential oil extraction

Pterodon emarginatus fruits (760 g) were crushed with distilled water. Then, the material was placed in a 5 L bottom flask and submitted to hydrodistillation during 3 h using a Clevenger-type apparatus. In the end, the oils were collected and stored at 4°C for further analyses.

Gas-chromatography analysis

Characterization of each essential oil was performed by gas chromatography coupled with mass spectrometry (GC-MS) using an Agilent 6890 gas chromatograph coupled to an Agilent 5973N mass spectrometer. Separation was accomplished with a HP-5MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm phase thickness). Essential oils samples were dissolved in dichloromethane at a ratio of 1:1000 and a volume of 1 µL was injected. Operating conditions were as follows: split ratio 1:20; Injector temperature 250 ºC; carrier gas: helium, 1.0 mL/min, constant flow; column temperature, 60 ºC (no hold), 3 ºC per min to 240 ºC. Mass spectra were acquired in electron ionization mode at 70 eV using a scan range of 40-450 m/z and a sampling rate of 3.15 scan/s. The ion source temperature was 230 ºC, mass analyzer 150 ºC and transfer line 260 ºC.

Essential oils relative compositions were obtained using gas chromatography coupled with flame ionization detection (GC-FID). Analyses were performed using an Agilent 7890A gas chromatograph and separation was accomplished with a HP-5MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm phase thickness). The detector (FID) was operated at 280 ºC. The injection procedure and conditions was the same as described above, except the carrier gas, which was hydrogen, 1.5 mL/min.

Compounds identification

Linear retention indices were calculated by injection of a series of n-alkanes (C7-C26) [57] using de same column and condition as above for GC analyses. Identification of peaks was performed by comparison of mass spectra with an electronic library database [58] and comparing their calculated linear retention indices with literature data [59].

Preparation of nanoemulsions

Nanoemulsions were prepared by low energy titration method [36]. Final mass was 50 g and nanoemulsions contained 2500 ppm of *P. emarginatus* essential oil. Essential oil and surfactants (sorbitan monooleate/polysorbate 80) were pooled together and stirred for 30 minutes. Then, water was added drop wise and the system was stirred for additional 60 min under 800 rpm.

Factors of influence on nanoemulsion formation

Surfactant mixtures with different hydrophile lipophile balance (HLB) were achieve by mixing sorbitan monooleate (HLB = 4.3) and polysorbate 80 (HLB 15) at different ratio according to following equation:

$$HLBm = [(HLB_{sm} \times m_{sm}) + (HLB_{p80} \times m_{p80})] / (m_{sm} + m_{p80})$$

Where: HLB_{sm} is HLB of sorbitan monooleate (4.3); HLB_{p80} is HLB of polysorbate 80 (15.0); m_{sm} and m_{p80} is the mass of each surfactant.

Influence of surfactant to oil ratio (SOR) was performed by using two different amounts of surfactant (SOR = 1 or 2). Surfactant to oil ratio was calculated as follows [38]:

$$SOR = m_s/m_o$$

Where: m_s is the mass of surfactant (s) at rHLB of the oil and m_o is the mass of *P. emarginatus* essential oil.

Characterization of nanoemulsions

Droplet size, polydispersity index and zeta potential of the nanoemulsions were determined using a Zetasizer ZS (Malvern, UK). Each sample was diluted with distilled water (1:20) for analysis. Measurements were performed in triplicate and results were expressed as the mean diameter ± standard deviation.

Particle growth

Evaluation of increase in droplet size was after 1 day of storage. Particle growth (PG) was performed according to the following equation [60]:

$$PG = 100 \times d(0) / d(1)$$

Where: d(0) is the mean droplet diameter just afterwards nanoemulsion preparation and d(1) is mean droplet size after 1 day of storage.

Larvicidal assay

Aedes aegypti larvae were obtained from the Arthropoda Laboratory (Universidade Federal do Amapá, Brazil). Biological assay was performed under controlled conditions, being fourth-instar larvae kept at 25±2 ºC, relative humidity of 75±5% and a 12h light:dark cycle. Experimental evaluation was performed according to World Health Organization protocol [61] with some modifications. All experiments were performed in triplicate with 10 forth-instar larvae in each sample. Nanoemulsion was diluted in distilled water at 25, 75, 150, 250, 500 ppm (concentration expressed as *P. emarginatus* essential oil content on aqueous media). Control group was constituted by deionized water. Mortality levels were recorded after 24 and 48 hours of exposure.

Statistical Analysis

Analysis of variance (Two-way ANOVA) followed by Tukey´s test or Bonferroni´s test was conducted using the Software GraphPad Prism 6.0 (San Diego, California, USA).

Differences were considered significant when $p \leq 0.05$. Probit analysis was performed with 95% confidence interval for LC50 determination.

Competing interests

All authors declare no conflict of interests.

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REFERENCES

1. WORLD HEALTH ORGANIZATION 2014. A global brief on vector-borne diseases. Geneva, Swizerland.

2. BRASIL, Ministério da Saúde 2016. Boletim epidemiológico. Secretária de vigilância em saúde, v. 47, n. 8.

3. WORLD HEALTH ORGANIZATION (WHO) 2009. Guidelines for diagnosis, treatment, prevention and control Geneva, Swizerland.

4. Rouis, Z., Laamari, A., Abid, N., Elaissi, A., Cioni, P.L., Flamini, G., Aouni, M.: Chemical composition and larvicidal activity of several essential oils from Hypericum species from Tunisia. Parasitol Res **112**, 699–705 (2013).

5. Feitosa, E.M.A., Arriaga, A.M.C., Santiago, G.M.P., Lemos, T.L.G., Oliveira, C.F., Vasconcelos, J.N., Lima, J.Q., Malcher, G.T., Nascimento, R.F., Braz-Filho, R.: Chemical Composition and Larvicidal Activity of *Rollinia leptopetala* (Annonaceae). J Braz Chem Soc **20,** 375-378 (2009).

6. Oliveira, G.L., Cardoso, S.K., Lara Júnior, C.L.R., Vieira, T.M., Guimarães, E.F., Figueiredo, L.S., Martins, E.R., Moreira, D.L., Kaplan, M.A.C.: Chemical study and larvicidal activity against *Aedes aegypti* of essential oil of *Piper aduncum* L. (Piperaceae). An Acad Bras Cienc**: 85,** 1227-1234 (2013).

7. *Pterodon* in **Flora do Brasil 2020**. Jardim Botânico do Rio de Janeiro. Available at: <http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB29840>. Accessed in18 Feb. 2016].

8. Hansen, D., Haraguchi, M., Alonso, A.: Pharmaceutical properties of 'sucupira' (*Pterodon* spp.). Braz J Pharm Sci **46**, 607-616 (2010).

9. Hoscheid, J., Cardoso, M.L.C.: Sucupira as a potential plant for arthritis treatment and other diseases. Arthritis **2015**, Article ID 379459, 1-12 (2015).

10. Mahajan, J.R., Monteiro, M.B.: New Diterpenoids from *Pterodon ernarginatus* Vog. J Chem Soc **1,** 520-525 (1973).

11. Alberti, T.B., Marcon, R., Bicca, M.A., Raposo, N.R.B., Calixto, J.B., Dutra, R.C.: Essential oil from *Pterodon emarginatus* seeds ameliorates experimental autoimmune encephalomyelitis by modulating Th1/Treg cell balance. J Ethnopharmacol **155,** 485-494 (2014).

12. Evangelista, G.L., de Souza, A.N.C., Santos, C.F., Cardoso, J.H.L., Lopes, E.A.B., dos Santos, M.V., Lahlou, S., Magalhães, P.J.C.: Essential oil of *Pterodon polygalaeflorus* inhibits electromechanical coupling on rat isolated trachea. J Ethnopharmacol **109**, 515-522 (2007).

13. Dutra, R.C., Trevizani, R., Pittela, F., Barbosa, N.R.: Antinociceptive activity of the essential oil and fractions of *Pterodon emarginatus* Vogel Seeds. Lat Am J Pharm **27**, 865-70 (2008).

14. Dutra, R.C., Fava, M.B., Alves, C.C.S., Ferreira, A.P., Barbosa, N.R.: Antiulcerogenic and anti-inflammatory activities of the essential oil from *Pterodon emarginatus* seeds. J Pharm Pharmacol **61**, 243-250 (2009).

15. Velozo, L.S.M., Martino, T., Vigliano, M.V., Pinto, F.A., Silva, G.P., Justo, M.G.A., Sabino, K.C.C., Coelho, M.G.P.: *Pterodon polygalaeflorus* essential oil modulates acute inflammation and B and T lymphocyte activation. The America Journal of Chinese Medicine **41**, 545-563 (2013).

16. Dutra, R.C., Pittela, F., Dittz, D., Marcon, R., Pimenta, D.S., Lopes, M.T.P., Raposo, N.R.B.: Chemical composition and cytotoxicity activity of the essential oil of *Pterodon emarginatus*. Braz J Pharmacogn **22**, 971-978 (2012).

17. Dutra, R.C., Braga, F.G., Coimbra, E.S., Silva, A.D., Barbosa, N.R.: Antimicrobial and leishmanicidal activities of seeds of *Pterodon emarginatus.* Braz J Pharmacogn **19**, 429-435 (2009).

18. Machado, R.R.P., Dutra, R.C., Raposo, N.R.B., Lesche, B., Gomes, M.S., Duarte, R.S., Soares, G.L.G., Kaplan, M.A.C.: Interferometry as a tool for evaluating effects of antimicrobial doses on *Mycobacterium bovis* growth. Tuberculosis **95**, 829-838 (2015).

19. Pimenta, A.T.A., Santiago, G.M.P., Arriaga, A.M.C., Menezes, G.H.A., Bezerra, S.B.: Estudo fitoquímico e avaliação da atividade larvicida de *Pterodon polygalaeflorus* Benth (Leguminosae) sobre *Aedes aegypti*. Braz J Pharmacogn **19,** 501-505 (2006).

20. Bruxel, F., Laux, M., Wild, L.B., Fraga, M., Koester, L.S., Teixeira, H.F.: Nanoemulsões como sistemas de liberação parenteral de fármacos. Quim. Nova **35**, 1827-1840 (2012).

21. McClements, D.J.: Edible nanoemulsions: fabrication, properties, and functional performance. Soft matter **7,** 2297-2316 (2011).

22. Tadros, T., Izquierdo P., Esquena, J., Solans, C.: Formation and stability of nano-emulsions. Adv Colloid Interface Sci **108-109,** 303-318 (2004).

23. Maali, A., Mosavian, M.T.H.: Praparation and application of nanoemulsion in the last decade. J Dispersion Sci *Technol* **34**, 92-105 (2013).

