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EMANUELA CARLA DOS SANTOS

MMP-1 NO REPARO DE ÚLCERAS BUCAIS DE RATOS TRATADOS COM A FRAÇÃO ALCALOIDE DO PAU PEREIRA

Curitiba
2016

EMANUELA CARLA DOS SANTOS

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do título de Mestre em Odontologia, Área de Concentração em Estomatologia.

Orientadora: Profª. Drª. Ana Maria Trindade Gregio.

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Resumo

Considerando que a fração alcaloide do Pau Pereira acelera a deposição de colágeno tipo I em úlceras de línguas de ratos tratados por 14 dias, e que a MMP-1 é a principal enzima envolvida na degradação do colágeno tipo III para que ocorra a deposição do colágeno tipo I, o objetivo desta pesquisa foi verificar imunoexpressão da metaloproteinase 1 (MMP-1) nessas úlceras. Foram utilizados 48 ratos machos (Wistar), que receberam anestesia e após induziu-se úlceras no dorso de língua usando micropunch. Os animais foram divididos em grupo tratado (50 microlitros de fração alcaloide de Pau Pereira em dimetilsulfóxido) e controle (50 microlitros de dimetilsufóxido), subdivididos de acordo com o tempo de tratamento: 2, 7, 14, 21 dias, sendo 3 aplicações diárias. Após este período, foram mortos por superdosagem anestésica e as línguas foram removidas, processadas e emblocadas em parafina. Os cortes histológicos foram dispostos em blocos de TMA (Tissue Microarray) e submetidas a imunohistoquímica para o anticorpo anti MMP-1. As lâminas foram escaneadas (Zeiss) e as imagens analisadas no Programa Image Pro PLUS, no qual obteve-se a porcentagem de área de expressão de MMP-1 por área de tecido conjuntivo. A expressão de MMP-1 aos 14 dias foi maior no grupo tratado com Pau Pereira, tendo diferença estatística significante, quando comparado ao grupo controle. Conclui-se que a fração alcaloide do extrato de Pau Pereira interfere na imunoexpressão da MMP-1 aos 14 dias do processo de reparo, podendo contribuir com a maior deposição de colágeno tipo I neste período.

Palavras-chave: úlcera oral; cicatrização; metaloproteinase-1 da matriz; Apocynaceae; *Geissospermum velosii.*

Introdução

O uso de plantas medicinais para tratamento de várias desordens sistêmicas e bucais vem crescendo em todo o mundo [1, 2]. Estima-se que 48% da população dos Estados Unidos utilizem tratamentos não convencionais, inclusive a fitoterapia, para tratamento de doenças [2]. No Brasil não existem números oficiais que estimem esse uso das plantas, mas, seguramente, segue essa tendência mundial. Nosso país é rico em biodiversidade, sendo a flora abastecida por diversas espécies vegetais com potencial terapêutico, desta forma é alvo de estudo pela comunidade científica. A floresta amazônica representa 30% de todas as florestas tropicais remanescentes no mundo, com 53 grandes ecossistemas [3]. Nela é possível encontrar uma grande variedade de ervas medicinais utilizadas pelos nativos [4].

"Pau Pereira" (*Geissospermum vellosi* ou *Geissospermun leave*), pertencente à família Apocynaceae, é uma árvore de porte médio encontrada principalmente na Amazônia. A sua casca é usada pela população nativa para o tratamento da malária, febre, distúrbios do estômago, constipação, tontura, estimulante sexual e para tratamento da dor [4-7]. Isto despertou interesse entre médicos e farmacêuticos já em 1838, quando Ezequiel Corrêa dos Santos isolou os princípios ativos e os descreveu como sendo alcaloides [8]. São os princípios ativos que conferem às plantas suas propriedades terapêuticas, sendo que não atuam isoladamente, e sim, em conjunto com outras substâncias, proporcionando aos fitoterápicos ação mais suave do que as medicações alopáticas [9 - 11].

Os alcaloides encontrados na casca do Pau Pereira são a geissosquizina, geissospermina, flavopereirina e geissoschizolina. Eles podem ser amplamente utilizados na medicina, como por exemplo, a flavopereirina, que tem atividade contra protozoários parasitas causadores da malária, ação anticancerígena, anti-inflamatória e também tem sido estudada para o tratamento da infecção pelo vírus HIV [7, 8, 12 - 15]. Lima et al. demonstraram sua propriedade anti-inflamatória, através de ação antiedematogênica no processo inflamatório [13]. Devido às suas inúmeras propriedades biológicas, esta planta vem sendo investigada para o tratamento de diversas alterações patológicas [7, 12, 14].

A úlcera bucal é uma das queixas mais frequentes na prática odontológica [16]. Esta lesão acomete indivíduos no mundo todo, podendo afetar até 25% da

população mundial [17]. As ulcerações bucais impactam negativamente na qualidade de vida das pessoas e bem-estar geral [18]. Elas são caracterizadas por um processo inflamatório localizado e doloroso, geralmente ovoide, com um centro necrótico branco-amarelado cercado por um halo eritematoso, com etiologia multifatorial ainda não esclarecida [19, 20]. Os fármacos tradicionais de eleição para o tratamento das úlceras bucais são os corticosteroides de uso tópico. Entretanto, apesar de usados topicamente, podem ser absorvidos e causar efeitos sistêmicos adversos que serão dependentes do modo, tempo e dose de administração. Estes fármacos tratam apenas os sintomas, não alterando o processo de cicatrização, que leva de 10-14 dias [21, 22].

O processo de cicatrização é complexo, muito bem organizado e depende de vários eventos intra e extracelulares. Dentre estes eventos, a deposição de colágeno é fundamental para este processo. Inicialmente o colágeno tipo III é depositado e, no período mais avançado do processo de reparo tecidual, é substituído pelo tipo I [23]. O presente grupo de pesquisa verificou em estudos prévios que a fração desta planta tem propriedades anti-inflamatórias, sendo capaz de reduzir o número de macrófagos e neutrófilos, e acelerar a cicatrização de úlceras através do aumento do número de fibroblastos e consequente deposição de fibras colágenas. Foi possível verificar maior deposição de colágeno I (colágeno maduro) decorridos 14 dias do momento em que ocorreu a lesão, quando comparado com o grupo controle. Esse colágeno tipo I conferiu maior resistência à ferida em período precoce do processo de cicatrização [24, 25]. Diante disto postulou-se um possível papel da metaloproteinase-1 (MMP-1) na degradação do colágeno tipo III permitindo a deposição de uma maior quantidade de colágeno tipo I.

As metaloproteinases (MMP's) são enzimas pertencentes à família das metaloendopeptidases e tem um papel central na cicatrização [26, 27]. Elas promovem a degradação de componentes da matriz extracelular, como colágeno intersticial, entre outros, participando ativamente da remodelação tecidual. As células que produzem as MMP's são os leucócitos polimorfonucleares, queratinócitos, macrófagos, fibroblastos e células mesenquimais [28]. MMP-1, MMP-8 e MMP-13 são as únicas com a capacidade de clivar o colágeno, chamadas também de colagenases, sendo que a MMP-1 tem maior afinidade em degradar o colágeno tipo III [26, 27].

Desta forma, esta pesquisa tem como objetivo verificar a imunoexpressão da MMP-1, em úlceras bucais de ratos tratados com a fração alcaloide do extrato de Pau Pereira.

Material e Método

Extração da Fração

A fração de Pau Pereira foi extraída no laboratório de fitoquímica da Universidade Federal do Paraná. As cascas de *G. Vellosii* foram colhidas em novembro de 2002, pela Químer Comercial Ltda, e identificadas por comparação com espécie registrada no Museu Botânico de Curitiba, sob número 36060. Todo o processo de extração do extrato bruto, assim como as frações e o reconhecimento químico destas frações, foram desenvolvidos de acordo com a técnica de Obdulio Gomez Miguel, que está descrito no artigo publicado "Evidence for a role of 5-HT(1A) receptor on antinociceptive action from Geissospermum vellosii" [29].

Para obtenção do extrato e da fração derivada da planta dispôs-se do seguinte processo: as cascas do espécime foram moídas e depositadas em cartucho de vidro sintetizado e submetidas à extração em Soxhlet (Bunker®, Piracicaba, Brasil), em etanol absoluto, até esgotamento total. Logo o extrato obtido foi filtrado com algodão e submetido à concentração em evaporador rotatório com pressão reduzida à temperatura de 50ºC e 90 rpm até redução em 1/5 de seu volume inicial. Em seguida os extratos foram armazenados, em frascos âmbares, no freezer por 24 horas e filtrados a vácuo em funil de Büchner. O extrato bruto etanólico foi transferido para recipiente de vidro e para o extrator de Soxhlet modificado. Posteriormente foi adicionado o diclorometano e extraído por um período de 4 horas, sofrendo partição líquido-líquido e obtendo-se a fração diclorometano. Parte da fração diclorometano foi submetida à partição líquido-líquido com acetato de etila e butanol, obtendo-se fração butanol, fração acetato de etila e fração hidroalcóolica remanescente. Utilizando a técnica de Miguel [29], 1(um) mL de extrato bruto etanólico e respectivas frações, foram colocadas em cadinhos pesados e encaminhados para estufa a 100ºC até peso constante para obter-se o teor de sólidos. O resultado foi apresentado em porcentagem, em peso do teor de sólidos em relação ao material vegetal. O extrato e respectivas frações foram divididos, uma parte foi seca e a outra parte

foi conservada em câmara fria, com intuito de eliminar fatores de alterações como luz, temperatura e contaminações microbiológicas.

Posteriormente a obtenção da fração, passou-se para o isolamento de constituintes químicos da fração diclorometano, a qual foi acrescida de sílica gel 60 (70 - 230 mesh) e levada à evaporação até secura (pastilha: fração + sílica). A pastilha da respectiva fração foi cromatografada por meio de uma coluna de três cm de diâmetro por 40 cm de altura com sílica gel 60 (70 - 230 mesh) modificada com bicarbonato de sódio como corrobora Prado, Faria e Padilha [30]. Utilizando fases móveis apropriadas com uma vazão de um ml por minuto, coletaram-se 44 frascos de 50 ml, os quais foram monitorados por cromatografia em camada delgada (CCD) em placas aluminizadas Merck® 60 F254. Para a CCD utilizou-se a fase móvel acetato de etila e tolueno na proporção de 90: 10 (v/v) com vapores de hidróxido de amônio e para revelar, uma solução ácida de Reagente de Dragendorff. Seguiu-se então para identificação; as substâncias isoladas foram submetidas à análise de ressonância magnética nuclear de 1H e 13C. Para as análises de 13C RMN, utilizou-se espectrofotômetro a 75 MHz e para as análises de 1H RMN, a 300 MHz. Os deslocamentos químicos foram expressos em ppm em relação ao sinal do TMS (tetrametilsilano – (CH3)4Si) como referência em δ 0 ppm. O solvente utilizado na espectroscopia 13C e 1H RMN foi dimetilsulfóxido deuterado. A fração diclorometano (66,7570 g) foi cromatografada em sílica gel modificada (66,6417 g). Utilizaram-se 100 ml de cada fase móvel, iniciando com hexano 100%, com acréscimo de 5% de diclorometano (95:05 - v/v), num gradiente de polaridade crescente, aumentando-se a proporção de diclorometano até 100% e acrescentando metanol na ordem de 10% até metanol 100%. Ao final, água destilada 100% foi acrescida para limpeza da coluna. Foram recolhidos 44 frascos com aproximadamente 50 ml de eluato. Um dos frascos foi submetido a uma solução gelada de metanol para purificação [29].

