UNIVERSIDADE ESTADUAL DE MARINGÁ
DEPARTAMENTO DE FARMÁCIA E FARMACOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Estudo Químico, Desenvolvimento de Metodologia Analítica e Avaliação da Estabilidade de Extratos de G*uazuma ulmifolia* Lam. (Sterculiaceae)

Gisely Cristiny Lopes

MARINGÁ, 2009

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Área de Concentração: Produtos Naturais Biologicamente Ativos

Estudo Químico, Desenvolvimento de Metodologia Analítica e Avaliação da Estabilidade de Extratos de G*uazuma ulmifolia* Lam. (Sterculiaceae)

Tese apresentada por **Gisely Cristiny Lopes** ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Estadual de Maringá como parte dos requisitos para a obtenção do Título de Doutor em Ciências Farmacêuticas.

ORIENTADOR: **Prof. Dr. João Carlos Palazzo de Mello**

MARINGÁ, 2009

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

L864e

Lopes, Gisely Cristiny
Estudo químico, desenvolvimento de metodologia analítica e avaliação da estabilidade de extratos de *Guazuma ulmifolia* Lam.(Sterculiaceae) / Gisely Cristiny Lopes. -- Maringá : [s.n.], 2009.
91 f. : il.

Orientador : Prof. Dr. João Carlos Palazzo de Mello.
Tese (doutorado) - Universidade Estadual de Maringá, Programa de Pós-graduação em Ciências Farmacêuticas, 2009.

1.*Guazuma ulmifolia* - Análise. 2. *Guazuma ulmifolia* - Mutamba - Análise fitoquímica. 3. *Guazuma ulmifolia* - Cromatografia líquida de alta eficiência. 4. *Guazuma ulmifolia* - Extratos - Estabilidade. I.Universidade Estadual de Maringá, Programa de Pós-graduação em Ciências Farmacêuticas. II. Título.

CDD 21.ed.615.321

AGRADECIMENTOS

A Deus, pela vida, eterno amor, força nos momentos de alegria e provação e pela oportunidade de sempre evoluir.

Aos meus pais, Paulo Trindade Lopes e Tania Sueli Stamato Lopes e aos meus irmãos Daniely e Paulo Cesar por todo o amor e paciência.

Agradeço ao Professor Dr. João Carlos Palazzo de Mello pela confiança, orientação e amizade.

Ao professor Dr. Marcos Luciano Bruschi pela valiosa contribuição.

Ao professor Dr. Antonio Medina Neto, pela colaboração, atenção e suporte nas análises térmicas.

Ao Dr. Heinrich Luftmann do Instituto de Química Orgânica da Universidade de Münster, Alemanha, pelos espectros de massas e dicroísmo circular e pela ajuda na interpretação.

Á Dra. Ivânia Terezinha Albrecht Schuquel do Departamento de Química da Universidade Estadual de Maringá pelos espectros de RMN.

Aos meus colegas e amigos que não pouparam esforços para que este trabalho fosse realizado, em especial a Renata Longhini, Paulo Victor Pires dos Santos, Claudio Novello e Glauber de Almeida.

Às minhas amigas Helena Francisca Mesquita de Carvalho, Monica Bordin Pessutto, Patrícia Érika Rabelo e Silva Corazza e Kelly Sivocy Teixeira pelo apoio nas horas incertas, quando o caminho a ser percorrido estava árido aos pés.

À Wanda e Walter Ribeiro pela amizade, carinho, alegria e disposição ao trabalho com que sempre me receberam.

À minha querida Irmã Benigna, pelo incentivo, amor e cuidado em todos os momentos de minha vida.

Ao Mestre Avelino Alves, pela confiança e amizade.

À coordenação e aos professores do Programa de Pós-Graduação em Ciências Farmacêuticas pelo profissionalismo e dedicação.

A todas as pessoas que de uma maneira ou de outra contribuíram para a realização deste trabalho.

A Capes pelo suporte financeiro.

"O futuro não é um lugar onde estamos indo, mas um lugar que estamos criando. O caminho para ele não é encontrado, mas construído e o ato de fazê-lo muda tanto o realizador quanto o destino."

Antoine de Saint-Exupery

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RESUMO

Guazuma ulmifolia Lam. (Sterculiaceae), popularmente conhecida como "mutamba", é uma planta de distribuição tropical encontrada a partir do México até o sul da América do Sul. Na medicina popular de vários países latino-americanos, é usada para o tratamento de queimaduras, diarréia, inflamações e na alopecia. A partir da casca *de G. ulmifolia*, nove compostos foram isolados e identificados, entre eles: *ent*-catequina, epicatequina (EP), *ent*-galocatequina, epigalocatequina, epiafzelequina-(4β → 8)-epicatequina, epicatequina-(4β → 8)-catequina (PB1), epicatequina-(4β → 8)-epicatequina (PB2), epicatequina-(4β → 8)-epigalocatequina, e o novo compostos 4'-*O*-metil-epiafzelequina. A análise do extrato semipurificado por cromatografia líquida de alta eficiência (CLAE) foi realizada utilizando coluna C18 (Phenomenex®, modelo Gemini, 5 μm) como fase estacionária, a 30 ºC, com uma vazão de 0,8 mL/min, a 210 nm. Os principais parâmetros de validação do método foram avaliados. Para a análise de correlação linear, curvas analíticas foram construídas com intervalos de 20,00-150,00 μg/mL para a procianidina B2 (PB2) e 10,00-110,00 μg/mL para a epicatequina (EP). Os coeficientes de correlação da análise de regressão linear ficaram entre 0,9981-0,9988, e os limites de detecção foram entre 2,89 e 2,54 μg/mL. O conteúdo de PB2 e EP foram determinadas com sucesso, com boa reprodutibilidade e boa recuperação. A exatidão do método foi de 103,00 e 104,01%, respectivamente para PB2 e EP. A fim de avaliar o comportamento de seus constituintes químicos frente a variações de temperatura e umidade, realizou-se um estudo preliminar de estabilidade. As propriedades físico-químicas e de compatibilidade do extrato com a presença ou não de dióxido de silício coloidal, foram determinadas durante 21 dias de armazenamento sob condições forçadas de temperatura (45±2 °C) e umidade (75±5%). A alteração máxima no conteúdo de EP foi observada em 21 dias no extrato seco + dióxido de silício coloidal. Análise térmica (TG) foi realizada para estabelecer parâmetros de compatibilidade com o excipiente, e mostrou que o dióxido de silício coloidal pode contribuir com a estabilidade o extrato seco das cascas de *G. ulmifolia*.

Palavras chaves: *Guazuma ulmifolia*, Sterculiaceae, proantocianidinas, CLAE, método de validação, estabilidade, análise térmica.