24. Solans, C., Solé, I.: Nano-emulsions: Formation by low-energy methods. Curr Opin Colloid Interface Sci **17,** 246-254 (2012).

25. Ghosh, V., Mukherjee, A., Chandrasekaran, N.: Optimization of Process Parameters to Formulate Nanoemulsion by Spontaneous Emulsification: Evaluation of Larvicidal Activity Against *Culex quinquefasciatus* Larva. BioNanoSci **4**, 157–165 (2014).

26. Rodrigues, E.C.R., Ferreira, A.M., Vilhena, J.C.E., Almeida, F.B., Cruz, R.A.S., Florentino, A.C., Souto, R.N.P., Carvalho, J.C.T., Fernandes, C.P. Development of a larvicidal nanoemulsion with Copaiba (Copaifera duckei) oleoresin. Braz J Pharmacog **24**, 99–705 (2014).

27. Sugumar, S.K.S., Clarke, M.J., Nirmala, B.K., Tyagi, A.M., Chandrasekaran. N.: Nanoemulsion of eucalyptus oil and its larvicidal activity against *Culex quinquefasciatus*. Bull Entomol Res **104**, 393–402 (2014).

28. Oliveira, A.E.M.F.M., Duarte, J.L., Amado, J.R.R., Cruz, R.A.S., Rocha, C.F., Souto, R.N.P., Ferreira, R.M.A., Santos, K., Conceição, E.C., Oliveira, L.A.R., Kelecom, A., Fernandes, C.P., Carvalho, J.C.T.: Development of a Larvicidal Nanoemulsion with *Pterodon emarginatus* Vogel Oil. Plos ONE **11**, e0145835. doi:10.1371/journal.pone.0145835 (2016).

29. Alves, S.F., Borges, L.L., de Paula, J.A.M., Vieira, R.F., Herri, P.H., do Couto, R.O., de Paula, J.R., Bara, M.T.F.: Chemical variability of the essential oils from fruits of *Pterodon emarginatus* in the Brazilian Cerrado. Braz J Pharmacogn **23**, 224-229 (2013).

30. Forgiarini, A., Esquena, J., González, C., Solans, C.: Studies of the relation between phase behavior and emulsification methods with nanoemulsion formation. Progr Colloid Polym Sci **115**, 36-39 (2000).

31. Wang, L., Dong, J., Chen, J., Eastoe, J., Li, X.: Design and optimization of a new self-nanoemulsifying drug delivery system. J Colloid Interf Sci **330**, 443–448 (2009).

32. Griffin, W.C.: Classification of surface-active agents by HLB. Journal of the Society of Cosmetic Chemists **1**, 311-326 (1949).

33. Orafidiya, L.O., Oladimeji, F.A.: Determination of the required HLB values of some essential oils. Int J Pharm **237**, 241–249 (2002).

34. Fernandes, C.P., Mascarenhas, M.P., Zibetti, F.M., Lima, B.G., Oliveira, R.P.R.F., Rocha, L., Falcão, D.Q.: HLB value, an important parameter for the development of essential oil phytopharmaceuticals. Braz J Pharmacogn **23**, 108-114 (2013).

35. Kentish, S., Wooster, T.J., Ashokkumar, M., Balachandran, S., Mawson, R., Simons, L.: The use of ultrasonics for nanoemulsion preparation. Innovative Food Science and Emerging Technologies **9**, 170–175 (2008).

36. Ostertag, F., Weiss, J., McClements, D.J.: Low-energy formation of edible nanoemulsions: Factors influencing droplet size produced by emulsion phase inversion. J Colloid Interface Sci **388**, 95–102 (2012).

37. Tan, T.B., Yussof, N.S., Abas, F., Mirhosseini, H., Nehdi, I.A., Tan, C.P.: Forming a lutein nanodispersion via solvent displacement method: The effects of processing parameters and emulsifiers with diferente stabilizing mechanisms. Food Chemistry **194**, 416–423 (2016).

38. Komaiko, J., McClements, D.J.: Low-energy formation of edible nanoemulsions by spontaneous emulsification: Factors influencing particle size. Journal of Food Engineering **146**, 122–128 (2015).

39. Silva, H.D., Cerqueira, M.A., Souza, B.W.S., Ribeiro, C., Avides, M.C., Quintas, M.A.C., Coimbra, J.S.R., Carneiro-da-Cunha, M.G., Vicente, A.A.: Nanoemulsions of β-Carotene using a high-energy emulsification-evaporation technique. J Food Eng **102**, 130-135 (2011).

40. Anjali, C.H., Sharma, Y., Mukherjee, A., Chandrasekaran, N.: Neem oil (*Azadirachta indica*) nanoemulsion - a potent larvicidal agent against *Culex quinquefasciatus*. Pest Manag Sci.: **68**, 158–163 (2012).

41. Saberi, A.H., Fang, Y., McClements, D.J.: Stabilization of vitamin E-enriched mini-emulsions: Influence oforganic and aqueous phase compositions. Colloids and Surfaces A: Physicochem Eng Aspects **449,** 65–73 (2014).

42. Saberi, A.H., Fang, Y., McClements, D.J.: Effect of glycerol on formation, stability, and properties of vitamin-E enriched nanoemulsions produced using spontaneous emulsification. J Colloid Interf Sci **411**, 105–113 (2013).

43. Saberi, A.H., Fang, Y., McClements, D.J.: Fabrication of vitamin E-enriched nanoemulsions by spontaneous emulsification: Effect of propylene glycol and ethanol on formation, stability, and properties. Food Res Int **54**, 812–820 (2013).

44. Duarte, J.L., Amado, J.R.R., Oliveira, A.E.M.F.M., Cruz, R.A.S., Ferreira, A.M., Souto, R.N.P., Falcão, D.Q., Carvalho, J.C.T., Fernandes, C.P.: Evaluation of larvicidal activity of a nanoemulsion of *Rosmarinus officinalis* essential oil. Braz J Pharmacog **25**, 189-192 (2015).

45. Ghosh, V., Mukherjee, A., Chandrasekaran, N.: Formulation and Characterization of Plant Essential Oil Based Nanoemulsion: Evaluation of its Larvicidal Activity Against *Aedes aegypti*. Asian Journal of Chemistry **25**: *S321-S323* (2013).

46. Gnanadesigan, M., Anand, M., Ravikumar, S., Maruthupandy, M., Vijayakumar, V., Selvam, S., Dhineshkumar, M., Kumaraguru, A.K.: Biosynthesis of silver nanoparticles by using mangrove plant extract and their potential mosquito larvicidal property. Asian Pacific Journal of Tropical Medicine, 799-803 (2011).

47. Govindarajan, M., Rajeswary, M., Veerakumar, K., Muthukumaran, U., Hoti, S.L., Benelli, G.: Green synthesis and characterization of silver nanoparticles fabricated using *Anisomeles indica*: mosquitocidal potential against malaria, dengue and Japanese encephaliti vectors. Experimental Parasitology **161**, 40–47 (2016).

48. Murugan, K., Dinesh, D., Paulpandi, M., Althbyani, A.D.M., Subramaniam, J., Madhiyazhagan, P., Wang, L., Suresh, U., Kumar, P.M., Mohan, J., Rajaganesh, R., Wei, H., Kalimuthu, K., Parajulee, M.N., Mehlhorn, H., Benelli, G.: Nanoparticles in the fight against mosquito-borne diseases: bioactivity of *Bruguiera cilindrica*-synthesized nanoparticles against dengue virus DEN-2 (*in vitro*) and its mosquito vector *Aedes aegypti* (Diptera: Culicidae). Parasitol Res **114**, 4349–4361 (2015).

49. Muthukumaran, U., Govindarajan, M., Rajeswary, M.: Green synthesis of silver nanoparticles from *Cassia roxburghii* – a most potent power for mosquito control. Parasitol Res **114**, 4385–4395 (2015).

50. Suganya, G., Karthi, S., Shivakumar, M.S.: Larvicidal potential of silver nanoparticles synthesized from *Leucas aspera* leaf extracts against dengue vector *Aedes aegypti*. Parasitol Res **113**, 1673–1679 (2014).

51. Sujitha, V., Murugan, K., Paulpandi, M., Panneerselvam, C., Suresh, U., Roni, M., Nicoletti, M., Higuchi, A., Madhiyazhagan, P., Subramaniam, J., Dinesh, D., Vadivalagan, C., Chandramohan, B., Alarfaj, A.A., Munusamy, M.A., Barnard, D.R.: Benelli G,Green-synthesized silver nanoparticles as a novel control tool against dengue virus (DEN-2). Parasitol Res **114**, 3315–3325 (2015).

52. Veerakumar, K., Govindarajan, M., Rajeswary, M., Muthukumaran, U.: Low-cost and eco-friendly green synthesis of silver nanoparticles using *Feronia elephantum* (Rutaceae) against *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Diptera: Culicidae). Parasitol Res **113**, 1775–1785 (2014).

53. Ismann, M.B., Miresmailli, S., Machial, C.: Commercial oportunities for pesticides based on plant essential oils in agriculture, industry and consumer products. Phytochem Rev **10**, 197-204 (2011).

54. Garelli, F.M., Espinosa, M.O., Weinberg, D., Trinelli, M.A., Gürtler, R.E., Water.: Use Practices Limit the Effectiveness of aTemephos-Based Aedes aegypti Larval Control Program in Northern Argentina. PLoS Negl Trop Dis. **5(3),** e991. doi: 10.1371/journal.pntd.0000991 (2011).

55. Lai, F., Wissing, S.A., Müller, R.H., Fadda, A.M.: *Artemisia arborescens* L essential oil-loaded solid lipid nanoparticles for potential agricultural application: Preparation and characterization. AAPS PharmSciTech **7**, E10 (2006).

56. Alves, S.F., Borges, L.L., Santos, T.O., de Paula, J.R., Conceição, E.C., Bara, M.T.F.: Microencapsulation of Essential Oil from Fruits of *Pterodon emarginatus* Using Gum Arabic and Maltodextrin as Wall Materials: Composition and Stability. Drying Technology **32**, 96–105 (2014).

57. Van Den Dool, H., Krats, P.D.J.A.: A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. J Chromatography A **11,** 463-471 (1963).

58. WILEY. (1999): Wiley registry of mass spectral data. 6th Ed. Wiley Interscience, New York.

59. Adams, R.P. (2007): Identification Of Essential Oil Components By Gas Chromatography/Mass Spectrometry. 4th ed. Carol Stream, IL, USA: Allured, 804p.

