HUMBERTO MOREIRA SPINDOLA

ATIVIDADE ANTINOCICEPTIVA E ANTITUMORAL DE COMPOSTOS ISOLADOS DA *Pterodon pubescens* Benth. *(Leguminosae- Papilionoidea)*

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Doutor em Odontologia na área de Farmacologia, Anestesiologia e Terapêutica.

Orientador: Prof.ª Drª Mary Ann Foglio
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A todos vocês...

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Epígrafe

"Quando nada parece dar certo, vou ver o cortador de pedras martelando sua rocha talvez 100 vezes, sem que uma única rachadura apareça. Mas na centésima primeira martelada a pedra se abre em duas, e eu sei que não foi aquela que conseguiu isso, mas todas as que vieram antes."

(Jacob Riis)

Resumo

A espécie vegetal *Pterodon pubescens* Benth. tem seu uso amplamente difundido na medicina popular. Neste trabalho, demonstramos de forma específica a atividade antinociceptiva (analgésica) e antitumoral de compostos isolados da espécie. Os resultados mais expressivos foram: 1) fracionamento, isolamento e identificação de compostos biomonitorados por ensaios experimentais *in vitro* e *in vivo*; 2) identificação dos compostos 6*α*-acetoxi-7*β*-hidroxivouacapano *(inédito),* éster 6*α,*7*β*-diidroxivouacapano-17*β*-oato de metila e 6*α,*7*β*-diidroxivouacapano-17*β*-metilenol com atividade antitumoral *in vitro* específica para uma linhagem celular de próstata (PC-3); 3) biodisponibilidade dos compostos geranilgeraniol e éster 6*α,*7*β*-diidroxivouacapano-17*β*-oato de metila com atividade antitumoral através de ensaios *in vivo*; 4) mecanismos de modulação analgésica dos compostos geranilgeraniol e éster 6*α,*7β-diidroxivouacapano-17*β*-oato de metila atribuídos a dor inflamatória, dor neurogênica, receptores gluatamatérgicos, receptores vanilóides, e possível exclusão de receptores opióides; 5) ação antialodínica e anti-hiperalgésica dos compostos geranilgeraniol e éster 6*α,*7*β*-diidroxivouacapano-17*β*-oato de metila*,* que demonstraram atividade medular e central; 6) mecanismos da ação antinociceptiva relacionados à síntese e/ou liberação de serotonina (5-HT) para o composto éster 6*α*,7*β*-diidroxivouacapano-17*β*-oato de metila e especificidade por receptores serotonérgicos 5-HT_3 e imidazólicos I_1 para o composto geranilgeraniol. Os resultados apresentados no presente trabalho permitiram determinar a eficácia dos compostos relacionada às atividades analgésica e antitumoral.

Abstract

Pterodon pubescens Benth. species is widespread used in folk medicine. In this study, we demonstrated the antinociceptive (analgesic) and anti-tumor activity of compounds isolated from species. The most relevant results were: 1) bioactivity-guided fractionation, isolation and identification of compounds using *in vitro* and *in vivo* assays; 2) identification of compounds 6*α*-acetoxy-7*β*-hydroxyvouacapan; 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester and 6*α,*7*β*-dihydroxyvouacapan-17*β*-metilenol with selectivity for prostate cell line (PC-3); 3) bioavailability of compounds geranylgeraniol and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester with antitumor activity confirmed by *in vivo* assays; 4) analgesic modulation mechanisms of compounds geranylgeraniol and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester related to inflammatory pain, neurogenic pain, glutamate receptors, vanilloid receptors, and possible exclusion of opioid receptors, 5) anti-allodynic and anti-hyperalgesic activities of compounds geranylgeraniol and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester demonstrating spinal and central activity; 6) the antinociceptive mechanisms related to the synthesis and / or release of serotonin (5-HT) for compound 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester, and specificity for serotonergic 5-HT_3 and imidazoline I_1 receptors for geranylgeraniol . The results presented in this study allowed us to determine the efficacy of compounds related to analgesic and anti-tumor activities, with potential for development as drugs.

Sumário

Trabalho apresentado sob forma alternativa (dividida em capítulos) de acordo com a Informação CCPG/ 002/ 06 (Anexo I) da Universidade Estadual de Campinas.

Introdução

A natureza é uma importante fonte de novas moléculas com potencial farmacológico para o tratamento de diversas moléstias. As plantas tem desempenhado importante papel no desenvolvimento de sofisticados medicamentos para a medicina tradicional. Registros da Mesopotâmia que datam de 2600 AC documentam o uso de milhares de plantas e derivados, e muitos desses registros até hoje ainda são utilizados na medicina para tratar tosses, resfriados, infecções, inflamações, dentre outras. (Cragg et al, 2009).

O uso de plantas como fonte de medicamentos em diversas culturas tem sido amplamente documentado, e a Organização Mundial da Saúde (OMS) estima que aproximadamente 65% da população mundial dependem de plantas tradicionais para os cuidados primários de saúde. No entanto, em países desenvolvidos os produtos naturais também desempenham um importante papel na promoção e manutenção da saúde (Cragg et al, 2009).

O interesse na pesquisa de novas substâncias ativas de origem vegetal tem aumentado significativamente. Várias empresas privadas e organizações governamentais têm instituído projetos de pesquisa nesta área. É importante salientar que na década de 70, nenhuma das 250 maiores companhias do ramo farmacêutico do mundo mantinha qualquer programa de pesquisa na área de produtos naturais e até o ano de 2005, pelo menos metade destas tinham introduzido este tipo de pesquisa como uma de suas prioridades (Fellows et al, 1995; Cragg e Newman, 2005).

Com relação ao desenvolvimento de medicamentos, este contexto é particularmente importante, uma vez que, as plantas medicinais constituem uma rica fonte de novas moléculas a serem exploradas tanto como drogas convencionais quanto como ferramentas bioquímicas para a elucidação de aspectos etiológicos de determinadas patologias (Lewis e Hanson, 1991; Newman

e Cragg, 2007). Outro aspecto importante é a obtenção de compostos que, apesar de apresentarem potencial terapêutico limitado, podem ser utilizados como matéria prima inicial para obtenção de produtos semi-sintéticos com atividade farmacológica mais pronunciada (Cordell et al, 2000).

Como exemplos, podem ser citados o desenvolvimento do atracúrio a partir dos alcalóides do curare, obtido de *Chondrodendron tometosum* e dos anestésicos locais a partir da cocaína (Waigh, 1988). Também a grande eficácia clínica de algumas substâncias antitumorais obtidas de plantas, como os alcalóides da vinca extraídas da *Catharanthus roseus* e os taxóides extraídos de *Taxus brevifolia* e *Taxus baccata* podem ser citadas. A década de 1990 registrou um aumento expressivo no interesse em drogas derivadas de espécies vegetais evidenciado pelo crescimento de publicações dessa linha de pesquisa nas principais revistas científicas das áreas de Química e Farmacologia (Rates et al, 2001; Calixto et al, 2003). A pesquisa com plantas que são empregadas na medicina popular é, portanto, uma das mais lógicas e produtivas maneiras de pesquisar uma nova droga para uso terapêutico (Elizabetsky et al, 1995).

O gênero *Pterodon* compreende 4 espécies nativas do Brasil: *P, abruptus Benth., P. apparucuri Pedersoli, P. pubescens* Benth, (*P. emarginatus Vog,)* e *P. polygalaeflorus* Benth. O estudo dessas espécies foi motivado pela comprovação da ação anti-cercariana e antimicrobiana *in vitro* do óleo obtido das sementes de *P. emarginatus* Vog., e de outras espécies (Fascio et al, 1976; Campos et al 1994).

A espécie vegetal, *Pterodon pubescens* Benth. -sinonímia botânica – *P. emarginatus* Vog., é uma árvore da família das *Leguminosae-Papillonoideae.* Conhecida popularmente como faveiro, sucupira-branca, fava-de-sucupira, sucupira ou sucupira-lisa, é utilizada no tratamento de reumatismo, dores de garganta, problemas da coluna, como tônico e depurativo. É encontrada principalmente no cerrado dos estados de Minas Gerais, São Paulo, Goiás e Mato Grosso do Sul. Esta espécie atinge de 8-16 metros, com tronco de 30-40

centímetros de diâmetro; possui folhas pinadas, com 20-36 folíolos de 3-4 centímetros de diâmetro (Lorenzi, 1998) (**fig. 1**).

Figura 1: Árvore e sementes da *Pterodon pubescens* Benth. localizados no município de São Carlos (SP) utilizadas no trabalho.

Em 1970, diterpenos foram obtidos por Mahajan e Monteiro, e no mesmo ano, terpenos foram isolados por Fascio e colaboradores (1976). Os diterpenos 14,15-epoxigeranilgeraniol e alguns derivados foram isolados de *Pterodon pubescens* Benth, provando serem eficientes agentes quimioprofiláticos contra infecções causadas por cercarias de *S. mansoni* quando aplicados topicamente na cauda de ratos (Dos Santos Filho et al, 1972).

Quatorze diterpenos furânicos foram identificados e isolados dos frutos do gênero *Pterodon.* O diterpeno furânico – vouacapano, 6α-7β-dihidroxivouacapano-17β-oato de sódio, foi isolado da espécie *P. polygalaeflorus* Benth, e sua atividade anti-inflamatória demonstrada através do modelo de pata induzido por carragenina (Nunan et al, 1985; Arriaga et al, 2000). A atividade antinociceptiva do vouacapano também foi estudada por Duarte e colaboradores em 1996, sugerindo uma possível ação catecolaminérgica desse composto.

A atividade anti-inflamatória do extrato hexânico das sementes de *P. pubescens* Benth, foi estudada por Carvalho e colaboradores sugerindo que a atividade anti-inflamatória do extrato hexânico estaria relacionada à inibição da liberação de prostaglandinas e outros mediadores envolvidos no sistema de cininas. Sugere ainda que, a atividade anti-inflamatória do extrato poderia estar relacionada à presença de compostos terpênicos (Carvalho et al, 1999).

Um estudo do extrato hidroalcoólico das sementes de *P. pubescens* Benth. confirmou seus efeitos em modelo de artrite experimental e a ausência de toxicidade subaguda (Coelho et al, 2001). Silva et al (2004), identificaram o diterpeno 6α,7β-dihidroxivouacapano, como responsável pela atividade antiedematogênica do óleo extraído das sementes de *P. pubescens*. Em 2005, Coelho e colaboradores demonstraram a ação antinociceptiva do extrato hidroalcoólico das sementes de *P. pubescens,* atribuída à presença de derivados vouacapânicos.

Em 2007, Calixto e colaboradores demonstraram a atividade antiplaquetária do geranylgeraniol extraído de sementes da *P. pubescens*, relacionando este efeito à inibição da ciclooxigenase na cascata de coagulação sanguínea. No

mesmo ano, Vieira e colaboradores (2007) demonstraram, em um ensaio preliminar, a atividade antiproliferativa do extrato bruto etanólico das sementes da *Pterodon* contra uma linhagem de melanoma humano.

Menna-Barreto e colaboradores (2008) determinaram a atividade contra *T. cruzi* de frações e do geranilgeraniol obtidos do extrato etanólico das sementes. Ainda em 2008, Cardoso e colaboradores demonstraram atividade supressora de linfócitos T e B, relacionado à atividade antiinflamatória do extrato etanólico. Spindola et al (2009) e Euzébio et al (2009) descreveram a atividade antiproliferativa contra diversas linhagens tumorais de furanoditerpenos obtidos da *P. pubescens.* Recentemente Spindola et al (2010) relataram o envolvimento dos compostos geranilgeraniol e do éster 6*α*-7*β*-diidroxivouacapano-17*β*-oato de metila, isolados da *P. pubescens,* na atividade antinociceptiva.

Este trabalho deu continuidade aos estudos desenvolvidos na dissertação de mestrado (Spindola, 2006), avaliando a atividade farmacológica da espécie, contribuindo com dados relacionados aos parâmetros de eficácia e segurança necessários para o desenvolvimento de novos medicamentos.

Proposição

Geral

- Avaliação das atividades antitumoral e antinociceptiva de compostos isolados das sementes da *Pterodon pubescens* Benth. utilizando técnicas *in vitro* e *in vivo*.

Específica

- *Capítulo 1:* Fracionamento, isolamento e identificação dos compostos, biomonitorados por ensaios *in vitro* de atividade antiproliferativa contra linhagens tumorais humanas;

- *Capítulo 2:* Estudo da atividade antitumoral *in vivo* (em camundongos) dos compostos previamente identificados com atividade anticancer *in vitro;*

- *Capítulo 3:* Estudo do envolvimento de frações e compostos obtidos a partir de sementes da *P.* pubescens com atividade antinociceptiva, utilizando modelos experimentais *in vivo* com camundongos.

- *Capítulo 4:* Avaliação de mecanismos da ação de compostos envolvidos na atividade antinociceptiva, através de ensaios *in vivo* em ratos e camundongos.

Capítulo 1

Furanoditerpenes from *Pterodon pubescens* Benth with selective *in vitro* anticancer activity for prostate cell line.

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Resumo:

O fracionamento biomonitorado do extrato diclorometânico das sementes de *Pterodon pubescens* Benth forneceu o 6α-acetóxi-7β-hidroxi-vouacapano **1** (inédito), além de quarto diterpenos furânicos (**2**, **3,** **4** e **5**). A atividade antiproliferativa dos compostos foi avaliada *in vitro* contra as linhagens de células tumorais humanas UACC-62 (melanoma), MCF-7 (mama), NCI-H460 (pulmão), OVCAR-03 (ovário), PC-3 (próstata), HT-29 (colon), 786-0 (rim), K562 (leucemia) e NCI-ADR/RES (ovário com fenótipo de resistência a múltiplos fármacos). Os resultados foram expressos em três concentrações efetivas GI_{50} (concentração para que ocorra 50% de inibição de crescimento), TGI (concentração que resulta em inibição total de crescimento) e LC_{50} (concentração que resulta em 50% de morte celular). A citotoxicidade *in vitro* foi avaliada também frente a uma linhagem de célula murina normal (3T3). Este é o primeiro relato de atividade anticâncer para os compostos **1**, **4** e **5**, que apresentaram grande seletividade, dependente da concentração, para PC-3. O composto **1** foi 26 vezes mais potente para inibir 50% do crescimento (GI_{50}) de PC-3, 15 vezes mais citostático (TGI) e 6 vezes menos tóxico (LC_{50}) quando comparado com doxorrubicina (controle).

Abstract

Activity guided fractionation of *Pterodon pubescens* Benth. methylene chloride-soluble fraction afforded novel 6α-acetoxi-7β-hydroxy-vouacapan **1** and four known diterpene furans **2, 3, 4, 5**. The compounds were evaluated for *in vitro* cytotoxic activities against human normal cells and tumour cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-03 (ovarian), PC-3 (prostate), HT-29 (colon), 786-0 (renal), K562 (leukemia) and NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance) Results were expressed by three concentration dependent parameters GI_{50} (concentration that produces 50% growth inhibition), TGI (concentration that produces total growth inhibition or cytostatic effect) and LC_{50} (concentration that produces -50% growth, a cytotoxicity parameter). Also, *in vitro* cytotoxicity was evaluated against 3T3 cell line (mouse embryonic fibroblasts). Antiproliferative properties of compounds **1**, **4** and **5** are herein reported for the first time. These compounds showed selectivity in a concentration-dependent way against human PC-3. Compound **1** demonstrated selectivity 26 fold more potent than the positive control, doxorubicin, for PC-3 (prostrate) cell line based on GI_{50} values, causing cytostatic effect (TGI value) at a concentration fifteen times less than positive control. Moreover comparison of 50% lethal concentration (LC_{50} value) with positive control (doxorubicin) suggested that compound **1** was less toxic.

Keywords: *Pterodon pubescens*, Leguminosae, furanoditerpenes, *in vitro* assay, prostate cell line, cytotoxicity.

Introduction

Throughout history, natural products have afforded a rich source of compounds that have found many applications in the fields of medicine, pharmacy and biology. Within the sphere of cancer, a number of important new commercialized drugs have been obtained from natural sources, by structural modification of natural compounds, or by the synthesis of new compounds, designed following a natural compound as model. Among the many compounds identified for cancer treatment Taxol, isolated from *Taxus brevifolia*, has proven to be an important chemotherapeutic agent.[1] Medicinal plants still play an important role as source of new targets for drug discovery. The huge structural diversity of natural compounds and their bioactivity potential have meant that several products isolated from plants, marine flora and microorganisms can serve as "lead" compounds for improvement of their therapeutic potential by molecular modification.[2, 3]

Pterodon genus compromises four species native to Brazil: *P. abruptus* Benth., *P. apparucuri* Pedersoli, *P. pubescens* Benth. (*P. emarginatus* Vog.) and *P. polygalaeflorus* Benth. Initially scientific studies of these plants were motivated by evidence that the seed's oil had cercaricidal [4] and anti-microbial activity.[5]

Pterodon pubescens Benth. (Leguminosae) known, as Sucupira Branca is widespread throughout Goiás, Minas Gerais and São Paulo states in Brazil. The seeds are commercially available in Brazilian medicinal flora market. Plant's crude alcoholic extracts are used in folk medicine as anti-inflammatory, analgesic and anti-rheumatic preparations.[5, 6]

Phytochemical studies of *Pterodon* genus have revealed the presence of alkaloids, isoflavones and diterpenes. Furan-diterpenes were identified and isolated from *Pterodon* fruits.[8-11] Some authors have suggested that furan-diterpenes possessing vouacapan skeleton are involved with anti-inflammatory properties of *Pterodon pubescens* seeds´ oil.[5, 12, 13] Diterpenes 6α-hydroxyvouacapan-7β-17β-lactone and 6α,7β-dihydroxyvouacapan-17β-oate

methyl ester, present in *P. emarginatus* and *P. polygalaeflorus* seeds, respectively, were previously found to be associated with anti-inflammatory activity of these species.[12] Another compound, acid 6α,7β-dihydroxyvouacapan-17β-oic, was suggested to be one of the possible compounds involved with anti-inflammatory activity, since this compound was identified in the active fraction that exhibited anti-edematogenic activity when tested in carrageenan-induced paw edema or in croton oil-induced ear edema assays.[13]

Evidence of biogenic amines involved with antinociceptive effect of a vouacapans extracted from *P. polygalaeflorus* Benth was studied by Duarte et al (1996) suggested that the pharmacological activity was triggered by catecholaminergic system.[14]

Coelho et al (2005) studied Pterodon seed extract's antinociceptive activity suggesting that both peripheral and central inhibitory mechanisms are involved.[15]

In the present study we report the isolation by activity-guided fractionation, identification and *in vitro* anticancer activities of vouacapans from *Pterodon pubescens* Benth that are herein reported for the first time.

Results and Discussion

Compounds 6α,7β-diacetoxyvouacapan **2**, 7β-diacetoxyvouacapan **3**, 6α,7β-dihydroxyvouacapan-17β-oate methyl ester **4**, and 6α,7β-dihydroxyvouacapan-17β-methylene-ol **5** (Figure **1**) were identified based on comparison of experimental ^{1}H and ^{13}C-NMR with reported spectral data.[9, 10, 16, 17]

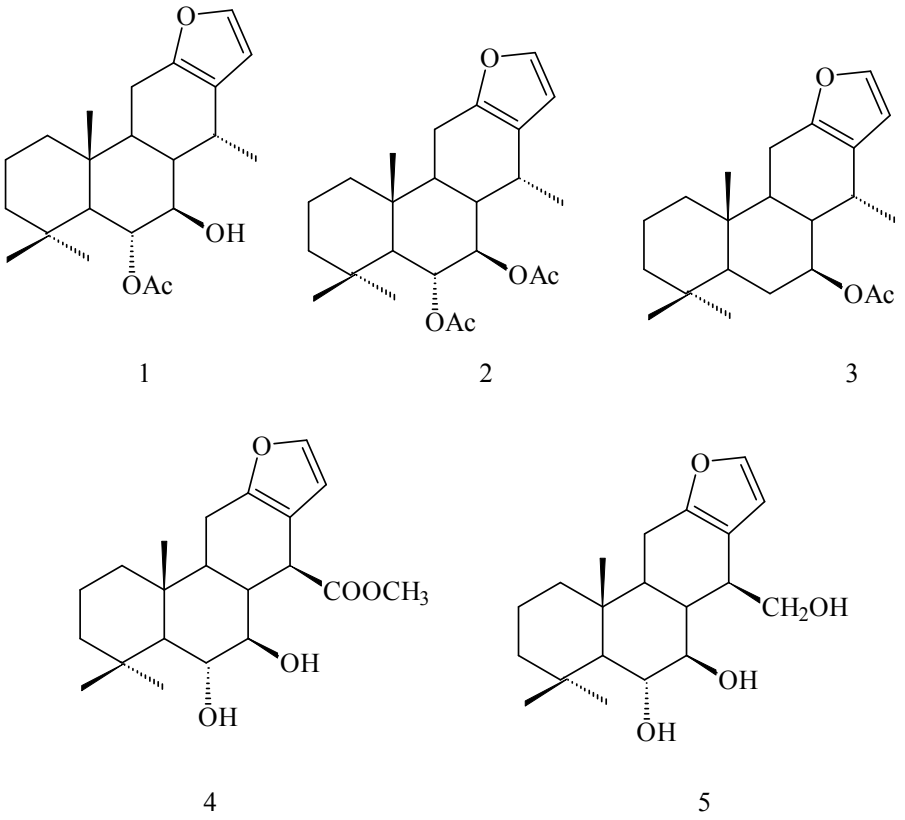


Figure 1: Chemical structures of vouacapans **1**, **2**, **3**, **4** and **5** isolated from *Pterodon pubescens* Benth. seeds.

Novel compound **1** was deduced as having an elemental formula $C_{22}H_{32}O_4$, by HREI-MS (observed $M^{+\cdot}$ = 360.23556, required $M^{+\cdot}$ = 360.23010), which indicated seven insaturation sites. Infrared absorptions at 3449 (OH) and 1713 (C=O) cm^{-1} provided evidences for hydroxyl and carbonyl functionalities. The ^{1}H-NMR spectral data (Table 1) showed a signal at δ_H 3.48 (1H, dd, *J* 9.7; 9.3 Hz, H-7) that presented correlations with hydrogens H-8 (δ_H 1.88) and H-6 (δ_H 5.2) in H-H

COSY experiment. This signal at δ_H 3.48 was attributed to a proton geminal to the hydroxyl group at C-7. Signal at δ_H 5.2 (1H, dd, *J* 11.7; 9.3Hz) was attributed hydrogen H-6, which was confirmed by correlations with hydrogens H-5 (δ_H 1.3) and H-7 (δ_H 3.48) in H-H COSY experiment. The coupling constant of hydrogens H-6 and H-7 was observed as 9.3 Hz indicating a *trans*-diaxial relationship. When compound **1** was acetylated with excess acetic anhydride /pyridine, this compound showed identical ^{1}H and ^{13}C-NMR spectral data to compound 6α,7β-diacetoxyvouacapan **2** [9] (HREI-MS 402.2630), suggesting that compound **1** has the same relative configuration to 6α,7β-diacetoxyvouacapan **2** with a hydroxy group attached β to C-7 whereas the acetyl group positioned α at C-6 (Table **1**).