ABSTRACT

Guazuma ulmifolia Lam. (Sterculiaceae), popularly known as "Mutamba", is a tropical-American plant found from Mexico to southern South America. In the popular medicine of several Latin-American countries, it is used for the treatment of burns, diarrhea, inflammations and alopecia. From the bark of *G. ulmifolia*, nine compounds were isolated and identified: *ent*-catechin, epicatechin, *ent*-gallocatechin, epigallocatechin, epiafzelechin-(4β→8)-epicatechin, procyanidins B1 and B2, epicatechin-(4β→8)-epigallocatechin, and the new compound 4'-*O*-methyl-epiafzelechin. Its structure was elucidated on the basis of spectral and literature data. HPLC analysis of the semipurified extract was performed using a Phenomenex® Gemini RP C_{18} column (5 µm) as stationary phase, at 30 ºC, with a flow rate of 0.8 mL min^{-1}, at a wavelength of 210 nm for detection and determination. The main validation parameters of the method were also determined. Calibration curves were found to be linear, with ranges of 20.00–150.00 µg mL^{-1} procyanidin B2 (PB2) and 10.00–110.00 µg mL^{-1} epicatechin (EP). The correlation coefficients of linear regression analysis were between 0.9981–0.9988, and the detection limits were between 2.89 and 2.54 µg mL^{-1}. The contents of PB2 and EP were successfully determined, with satisfactory reproducibility and recovery. Recoveries of the PB2 and EP were 103.00 and 104.01%, respectively. The method was successfully applied to the determination of procyanidins in bark of *G. ulmifolia*. The preliminary stability of the dried extracts from bark of *G. ulmifolia* containing or not colloidal silicon dioxide (CSD) was evaluated. The physical–chemical properties and compatibility of CSD in the extract were evaluated for 21 days of storage under stress conditions of temperature (45±2 °C) and humidity (75±5%). Thermal analysis (TG) was supplemented using a selective high-performance liquid chromatography (HPLC) for determination of stability of the characteristic constituents (chemical markers), namely PB2 and EP. The results showed that PB2 is an appropriate compound to use as chemical marker in control quality of dried extracts of *G. ulmifolia*. The stress study showed that there was no significant difference between the two extracts. On the other hand, considering the TG data and the high temperatures involved, the results suggest that CSD would be increase the stability of dried extracts of *G. ulmifolia*.

Keywords: *Guazuma ulmifolia*, Sterculiaceae, proanthocyanidins, HPLC, validation method, stability test, thermal analysis.

CAPÍTULO I

1.1. INTRODUÇÃO

Há milhões de anos a natureza vem adaptando-se para permitir a sobrevivência das mais diversas espécies vegetais e animais em ecossistemas variados e muitas vezes hostis. Do ponto de vista bioquímico as espécies vegetais adaptaram-se a estes ambientes devido à presença de substâncias capazes de exercer as mais diferentes funções nos organismos vegetais, entre elas, os metabólitos secundários, que apresentam estrutura complexa, baixo peso molecular e marcante atividade biológica.

Embora não sejam os responsáveis diretos pela organização e vida celular, os metabólitos secundários têm papel fundamental na atração de polinizadores, proteção contra os raios UV, defesa celular contra herbívoros e microorganismos, entre outras atividades, indispensáveis para a adaptação do vegetal a seu meio (GOTTLIEB *et al.,* 2000).

A importância dos metabólitos secundários, particularmente na área farmacêutica, o estudo da sua biogênese e regulação tem crescido de maneira notável nos últimos anos. As recentes estratégias para a síntese de novos fármacos têm por base a diversidade estrutural, encontrada na flora, como alicerce fundamental na pesquisa para atingir diferentes alvos biológicos, portanto, os pesquisadores voltaram seus estudos aos produtos naturais, considerando que durante milhares de anos de evolução biológica a seleção natural realizou um processo de química combinatória realmente inigualável (YUNES *et al.,* 2001).

As oportunidades para a identificação de produtos com possível utilização econômica aumentam com a diversidade das espécies. Ao se considerar a perspectiva de obtenção de novos fármacos, dois aspectos distinguem os produtos de origem natural dos sintéticos: a diversidade molecular e a função biológica. A indústria farmacêutica na busca de moléculas biologicamente ativas tem levado em consideração estudos etnofarmacológicos, considerando o fato que a seleção de espécies vegetais para a pesquisa e desenvolvimento, baseada na

alegação de um dado efeito terapêutico em humanos pode constituir-se num valioso atalho para a descoberta de fármacos, visto que o uso tradicional pode ser encarado como uma pré-triagem quanto à utilidade terapêutica da espécie em questão (ELISABETSKY, 2003).

Os resultados da pesquisa com plantas medicinais podem ser desdobrados em vários níveis. Individualmente, a descoberta de novos fármacos, ou fármacos acessíveis, pode determinar a melhoria da qualidade de vida em doenças crônicas ou a própria sobrevivência do paciente afetado. Socialmente, a descoberta de fontes naturais e locais de compostos químicos usualmente importados e/ou o desenvolvimento de fitoterápicos de fabricação nacional, podem ter conseqüências econômicas significativas, além de possibilitar a autonomia de cada país no gerenciamento de suas políticas de saúde (ELISABETSKY, 2005).

A importância histórica das substâncias ativas obtidas a partir de plantas para o desenvolvimento de fármacos (*Digitalis purpurea,* digoxina*; Papaver somniferum,* morfina; *Cinchona calisaya,* quinina; *Atropa belladona*, atropina entre outras), não representou apenas o surgimento de um novo grupo de fármacos, mas originou, não somente, a identificação de uma nova possibilidade de intervenção terapêutica, como também, avanços significativos na geração de tecnologia quanto à elucidação estrutural, técnicas cromatográficas e novos *screening* para a elucidação de alvos moleculares específicos. Estes avanços permitiram maior rapidez e eficiência na busca de moléculas biologicamente ativas.

Portanto, bases científicas e tecnológicas possibilitam a produção regular e reprodutível de extratos vegetais com as mesmas características farmacêuticas, bem como fornecem a confirmação de segurança e eficácia destas.

Entre os metabólitos secundários de importância econômica, os compostos fenólicos têm se mostrado especialmente efetivos em seus efeitos medicinais e se encontram amplamente distribuídos em muitas espécies de plantas tropicais. Taninos são substâncias fenólicas solúveis em água, com massa molecular entre 500 a 3.000 Dalton, que apresentam

habilidade de formar complexos insolúveis em água com alcalóides, gelatinas e outras proteínas. Acredita-se que as atividades farmacológicas dos taninos são devidas, pelo menos em parte, a três características gerais: 1- Complexação com íons metálicos; 2- Atividade antioxidante, seqüestradora de radicais livres; 3- Habilidade de complexar com proteínas e polissacarídeos. Sendo um grupo de substâncias conhecidas por possuir atividade anti-séptica e por agir no sistema digestivo, sendo úteis no tratamento de diarréias, desordens estomacais, feridas, queimaduras e inflamações (HASLAM *el. al*., 1989; LOPES *et al.*, 2005).