Amostra

A pesquisa foi aprovada pelo Comitê de Ética em Uso de Animais (CEUA-01043/2016 - PUCPR). Foram utilizados 48 ratos machos Wistar, com idade entre 45-50 dias e cerca de massa corporal 200 gramas do Biotério Central da Pontifícia Universidade Católica do Paraná. Os animais foram alimentados com

Nuvilab® CR-1 (Nuvital Nutrientres S.A.) e água ad libitum, com um ciclo de luz-escuro de 12 horas e temperatura ambiente (25 ± 1 ° C).

Indução de Úlceras

Os animais foram previamente anestesiados com Tiopental Sódico (30mg/Kg, Cristália, Itapira, Brasil), via intraperitoneal. As lesões na cavidade bucal foram induzidas mecanicamente na região central do dorso da língua, por meio de um *punch* (R-806-9-4; Richter®, São Paulo, Brasil) de 4,0 mm. A técnica consistiu em fazer movimentos de rotação e pressão com o instrumento, aprofundando-o até se notar resistência [31]. Durante os quatro primeiros dias após indução das úlceras, os animais receberam via intramuscular dipirona sódica (20 mg/kg; Boehringer Ingelheim do Brasil Química e Farmacêutica Ltda, Itapecerica da Serra, Brasil).

Tratamento Experimental

Após a indução mecânica de úlceras, os animais foram distribuídos aleatoriamente por oito grupos de seis espécimes. Quatro grupos serviram como controles (GC2, GC7, GC14, GC21) e apenas receberam a aplicação tópica de 50 microlitros da solução de dimetilsufóxido – DMSO (Galena®, Campinas, Brasil), duas vezes por dia. Outros quatro grupos (GE2, GE7, GE14 e GE21) receberam duas doses diárias de 50 microlitros de solução tópica de Pau Pereira. A aplicação era realizada utilizando Microbrush® (Microbrush® INTERNATIONAL Grafton, Wisconsin, Estados Unidos da América). Os animais foram mortos por overdose de tiopental de sódio (100mg/Kg, Cristália, Itapira, Brasil) aos 2, 7, 14 e 21 dias, por via intraperitoneal.

Preparação das Lâminas

Após a morte dos ratos, as línguas foram removidas com lâmina de bisturi número 15 e os espécimes armazenados em solução fixadora de formalina a 10% por 24 horas. Posteriormente foram seccionados na região mediana do seu dorso no sentido longitudinal, processados e emblocados em parafina. Foram obtidos cortes histológicos com 4 micrômetros de espessura, sendo corados por hematoxilina e eosina. A área da úlcera foi delimitada com caneta para retroprojetor azul 1.0 milímetro (Pilot do Brasil®, São Paulo, Brasil) e as amostras

foram extraídas do bloco doador usando uma broca trefina (Neodent®, Curitiba, Brasil), com 3 mm de diâmetro, acoplada em um motor de suspensão de 130 watts de Bethil (Prometal Ind. Met. LTDA®, Marília, Brasil) e foram posicionadas no bloco receptor de acordo com o mapa cartesiano, previamente confeccionado, com a localização de cada amostra para TMA (tissue microarray).

Reação Imunohistoquímica para MMP-1

Os cortes histológicos foram desparafinados, (xileno, Biotec, Curitiba, Brasil), hidratados (álcool na diminuição soluções, Biotec®, Curitiba, Brasil) e incubados em solução de peróxido de hidrogênio (Biotec®, Curitiba, Brasil) e metanol 5% (Biotec®, Curitiba, Brasil). Este material foi submetido para a recuperação de antígeno com Immuno Retriver (Dako®, Carpinteria, CA), seguido por incubação com anti-MMP-1 (clone : EP1252Y Abcam, Cambridge, MA) a 4 ° C durante a noite. A diluição do anticorpo em diluente de anticorpo com componentes redutores (DAKO Carpinteria, CA, código S302283) foi de 1: 200. A detecção foi realizada com Advance Link (Dako, Carpinteria, CA código K406889), seguido pela enzima Advance (DAKO Corporation, Carpinteria, CA, K406889 código), e por cromogênio 3,3'-tetraidrocloreto de diaminobenzidina (DAB - Sigma Chemical®, St. Louis, código D7679). As lâminas foram contra-coradas por hematoxilina de Harris (Biotec®, Curitiba, Brasil), desidratadas (etanol) e diafanizadas (xileno).

Análise da Imunoexpressão

As lâminas foram escaneadas com o escâner de lâminas Zeiss Axio Scan.Z1 (Zeiss®, Oberkochen, Alemanha) analisadas por um único observador. Uma imagem foi capturada de cada corte através do programa ZEN 2 (Blue Edition® Carls Zeiss Microscopia GmbH, 2011), na magnificação de 200X. De cada imagem, epitélio, fibras musculares, tecido adiposo e artefatos de imagem foram removidos com Adobe Photoshop® CS6 extended version 13.0. As estruturas ou células coradas de marrom foram consideradas imunopositivas. As imagens foram analisadas no software Image-Pro Plus 4.5 (Media Cybermetics®, Silver Spring, MD) e as medidas foram obtidas por segmentação da imagem, obtendo-se a área de MMP-1 em micrometros quadrados e da área total de

tecido conjuntivo. Para calcular a porcentagem de área imunopositiva foi utilizada a seguinte fórmula:

$$\frac{Área\ imunopositiva\ * 100}{Área\ Total\ em\ \mu m^2}$$

Os valores do colágeno tipo I foram obtidos do trabalho prévio [20].

Análise Estatística

Na análise estatística foi utilizado o programa IBM SPSS Statistics 23.0 (SPSS Inc, Chicago, Illinois, USA). Foi realizado o Teste de Normalidade de Kolmogorov-Smirnov, no qual se observou que a variável MMP-1 apresentou distribuição normal. Por isto, visando comparar os valores médios da variável nos grupos que apresentaram diferença estatisticamente significante realizou-se o teste ANOVA a dois critérios modelo fatorial completo. O teste de homogeneidade de variâncias de Levene revelou que a variável apresentou variância heterogênea. Portanto, as comparações entre os grupos foram feitas utilizando-se o teste de comparações múltiplas paramétricas de Games-Howell. O nível de significância adotado em todos os testes foi de 5% ($p<0,05$).

Resultados

A expressão de MMP-1 foi significativamente menor no grupo controle (Figura 1A), quando comparado ao grupo experimental (Figura 1B), aos 14 dias. O mesmo foi observado para colágeno tipo I (Tabela 1).

Tabela 1. Porcentagem de expressão de colágeno tipo I [25] e imunoexpressão de MMP-1 nos grupos controle e experimental, aos 2, 7, 14 e 21 dias.

	Tempo ***GRUPO***	***2 dias*** (M±DP)	***7 dias*** (M±DP)	***14 dias*** (M±DP)	***21 dias*** (M±DP)
Colágeno tipo I [20]	C (%)	35,44 ± 11,68	32,36 ± 3,25	26,06 ± 0,37	33,11 ± 7,97
	E (%)	29,96 ± 11,54	31,58 ± 8,18	28,01 ± 2,09	35,97 ± 5,60
	Valor de p*	0,94	1,0	**0,03**	0,98
MMP-1	C (%)	2,52 ± 1,48	5,44 ± 2,41	2,62 ± 1,58	3,18 ± 0,66
	E (%)	3,05 ± 1,15	5,63 ± 2,96	5,01 ± 2,73	3,95 ± 1,72
	Valor de p*	0,96	1,0	**0,02**	0,84

Teste de ANOVA p<0.05; * Teste de Games-Howell; Em negrito valores de p<0,05; M= média; DP=desvio padrão; C= controle; E= experimental.