The activity-guided fractionation of methylene chloride soluble fraction was monitored by *in vitro* anticancer activity assay in UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-3 (ovarian), PC-3 (prostate), HT-29 (colon), 786-0 (renal), K562 (leukemia) and NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance) cancer cell lines. A 48-h SRB (Sulforhodamine B) cell viability assay was performed to determine growth inhibition and cytotoxic properties of fractions and compounds. Cells were treated with at least four different concentrations levels (0.25 to 250 μg mL^{-1}) with determination of three endpoints, concentration inhibiting the growth of 50% of the cells (GI_{50}), concentration for total growth inhibition (TGI) and concentration needed to kill 50% of the cells (Table **2**).[18] Compounds **2** and **3** were equally not potent based on GI_{50}, TGI and LC_{50} values.

Table 1: ^{1}H and ^{13}C NMR (11 Tesla, $CDCl_3$/TMS) data for diterpene **1** [a].

Atom	C type	δ ^{13}C	δ ^{1}H	δ ^{1}H x ^{1}H COSY
C-1	CH_2	39.9	1.06; 1.76	
C-2	CH_2	18.3	1.38; 1.58	
C-3	CH_2	43.6	1.3; 1.55	
C-4	C^o	33.1	-	
C-5	CH	54.6	1.3 (d, *J* 11.7 Hz)	5.2
C-6	CH	76.4	5.2 (dd, *J* 11.7; 9.3Hz)	3.48; 1.3
C-7	CH	75.8	3.48 (dd, *J* 9.7; 9.3 Hz)	5.2; 1.88
C-8	CH	43.2	1.88 (td, *J* 9.7, 5.1 Hz)	3.48;3.13;1.92
C-9	CH	43.3	1.92 (ddd, *J* 12.3; 10.5; 5.1 Hz)	1.88; 0.95
C-10	C^o	38.6	-	
C-11	CH_2	22.6	0.95 (dd, *J* 12.3 Hz); 2.15 (dd, *J* 10.5 Hz)	1.92
C-12	C^o	148.7	-	
C-13	C^o	121.7	-	
C-14	CH	27.4	3.13 (d, *J* 5.1 Hz)	1.88
C-15	CH	109.6	6.21 (d, *J* 3.5 Hz)	7.25
C-16	CH	140.6	7.25 (d, *J* 3.5 Hz)	6.21
C-17	CH_3	16.9	0.99	
C-18	CH_3	36.4	1.08	
C-19	CH_3	22.7	0.95	
C-20	CH_3	15.8	0.99	

[a] Chemical shifts are in δ (ppm)

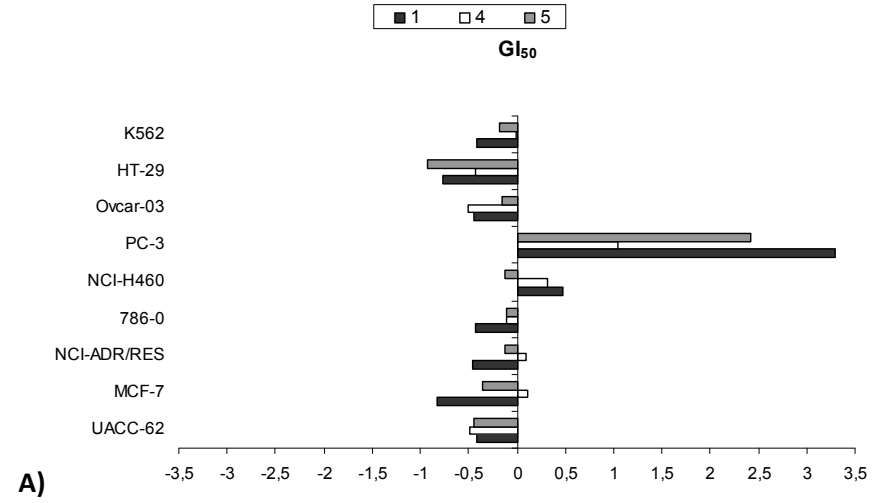
Table 2: Cytotoxic activity of compounds **1**. **2**. **3**. **4** and **5**.

Cell lines		1	2	3	4	5	Doxorrubicin
UACC-62	**GI_{50}**	27.8	71.95	>250	12.7	6.23	0.02
	TGI	64.47	>250	>250	39.58	118.3	0.19
	LC_{50}	198.3	>250	>250	241.9	250	1.36
MCF-7	**GI_{50}**	69.7	148.1	212.6	3.2	5.18	0.20
	TGI	172.88	>250	>250	20.97	105.3	2.69
	LC_{50}	179.6	>250	>250	185.3	250	40.40
NCI-ADR/RES	**GI_{50}**	30.5	>250	>250	3.3	3.02	0.09
	TGI	96.72	>250	>250	11.56	21.43	5.31
	LC_{50}	230.1	>250	>250	23.47	223.2	36.23
786-0	**GI_{50}**	28.5	86.16	196.6	5.2	2.86	0.03
	TGI	59.30	>250	>250	24.29	14.41	0.26
	LC_{50}	173.8	>250	>250	32.85	30.85	12.92
NCI-H460	**GI_{50}**	3.50	122.3	21.43	2.0	2.98	0.02
	TGI	48.34	>250	>250	47.08	77.48	0.11
	LC_{50}	228.8	>250	>250	250	250	2.14
PC-3	**GI_{50}**	0.0053	0.006	0.50	0.37	0.0085	0.14
	TGI	0.38	>250	>250	0.27	0.17	4.53
	LC_{50}	28.02	>250	214.4	15.80	4.85	5.90
Ovcar-03	**GI_{50}**	29.3	>250	>250	13.05	3.14	0.20
	TGI	87.52	>250	>250	49.66	24.02	12.91
	LC_{50}	243.4	>250	>250	240.0	178.6	250
HT-29	**GI_{50}**	60.9	>250	>250	10.8	19.04	0.25
	TGI	200.7	>250	>250	37.97	35.83	2.26
	LC_{50}	248.7	>250	>250	245.9	218.3	37.59
K562	**GI_{50}**	27.7	>250	132.8	4.1	3.37	0.22
	TGI	80.97	>250	>250	26.29	36.16	2.02
	LC_{50}	232.1	>250	>250	213.1	250	25.82

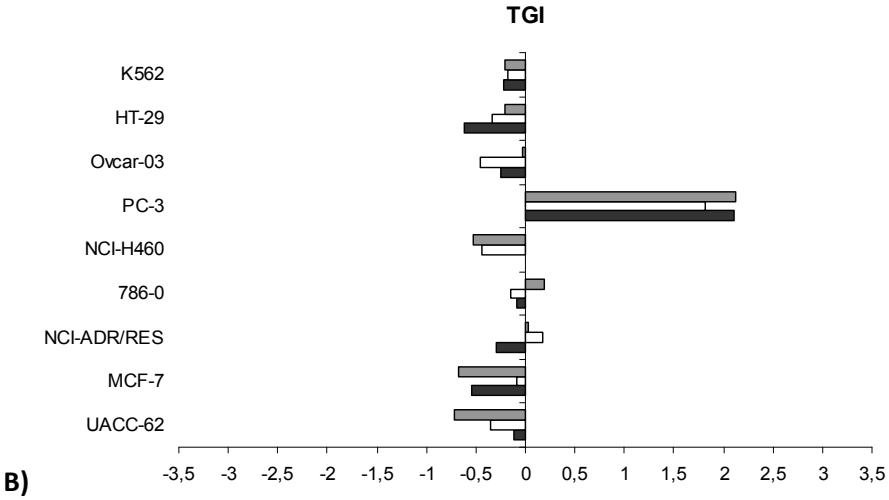
UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung non-small cells), OVCAR-3 (ovarian), PC-3 (prostate), HT29 (colon), 786-0 (renal), K562 (leukemia) and NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance). GI_{50}: concentration ($\mu g\ mL^{-1}$) inhibiting the growth of 50% of the cells; TGI: concentration ($\mu g\ mL^{-1}$) total growth inhibition; LC_{50}: concentration ($\mu g\ mL^{-1}$) need to kill 50% of the cells [18].

A mean graph for compounds **1**-**5** corroborated the selectivity of compounds **1**, **4** and **5** for PC-3 human prostate cancer cell lines (Figure 2). The mean graph was developed by NCI emphasize differential effects of test compounds on various human tumor cell lines. This graph is generated from a set of GI_{50}, TGI, or LC_{50} values. Positive values project to the right of the vertical line and represent cellular sensitivities to the test agent that exceed the mean. Negative values project to the left and represent cell line sensitivities to the test agent that are less than the average value.[19] Based on the three graphics, PC-3 cell line was high sensible to compounds **1, 4** and **5** . The interesting thing to notice is that compound **1** was more potent than **4** and **5** to inhibit cellular growth in 50% (GI_{50}, Figure 2A), whereas compounds **1** and **5** showed almost same potency in causing cytostatic (TGI, Figure 2B). On the other hand, when cytotoxicity parameter (LC_{50}, Figure 2C) was evaluated, compound **5** was more toxic whereas **1** and **4** were similarly toxics to PC-3 cell line. This high selectivity to PC-3 cell line suggests that furanoditerpenes **1**, **4** and **5** may share a similar action mechanism, probably evolving androgenic receptors. Compound **4** was able to reduce in 50% cellular growth of MCF-7 and NCI-H460 cell lines and also presented activity against NCI/ADR-RES cell line measured by all three parameters.

Compound **1** demonstrated selectivity 26 fold more potent than the positive control (doxorubicin) for PC-3 (prostrate) cell line based on GI_{50} values, causing cytostatic effect (TGI value) at a concentration fifteen times inferior than positive control (doxorubicin). Moreover comparison of 50% lethal concentration (LC_{50} value) with positive control (doxorubicin) demonstrated compound **1** to be less toxic.



A)



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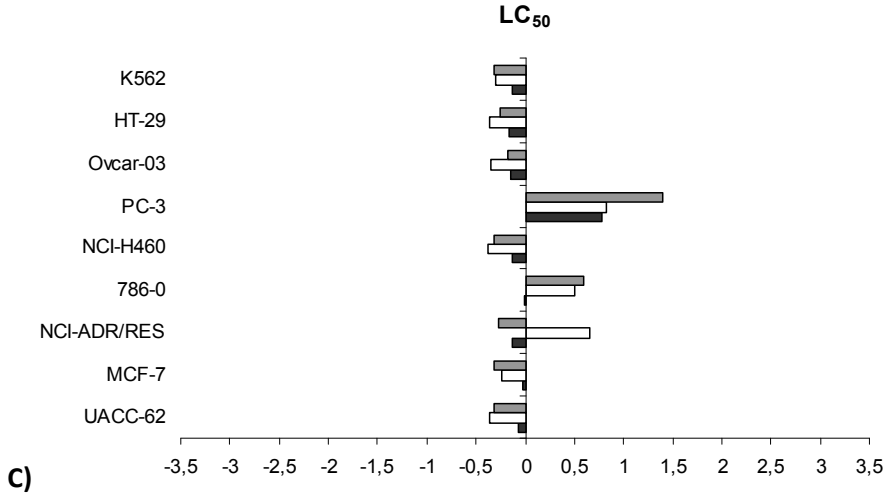

C)

Diferential Cytotoxicity

(Relative deviation from mean)

Figure 2: Patterns of differential cytotoxicity toward human tumor cell lines. A mean graph for compounds **1**, **4** and **5** is shown. The midline of each portion represents the mean for GI_{50} (**A**), TGI (**B**) and LC_{50} (**C**) endpoint, calculated across nine cell lines: UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT29 (colon), and K562 (leukemia). This mean value is then subtracted from the value for each individual cell line and plotted. Cell lines more sensitive are visualized as bars deflecting to the right, whereas more resistant cell lines have bars extending to the left of the mean.[19]

Cytotoxicity in normal cell lines of compounds **1, 4, 5** were evaluated against 3T3 cell line (mouse embryonic fibroblasts) assessing mitochondrial functions by MTT reduction with succinate dehydrogenase in order to obtain cell viability. Compound **1** (IC_{50} = 34.33 $\mu g\ mL^{-1}$) demonstrated to be slightly less cytotoxic than compounds **4** (IC_{50} = 22.83 $\mu g\ mL^{-1}$) and **5** (IC_{50} = 23.55 $\mu g\ mL^{-1}$). All these values were higher than almost all GI_{50} and TGI obtained for promissory compounds.

Cyproterone, a steroid formed by the mevalonate pathway is a known drug used as anti-androgen for prostrate cancer treatment.[20] Cyproterone is a steroidal antiandrogen agent that inhibits the action of adrenal and testicular androgens on prostate cells, seminal vesicles, testes, and the vas deferens. Additionally causes a centrally mediated reduction in testicular secretion of androgens. This drug is indicated for treatment of prostate cancer, androgen induced disorders of the skin (acne, seborrhoea, hirsutism, alopecia), precocious puberty and sexual disorders in men.[21,22]

Male rats treated during two weeks with 100 and 300 mg kg^{-1} doses of *Pterodon pubescens* dichloromethane crude extract decreased body weight gain by 57 and 75% respectively.[23] That difference in body weight gain may have a relationship with antiandrogen activity of vouacapan type compounds found in the crude dichloromethane extract. Decrease of mean final body weight was also observed with cyproterone after 15 days treatment.[24] This data corroborates with

the hypothesis that vouacapan type compounds interact with testosterone receptors. Further animal studies shall evaluate these findings.

Considering that compounds **1**, **4** and **5**, furan-diterpenoid, also originates from the mevalonate biosynthetic pathway[20], PC-3 human tumor cell line inhibition observed might arise by similar pharmacological mechanism such as Cyproterone. Therefore compounds **1**, **4**, **5** are interesting pharmacophores capable of providing new insights to the understanding of agonist versus antagonist properties of androgens, leading to the development of new anticancer chemotherapeutics agents.

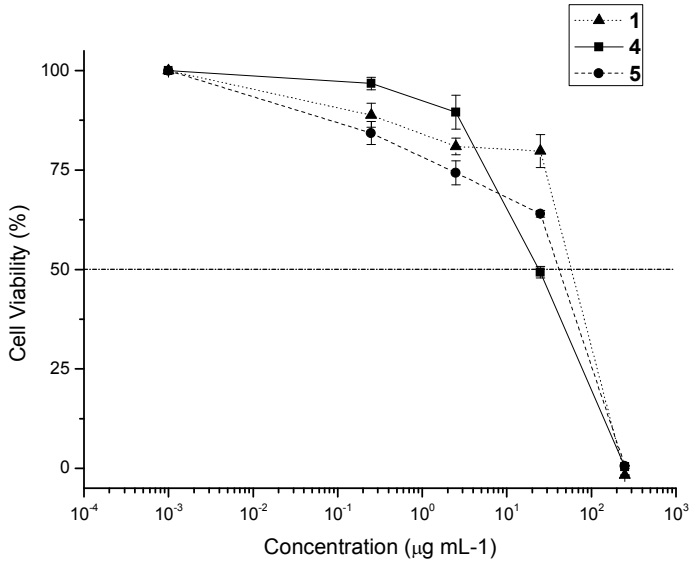

Figure 3: Cell viability test with compounds **1, 4,** and **5** on 3T3 cell lines (mouse fibroblast) after 48 hours exposure.

Experimental

General Experimental Procedures:

IR spectra: JASCO-FT/IR-410 spectrometer. ^{1}H, ^{13}C NMR and 2D experiments: Varian Inova-500 spectrometer (11 tesla). Chemical shifts were recorded in $CDCl_3$ solutions and quoted relative to TMS (δ 0.0, ^{1}H NMR) and $CHCl_3$ (δ 77.0, ^{13}C NMR). High-resolution electron ionization mass spectroscopy (HREIMS) was recorded on a VG-AutoSpec High Resolution Mass Spectrometer (70 eV) using direct probe. Column chromatography (CC): silica gel (0.063 x 0.200 mm, Merck®). TLC (thin layer chromatography): precoated plates (775554 Merck®), UV detection and anisaldehyde solution.

Plant Material:

P. pubescens Benth. seeds were collected in Pedregulho (SP) and São Carlos (SP) cities, in march 2004. Prof. Dr. Jorge Yoshio Tamashiro from IB-UNICAMP (department of botany) identified the plant species. A voucher specimen was deposited at Universidade Estadual de Campinas (UEC) Herbarium, under numbers 1398 and 1402.

Preparation of plant extract (EB) and fraction purification:

Freeze-dried seeds (100g) were grinded prior to use on a Stephen mill (model UM 40) and extracted with dichloromethane three times during two hour periods, with 5:1 solvent/plant ratio, at room temperature. The extract was dried over anhydride Na_2SO_4 filtered and concentrated under vacuum (Buchi RE 120), with 32% yield of crude seed oil extract (EB).

This crude oil (18.2g) was purified on pre-column chromatography using silica gel (Merck 7734) (5 x 60 cm) with hexane [FR1] (0-450 mL); hexane/ethyl acetate (95:5), [FR2] (500-900 mL); hexane/ethyl acetate 1(80:20) [FR3-4] (1000 x 1350 mL); hexane/ethyl acetate (60:40) (1400 x 1800 mL) [FR5-6]; hexane/ethyl acetate (40:60) (1900 x 2300 mL) [FR7]; rest flushed with methanol. The resulting

fractions were monitored by thin layer chromatography (TLC), exposed with anysaldehyde reagent (50 mL acetic acid, 0.5 mL sulfuric acid and 0.5 mL anysaldehyde) followed by heating at 110^{o}C. According to TLC profile the fractions were group and submitted to biological assay. The *in vitro* anticancer model on nine human cell lines determined the fractions that were further purified. Among the fractions isolated by column chromatography, Fraction 7 presented the best anticancer *in vitro* activity (data not shown).

Fraction 7 (10g) was successively chromatographed by CC on silica-gel (70-230 mesh) (5 x 60 cm) and eluted with hexane/ dichloromethane (7:3) (900-1800 mL) yielded **3** (333mg, 3% yield); R_f **3** 0.75; hexane/ dichloromethane (6:4) (1900-2600 mL) yielded **2** (990 mg, 9.9% yield), R_f **2** 0.56; hexane/ dichloromethane (2:8) (2650- 3100 mL) yielded **1** (963 mg, 9.63% yield), R_f **1** 0.29; (3350-4100 mL) yielded **4** (1.2 g, 12% yield) R_f **4** 0.17 and **5** (0.3g, 3% yield) R_f **5** 0.14.

6α-acetoxy-7β-hydroxy-vouacapan (**1**):

White crystal; mp 168-171ºC, $[\alpha]^{20}_{D}$: +39.4º ($CHCl_3$; *c* 0.0094); FTIR ν_{max} (cm^{-1}): 3449, 1713; ^{1}H and ^{13}C NMR (11 Tesla, $CDCl_3$/TMS): see Table 1.

6α7β-diacetoxyvouacapan (**2**):

White crystal; mp 167.3-168.0ºC; FTIR, ^{1}H and ^{13}C NMR data are in agreement with those reported in the literature. [9, 10, 16, 17]

7β-diacetoxyvouacapan (**3**):

White crystal; mp 125.6-127.2ºC; FTIR, ^{1}H and ^{13}C NMR data are in agreement with those reported in the literature. [9, 10, 16, 17]

6α,7β-dihydroxyvouacapan-17β-oate methyl ester (**4**):

Colorless oil; FTIR, ^{1}H and ^{13}C NMR data are in agreement with those reported in the literature. [9, 10, 16, 17]

6α7β-dihydroxyvouacapan-17β-methylene-ol (**5**):

Colorless oil; FTIR, ^{1}H and ^{13}C NMR data are in agreement with those reported in the literature. [9, 10, 16, 17]

Chromatographic analysis:

The GC/MS analysis were carried out using a HP-6890/5975 system equipped with a J&W Scientific DB-5 fused capillary column (25 m x 0.2 mm x 0.33 m). Temperature program: 60°C (5°C min^{-1}) – 300°C (10 min.), Injector 250°C, detector 300°C. Helium was used as carrier gas (0.7 bar, 1 ml min^{-1}). The MS were taken at 70eV. Scanning speed was 0.84 scans s^{-1}, from 40 to 550. Sample volume was 1 µl. Split: 1:40.

In vitro anticancer activity assay:

Human tumour cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-03 (ovarian), PC-3 (prostate), HT-29 (colon), 786-0 (renal), K562 (leukemia) and NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance) were kindly provided by National Cancer Institute (NCI). Stock cultures were grown in medium containing 5 mL RPMI 1640 (GIBCO BRL) supplemented with 5% fetal bovine serum. Gentamicine (50 μg mL^{-1}) was added to experimental cultures. Cells in 96-well plates (100 μL cells $well^{-1}$) were exposed to sample concentrations in DMSO/RPMI (0.25, 2.5, 25 and 250 μg mL^{-1}) at 37°C, 5% of CO_2 in air for 48h. Final DMSO concentration did not affect cell viability. Afterwards cells were fixed with 50% trichloroacetic acid and cell proliferation determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay. Using the concentration-response curve for each cell line, GI_{50} (concentration that produces 50% growth inhibition), TGI (concentration that produces total growth inhibition or cytostatic effect) and LC_{50} (concentration that produces −50% growth, a cytotoxicity parameter) were determined through non-linear regression analysis (Table **2**) using software ORIGIN 7.5 (OriginLab Corporation). [18]

In vitro Cytotoxicity assay:

Cell line 3T3 (mouse embryonic fibroblasts) was grown in medium containing 5 mL DMEM (glucose 4.5g L^{-1}, glutamine 4mM) (LGC Biotecnologia) supplemented with 10% fetal bovine serum. Gentamicine (50 μg mL^{-1}) was added to experimental cultures. Cells in 96-well plates (100 μL cells $well^{-1}$, 1 x 10^4 cell mL^{-1}) were exposed to sample concentrations in DMSO/RPMI (0.25, 2.5, 25 and 250 μg mL^{-1}) at 37°C, 5% of CO_2 in air for 48h before MTT assay to access cell viability.[25] Cells not exposed to samples were used as control. Final DMSO concentration did not affect cell viability. IC_{50} (concentration reducing cell viability in 50%) was determined through non-linear regression analysis using software ORIGIN 7.5 (OriginLab Corporation).

Conclusion

Considering the data presented herein the chemotherapeutic potential of compounds **1**, **4** and **5** were determined as possible candidates of new agents with high selectivity for prostate cancer. Further *in vivo* studies and *in vitro* assays are needed to establish pharmacological mechanism, toxicity and production viability.

Acknowledgements

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Supplementary Information:

NMR spectral data of compounds **1**-**5** are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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Capítulo 2

Further investigation on the antineoplasic activity of compounds isolated from *Pterodon pubescens* Benth.: a preliminary *in vivo* study in Solid Ehrlich Carcinoma in mice.

Trabalho a ser submetido para publicação como complementação dos resultados apresentados no Capítulo 1

(sujeito a alterações na versão final)

Abstract

BACKGROUND: *Pterodon pubescens* Benth. crude alcoholic extract is used for pain relief, inflammation disorders, as tonic, and depurative preparations. Previous findings of our group demonstrated *in vitro* antiproliferative activity of compounds isolated from *P. pubescens* against several human tumor cell lines, with important selectivity for prostate cell lines (PC-03). The aim of this study was to evaluate the possible systemic antineoplasic activity attributed to compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) isolated from *P. pubescens,* using the established *in vivo* Ehrlich's Solid Carcinoma experimental model in mice paw. RESULTS: Compounds C1 and C2 demonstrated potential *in vivo* antineoplasic activity by intraperitoneal (i.p.) administration with different treatment schemes demonstrated by the measurement of tumor volume decrease throughout treatment. For scheme 1 both 30 mg/kg and 100 mg/kg doses were evaluated as follows: pre-treatment before cell inoculation with test samples, followed by treatment every three days with test samples. Under scheme 2, no pretreatment with test samples (30 mg/kg and 100 mg/kg) were employed beginning treatment after cell inoculation every three days. For scheme 3, only a 30 mg/kg test sample was administered daily after cell inoculation. All results were comparable to positive control group 5-Fluoruracil, a known chemotherapeutics agent. CONCLUSION: The present study showed the antineoplasic activity observed for compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) isolated from *P. pubescens* Benth. against the Ehrlich solid carcinoma in mice, corroborating the potential use of these compounds as future chemotherapy agents.