Guazuma ulmifolia Lam. (Sterculiaceae), popularmente conhecida como Mutamba no Brasil, tem origem na América Tropical, e se estende desde o México até a América do Sul.

Tradicionalmente seu uso tem sido relatado na medicina popular de vários países, entre eles: Belize, Brasil, Colômbia, Cuba, Republica Dominicana, Guatemala, Haiti, Jamaica, México, Panamá e Peru. É usada popularmente no tratamento de bronquite, queimadura, diarréia, asma, elefantíase, inflamações e na alopecia.

O estudo da composição química de *G. ulmifolia* Lam. aponta até o presente, cascas ricas em taninos e proantocianidinas (HÖR *et al.,* 1996; GALINA, 2003; ROCHA, 2005), substâncias estas que têm sido relatadas por suas atividades biológicas diversas, entre elas: atividade antitumoral (KASHIWADA *et al.,* 1992), atividade neurológica (SHIMADA *et al*., 2001), atividade cardiovascular (CHENG *et al*., 1993), atividade antiviral (FELIPE *et al.,* 2002), atividade antioxidante (SHAHAT *et al.,* 2002) e promotora do crescimento capilar (KAMIMURA *et al*. 2002; TAKAHASHI *et al.,* 2001; TAKAHASHI *et al.,*1999a; TAKAHASHI *et al.,* 1999b; TAKAHASHI *et al.,* 1998).

Considerando os estudos descritos, e os estudos etnofarmacológicos, o presente trabalho é centrado na avaliação de extratos de *Guazuma ulmifolia* Lam. A estratégia do trabalho foi baseada na elucidação química do extrato semipurificado, no desenvolvimento de

metodologia analítica para fins de controle de qualidade dos extratos e na avaliação preliminar da estabilidade do extrato seco na presença ou ausência de adjuvante farmacêutico.

1.2. REVISÃO DE LITERATURA

1.2.1. *Guazuma ulmifolia* Lam.

1.2.1.1. Generalidades

O nome vulgar mais empregado no Brasil é mutamba, porém é conhecida localmente como embira e/ou embireira em Marajó; embirú no Rio Grande do Sul; mutamba-verdadeira, camaca, no Pará; envieira, mutambo, pau-de-bicho, em Mato Grosso, guaxima-macho e araticum-bravo em São Paulo.

Devido a sua ampla distribuição na América Tropical recebe sinonímias como: guácimo, na Venezuela, Honduras e El Salvador; guácima, guácima cimarrona, na República Dominicana; guácima de caballo, em Cuba; guacimilla, majaqua de toro, guazima, no México; chicharrón em El Salvador; guacimillo, na Nicarágua; guácimo blanco, na Costa Rica; canlote, na Colômbia; Iumanasi, papauillo, no Peru; coco, na Bolívia; cambaacá, guazuma, na Argentina.

1.2.1.2. Descrição botânica

Guazuma ulmifolia Lam., é uma planta da família Sterculiaceae, cujo gênero está distribuído em regiões tropicais, penetrando em zonas secas. Francis (2006), apresenta um mapa com a distribuição geográfica dessa planta na América Latina (fig. 1).

Fonte: Francis, 2006

Fig. 1 – Distribuição natural da *Guazuma ulmifolia* Lam. na América Latina

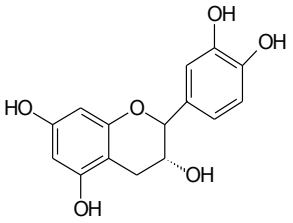
De acordo com Corrêa (1974), a mutamba é uma planta arbórea, com 6 a 16 m de altura, tronco com diâmetro que varia entre 30-60 cm, com numerosos ramos e de copa larga. As folhas são simples e alternas, com configuração oval, brilhantes e recortadas nas margens. A lâmina foliar apresenta-se do tipo membranácea, com 8 a 15 cm de comprimento e 4 a 7 cm de largura, têm coloração verde clara, sendo concolor, oblongada, com base redonda e levemente cordiforme e ápice agudo e acuminado. A nervação é do tipo peninérvea, com cinco nervuras na inserção do pecíolo, proeminentes na face inferior. As nervuras secundárias apresentam-se em intensa anastomose. Tricomas estrelados são encontrados por toda a lamina foliar principalmente na nervura principal, sendo mais abundantes na superfície abaxial. O pecíolo, marrom-esverdeado, tem 1,5 a 3 cm de comprimento, com secção transversal circular, é levemente estriado e muito piloso. As flores apresentam-se em inflorescências racemosas ou paniculadas, axilar; o pedúnculo, com 0,6 a 1,5 cm de comprimento, apresenta coloração marrom, revestido de tricomas estrelados. As flores são actinomorfas, pediceladas; o cálice é trilobado, dialissepalo, de coloração creme, sendo piloso. A corola é dialipétala, pentâmera, alva, as pétalas são curvadas para o lado do gineceu, apresentando finos prolongamentos levemente delineados com aproximadamente 5 mm de comprimento, pilosos interna e externamente. O androceu apresenta seis estames soldados a base e entre si; os filetes possuem, em media, 2 mm de comprimento e as anteras, 0,5 mm. O ovário é supero, com aproximadamente 1 mm de comprimento, possuindo vários óvulos por lóculo. Os estigmas, em numero de 5, com 1 mm de comprimento, apresentam nectários acima do ovário. O fruto é pedicelo, com 2 a 3,5 cm de comprimento. Quando seco, possui 2,1 a 2,6 cm de diâmetro e 6 a 10 cm de circunferência, é deiscente, capsular, possuindo forma arredondada com projeções pontiagudas por toda a superfície, coloração negra.

1.2.1.3. Avaliação do conteúdo químico

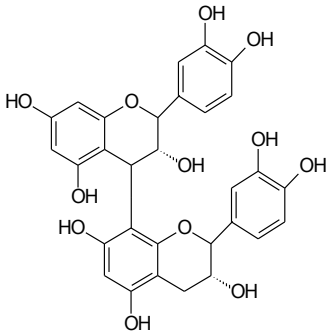
A análise química preliminar de extratos de cascas de *Guazuma ulmifolia* Lam., indicaram a presença de proantocianidinas com vários graus de polimerização (HÖR *et al.*, 1995; CABALLERO-GEORGE *et al.*, 2002; HEINRICH, 2003).

A partir do extrato bruto etanólico das cascas, com posterior partição em acetato de etila e purificação em coluna de Sephadex® LH-20, foram isolados monômeros, dímeros e polímeros de proantocianidinas (fig. 2). Nos polímeros as unidades de flavanóides com ligação do tipo (4→8) predominavam sobre as do tipo (4→6) (HÖR *et al.*, 1996).