60. Guttoff, M., Saberi, A.H., McClements, D.J.: Formation of vitamin D nanoemulsion-based delivery systems by spontaneous emulsification: Factors affecting particle size and stability. Food Chemistry **171**, 117–122 (2015).

61. WHO. World Health Organization 2005. Guidelines for laboratory and field testing of mosquito larvicides. World health organization Communicable disease control, prevention and eradication Who pesticide evaluation scheme. Geneva, Swizerland.

FIGURES

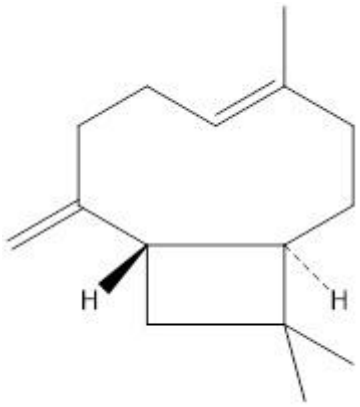

Figure 1. Chemical structure of the sesquiterpene β-caryophyllene identified as the main constituent of P. emarginatus essential oil.

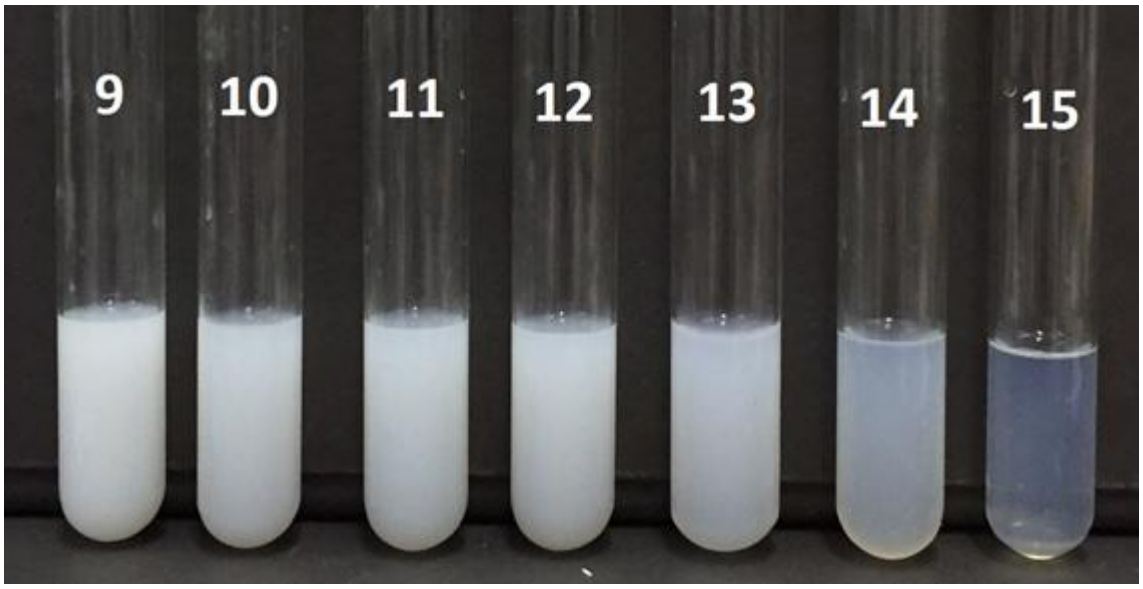

Figure 2. Nanoemulsions prepared with Pterodon emarginatus essential oil at different surfactant ratios (HLB 9-15).

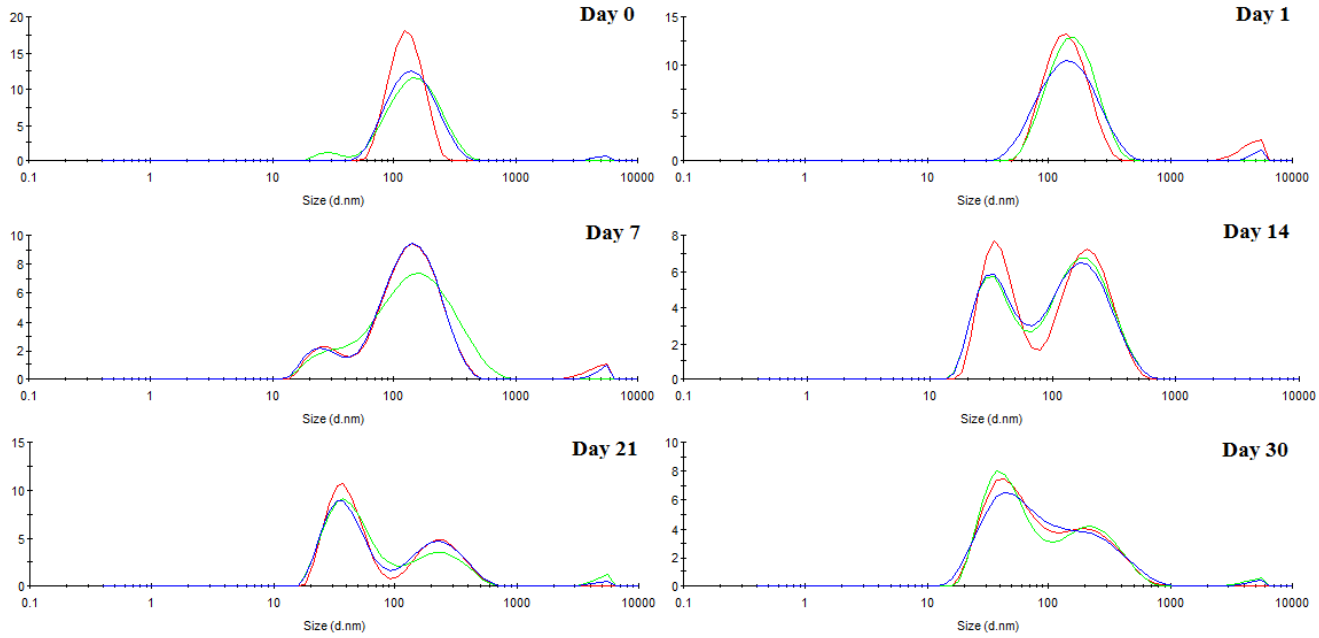

Figure 3. Mean droplet size - Day 0: 128.0±6.217; Day 1: 133.6±1.850; Day 7: 95.4±0.869; Day 14: 66.33±1.019; Day 21: 55.60±0.5631; Day 30: 62.63±1.323. Polydispersity index, Day 0: 0.250±0.011; Day 1: 0.288±0.038; Day 7: 0.441±0.002; Day 14: 0.477±0.023; Day 21: 0.416±0.015; Day 30: 0.412±0.019.

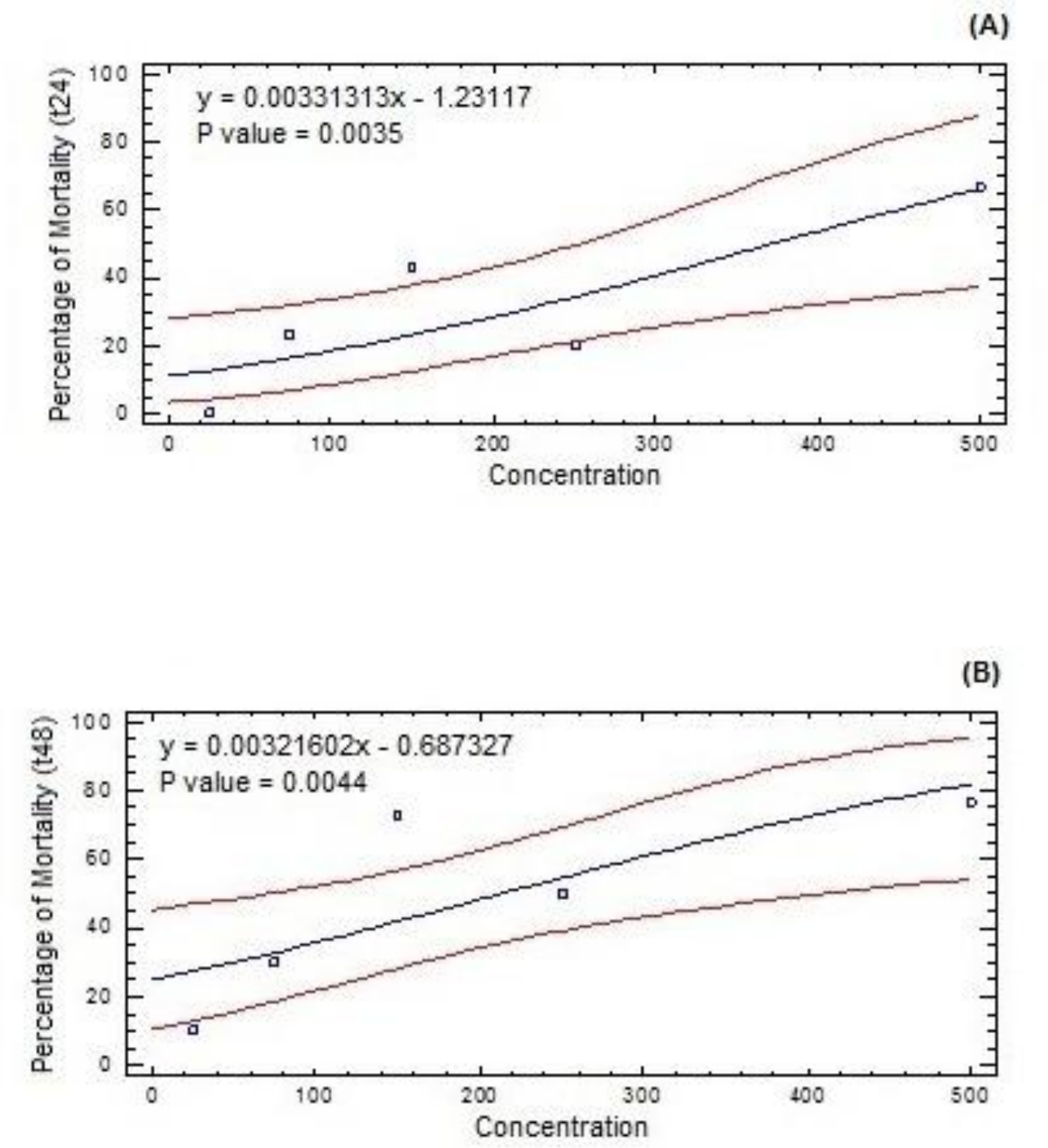

Figure 4. Plot of fitted model with 95.0 % of confidence limits after treatment of Aedes aegypti larvae with Pterodon emarginatus essential oil-based nanoemulsions.