1. **Introduction**

Plants have a long history of use for cancer treatment. Hartwell (1982), in his review of plants used against cancer, lists more than 3000 plant species that have reportedly been used (Cragg et al, 2009). Cancer is responsible for 12% of the world's mortality and the second-leading cause of death in the western world. Limited chances for cure by chemotherapy are a major contributing factor to this situation. Despite much progress in recent years, a key problem in tumor chemotherapy with cytostatic compounds is the side effects and development of drug resistance. Most established drugs have low specificity toward tumor cells and therefore the identification of improved anti-tumor drugs is urgently needed (Effert et al, 2007)

Several approaches have been designed to search for novel antitumor compounds. Natural products such as marine organisms, terrestrial plants and animals are good sources for novel drugs. Prominent examples for the success of natural products originally obtained from plants are the *Vinca* alkaloids from *Catharanthus roseus* G. Don. (Apocynaceae), the DNA topoisomerase I inhibitor camptothecin from from *Camptotheca acuminate* Decne. (Nyssaceae), the terpene paclitaxel from *Taxus brevifollia* Nutt. (Taxaceae), or the lignin podophyllotoxin isolated from *Podophyllum peltatum* L. (Berberidaceae) (Effert et al, 2008).

Between the years of 1980s and 2002, 69% of new anticancer drugs approved were either natural products or developed based on knowledge from natural products. The long-lasting experience of traditional folk medicines may facilitate the identification of novel agents. Most of theses potentially useful plant products are substances from secondary metabolic pathways and most of them are protective agents against herbivores and pathogens or growth regulators (Newman and Cragg, 2007).

In this context, the Brazilian native genus *Pterodon* (used in folk medicine) which compromise four species [*P. abruptus* Benth., *P. apparucuri* Pedersoli, *P. pubescens* Benth. (*P. emarginatus* Vog.) and *P. polygalaeflorus* Benth.], has many

pharmacological activities such as cercaricidal, anti-microbial, anti-inflammatory, antinociceptive and antitumor activity. Phytochemical studies of *Pterodon* genus have revealed the presence of alkaloids, isoflavones and diterpenes. Furanoditerpenes identified and isolated from *Pterodon* have been reported as the major compounds responsible for several pharmacological actions of this species (Nunan et al, 1982; Duarte et al, 1996; Silva et al, 2004; Calixto et al, 2007).

We have previously reported the isolation of vouacapans from *P. pubescens* Benth through *in vitro* anticancer activity-guided fractionation. That work identified three compounds (6α-acetoxy-7β-hydroxyvouacapan, 6α,7β-dihydroxyvouacapan-17β-oate methyl ester and 6α,7β-dihydroxyvouacapan-17β-methylene-ol) with potential anticancer activity with high selectivity for prostate cancer (PC-3) (Spindola et al, 2009).

The present study, demonstrated for the first time the *in vivo* antineoplasic activity of 6α,7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol isolated from *P. pubescens* Benth., with Ehrlich Solid Carcinoma assay in mice paw. This experimental assay has been used as a transplantable tumor model to investigate the antineoplasic effects of several chemical compounds.

2. **Materials and Methods**

2.1. **Plant material**

Seeds were collected in São Paulo State (São Carlos city), in March 2006. Dr. Jorge Yoshio Tamashiro from IB-UNICAMP (Department of Botany) identified the plant specie. A voucher specimen was deposited at State University of Campinas (UEC) Herbarium, under number 1402.

2.2. **Compound isolation**

The isolation of compounds geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) was as previously reported (Spindola et al, 2009; 2010). Briefly, compounds C1 and C2 were obtained by successive column chromatography purifications from the crude dichloromethane extract produced from *P. pubescens* seeds.

2.3. **Animals**

Male Swiss and Balb-C mice with 25-35 g body weight were kept at 25 ± 2ºC in 12 h light-dark cycles (light phase started at 7:00 am) maintained in the laboratory (10 animals per cage) with water and food ad libitum, at least for 7 days prior to assays . Groups of eight mice were used and the studies were carried out in accordance with current guidelines for the veterinary care of laboratory animals [Voipio et al, 2008] and were performed under the consent and surveillance of Unicamp's Institute of Biology Ethics Committee for Animal Research (1076-1).

2.4. **Drugs**

Compounds C1, C2 and 5-fluoruracil (5-FU) were suspended in a vehicle made of saline solution (NaCl 0.9% in distilled water) and Tween^{80} 0.5%. The cells were suspended in phosphate buffer saline (PBS- pH 7.0).

2.5. ***In vivo*** **antineoplasic assay: Ehrlich solid carcinoma in mice paw**

The Ehrlich ascitic tumor (EAT), derived from a spontaneous murine mammary adenocarcinoma, was maintained in the ascitic form by sequential passages in *Swiss* mice, by means of weekly i.p. transplantations of 5×10^5 tumor cells, in order to prepare cells for the following test. The ascitic fluid was removed by opening the belly and carefully collecting all the fluid with the help of a sterile 3 mL syringe. Ascitic tumor cell counts were performed in a Neubauer hemocitometer, and the total number was determined by the Trypan blue dye exclusion method, with tumor cell viability always higher than 90%. The cells were then diluted in 0.9% phosphate buffer saline (PBS) for final inoculation density (2.5×10^6 cells/mL).

For the solid form implantation, 2.5×10^6 viable tumor cells in a volume of 60 µL were injected in sub-plantar site of the right hind paw of *Balb-C* mice (Kleeb et al, 1997). After tumor cell inoculation, the foot volume was measured every three days using a plethysmometer apparatus (Panlab, Spain) during fifteen days when the animals were sacrificed. The tumor growth was measured considering the following formula:

Volume measured – Basal volume = Tumor volume

2.5.1. **Treatment schedules and dosing**

The treatment schedules and doses used were determined during the standardization and validation of this model in our laboratory. For this purpose, five known chemotherapeutic agents were used (taxol, doxorrubicin, 5-fluoruracil, ciproterone and vincristine) in order to determine the best one to be used in the experiments (not shown). Compound 5-fluorouracil (5-FU) was selected as positive control considering the effectiveness from the beginning of the tumor growth until the end of the experiment. For this validation, 5-FU was administered in three different ways, as follows: one treatment (day 1) 75 mg/kg i.p.; two treatments (days 1 and 8) 50 mg/kg i.p.; and four treatments (days 1, 4, 7 and 10) 25 mg/kg i.p.

In order to investigate the potential antineoplasic *in vivo* activity of compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2), were both compared to 5-FU in the same test, and administered to animals in different doses (based on preliminary studies) and schedules, as follows:

i) *Compounds C1 and C2 given (i.p.) 48h and 24h (pre-treatment) before cell inoculation (Day 0) using 30 mg/kg and 100 mg/kg doses, administered every 3 days;*
ii) *Compounds C1 and C2 given (i.p.) on the day after inoculation (Day 1) using 30 mg/kg and 100 mg/kg doses, administered every 3 days;*
iii) *Compounds C1 and C2 given (i.p.) daily using 30 mg/kg doses, starting on the day after cell inoculation (Day 1).*
iv) Compound *5-FU given (i.p.) every 3 days using 20 mg/kg doses, starting on the day after inoculation (Day 1).*

2.6. **Statistical analysis**

All results were submitted to one way analysis of variance (ANOVA), considering as critical level $p \leq 0.05$ to evaluate significant difference between the control and treated groups, followed by Duncan´s Test , using StatSoft® software. Graphs were designed using the Origin® software.

3. **Results**

3.1. **Tumor growth curves for 5-FU**

Results presented in **fig.1** showed that the chemotherapeutic agent 5-Fluoruracil (5-FU) given 1 day (75 mg/kg, i.p.), 2 days (50 mg/kg, i.p.), and 4 days (25 mg/kg, i.p.) produced a significantly antineoplasic activity revealed by decreasing tumor volume compared to control group (vehicle). ANOVA revealed a significant difference among groups: Day 3 [$F_{(3,36)}$= 6.73, p ≤ 0.01]; Day 6 [$F_{(3,36)}$= 7.97, p ≤ 0.001]; Day 9 [$F_{(3,36)}$= 18.23, p ≤ 0.001]; Day 12 [$F_{(3,36)}$= 10.90, p ≤ 0.001]; Day 15 [$F_{(3,36)}$= 14.19, p ≤ 0.001].

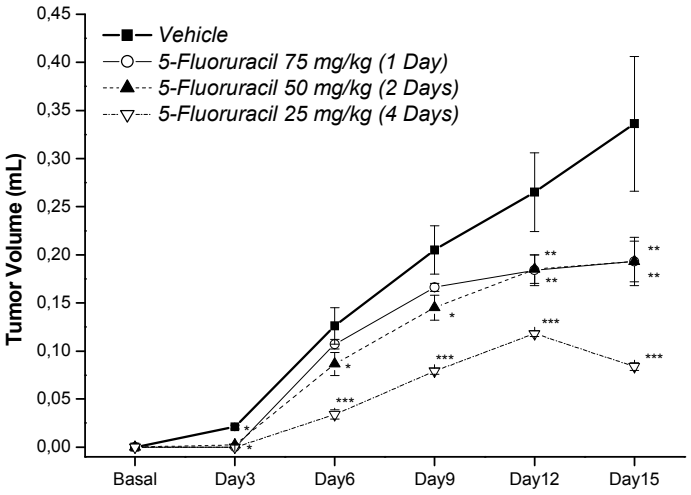

Figure 1: Graph demonstrating the chemotherapeutic effect of 5-Fluouracil (5-FU) against the Ehrlich solid carcinoma in mice paw. Compound 5-FU given by i.p. route either on day 1 (75 mg/kg), days 1, and 8 (50 mg/kg), or days 1, 4, 8 and 11 (25 mg/kg) during the 15 days of the experiment. Each point represents the means of 10 animals per group/day. The error bar indicates the S.E.M. $^{*}P$<0.05, $^{**}P$<0.01 and $^{***}P$<0.001 compared with control values (vehicle group).

3.2. **Tumor growth curves for compounds C1 and C2 compared to 5-FU**

The following results were obtained in the same experimental model, but, for best comprehension and discussion, we divided into four graphs depending on the compound tested and the treatment schedule. For each phase, ANOVA revealed a significant difference among groups, as follows: Day 3 [$F_{(9,70)}$= 2.36, $p \leq 0.05$]; Day 6 [$F_{(9,70)}$=7.02, $p \leq 0.001$]; Day 9 [$F_{(9,70)}$= 9.48, $p \leq 0.001$]; Day 12 [$F_{(9,70)}$= 8.77, $p \leq 0.001$]; Day 15 [$F_{(9,70)}$ = 9.74, $p \leq 0.001$].

3.2.1. **Effect of pre-treatment with compound C1:**

Results presented in **fig.2** showed that compound C1 given (i.p.) either on 30 mg/kg or 100 mg/kg doses, 48h and 24h before cell inoculation, and every 3 days during the experiment, produced a significantly antineoplasic activity decreasing tumor volume compared to control group (vehicle). Curves compared to the positive control 5-FU (20 mg/kg) given every 3 days.

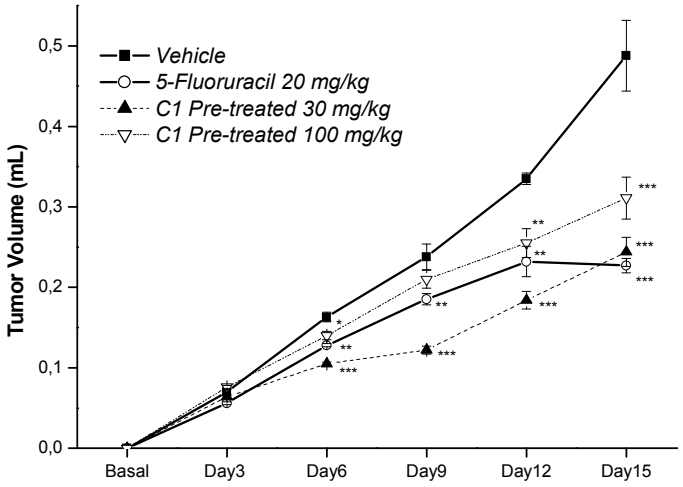

Figure 2: Graph demonstrating the chemotherapeutic effects of geranylgeraniol (C1) treated i.p. 48h and 24h prior to cell inoculation, administered every 3 days (30 mg/kg and 100 mg/kg, i.p.) against the Ehrlich solid carcinoma in mice paw.

Control group 5-FU given every 3 days (20 mg/kg, i.p.) during the 15 days of the experiment. Each point represents the means of 8 to 10 animals per group/day. The error bar indicates S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with control values (vehicle group).

3.2.2. **Effect of pre-treatment with compound C2:**

Results presented in **fig.3** showed that compound C2 given (i.p.) either on 30 mg/kg or 100 mg/kg doses, 48h and 24h before cell inoculation and every 3 days during the experiment, produced a significantly antineoplasic activity decreasing tumor volume compared to control group (vehicle). Curves compared to the positive control 5-FU (20 mg/kg) given every 3 days.

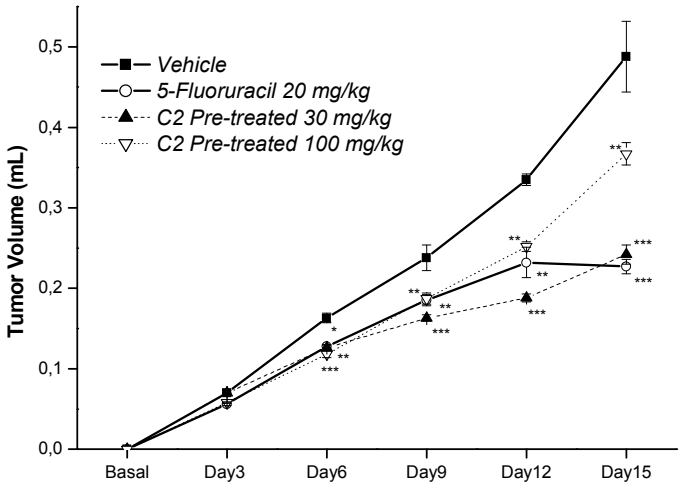

Figure 3: Graph demonstrating the chemotherapeutic effects of 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) treated i.p. 48h and 24h prior to cell inoculation, administered every 3 days (30 mg/kg and 100 mg/kg) against the Ehrlich solid carcinoma in mice paw. Control group 5-FU given every 3 days (20 mg/kg, i.p.) during the 15 days of the experiment. Each point represents the means of 8 to 10 animals per group/day. The error bar indicates S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with control values (vehicle group).

3.2.3. **Effect of compounds C1 and C2 (100 mg/kg) given every 3 days**

Results presented in **fig.4** showed that compounds C1 and C2 given (i.p.) in 100 mg/kg doses, every 3 days during the experiment, produced a significantly antineoplasic activity decreasing tumor volume compared to control group (vehicle). Curves compared to the positive control 5-FU (20 mg/kg) given every 3 days.

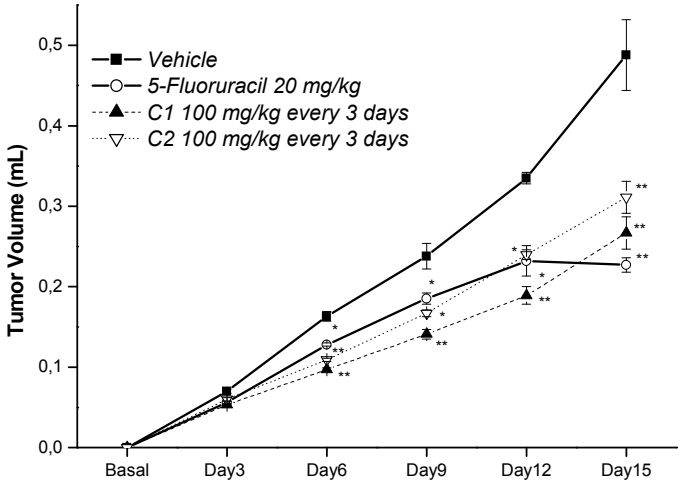

Figure 4: Graph demonstrating the chemotherapeutic effects of geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) treated every 3 days (100 mg/kg, i.p.) against the Ehrlich solid carcinoma in mice paw. Control group 5-FU given every 3 days (20 mg/kg, i.p.) during the 15 days of the experiment. Each point represents the means of 8 to 10 animals per group/day. The error bar indicates S.E.M. $^{*}P<0.01$, $^{**}P<0.001$ compared with control values (vehicle group).

3.2.4. **Effect of compounds C1 and C2 given Daily 30 mg/kg**

Results presented in **fig.5** showed that compounds C1 and C2 given daily (i.p.) in 30 mg/kg doses during the experiment, produced a significantly antineoplasic activity decreasing tumor volume compared to control group (vehicle). Curves compared to the positive control 5-FU (20 mg/kg) given every 3 days.

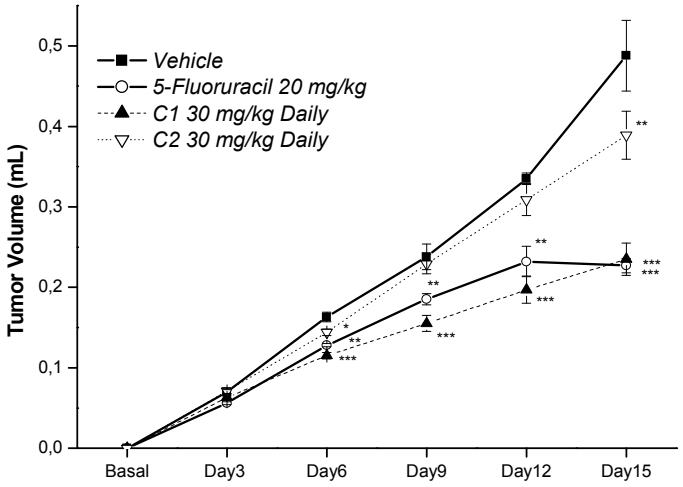

Figure 5: Graph demonstrating the chemotherapeutic effects of geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) treated daily (30 mg/kg, i.p.) against the Ehrlich solid carcinoma in mice paw. Control group 5-FU given every 3 days (20 mg/kg, i.p.) during the 15 days of the experiment. Each point represents the means of 8 to 10 animals per group/day. The error bar indicates S.E.M. $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.001$ compared with control values (vehicle group).

4. **Discussion**

Further evaluation of the antineoplasic potential of natural products identified from *in vitro* assays, requires the use of *in vivo* assays. We previously reported (Spindola et al, 2009) the *in vitro* antiproliferative activity against tumor cells attributed to Furanoditerpenes obtained from *P. pubescens.* Herein we report the potential *in vivo* antineoplasic activity for some of these compounds, in order to evaluate if the systemic administration is capable of maintaining the antitumor activity.

There are too many levels in the cell cycle that drugs may act for antineoplasic activity, such as: tubulin interactive agents (e.g. taxanes), inhibitor of topoisomerase I and II (e.g. campthotecin), inhibitors of histone deacetylases (e.g. trichostatin A), protein kinase inhibitors (e.g. flavopiridol), inhibitors of heat shock protein 90 (e.g. geldanamycin), proteasome inhibitors (e.g. dipeptidyl peptide baronate), DNA interactive agents- non-topoisomerase I and II inhibitors (e.g. ecteinascidin), agents that activate caspase and/or induce apoptosis (e.g. naphtoquinone β- lapachol), and inhibitors of hypoxia inducible factors (Cragg et al, 2009).

The systemic antineoplasic activity was evaluated using the Ehrlich solid carcinoma in mice. Ehrlich tumor is a rapidly growing carcinoma with very aggressive behavior (Segura et al, 2000). This tumor is able to grow in almost all mice strains which suggest that the recognition and immune responses to this tumor are independent of major histocompatibility complex (MHC) (Chen and Watikins, 1970). This characteristic suggests that the controlling of Ehrlich tumor is related more with innate immunity, specially the inflammatory response, than with T cell responses.

Previous reports have demonstrated that the neutrophilic inflammatory response is essential for controlling Ehrlich tumor. However, the high influx of these cells promotes tumor development (Bergami-Santos et al, 2004). This effect is probably related with the angiogenesis and growing factors induced by

inflammation that are necessary for tumor development. The Ehrlich ascitic tumor implantation induces per se a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascitic fluid formation (Fecchio et al, 1990). The ascitic fluid is essential for tumor growing since this fluid constitutes the direct nutritional source for tumor cells (Gupta et al, 2004).

To investigate if the antiproliferative activity of compounds C1 and C2 had a straight relationship with direct tumor cells contact (*in vitro*), the Ehrlich tumor cells were inoculated in the footpad of mice, and compounds were administered by i.p. route, in order to evaluate the ability to decrease tumor growth. Some reports used the ascite Ehrlich carcinoma to evaluate antineoplasic activity of drugs (Nascimento et al, 2006), however the solid form was chosen considering the shorter experimental period requires, minimizing animals suffering. Results reported in the present study showed that the antineoplasic activity of compounds C1 and C2 were maintained using different doses and treatment schemes. One interesting finding was the evaluation that the pre- treatment, two consecutive days prior to cell inoculation, with test samples, did not show a better effect comparing to the post cell inoculation treatment, suggesting that compounds may have either a cytotoxic activity directly on the cells, or acting indirectly by angiogenesis inhibition. However, increasing doses of test compounds did not demonstrate a better activity, suggesting that higher doses for both compounds might be toxic, inducing less activity for immune system. This hypothesis may be confirmed by the fact that the daily treatment using low doses for both compounds produced less activity comparing to other groups. Whereas, the higher doses given every three days demonstrated effectiveness against tumor growth. These results were comparable with those to the known chemotherapy agent 5-Fluoruracil, an anti-metabolic compound which acts on RNA polymerase (Hill, 1970).

Some reports mentioned that terpenes may posses antioxidant activity, and consequently involved in anti-tumor activities (Jiau-Jian and Larry, 1997). The Ehrlich tumor growth induces an inhibition of superoxide dismutase and catalase

enzymes (Sun et al, 1989) which are fundamental in the elimination of free radicals as superoxide and hydrogen peroxide (Rushmore and Picket, 1993). In Ehrlich tumor-bearing mice the antioxidant acts by a mechanism that involves modulating lipid peroxidation and augmenting antioxidant defense system (Gupta et al., 2004b). Whereas, previous reports have shown the anti-inflammatory activity of compounds extracted from *P. pubescens,* also mentioning mechanisms involved directly over inhibition of inflammation components such as Cox-1, Cox-2, and prostaglandins (Calixto et al, 2007).

Inflammation is a physiologic process in response to tissue damage, whether resulting from physical injury, ischemic injury, infection, exposure to toxins, or other types of trauma. The relationship between inflammation and cancer is highly complex. In 1863, Virchow hypothesized that the origin of cancer was at sites of chronic inflammation. Virchow's hypothesis was based on the presumption that some classes of irritants, together with the tissue injury and ensuing inflammation they cause, enhance cell proliferation (Coussens and Werb, 2002). Although reports have shown that proliferation of cells alone do not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA damage–promoting agents certainly potentiates and/ or promotes neoplasic risk (Lu et al, 2006; Phillip et al, 2004). Now hypothesis have been made that the development of cancer from inflammation might be a process driven by inflammatory cells as well as a variety of mediators, including cytokines, chemokines, and enzymes, which altogether establish the inflammatory microenvironment (Nathan, 2002; Hanahan and Weinberg, 2000).