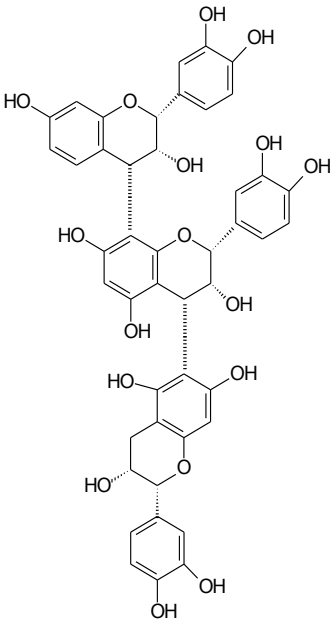
Galina (2003) isolou a partir do extrato acetônico das cascas de *G. ulmifolia* Lam. três substâncias fenólicas do tipo taninos condensados: epicatequima, procianidina B_2 [epicatequina-(4β→8)-epicatequina] e epiafzelequina-(4β→8)-equicatequina. As estruturas foram elucidadas através de análise espectroscópica em RMN ^{1}H, RMN ^{13}C e EI- e DCI-MS.



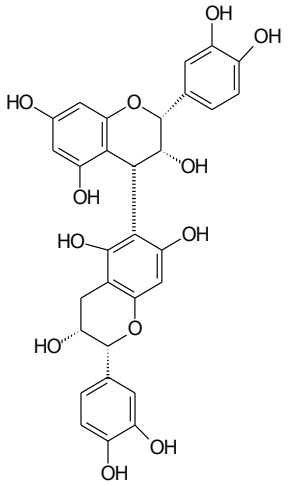
Epicatequina



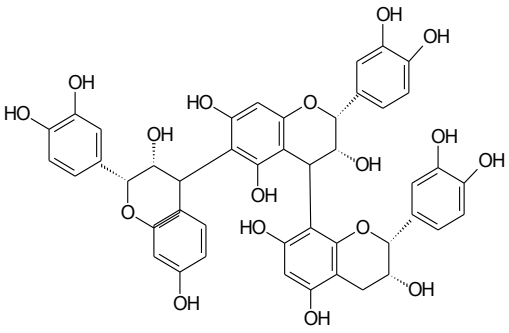
Epicatequina-(4β→8)-epicatequina



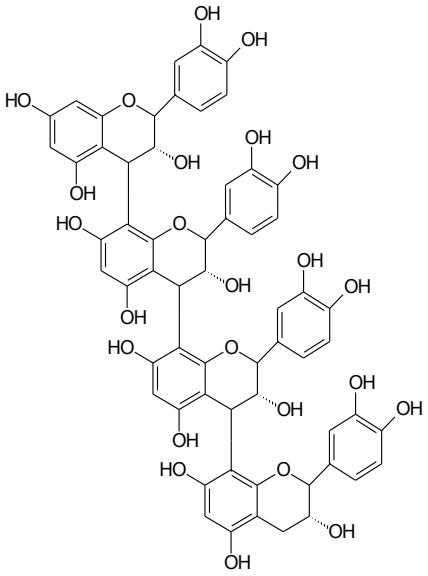
Epicatequina-(4β→8)-epicatequina-(4β→6)-epicatequina



Epicatequina-(4β→6)-epicatequina



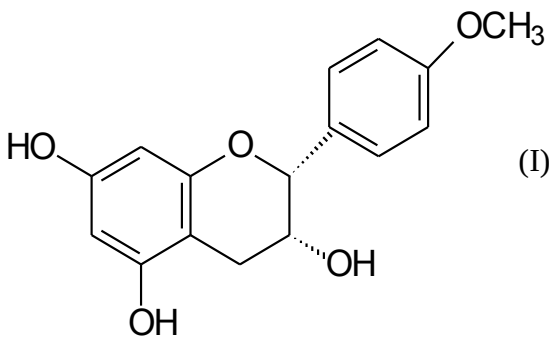
Epicatequina-(4β→6)-epicatequina-(4β→8)-epicatequina



Epicatequina-(4β→8)-epicatequina-(4β→8)-epicatequina-(4β→8)-epicatequina

Figura 2 - Proantocianidinas isolados das cascas de *Guazuma ulmifolia* Lam.

Rocha (2005), realizou estudo químico de extratos semipurificados das cascas de *G. ulmifolia* Lam., demonstrando que as formas de extração e fracionamento utilizadas foram adequadas ao isolamento de taninos condensados. Foram isoladas e identificadas as substâncias: catequina, epicatequina, galocatequina, epigalocatequina, 4'-*O*-metil-epiafzelequina, epiafzelequina-(4β→8)-epicatequina, epicatequina-(4β→8)-catequina, epicatequina-(4β→8)-epicatequina e epicatequina-(4β→8)-epigalocatequina. Entre essas, a substância 4'-*O*-metil-epiafzelequina (I), foi identificada em material vegetal pela primeira vez.



(I)

Recentemente, Seigler *et al.* (2005), isolaram e identificaram glicosídeos cianogênicos em folhas de *G. ulmifolia* Lam. Suas estruturas foram elucidadas através de análise espectroscópica em RMN ^{1}H, COSY-1D, TOCSY, NOESY, HETCOR, RMN ^{13}C e DCI-MS, além de métodos ópticos e cristalografia. Segundo os autores, a ocorrência não é comum na espécie, mas tem sido relatada em outros membros da família. Porém, não são substâncias características da família Sterculiaceae.

1.2.1.4. Etnofarmacologia e Farmacologia

O uso da mutamba é praticado pelos povos indígenas da América Central e do Sul por suas propriedades curativas.

As folhas e cascas de *G. ulmifolia* Lam., são usadas na medicina popular na forma de chá e decocto respectivamente, como: antibacteriana, antidesentérica, antifúngica, anti-inflamatória, adstringente, depurativa, diaforética, emoliente, hepatoproterora, expectorante e para queda de cabelo (TECHNICAL DATA, 2002).

Pesquisas etnofarmacológicas no México (DOMINGUEZ *et al.,* 1985*;* ZAMORRA-MARTINEZ *et al.,* 1992; HEINRICH *et al.,* 1992a; HEINRICH *et al.,* 1992b; ALARCON-AGUILARA *et al.,* 1998), Guatemala (CÁCERES *et al.,* 1987a; CÁCERES *et al.,*1987b; CÁCERES *et al.,* 1990; CÁCERES *et al.,* 1993, NAVARRO *et al.,* 2003), Haiti (WENIGER *et al.,* 1986), Belize (CAMPORESE *et al.,* 2003) e Panamá (CABALLERO-GEORGE *et al.,* 2001) descrevem a longa história de uso da mutamba pela população local.