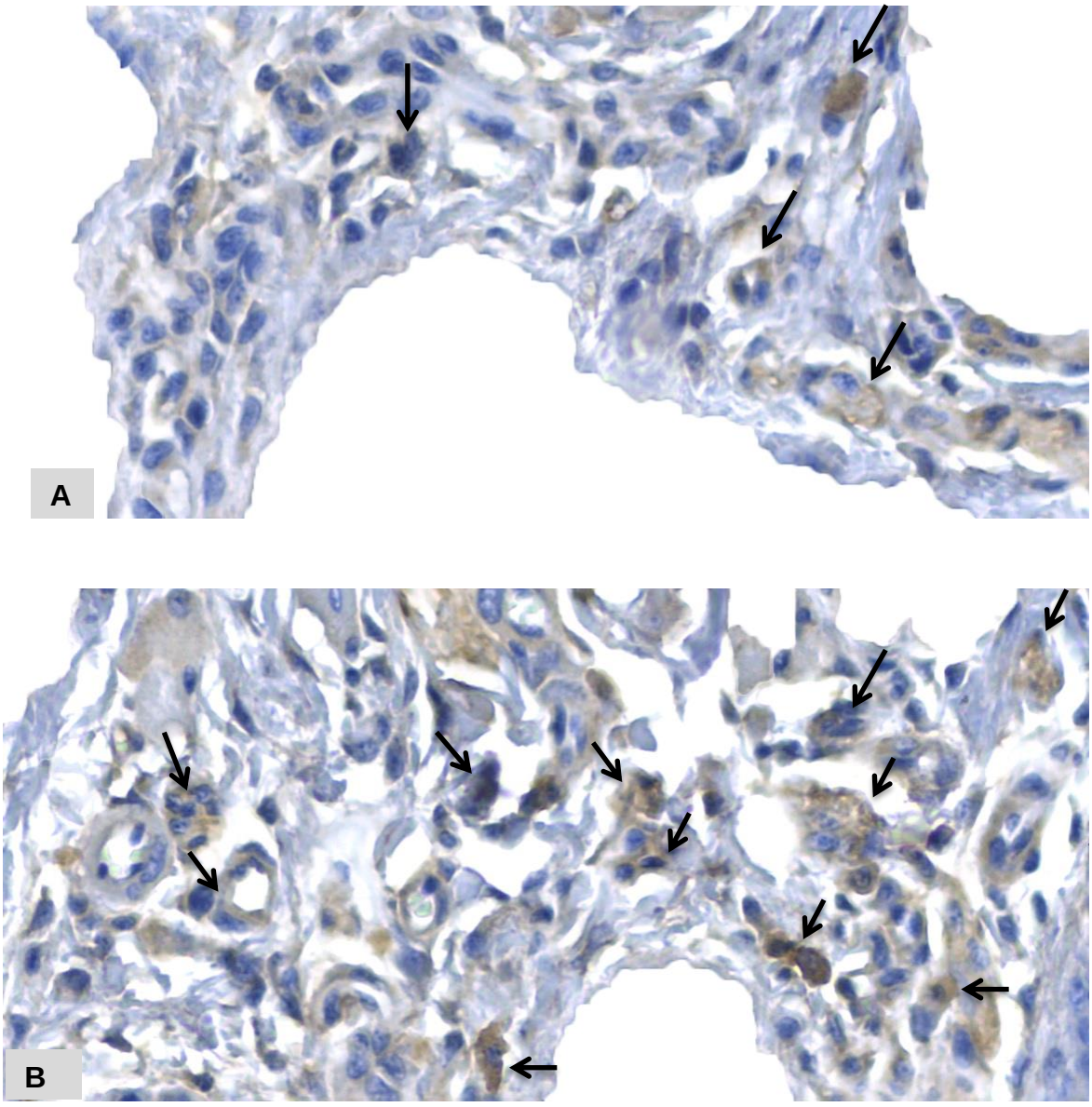

Figura A. Fotomicrografia do tecido de granulação aos 14 dias, A) grupo controle e **B)** grupo Pau Pereira revelando células e estruturas imunopositivas para MMP-1 em marrom (setas, magnificação de 200x).

Discussão

O presente estudo traz como resultado principal a porcentagem elevada das MMP-1 aos 14 dias, quando comparado ao grupo controle. As colagenases são fundamentais no processo de cicatrização, elas degradam as fibras colágenas, possibilitando a remodelação. A MMP-1 é a colagenase responsável pela degradação do colágeno tipo III. Durante o processo de uma injúria normalmente ela é produzida e secretada em estágios mais tardios, na fase de remodelação da cicatrização [26]. Este fato explica o efeito cicatrizante da fração

de Pau Pereira que acelerou a degradação do colágeno tipo III, através da MMP-1, sendo que esta tem maior predileção por degradar o colágeno imaturo, já em fase precoce do processo de cicatrização, permitindo a substituição do mesmo por colágeno do tipo I, conferindo maior resistência à ferida aos 14 dias [25].

Na ausência de estudos que avaliem a influência dos alcaloides do Pau Pereira na expressão da MMP-1, buscou-se na literatura outros alcaloides que poderiam intervir na expressão desta enzima. Assim como este estudo, que verificou aumento na porcentagem de MMP-1 em úlceras bucais de animais tratados com a fração alcaloide, Hu et al. também constatou aumento desta metaloproteinase frente ao uso do alcaloide hidroxi-camptotecina em fibrose pulmonar induzida em ratos [32]. Chung et al. notou aumento na expressão de MMP-1 quando estudou a ação da colchicina em fibroblastos de derme humana [33]. Lee et al. analisou a expressão da MMP-1 no ligamento periodontal de humanos diante do uso da nicotina, o alcaloide que constitui o princípio ativo do tabaco, e percebeu que esta substância tem uma regulação positiva das MMP's, incluindo a MMP-1 [34]. Estes estudos confluem para o fato que os alcaloides tem capacidade de influenciar a expressão da metaloproteinase-1.

No processo de cicatrização os biomarcadores com maior potencial são as citocinas e proteases, dentre elas as metaloproteinases [35]. Snyder et al. e Chromy et al. estudaram as MMP's em processo de reparo e postularam que as MMP's possam ser os biomarcadores adequados para estimar o comportamento do processo de cicatrização. Os autores afirmam que no processo normal de cura níveis mais elevados de colagenases são necessários para remodelação [36, 37]. Isto reforça que o maior nível de MMP-1 encontrada aos 14 dias é favorável a este processo.

Estudos correlacionam mudanças nos níveis de certas proteínas do hospedeiro, incluindo citocinas, quimiocinas, MMP's juntamente com outras proteases, com proteínas marcadoras de inflamação [38]. Além de estarem relacionadas à remodelação, as metaloproteinases também participam na regulação de outros mecanismos presentes no processo de reparo. Segundo Gill et al., a produção de alguns fatores que influenciam a inflamação é controlada por proteínas, incluindo as MMP's. Elas podem estimular ou degradar mediadores inflamatórios, como citocinas, quimiocinas e controlar atividade quimiotática, possibilitando migração celular. Estudos levam a conclusão de que

as MMP’s não participam somente da degradação da matriz extracelular, mas também tem papel fundamental na regulação da inflamação [27, 38]. Estes achados corroboram com o trabalho de Fosquiera et al., que demonstrou alterações no número de células envolvidas no processo inflamatório, frente a ação da fração do Pau Pereira [24]. Estudos correlacionando MMP-1 com modulação inflamatória e o alcaloide precisam ser realizados com a finalidade de esclarecer essa possível interação.

Dentro das Limitações deste estudo podemos destacar o uso de animais, o que pode alterar a resposta inflamatório e imunológica.

A metaloproteinase-1 apresenta-se em níveis mais elevados nos animais submetidos ao tratamento com a fração, aos 14 dias comparando com o grupo controle. Sendo assim, a fração alcaloide do extrato da casca da árvore Pau-Pereira é capaz de interferir na expressão da metaloproteinase-1. Tal fato associado a resultados prévios mostra que esta substância é facilitadora do processo de cicatrização de úlceras em línguas de ratos aos 14 dias.

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ARTIGO EM INGLÊS

Title page

MMP-1 in the repair of oral ulcers of rats treated with the alkaloid fraction of Pau Pereira.

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Abstract

Considering that the alkaloid fraction of Pau Pereira accelerates the deposition of type I collagen in rat tongue ulcers at 14 days, the objective of this study was to verify metalloproteinase 1 (MMP-1) immunoexpression in these ulcers. MMP-1 are the main enzymes involved in the degradation of type III collagen that allows type I collagen deposition. 48 male rats (Wistar) were used, which received anesthesia and then ulcers were induced on the back of the tongue using micropunch. The animals were divided into treated (50 microlitres of Pau Pereira alkaloid fraction in dimethyl sulfoxide solution) and control (50 microlitres of dimethyl sulfoxide), three times per day, and subdivided according to the treatment time: 2, 7, 14, 21 days. After this period, they were killed by anesthetic overdosage and the tongues were removed, processed and embedded in paraffin. The samples were placed in TMA blocks (Tissue Microarray) and submitted to immunohistochemistry. The slides were scanned (Zeiss) and the images analyzed in the Image Pro PLUS Program, in which the percentage of MMP-1 expression per area of connective tissue was obtained. The expression of MMP-1 at 14 days was greater, with a statistically significant difference when compared to the control group. These results suggests that the alkaloid fraction of the Pau Pereira extract interferes in the MMP-1 immunoexpression at 14 days of the repair process, and may contribute to the higher deposition of type I collagen in this period.

Key-words: oral ulcer; wound healing; matrix metalloproteinase-1; Apocynaceae; *Geissospermum velosii.*

Introduction

The use of medicinal plants for the treatment of various systemic and oral disorders has been growing worldwide [1, 2]. It is estimated that 48% of the United States population uses non-conventional treatments, including phytotherapy, for diseases treatment [2]. In Brazil, there is no official number estimating the use of plants, but surely, it follows this world trend. Brazil is rich in biodiversity, our flora is supplied by several species with therapeutic potential, being the target of study by the scientific community. The Amazon forest represents 30% of all remaining tropical forests in the world, with 53 large ecosystems [3]. It is possible to find a great variety of medicinal herbs used by the natives [4].

"Pau Pereira" (Geissospermum vellosi or Geissospermun leave), that belongs to the Apocynaceae family, is a medium sized tree found mainly in the Amazon. Its bark is used by the native population for the treatment of malaria, fever, stomach disorders, constipation, dizziness, sexual stimulant and for the treatment of pain [4-7]. This aroused interest among doctors and pharmacists in earlies 1838, when Ezequiel Corrêa dos Santos isolated the active principles and described them as alkaloids [8]. The active principles give plants their therapeutic properties, and they do not act alone, but in conjunction with other substances, providing phytotherapics milder action than allopathic medications [9-11].

The alkaloids found in the bark of Pau Pereira are geissosquizine, geissospermine, flavopereirine and geissoschizoline. They can be widely used in medicine, for example, flavopereirine, which has activity against the protozoans parasites that causes malaria, anticancer, anti-inflammatory action and has also been studied for the treatment of HIV virus infection [12,13,17 , 18]. Lima et al. (2016) demonstrated its anti-inflammatory properties through antiedematogenic action in the inflammatory process [16]. Due to its various biological properties, this plant has been investigated for the treatment of various pathological changes [12,15,17].

Oral ulceration is one of the most frequent complaints in dental practice [16]. This lesion affects individuals worldwide, affecting up to 25% of the world population [17]. Oral ulcerations impact negatively people's quality of life and

general well-being [18]. They are characterized by a painful and localized inflammatory process, usually ovoid, with a white-yellow necrotic center surrounded by an erythematous halo, its etiology is multifactorial, but it is not clarified yet [19 20]. The traditional drugs of choice for the treatment of oral ulcers are topical corticosteroids. However, although they are used topically, they may be absorbed and can cause adverse systemic effects which will be dependent on the mode, time and dose of administration. These drugs only treat the symptoms, not altering the healing process, which takes 10-14 days [21, 22].

Wound healing process is complex, very well organized and depends on several intra and extracellular events. Among these events, the deposition of collagen is fundamental to this process. Initially type III collagen is deposited and, in advanced period of the tissue repair process, is replaced by type I [23]. Our research group verified in previous studies that the fraction of this plant has anti-inflammatory properties, being able to reduce the number of macrophages and neutrophils, and to accelerate the healing of ulcers through the increase of fibroblasts and consequent deposition of collagen fibers. It was possible to verify greater deposition of collagen I (mature collagen) after 14 days from the moment of injury when compared to the control group. The type I collagen conferred greater wound resistance at an early stage of the healing process [24, 25]. In view of this, a possible role of metalloproteinase-1 (MMP-1) was postulated in the degradation of type III collagen allowing the deposition of a greater amount of type I collagen.