The exact mechanism by which geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β–oate (C2) methyl ester mediates their *in vivo* antineoplasic effect is unknown. However the mechanism seems not to be involved with antioxidant activity, but rather, with anti-inflammatory and caspase induction (C1) or anti-angiogenic, and anti-androgenic activity (C2). This suggestion derives from the fact that compound C1 has demonstrated to posses pronounced anti-inflammatory activity (Silva 2004) and induction of caspase (inducing apoptosis),

and compound C2 demonstrated activity against prostate tumor proliferation (Spindola et al, 2009). Further investigation will be undertaken to evaluate the exact mechanisms by which compounds geranylgeraniol and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester acts on *in vivo* antineoplasic activity.

5. **Conclusion**

The present study showed the antineoplasic activity observed for compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) isolated from *Pterodon pubescens* Benth. against the Ehrlich solid carcinoma in mice, corroborating the potential use of these compounds as future chemotherapy agents.

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Capítulo 3

Antinociceptive effect of geranylgeraniol and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester isolated from *Pterodon pubescens* Benth

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Abstract

BACKGROUND: *Pterodon pubescens* Benth seeds are commercially available in the Brazilian medicinal plant street market. The crude alcoholic extracts of this plant are used in folk medicine as anti-inflammatory, analgesic, and anti-rheumatic preparations. The aim of this study was to evaluate the contribution of geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) isolated from *Pterodon pubescens* Benth. to the antinociceptive activity of the crude extract. RESULTS: Compounds C1 and C2 demonstrated activity against writhing with intraperitoneal (i.p.) and oral (p.o.) routes, capsaicin (i.p. and p.o.), glutamate (i.p.), and in the hot-plate (p.o.) tests, demonstrating their contribution to the antinociceptive activity of crude *Pterodon pubescens* Benth extracts. The observed activity of compounds C1 and C2 may be related to vanilloid receptors VR1, and/or glutamate peripheral receptors. In hot-plate model, the antinociceptive activity was maintained when naloxone chloride (opioid antagonist) was administered prior to treatment with compounds suggesting that C1 and C2 (p.o.) do not exert their antinociceptive effects in the hot-plate test via opioid receptors. The findings presented herein also suggest that compounds within the crude *Pterodon pubescens* Benth. extract may exert a synergistic interactive effect, since the crude extract (300 $mg.kg^{-1}$) containing lower concentrations of compounds C1 (11.5%- 34.6 $mg.\ kg^{-1}$) and C2 (1.5% - 4.7 $mg.kg^{-1}$) gave statistically the same effect to the pure compounds when tested separately (C1=C2=300 $mg.kg^{-1}$) in writhing experimental model with p.o. administration. Further studies will be undertaken to establish more specifically the mechanisms of action for compounds C1 and C2. Possible synergistic interactions will be evaluated employing the Isobole method. CONCLUSION: These results allowed us to establish a relationship between the popular use of *Pterodon pubescens* seeds for pain relief and the activity of two major compounds isolated from this species which demonstrated antinociceptive activity. Various "*in vivo*" experimental models corroborate the folk use of this species for different pain and inflammation disorders.

1. **Background**

Pterodon pubescens Benth. (Leguminosae), known as sucupira, is widespread throughout the Brazilian states of Goiás, Minas Gerais and São Paulo. Sucupira seeds are commercially available in the Brazilian medicinal plant market. The crude alcoholic extracts of this plant are used in folk medicine as anti-inflammatory, analgesic and anti-rheumatic preparations [1,2]. Phytochemical studies of the *Pterodon* genus have shown the presence of alkaloids, isoflavones and diterpenes. Furanoditerpenes were identified and isolated from *Pterodon* fruits [3,4,5,6,7]. Studies have suggested that furanoditerpenes possessing the vouacapan skeleton contribute to the anti-inflammatory and antinociceptive properties of *Pterodon pubescens* seed oil [8,9,10,11,12]. Diterpenes 6α-hydroxyvouacapan-7β-17β-lactone and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester, found in *P. emarginatus* and *P. polygalaeflorus* seeds were previously reported to be associated with the anti-inflammatory activity of these species [8]. Herein we report the antinociceptive activity of 6α,7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol isolated from *Pterodon pubescens* Benth. when evaluated in writhing, capsaicin, glutamate and hot-plate animal experimental models.

2. Results and Discussion

Some authors have reported the antinociceptive activity of the crude extract and fractions obtained from *P. pubescens* and established a relationship with anti-inflammatory activity [10, 12]. This report evaluated for the first time the contribution of geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2), isolated from *P. pubescens* (**Fig.1**), to the antinociceptive activity using various experimental models to evaluate a distinct pain modulation.

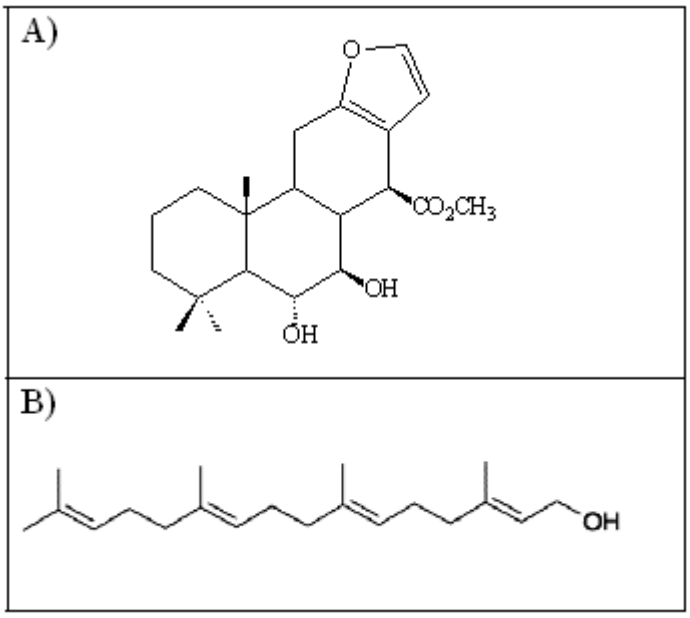

Figure 1: Chemical structures of compounds A) 6α, 7β-dihydroxyvouacapan-17β–oate methyl ester (C2), and B) geranylgeraniol (C1).

Calixto et al [13] demonstrated the antiplatelet activity of geranylgeraniol attributed to cyclooxygenase enzyme inhibition, but did not mention data on antinociceptive activity. Some vouacapan compounds have been suggested to have a relationship with *P. pubescens´* antinociceptive activity. Nevertheless pain modulation by this species has never been reported, being described herein for the first time. The following screening results demonstrated the activity and general mechanisms involved in antinociception caused by geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2).

The most relevant additional findings of the present work are that, (i) compounds C1 and C2 may present synergistic activity; (ii) both intraperitoneal (i.p.) and oral (p.o.) treatment of compounds C1 and C2 reduced reactivity to the writhing test demonstrating differences in potency related to the route of administration; (iii) both compounds C1 and C2 demonstrated possible activity related to vanilloid receptors and/or glutamate peripheral receptors, with C2 being more potent by the i.p. route; (iv) the antinociceptive activity of compounds C1 and C2 (p.o.) do not appear to exert their antinociceptive effects in the hot-plate test via opioid receptors.

Initially, the open field test was performed in order to exclude the possibility that the antinociceptive action of geranylgeraniol (C1) and 6α, 7β-dihydroxyvouacapan-17β-oate methyl ester (C2) could be related to non-specific disturbances in the locomotor activity of the animals. Treatment with compounds C1 and C2 (30 $mg.kg^{-1}$, i.p.) did not cause any significant change in the ambulation of mice when tested in the open field. However, pentobarbital (35 $mg.kg^{-1}$, i.p.) significantly ($p < 0.001$) reduced the locomotor activity of animals in this test. The mean number of crossings was 48.2 ± 2.2, 13.7 ± 6.1, 50 ± 4.4 and 42 ± 3.6 for vehicle, pentobarbital, C1 and C2, respectively.

After these results, the acetic acid writhing test model was employed. This is a convenient stimulus assay for screening, because the intensity of response depends on the interaction of several factors, neurotransmitters and neuromodulators that determine nociception, such as kinines, acetylcholine, substance P and prostaglandins. Therefore, this model is responsive to analgesic substances possessing with the most varied action mechanisms [14,15], being sensitive to drugs with analgesic activity such as aspirin, kinin receptor antagonists (bradykinin, kallidin or T-kinin) and opioid analgesics with central or peripheral action [16,17]. This model permitted evaluation of antinociceptive activity caused by both neurogenic and/or inflammatory pain. This assay was used during the initial studies with compounds C1 and C2 in order to establish differences in potency using different routes of administration.

The antinociceptive activity of compounds C1 and C2 were compared with the dichloromethane extract (EB) (p.o.) in the writhing test using 300 $mg.kg^{-1}$ doses. The reductions in the number of abdominal constrictions were 62%, 64% and 66% for EB, C1 and C2 respectively ($p \leq 0.001$) (not shown). These results suggested a possible synergistic activity among compounds C1 and C2 [these compounds are only at concentration: C1 (11.6%- 34.6 mg. kg^{-1}) and C2 (1.6% - 4.7 $mg.kg^{-1}$) in the EB fraction]. Accordingly, dose-response curves for i.p. and p.o. administered compounds were determined in the writhing test to calculate ED_{50} values and possible differences caused by administration routes.

The p.o. administration of compound C1 showed dose-related activity reducing by 51%, 57%, and 75% ($p \leq 0.01$) the abdominal constrictions with 30 $mg.kg^{-1}$, 100 $mg.kg^{-1}$, and 300 $mg.kg^{-1}$ doses respectively, presenting ED_{50}= 26.7 $mg.kg^{-1}$ (**Fig.2**).

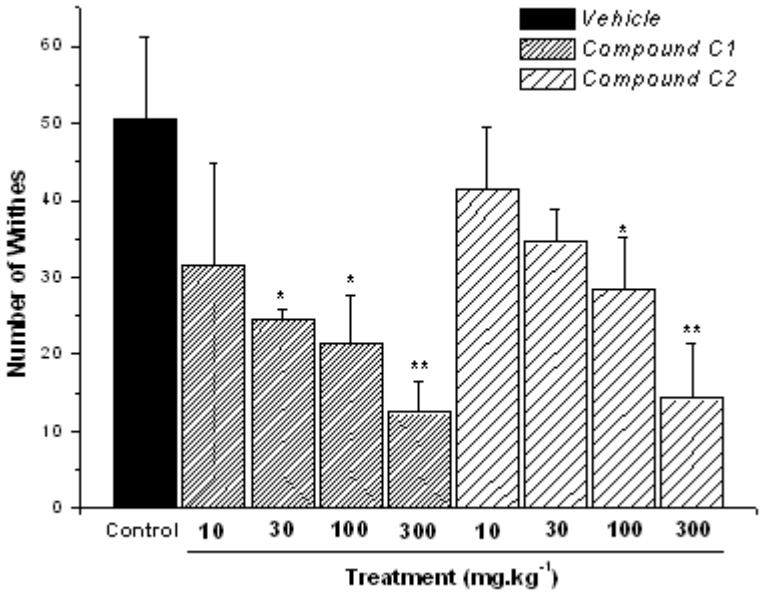

Figure 2: Abdominal constrictions induced by acetic acid (0.8% in saline) in mice previously administered (60 min) p.o. with control vehicle (10 $mL.kg^{-1}$), or compounds C1 and C2 (dose-response). Results expressed as mean ± SEM of up to 8 animals for experimental groups (*$p \leq 0.05$; **$p \leq 0.01$).

The same treatment with compound C2 reduced constrictions by 43% (100 $mg.kg^{-1}$) and 71% (300 $mg.kg^{-1}$) with ED_{50}= 35.6 $mg.kg^{-1}$. These results showed that compound C1 was more potent than C2 when given by p.o. administration. The dose-related activity for compounds administered i.p. (systemic route) in the writhing test, demonstrated that compound C1 reduced constrictions by 58% and 98% ($p \leq 0.001$) with 100 $mg.kg^{-1}$ and 300 $mg.kg^{-1}$ doses respectively (ED_{50}= 22.4 $mg.kg^{-1}$) (**Fig.3**). Compound C2 reduced constrictions by 84%, 90%, and 98% with 30 $mg.kg^{-1}$, 100 $mg.kg^{-1}$, and 300 $mg.kg^{-1}$ doses respectively, showing ED_{50}= 11.5 $mg.kg^{-1}$, more potent than C1. In the i.p. tests, the numbers of abdominal constrictions of the control group were fewer compared to the control group of the p.o. route, maybe caused by injection-stress of the animals.

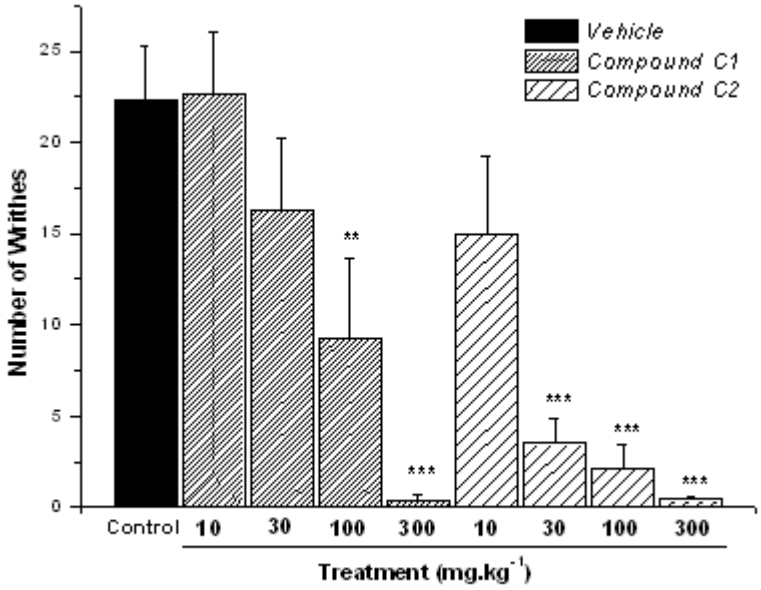

Figure 3: Abdominal constrictions induced by acetic acid (0.8% in saline) in mice previously administered (30 min) i.p. with control vehicle (10 $mL.kg^{-1}$), or compounds C1 and C2 (dose-response). Results expressed as mean ± SEM of up to 8 animals for experimental groups (**$p \leq 0.01$; ***$p \leq 0.001$).

In order to evaluate the antinociceptive response of compounds C1 and C2 in neurogenic pain caused by direct chemical stimulation on nociceptors, the capsaicin test was evaluated. The active principle, Capsaicin, from *Capsicum* genus peppers, is the painful substance used to determine if compounds possessing antinociceptive activity act by the vanilloid VR1 receptors. The animal response is caused by release of neuropeptides, such as substance P, neurokinines, somastotastin, peptide related to the calcitonin gene (CGRP) with participation of nociceptive afferent C fibers, and in part, A fibers [18,19]. The induced nociceptive process with capsaicin is related to the activation of the tachykininergic system and seems to be mediated by activation of a specific receptor, whose presence has been confirmed with capsazepine, a competitive vanilloid antagonist. Evidence also suggests that bradykinin (BK) acting through B_2 receptors seems to be involved in the neurogenic nociception caused by capsaicin in mice [20].

In this test, i.p. administration of compound C1 reduced by 43%, 48%, 58%, and 67% ($p \leq 0.01$) with 10 $mg.kg^{-1}$, 30 $mg.kg^{-1}$, 100 $mg.kg^{-1}$, and 300$mg.kg^{-1}$ doses respectively (ED_{50}= 34.1 $mg.kg^{-1}$) (**Fig.4**).

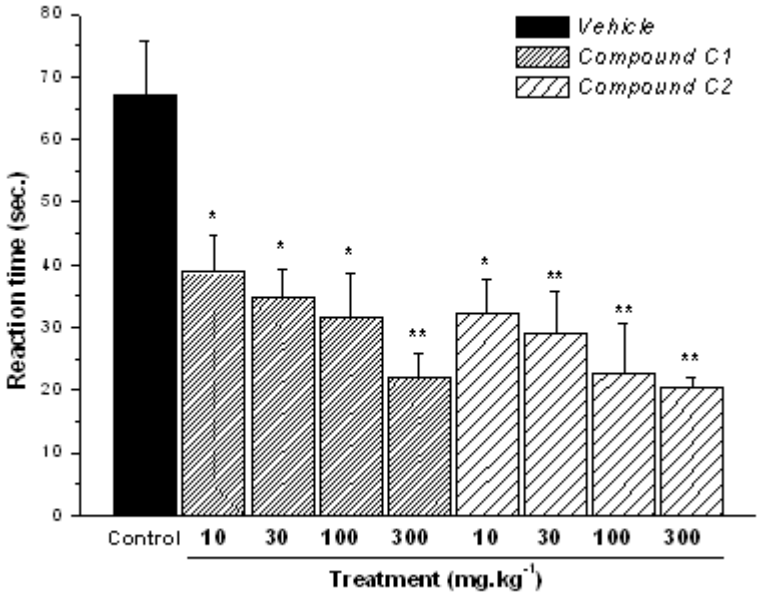

Figure 4: Reactivity time to the intraplantar application of capsaicin (1.6 µg.paw^{-1}) in the hind paw of mice previously treated (30 min) i.p. with control vehicle (10 mL.kg^{-1}), or compounds C1 and C2 (dose-response curve). Results expressed as mean ± SEM of up to 8 animals for experimental groups (* $p \leq 0.05$; ** $p \leq 0.01$).

Compound C2 showed dose-related activity too, reducing by 51%, 56%, 66%, and 69% ($p \leq 0.01$) the reaction time, with 10 mg.kg^{-1}, 30 mg.kg^{-1}, 100 mg.kg^{-1}, and 300 mg.kg^{-1} doses respectively (ED_{50}= 27.8 mg.kg^{-1}). These results showed that the antinociceptive activity of compounds C1 and C2 may be related to the tachykininergic system or vanilloid VR1 receptors. The same experimental model was tested with p.o. administration of compounds C1 (300 mg.kg^{-1}) and C2 (300 mg.kg^{-1}) employing morphine (20 mg.kg^{-1}) as positive control, in order to evaluate the activity of compounds on this route (**Fig.5**). Compound C2 reduced reaction time by 82% whereas C1 reduced by 65%, with the positive control demonstrating reduction by 52% ($p \leq 0.001$). This assay demonstrated that compound C2 was more potent in neurogenic pain modulated by vanilloid receptors than compound C1, corroborating the previous result with intraperitoneal administration.

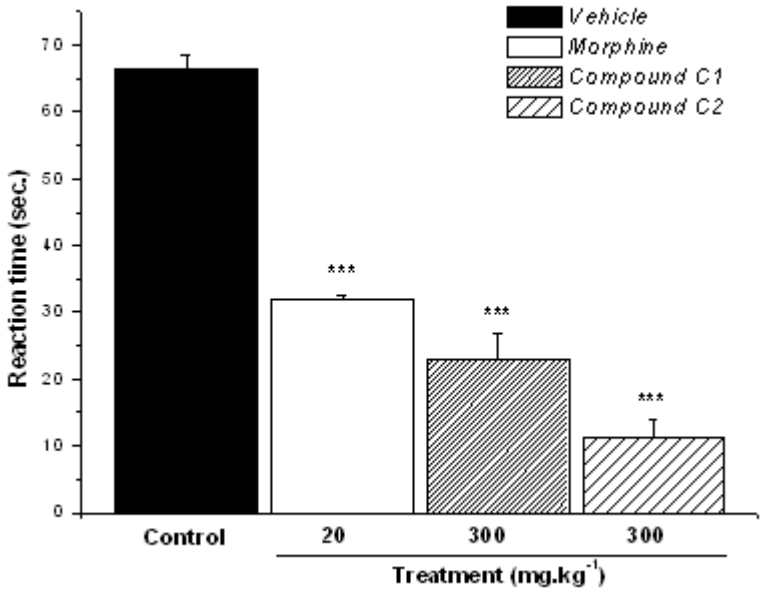

Figure 5: Reactivity time to the intraplantar application of capsaicin (1.6 µg.paw^{-1}) in the hind paw of mice previously treated (30 min) p.o. with control vehicle (10 mL.kg^{-1}), morphine (20 mg.kg^{-1}), or compounds C1 and C2 (300 mg.kg^{-1}). Results expressed as mean ± SEM of up to 8 animals for experimental groups (***p ≤ 0.001).

Once we had demonstrated the activity of compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) with the writhing and capsaicin tests, we considered the glutamate test. This model is based on the activation of peripheral glutamate receptors responsible for several types of pain sensation. The nociceptive response induced by glutamate appears to involve peripheral, spinal and supraspinal sites of action and is mediated by both NMDA and non-NMDA receptors as well as by the release of nitric oxide or by some nitric oxide-related substances. Nitric oxide inhibitors and both NMDA and non-NMDA receptor antagonists, and other drugs have been previously reported to inhibit the acetic-acid and capsaicin- induced nociceptive response [21].

Our results showed that i.p. administration of compounds C1 and C2 produced a significant dose-related inhibition of the nociceptive response caused

by intraplantar injection of glutamate into the mouse´s hind paw. In this test, the p.o. route was not evaluated because of possible changes of pharmacokinetic, metabolic or distribution parameters, which could disrupt the interaction among compounds and receptors (considering the wide receptors distribution). Compound C1 (i.p.), with 10 $mg.kg^{-1}$, 30 $mg.kg^{-1}$, 100 $mg.kg^{-1}$, and 300 $mg.kg^{-1}$ doses reduced reaction time by 67%, 72%, 75%, and 80% respectively ($p \leq 0.001$), corresponding to on ED_{50}= 57.4 $mg.kg^{-1}$ (**Fig.6**).

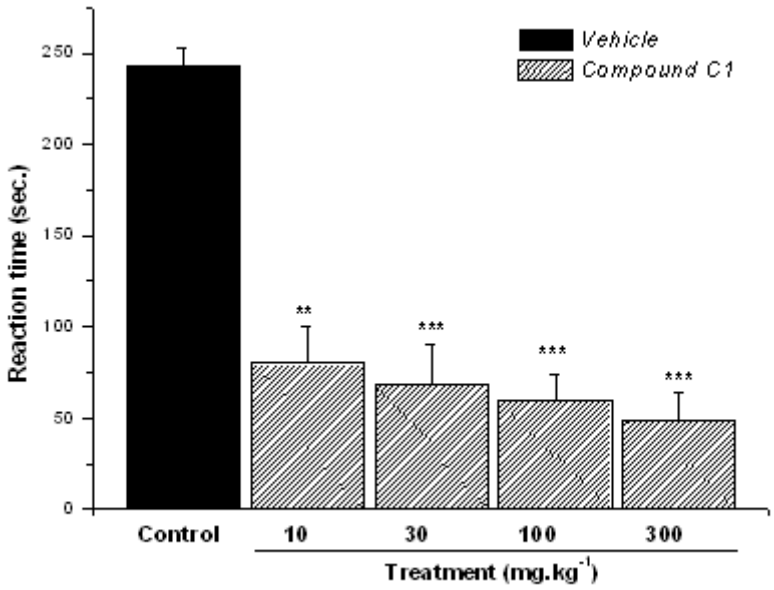

Figure 6: Reactivity time to the intraplantar application of glutamate (10 µmol.paw^{-1}) in the hind paw of mice previously treated (30 min) i.p. with control vehicle (10 $mL.kg^{-1}$) or compound C1 (dose-response curve). Results expressed as mean ± SEM of up to 8 animals for experimental groups (**$p \leq 0.01$ ***$p \leq 0.001$).