Cáceres *et al.* (1990), relacionaram através de seus estudos, que o uso bem sucedido de *Guazuma ulmifolia* Lam. pela população local na Guatemala, em desordens gastrintestinais e infecções microbianas, está relacionada à ação inibitória sobre o crescimento das enterobactérias patogênicas: *Escherichia coli, Salmonella enteritides, Salmonella typhi, Shigella dysentereae* e *Shigella flexneri*.

Através de ensaios, utilizando-se coelhos machos, foi constatado que a administração do decocto das folhas de *G. ulmifolia* Lam., levou a uma significativa queda do pico hiperglucêmico em relação ao controle. Os resultados obtidos sugerem atividade hipoglucemiante, colocando o extrato de mutamba como excelente candidato a futuros estudos para a determinação dos mecanismos de atividade sobre a glucemia (ALARCON-AGUILARAI *et al.,* 1998). Recentemente, Alonso-Castro e Salazar-Olivo (2008) avaliaram ação anti-diabética de extratos de cascas *G. ulmifolia*. Os autores concluíram que a mutamba

exerce os seus efeitos anti-diabéticos, estimulando a captação de glucose em adipócitos, insulino-sensíveis e/ou insulino-resistentes, sem induzir a adipogênese.

Navarro *et al.* (1996) avaliaram as propriedades antimicrobiana e antioxidante do extrato metanólico dos frutos de *G. ulmifolia*. O estudo revelou atividade significante contra *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa.* No teste com DPPH os resultados foram satisfatórios, indicando atividade antioxidante.

Na avaliação da atividade antibacteriana (MIC), utilizando o método da microdiluição em placa, extratos hexânico, metanólico e clorofórmico liofilizados obtido de cascas de *G. ulmifolia* Lam., foram testados contra *Escherichia coli, Pseudonomas aeruginosa, Staphylococcus aureus* e *Enterococcus faecalis.* Os resultados indicaram que o extrato hexânico inibiu o crescimento de *E. coli*, e o extrato metanólico de *P. aeruginosa*, sugerindo atividade antibacteriana contra esses microorganismos (CAMPORESE *et al.,* 2003).

Felipe *et al.* (2006) avaliaram a atividade antiviral dos extratos acetônicos obtidos a partir das cascas de *Guazuma ulmifolia*. A atividade antiviral foi monitorada através de ensaio em placa e imunofluorescência indireta. Os resultados indicaram que os extratos testados, bloquearam a síntese de antígenos virais em culturas de células Hep-2 infectadas com vírus pólio-1 e herpes bovino-1.

Galina (2003), relatou a ação bacteriostática e bactericida de extratos bruto e semipurificado das cascas de *G. ulmifolia* Lam., sobre o crescimento de bactérias Gram-positivas e Gram-negativas, confirmando a atividade antibacteriana contra as cepas testadas. O extrato semipurificado mostrou concentração mínima inibitória (MIC) de 250 μg/mL frente à *Pseudononas aeruginosa* e de 62,5 μg/mL frente à *Staphylococcus aureus.*

O extrato aquoso obtido a partir de cascas de *G. ulmifolia* Lam. foi avaliado sobre a secreção gástrica de coelhos. Os resultados permitiram concluir que a secreção gástrica estimulada por toxina colérica foi completamente inibida pela ação do extrato. No entanto, a

secreção estimulada por prostaglandina E2 não sofreu alteração (HÖR *et al.*, 1995). O estudo revela a possível complexação dos taninos condensados (HÖR *et al.*, 1996) com as toxinas, explicando seu uso popular em diarréias. Recentemente, Berenguer *et al.* (2007), demonstraram o efeito gastro-protetor do extrato etanólico das folhas e flores de *Guazuma ulmifolia,* em modelo de úlcera gástrica induzida por diclofenaco em ratos Wistar, utilizando o omeprazol como substância referência. Após a análise estatística dos resultados, os autores observaram que: a) a área ulcerada de uma maneira em geral é dose-dependente; b) as menores doses do extrato diminuíram significativamente os níveis de lipoperoxidases; c) houve um ligeiro aumento nos níveis de prostaglandina E2 em todas as doses. Assim, foi possível concluir que extratos obtidos a partir de estruturas aéreas de *Guazuma ulmifolia* protegeram efetivamente a mucosa gástrica.

Magos *et al.* (2008) demonstraram a ação cardiovascular da fração enriquecida em procianidinas do extrato de cascas de *G. ulmifolia*, em diferentes modelos de atividade hipotensora, corroborando assim com os trabalhos de Caballero-George *et al.* (2002), que avaliaram o efeito do extrato acetônico liofilizado das cascas de *G. ulmifolia* Lam. sobre a ligação da Angiotensina II [^{3}H] com o receptor humano AT1 *in vitro*. Este estudo indica que as proantocianidinas presentes no extrato, possam competir molecularmente com os ligantes específicos do receptor de AT1. O trabalho sugere que o extrato tenha efeito hipotensor e confirma o uso tradicional na hipertensão arterial (CABALLERO-GEORGE *et al.*, 2002). Em trabalho anterior, Caballero-George *et al.* (2001), realizaram estudo sobre as propriedades ligantes de substâncias da flora medicinal do Panamá, a alvos moleculares específicos.

Rocha (2005) utilizando-se do método do fosfomolibdênio e do radical DPPH, demonstrou a capacidade antioxidante dos extratos bruto e semipurificados das cascas de *G. ulmifolia* Lam., recomendando a utilização de várias técnicas e metodologias diferentes para descobrir em qual classe de antioxidante se enquadra a amostra testada.

O extrato bruto de cascas de *G. ulmifolia* Lam. incorporado a uma loção capilar hidroalcoólica, foi avaliado frente a uma loção controle, no estimulo ao crescimento capilar em ratos Wistar. A avaliação morfológica realizada através das análises morfométricas e da proliferação celular epitelial não evidenciaram o desenvolvimento do folículo piloso (ROCHA, 2005). No entanto foi demonstrada a permeação da formulação farmacêutica através de métodos de espectroscopia fotoacústica (ROCHA *et al*., 2007).

1.3. OBJETIVOS

O objetivo geral desse trabalho foi estudar quimicamente as cascas da espécie *Guazuma ulmifolia* Lam. (Sterculiaceae) visando obter um extrato semipurificado, com conteúdo químico conhecido.

A busca para tal condição foi orientada em três etapas: estudo fitoquímico do extrato, por meio de métodos cromatográficos e determinação estrutural das substâncias químicas isoladas; desenvolvimento e validação de metodologia analítica por cromatografia líquida de alta eficiência, visando a quantificação dos principais marcadores nos extratos e a avaliação preliminar da estabilidade, através de teste de stress acelerado com acompanhamento dos extratos por análises físicas.