Table 1. Chemical constituents from of essential oil from fruits of *P. emarginatus*.

RI_{exp}	RI_{lit}	**Identification**	**%**
1352	1345	α-cubebene	0.5
1380	1374	α-copaene	2.7
1395	1389	β-elemene	5.8
1423	1417	β-caryophyllene	25.8
1456	1448	*cis*-muurola-3,5-diene	0.2
1460	1452	α-humulene	6.0
1467	1458	*allo*-aromadendrene	2.1
1478	1475	*trans*-cadina-1(6),3-diene	0.4
1481	1478	γ-muurolene	1.3
1487	1484	germacrene D	7.4
1493	1489	β-selinene	0.3
1499	1493	*trans*-muurola-4(14),5-diene	0.5
1503	1500	Bicyclogermacrene	5.6
1519	1513	γ-cadinene	3.6
1528	1522	δ-cadinene	9.2
1537	1533	*trans*-cadina-1,4-diene	0.2

1542	1537	α-cadinene	0.6
1560	-	n.i.*	0.2
1585	1577	Spathulenol	2.4
1592	1582	caryophyllene oxide	2.9
1618	1608	humulene epoxide II	0.6
1648	1638	*epi*-α-cadinol	1.2
1661	1652	α-cadinol	0.7
1721	1714	(2*E,6Z*)-farnesol	0.4
1893	-	n.i.	9.5
2312	-	di-octyl adipate** (artifact)	10.2
		Total identified:	90.3

***** n.i. not identified

** Tentatively identified.

Table 2. Size distribution of nanoemulsions prepared with essential oil from fruits of *P. emarginatus*.

	Day 0		Day 1		Particle Growth
	Size (nm)	PdI	Size (nm)	PdI	
9	278.0±37.6	0.424±0.05	232.1±3.83	0.458±0.05	83.49
10	320.6±71.6	0.406±0.02	332.8±20.4	0.427±0.02	103.8
11	215.8±25.7	0.517±0.07	245.8±20.1	0.531±0.05	113.90
12	290.1±59.2	0.531±0.18	302.0±34.0	0.446±0.01	104.10
13	245.2±39.1	0.482±0.11	193.9±13.7	0.424±0.01	78.83
14	213.8±4.38	0.508±0.08	169.3±6.40	0.435±0.00	79.18
15	128.0±6.2	0.250±0.0	133.6±1.8	0.288±0.03	104.37

Table 3. Mortality levels induced by treatment of *Aedes aegypti* larvae with *Pterodon emarginatus* essential oil-based nanoemulsions.

Exposure time (h)	25ppm	75 ppm	150 ppm	250 ppm	500 ppm	Control
24	0 ± 0[a]	23.3 ± 4.7[a]	43.3 ± 12.5a	17 ± 23.3[a]	66.7 ± 12.5c	0 ± 0[a]
48	10 ± 8.2[a]	30 ± 0[a]	73.3 ± 9.4c	50 ± 8.2b	76.7 ± 12.4c	0 ± 0[a]

[a] No significance – $p>0.05$; Significance: a – $p< 0.01$; b – $p<0.001$; c – $p<0.0001$

Control – non-treated larvae. Data is expressed as mean ± standard deviation.

Concentrations expressed as function of essential oil content on nanoemulsions

INVESTIGATION OF POTENTIAL RESIDUAL ACTIVITY OF A NOVEL AQUEOUS NANODISPERSION PREPARED WITH VOUACAPAN DITERPENE ISOLATED FROM *Pterodon emarginatus* OLEORESIN

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Communication

Investigation of Potential Residual Larvicidal Activity of a Novel Aqueous Nanodispersion Prepared with Vouacapan Diterpene Isolated from *Pterodon emarginatus* Oleoresin

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Abstract: Species from the genus *Pterodon* (Fabaceae) are a great source of vouacapan diterpenes with a wide range of biological activities, including a larvicidal property against *Aedes aegypti*. However, poor water solubility of these terpenoids make development of viable aqueous larvicidal agents a major technological challenge. Nowadays, nanotechnology is recognized as a leading area for generating novel larvicidal products, including those with natural compounds. On this context, aqueous nanodispersions allow better availability of lipophilic compounds in water media and provide additional advantages, such as enhanced physical, chemical stability and even controlled release. To our knowledge, no efforts were carried out for this purpose with isolated diterpene from this *P. emarginatus*. The present study aimed to isolate a diterpene from this source and obtain an aqueous nanodispersion with residual larvicidal activity. Our results suggest that this nanoproduct has potential as a low-cost and ecofriendly agent on integrated programs for vector control. Moreover, we suggest a retro diels alder mechanism for fragmentarion that may be useful for identification of another vouacapan diterpenes.

Keywords: *Aedes aegypti*, Fabaceae; methyl 6α,7β-dihydroxyvouacapan-17 β-oate, nanotechnology; sucupira-branca

1. Introduction

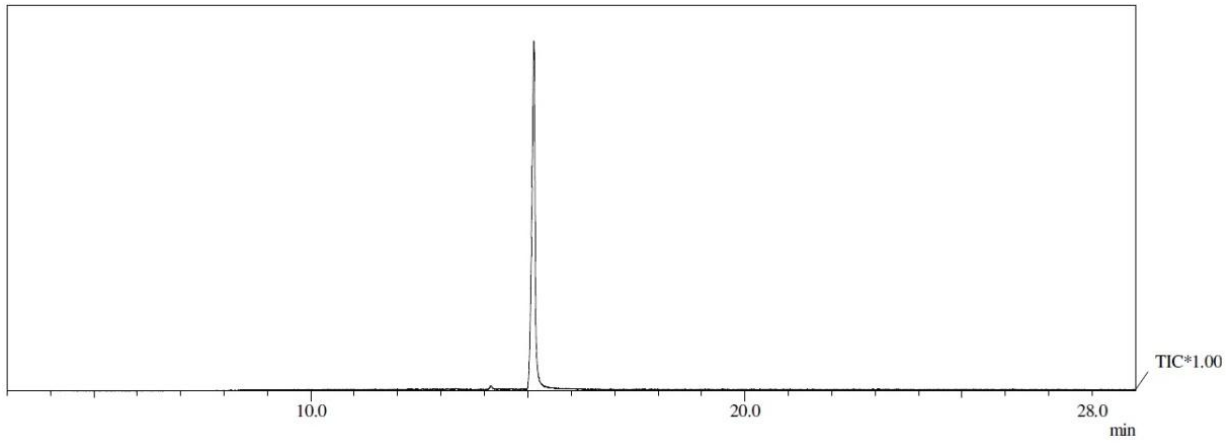
Diterpenes are a large class of terpenoids containing 20 atoms of carbon (4 isoprene units) [1]. They can be acyclic, bicyclic, tricyclic, tetracyclic, macrocyclic or miscellaneous and were isolated from several sources [2]. The genus *Pterodon* belongs to the family Fabaceae and it is native to Brazil [3]. *Pterodon* species are commonly known as sucupira and their fruits have an oleoresin with a wide range of biological activities, including a larvicidal action against *Aedes aegypti.* This property was partially attributed to the vouacapan diterpenes, that are considered the main bioactive compounds of *Pterodon sp* [4,5].

Aedes aegypti is the main vector of dengue, a tropical disease that affects million of people worldwide. Moreover, recent cases of some uncommon diseases transmitted by this vector emerged, such as zika and chikungunya fever [6,7]. A major problem for viable larvicidal products using some natural products, including terpenoids, rely on the fact that most of them have limited solubility in water. On this context, nanotechnology emerged as a promising area for development of viable bioactive larvicidal agents [8]. Nanostructured insecticides also have been considered ecofriendly alternatives for safer programs of mosquito control. Potential less impairment to human health and less induction of resistance put nanotechnology in the spotlight of pesticide research [9]. Most of studies in this field are related to silver or other metallic phytofabricated nanoparticles, which presented good signals of effectiveness. However, few information about toxicity to non-target organism has been observed for these nanoproducts [10]. Recent study performed with an aqueous nanoemulsion prepared with oleoresin from fruits of *P. emarginatus*, a remarkable source of vouacapan diterpenes, showed a promising larvicidal action against *A. aegypti*. Good indicative of safety for mammals was observed, being considered a great advantage. Moreover, low cost of the process and utilization of non-ionic surfactants in this nanoformulations, in addition to absence of metals in the formulations, suggested the potential of sucupira-based aqueous nanodispersions against this vector [11].

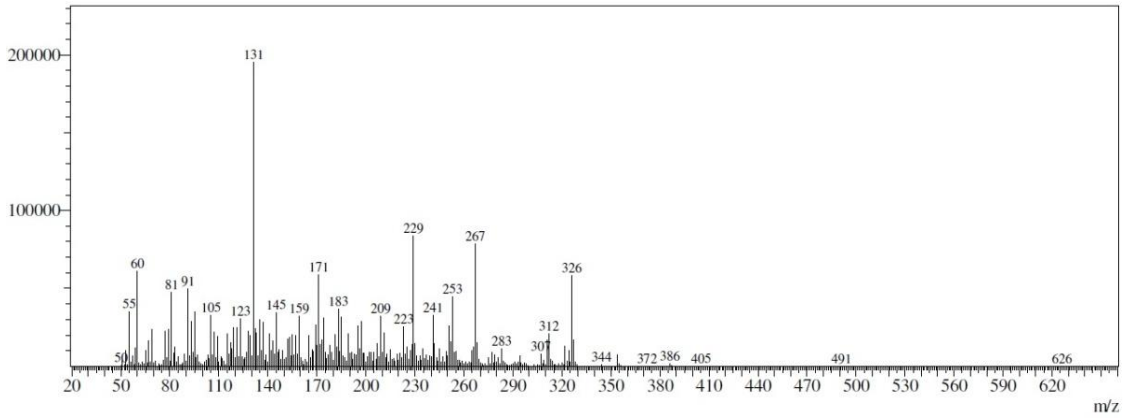
The vouacapan diterpenes 6α-acetoxivouacapane [12], 6α-hydroxivouacapan-7β,7β-lactone, 6α,7β-dihydroxivouacapan-17β-oic acid and methyl 6α,7β-dihydroxivouacapan-17β-oate [13] were investigated in order to verify its larvicidal potential against *A. aegypti*. However, to our knowledge no study was carried out in order to obtain an aqueous nanodispersion using a vouacapan diterpene from *Pterodon* species. Moreover, these investigations just focused on estimation of lethal concentrations using short time experiments. Thus, the aim of the present study was to generate a viable aqueous nanodispersion with diterpene isolated from oleoresin of *P. emarginatus* fruits by a low-energy, low-cost and ecofriendly method and investigate the residual activity against *A. aegypti* larvae. A critical investigation about fragmentation pattern of the compound was also performed.