Compound C2 (i.p.) showed ED_{50}= 35.1 $mg.kg^{-1}$, reducing by 62%, 83%, and 96% the reaction time with 30 $mg.kg^{-1}$, 100 $mg.kg^{-1}$, and 300 $mg.kg^{-1}$ doses ($p \leq 0.001$) respectively (**Fig.7**), again more potent than C1 in peripheral neurogenic modulation. Thus, these previous findings and the present results suggested that the antinociceptive action of compounds C1 and C2 in the acetic acid, capsaicin, and glutamate tests could be the result of both the inhibition of NOS and the

blockade of glutamate receptors. Further studies are being undertaken to confirm receptors involved on this activity.

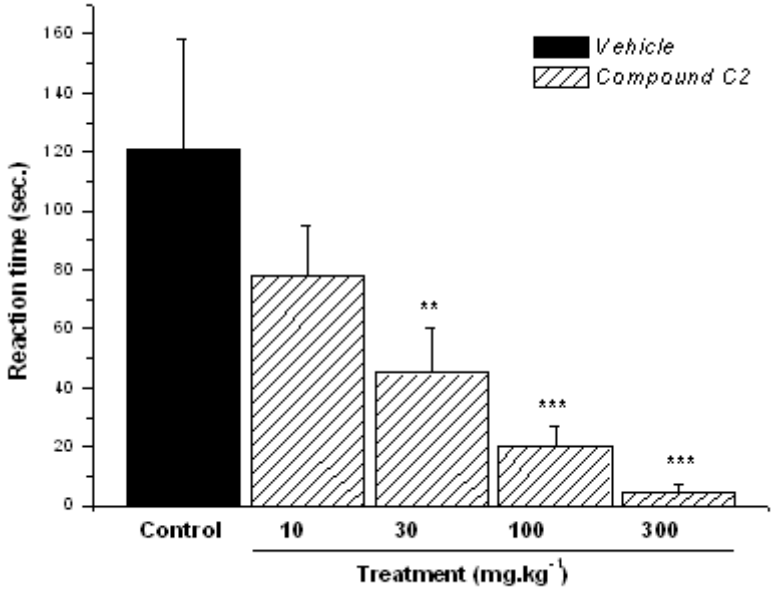

Figure 7: Reactivity time to the intraplantar application of glutamate (10 µmol.paw^{-1}) in the hind paw of mice previously treated (30 min) i.p. with control vehicle (10 mL.kg^{-1}) or compound C2 (dose-response curve). Results expressed as mean ± SEM of up to 8 animals for experimental groups (**p ≤ 0.01 ***p ≤ 0.001).

Compounds C1 and C2 were evaluated in the hot-plate thermal nociception model by p.o. route. This route was chosen based on preliminary set of experiments which showed to induce less stress to animals. The hot-plate test is a neurogenic-modulated model that produces, at constant temperature, two kinds of behavioral response, which are paw licking and jumping. Both of these are considered to be supraspinally- integrated responses [22]. In order to evaluate the antinociceptive activity of compounds C1 and C2 mediated by opioid receptors, the hot plate test was carried out using the opioid antagonist naloxone hydrochloride. The p.o. doses for compounds C1 and C2 were chosen based on preliminary studies in our laboratory. Pre-treatment with naloxone (1 mg.kg^{-1}), did not reverse the antinociceptive effect of both compounds when the animals were treated p.o.

with 100 mg.kg^{-1} doses ($p \leq 0.001$), especially at 60 minutes after challenge, suggesting that these compounds do not possess similar action to morphine or derivatives when evaluated in this assay (**Table 1**).

Table 1: Time elapsed (sec.) for nociceptive response in Hot Plate Test in mice related to the time of experiment.

Treatment	Time elapsed after treatment (means ± SD) / Increase in response time compared to control (%)									
	N	0 min	30 min		60 min		90 min		120 min	
Control	7	3.4 ± 1.2	3.4 ± 1.5	NS	5.2 ± 0.92	NS	7.04 ± 0.75	NS	6.0 ± 0.95	NS
Morphine 20mg.kg^{-1}	6	2.07 ± 0.65	6.9 ± 0.76	**203%****	8.15 ± 1.52	**163.5%***	13.35 ± 4.05	**189.6%****	6.8 ± 2.11	NS
Naloxone 1mg.kg^{1}/control	6	3.0 ± 0.39	4.72 ± 0.77	NS	5.3 ± 0.7	NS	6.25 ± 1.23	NS	6.65 ± 0.9	NS
Naloxone 1mg.kg^{1} /Morphine20 mg.kg^{1}	8	3.6 ± 1.12	4.81 ± 1.42	NS	7.07 ± 2.23	NS	5.2 ± 1.53	NS	6.0 ± 1.27	NS
C2 100 mg.kg^{-1}	8	3.5 ± 1.0	4.88 ± 2.21	NS	12.35 ± 1.24	**237.5%****	11.5 ± 2.77	**163.4%****	7.38 ± 0.76	NS
Naloxone1mg.kg^{1} / C2 100 mg.kg^{1}	7	3.4 ± 1.2	4.21 ± 1.8	NS	11.5 ± 2.11	**221.2%****	9.41 ± 2.69	NS	6.65 ± 2.8	NS
C1 100 mg.kg^{-1}	8	3.1 ± 1.2	6.16 ± 2.0	**179%****	10.66 ± 2.13	**205%****	8.98 ± 1.48	NS	9.2 ± 2.4	**153.3%****
Naloxone1mg.kg^{1} / C1 100 mg.kg^{1}	8	3.5 ± 1.3	5.0 ± 1.68	NS	13.42 ± 2.24	**258%****	12.06 ± 2.27	**171.3%****	6.7 ± 2.12	NS

Statistical Analysis ANOVA

DUNCAN´S Test: *p<0.05; **p<0.01

NS: no significant result

Morphine, Naloxone, C1, C2 and vehicle given orally.

3. Conclusion

Both i.p. and p.o. treatment with compounds C1 and C2 reduced response in the writhing, Capsaicin, Glutamate, and hot-plate tests demonstrating their contribution to the antinociceptive activity of crude *Pterodon pubescens* Benth extracts.

Compounds, C1 and C2, demonstrated activity in models demonstrative of vanilloid receptors and glutamate peripheral receptors. In the hot-plate model, the antinociceptive activity was maintained when naloxone hydrochloride, an opioid antagonist, was administered prior to sample dosing suggesting that compounds C1 and C2 do not exert their antinociceptive effects in the hot-plate test via opioid receptors.

The findings presented herein also suggested that crude *Pterodon pubescens* Benth extract probably is a mixture of substances with synergistic interactive effect, since the crude extract (300 $mg.kg^{-1}$) with lower concentrations of compounds C1 (11.6%- 34.6 mg. kg^{-1}) and C2 (1.6% - 4.7 $mg.kg^{-1}$) presented statistically analogous effects to those of the pure compounds when tested separately (C1=C2=300 $mg.kg^{-1}$) with p.o. administration in the writhing experimental model.

Further studies will be undertaken to establish the mechanisms of action for compounds C1 and C2. The synergistic interactions will be evaluated employing the Isobole method [23].

4. Methods

4.1. *Plant Material*

Seeds were collected in Pedregulho (SP) and São Carlos (SP) cities, in March 2004. Prof. Dr. Jorge Yoshio Tamashiro from IB-UNICAMP (Department of Botany) identified the plant species. A voucher specimen was deposited in the State University of Campinas (UEC) Herbarium, under number 1398.

4.2. *Compound Isolation*

Freeze-dried Seeds (100 g) were ground prior to use in a Stephen mill (model UM 40) and extracted with dichloromethane at room temperature yielding 32% extractable. A portion of the crude dichloromethane extract (32 g) was chromatographed over silica gel (192 g) and eluted sequentially with hexane [F1] (3x 150 ml); hexane/ethyl acetate 5% [F2] (3 x 150 mL); hexane/ethyl acetate 15% [F3] (3 x 150 mL); ethyl acetate (3 x 150 mL) [F4]; methanol (3 x 150 mL) [F5]. The crude extract fractions were monitored by thin layer chromatography (TLC), visualized with anisaldehyde reagent (50 mL acetic acid, 0.5 mL sulfuric acid and 0.5 mL anisaldehyde) followed by heating at 110^{o}C. Similar fractions were grouped according to their thin layer chromatography profile. Antinociceptive assay indicated that fractions F3 and F4 (10 g) showed activity and they were successively chromatographed by CC on silica-gel (70-230 mesh) (5 x 60 cm) providing 6α,7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol with spectral data (FTIR, ^{1}H and ^{13}C NMR data) in accordance with those reported previously [7].

4.3. *Chromatographic analysis*

The GC/MS analysis was carried out using a HP-6890/5975 system equipped with a J&W Scientific DB-5 fused capillary column (25 m x 0.2 mm x 0.33 m). Temperature program: 60°C (5^{o}C min^{-1}) – 300°C (10 min.), injector 250°C, detector 300°C. Helium was used as carrier gas (0.7 bar, 1 ml min^{-1}). The MS were taken at 70eV. Scanning speed was 0.84 scans s^{-1}, from 40 to 550. Sample volume was 1 µl. Split: 1:40.

Quantification of 6α,7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol in crude *P. pubescens* extract were obtained by internal standard method [24] using butylphtalate as internal standard with authentic 6α, 7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol samples. Analysis determined 1.6% of 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) and 11.6% of geranylgeraniol (C1).

4.4. *High-resolution eletrospray ionization mass spectroscopy (HRESI-MS)*

HRESI-MS was recorded on a Q-Tof Mass Spectrometer (Micromass – U.K.) using direct infusion of a 10 µL.min^{-1} MeOH + 0,1 % formic acid solution and ionization by electrospray in the positive ion mode. Major operation conditions were as follow: capillary voltage of 3.5 kV, source temperature of 100 °C, desolvation temperature of 100 °C and cone voltage of 35 V.

4.5. *Animals*

Male Swiss mice with 25-35 g body weight were kept at 25 ± 2ºC in 12 h light-dark cycles (light phase started at 7:00 am) maintained (10 animals per cage) with water and food ad libitum, at least for 7 days prior to assays . Animals were fasted 12 hours prior to oral administration of compounds, in order to avoid possible pharmacokinetic interactions. Separate groups of mice were used for each analgesic test and route of administration, and animals were used only once in experiments. Studies were carried out in accordance with current guidelines for the veterinary care of laboratory animals [25] and were performed under the consent and surveillance of Unicamp's Institute of Biology Ethics Committee for Animal Research (766-1).

4.6. *Drugs*

All drugs and compounds C1 and C2 were diluted in vehicle made of Tween^{80} 1% (Sigma-Aldrich, U.S.A) in saline solution 0.9% (NaCl diluted in distilled water). Reagents (capsaicin and glutamic acid) diluted in phosphate buffer solution

(PBS, pH 6.8). The following drugs and reagents were used: pentobarbital (Cristália- Brazil), acetic acid, capsaicin, glutamic acid, naloxone hydrochloride (Sigma-Aldrich, U.S.A) and morphine hydrochloride (FHC, Brazil).

4.7. *Evaluation of locomotor activity*

The open-field test was used to exclude the possibility that the antinociceptive action of compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) could be resultant from non-specific disturbances in the locomotor activity of the animals. The ambulatory behavior was assessed in an open-field test as described previously [17] with few changes. The apparatus consisted of a plastic box measuring 45 x 45 x 20 cm, with the floor divided into 9 equal squares (15 x 15 cm). The number of squares crossed with all paws (crossing) was counted in a 3-min session. Mice were treated intraperitoneally (i.p.) with compounds C1 and C2 (30 $mg.kg^{-1}$, i.p.), pentobarbital (35 $mg.kg^{-1}$) or vehicle 30 min beforehand. Results expressed as mean ± S.E.M. of 4 animals per group.

4.8. *Writhing test*

The writhing test was carried out as described by Koster et al [26] with few changes. Groups of mice (*n*=8) were treated orally (p.o.) or i.p with vehicle (10 $mL.kg^{-1}$) or compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) using four doses 10, 30, 100, and 300 $mg.kg^{-1}$ to determine ED_{50}. Dose-related results do not need positive control. Writhing was induced by an i.p. injection of 0.8% acetic acid solution (0.1 $mL.10g^{-1}$), 30 or 60 min after treatment (i.p. and p.o. respectively). After injection of the acetic acid solution, the numbers of writhings (abdominal constrictions) were cumulatively counted over 15 minutes, for nociception evaluation. Data represent the average of the total writhing observed per dose concentration.

4.9. *Capsaicin Test*

The procedure used was according to Santos and Calixto [27] with few changes. Different groups of animals (n=8) were treated p.o. with compounds C1 and C2 (300 $mg.kg^{-1}$), morphine hydrochloride (20 $mg.kg^{-1}$), or vehicle, or i.p. with compounds C1 and C2 (10, 30, 100, 300 $mg.kg^{-1}$) or vehicle. No need to use positive control to determine ED_{50}. After 60 or 30 minutes (respectively), 50 µL of capsaicin (1.6 $µg.paw^{-1}$ prepared in PBS) was injected in the ventral surface of the right hind paw. The time that the animals spent licking the injected paw, for the first 5 minutes post capsaicin injection, was recorded with a chronometer and considered as indicative of nociception.

4.10. *Glutamate test*

The glutamate test was carried out as described by Ellson et al [28] with adaptations. Different groups of animals (n=8) were treated i.p. with compounds C1 and C2 (10, 30, 100 and 300 $mg.kg^{-1}$) (dose- related to determine ED_{50}). No need to use positive control to determine ED_{50}. Animals received 20 µL of glutamate solution (glutamic acid in PBS) 30 min after compound treatment, injected intraplantar (i.pl.) in the ventral surface of right hind paw (10 $µmol.paw^{-1}$), and were observed individually for 15 min following glutamate injection. The amount of time spent licking the injected paw was chronometered and was considered as indicative of nociception.

4.11. *Hot Plate Test*

The hot plate test was performed according to Woolfe and Mac Donald (1944) [29]. Each animal group (*n*=16) were treated p.o. with compounds C1 (100 $mg.kg^{-1}$), C2 (100 $mg.kg^{-1}$), morphine hydrochloride (20 $mg.kg^{-1}$), or vehicle, and a half of these groups (*n*=8) were pre-treated (20 min) p.o. with naloxone hydrochloride (1 $mg.kg^{-1}$) or vehicle (oral administration was used to reduce the stress caused by injection), before thermal algesic stimulation. Mice were placed on the hot plate which was kept at 56ºC ± 0.1ºC, and the reaction time was noted

by observing either the licking of the hind paws, jumping, or the rotation movements at 30, 60, 90, and 120 minutes after administration. For this test, the animals were selected 24hs before using the same algesic stimulation (cut off 20 sec.).

4.12. *Statistical Analysis*

All results were submitted to one way analysis of variance (ANOVA), considering as critical level $p \leq 0.05$ to evaluate significant difference between the control and treated groups, followed by Duncan´s Test , using StatSoft® software.

Authors' contributions

HMS (PhD student) carried out pharmacological studies on antinociceptive experimental models developing new techniques in the laboratory and drafted the manuscript; LS (Master student) carried out Isolation of 6α,7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol and gave assistance with pharmacological studies in antinociceptive experimental models; CD (Master Student) carried out initial assays with pharmacological studies with anti-inflammatory experimental models; RAFR carried out the large scale production of authentic samples of 6α,7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol; IMOS carried out Analytical support for 6α, 7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol quantification; JEC as co-supervisor, participated in the design of the study; JYT carried out plant identification; MNE and EC were responsible for HREIMS experiments; MAF Supervisor, conceived design of study, structure elucidation, discussion of pharmacological results and coordination. All authors read and approved the final manuscript.

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Capítulo 4

Geranylgeraniol and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester isolated from *Pterodon pubescens* Benth.: Further investigation of the Antinociceptive Mechanisms of Action.

Trabalho a ser submetido para publicação como complementação dos resultados apresentados no Capítulo 3

(sujeito a alterações na versão final)

Abstract

BACKGROUD: The crude alcoholic extracts obtained from *Pterodon pubescens* Benth. seeds, is widely used in Brazilian folk medicine as anti-inflammatory, analgesic, anti-rheumatic, as tonic, and depurative preparations. We have previously demonstrated the antinociceptive activity on writhing, capsaicin, glutamate, and hot-plate tests of two compounds isolated from *P. pubescens*: geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2). This work is a continuation of the previous one, concerning the possible mechanisms of action for both compounds C1 and C2, and differences between them. RESULTS AND CONCLUSION: The present study demonstrated that: i) compounds C1 and C2 produced a significantly anti-allodynic activity in the acute phase in the CFA-induced persistent pain model; ii) compound C1 produced a significantly anti-hypernociception activity in the carrageenan-induced pain model; *iii*) compound C2 significantly lost activity after PCPA treatment suggesting that 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester mechanisms could have a relationship either to the synthesis or release of serotonin; *iv*) compound C1 significantly lost activity after ondansentron treatment suggesting the geranylgeraniol's participation on the 5-HT_3 serotonin receptors; *v*) compound C1 significantly lost activity after efaroxan treatment suggesting this compound's participation on the imidazoline I_1 receptors; and *vi*) both compounds C1 and C2 do not appear to exert their activity via 5-HT_{1A}, 5-HT_{2A}, α_2-adrenoceptor, imidazoline I_2, NO, $GABA_A$ and acetylcholine muscarinic and nicotinic receptors when evaluated in the acetic acid-induced nociception.

1. Introduction

Pterodon pubescens Benth. (Leguminosae) seeds are commercially available in Brazilian medicinal flora market and the crude alcoholic extracts of this plant is used in folk medicine as anti-inflammatory, analgesic and anti-rheumatic preparations (Lorenzi, 1998; Pio Correa et al, 1975). Phytochemical studies of *Pterodon* genus have shown the presence of alkaloids, isoflavones and diterpenes. Furan diterpenes were identified and isolated from *Pterodon* species (Mahjan and Monteiro, 1973; Fascio et al, 1975; Campos et al, 1994; Arriaga et al, 2000; Spindola et al, 2009). Our previous studies and those by other research groups have demonstrated that furanoditerpenes possessing vouacapan skeleton are involved with the anti-inflammatory, antinociceptive and antiproliferative properties of *Pterodon pubescens* seed's oil (Nunan et al, 1982; Carvalho et al, 1999; Silva et al, 2004; Spindola et al, 2009, 2010). Diterpenes 6α-hydroxyvouacapan-7β-17β-lactone and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester, found in *P. emarginatus* and *P. polygalaeflorus* seeds, respectively, were previously reported to be associated with the anti-inflammatory activity of these species (Nunan et al, 1982). Some authors (Silva et al, 2004; Duarte et al, 1996; Coelho et al, 2005; Spindola et al, 2010) have suggested participation of vouacapan compounds in antinociceptive and anti-inflammatory activity. The antiproliferative activity against melanoma and prostate tumor cells was reported by Vieira et al (2008) and Spindola et al (2009) respectively.

We have previously shown the antinociceptive modulation of compounds 6α,7β-dihydroxyvouacapan-17β-oate methyl ester and geranylgeraniol isolated from *Pterodon pubescens* Benth. when evaluated on writhing, capsaicin, glutamate and hot-plate animal experimental models (Spindola et al, 2010). In the present study, we examined the possible activity on hyperalgesia and allodynia for both compounds, and the potential mechanisms involved in the antinociceptive profile in the writhing test.

2. Materials and Methods

2.1. *Phytochemistry*

2.1.1. **Plant material**

Seeds were collected in São Paulo state (São Carlos city), in March 2006. Prof. Dr. Jorge Yoshio Tamashiro from IB-UNICAMP (Department of Botany) identified the plant species. A voucher specimen was deposited at State University of Campinas (UEC) Herbarium, under number 1402.

2.1.2. **Compounds isolation**

The compounds were isolated and identified in previous work by our group (Spindola et al, 2009; 2010). Briefly, compounds C1 and C2 were obtained under successive column chromatography of the crude dichloromethane extract produced from *P. pubescens* seeds.

2.2. *Pharmacology*

2.2.1. **Drugs**

All drugs and compounds C1 and C2 were diluted with Tween^{80} 1% (Sigma-Aldrich, U.S.A) in saline solution 0.9% (NaCl diluted in distilled water). The following substances were used: Acetic acid, Complete Freund 's Adjuvant (CFA), *p*-chlorophenylalanine methyl ester hydrochloride (PCPA), Pindolol, Ketanserin, Ondansentron hydrochloride, Yohimbine hydrochloride, Clonidine hydrochloride, N$^{\omega}$-nitro-L-Arginine, L-Arginine hydrochloride, Efaroxan hydrochloride, Idazoxan hydrochloride, Bicuculline methiodide, Atropine, Mecamylamine and Carrageenan (Sigma- Aldrich, U.S.A).

2.2.2. **Animals**

Male *Swiss* mice (25-35 g) and *Wistar* rats (150- 250g) were kept at 25 ± 2ºC in 12 h light-dark cycles (light phase started at 7:00 am) and maintained in animal facilities (10 and 5 animals per cage, respectively) with water and food ad libitum, at least for 7 days prior to assays. Separate groups of mice and rats were used only once for each experiment. Studies were carried out in accordance with current guidelines for the veterinary care of laboratory animals (Voipio et al, 2008) and were performed under the consent and surveillance of Unicamp's Institute of Biology Ethics Committee for Animal Research (1076-1).

2.2.3. **Mechanical Allodynia induced by *Complete Freund Adjuvant* (CFA)**

The procedures were developed and standardized in our laboratory based on the previously described method (Villetti et al, 2003) with changes on protocol and data analysis. Different groups of rats (n=5) were used, and inflammation was induced with a solution of CFA (1 mg/ml of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate) injected (0.1 mL) into the plantar surface of the right rind paw. The left hind paw received the same volume of saline solution (NaCl 0.9% diluted in distilled water) in order to equalize the sensibility of animals caused by injection. Mechanical allodynia was assessed using the Dynamic Plantar Anaestesiomether apparatus (Ugo Basile, mod- 37450- Italy) which consist on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. A steel rod (diameter 0.5 mm) was pushed against the hind paw with ascending force (touch stimulator). The force ranged from 0 to 35g over a 20-s period. When the animal withdrew the hind paw, the mechanical stimulus was automatically stopped, and the force at which the animal withdrew the paw was recorded to the nearest 0.1g. An allodynia score was determined after four consecutive measurements using the touch stimulator sequentially in the left and right hind paw and calculated considering the formula:

Left hind paw value / Right hind paw value = Allodynia score

The basal score was measured before CFA injection on day 0, and best animals considered to be tested were the animals with means value nearest to 1 (demonstrating no significant differences among both paw stimuli). After CFA injection, measurements were made considering three different phases, as follows: 4h on day 0 (acute pain); 24 h on day 1 (sub-acute pain) and on day 14 (chronic pain). Vehicle (10 ml/kg) or compounds C1 and C2 were administered (30 mg/kg, i.p.) 30 min prior to the touch stimulation, in order to evaluate the possible anti-allodynic activity observed for each phase. The aim of this test was to evaluate the activity of compounds using the same doses used in the following tests for evaluation of the potential antinociceptive mechanisms, therefore a positive control was not employed.