Metalloproteinases (MMPs) are enzymes belonging to the family of metalloendopeptidases and play a central role in healing [26, 27]. They promote the degradation of components of the extracellular matrix, such as interstitial collagen, among others, participating actively in tissue remodeling. The cells that produce MMP's are polymorphonuclear leukocytes, keratinocytes, macrophages, fibroblasts and mesenchymal cells [28]. MMP-1, MMP-8 and MMP-13 are the only ones with the ability to cleave collagen, also called collagenases, and MMP-1 has a greater affinity for type III collagen [26,27].

Thus, this research aims to verify the immunoexpression of MMP-1 in oral ulcers of rats treated with the alkaloid fraction of the Pau Pereira extract.

Material and Methods

Fraction Extraction

The fraction of Pau Pereira was extracted at the phytochemical laboratory of the Federal University of Parana. Barks G. vellosii were taken in November 2002 by Quimer Comercial Ltda, and identified by comparison with species recorded in Curitiba Botanical Museum – registration number 36060 (Brazil). The extraction process of the crude extract, as well as the fractions and chemical recognition of those fractions, were developed according to Obdulio Gomez Miguel technique, as described in the published article “Evidence for a role of 5-HT(1A) receptor on antinociceptive action from Geissospermum vellosii” [29].

To obtain the extract and the plant derived fraction we have the following process: Specimen barks were grinded and deposited in a synthesized glass cartridge and the extraction happened in Soxhlet (Bunker®, Piracicaba, Brasil), in absolute ethanol, until complete exhaustion. Then the extract was filtered with cotton and subjected to concentration in a rotary evaporator with reduced pressure at 50 ° C and 90 rpm to reduce by 1/5 of its initial volume. Then the extracts were stored in amber bottles in a freezer for 24 hours and vacuum filtered on Buchner funnel. The crude ethanol extract was transferred to the glass container and the modified Soxhlet extractor. Thereafter it was added the dichloromethane and extracted for a period of 4 hours, undergoing liquid-liquid partition and obtaining the fraction of dichloromethane. Part of the dichloromethane fraction was subjected to liquid-liquid partition with ethyl acetate and butanol, yielding butanol fraction, ethyl acetate fraction and remainder fraction hydroalcoholic. Using MIGUEL [29] technique, one (1) ml of crude ethanolic extract and its fractions were put in crucibles put into an oven at 100ºC until the weight be constant to obtain the solids content. The result was shown in percentage by weight of the solids content in the plant material. The extract and its fractions were divided, one part was dry and the other part was kept in cold storage, in order to eliminate changes in factors such as light, temperature and microbiological contamination.

It was followed by the isolation of chemical constituents fraction of dichloromethane, which was added silica gel 60 (70 - 230 mesh) and brought to evaporation till dryness (tablet: fraction + silica). The tablet of the respective

fraction was chromatographed through a column of 3 cm diameter by 40 cm height with silica gel 60 (70 - 230 mesh) modified with sodium bicarbonate as confirmed by Prado, Faria and Padilha [30] with modifications. Using appropriate mobile phase with a flow rate of 1 ml per minute, were collected from 44 bottles of 50 ml, which were monitored by thin layer chromatography (TLC) on aluminized Merck® 60 F254 plates. For the TLC used the mobile phase ethyl acetate and toluene in the ratio 90: 10 (v / v) with ammonium hydroxide vapors and, to reveal, an acidic solution Dragendorff reagent. There followed for identification; the isolated substances were subjected to nuclear magnetic resonance analysis of $_{1}H$ and $_{13}C$. . For analysis of $_{13}C$ RMN spectrophotometer was used at 75 MHz and for $_{1}H$ RMN analysis, at 300 MHz. Chemical shifts were expressed in ppm relative to TMS signal (tetramethylsilane - $(CH_3)_4Si$) as reference δ 0 ppm. The solvent used in spectroscopy $_{13}C$ and $_{1}H$ RMN were deuterated dimethyl sulfoxide. The dichloromethane fraction (66.7570 g) was chromatographed on silica gel modified (66.6417 g). 100 ml were used for each mobile phase, starting with 100% hexane with addition of 5% dichloromethane (95:05 - v / v) in an increasingly polar gradient, increasing the proportion of dichloromethane to 100% and adding methanol in the order of 10% to 100% methanol. Finally, distilled water 100% was added to the column cleaning. 44 bottles were collected with about 50 ml of eluate. One of them was subjected to a cold solution of methanol for purification [29].

Sample

The research was approved by the Committee on Ethics in the Use of Animals (CEUA-01043/2016 - PUCPR). A total of 48 male Wistar rats, aged 45-50 days and about 200 grams body mass, were used at the Central Biotério of the Pontifical Catholic University of Paraná. The animals were fed Nuvilab® CR-1 (Nuvital Nutrientres S.A.) and water ad libitum, with a dark-light cycle of 12 hours and room temperature (25 ± 1 ° C).

Induction of Ulcers

The animals were previously anesthetized with Sodium Thiopental (30mg / kg, Cristália, Itapira, Brazil), intraperitoneally. The lesions in the oral cavity were mechanically induced in the central region of the tongue dorsum, using a 4.0 mm punch (R-806-9-4; Richter®, São Paulo, Brazil). The technique consisted in

making movements of rotation and pressure with the instrument, deepening it until resistance [31]. During the first four days after induction of ulcers, the animals received intramuscular dipyrone sodium (20 mg / kg; Boehringer Ingelheim of Brazil Chemical and Pharmaceutical Ltda, Itapecerica da Serra, Brazil).

Experimental Treatment

After mechanical induction of ulcers, the animals were randomly distributed in eight groups of six specimens. Four groups served as controls (GC2, GC7, GC14, GC21) and received only the topical application of 50 microliters of the dimethylsufoxide-DMSO solution (Galena®, Campinas, Brazil) twice daily. Four other groups (GE2, GE7, GE14 and GE21) received two daily doses of 50 microliters of Pau Pereira topical solution. The application was performed using Microbrush® (Microbrush® INTERNATIONAL Grafton, Wisconsin, United States of America). The animals were killed by overdose of sodium thiopental (100mg / kg, Cristália, Itapira, Brazil) at 2, 7, 14 and 21 days, intraperitoneally.

Slides Preparation

After the mice died, the tongues were removed with a 15-knife scalpel and the specimens were stored in 10% formalin-fixing solution for 24 hours. Later, they were sectioned in the medial region of their back in the longitudinal direction, processed and embedded in paraffin. Histological sections with 4 micrometers thickness were obtained, stained with hematoxylin and eosin. The ulcer area was delimited with a 1.0 mm blue pen for overhead projector (Pilot do Brasil®, São Paulo, Brazil) and the samples were extracted from donor block using a trephine drill (Neodent®, Curitiba, Brazil), 3 mm in diameter, coupled in 130-watt suspension motor from Bethil (Prometal Ind. Met. LTDA®, Marília, Brazil) and were positioned on the receptor block according to the previously made cartesian map with the location of each TMA (tissue microarray) sample.

Immunohistochemical Reaction for MMP-1

The histological sections were dewaxed, (xylene, Biotec®, Curitiba, Brazil), hydrated (alcohol in decreasing solutions, Biotec®, Curitiba, Brazil) and incubated in hydrogen peroxide solution (Biotec®, Curitiba, Brazil) and 5% methanol (Biotec ®, Curitiba, Brazil). This material was subjected to antigen retrieval with Immuno

Retriver (Dako®, Carpinteria, CA), followed by incubation with anti-MMP-1 (clone: EP1252Y Abcam, Cambridge, MA) at 4 ° C overnight. Dilution of the antibody in antibody diluent with reducing components (DAKO Carpinteria, CA, code S302283) was 1: 200 for MMP-1. The detection was performed with Advance Link (Dako, Carpinteria, CA code K406889), followed by the enzyme Advance (DAKO Corporation, Carpinteria, CA, K406889 code), and by chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB - Sigma Chemical®, St. Louis, code D7679). The slides were counterstained by Harris hematoxylin (Biotec®, Curitiba, Brazil), dehydrated (ethanol) and diaphanized (xylene).

Immunoexpression Analysis

The slides were scanned with the Zeiss Axio Scan.Z1 slide scanner (Zeiss®, Oberkochen, Germany) analyzed by a single observer. An image was captured from each sample through the ZEN 2 program (Blue Edition® Carls Zeiss Microscopy GmbH, 2011), at magnification of 200X. From each image, epithelium, muscle fibers, adipose tissue and image artifacts were removed with Adobe Photoshop® CS6 extended version 13.0. Structures or cells stained with brown were considered immunopositive. The images were analyzed in Image-Pro Plus 4.5 software (Media Cybermetics®, Silver Spring, MD) and measurements were obtained by segmentation of the MMP-1 area in square micrometers and the total area of connective tissue. To calculate the percentage of immunopositive area the following formula was used:

$$\frac{Imunopositive\ area\ *100}{Total\ área\ in\ \mu m^2}$$

The values of type I collagen were obtained from previous work [20].

Statistical Analysis

In the statistical analysis we used the program IBM SPSS Statistics 23.0 (SPSS Inc, Chicago, Illinois, USA). The Kolmogorov-Smirnov Normality Test was performed, in which it was observed that the MMP-1 variable presented a normal distribution. Therefore, in order to compare the mean values of the variable in the groups that presented a statistically significant difference, the ANOVA test was applied to two criteria: a complete factorial model. The Levene variance homogeneity test revealed that the variable presented heterogeneous variance.

Therefore, comparisons between the groups were made using Games-Howell's multiple parametric comparison test. The level of significance adopted in all tests was 5% ($p < 0.05$).

Results

MMP-1 expression was significantly lower in the control group (Figure 1A), when compared to the experimental group (Figure 1B) at 14 days. The same was observed for type I collagen (Table 1).

Table 1. Percentage of type I collagen expression [25] and MMP-1 immunoexpression in the control and experimental groups at 2, 7, 14 and 21 days.

	Time	***2 days***	***7 days***	***14 days***	***21 days***
	GROUP	(M±SD)	(M±SD)	(M±SD)	(M±SD)
Tipo I Colagen [20]	C (%)	35,44 ± 11,68	32,36 ± 3,25	26,06 ± 0,37	33,11 ± 7,97
	E (%)	29,96 ± 11,54	31,58 ± 8,18	28,01 ± 2,09	35,97 ± 5,60
	p value*	0,94	1,0	**0,03**	0,98
MMP-1	C (%)	2,52 ± 1,48	5,44 ± 2,41	2,62 ± 1,58	3,18 ± 0,66
	E (%)	3,05 ± 1,15	5,63 ± 2,96	5,01 ± 2,73	3,95 ± 1,72
	P value*	0,96	1,0	**0,02**	0,84

ANOVA test $p<0.05$; * Games-Howell test; **Values of p<0,05**; M = mean; SD = standard deviation; C = control; E = experimental.