Este trabalho envolveu: 1) preparação dos extratos vegetais obtidos a partir de cascas; 2) obtenção de frações semipurificadas; 3) isolamento das substâncias através de métodos cromatográficos; 4) identificação das estruturas químicas por métodos espectroscópicos; 5) determinação do perfil químico por cromatografia liquida de alta eficiência; 6) validação de metodologia analítica; 7) avaliação da estabilidade de extratos submetidos a condições forçadas de temperatura e umidade por cromatografia líquida de alta eficiência e 8) análise térmica.

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CAPÍTULO II

CONDENSED TANNINS FROM THE BARK OF *GUAZUMA ULMIFOLIA*

(ARTIGO SUBMETIDO AO PERÍODICO JOURNAL OF THE BRAZILIAN CHEMICAL SOCIETY)

Condensed Tannins from the Bark of *Guazuma ulmifolia* Lam. (Sterculiaceae)

Gisely C. Lopes[1], Juliana C. B. Rocha[1], Glauber C. de Almeida[2], João C. P. de Mello[1]*

[1]Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900 Maringá-PR, Brazil

[2]Acadêmico de Farmácia, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900 Maringá-PR, Brazil

[*]Corresponding author. Tel.: + 55 4432614816; fax: + 55 4432614999
E-mail address: mello@uem.br (J. C. P. de Mello).

Condensed Tannins from the Bark of *Guazuma ulmifolia*

Das cascas de *Guazuma ulmifolia* Lam. (Sterculiaceae) foram isolados e identificados nove compostos: *ent*-catequina, epicatequina, *ent*-galocatequina, epigalocatequina, epiafzelequina-(4β→8)-epicatequina, epicatequina-(4β→8)-catequina (procianidina B1), epicatequina-(4β→8)-epicatequina (procianidina B2), epicatequina-(4β→8)-epigalocatequina, e a nova substância 4'-*O*-metil-epiafzelequina. Suas estruturas foram elucidadas com base em dados espectrais e de literatura. A "impressão digital" de um extrato semipurificado por cromatografia líquida de alta eficiência foi realizada em coluna C18, com uma mistura de acetonitrila (0,05% de ácido trifluoroacético): água (0,05% de ácido trifluoroacético) (v/v) com vazão de 0,8 mL min^{-1}, a amostra foi de 20 μL e o comprimento de onda 210 nm.

From the bark of *Guazuma ulmifolia* Lam. (Sterculiaceae), nine compounds were isolated and identified: *ent*-catechin, epicatechin, *ent*-gallocatechin, epigallocatechin, epiafzelechin-(4β→8)-epicatechin, epicatechin-(4β→8)-catechin (procyanidin B1), epicatechin-(4β→8)-epicatechin (procyanidin B2), epicatechin-(4β→8)-epigallocatechin, and the new compound 4'-*O*-methyl-epiafzelechin. Their structures were elucidated on the basis of spectral and literature data. HPLC fingerprint analysis of the semipurified extract was performed on a C18 column, with a mixture of acetonitrile (0.05% trifluoroacetic acid):water (0.05% trifluoroacetic acid) (v/v) with a flow rate of 0.8 mL min^{-1}. The sample injection volume was 20 μL and the wavelength was 210 nm.

Keywords: *Guazuma ulmifolia*; condensed tannins; HPLC; fingerprint, Sterculiaceae

Introduction

Despite extensive destruction, it is believed that the rainforests still preserve 30 million individual species, roughly half of all life forms on earth and 2/3 of all plants, without mentioning the importance of these forests to the earth's weather and atmosphere. In South America, the Amazon tropical forest covers approximately 665 million hectares, of which 60% lies within Brazil, covering 59% of the country's territory, in nine states: Acre, Amapá, Amazonas, Maranhão, Mato Grosso, Pará, Rondônia, Roraima and Tocantins.[1]

The importance of conserving the Amazon tropical forest is not limited only to the animal and plant species themselves, but also to the rich knowledge of the local people about the use of medicinal plants. Their knowledge stems as much from the necessity for alternative treatments because of low purchasing power and the difficulty of access to medical assistance, as from the profound cultural influence of the indigenous peoples of the region.

It is known that rapid social, cultural and economic changes strongly affect local knowledge of how to use natural resources. The problems stemming from this cultural loss are irreversible, and this loss reduced the possibilities of sustainably developing a region based on local experience.[2]

The discussion of alternatives for development and their relationship with the productive use of biodiversity is recent in Brazil. A proposal for sustainable development seeks to ally the need to protect the environment with the principal of equity with present and future human generations, through effective inclusion of the environment in socio-economic decisions. However, it is no simple matter to develop a strategy, which requires many solutions acting in parallel, including the demarcation of forestal preserves, projects for renewable forest harvesting, and domestication of local species of economic importance,

envisaging local productive networks. Thus, surrounding the interest in biodiversity are many economic, ecological, ethical and heritage factors.[3]

In this context, many endemic and non-native plant species in Amazonia have been studied scientifically, taking into consideration the knowledge of the local population, with a view toward obtaining new phytotherapeutic and cosmetic pharmaceuticals.[4-9]

Guazuma ulmifolia Lam. is a middle-sized tree, belonging to the family Sterculiaceae, which occurs naturally throughout Latin America.[10] In Brazil, where it is popularly known as mutamba, this species extends from the Amazon region to the state of Paraná.[11] It is pantropical, semideciduous, heliophytic, a pioneer, and is characteristic of second-growth broad-leaf forests. In Brazil, *G. ulmifolia* has been studied for its important role for the recovery of degraded areas.[10,11]

In popular medicine, *G. ulmifolia* is traditionally used in several countries including Brazil,[12] Guatemala,[13-15] Haiti,[16] Mexico,[17-19] and Belize[20] to treat bronchitis, burns, diarrhea, asthma, inflammations, and alopecia.

Previous investigations of the chemical composition of *G. ulmifolia* have indicated the occurrence of flavan-3-ols and procyanidins,[21] nitrile glucoside menisdaurin.[22] The anti-diabetic properties,[23] hypotensive and vasorelaxant activity,[24] antiulcer,[25,26] anti-bacterial activities[20,27] and antiviral activity[28] of the bark, aerial parts, fruits, crude extract, and fractions were attributed to the presence of proanthocyanidins.

The aim of the present study was to investigate the chemical profile of the bark of *Guazuma ulmifolia* Lam., and to develop a HPLC-UV fingerprint and characterize its major active chemical constituents.