2.2.4. **Mechanical Hyperalgesia induced by carrageenan**

The procedures used were similar to those described previously (Randall and Selitto, 1957) with some changes on protocol and data analysis. Different groups of rats (n = 6) were submitted to the pressure stimulus (0 to 500g) in the right hind paw using an Analgesy-meter (Ugo Basile, mod 37215/372116, Italy) prior to the carrageenan injection, in order to determine the basal value. After this first measurement, animals received the sub plantar injection of 0.1 mL of carrageenan (2.5% in saline) into the right hind paw surface. After 2:30h, they were submitted to pressure again, in order to evaluate if the hypernociceptive state was reached. Animals were than treated with vehicle (negative control), indomethacin (30 mg/kg, i.p. – positive control) and compounds C1 and C2 (30 mg/kg, i.p.), and the mechanical hyperalgesia was evaluated after 30 min, 1h, 2h and 3h. Doses for compounds were defined according to previous experiments (Spindola et al, 2010). The value demonstrating mechanical hyperalgesia was obtained after one measurement at each time, and results were shown as decreased percentage compared to the pressure supported in the basal (100%) value for each group.

2.2.5. **Abdominal constriction induced by acetic acid (Writhing test)**

The abdominal constrictions were induced according to procedures previously described (Spindola et al, 2010), and resulted in contraction of the abdominal muscle concomitant with a stretching of the hind limbs in response to an i.p. injection of acetic acid 0.8% (10 mL/kg) at the time of the test. The number of abdominal constrictions during 15 min was indicative of nociception.

2.2.5.1. **Investigation of the antinociceptive mechanisms of compounds C1 and C2**

To address some of the antinociceptive mechanisms of C1 and C2, male Swiss mice were pre-treated with different receptor antagonists in the writhing test. The response thresholds were measured 30 min after the second injection. The doses of each receptor agonists (including C1 and C2) and antagonists were selected on the basis of other experiments in the literature (Santos et al, 2005; Dalbó et al, 2006; Yue CQ et al, 2007) and our preliminary experiments (Spindola et al, 2010).

2.2.5.1.1. ***Involvement of serotonergic system***

To explore the possible participation of the serotonergic system in the antinociceptive action of compounds C1 and C2, mice were pre-treated with either PCPA (a 5-HT synthesis inhibitor, once a day, during 4 consecutive days before test, 100 mg/kg, i.p.), pindolol (a 5-HT_{1A} receptor antagonist, 1 mg/kg, i.p.), ketanserin (a 5-HT_{2A} receptor antagonist, 0.3 mg/kg, i.p.), ondansentron (a 5-HT_3 receptor antagonist, 0.2 mg/kg, i.p.) or vehicle (10 mL/kg) and 20 min later received compounds C1 and C2 (30 mg/kg, i.p.) or vehicle, 30 min before acetic acid injection.

2.2.5.1.2. ***Involvement of α_2- adrenergic system***

In order to evaluate the possible participation of the α_2- adrenergic receptors in the antinociceptive effect of compounds C1 and C2, animals were pre-treated

with either yohimbine (an α_2- adrenoceptor antagonist, 0.2 mg/kg, i.p.) or vehicle (10 mL/kg), and then, 20 min later, received compounds C1 and C2 (30 mg/kg, i.p.), clonidine (an α_2- adrenoceptor agonist, 0.1 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.), 30 min before acetic acid injection.

2.2.5.1.3. ***Involvement of imidazoline system***

With the purpose of evaluating the involvement of imidazoline receptors in the antinociceptive action of compounds C1 and C2, mice were pre-treated with efaroxan (a mixed I_1 imidazoline/ α_2 – adrenoceptor antagonist, 2 mg/kg, i.p.), idazoxan (a mixed I_2 imidazoline / α_2 – adrenoceptor antagonist, 10 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.), 20 min before administration of compounds C1 and C2 (30 mg/kg), clonidine (an α_2- adrenoceptor agonist, 0.1 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.), and then, 30 min later injection of acetic acid.

2.2.5.1.4. ***Involvement of the nitric oxide system***

To investigate the role played by L-Arginine/ nitric oxide pathway in the antinociception caused by compounds C1 and C2 in the writhing test, mice were pre-treated with L-Arginine (L-ARG; precursor of nitric oxide, 50 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.) 20 minutes prior to the treatment with compounds C1 and C2 (30 mg/kg, i.p.), N^{ω}- nitro-L-Arginine (L-NAME; nitric oxide synthesis inhibitor, 75 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.). After 30 min animals received the acetic acid injection.

2.2.5.1.5. ***Involvement of $GABA_A$ system***

The involvement of $GABA_A$ inhibitor receptor in the antinociceptive activity of compounds C1 and C2, mice were pre-treated with biccuculine ($GABA_A$ receptor antagonist, 0.7 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.) 20 min before compounds C1 and C2 administration (30 mg/kg, i.p.) or vehicle, 30 min prior to the acetic acid-induced nociception.

2.2.5.1.6. ***Involvement of neuronal nicotinic acetylcholine system***

In order to evaluate the involvement of nicotinic receptors in the antinociceptive action of compounds C1 and C2, mice were pre-treated with mecamylamine (a central and peripheral neuronal nicotinic acetylcholine receptor antagonist, 2 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.), 20 min before injection of compounds C1 and C2 (30 mg/kg) or vehicle (10 mL/kg), 30 min prior to acetic acid administration.

2.2.5.1.7. ***Involvement of muscarinic acetylcholine system***

To asses the involvement of muscarinic receptors in the antinociceptive activity of compounds C1 and C2, animals were pre-treated with atropine (a central and peripheral acetylcholine muscarinic antagonist, 5 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.), 20 minutes before C1 and C2 (30 mg/kg) or vehicle administration, and after 30 min, mice received the acetic acid injection.

2.2.6. **Statistical analysis**

All results were submitted to one way analysis of variance (ANOVA), considering as critical level $p \leq 0.05$ to evaluate significant difference between the control and treated groups, followed by Duncan´s Test , using StatSoft® software. Graphs were designed using the Origin® software.

3. Results

3.1. **Mechanical allodynia**

Results presented in **Fig.1** showed that compounds C1 and C2 (30 mg/kg, i.p.) produced a significantly anti-allodynic activity reducing the allodynic score compared to control (vehicle) in the acute phase (4h post CFA) of the test. ANOVA revealed a significant difference between groups in this phase [$F_{(2,\ 12)}$= 2,82; P ≤ 0.05]. In the sub-chronic and chronic phases compounds did not exert activity.

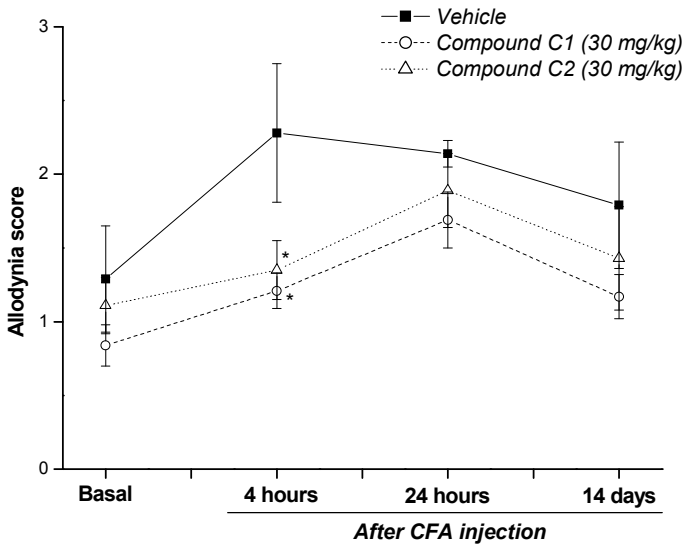

Figure 1: Graph demonstrating decrease on allodynia score related to the touch stimulation exerted on the surface of the left and right hind paws caused by injection of saline solution and CFA (0.1 mL) respectively, producing persistent pain sensitization in rats treated intraperitoneally with compounds geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) compared to the control group (vehicle, 10 mL/kg). Results expressed as reduction score means ± SEM of 5 animals for experimental groups (* p ≤ 0.05).

3.2. **Mechanical hyperalgesia**

Results presented in **Fig.2** showed that control indomethacin (30 mg/kg, i.p.) and compound C1 (30 mg/kg, i.p.), but not C2, produced a significantly anti-hypernociception activity increasing the percentage stimulus compared to control group (vehicle) after 1h, 2h and 3h after carrageenan injection. ANOVA revealed a significant difference between groups: 1h [$F_{(3,16)}$= 3.78; P ≤ 0.05]; 2h [$F_{(3,16)}$= 8.44; P ≤ 0.001]; 3h [$F_{(3,16)}$= 6.08; P ≤ 0.01].

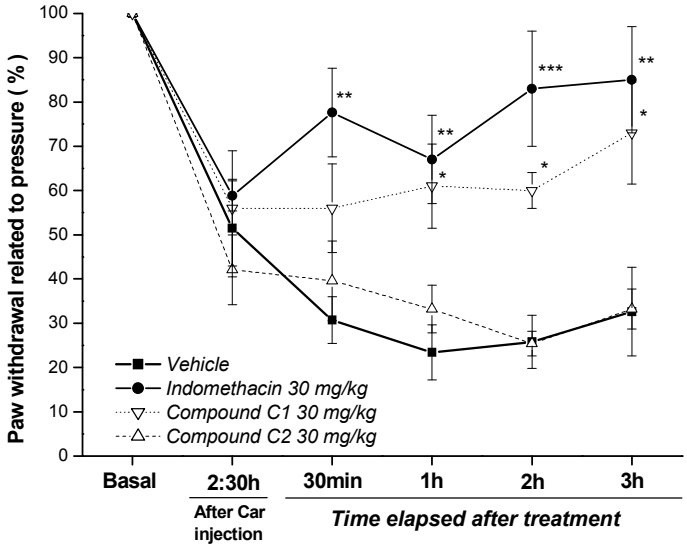

Figure 2: Graph demonstrating results of paw withdrawal related to pressure exerted in the right hind paw in response to the hypernociception caused by injection of carrageenan (2.5%, 0.1 mL) in rats treated intraperitoneally with indomethacin, compounds geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) compared to the control group (vehicle). Results expressed as reduction percentage means ± SEM of 5 animals for experimental groups compared to the basal values (considered 100%) (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001).

3.3. **Evaluation of some potential antinociceptive mechanisms of geranylgeraniol and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester:**

3.3.1. ***Participation of serotonergic system***

Results presented in **Fig.3** showed that compounds C1 and C2 (30 mg/kg) produced a significantly antinociception reducing writhes in mice, compared to control group (vehicle only). The pre-treatment (once a day) during four consecutive days, with the 5-HT synthesis inhibitor PCPA (100 mg/kg, i.p.) significantly reversed the antinociception caused by compound C2, but not for C1. ANOVA revealed a significant difference between groups [$F_{(5,30)} = 8.16$; $P \leq 0.001$].

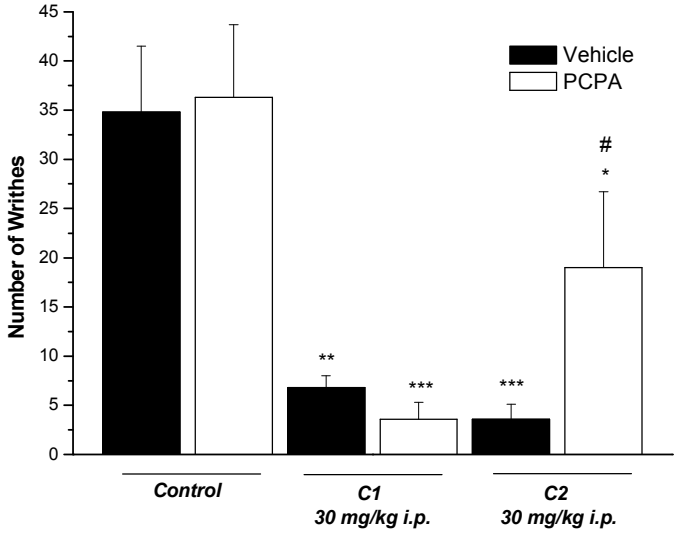

Figure 3: Graph demonstrating effect of pre-treatment of animals with PCPA (100 mg/kg, 4 consecutive days, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with corresponding control values (injected with vehicle alone); $^{\#}P \leq 0.05$ comparing to the respective agonist group (reversing effect of compounds C1 or C2).

Results presented in **Fig.4** showed that compounds C1 and C2 (30 mg/kg) produced a significantly antinociception reducing writhes in mice, compared to control group (vehicle only). The pre-treatment with the 5-HT_{1A} receptor antagonist pindolol (1 mg/kg, i.p.), did not reverse antinociception caused by both compounds C1 and C2. ANOVA revealed a significant difference between groups [$F_{(5,30)}$ = 45.22; $P \leq 0.001$].

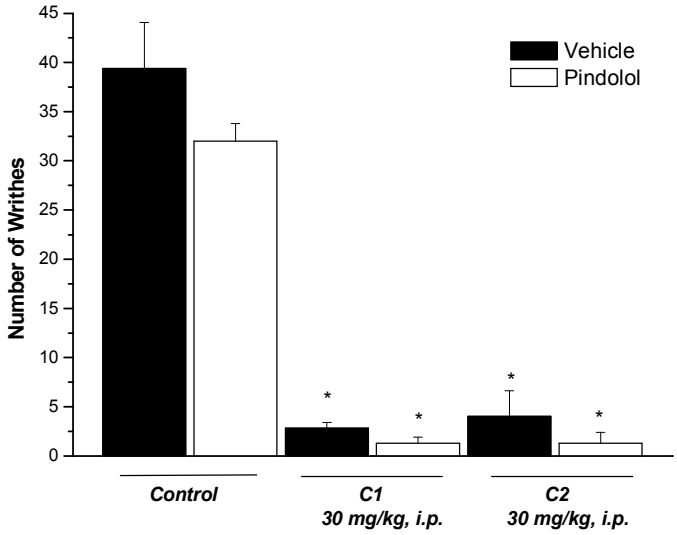

Figure 4: Graph demonstrating effect of pre-treatment of animals with pindolol (1 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.001$ compared with corresponding control values (injected with vehicle alone).

Results presented in **Fig.5** showed that compounds C1 and C2 (30 mg/kg) produced a significantly antinociception reducing writhes in mice, compared to control group (vehicle only). The pre-treatment with the 5-HT_{2A} receptor antagonist ketanserin (0.3 mg/kg, i.p.), did not reverse antinociception caused by both compounds C1 and C2. ANOVA revealed a significant difference between groups [$F_{(5,27)}$ = 31.72; $P \leq 0.001$].

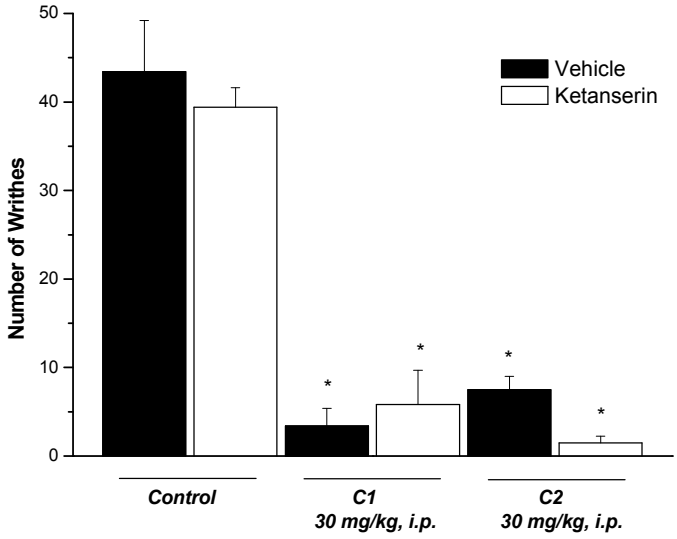

Figure 5: Graph demonstrating effect of pre-treatment of animals with ketanserin (0.3 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.001$ compared with corresponding control values (injected with vehicle alone).

Results presented in **Fig.6** showed that compounds C1 and C2 (30 mg/kg) produced a significantly antinociception reducing writhes in mice, compared to control group (vehicle only). The pre-treatment with the 5-HT_3 receptor antagonist ondansentron (0.2 mg/kg, i.p.) significantly reversed antinociception caused by compound C1, but not for C2. ANOVA revealed a significant difference between groups [$F_{(5,30)} = 4.62$; $P \leq 0.01$].

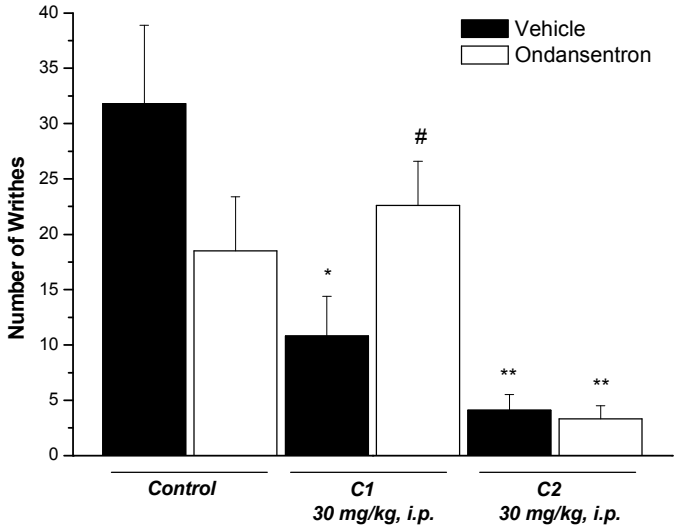

Figure 6: Graph demonstrating effect of pre-treatment of animals with ondansentron (0.2 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, compared with corresponding control values (injected with vehicle alone); $^{\#}P \leq 0.05$ comparing to the respective agonist group (reversing effect of compounds C1 or C2).

3.3.2. ***Participation of α_2- adrenergic system***

Fig.7 demonstrated that compounds C1, C2 (30 mg/kg) and the α_2-adrenoceptor agonist clonidine (0.1 mg/kg, i.p.) produced a significantly antinociception reducing writhes in mice, compared to control group (vehicle only). The pre-treatment with the α_2- adrenoceptor antagonist yohimbine (0.2 mg/kg, i.p.) reversed antinociception caused by clonidine, but not for compounds C1 and C2. ANOVA revealed a significant difference between groups [$F_{(7,38)} = 4.24$; $P \leq 0.01$].

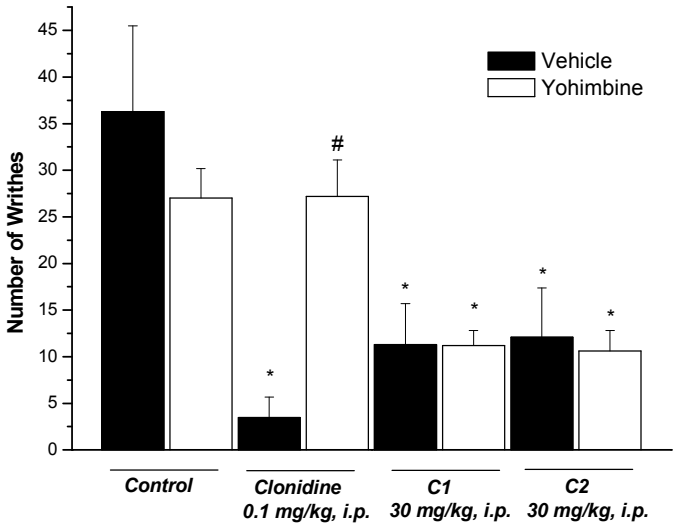

Figure 7: Graph demonstrating effect of pre-treatment of animals with yohimbine (0.2 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.001$compared with corresponding control values (injected with vehicle alone); $^{\#}P \leq 0.05$ comparing to the respective agonist group (reversing effect of compounds C1, C2 or clonidine 0.1 mg/kg).

3.3.3. ***Participation of imidazoline system***

Results showed in **Fig.8** demonstrated that compounds C1, C2 (30 mg/kg) and the α_2- adrenoceptor agonist clonidine (0.1 mg/kg, i.p.) produced a significantly antinociception reducing writhes in mice, compared to control group (vehicle only). The pre-treatment with the mixed I_1 imidazoline/ α_2 – adrenoceptor antagonist efaroxan (2 mg/kg, i.p) significantly reversed antinociception caused by clonidine (α_2- adrenoceptor agonist, 0.1 mg/kg, i.p.) and compound C1 (30 mg/kg, i.p.), but not for C2. On the other hand, the pre-treatment with the mixed I_2 imidazoline / α_2-adrenoceptor antagonist idazoxan (10 mg/kg, i.p.), reversed only antinociception caused by clonidine. ANOVA revealed a significant difference between groups [$F_{(9,50)}$ = 26.98; $P \leq 0.001$].

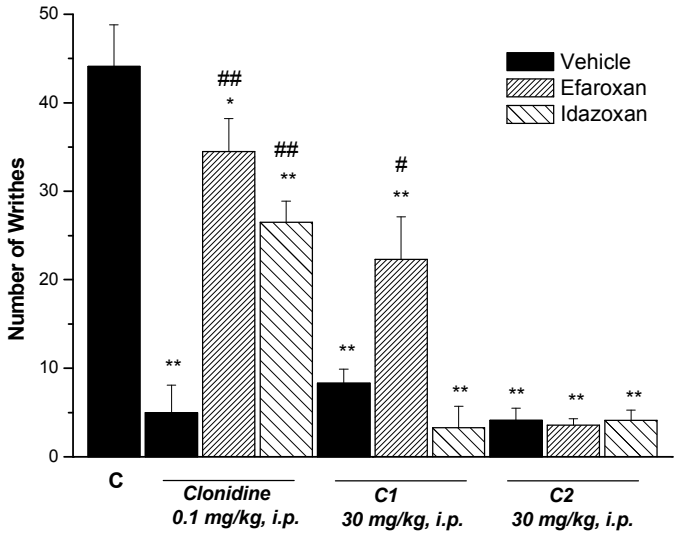

Figure 8: Graph demonstrating effect of pre-treatment of animals with efaroxan (2 mg/kg, i.p.) and idazoxan (10 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$ compared with corresponding control values (injected with vehicle alone); $^{\#}P \leq 0.01$, $^{\#\#}P \leq 0.001$ comparing to the respective agonist group (reversing effect of compounds C1, C2 or clonidine 0.1 mg/kg).

3.3.4. ***Participation of nitric oxide system***

Fig.9 showed that compounds C1, C2 (30 mg/kg, i.p.) and the nitric oxide synthesis inhibitor L-NAME (75 mg/kg, i.p.), produced a significantly antinociception reducing writhes in mice compared to control group (vehicle only). The pre-treatment with the oxide nitric precursor L-Arginine (50 mg/kg, i.p.) significantly reversed L-NAME antinociception activity, but not for compounds C1 and C2. ANOVA revealed a significant difference between groups [$F_{(6,35)}$ = 8.58; $P \leq 0.001$].

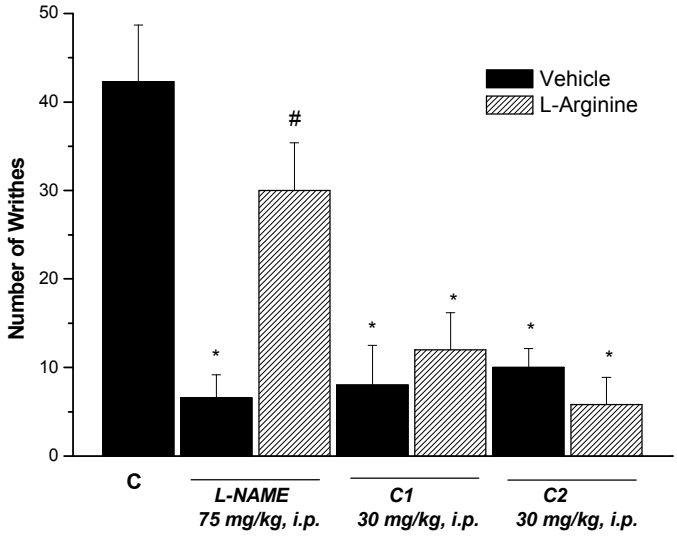

Figure 9: Graph demonstrating effect of pre-treatment of animals with L-Arginine (50 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.001$ compared with corresponding control values (injected with vehicle alone); $^{\#}P \leq 0.01$ comparing to the respective agonist group (reversing effect of compounds C1, C2 or L-NAME).