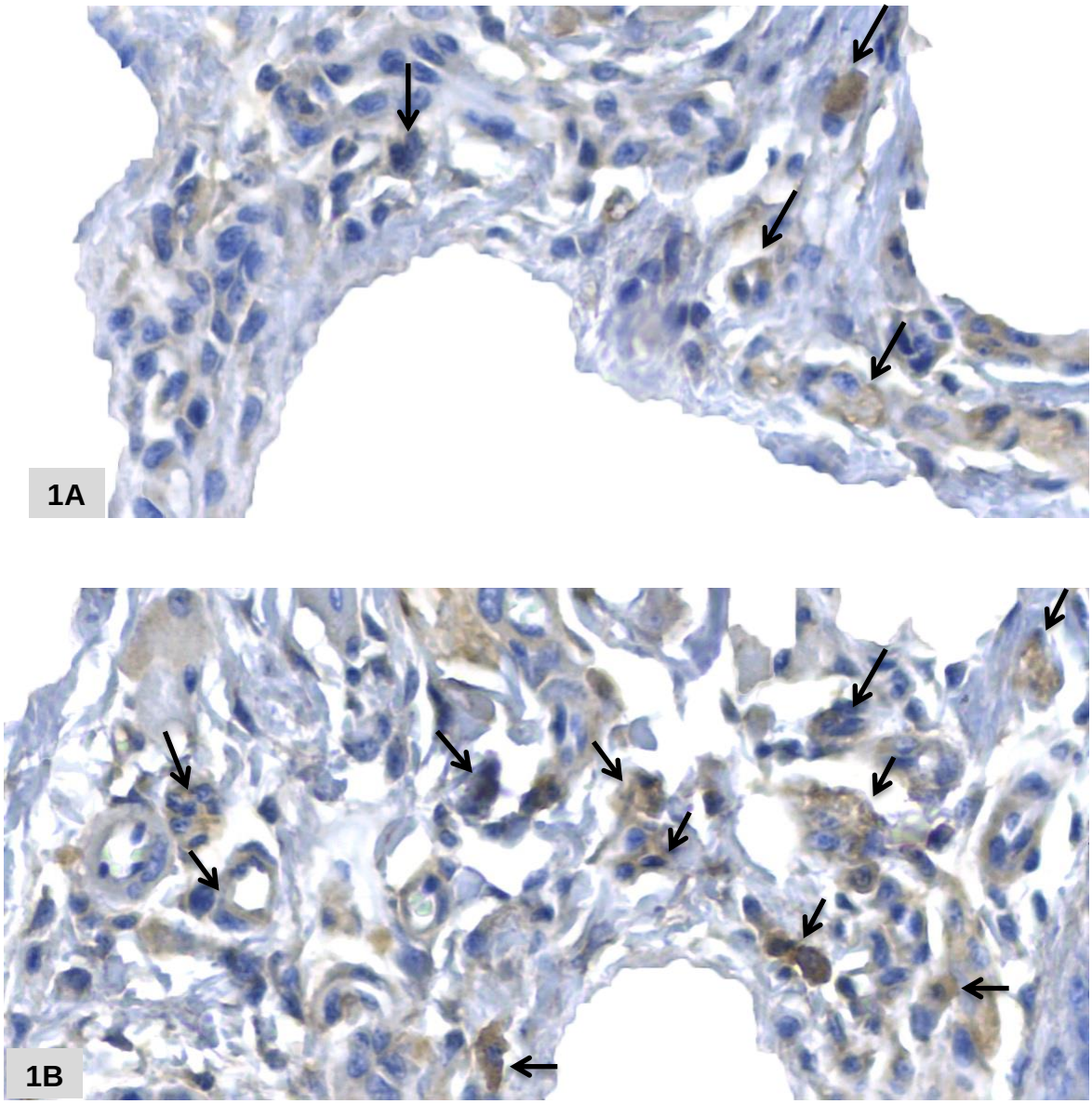

Figura 1. Granulation tissue at 14 days, A) control group and B) Pau Pereira group showing immunopositive cells and structures for MMP-1 in brown (arrows, magnification of 200x).

Discussion

The main result of the present research is high percentage of metalloproteinases-1 at 14 days, when compared to the control group. The collagenases are fundamental in the healing process, they degrade the collagen fibers, allowing the remodeling. MMP-1 is the collagenase responsible for the degradation of type III collagen. During the process of an injury it is normally produced and secreted in later stages in the remodeling phase of healing process [26]. This fact explains the healing effect of the Pau Pereira fraction that

accelerated the degradation of type III collagen through MMP-1, which has preferential activity against immature collagen, already in the early stage of the healing process, allowing its substitution by type I collagen, conferring greater resistance to the wound at 14 days [25].

In the absence of studies evaluating the influence of Pau Pereira alkaloids on the expression of MMP-1, other alkaloids that could intervene in the expression of this enzyme were sought in the literature. As well as this study, which verified an increase in the percentage of MMP-1 in oral ulcers of animals treated with the alkaloid fraction, Hu et al. also found an increase of this metaloproteinase against the use of the hydroxy-camptothecin alkaloid in pulmonary fibrosis induced in rats [32]. Chung et al. found an increase in MMP-1 expression when studying the action of colchicine on human dermal fibroblasts [33]. Lee et al. analyzed the MMP-1 expression in the periodontal ligament of the humans in the presence of nicotine, the alkaloid that constitutes the active principle of the tobacco, and noticed that this substance has a positive regulation of MMPs, including MMP-1. These studies conclude that alkaloids have the potential to influence the expression of metalloproteinase-1.

In the healing process the biomarkers with the greatest potential are the cytokines and proteases, among them the metalloproteinases [35]. Snyder et al. and Chromy et al. studied MMPs in the repair process and postulate that MMP's may be the appropriate biomarkers to estimate the behavior of the healing process. The authors say that in the normal healing process higher levels of collagenases are required for remodeling [36,37]. This reinforces that the highest level of MMP-1 found at 14 days is propitious for this process.

Studies correlate changes in the levels of certain host proteins, including cytokines, chemokines, MMP's along with other proteases, with inflammatory markers [38]. Besides being related to the remodeling, the metalloproteinases also participate in the regulation of other mechanisms present in the repair process. According to Gill et al., the production of some factors that influence inflammation is controlled by proteins, including MMP's. They can stimulate or degrade inflammatory mediators, such as cytokines, chemokines, and control chemotactic activity, enabling cellular migration. Studies lead to the conclusion that MMPs do not only participate in the degradation of the extracellular matrix, but also play an important role in the regulation of inflammation [27, 38]. These

findings corroborate with Fosquiera et al., which demonstrated changes in the number of cells involved in the inflammatory process, compared to the action of the Pau Pereira fraction [24]. Studies correlating MMP-1 with inflammatory modulation and the alkaloid need to be performed with the purpose of clarifying this possible interaction.

Within the limitations of this study we can highlight the use of animals, which may alter the inflammatory and immune response.

Metalloproteinase-1 is present in higher levels in the animals treated with the fraction at 14 days then the control group. Thus, the alkaloid fraction of the bark extract from the Pau-Pereira tree is capable of interfering with the expression of metalloproteinase-1. Such fact associated with previous results shows that this substance facilitates the healing process of ulcers in rat tongues.

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To the partners of the Phytochemical Laboratory of the Federal University of Paraná.

The authors declare that there is no conflict of interest.

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ANEXO

Parecer de comitê de ética

PUCPR

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa e Pós-Graduação
Comitê de Ética em Pesquisa no Uso de Animais

Curitiba, 11 de Agosto de 2016.

PARECER DE PROTOCOLO DE PESQUISA

REGISTRO DO PROJETO: 01043/2016 – 1ª versão

TÍTULO DO PROJETO: Análise do processo de reparo de lesão bucal de ratos tratados com extrato de pau pereira – Análise Histopatológica.

PESQUISADOR RESPONSÁVEL: Ana Maia Trindade Grégio

EQUIPE DE PESQUISA: Emanuela Carla dos Santos, Eliana Cristina Fosquiera, Thayse da Silva Arcenio, Luana Pereira dos Santos

INSTITUIÇÃO

Pontifícia Universidade Católica do Paraná

ESCOLA / CURSO:

Escola de Ciências da Vida / Odontologia

VIGÊNCIA DO PROJETO	2016 a 2017	**QUANTIDADE DE ANIMAIS**	DADOS JÁ COLETADOS
ESPECIE/LINHAGEM	*Ratos norvegicus* (Ratos)	**Nº SISBIO** (Somente animais de vida livre)	Não se aplica
SEXO	DADOS JÁ COLETADOS	**ATIVIDADES** (Somente animais de vida livre)	Não se aplica
IDADE / PESO	DADOS JÁ COLETADOS	**ESPECIÉ – GRUPO TAXONÔMICOS** (Somente animais de vida livre)	Não se aplica
ORIGEM DO ANIMAL	Pesquisas anteriores	**LOCAL (IS)** (Somente animais de vida livre)	Não se aplica

O colegiado do CEUA certifica que este protocolo que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto homem), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794/2018 e Decreto nº 6.899/2009, e com as normas editadas pelo CONCEA e foi **APROVADO** pela CEUA - PUCPR em reunião de ***11.08.2016.*** Se houver mudança do protocolo o pesquisador deve enviar um relatório á CEUA descrevendo de forma clara e sucinta, a parte do protocolo a ser modificado e as suas justificativas. Se a pesquisa, ou parte dela for realizada em outras instituições, cabe ao pesquisador não iniciá-la antes de receber a autorização formal para a sua realização. O documento que autoriza o início da pesquisa deve ser carimbado e assinado pelo responsável da instituição e deve ser mantido em poder do pesquisador responsável, podendo ser requerido por esta CEUA em qualquer tempo. **Lembramos ao pesquisador que é obrigatório encaminhar o relatório anual parcial e relatório final da pesquisa a esta CEUA.**

Atenciosamente,

Profa. Dra. Marta Luciane Fischer
Coordenadora
Comissão de Ética no Uso de Animais

Comissão de Ética no Uso de Animais PUCPR

Rua Imaculada Conceição, 1155 Prado Velho CEP 80.215-901 Curitiba Paraná Brasil
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Análise estatística

Testes de Normalidade

Grupo x Tempo		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Estatística	gl	Valor p	Estatística	gl	Valor p
Percentagem da imunoexpressão da MMP-1	Controle / 2 dias	,170	12	,200*	,918	12	,273
	Controle / 7 dias	,289	7	,080	,877	7	,213
	Controle / 14 dias	,179	16	,182	,913	16	,132
	Controle / 21 dias	,273	5	,200*	,857	5	,218
	Experimental / 2 dias	,210	13	,120	,944	13	,507
	Experimental / 7 dias	,198	8	,200*	,931	8	,525
	Experimental / 14 dias	,108	25	,200*	,960	25	,415
	Experimental / 21 dias	,122	14	,200*	,971	14	,889