2. Results

Gas-chromatographic analysis of the product obtained from *P. emarginatus* oleoresin revealed the presence of a remarkable signal with high abundance at 15.13 min (Figure 1a), suggesting the isolation of the compound (**1**). Mass spectra (Figure 1b) indicated the presence of a peak at *m*/*z* 344, due to loss of H_2O. The peak observed at *m*/*z* 326 was associated to an additional loss of H_2O. Loss of CH_2 gave the peak at *m*/*z* 312, while alternative loss of CO_2Me gave peak at *m*/*z* 267. Peak at *m*/*z* 159 was observed and we suggest that it is associated to a fragment formed after ring opening, being explained by a retro Diels-Alder fragmentation (Figure 2).



(a)



(b)

Figure 1. (a) Total ion chromatogram presenting the peak of methyl 6α,7β-dihydroxyvouacapan-17 β -oate isolated from *Pterodon emarginatus* oleoresin. (b) Mass spectra of methyl 6α,7β-dihydroxyvouacapan-17 β -oate isolated from *P. emarginatus* oleoresin.

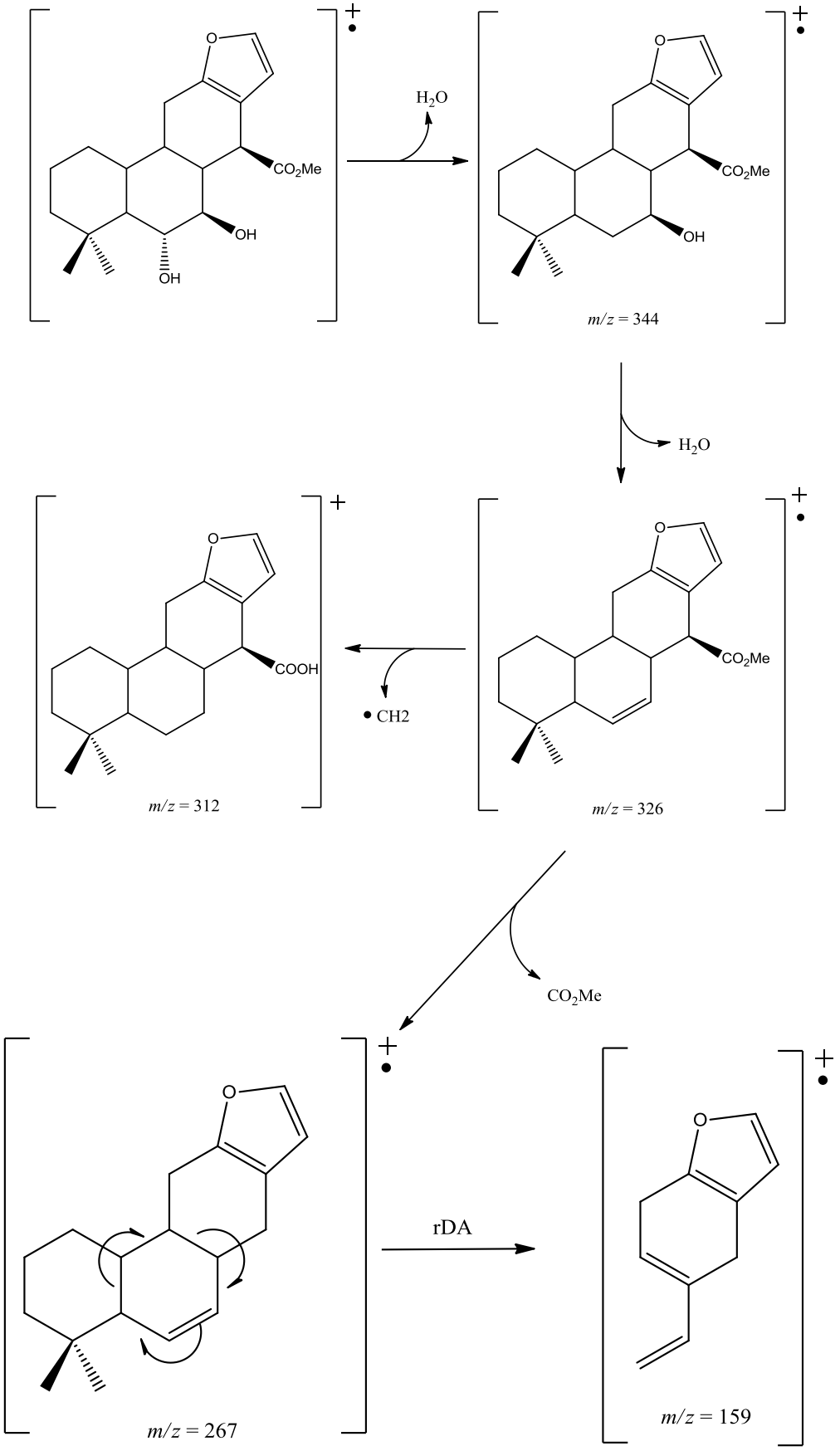


Figure 2. Proposed fragmentation route for methyl 6α,7β-dihydroxyvouacapan-17 β –oate. rDA represents a retro Diels-Alder mechanism for fragmentation.

Analysis of mass spectra pattern of terpenoids using retro Diels-Alder reaction is a powerful diagnostic tool for compound identification and has been extensively studied for some classes, especially for pentacyclic and tetracyclic triterpenes [14]. Several studies using gas-chromatography coupled to mass spectrometer were carried with sucupira-branca oleoresin. Fragmentation pattern of compounds due to sequential cleavage after electron ionization and comparison of these data with literature have been the major approach for identification of the vouacapan diterpenes from this plants. However, most of then focuses on comparison to previous data without any further discuss about peaks formation in the mass spectra [15-17]. In addition, some noise may also occur if the oleoresin is analyzed without a previous treatment. Despite GC-MS has been mostly used for identification of sucupira vouacapan diterpenes, efforts for better understanding of their fragmentation pattern are restricted to a study carried out using combination of techniques such as ESI-MS, HRESI-MS and ESI-MS/MS [18]. To our knowledge, this is the first time that a proposal route for fragments from a sucupira vouacapan diterpene is performed based on a retro Diels-Alder mechanism. This analysis and comparison to literature [19] data suggest the isolation of methyl 6α,7β-dihydroxyvouacapan-17 β –oate (1) (Figure 3).

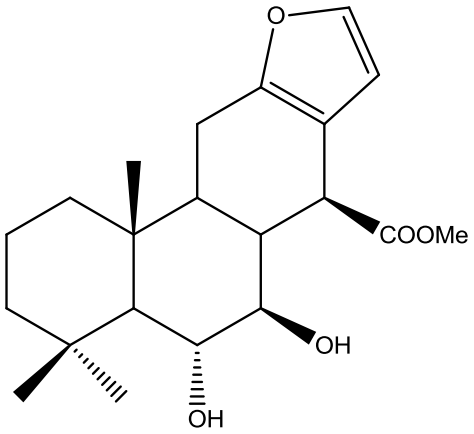


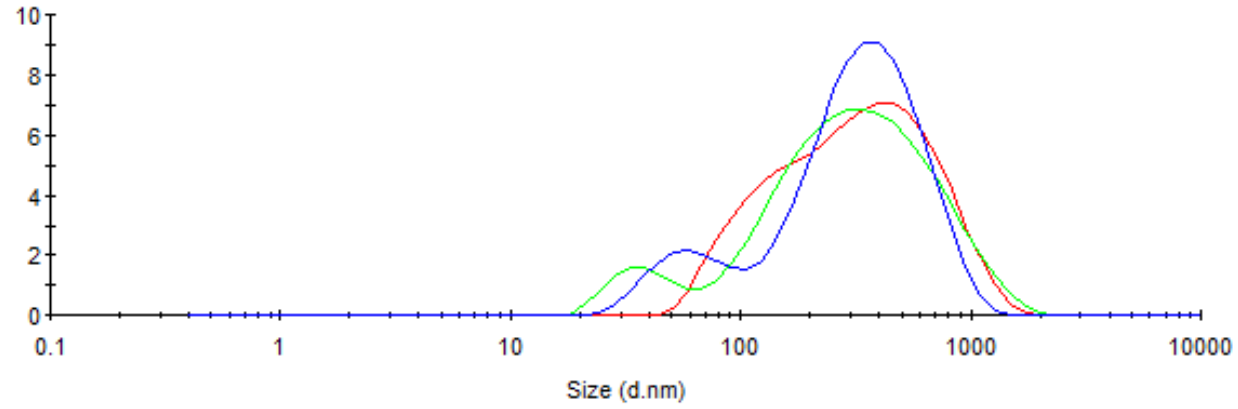
Figure 3. Chemical structure of diterpene methyl 6α,7β-dihydroxyvouacapan-17 β –oate isolated from oleoresin of *P. emarginatus* fruits.

Preparation of methyl 6α,7β-dihydroxyvouacapan-17 β –oate nanodispersion was performed by solvent displacement method. Particle size distribution of the diterpene-based nanodispersion and blank nanodispersion (without diterpene) are shown in Figure 4. Table 1 shows mean droplet size, polydispersity index and zeta potential of both nanodispersions. Blank nanodispersion showed lower mean droplet size (186.8±4.62 nm), when compared to diterpene nanodispersion (445.3±6.07 nm). However, diterpene nanodispersion presented narrower polydispersity index. Pdi values around 0.500 are associated to relatively broad distribution [20]. Zeta potential of both nanodispersions were negative, being the value observed for diterpene

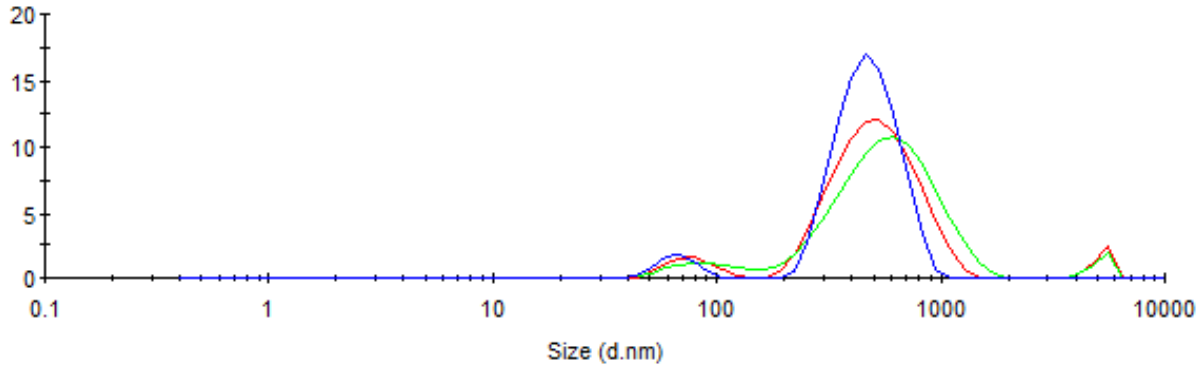
nanodispersion higher (in module). Physically stable aqueous nanodispersions are associated to zeta potential lower than -30 mV [21].