3.3.5. ***Participation of $GABA_A$ system***

Results demonstrated in **Fig.10** showed that compounds C1 and C2 (30 mg/kg, i.p.) produced a significantly antinociception reducing writhes in mice compared to control group (vehicle only). The pre-treatment with the $GABA_A$ receptor antagonist bicuculline (0.7 mg/kg, i.p.), did not reverse antinociception caused by neither compound C1, nor C2. ANOVA revealed a significant difference between groups [$F_{(5,32)} = 13.58$; $P \leq 0.001$].

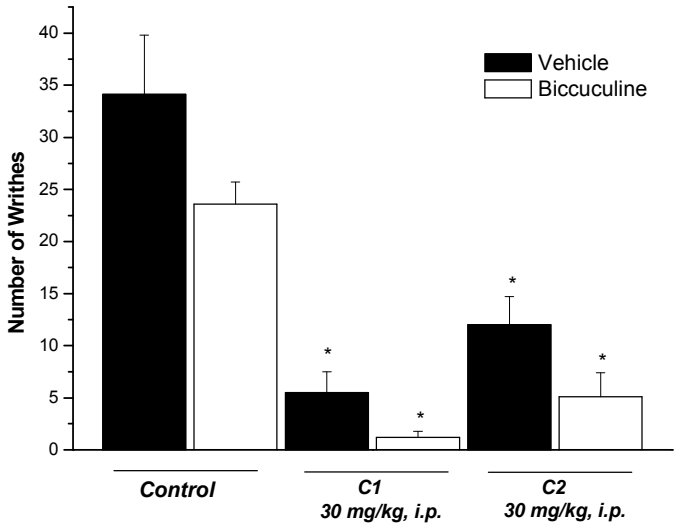

Figure 10: Graph demonstrating effect of pre-treatment of animals with bicuculline (0.7 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.001$ compared with corresponding control values (injected with vehicle alone).

3.3.6. ***Participation of neuronal muscarinic acetylcholine system***

As showed in **Fig.11**, compounds C1 and C2 (30 mg/kg, i.p.) produced a significantly antinociception reducing writhes in mice compared to control group (vehicle only). The pre-treatment with the central and peripheral acetylcholine muscarinic antagonist atropine (5 mg/kg, i.p.), did not reverse antinociception observed for both compounds C1 and C2 (30 mg/kg, i.p.). ANOVA revealed a significant difference between groups [$F_{(5,28)} = 14.45$; $P \leq 0.001$].

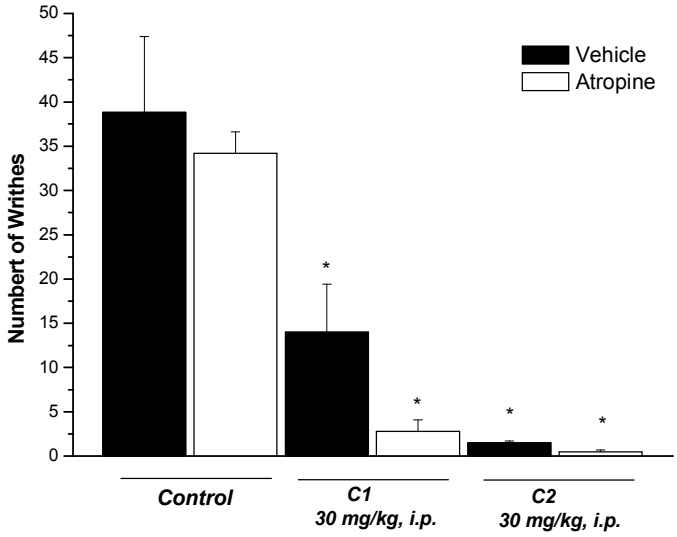

Figure 11: Graph demonstrating effect of pre-treatment of animals with atropine (5 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.001$ compared with corresponding control values (injected with vehicle alone).

3.3.7. ***Participation of neuronal nicotinic acetylcholine system***

Fig. 12 showed that compounds C1 and C2 (30 mg/kg, i.p.) produced a significantly antinociception reducing writhes in mice compared to control group (vehicle only). The pre-treatment with central and peripheral neuronal nicotinic acetylcholine receptor antagonist mecamylamine (2 mg/kg, i.p), did not reverse antinociception observed for both compounds C1 and C2 (30 mg/kg, i.p.). ANOVA revealed a significant difference between groups [$F_{(5,27)} = 8.95$; $P \leq 0.001$].

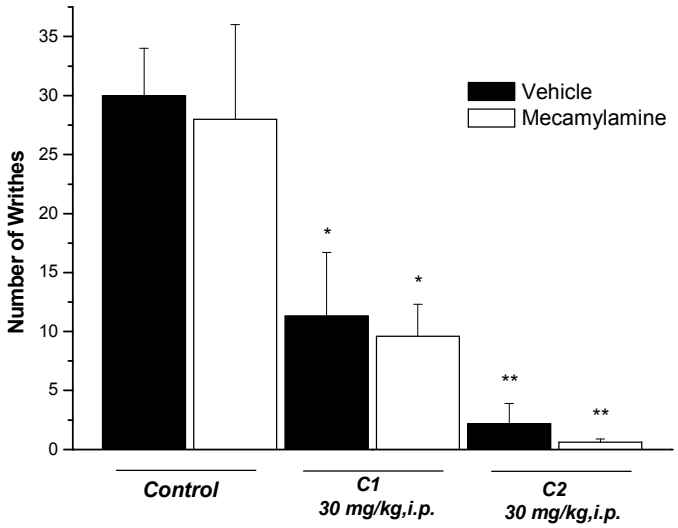

Figure 12: Graph demonstrating effect of pre-treatment of animals with mecamylamine (2 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (30 mg/kg) against the acetic acid-induced writhing in mice. Each column represents the mean of 6-8 animals and the error bar indicate the S.E.M. $^{*}P \leq 0.01$, $^{**}P \leq 0.001$ compared with corresponding control values (injected with vehicle alone).

4. Discussion

We have recently reported some of the antinociceptive modulation mechanisms for compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) isolated from *P. pubescens* Benth. using different animal experimental models such as acetic acid-induced nociception (writhing test), capsaicin-induced nociception, glutamate- induced nociception and hot plate test. These previous results suggested that: compounds C1 and C2 may present synergistic activity; both i.p. and p.o. treatment of compounds C1 and C2 reduced reactivity to the writhing test demonstrating differences in potency related to the route of administration; both compounds C1 and C2 demonstrated possible activity related to vanilloid receptors and/or glutamate peripheral receptors (with C2 being more potent by the i.p. route); and finally, the antinociceptive activity of compounds C1 and C2 (p.o.) do not appear to exert their effects in the hot-plate test via opioid receptors (Spindola et al, 2010).

Those results prompted us to continue the investigation on some of the potential mechanisms of action for both compounds, trying to establish differences among their antinociceptive profiles, in order to determine which kind of pain disorders they are able to combat. In the present study, we investigated the potential anti-allodynic, anti-hypernociceptive, and some antinociceptive mechanisms (using agonists and antagonists) for both compounds C1 and C2 in the writhing test. The most relevant findings of this work are: i) compounds C1 and C2 produced a significantly anti-allodynic activity in the acute phase on the CFA-induced persistent pain model; ii) compound C1 produced a significantly anti-hypernociception activity on the carrageenan-induced pain model; iii) the antinociceptive action of compounds C1 and C2 in the writhing test was reversed by treatment with antagonists of several systems involved in pain.

In order to evaluate some of the mechanisms and differences involved in the antinociception caused by compounds geranylgeraniol (C1) and 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2), we attempted to analyze different pathways of pain sensitization using three experimental approaches: the allodynic

response caused by i.pl. injection of CFA; the hypernociception response caused by i.pl. carrageenan injection; and the blockade of several receptors with specific antagonists in a chemical-induced visceral nociception model (writhing test). All these procedures allowed us to define specifically mechanisms and differences among compounds C1 and C2, corroborating our previous results. The doses used were based on ED_{50} determined in preliminary studies (Spindola et al, 2010). In order to respect the ethics for animal research, the minimum numbers of animals were used. For this purpose, we standardized the use of a 30 mg/kg doses for compounds C1 and C2 in each experimental design, and this fixed dose was considered the best to observe the differences among the compounds activity.

Glial (or microglial) activation is a common feature of many CNS diseases and has been regarded as the hallmark of stereotypical tissue reaction to cell death, injury or infection (Kreutzberg et al, 1996). Glia undergoes different active states depending on the stimulus that triggers activation (Watkins and Maier, 2003). Increased evidence has demonstrated that glial activation is a process actively involved in neurodegeneration or neuroprotection (Vila et al, 2001). Astrocytes and microglia in the spinal cord have been recognized as potential active participants in the initiation and maintenance of pain facilitation induced by inflammation and damage to peripheral tissues, peripheral nerves, spinal nerves and spinal cord (Watkins et al, 2001; Raghavendra et al, 2004). Upon activation, glia releases a variety of algesic substances that may potentiate pain transmission by neurons. Of these glial products, proinflammatory cytokines were shown to be common mediators of allodynia and hyperalgesia (Raghavendra & DeLeo, 2003; Sommer et al, 2003; Watkins & Maier, 2003). The role of glia and cytokines in the initiation and maintenance of pain states, and the time course and region of the activation in the CNS, has been reported (DeLeo & Yezierski, 2001; Watkins et al, 2001; Watkins & Maier, 2003; Raghavendra et al, 2004).

Persistent pain caused by intraplantar injection of CFA involves central sensitization due to the release of multiple inflammatory and pain mediators that in

turn account for the increase in sensitivity of both peripheral sensory afferents at the site of the injury, and in the central nervous system is well recognized (Sammad et al, 2001). Raghavendra (2004) demonstrated that in CFA-injected rats, occur an increased expression of mRNA for Mac-1 (glial marker) 4 h after inflammation (acute phase), and the immune-reactivity was also enhanced in the CNS after the acute and sub-acute phase of inflammation, demonstrating microglial activation. Another important microglial marker is TLR4, which is a pattern recognition receptor involved in the innate immune response to various microorganisms and other endogenous and exogenous stress factors, such as nociception (Medzhitov et al, 2001; Vabulas et al, 2002). Also, TLR4 has been involved in the recognition of eukaryotic proteins, which appear after destruction of cells, tissue, or fibrinogen (Smiley et al, 2001; Vabulas et al, 2002).

Some studies established that in the CNS, TLR4 is almost exclusively expressed by microglia, and activation induces innate immune responses that trigger neurodegeneration (Lehnardt et al, 2003). Because activation of TLR4 induces the release of pro-inflammatory cytokines, and pro-inflammatory cytokines have been implicated in the development and maintenance of persistent pain states, targeting the TLR4 pathway may have pharmacological efficacy to overcome pathological pain processes (Watkins et al, 2001; Raghavendra and DeLeo, 2003). Results shown in **fig.1** demonstrated the activeness of compounds C1 and C2 on the first phase (acute) of the allodynic response (but not for sub-acute and chronic), suggesting that both may have their antinociceptive activity related to microglia pathways in the CNS, corroborating our previous results intending to elucidate their central activity.

Carrageenan-induced hind paw inflammation is a neutrophil-mediated acute inflammatory response that produces hind paw swelling, erythematic and localized hyperthermia (Cunha et al, 1992; 2005). The inflammatory response has become a widely used model of acute inflammation, involving macrophages, mast cells, and endothelial cells (Winter et al, 1962). The carrageenan inflamed hind paw also is

painful, and was first measured by the method of Randall and Selitto (1957). There are several evidences demonstrating that cytokines mediate the neutrophil migration into the inflammatory site, but, anti-inflammatory cytokines such as IL-10, down modulate this process (Schein et al, 2002; Kotenko et al, 2002). Interleukin IL-1β and TNF-α mediate the neutrophil migration observed in several experimental models and also in human inflammatory diseases. Carrageenan-induced inflammation of localized tissues has been shown to affect systemic blood cell reactivity and appeared to sensitize leucocytes to a lipopolysaccharide challenge and to enhance the production of TNF-α (Ogata et al, 1999).

In order to investigate whether compounds C1 and C2 could either inhibit the production of the cytokines or increase the production of the anti-inflammatory cytokines, both were investigated on the carrageenan-induced acute inflammatory model in rats. **Fig. 2** shows that only indomethacin control and compound geranylgeraniol (C1) were effective in this assay, presenting an important difference comparing to compound 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2), demonstrating that the antinociceptive activity of C1 may be related to activation of cytokines or interleukins pathways, when evaluated on this assay. These findings encouraged us to evaluate some specific receptors on different pain systems that compounds C1 and C2 may be acting, using the acetic acid-induced visceral pain in mice (writing test).

In the spinal cord, the nociceptive information coming from gut, skin and other organs is submitted to a modulation by a great variety of transmitters that will filter and modulate the transmission of nociceptive impulses to the brain (Millan, 2002). These modulating substances are able to act as pro (descending facilitation) or antinociceptive (descending inhibition), depending on diverse factors, such as the type and the intensity of the stimulation, the central region activated, receptor type, and others. The neurons projected by the central areas responsible for the control of the perception of pain, contain several transmitters including noradrenaline (NA), serotonin (5-HT), acetylcholine (ACh), γ hidroxi-butyric acid (GABA), nitric oxide (NO), glutamate (GLU), dopamine (DA), imidazoline and

others (Dalbó et al, 2006). In order to evaluate some antinociceptive mechanisms for both compounds C1 and C2, the following pathways were assessed: serotonergic, α_2-adrenergic, imidazoline, nitric oxide, inhibitory $GABA_A$, neuronal nicotinic acetylcholine and muscarinic acetylcholine systems.

Serotonin was first identified in the gastrointestinal tract and subsequently also in the CNS where this substance has shown to play a neurotransmitter role, and is mainly degraded by oxidative deamination by monoaminooxidase to 5-hydroxyindoleacetic acid (5-HT). The cell bodies of the brains 5-HT neurons are located in large clusters close to the midline in the raphe nuclei in the mesencephalon and project diffusely to the forebrain and spinal cord. Although 5-HT has long been considered of importance in the control of nociception, only with discovery of multiple 5-HT receptor types has the complexity of the role of this compound become clearer (Furst, 1999). Generally, three major classes of 5-HT receptors have been identified: 5-HT_1, 5-HT_2 and 5-HT_3. These receptors have been further subdivided into A, B, C and D subtypes and were shown to be present within the spinal cord and to play some role in pain transmission (Hoyer et al, 1994; Millan & Colpaert, 1991; Roberts et al, 1988; Zemlan et al, 1988).

In the CNS, 5-HT neurons are involved in nociceptive transmission as well as in the pain inhibition induced by opioid agonists. Serotonin was also observed to produce an inhibitory effect on pain transmission in the spinal cord and in the brain (Yaksh et al, 1979). This inhibitory effect might be mediated by 5-HT_1 and 5-HT_2-type receptors influencing the descending inhibitory system (Sawynok et al, 1996; Bjorkman et al, 1995). The dorsal raphe nucleus (DRN) has been implicated in the regulatory effect of antidepressants in mood disorders (Artigas et al, 1996) as well as in pain modulation and the magnus raphe nucleus (MRN) is probably the most important serotonergic nucleus modulating the descending control of pain transmission (Basbaum and Fields, 1984 ; Millan, 2002).

Our findings demonstrated that compounds C1 and C2 may exert their antinociceptive activity, at least in part, via serotonergic system, with peculiar differences among them. This assertion is supported by the demonstration that

depletion of endogenous serotonin with the tryptophan hydroxylase inhibitor PCPA, at a dose known to decrease the cortical content of serotonin, largely antagonized the antinociception of compound 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester (C2) (but not for C1), suggesting that the compounds action could be due to the synthesis or release of 5-HT (**fig.3)**. On the other hand, the selective antagonist of 5-HT_3 receptors, ondansentron, consistently reversed the antinociception caused by systemic administration of geranylgeraniol (C1) when evaluated in the writhing test (**fig.6)**. In contrast, both compounds did not seems to exert their antinociceptive activity acting on 5-HT_{1A} or 5-HT_{2A}, considering that the pretreatment with the specific antagonists pindolol and ketanserin (respectively) did not block the antinociceptive action of both compounds on the same model **(figs.4 and 5)**. These results allowed us to speculate that C1 may be acting directly on the 5-HT_3 receptor, and that C2 may be acting by increasing or decreasing the amount of serotonin in the CNS.

The α_2-Adrenoceptor agonists share many actions with the opiates (sedation, analgesia). Studies in both animals and humans have shown that clonidine, the α_2-adrenoceptor agonist, not only produce significant analgesia, but also potentiate the analgesia produced by opiates. The analgesic effect of clonidine is probably mediated by activation of α_2-acrenoceptors in the LC and in the dorsal horn of the spinal cord especially in the substantia gelatinosa where this compound depresses nociceptive transmission from primary afferent pain fibers to secondary afferent neurons for signaling to higher centers. There are opinions that the antinociceptive effects of systematically and intraspinally administered α_2-adrenoceptor agonists were due to a direct spinal action and not to an activation of descending inhibitory pathways, suggesting that may be mediated by a specific receptor subtype (Yaksh et al, 1986).

We next investigated the possible participation of the α_2-adrenoceptors in the antinociceptive activity of compounds C1 and C2. Results presented in **fig. 7** showed that neither C1 nor C2 seems to act by α_2-adrenoceptors on this assay. This assertion derives from the fact that the pretreatment with yohimbine, a α_2-

adrenoceptor antagonist, did not reverse the antinociception caused by both compounds in the writhing test.

Of the reported imidazoline receptors, the I_1- receptor has the best understood physiologic actions. The I_1- receptor has been implicated in the hypotension produced by clonidine and other imidazolines when injected into the rostral ventrolateral medulla (RVLM) or when given peripherally (Ernsberger et al, 1997; Head at al, 1997). Other physiologic functions in which the I_1- receptor has been implicated include modulation of gastric acid secretion, analgesia and modulation of electrolyte secretion in the rat kidney (Smyth at al, 1995). Ernsberger's group (2004) reported that monoxidine, a relatively selective I_1- site ligand, stimulates diacylglycerol accumulation via activation of phosphatidylcholine-specific phospholipase C in PC-12 cells (Separovic et al, 1996; 1997). Monoxidine acts in a concentration- dependent fashion and is blocked by efaroxan, a putative selective I_1-site antagonist (Berdeu et al, 1995; Olmos et al, 1995). Monoxidine stimulates phosphatidylcholine-specific phospholipase C activity and diacylglycerol accuymulation at concentrations that much better reflect the reported affinity for I_1-sites.

Musgrave (1998) suggested that some of the reported non-adrenergic affects of imidazolines are due to blockade of ion channels, including the nicotinic acetylcholine receptor, the $5HT_3$ receptor (Molderings et al, 1996), the K^+_{ATP} channel (Shepherd at al, 1996; Dunne et al, 1991), and the NMDA channel (Olmos et al, 1996). Imidazoline-induced channel blockade is specific to the ligand-gated ion channel family, as imidazolines do not inhibit voltage-operated calcium channels or nonselective cation channels (Musgrave and Seifert, 1995).

Several I_1-receptor ligands inhibit the influx of [^{14}C] guanidinium stimulated by 5HT into mouse neuroblastoma cells. 5HT-stimulated [^{14}C] guanidinium influx occurs mostly via the intrinsic ion channel of $5HT_3$ receptors under these conditions. I_1 receptor ligands inhibited 5HT-stimulated [^{14}C] guanidinium influx in a concentration-dependent manner. However, the affinity and order of potency of these ligands did not match either I_1 or I_2- receptors (Moldernings et al, 1996).

Furthermore, the results of the present study provided consistent evidence supporting the involvement of imidazoline I_1 receptors in the antinociception caused by compound C1, but not for C2, evident by the fact that efaroxan (the preferential I_1 imidazoline receptor antagonist) (Eglen et al, 1998), at a dose similar to that known to prevent antinociception induced by the imidazoline I_1 receptor agonist moxonidine (Shannon and Lutz, 2000), consistently attenuated both clonidine and geranylgeraniol (C1) induced antinociception in the writhing test (**fig 8)**. Contrasting, imidazoline I_2 receptors seems not to be involved in the antinociception of both compounds C1 and C2, considering that the pretreatment with the preferential I_2 imidazoline receptor antagonist idazoxan, largely failed to prevent the antinociception of both compounds. The present data allowed us to speculate that the antinociception caused by geranylgeraniol is probably, at least in part, linked to an interaction with imidazoline I_1 receptors.

Another approach of the present study was the evaluation of the possible participation of the nitric oxide pathway in the antinociception caused by compounds C1 and C2 on the writhing test. In several peripheral nerves, in the dorsal horn and possibly in other parts of the CNS, NO might be formed presynaptically and act as a nonadrenergic-noncholinergic neurotransmitter. Among their effects, NO has been implicated in the control of neuronal development and of synaptic plasticity in the CNS, as well as in appetite and nociception. Nitric oxide biosynthesis inhibitors (L-NAME) produced antinociceptive affects in mice (Furst, 1999; Millan, 2002). **Fig 9** demonstrated that neither C1 nor C2 seems to be related to this system on the writhing test. This reasoning derives from the fact that pretreatment of animals with the substrate for NOS, L-Arginine, at a dose that produced no significant effect on writhing test, did not reverse antinociception caused by compounds C1 and C2, like observed with the nitric oxide inhibitor L-NAME.

Amino acids are present in high concentration in the CNS and are potent modifiers of neuronal excitability at various levels. Neutral amino acids like glicine and γ-aminobutyric acid (GABA) are generally inhibitory, and they increase

membrane permeability to chloride ions, thus mimicking the inhibitory postsynaptic potential (IPSP). There are two main types of GABA receptors: $GABA_A$ and $GABA_B$. These neurons from axo-axonic synapses with primary sensory nerve terminals are responsible for presynaptic inhibition. The concentration of GABA is the highest in the dorsal horn of the spinal cord, where this substance is a major inhibitory transmitter (Furst 1999). In order to evaluated the possible role of the inhibitory amino acid $GABA_A$ receptor in the antinociception caused by compounds C1 and C2, both were tested in the writhing test using the specific antagonist for $GABA_A$ receptors bicuculline. The blockage of these receptors did not reverse antinociception caused by compounds C1 and C2 **(fig 10)**, suggesting that their activity seems not to be related to these receptors in the writhing test.

Finally, we considered the acetylcholine muscarinic and nicotinic systems. Numerous studies have implicated the role of the central cholinergic system in nociception and in the mechanism of action of opioids. The types of opioid-receptor-induced presynaptic inhibition of ACh release depends on the type and origin of the cholinergic nerves concerned (Herz et al, 1993). Many papers suggest that ACh is an endogenous antinociceptive compound that may act by monoaminergic pathways. The possibility that these spinal pathways may mediate the response to ACh is suggested by the finding that the increase in tail flick latency observed in mice treated with cholinomimetic agents was markedly attenuated by spinal transection. Paradoxically, some reports have suggested that blockade of the cholinergic system also results in an antinociceptive effect in animal studies (Bannon et al, 1998; Dewey et al, 1969; Haubrich et al, 1984).