*. Este é um limite inferior da significância verdadeira.

a. Correlação de Significância de Lilliefors

Descritivas

Percentagem da imunoexpressão da MMP-1

	N	Média	Desvio Padrão	Erro Padrão	Intervalo de confiança de 95% para média		Mínimo	Máximo
					Limite inferior	Limite superior		
Controle	40	3,158432	1,9271167	,3047039	2,542110	3,774754	,6674	9,9313
Experimental	60	4,428457	2,4146758	,3117333	3,804680	5,052234	,5901	10,8289
Total	100	3,920447	2,3084329	,2308433	3,462404	4,378490	,5901	10,8289

Teste de Homogeneidade de Variâncias

Percentagem da imunoexpressão da MMP-1

Estatística de Levene	gl1	gl2	Valor p
2,474	1	98	0,1190

Descritivas

Percentagem da imunoexpressão da MMP-1

	N	Média	Desvio Padrão	Erro Padrão	Intervalo de confiança de 95% para média		Mínimo	Máximo
					Limite inferior	Limite superior		
2 dias	25	2,801716	1,3251991	,2650398	2,254700	3,348731	,6674	5,4971
7 dias	15	5,547063	2,6283912	,6786477	4,091508	7,002617	1,8124	9,9802
14 dias	41	4,084325	2,6142352	,4082749	3,259170	4,909479	,5901	10,8289
21 dias	19	3,754660	1,5405990	,3534377	3,012115	4,497205	1,1076	6,8475
Total	100	3,920447	2,3084329	,2308433	3,462404	4,378490	,5901	10,8289

Teste de Homogeneidade de Variâncias

Percentagem da imunoexpressão da MMP-1

Estatística de Levene	gl1	gl2	Valor p
5,422	3	96	0,0017

Descritivas

Percentagem da imunoexpressão da MMP-1

	N	Média	Desvio Padrão	Erro Padrão	Intervalo de confiança de 95% para média		Mínimo	Máximo
					Limite inferior	Limite superior		
Controle / 2 dias	12	2,523110	1,4891350	,4298763	1,576959	3,469261	,6674	5,4971
Controle / 7 dias	7	5,445972	2,4154275	,9129458	3,212074	7,679870	2,8068	9,9313
Controle / 14 dias	16	2,624700	1,5841983	,3960496	1,780541	3,468860	,7575	5,5534
Controle / 21 dias	5	3,188589	,6628864	,2964518	2,365507	4,011671	2,0785	3,7633
Experimental / 2 dias	13	3,058890	1,1533882	,3198923	2,361904	3,755876	1,3772	5,0383
Experimental / 7 dias	8	5,635517	2,9659567	1,0486241	3,155915	8,115119	1,8124	9,9802
Experimental / 14 dias	25	5,018484	2,7372383	,5474477	3,888608	6,148361	,5901	10,8289
Experimental / 21 dias	14	3,956828	1,7273803	,4616618	2,959469	4,954188	1,1076	6,8475
Total	100	3,920447	2,3084329	,2308433	3,462404	4,378490	,5901	10,8289

Teste de Homogeneidade de Variâncias

Percentagem da imunoexpressão da MMP-1

Estatística de Levene	gl1	gl2	Valor p
2,759	7	92	0,0120

Comparações múltiplas

Variável dependente: Percentagem da imunoexpressão da MMP-1

Games-Howell

(I) Tempo		Diferença média (I-J)	Erro Padrão	Valor p	Intervalo de Confiança 95%	
					Limite inferior	Limite superior
2 dias	7 dias	-2,7453473*	,7285663	0,0068	-4,800693	-,690002
	14 dias	-1,2826091	,4867592	0,0506	-2,567500	,002282
	21 dias	-,9529444	,4417740	0,1551	-2,143520	,237631
7 dias	2 dias	2,7453473*	,7285663	0,0068	,690002	4,800693
	14 dias	1,4627382	,7919919	0,2762	-,716788	3,642264
	21 dias	1,7924029	,7651672	0,1196	-,337064	3,921869

14 dias	2 dias	1,2826091	,4867592	0,0506	-,002282	2,567500
	7 dias	-1,4627382	,7919919	0,2762	-3,642264	,716788
	21 dias	,3296647	,5400061	0,9283	-1,101446	1,760776
21 dias	2 dias	,9529444	,4417740	0,1551	-,237631	2,143520
	7 dias	-1,7924029	,7651672	0,1196	-3,921869	,337064
	14 dias	-,3296647	,5400061	0,9283	-1,760776	1,101446

*. A diferença média é significativa no nível 0.05.

Comparações múltiplas

Variável dependente: Percentagem da imunoexpressão da MMP-1

Games-Howell

(I) Grupo x Tempo		Diferença média (I-J)	Erro Padrão	Valor p	Intervalo de Confiança 95%	
					Limite inferior	Limite superior
Controle / 2 dias	Controle / 7 dias	-2,9228620	1,0090905	0,1846	-6,828291	,982567
	Controle / 14 dias	-,1015904	,5845074	1,0000	-2,033238	1,830057
	Controle / 21 dias	-,6654791	,5221851	0,8947	-2,493594	1,162636
	Experimental / 2 dias	-,5357799	,5358402	0,9693	-2,335449	1,263889
	Experimental / 7 dias	-3,1124072	1,1333164	0,2175	-7,423459	1,198644
	Experimental / 14 dias	-2,4953742*	,6960550	0,0206	-4,740141	-,250608
	Experimental / 21 dias	-1,4337181	,6308131	0,3474	-3,522944	,655507
Controle / 7 dias	Controle / 2 dias	2,9228620	1,0090905	0,1846	-,982567	6,828291
	Controle / 14 dias	2,8212717	,9951509	0,2040	-1,072295	6,714838
	Controle / 21 dias	2,2573830	,9598717	0,3771	-1,654158	6,168924
	Experimental / 2 dias	2,3870822	,9673681	0,3290	-1,508990	6,283154
	Experimental / 7 dias	-,1895452	1,3903533	1,0000	-5,156498	4,777408
	Experimental / 14 dias	,4274879	1,0645041	0,9999	-3,506468	4,361444
	Experimental / 21 dias	1,4891439	1,0230355	0,8126	-2,421612	5,399900
Controle / 14 dias	Controle / 2 dias	,1015904	,5845074	1,0000	-1,830057	2,033238
	Controle / 7 dias	-2,8212717	,9951509	0,2040	-6,714838	1,072295
	Controle / 21 dias	-,5638887	,4947110	0,9381	-2,266179	1,138401
	Experimental / 2 dias	-,4341895	,5091035	0,9879	-2,105077	1,236698
	Experimental / 7 dias	-3,0108169	1,1209227	0,2385	-7,310170	1,288536
	Experimental / 14 dias	-2,3937838*	,6756879	0,0212	-4,557410	-,230158
	Experimental / 21 dias	-1,3321277	,6082655	0,3885	-3,328897	,664642

Controle / 21 dias	Controle / 2 dias	,6654791	,5221851	0,8947	-1,162636	2,493594
	Controle / 7 dias	-2,2573830	,9598717	0,3771	-6,168924	1,654158
	Controle / 14 dias	,5638887	,4947110	0,9381	-1,138401	2,266179
	Experimental / 2 dias	,1296992	,4361362	1,0000	-1,429288	1,688687
	Experimental / 7 dias	-2,4469282	1,0897229	0,4141	-6,748560	1,854704
	Experimental / 14 dias	-1,8298951	,6225613	0,1043	-3,874721	,214931
	Experimental / 21 dias	-,7682390	,5486486	0,8450	-2,657142	1,120664
Experimental / 2 dias	Controle / 2 dias	,5357799	,5358402	0,9693	-1,263889	2,335449
	Controle / 7 dias	-2,3870822	,9673681	0,3290	-6,283154	1,508990
	Controle / 14 dias	,4341895	,5091035	0,9879	-1,236698	2,105077
	Controle / 21 dias	-,1296992	,4361362	1,0000	-1,688687	1,429288
	Experimental / 7 dias	-2,5766273	1,0963318	0,3668	-6,870186	1,716931
	Experimental / 14 dias	-1,9595943*	,6084819	0,0069	-3,366700	-,552488
	Experimental / 21 dias	-,8979382	,5616607	0,7463	-2,766897	,971021
Experimental / 7 dias	Controle / 2 dias	3,1124072	1,1333164	0,2175	-1,198644	7,423459
	Controle / 7 dias	,1895452	1,3903533	1,0000	-4,777408	5,156498
	Controle / 14 dias	3,0108169	1,1209227	0,2385	-1,288536	7,310170
	Controle / 21 dias	2,4469282	1,0897229	0,4141	-1,854704	6,748560
	Experimental / 2 dias	2,5766273	1,0963318	0,3668	-1,716931	6,870186
	Experimental / 14 dias	,6170331	1,1829249	0,9992	-3,727073	4,961139
	Experimental / 21 dias	1,6786891	1,1457504	0,8089	-2,639491	5,996869
Experimental / 14 dias	Controle / 2 dias	2,4953742*	,6960550	0,0206	,250608	4,740141
	Controle / 7 dias	-,4274879	1,0645041	0,9999	-4,361444	3,506468
	Controle / 14 dias	2,3937838*	,6756879	0,0212	,230158	4,557410
	Controle / 21 dias	1,8298951	,6225613	0,1043	-,214931	3,874721
	Experimental / 2 dias	1,9595943*	,6084819	0,0069	,552488	3,366700
	Experimental / 7 dias	-,6170331	1,1829249	0,9992	-4,961139	3,727073
	Experimental / 21 dias	1,0616561	,7161219	0,8115	-1,239720	3,363032
Experimental / 21 dias	Controle / 2 dias	1,4337181	,6308131	0,3474	-,655507	3,522944
	Controle / 7 dias	-1,4891439	1,0230355	0,8126	-5,399900	2,421612
	Controle / 14 dias	1,3321277	,6082655	0,3885	-,664642	3,328897
	Controle / 21 dias	,7682390	,5486486	0,8450	-1,120664	2,657142
	Experimental / 2 dias	,8979382	,5616607		-,971021	2,766897

			0,7463		
Experimental / 7 dias	-1,6786891	1,1457504	0,8089	-5,996869	2,639491
Experimental / 14 dias	-1,0616561	,7161219	0,8115	-3,363032	1,239720

*. A diferença média é significativa no nível 0.05.