Table 1. Physical characterization of nanodispersion prepared with the diterpene methyl 6α,7β-dihydroxyvouacapan-17 β –oate isolated from oleoresin of *Pterodon emarginatus* fruits. Blank nanodispersion was prepared solely with the surfactant (polyethylene glycol 400 monooleate)

Parameters	Blank nanodispersion	Diterpene nanodispersion
Size (nm)	186.8±4.62	445.3±6.07
Polydispersity index	0.540±0.042	0.472±0.043
Zeta potential (mV)	-29.7±0.473	-40.0±2.69



(a)



(b)

Figure 4. Particle size distribution of (a) blank nanodispersion and (b) nanodispersion prepared with 6α,7β-dihydroxyvouacapanoate isolated from *P. emarginatus* oleoresin.

Figure 5 shows results of residual larvicidal activity after treatment with aqueous nanodispersion prepared with methyl 6α,7β-dihydroxivouacapan-17β-oate (200 ppm expressed as diterpene content in aqueous media) isolated from oleoresin of *P. emarginatus* fruits. After the first cycle, higher mortality level was observed for

treated group (40.0±8.2 %) followed by blank group (20.0±14.1 %), while almost no mortality was observed in control group (3.33±4.7 %). Then, increment of mortality level was observed for treated group, reaching a maximum after second cycle (56.67±17.0 %). This result presents statistical significant difference, when compared to blank and control groups ($p<0.0001$), which presented almost no mortality after second cycle. No significant differences were observed between mortality levels of control and blank groups ($p>0.05$), from second cycle to the end of experiment ($p>0.05$). After third cycle ,a slight decrease in mortality level was observed for treated group, which reached 53.3±4.7 % ($p<0.0001$). Then, higher decrease was observed, reaching 26.67±24.9 % after fourth cycle ($p<0.05$). No significant statistical difference was observed in mortality level of treated group after fifth cycle (23.33±12.5 %), when compared to control and blank groups ($p>0.05$). Comparison among cycles for each experimental group was performed and revealed that no significant differences were observed for blank or control groups. Regarding treated group, the mortality level after second cycle was significantly different from mortality observed after fourth ($p<0.05$) and fifth ($p<0.01$) cycles. It was also observed significant difference among mortality level after third cycle, when compared to fourth and fifth cycles ($p<0.05$). Preparation of microparticles with *Copaifera sp* (Fabaceae) oleoresin was performed in order to enhance water solubility and residual effect of this product against *Aedes aegypti* larvae. At 400 ppm (expressed as copaiba oleoresin content), mortality levels around 40 % were observed from 8 to 16 days of treatment. Then, a decrease to mortality levels around 20% was observed [22].

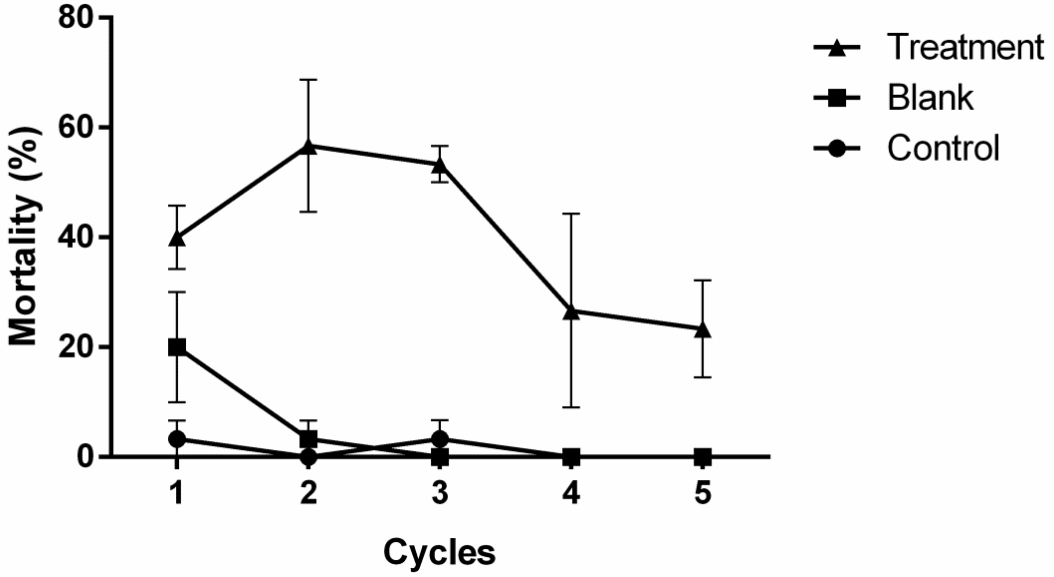


Figure 5. Residual larvicidal activity against *Aedes aegypti*. Treatment – larvae treated with aqueous nanodispersion prepared with the diterpene methyl 6α,7β-dihydroxyvouacapanoate (1) isolated from *P. emarginatus* oleoresin. Blank – larvae treated with aqueous nanodispersions prepared solely with surfactant and without the diterpene. Control – untreated larvae.

Metallic nanoparticles are the main type of natural product-based nanoformulations that have been prepared for mosquito larvae control. Several of them were considered active larvicidal agents against tropical disease vectors, including *A. aegypti*. Often, they are related to more polar derivatives such as aqueous extracts [23-28]. However, utilization of metal salts (eg. silver nitrate) enhances costs of process. In addition, they may not be ecofriendly if they bioaccumulates, being potentially toxic to non-target organism. Polymeric micro- or nanocapsules, defined according to mean particle diameter, are also considered promising for preparation of natural product-larvicidal agents [22]. However, these techniques involve utilization of organic volatile solvents that are potentially toxic, such as hexane, dichloromethane, acetone, ethyl acetate, among others, in addition to elevate costs of coating polymers. Oil in water nanoemulsions containing natural products were also evaluated against *A. aegypti*. In most of cases, the bioactive compounds were presented as a natural oil and solubilization of the compounds prior to emulsification was not necessary [29-31]. However, studies regarding evaluation of larvicidal activity of nanoemulsions with isolated natural products for this purpose are scarce. All nanoformulations containing natural products as encapsulated larvicidal bioactive ingredients that are disperse in water can be classified as nanodispersions. The aqueous nanodispersion with methyl 6α,7β-dihydroxyvouacapan-17 β –oate was prepared without metallic or polymeric nanoencapsulation and neither using an oil for solubilization of the bioactive diterpene. The utilization of ethanol as a "green solvent", in addition to low cost method is a great advantage and ours results for larvicidal activity confirm the potential of this natural product for *A. aegypti* control.

3. Materials and Methods

3.1. Isolation of the Diterpene

A diterpene fraction previously obtained from hexane extract of fruits from *P. emarginatus* [32] was used for isolation procedure. 3.7 g of the diterpene fraction was purified through column chromatography using silica gel as stationary phase. Elution was performed with hexane (500 mL), hexane:$CHCl_3$ (1:1; 500 mL). $CHCl_3$ (500 mL), $CHCl_3$:ethanol (1:1; 500 mL) and ethanol (500 mL). Crystals from ethanol fraction were filtered and washed with acetone. From the acetone filtrate, crystallization afforded 600 mg of vouacapan diterpene (**1**).

3.2. Gas-chromatographic conditions and identification of diterpene

GCMS-QP2010 (SHIMADZU) gas chromatograph equipped with a mass spectrometer using electron ionization was used, according to these experimental

conditions: injector temperature, 270 °C; detector temperature, 270°C; carrier gas, Helium; flow rate 1 mL/min; split injection with split ratio 1:40. The oven temperature was programmed from 180 °C, with an increase of 10 °C/min to 270 °C, ending with a 20 min isothermal at this temperature. One microliter of the sample, dissolved in $CHCl_3$ (1 : 100 mg/μL), was injected into a RTX-5MS column (i.d. = 0.25 mm, length 30 m, film thickness = 0.25 μm). Mass spectrometry (MS) conditions were ionization voltage, 70 eV and scan rate, 1 scan/s. Mass range was *m*/*z* from 50 to 750). Identification was performed by comparison to literature data and proposal fragmentation.

3.3. Preparation of diterpene-based aqueous nanodispersion

The aqueous diterpene nanodispersion was prepared by a solvent displacement method [33] with some modifications. Stock solution of methyl 6α,7β-dihydroxyvouacapan-17 β –oate in absolute ethanol was prepared at 40 mg/mL. Then, 400 mg of polyethylene glycol 400 monooleate was added to 2 mL of the diterpene solution and homogenized to generate the organic phase. Water pre-heated at 80 °C was slowly added to the organic phase and the system was stirred at 400 rpm for 30 minutes. Removal of organic solvent was performed under reduced pressure using a rotary evaporator. Further addition of water was performed to restore the initial final mass of 40 g. Final concentration of the diterpene in the nanodispersion was 2000 μg/mL. Blank nanodispersion was prepared under the same conditions without using the diterpene. Nanodispersions were assayed immediately after preparation.

3.4. Particle size distribution and zeta potential measurements

Physical characterization of the nanodispersion was performed using a Zetasizer Nano ZS. Nanodispersion was diluted with deionized water (1:25, v/v). Measurements of droplet size, polydispersity index and zeta potential were performed in triplicate. Data was expressed as the mean ± stander deviation.

3.5. Residual larvicidal activity

The biological assay was performed using 4th instar larvae of *Aedes aegypti*, obtained from the Arthropoda Laboratory (Universidade Federal do Amapá, Brazil). Experiments were performed under controlled conditions as follows: water temperature was 25 ± 2 °C ; room relative humidity was 75 ± 5 % and 12 h dark:light cycle was used. All experiments were performed in triplicate with 10 larvae in each replicate (n=30). Mortality levels were recorded after each cycle (48 h). The treated group was performed using diterpene nanodispersion at 200 ppm (expressed as methyl 6α,7β-dihydroxyvouacapan-17 β –oate content in aqueous media). Residual effect was performed according to a previous published protocol [22] with some modifications. After each cycle, the aqueous media was filtered and new larvae (total

of 10 per replicate) were added. Control group was constituted by water and blank group was constituted by surfactant nanodispersion without diterpene.