Some author mentioned that attenuation of antinociceptive action of fluoxetine by atropine in mice indicates that muscarinic receptors play an important role in the modulation of pain perception by serotonin. This is in support with other reports showing that intrathecal administration of atropine prior to 5-HT_2 agonists significantly attenuated the anti-allodynic effect of nerve ligation model (Obata et al, 2001). Further, intraperitoneal administration of atropine is reported to block the antinociceptive action of 5-HT_1 receptor agonists tested in the hot plate method

(Galeotti et al, 1997). A possible mechanism may include modulation of acetylcholine release from cholinergic interneurons in the spinal and supraspinal sites in the central nervous system (Ramirez, 1997). The present study shows clearly that neither compound C1 or C2 seem to be involved with the muscarinic acetylcholine system on the writhing test (**fig 11)**. This assumption derives from the fact that the treatment of atropine prior to the compounds administration, did not reverse neither C1 nor C2 antinociceptive activity, corroborating the dependence of 5-HT attributed to both compounds action, independently of muscarinic receptors on this assay.

The antinociceptive affect of nicotine was reported as early as 1932 (Davis et al, 1932). Some investigations have shown that injection of nicotine into the NRM of rats could produce an antinociceptive effect of both the tail flick and hot plate assays. Furthermore, ABT-594 (a derivative of epibatidin, the nicotine-like antinociceptive alkaloid isolated from *Ecuadorian frog*) produced strong spinal and supraspinal analgesic action, mediated by neuronal nicotinic acetylcholine ($_n$ACh) receptors (Bannon et al, 1998). Since the fact that norepinephrine (NE) released from noradrenergic nerve terminals is able to exert antinociception and that nicotinic ACh receptor agonists (e.g., nicotine, epibatidin) release NE from axon terminals (Serchen et al, 1997; Zhang et al, 1997) is generally accepted, the suggestion that there is an interaction between the analgesic action of nicotinic receptor agonists and their NE-releasing property seems plausible. The final result of the present work, was the finding that compounds C1 and C2 appear not to be involved with the neuronal nicotinic acetylcholine receptors in the acetic acid-induced nociception, considering that the pretreatment with the $_n$ACh antagonist, mecamylamine, did not block the antinociception produced by geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) isolated from *Pterodon pubescens* Benth. Further studies will be undertaken to determine the possible role of glutamate, NMDA, AMPA, trans-ACPD, kainate, P substantia, histamine and bradykinin receptors participation in the antinociception caused by C1 and C2, via intrathecal route.

5. Conclusion

The present study demonstrated that: ***1***) compounds C1 and C2 produced a significantly anti-allodynic activity in the acute phase on the CFA-induced persistent pain model; ***2***) compound C1 produced a significantly anti-hypernociception activity on the carrageenan-induced pain model; ***3***) compound C2 significantly lost activity after PCPA treatment suggesting that 6*α*,7*β*-dihydroxyvouacapan-17*β*-oate methyl ester mechanisms could be related either to the synthesis or liberation of serotonin; ***4***) compound C1 significantly lost activity after ondansentron treatment suggesting geranylgeraniol's participation on the 5-TH_3 serotonin receptors; ***5***) compound C1 significantly lost activity after efaroxan treatment suggesting this compound's participation on the imidazoline I_1 receptors; and ***6***) both compounds C1 and C2 do not appear to exert their activity via 5-HT_{1A}, 5-HT_{2A}, α_2-adrenoceptor, imidazoline I_2, NO, $GABA_A$ and acetylcholine muscarinic and nicotinic receptors when evaluated in the acetic acid-induced nociception.

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Discussão geral

O uso popular da infusão das sementes da *Pterodon pubescens* Benth. para dor e inflamação, tem sido base para os estudos acerca dessa espécie. Pesquisadores tem desenvolvido métodos para identificar e avaliar a atividade farmacológica de frações e compostos oriundos do gênero *Pterodon.* Nosso grupo de pesquisa contribuiu com estudos sobre as atividades analgésica e antitumoral, de forma específica, com resultados inéditos que agregam conhecimento para viabilizar o desenvolvimento de novos medicamentos.

As atividades antitumoral e analgésica (antinociceptiva) das frações e compostos isolados da *P. pubescens* foram avaliadas de forma simultânea durante todo o desenvolvimento deste trabalho, sendo possível determinar grupos de compostos com pronunciada atividade farmacológica através de ensaios *in vitro* e *in vivo*. Com características multidisciplinares, o projeto foi desenvolvido no Centro Pluridisciplinar de Pesquisas Químicas Biológicas e Agrícolas (CPQBA) da Unicamp, nas Divisões de Fitoquímica (DFITO) e Farmacologia e Toxicologia (DFT). Na DFITO foram desenvolvidos os processos de isolamento e identificação dos compostos, e na DFT, os ensaios de atividade farmacológica.

Trabalhando com técnicas *in vitro* de avaliação de atividade antiproliferativa contra linhagens tumorais humanas, os ensaios de Sulforrodamina B (SRB) e 3-[4,5-Dimetiltiazol-2yl]-2,5 Difeniltetrazol bromida (MTT) foram realizados com base no método utilizado pelo *National Cancer institute* (NCI-USA) para triagem de moléculas com potencial atividade antitumoral. Desde 1996 a DFT tem realizado ensaios de cancer *in vivo*. Este trabalho contribuiu com o desenvolvimento de novas técnicas, dentre elas, o Tumor Sólido de Ehrlich na pata de camundongos, para avaliação de crescimento tumoral em organismo vivo. Juntas as técnicas *in*

vitro e *in vivo* constituem uma importante ferramenta de avaliação de atividade antitumoral experimental.

Compostos que possuam atividade antiinflamatória, podem ter ação antiangiogênica e também auxiliar na diminuição da resistência de células tumorais a agentes conhecidos, tornando-os potenciais agentes quimioterápicos de forma direta ou indireta. Alguns trabalhos relatam a atividade anti-inflamatória de frações e compostos obtidos da *P. pubescens.* Os dados da literatura (capítulos 1 e 2), somados aos nossos estudos preliminares (Spindola, 2006) foram motivadores para a avaliação da atividade antitumoral da espécie, considerando a forte relação entre as ações anti-inflamatória e antitumoral.

O fracionamento biomonitorado por ensaios de atividade antiproliferativa contra células tumorais humanas *in vitro* foi fundamental para o desenvolvimento da pesquisa, à medida que, durante o processo de isolamento de compostos, a quantidade obtida é, por vezes, insuficiente para ser submetida a ensaios *in vivo.* Desta forma, pequenas quantidades dos compostos isolados, foram avaliadas em ensaios *in vitro,* permitindo identificação de frações e compostos com pronunciada atividade farmacológica. O trabalho referente ao *capítulo 1 demonstrou* o isolamento e identificação dos compostos 6*α*-acetoxi-7*β*-hidroxivouacapano (inédito), éster 6*α*,7*β*-diidroxivouacapano-17*β* oato de metila e 6*α*,7*β*-diidroxivouacapano-17*β*- metilenol. Estes resultados foram de suma importância para seleção das moléculas a serem submetidas aos ensaios *in vivo* de atividade antitumoral, avaliando parâmetros, tanto farmacocinéticos e farmacodinâmicos, quanto de eficácia e segurança.

Seguindo a triagem, dois dos compostos isolados, em maior quantidade, denominados geranilgeraniol e éster 6*α,*7β-diidroxivouacapano-17*β* oato de metila, foram submetidos aos ensaios *in vivo*, com intuito de avaliar uma possível ação antitumoral sistêmica (farmacodinâmica). Para tanto, foi desenvolvido o Tumor de Ehrlich sólido na pata de camundongos, método implantado com auxílio deste projeto. Para se obter resultados confiáveis de um novo método, foram necessários experimentos preliminares para a padronização do mesmo. Neste

período foi definida a espécie de animal a serem utilizadas no modelo experimental, as técnicas para administração de drogas e amostras, além de parâmetros para mensurar os resultados observados, definindo as doses, densidades de inoculação das células tumorais e esquemas de tratamento. Desta forma, foi possível determinar importantes parâmetros relacionados à técnica, tais como reação dos animais e crescimento tumoral, reduzindo tempo, custos e uso de animais, devido à reprodutibilidade dos resultados.

O critério da escolha camundongos da linhagem Balb-C foi considerando por esta ser isogênica garantindo resultados mais homogêneos. Foi adotada administração intraperitoneal das drogas e amostras, pois esta via demonstrou maior reprodutibilidade das respostas observadas. As doses utilizadas foram determinadas a partir de ensaios utilizando diferentes esquemas de tratamentos. Estes ensaios preliminares permitiram detectar a condição de tratamento que consumia o mínimo de amostras, aliado a resultados estatisticamente significativos.

Para a escolha do controle positivo utilizado no modelo experimental foram avaliados os compostos taxol, vincristina, doxorrubicina, ciproterona, e 5-fluorouracil para verificar aquele que demonstrava maior inibição do crescimento tumoral num menor tempo. Os resultados estatísticos demonstraram que o composto 5-fluorouracil foi capaz de diminuir o crescimento tumoral a partir do 4^{o} dia experimental, portanto este foi padronizado como nosso controle positivo no ensaio.

Para determinar a densidade de inoculação foi realizada curva dose resposta da suspensão celular inoculada nos animais. Para isto foram inoculados diferentes grupos de animais com diferentes concentrações de células tumorais. O grupo que foi considerado o melhor foi aquele que apresentou um maior crescimento tumoral e homogeneidade no tempo experimental ($2,5\ 10^{6}$ celulas/ $60\mu l$). O esquema de tratamento adotado foi selecionado considerando a toxicidade aliado a eficácia das amostras.

O*s* resultados obtidos no *capítulo 2* corroboram a potencial atividade antitumoral atribuída aos compostos terpênicos isolados da *P. pubescens* (geranilgeraniol e éster 6*α,*7*β*-diidroxivouacapano-17*β* oato de metila*),* evidenciados pelo decréscimo do crescimento tumoral, mensurado pela diferença do volume da pata dos animais.

Os métodos *in vivo* para avaliação de atividade analgésica (resposta comportamental à dor- nocicepção) tem sido amplamente utilizados, pois representam importantes aspectos relacionados ao processo de analgesia, envolvendo diferentes locais anatômicos, neurotransmissores e receptores. Diferentes técnicas experimentais são utilizadas para avaliar os processos fisiopatológicos envolvidos na resposta à dor (analgesia). A nocicepção é mensurada através da resposta comportamental induzida por estímulos químicos, térmicos ou mecânicos, desta forma, foi necessário desenvolvimento de diferentes modelos experimentais. Este trabalho contribuiu com o desenvolvimento de novas técnicas e equipamentos essenciais para avaliação da potencial atividade analgésica de uma molécula. As técnicas foram desenvolvidas a partir de métodos clássicos previamente utilizados, porém de forma mais refinada, através do uso de agonistas e antagonistas receptor-específicos, e o desenvolvimento de técnicas que avaliam desde a mais sensível reação à dor (alodínia), até a dor na sua forma mais intensa (hiperalgesia). Os agonistas e antagonistas específicos no teste das contorções abdominais são necessários para bloquear ou ativar receptores envolvidos no processo de analgesia, determinando possíveis mecanismos de ação. Estes procedimentos foram previamente padronizados através de curvas-dose resposta a fim de determinar as melhores doses a serem utilizadas. Dentre as novas técnicas empregadas, estão as de avaliação de alodínia e hiperalgesia. Os métodos foram definidos com base na literatura, possibilitando a aquisição de importantes equipamentos para avaliação de atividade antialodínica (Aestesiômetro) e anti-hipernociceptiva (Analgesímetro).

Alguns trabalhos demonstram a ação analgésica de compostos extraídos do gênero *Pterodon*. Resultados preliminares (Spindola, 2006) demonstraram a

caracterização da atividade antinociceptiva do extrato bruto e frações da *P. pubescens*. A partir destes resultados, foi possível biomonitorar compostos isolados que apresentaram bom rendimento e atividade antinociceptiva pronunciada nos ensaios *in vivo*. Dois dos compostos identificados foram selecionados para dar sequência ao estudo das propriedades analgésicas da espécie: geranilgeraniol (C1) e éster 6*α,*7*β*-diidroxivouacapano- 17*β* oato de metila (C2), os mesmos discutidos no *capítulo 2.*

Primeiramente, foi caracterizada a atividade antinociceptiva utilizando diferentes métodos experimentais e vias de administração, a fim de observar possíveis diferenças farmacocinéticas e farmacodinâmicas entre os compostos. Para tanto, foram utilizadas técnicas que avaliam diferentes mecanismos modulatórios de analgesia (*capítulo 3*). Os resultados elucidaram importantes vias de modulação atribuídas aos compostos C1 e C2, sugerindo possíveis mecanismos de ação, importantes na determinação de eficácia e segurança dos compostos. Dando sequência aos estudos da atividade antinociceptiva dos compostos C1 e C2, o *capitulo 4* relatou de forma específica alguns possíveis mecanismos de ação atribuídos a estes. Dentre os mecanismos avaliados, determinou-se a participação da serotonina como um dos principais neurotransmissores envolvidos na mediação antinociceptiva dos compostos.

Os resultados demonstraram importantes mecanismos de antinocicepção, bem como diferenças farmacodinâmicas entre os compostos, tornando-os importantes agentes a serem desenvolvidos, também como analgésicos.

Conclusões

O presente trabalho demonstrou as atividades antinociceptiva e antitumoral de compostos isolados do extrato bruto diclorometânico de sementes da *Pterodon pubescens* Benth. Os resultados obtidos determinaram importantes aspectos relacionados à farmacocinética e farmacodinâmica destes compostos, demonstrando:

1) Potencial quimioterápico em estudos in vitro atribuído aos compostos 6*α* acetoxi-7β-hidroxivouacapano (inédito), éster 6*α,*7*β*-diidroxivouacapano-17*β* oato de metila e 6*α*,7*β*-diidroxivouacapano-17*β*-metilenol contra diversas linhagens tumorais, com ênfase na atividade antitumoral específica para a linhagem de próstata (PC-3), através do ensaio de sulforrodamina B (SRB);

2) Potencial quimioterápico em estudos in vivo atribuído aos compostos geranilgeraniol e éster 6*α,*7*β*-diidroxivouacapano-17*β* oato de metila, utilizando o modelo de Tumor Sólido de Ehrlich na pata de camundongos;

3) Importantes mecanismos da modulação antinociceptiva atribuída aos compostos geranilgeraniol e éster 6*α,*7*β*-diidroxivouacapano-17*β* oato de metila através de diferentes modelos experimentais in vivo: contorções abdominais induzidas por ácido acético, teste da capsaicina, teste do glutamato e teste da placa quente;

4) Importantes mecanismos da ação antinociceptiva receptor-específica atribuída aos compostos geranilgeraniol e éster 6*α,*7*β*-diidroxivouacapano- 17*β* oato de metila através de diferentes modelos experimentais in vivo: alodinia induzida por

CFA, hiperalgesia induzida por carragenina, bloqueio de receptores específicos no teste das contorções abdominais induzidas por ácido acético, revelando principalmente atividade relacionada a receptores imidazólicos e serotonérgicos.

Dentre os parâmetros de eficácia, segurança e padronização necessários para o desenvolvimento de um novo medicamento, este estudo forneceu dados relevantes sobre eficácia dos extratos, frações e compostos isolados da espécie estudada contribuindo com dados para viabilizar um futuro medicamento fitoterápico.

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Anexos

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Comissão de Ética na Experimentação Animal
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CERTIFICADO

Certificamos que o Protocolo nº **766-1**, sobre "**AVALIAÇÃO DA ATIVIDADE ANTINOCICEPTIVA DE FAÇÕES RESULTANTES DO ÓLEO DAS SEMENTES DE PTERODON PUBESCENS BENTH**" sob a responsabilidade de **Profa. Dra. Mary Ann Foglio / Humberto Moreira Spindola** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 17 de fevereiro de 2005.

CERTIFICATE

We certify that the protocol nº **766-1**, entitled "**PTERODON PUBESCENS SEED'S CRUDE OIL AND FRACTIONS' ANTICONCEPTIVE ACTIVITY EVALUATION**", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on February 17, 2005.

Campinas, 17 de fevereiro de 2005.

Profa. Dra. Liana Verinaud
Presidente - CEEA/IB/UNICAMP

Fátima Alonso
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CERTIFICADO

Certificamos que o Protocolo nº **1076-1**, sobre "**ATIVIDADE ANTI-INFLAMATÓRIA, ANTINOCICEPTIVA E ANTICANCER DE FRAÇÕES E PRINCÍPIOS ATIVOS OBTIDOS DO ÓLEO DE *PTERODON PUBESCENS* BENTH**", sob a responsabilidade de **Profa. Dra. Mary Ann Foglio / Humberto Moreira Spindola**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em 30 de agosto de 2006.

CERTIFICATE

We certify that the protocol nº **1076-1**, entitled "**ANTIINFLAMMATORY, ANTINOCICEPTIVE AND ANTICANCER ACTIVITIES OF FRACTIONS AND ACTIVE PRINCIPLES OBTAINED FROM *PTERODON PUBESCENS* BENTH SEEDS**", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on August 30, 2006.

Campinas, 30 de agosto de 2006.

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Short Report

Furanoditerpenes from *Pterodon pubescens* Benth with Selective *in vitro* Anticancer Activity for Prostate Cell Line

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O fracionamento biomonitorado do extrato diclorometânico das sementes de *Pterodon pubescens* Benth forneceu o 6α-acetóxi-7β-hidróxi-vouacapano **1** (inédito), além de quatro diterpenos furânicos (**2**, **3, 4** e **5**). A atividade antiproliferativa dos compostos foi avaliada *in vitro* contra as linhagens de células tumorais humanas UACC-62 (melanoma), MCF-7 (mama), NCI-H460 (pulmão), OVCAR-03 (ovário), PC-3 (próstata), HT-29 (colon), 786-0 (rim), K562 (leucemia) e NCI-ADR/RES (ovário com fenótipo de resistência a múltiplos fármacos). Os resultados foram expressos em três concentrações efetivas GI_{50} (concentração para que ocorra 50% de inibição de crescimento), TGI (concentração que resulta em inibição total de crescimento) e LC_{50} (concentração que resulta em 50% de morte celular). A citotoxicidade *in vitro* foi avaliada também frente a uma linhagem de célula murina normal (3T3). Este é o primeiro relato de atividade anticâncer para os compostos **1**, **4** e **5**, que apresentaram grande seletividade, dependente da concentração, para PC-3. O composto **1** foi 26 vezes mais potente para inibir 50% do crescimento (GI_{50}) de PC-3, 15 vezes mais citostático (TGI) e 6 vezes menos tóxico (LC_{50}) quando comparado com Doxorrubicina (controle).

Activity guided fractionation of *Pterodon pubescens* Benth. methylene chloride-soluble fraction afforded novel 6α-acetoxi 7β-hydroxy-vouacapan **1** and four known diterpene furans **2**, **3**, **4**, **5**. The compounds were evaluated for *in vitro* cytotoxic activities against human normal cells and tumour cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-03 (ovarian), PC-3 (prostate), HT-29 (colon), 786-0 (renal), K562 (leukemia) and NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance). Results were expressed by three concentration dependent parameters GI_{50} (concentration that produces 50% growth inhibition), TGI (concentration that produces total growth inhibition or cytostatic effect) and LC_{50} (concentration that produces −50% growth, a cytotoxicity parameter). Also, *in vitro* cytotoxicity was evaluated against 3T3 cell line (mouse embryonic fibroblasts). Antiproliferative properties of compounds **1**, **4** and **5** are herein reported for the first time. These compounds showed selectivity in a concentration-dependent way against human PC-3. Compound **1** demonstrated selectivity 26 fold more potent than the positive control, doxorubicin, for PC-3 (prostrate) cell line based on GI_{50} values, causing cytostatic effect (TGI value) at a concentration fifteen times less than positive control. Moreover comparison of 50% lethal concentration (LC_{50} value) with positive control (doxorubicin) suggested that compound **1** was less toxic.

Keywords: *Pterodon pubescens*, leguminosae, furanoditerpenes, *in vitro* assay, prostate cell line, cytotoxicity

Intoduction

Throughout history, natural products have afforded a rich source of compounds that have found many applications in the fields of medicine, pharmacy and biology. Within the sphere of cancer, a number of important new commercialized drugs have been obtained from natural sources, by structural modification of natural compounds, or by the synthesis of new compounds, designed following a natural compound as model.

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RESEARCH ARTICLE Open Access

Antinociceptive effect of geranylgeraniol and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester isolated from *Pterodon pubescens* Benth

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Abstract

Background: *Pterodon pubescens* Benth seeds are commercially available in the Brazilian medicinal plant street market. The crude alcoholic extracts of this plant are used in folk medicine as anti inflammatory, analgesic and anti-rheumatic preparations. The aim of this study was to evaluate the contribution of geranylgeraniol (C1) and 6α, 7β-dihydroxyvouacapan-17β-oate methyl ester (C2) isolated from *Pterodon pubescens* Benth. to the antinociceptive activity of the crude extract.

Results: Compounds C1 and C2 demonstrated activity against writhing with intraperitoneal (i.p.) and oral (p.o.) routes, capsaicin (i.p. and p.o.), glutamate (i.p.), and in the hot-plate (p.o.) tests, demonstrating their contribution to the antinociceptive activity of crude *Pterodon pubescens* Benth extracts. The observed activity of compounds C1 and C2 may be related to vanilloid receptors VR1, and/or glutamate peripheral receptors. In hot-plate model, the antinociceptive activity was maintained when naloxone chloride (opioid antagonist) was administered prior to treatment with compounds suggesting that C1 and C2 (p.o.) do not exert their antinociceptive effects in the hot-plate test via opioid receptors. The findings presented herein also suggest that compounds within the crude *Pterodon pubescens* Benth. extract may exert a synergistic interactive effect, since the crude extract (300 mg.kg^{-1}) containing lower concentrations of compounds C1 (1.5%= 34.6 mg. kg^{-1}) and C2 (1.5% = 4,7 mg.kg^{-1}) gave statistically the same effect to the pure compounds when tested separately (C1 + C2 = 300 mg.kg^{-1}) in writhing experimental model with p.o. administration. Further studies will be undertaken to establish more specifically the mechanisms of action for compounds C1 and C2. Possible synergistic interactions will be evaluated employing the Isobole method.

Conclusion: These results allowed us to establish a relationship between the popular use of *Pterodon pubescens* seeds for pain relief and the activity of two major compounds isolated from this species which demonstrated antinociceptive activity. Various "in vivo" experimental models corroborate the folk use of this species for different pain and inflammation disorders.

Background

Pterodon pubescens

Benth. (Leguminosae), known as *sucupira*, is widespread throughout the Brazilian states of Goiás, Minas Gerais and São Paulo. Sucupira seeds are commercially available in the Brazilian medicinal plant market. The crude alcoholic extracts of this plant are used in folk medicine as anti-inflammatory, analgesic and anti-rheumatic preparations [1,2]. Phytochemical studies of the *Pterodon* genus have shown the presence of alkaloids, isoflavones and diterpenes. Furanditerpenes were identified and isolated from *Pterodon* fruits [3-7]. Studies have suggested that furanditerpenes possessing the vouacapan skeleton contribute to the anti-inflammatory and antinociceptive properties of *Pterodon pubescens* seed oil [8-12]. Diterpenes 6α-hydroxyvouacapan-7β-17β-lactone and 6α, 7β-dihydroxyvouacapan-17β-oate methyl ester, found in *P. emarginatus* and *P. polygalaeflorus* seeds were

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