Normas para publicação

Planta Medica – Guidelines for Authors

1. Editorial Policy

1.1 Aims and Scope

PLANTA MEDICA – Journal of Medicinal Plant and Natural Product Research is published in 18 issues a year. The following areas of medicinal plant and natural product research are covered:

1. Biological and Pharmacological Activities
2. Natural Product Chemistry & Analytical Studies
3. Pharmacokinetic Investigations
4. Formulation and Delivery Systems of Natural Products

Contributions are not normally considered for publication and will be immediately rejected if:

- The manuscript does not fall into any of the above areas
- Activity data are reported without comparison to a recognized positive control
- Extracts have not been characterized by analysis of their major constituents (e.g. HPLC, GC, NMR)
- Predictable bioactivity is reported (e.g. antioxidant properties of phenolic compounds)

1.2 General terms of publication

Only papers of highest scientific quality, concisely written and complying with these Guidelines for Authors can be considered for publication. All contributions are peer-reviewed by independent referees.

Submission of a manuscript to Planta Medica implies that it represents original research not previously published and that it is not being considered for publication elsewhere. The corresponding author must

declare that the manuscript is submitted on behalf of all authors. Copyright belongs to the publisher upon acceptance of the manuscript.

Important: Publication in Planta Medica is free of charge.

The language of publication is English. British or American spelling is accepted, but should be consistent throughout the manuscript. Important: Incorrect English can result in the immediate rejection of your manuscript whereas correct English will facilitate a speedy publication process. It is in your own interest to ensure that your paper has been read by a native English speaker; alternatively, you should use a copy-editing service like "American Journal Experts" if you have concerns about the English in your manuscript. Manuscripts which do not meet acceptable standards will be returned to the authors.

Authors investigating the chemistry of a single species should aim to publish their results in a single manuscript rather than in a series of papers. Manuscripts should not report fragmentary parts of a larger study.

Pharmacological investigations of extracts require detailed extract characterization (see 4.7.7).

Submission of a manuscript signifies acceptance of the journal's Guidelines for Authors. Submissions which are not in line with these principles will be returned directly to the authors by the Editorial Office.

A statement clarifying the conflicts of interests of all authors must be included at the end of the manuscript (before the references); this will be published. Conflicts of interest also need to be declared during the submission process. Declaration of conflicts of interest is mandatory; if none, this also needs to be stated.

Planta Medica takes biodiversity and the protection of species very seriously. We support CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) as well as The Rio de Janeiro Convention on Biological Diversity and we expect that during the conduct of the scientific research leading up to the results submitted to Planta Medica these conventions as well as the local rules and regulations have been adhered to.

2. Submission of Manuscripts

Manuscripts can be submitted exclusively online at http://mc.manuscriptcentral.com/plamed. Submissions of hardcopy manuscripts or by e-mail will not be accepted.

A sample manuscript (for Original Papers) is available at http://mc.manuscriptcentral.com/plamed → Instructions and Forms, and at www.thieme.de/plantamedica. In addition to the Guidelines, authors are urged to follow these formats when preparing a manuscript.

Commonly used file formats (Doc, DOCX, RTF) should be used for preparation of the manuscripts. PDF files are not accepted. The manuscript has to be accompanied by a cover letter, in which the au- thors briefly explain the significance of their findings and the interest to the readership of Planta Medica.

The manuscript (main text, including tables) should be submitted as one file. All figures should be submitted separately (detailed layout requirements see 4.12.). Figures will be automatically rendered in color online and black and white in print. Color reproduction in print will be subject to fees of EUR 600 for the first color figure and EUR 120 for any further figure (incl. 19% VAT). Authors are strongly encouraged to provide non-essential but useful data, figures and tables as Supporting Information (see 4.14).

3. Types of Contribution

3.1. Original Papers are research articles describing original experimental results. The material should be arranged in the order: Title Page/Abstract/Keywords/Abbreviations/Introduction/Results and Discussion/Materials and Methods/Acknowledgements/Conflicts of Interest/References/Figure Legends/Tables. Figures should be up- loaded as separate files (see section 4.12., Graphics). Results and Discussion sections may appear as two separate parts or as a combined "Results and Discussion" section. The normal length of the main text of an Original Paper, excluding references, tables, figures and figure legends, is < 4,000 words. Original papers should not contain more than 45 references.

3.2. Rapid Communications are intended for the publication of exceptionally significant new and original results, such as unusual structures, bioactivities and innovative analytical techniques that de- serve rapid publication, in the format of an Original Paper. If authors want their submission to be considered as a Rapid Communication, they should provide a justification statement for this with their manuscript. However, also regular submissions can be elected by the Editors for rapid communication after the review process.

3.3. Reviews will generally be invited by the Editor-in-Chief. They should be as concise as possible and do not need to include experimental details. The main purpose of reviews is to provide a concise, accurate introduction to the subject matter and inform the reader critically of the latest developments in this area. All reviews should contain an abstract.

3.4. Minireviews and Perspectives will generally be invited by the Editor-in-Chief. Minireviews provide concise and critical updates on a subject of high interest. Perspectives are written by leading experts in an emerging field and provide a concise assessment of the current state-of-the-art and an outlook on future developments. The normal length of the main text of Minireviews and Perspectives, excluding references, tables, figures and figure legends, is about 2000 words.

3.5. Editorials addressing topical issues of general interest to the readership of Planta Medica will be published on an irregular basis. They are written by the Editor-in-Chief, other Editors, or by experts on a specific issue in the form of an Invited Editorial.

4. Preparation of Manuscripts

Please note that papers published in Planta Medica follow the IRDMACR structure:

Introduction, Results and Discussion, Materials and Methods, Acknowledgements, Conflict of Interest Statement, References.

In addition to the Guidelines, authors should consult the sample manuscript (for Original Papers) at https://www.thieme.de/statics/dokumente/thieme/final/de/dokumente/zw_thieme-en/plantame-dica_Sample_Manuscript prior to preparing their contribution. Com- monly used file formats (DOC, DOCX, RTF) should be used for prepa- ration of the manuscripts.

For submission of all manuscripts, follow the instructions of the on- line submission system. For general instructions on ScholarOne, please follow this link: http://mchelp.manuscriptcentral.com/ge- thelpnow/training/author/

Before submission, prepare the cover letter, and keep ready all information on the manuscript (title, full name and affiliation of all authors, abstract, name of all files to be submitted). The author submitting the manuscript will be corresponding author.

4.1. The Title Page must contain the title of the manuscript (title should not exceed 20 words), the full names referenced by numerical superscripts with affiliation and addresses of all authors, and the full address of the corresponding author, including e-mail, phone, and FAX number.

4.2. The Abstract should contain brief information on purpose, methods, results and conclusion (without subheadings). Abstracts should not exceed 250 words. Please note that during the upload of the manuscript files you will be asked to insert the abstract. This abstract needs to be identical to the abstract in the manuscript file itself.

4.3 The Keywords should include the scientific name and family of the organism(s) investigated. 4 – 6 keywords should be listed.

4.4. Abbreviations should generally be used sparingly. Abbreviations should be introduced only when repeatedly used. Standard abbreviations such as m. p., b. p., K, s, min, h, µL, mL, µg, mg, g, kg, nm, mm, cm, ppm, mmol, HPLC, TLC, GC, UV, CD, IR, MS, NMR, ELISA, PCR can be used throughout the manuscript (for a more extensive list follow this link: https://www.thieme.de/de/planta-medica/authors-5605.htm). Non-standard abbreviations must be defined in the text following their first use. Provide a list of all nonstandard abbreviations after the keywords. Define all symbols used in equations and formulas. If symbols are used extensively, provide a list of all symbols together with the list of abbreviations.

4.5. The Introduction should state the purpose of the investigation and relate to current knowledge in the specific topic addressed.

4.6. Results should be presented in a concise manner. The Discussion should provide an interpretation of the data and relate them to existing knowledge. The discussion should not be a repetition of the results. Results and Discussion may be combined. No subheadings are allowed within these sections. There should be no separate conclusions paragraph, the conclusion should be incorporated into the discussion.

4.7. In Materials and Methods specific details about test materials and test compounds, instrumentation and experimental protocols should be given. This section should contain sufficient details so that others are able to reproduce the experiment(s). Purity (%) of all reference and standard compounds should be mentioned, as well as the method of how it was determined. Previously reported methods should be referenced only. Suppliers for major equipment, cell lines, chemicals, biochemical reagents

and major disposables should be indicated. It should read in the manuscript for example ‘ Quercetin (purity > 98 %) was purchased from Sigma’ and not …….‘was purchased from Sigma (St. Louis, USA)’.

4.7.1. Documentation of plants and other organisms or starting materials. Use the correct scientific nomenclature. For plants, the Index Kewensis (electronic Plant Information Centre ePIC, Royal Botanic Gardens, Kew, UK: http://www.kew.org/epic), and/or the International Code of Botanical Nomenclature (www.bgbm.fu-berlin.de/ iapt/nomenclature/code/tokyo-e/default.htm) should be followed. Give the scientific name (in italics), the author of this name and the family. Indicate the person who identified the material as well as date and place of collection. The manuscript must include references to voucher specimens of the plants (deposited in a major regional herbarium) or the material examined including their registration number(s). It should be mentioned which plant parts have been used.

4.7.2. Description of the preparation of extracts and isolation of compounds. The kind and amount of starting material, solvents (including volumes) and extraction methods (including temperature and extraction time) must be indicated. The description of chromatographic systems should contain the quantitative information that al- lows the reader to repeat the work. Column dimensions, stationary phase, particle size, mobile phase composition, flow rate, sample amount, and elution volumes (or retention times, k’ values) of fractions should be given. E. g.: “MPLC on silica gel (40 – 63 µm; 2 × 50 cm), MeOH/EtOAc 8: 2, 3 mL/min; tR of 1: 60 – 70 mL, 2:120 – 140 mL, 3: 145 – 175 mL; detection of eluates by TLC (SiO2, MeOH/H2O 9 : 1; Dragendorff reagent), Rf 1: 0.35, 2: 0.55, 3: 0.73).” When using gradients the volumes of solvents should be presented; fractions should be defined by their elution volume. Similar information is necessary for HPLC, GLC, DCCC, MLCC and all other methods of purification. Figures of chromatograms will only be accepted if they are essential for understanding the methods or the results described. GC identifications of constituents of essential oils must be supported by retention indices on a polar and an apolar column. Identification by GC-MS is preferred.

4.7.3. Chemical nomenclature used should be based on the systematic rules adopted by Chemical Abstracts and IUPAC. Trivial names should be avoided unless they are definitely advantageous over the corresponding

systematic names. Trivial names are not accepted for close analogues and derivatives of known compounds. For reference drug substances the INN names should be used.

4.7.4. Physico-chemical characterisation of compounds. Data provided for new compounds should enable an unambiguous identification of the substance and have to appear in the following order, if available: visual appearance, chromatographic mobility in TLC, GC, or HPLC, mp, UV-vis, specific optical rotation, CD, IR, 1H-NMR, 13C-NMR, low resolution MS, high resolution MS, elemental analysis.