3.6. Statistical Analysis

Analysis of variance (Two-way ANOVA) followed by Tukey´s test was conducted using the Software GraphPad Prism 6.0 (San Diego, California, USA). Differences were considered significant when $p \leq 0.05$.

4. Conclusions

Detailed study of diterpene fragmentation have been neglected in most of previous studies with *Pterodon* genus. Our study performed a critical investigation about the fragmentation, proving to be a valuable tool for correct identification and moreover, key fragment due to a retro Diels-Alder mechanism was assigned. This reaction is considered very important for terpeneoids analysis and may be useful for further studies with vouacapan diterpenes. We also presented a low-cost and ecofriendly method using a "green-solvent" to generate viable nanodispersions with a diterpene from *P. emarginatus*. Satisfactory mean droplet size, polydispersity index and zeta potential was observed, suggesting that the method was able to satisfactorily generate the nanodispersion. Considering that it presented residual activity, controlled release of this larvicidal natural compound may be involved and therefore it may be useful for further integrated control programs against *A. aegypti*, the main vector of tropical diseases.

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Author Contributions: I.R.S. contributed to isolation of diterpene; J.L.D. performed nanodispersion characterization; R.A.S.C. contributed to diterpene identification; R.N.P.S. and R.M.A.F. contributed to larvicidal assay; E.C.C. contributed to nanodispersion preparation; J.C.T.C. and C.P.F. conceived and designed the experiments; C.P.F. and A.E.M.F.M.O. wrote the paper and contributed to all experiments and data interpretation.

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Dewick, P.M. **2009**. Medicinal Natural Products – A biosynthetic approach, 3rd edition, John Wiley & Sons, Chichester.

[2] Hanson, J. R. Diterpenoids. *Nat Prod Rep*, **2003**, 20, 70-78.

[3] *Pterodon* in **Flora do Bras il 2020**. Jardim Botânico do Rio de Janeiro. Avaiable at: <http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB29840>. Accessed on: 10 Jul. 2016

[4] Hansen, D.; Haraguchi, M.; Alonso, A. Pharmaceutical properties of `sucupira` (*Pterodon spp.*). *Brazilian Journal of Pharmaceutical Sciences*, **2010**, 46, 607-616.

[5] Hoscheid, J.; Cardoso, M. L. C. Sucupira as a potential plant for arthritis treatment and other diseases *Arthritis*, **2015**, Article ID 379459, 12 pages.

[6] WORLD HEALTH ORGANIZATION 2014. A global brief on vector-borne diseases. Geneva, Swizerland.

[7] BRASIL, Ministério da Saúde 2016. Boletim epidemiológico. Secretária de vigilância em saúde, v. 47, n. 8.

[8] Oliveira, J. L.; Campos, E. V. R.; Bakshi, M.; Abhilash, P. C.; Fraceto, L. F. Application of nanotechnology for the encapsulation of botanical insecticides for sustainable agriculture: prospects and promises. *Biotechnology Advances*, **2014**, 32, 1550-1561.

[9] Benelli, G. Research in mosquito control: current challenges for a brighter future. *Parasitol Res*, **2015**, 114, 2801–2805.

[10] Benelli, G. Plant-mediated biosynthesis of nanoparticles as an emerging tool against mosquitoes of medical and veterinary importance: a review. *Parasitol Res*., **2016**, 115, 23-34.

[11] Oliveira, A. E. M. F. M.; Duarte, J. L.; Amado, J. R. R.; Cruz, R. A. S.; Rocha, C. F.; Souto, R. N. P.; Ferreira, R. M. A.; Santos, K.; Conceição, E. C.; Oliveira, L. A. R.; Kelecom, A.; Fernandes, C. P.; Carvalho, J. C. T. Development of a larvicidal nanoemulsion with *Pterodon emarginatus* Vogel oil. *Plos One*, **2016**, 11, e0145835.

[12] Pimenta, A. T. A.; Santiago, G. M. P.; Arriaga, A. M. C.; Menezes, G. H. A.; Bezerra, S. B. Estudo fitoquímico e avaliação da atividade larvicida de *Pterodon polygalaeflorus* Benth (Leguminosae) sobre *Aedes aegypti. Revista Brasileira de Farmacognosia*, **2006**, 16, 501-505.

[13] De Omena, M. C.; Bento, E. S.; De Paula, J. E.; Sant´Ana, A. E. G. Larvicidal diterpenes from *Pterodon polygalaefloerus*. *Vector-Borne and Zoonotic Diseases*, **2006**, 6, 216-222.

[14] Assimopoulou, A.N; Papageorgiou, V.P. GC-MS analysis of penta- and tetra-cyclic triterpenes from resins of *Pistacia* species. Part I. *Pistacia lentiscus* var. Chia. *Biomed Chromatogr,* **2005**, 19, 285-311.

[15] Coelho, L.P.; Reis, P.A.; De Castro, F.L.; Gayer, C.R.M.; Lopes, S.C.; Silva, M. C.S.; Sabino, K.C.C.; Todeschini, A. R.; Coelho, M.G.P. Antinociceptive properties of ethanolic extract and fractions of *Pterodon pubescens* Benth. Seeds. *J Ethnopharmacol,* **2005**, 98, 109–116.

[16] Nucci, C.; Mazzardo-Martins, L.; Stramosk, J.; Brethanha, L.C.; Pizzolatti, M.G.; Santos, A.R.S.; Martins, D.F. Oleaginous extract from the fruits Pterodon pubescens Benth induces antinociception in animal models of acute and chronic pain. *J Ethnopharmacol*, **2012**, 143, 170-178.

[17] Oliveira, P. C.; Conceição, E. C.; Oliveira, P. A.; Nascimento, M. V.; Costa, E. A.; Paula, J. R.; Bara, M. T. Obtaining a dry extract of *Pterodon emarginatus* (Fabaceae) fruits by spray-drying. *Journal of Pharmacy Research*, **2012**, 5, 641-645.

[18] Cabral, E. C.; Sevart, L.; Spindola, H. M.; Coelho, M. B.; Sousa, I. M. O.; Queiroz, N. C. A.; Foglio, M. A.; Eberlin, M. N.; Riveros, J. M. *Pterodon pubescens* Oil: Characterisation, Certification of Origin and Quality Control via Mass Spectrometry Fingerprinting Analysis. *Phytochem Analysis*, **2012**, 24, 184–192.

[19] Silva, M. C. C.; Gayer, C. R. M.; Lopes, C. S.; Calixto, N. O.; Reis, P. A.; Passaes, C. P. B.; Paes, M. C.; Dalmau, S. R.; Sabino, K. C. C.; Todeschini, A. R.; Coelho, M. G. P. Acute and topic anti-edematogenic fractions isolated from the seeds of *Pterodon pubescens*. *Journal of Pharmacy and Pharmacology*, **2004**, 55, 135-141.

[20] Cheong, J. N.; Tan, C. P.; Man, Y. B. C.; Misran, M. α-tocopherol nanodispersions: preparation, characterization and stability evaluation. *Journal of Food Engineering,* **2008**, 89, 204-209.
[21] Kelmann, R. G.; Kuminek, G.; Teixeira, H. F.; Koester, L. S. Carbamazepine parenteral nanoemulsions prepared by spontaneous emulsification process. *International Journal of Pharmaceutics*, **2007**, 342, 231-239.
[22] Kanis, L. A.; Prophiro, J. S.; Vieira, E. S.; Nascimento, M. P.; Zepon, K. M.; Kulkamp-Guerreiro, I. C.; Silva, O. S. Larvicidal activity of *Copaifera sp.* (Leguminosae) oleoresin microcapsules against *Aedes aegypti* (Diptera: Culicidae) larvae. *Parasitol Res*, **2012**, 110, 1173-1178.
[23] Angajala, G.; Ramya, R.; Subashini, R. In-vitro anti-inflammatory and mosquito larvicidal efficacy of nickel nanoparticles phytofabricated from aqueous leaf extracts of *Aegle marmelos* Correa. *Acta Tropica*, **2014**, 135, 19–26.
[24] Gnanadesigan, M.; Anand, M.; Ravikumar, S.; Maruthupandy, M.; Vijayakumar, V.; Selvam, S.; Dhineshkumar, M.; Kumaraguru, A.K.. Biosynthesis of silver nanoparticles by using mangrove plant extract and their potential mosquito larvicidal property. *Asian Pacific Journal of Tropical Medicine*, **2011**, 799-803.
[25] Muthukumaran, U.; Govindarajan, M.; Rajeswary, M. Green synthesis of silver nanoparticles from *Cassia roxburghii* – a most potent power for mosquito control. Parasitology *Research*, **2015**, 114, 4385–4395.
[26] Roni, M.; Murugan, K.; Panneerselvam, C.; Subramaniam, J.; Nicoletti, M.; Madhiyazhagan, P.; Dinesh, D.; Suresh, U.; Khater, H.F.; Wei, H.; Canale, A.; Alarfaj, A.A.; Munusamy, M.A.; Higuchi, A.; Benelli, G.; Characterization and biotoxicity of *Hypnea musciformis*-synthesized silver nanoparticles as potential eco-friendly control tool against *Aedes aegypti* and *Plutella xylostella*. *Ecotoxicology and Environmental Safety*, **2015**, 121, 31–38.
[27] Suganya, G.; Karthi, S.; Shivakumar, M.S. Larvicidal potential of silver nanoparticles synthesized from *Leucas aspera* leaf extracts against dengue vector *Aedes aegypti*. *Parasitol Res*, **2014**, 113, 1673–1679.
[28] Sujitha, V.; Murugan, K.; Paulpandi, M.; Panneerselvam, C.; Suresh, U.; Roni, M.; Nicoletti, M.; Higuchi, A.; Madhiyazhagan, P.; Subramaniam, J.; Dinesh, D.; Vadivalagan, C.; Chandramohan, B.; Alarfaj, A.A.; Munusamy, M.A.; Barnard, D.R.; Benelli, G. Green-synthesized silver nanoparticles as a novel control tool against dengue virus (DEN-2) and its primary vector *Aedes aegypti. Parasitology Research*, **2015**, 114, 3315–3325.
[29] Ghosh, V.; Mukherjee, A.; Chandrasekaran, N. Formulation and characterization of plant essential oil based nanoemulsion: evaluation of its larvicidal activity against *Aedes aegypti Asian Journal of Chemistry*, **2013**, 25, 321-323.
[30] Duarte, J.L.; Amado, J.R.R.; Oliveira, A.E.M.F.M.; Cruz, R.A.S.; Ferreira, A.M.; Souto, R.N.P.;. Falcão, D.Q.; Carvalho, J.C.T.; Fernandes, C.P. Evaluation of larvicidal activity of a nanoemulsion of *Rosmarinus officinalis* essential oil. *Braz J Pharmacogn*, **2015**, 25, 189-192.
[31] Rodrigues, E.C.R.; Ferreira, A.M.; Vilhena, J.C.E.; Almeida, F.B.; Cruz, R.A.S.; Florentino, A.C.; Souto, R.N.P.; Carvalho, J.C.T.; Fernandes, C.P. Development of a larvicidal nanoemulsion with copaiba (*Copaifera duckei*) oleoresin. *Braz J Pharmacogn*, **2014**, 24, 699-705.
[32] Galceran, C. B.; Sertie, J. A. A.; Lima, C. S.; Carvalho, J. C. T. Anti-inflammatory and analgesic effects of 6α,7β-dihydroxy-vouacapan-17β-oic acid isolated from *Pterodon emarginatus* Vog. fruits. *Inflammopharmacol*, **2011**, 19, 139-143.
[33] Leong, W. F.,; Lai, O. M.; Long, K.; Man, Y. B. C.; Misran, M.; Tan, C. P. Preparation and characterization of water-soluble phytosterol nanodispersions. *Food Chemistry*, **2011**, 77-83.