Results and Discussion

The ethyl acetate-soluble fraction obtained from the aqueous acetone extract of the air-dried bark was chromatographed on a Sephadex LH-20 column. Fractions containing proanthocyanidins were further purified by multi-layer-coil counter-chromatography (MLCCC) to give known and some rare compounds including *ent*-catechin (**1**), epicatechin (**2**), *ent*-gallocatechin (**3**), epigallocatechin (**4**), epiafzelechin-(4β→8)-epicatechin (**5**), epicatechin-(4β→8)-catechin (PB1) (**6**), epicatechin-(4β→8)-epicatechin (PB2) (**7**) and epicatechin-(4β→8)-epigallocatechin (**8**). These were readily identified by comparison of spectroscopic data of the peracetates (^{1}H NMR, ESI-MS, $[\alpha]_D^{20^\circ}$) with authentic material.[29-37] The new compound 4'-*O*-methyl-epiafzelechin **(9)** discussed below was established by physical properties [^{1}H NMR, ESI-MS, $[\alpha]_D^{20^\circ}$] of the corresponding peracetate derivative.

Compound **(9a)** was visualized as blue spot by spraying with $FeCl_3$ reagent and showed a parent ion at *m/z* 437.5 $[M+Na]^+$ in the ESI-mass spectrum of the corresponding peracetate, suggesting a monomeric flavan-3-ol. The ^{1}H NMR spectral ($CDCl_3$) data showed one three-proton singlet at δ 3.89, indicating a methoxyl group. All heterocyclic protons could readily be assigned from the ^{1}H-^{1}H-COSY spectrum. The compound showed a specific rotation of -30°, and showed the typical spin systems of a 4',5,7-trihydroxyflavan-3-ol framework, i.e., a two-spin AB-system for the A-ring, a four-spin AA^1BB^1-system for the B-ring, and a four-spin AMXY-system for the protons of the heterocyclic ring. The 2,3-*cis* relative configuration was evident from the $^3J_{2,3}$ value of ~1.0 Hz for the broadened 2-H resonance at δ 5.12. The circular dichroism (CD) spectrum in methanol exhibited a high-amplitude negative Cotton effect at 280 nm for the ^1L_b transition and a positive Cotton effect at 240 nm for the ^1L_a transition, hence unequivocally indicating a 2*R*,3*R* absolute

configuration and confirming the structure of compound **(9)** as 4'–*O*-methyl-epiafzelechin from the natural source. This compound is described here for the first time.

Compound 9

An HPLC fingerprint method developed for *Guazuma ulmifolia* can be conveniently employed for quality-control analysis. The experimental conditions chosen were those giving the most chemical information about the herbal medicine in the chromatograms. The column, mobile phase, detection wavelength, and conditions for gradient elution were all investigated.

The chromatographic separations were performed on a C18 analytical column, according to published methods.[38,39] To obtain good separation, acetonitrile-water and methanol-water containing acid were investigated as mobile phases. With methanol-water the peaks of compounds **6** and **7** always coeluted. More compounds were separated by the use of acetonitrile-water containing 0.05% trifluoroacetic acid. To obtain chromatograms with good resolution of adjacent peaks, different flow rates (0.6, 0.8, and 0.9 mL min^{-1}) were also investigated. Good separation was obtained by a flow rate of 0.8 mL min^{-1}.

The elimination of high-molecular-weight phenolic compounds from the plant extract is critically important, because of the interaction of these compounds with the stationary phase. This interaction can seriously damage the analytical column, interfering with the chromatographic process. Currently, the most widely employed sample-preparation methodologies are solid-phase extraction and liquid-liquid extraction. Therefore, in this study, the extraction of proanthocyanidin compounds from *G. ulmifolia* was optimized by using a mixture of water:ethyl acetate. The utilization of the simple one-step liquid-liquid extraction method should completely extract the target constituents from the matrix. The extraction efficiency was evaluated by HPLC, and the results demonstrated the reliability of the process.

The choice of detection wavelength is a crucial step in developing a reliable fingerprint. A UV detector was used in the current study. The spectra of all the main peaks were investigated by use of the diode-array detector, and 210 nm was selected as the detection wavelength to obtain a sufficiently large number of detectable peaks in the chromatograms. Fig. 1 shows the chromatograms of the sample solution at 210 and 280 nm. Comparing the absorbances at the two wavelengths, the absorbances at 210 nm were higher than those at 280 nm for all compounds in the system. Thus, chromatograms recorded at 210 nm showed considerable improvement in the signal-to-noise ratio.

The total analysis time for each run was 32 min. Good separations with a short run time were observed (Fig. 2). The system suitability results are given in Table 1.

Fig. 1

Fig. 2

Method precision was based on replicated analyses of samples, with reported relative standard deviations (RSD) less than 5% for relative retention time (RRT) and relative peak area (RPA) of all peaks. The reproducibility of the method was assessed by means of six replicated sample solutions extracted from a single batch of *G. ulmifolia*. The corresponding RSD of RRT and RPA were less than 5% over the investigation. The stability test was performed with a sample solution left to stand for 24 h. The results obtained in the study of the solution (both time zero and the sample solution after 24 h) indicated that the solutions were stable for 24 h, because during this time the areas of the peaks did not decrease below the minimum percentage of 90% of the initial area. The data were assessed by Student's *t* test and ANOVA, and showed no significant differences ($p<0.05\%$). The results showed that the method developed is a straightforward, sensitive, and selective tool with good accuracy and

reproducibility, which can be readily utilized as a suitable method for quality control of *G. ulmifolia*.

Table 1

Experimental

Plant material

Bark of *Guazuma ulmifolia* Lam., Sterculiaceae, was collected in December 2004, in the city of Ibiporã, State of Paraná, Brazil (S 23°18'15.2"; W 050°58' 32.7"; 396 m altitude; Garmin v.2.24). The species was identified by Prof. Dr. Cássia Mônica Sakuragui. Voucher specimens are deposited in the herbarium of the Department of Biology of the State University of Maringá under number HUM 10.491. This specie occur in the Amazonian region.

Isolation and purification

Air-dried stem bark (2,000 g) was exhaustively extracted with Me_2CO-H_2O (7:3; 20 L) by turbo-extraction (Ultra-turrax® UTC115KT; 20 min; t≤40 ºC), and the combined extracts were filtered and evaporated under reduced pressure to 1.0 L and lyophilized (229.6 g, CE). 200 g of this fraction was redissolved in 2.5 L H_2O and extracted with EtOAc (35 L). After evaporation of solvents, the EtOAc fraction (GU) and the remaining H_2O phase (GU-1) gave dark-brown solids of 24 and 174 g, respectively. A portion (19 g) of the GU was subjected to CC on Sephadex LH-20 [710×50 mm; eluents: 20% EtOH (3.2 L), 30% EtOH (1.8 L), 40% EtOH (4.8 L), 50% EtOH (3.9 L), EtOH (1.6 L), 50% MeOH (2.3 L), and 70% Me_2CO (6 L); 10 mL fractions] afforded the following 29 fractions (indicated below with