Note that for specific optical rotation [α]D temp, the symbol c is defined as mass of substance (in g) in 100 mL of solution. For specific optical rotation no unit should be given; the "degree" symbol "°" should not be used. In case of spectroscopic work on known substances refer, if possible, to published data; the manuscript should then contain the following indication: Copies of the original spectra are obtainable from the corresponding author. Original spectra for new compounds should be provided as Supporting Information (see 4.14), IR, NMR, mass, and UV spectra should normally not be given in the manuscript as figures, unless the listing of characteristic signals is not sufficient. 4.7.5. X-Ray crystallographic data must include a line drawing of the structure, a perspective drawing, and a discussion of bond lengths and angles. A supplement describing full details of the structure and methods and means of its determination in a form suitable for deposition must be submitted to the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (fax: + 44 (0) 122333 60 33 or e-mail: deposit@ccdc.cam.ac.uk). Deposition of the data has to be prior to submission of the manuscript, and appropriate reference has to be made in the Materials and Methods section, including the deposition number.

4.7.6. Analytical studies. Key data on method validation must be provided and should typically include information on specificity, linearity, limit of detection, limit of quantification, accuracy, precision, intermediate precision, and some robustness studies. Information on the purity of reference compounds, and on the methods used for the determination of purity must be given. Recoveries of extraction and sample pre-purification steps have to be indicated. Adequate statistical treatment of data is required. For more information regarding validation issues, prospective

authors should also refer to ICH guidelines. Analytical studies of a routine nature will not be considered for publication.

4.7.7. Pharmacological investigations. Planta Medica will only consider manuscripts in which conclusions are based on adequate statistics that incorporate the appropriate tests of significance, account for the type of data distribution, and are based on the number of experimental observations required for the application of the respective statistical method. In each case, positive controls (reference compounds) have to be used and the dose-activity dependence should be shown. If IC50 values are given, the dose-response relation should be displayed graphically at least as supplementary data, and the method of calculation should be given. Authors should be conscious of the differences between EC50, IC50, TC50, LC50, ED50, LD50 values. Compounds should follow accepted guidelines when represented as "active". For example, the cytotoxic effect of a pure substance when tested against a cancer cell line would exhibit an IC50 value of < 10 µM. Authors should pay attention to the following definitions: Compounds that suppress the growth of, or kill, isolated tumor cell lines grown in culture should be referred to as either "cytostatic" or "cytotoxic", as appropriate. Only compounds that inhibit the growth of tumors in animal-based models should be called "antitumor". The term "anticancer" should be reserved for compounds that show specific activity in human-based clinical studies.

When working with experimental animals, reference must be made to principles of laboratory animal care or similar regulations and to approval by the local ethical committee. The protocol approval number and the exact date of approval (e.g. January 1st 2016) must be provided.

Pharmacological investigations of extracts require detailed extract characterization. This includes botanical characterization of plant material, solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). For starting material coming from a company/commercially obtained sample the batch/Lot. Number has to be provided. The drug to extract ratio (DER) must be given. Chromatographic profiling (e.g. HPLC profile with a reference compound recorded at different wavelengths) should be carried out, with at least the major peaks identified, or qualitative and quantitative information on active or typical constituents should be provided. Altogether the phytochemical standardization of an extract and/or fraction(s) requires state-of-the-art methods.

4.7.8. Biological screening. Papers dealing with the biological screening of a meaningful number of extracts of plants or other organisms can be considered for publication in Planta Medica. Identification of the material should properly be documented, and preparation of the extracts should clearly be described (see above, sections 4.7.1 and 4.7.7). Biological activities should be reported by listing IC50 or EC50 values, or a dose-response relationship should be shown by using at least two test concentrations. Positive controls (reference compounds) should be included. Results should be presented in a concise format, and the discussion should be kept to a minimum.

4.8. Acknowledgements should list persons who made minor contributions to the investigation and organizations providing support.

4.9. Conflict of Interest Disclosure. A statement describing any financial conflicts of interest or lack thereof is published with each manuscript. The statement should describe all potential sources of bias, including affiliations, funding sources, and financial or management relationships, that may constitute conflicts of interest (please see the ACS Ethical Guidelines to Publication of Chemical Research). The statement will be published in the final article. If no conflict of interest is declared, the following statement will be published in the article: "The authors declare no conflict of interest."

4.10. References, including those in tables and figure legends, should be numbered in the order in which they are cited in the text, using arabic numbers between square brackets, e.g. [1]; for multiple references, e.g. [1 – 3] or [1, 2, 5]. The list of references should be arranged consecutively according to the numbers in the text. Use Index Medicus abbreviations for journal titles. Authors bear complete responsibility for the accuracy of the references. Original Research articles should not have more than 45 references.

The following examples illustrate the format for references:

a) Journals

Trute A, Nahrstedt A. Separation of rosmarinic acid enantiomers by three different chromatographic methods and the determination of rosmarinic acid in Hedera helix. Phytochem Anal 1996; 7: 204 – 208

Article in press without doi:

Lim EK, Ashford DA, Hou B, Jackson RG, Bowles DJ. Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. Biotech Bioeng, in press

Note: If reference is made to papers that are in press, authors are requested to add the galley proof or acceptance letter to the online submission. Avoid references to unpublished personal communications. These have to be included in the body of the text as 'unpublished data'.

Article in press with doi:

Lim EK, Bowles DJ. A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J, advance online publication 8 July 2004; doi: 10.1038/sj.emboj.7600295

b) Books

Citation to complete book:

Mabberley DJ. The plant book, 2nd edition. Cambridge: Cambridge University Press; 1997: 520 – 521

Citation to article within a book:

Lechtenberg M, Nahrstedt A. Cyanogenic glycosides. In: Ikan R, editor. Naturally occurring glycosides. Chichester: Wiley & Sons; 1999:147 – 191

Lorberg A, Hall MN. TOR: the first ten years. In: Thomas G, Sabatini DM, Hall MN, editors. TOR – target of rapamycin. Heidelberg: Spring- er Verlag; 2004: 1 – 18

Multi-volume books and encyclopedias:

Warren SA. Mental retardation and environment. In: International encyclopedia of psychiatry, psychology, psychoanalysis and neurology, Vol. 7. New York: Aesculapius Publishers; 1977: 202–207 Pharmacopoeia of China, Part 1. Beijing: People's Health Press; 1977: 531–534.

c) PhD and Diploma Theses

Dettmers JM. Assessing the trophic cascade in reservoirs: the role of an introduced predator [dissertation]. Columbus: Ohio State University; 1995.

d) Patents

Cookson AH. Particle trap for compressed gas insulated transmission system. US Patent 4 554 399; 1985

e) Conference Paper

Okada K, Kamiya Y, Saito T, Nakagawa T, Kaawamukai M. Localization and expression of geranylgeranyldiphosphate synthases in Arabidopsis thaliana. Annual Meeting of the American Society of Plant Physiologists, Baltimore, MD; 1999

f) Electronic Sources

Agatep R, Kirkpatrick RD, Parchaliuk DL,Woods RA, Gietz RD. Transformation of S. cerevisiae by the lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol. Technical tips online. Available at http://research.bmn.com/tto. Accessed September 22, 2005.

If no author is given, the title is used as the first element of the citation.

4.11. Chemical structures should be prepared with ChemDraw or a similar program using the following settings: bond lengths 0.508 cm, bond width 0.021 cm, bold bond width 0.071 cm, bond spacing 18 % of length, hash spacing 0.088 cm, atom labels Arial 10, compound numbers Arial 10 bold. These settings correspond to American Chemical Society document settings preset in ChemDraw. The configuration of all stereocenters present should be indicated; use of bold and dashed lines rather than solid and dashed wedges is recommended. They will be reproduced without reduction and the charts should be prepared with maximum widths of up to 8.5 cm for single column print and up to 17.5 cm for double column print. Authors using other drawing packages should modify their program's parameters so that they reflect the above guidelines.

4.12. Graphics: Figures are numbered with Arabic numerals. The quality of the illustrations depends on the quality of the originals provided. Graphics cannot be modified or enhanced by the journal production staff. The graphics must be submitted as separate files. The legend should not appear under the figures but should be included as a separate figure legend after the references in the manuscript file. The figure legend needs to be self-explanatory.

Acceptable file formats are TIFF, EPS (vector artwork), or CDX (Chem-Draw file). Labeling of all figure parts should be present, and the parts should be assembled into a single graphic.

TIFF files should have the following resolution requirements:

Line Art (Black and White, Color: 800 – 1200 dpi
Greyscale Art: 600 dpi
Color Art (RGB mode):3 00 dpi

For best results, illustrations should be submitted in the actual size at which they should appear in the Journal. Consistently sized letters and labels in graphics throughout the manuscript will help ensure consistent graphic presentation for publication. Lettering should be no smaller than 5 points. (Arial type works well for lettering.) Lines should be exactly 0.5 point. Lettering and lines should be of uniform density. If artwork that should be reduced must be submitted, larger lettering and thicker lines should be used so that, when reduced, the artwork meets the above-mentioned parameters.

Complex textures and shading to achieve a three-dimensional effect should be avoided. Different grey scale tones to show group differences are preferred.

4.13. Tables: These should be numbered consecutively with Arabic numerals. Tables should be placed in the manuscript or uploaded as separate file (file format DOC, DOCX) after the figure legends. Foot- notes in tables should be given lowercase letter designations and be cited in the table by italic superscript letters. The sequence of letters should proceed by line rather than by column. If a footnote is cited both in the text and in a table, insert a lettered footnote in the table to refer to the numbered footnote in the text.

Each table should be provided with a descriptive heading, which, together with the individual column headings, should make the table, as nearly as possible, self-explanatory. Arrangements that leave many columns partially filled or that contain much blank space should be avoided. The table legend should appear directly under the tables.

4.14. Supporting Information: To keep articles as concise and at the same time as informative as possible, authors are strongly encouraged to

submit part of their tables and figures as Supporting Information. The following type of data will be preferentially published as Supporting Information rather than in the print article: Spectra, chromatograms, structural drawings outlining NMR correlations, experimental procedures of secondary importance, tables summarizing data that are non-quintessential but useful to the understanding of an article. Tables, figures and text provided as Supporting Information must be referred to in the manuscript as follows: (Table 1S, Sup- porting Information, etc.). The cover page for Supporting Information should be identical to the cover page of the manuscript. Legends for Figures and Tables must appear directly on the respective figure pages. Pages have to be numbered consecutively. All figures and tables should be referenced in the main manuscript. Supporting Information has to be submitted as a separate file.

Supporting Information is published on the journals homepage at http://www.thieme-connect.de/ejournals/toc/plantamedica.

5. Proofs and Reprints: Galley proofs will be sent to the corresponding author as a PDF file. An electronic author reprint will be supplied free of charge after online publication.