Sample Availability: Sample of the compound **1** is available from the authors.

10. CONSIDERAÇÕES FINAIS

Diversos estudos têm focado na investigação de atividades biológicas de produtos de origem natural, incluindo derivados da espécie Pterodon emarginatus. Contudo, a intrínseca baixa solubilidade do oleorresina, óleo essencial e diterpenos provenientes dos frutos dessa espécie são um entrave tecnológico. Embora a nanotecnologia seja considerada hoje uma área emergente, muitos estudos focam na obtenção de nanformulações empregando técnicas de alto aporte energético, o que torna o processo oneroso e dificultando sua aplicação em âmbito nacional. Neste trabalho, foi possível obter nanoemulsões e nanodispersões com indicativos de estabilidade e eficácia frente a larvas de Aedes aegypti, mostrando que foi possível obter êxito em disponibilizar as substâncias ativas em água. Adicionalmente, esse modelo abre perspectiva para o uso desses produtos em práticas integradas de controle desse vetor de doenças consideradas um problema grave de saúde pública no país. A simplicidade dos métodos envolvidos, utilizandose técnicas que podem ser aplicadas na indústria nacional também contribuem para o uso sustentável e valorização dessa espécie, com real possibilidade de aplicação em âmbito nacional.

ANEXO 1 – PARECER DO COMITÊ DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE FEDERAL DO AMAPÁ
PRÓ-REITORIA DE PESQUISA E PÓS- GRADUAÇÃO
COMITE DE ÉTICA NO USO DE ANIMAIS – CEUA – UNIFAP

CERTIFICADO

A Comissão de Ética no Uso de Animais da Universidade Federal do Amapá **APROVOU,** na reunião de 07 de agosto de 2014, o parecer referente ao protocolo no. **0018/2014** e certifica que o Projeto de Pesquisa intitulado "**Desenvolvimento de Nanoemulsão Inseticida com Óleo de Sucupira (*Pterodon emarginatus* vogel.)**" coordenado por **Anna Eliza Maciel de Faria Mota Oliveira**, está de acordo com os princípios de ética e bem estar animal.

CERTIFICATE

The Ethics Committee on Animal Use of the Amapá Federal University **APPROVED** at the meeting of 07 August 2014, the final decision about the Protocol **0018/2014** and certify that the research project entitled "**Desenvolvimento de Nanoemulsão Inseticida com Óleo de Sucupira (*Pterodon emarginatus* vogel.)**" coordinated by **Anna Eliza Maciel de Faria Mota Oliveira**, is in accordance with the principles of ethics and animal welfare.

Macapá, 07 de agosto de 2014

Prof. Tit. José Carlos Tavares Carvalho
Presidente CEUA-UNIFAP
Port. No. 1733/2014

Universidade Federal do Amapá
Pró-Reitoria de Pesquisa e Pós- Graduação
Comitê de Ética no Uso de Animais – CEUA – UNIFAP
Rod. Juscelino Juscelino Kubitschek, km 02 – Campus Marco Zero.
Macapá - AP, 68903-419 email: farmacos@unifap.br
Fone (96)4009-2907

ANEXO 2 – APRESENTAÇÃO ORAL DE TRABALHO

UNIFAP
UNIVERSIDADE FEDERAL DO AMAPÁ

Certificado

Certificamos que **Anna Eliza Maciel de Faria Mota Oliveira - PPGIF** realizou a apresentação oral do trabalho **"Desenvolvimento de uma nanoemulsão larvicida com Óleo de Sucupira (*Pterodon emarginatus vogel*)"** na *Semana Nacional de Ciência e Tecnologia 2015 – SNCT*, que ocorreu no período de 19 a 23 de outubro de 2015 na Universidade Federal do Amapá.

Profa Dra. Helena **Cristina G. Queiroz Simões**
Pró-Reitora de Pesquisa e Pós-Graduação
Portaria nº 1324/2014 - UNIFAP

ANEXO 3 – APRESENTAÇÃO EM CONGRESSOS

VI ENCONTRO DO PPGBIO

A produção científica como ferramenta de manutenção da biodiversidade

CERTIFICADO

Certificamos que o trabalho "**DESENVOLVIMENTO DE PRODUTO NANOBIOTECNOLÓGICO A BASE DE SUCUPIRA-BRANCA**" foi apresentado em forma de Pôster durante o VI Encontro do Programa de Pós-Graduação em Biodiversidade Tropical - PPGBIO no período de 04 a 06 de novembro de 2015, na Universidade Federal do Amapá, Macapá - AP, Brasil.

OLIVEIRA, A.E.M.F.M.
CRUZ, R.A.S
FERREIRA, R.M.A.
CAIO P. FERNANDES
DUARTE, J. L.
ROCHA, C.F.
CONCEIÇÃO, E.C.
JOSÉ C.T. CARVALHO
AMADO, J.R.R
SOUTO, R.N.P
OLIVEIRA, L.A.R.

Celeste Salinero
Comissão Científica
do VI Encontro do PPGBIO

Taires Peniche
Coordenação
do VI Encontro do PPGBIO

Organização

CARTA DE ACEITE

Comunicamos que o trabalho 'NANOEMULSÃO À BASE DA OLEORESINA OBTIDA DE PTERODON EMARGINATUS COMO UMA FERRAMENTA PROMISSORA PARA O CONTROLE DO CULEX QUINQUEFASCIATUS ' de autoria de HÉRICA. N. OLIVEIRA, JONATAS L. DUARTE, RODRIGO A.S. CRUZ, RAIMUNDO.N.P.SOUTO, RICARDO M.A. FERREIRA, EDEMILSON C. DA CONCEIÇÃO, LEANDRA A.R. DE OLIVEIRA, SILVIA M.M. FAUSTINO, ALEXANDRO C. FLORENTINO, JOSÉ C.T. CARVALHO, CAIO P. FERNANDES, ANNA E.M.F.M. OLIVEIRA, foi aceito para apresentação em forma de pôster na sessão 'Tecnologia e Desenvolvimento de Produtos Fitoterápicos' do XXIV Simpósio de Plantas Medicinais do Brasil – 2016, que será realizado no Minascentro, em Belo Horizonte, MG, no período de 21 a 24 de setembro de 2016.

Prof Dr Wanderlei das Graças Leite Brandão
FAFAR & MHNJB - UFMG
Presidente do XXIV Simpósio de Plantas Medicinais do Brasil

Prof Dr João Paulo Viana Leite
DBB - UFV
Presidente da Comissão Científica do XXIVSPMB

CARTA DE ACEITE

Comunicamos que o trabalho 'ÓLEO ESSENCIAL DE PTERODON EMARGINATUS COMO POTENCIAL MATÉRIA PRIMA NATURAL PARA O DESENVOLVIMENTO DE NANOEMULSÕES LARVICIDA.' de autoria de DESIRANE C. BEZERRA, JONATAS L. DUARTE, RODRIGO A.S. CRUZ, RAIMUNDO N.P. SOUTO, RICARDO M.A. FERREIRA, JEANE NOGUEIRA, EDEMILSON C. DA CONCEIÇÃO, SUZANA LEITÃO, HUMBERTO BIZZO, PAOLA E. GAMA, JOSÉ C.T. CARVALHO, CAIO P. FERNANDES, ANNA E.M.F.M. OLIVEIRA., foi aceito para apresentação em forma de pôster na sessão 'Tecnologia e Desenvolvimento de Produtos Fitoterápicos' do XXIV Simpósio de Plantas Medicinais do Brasil – 2016, que será realizado no Minascentro, em Belo Horizonte-MG, no período de 21 a 24 de setembro de 2016.

Belo Horizonte, 31/03/2016

Profª Drª Maria das Graças Lins Brandão
FAFAR & MHNJB - UFMG
Presidente do XXIV Simpósio de Plantas Medicinais do Brasil

Prof Dr João Paulo Viana Leite
DBB - UFV
Presidente da Comissão Científica do XXIVSPMB

ANEXO 4 – PRÊMIO

Programa Amazônia 2020

Santander UNIVERSIDADES

PROGRAMA AMAZÔNIA 2020

O Programa Amazônia 2020 tem como escopo apoiar as Universidades Federais da Região Norte por meio de um programa exclusivo de incentivo que promova a internacionalização das universidades, permita o intercâmbio de conhecimento, contribua para a formação do docente, incentive a pesquisa científica, o empreendedorismo sustentável e a transferência tecnológica. Além de inserir a educação financeira como ferramenta para a conscientização, a gestão responsável de recursos e a melhoria da renda.

	SIGLA	NOME INSTITUIÇÃO DE ENSINO SUPERIOR	NOME DO LÍDER DO PROJETO	NOME DO PROJETO	NOME DO PROFESSOR ORIENTADOR
1º lugar	UFAM	Universidade Federal do Amazonas	Diego Ken Osoegawa	NUYA'RLITUA: A fibra do coração	Ivani Ferreira de Faria
2º lugar	UNIFAP	Universidade Federal do Amapá	Anna Eliza Maciel de Faria Mota Oliveira	Produção de nano emulsões inseticidas a base de óleos de sucupira-branca no estado do Amapá	José Carlos Tavares Carvalho
3º lugar	UFT	Universidade Federal do Tocantins	Allison Daniel Fernandes Coelho Souza	Uso do carvão ativado granular a partir do coco babaçu na remoção de farmacos em estações de tratamento de esgoto	Thiago Costa Gonçalves Portelinha