Roman numerals). Fraction XII (706 mg) was separated on MLCCC, with the solvent system EtOAc-n-PrOH-H_20 (35:2:2) on a P.C. Inc. ITO Multi-layer Coil Separator-Extractor, flow rate 1.0 mL min^{-1}, using the upper layer as mobile phase, giving rise to 5 subfractions. A portion of subfraction XII-4 (31 mg) was acetylated and purified by TLC preparation to yield the peracetate resulting in epigallocatechin (1.4 mg) and gallocatechin (2.2 mg) (these subfractions are indicated below by ordinal numbers). Fraction XIII (376 mg) was submitted to the MLCCC as mentioned above, giving rise to 5 subfractions. A portion of subfraction XIII-1 (47.8 mg) was acetylated and purified by TLC preparation to yield the peracetate of 4'-*O*-methyl-epiafzelechin (2.2 mg) **(9a)**. Another portion of subfraction XIII-2 (123.1 mg) was acetylated and purified by TLC preparation to yield the peracetates of catechin (14.2 mg) and epicatechin (13.1 mg). Fraction XIV (1,156.5 mg) was separated on MLCCC as mentioned above, giving 5 subfractions. Subfraction XIV-1 (8.1 mg) was acetylated and purified by TLC preparation to yield the peracetate of epicatechin-(4β→8)-epigallocatechin (4.9 mg). Subfraction XIV-2 (198.1 mg) was acetylated and yielded epiafzelechin-(4β→8)-epicatechin. Subfraction XIV-3 (711.2 mg) was acetylated and yielded epicatechin-(4β→8)-epicatechin (PB2). A portion of subfraction XIV-4 (41 mg) was acetylated and purified by TLC preparation to yield the peracetate of epicatechin-(4β→8)-catechin (PB1) (4.8 mg).

General

^{1}H NMR spectra were recorded in $CDCl_3$ on a Varian Mercury 300BB (300 MHz) and Varian Inova (500 MHz) at ambient temperature with TMS as the internal standard. CD data were obtained in MeOH on a Jasco J-815. Polarimetry was measured in a Perkin-Elmer 241. ESI-MS mass spectrometer: Quattro LCZ, Firma Waters, was measured in the positive-ion mode. Compounds were revealed by spraying with vanillin-HCl reagent and 1% ethanol

$FeCl_3$ solution on TLC. Analytical TLC was carried out on precoated aluminum sheets (Kieselgel 60 F_{254}, 0.2 mm, Merck) using EtOAc:HCOOH:H_2O (90:5:5). Preparative TLC was performed on silica-gel plates (Kieselgel 60 F_{254}, 0.5 mm, Merck) using toluene:Me_2CO (60:40). Acetylation was performed in pyridine-Ac_2O (1:1.2) at ambient temperature for 24 h.

Compound identification:

ent-Catechin (**1**): ESI-MS *m/z* 313.1 $[M+Na]^+$; $[\alpha]_D^{20^o}$ = -20.1º (methanol; *c* 0.02); ^1H NMR (300 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.30 (5xOAc, m), 2.66 [1H, dd, *J*=16.8, 6.6, H-4β (C)], 2.87 [1H, dd, *J*=16.8, 5.1, H-4α (C)], 5.15 [1H, d, *J*=8.9, H-2 (C)], 5.25 [1H, ddd, *J*=8.9, 6.6, 5.1, H-3 (C)], 6.59 [1H, d, *J*=2.1, H-6 (A)], 6.66 [1H, d, *J*=2.1, H-8 (A)], 7.17 [1H, d, *J*=8.1, H-5' (B)], 7.28 [1H, dd, *J*=8.1, 2.1, H-6' (B)], 7.28 [1H, d, *J*=2.1, H-2' (B)].

Epicatechin (**2**): ESI-MS *m/z* 313.2 $[M+Na]^+$; $[\alpha]_D^{20^o}$ = -42º (methanol; *c* 0.004); ^1H NMR (300 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.30 (5xOAc, m), 2.87 [1H, dd, *J*=17.7, 2.1, H-4β (C)], 2.98 [1H, dd, *J*=17.7, 4.2, H-4α (C)], 5.11 [1H, s, *J*=<1, H-2 (C)], 5.39 [1H, m, *J*=<1, H-3 (C)], 6.57 [1H, d, *J*=2.1, H-6 (A)], 6.67 [1H, d, *J*=2.1, H-8 (A)], 7.20 [1H, d, *J*=8.4, H-5' (B)], 7.27 [1H, dd, *J*=8.4, 1.8, H-6' (B)], 7.36 [1H, d, *J*=1.8, H-2' (B)].

ent-Gallocatechin (**3**): ESI-MS *m/z* 329.3 $[M+Na]^+$; $[\alpha]_D^{20^o}$ = -16.8º (methanol; *c* 0.005); ^1H NMR (300 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.30 (6xOAc, m), 2.66 [1H, dd, *J*=16.8, 6.6, H-4β (C)], 2.91 [1H, dd, *J*=16.8, 5.1, H-4α (C)], 5.12 [1H, d, *J*=6.3, H-2 (C)], 5.21 [1H, ddd, *J*=6.6, 6.3, 5.1, H-3 (C)], 6.60 [1H, d, *J*=2.1, H-6 (A)], 6.66 [1H, d, *J*=2.1, H-8 (A)], 7.12 [1H, s, H-2'/H-6' (B)].

Epigallocatechin (**4**): ESI-MS *m/z* 329.1 $[M+Na]^+$; $[\alpha]_D^{20^o}$ = -30º (methanol; *c* 0.02); ^1H NMR (300 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.30 (6xOAc, m), 2.89-3.04 [1H, m, H-

4β (C)/H-4α (C)], 5.09 [1H, s, H-2 (C)], 5.38 [1H, m, H-3 (C)], 6.57 [1H, d, *J*=2.1, H-6 (A)], 6.67 [1H, d, *J*=2.1, H-8 (A)], 7.23 [1H, s, H-2/H-6' (B)].

Epiafzelechin-(4β→8)-epicatechin (**5**): ESI-MS *m/z* 963.2 $[M+Na]^+$; CD (MeOH): $[\Theta]_{240}$=+13,000 $[\Theta]_{280}$=-6,500; $[\alpha]_D^{20^o} = +12^o$ (methanol; *c* 0.001); ^{1}H NMR (500 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.30 (9xOAc, m), 2.87-2.91 [1H, m, H-4β (F)], 2.87-2.91 [1H, m, H-4α (F)], 4.57 [1H, s H-2 (F)], 4.44 [1H, d, H-4 (C)], 5.17 [1H, m, H-3 (F)], 5.19 [1H, m, H-3 (C)], 5.60 [1H, s, H-2 (C)], 6.00 [1H, d, *J*=2.1, H-6 (A)], 6.22 [1H, d, *J*=2.1, H-8 (A)], 6.62 [1H, s, H-6 (D)], 6.90-7.4- [2H, m, H2'/H5'/H6' (E)], 7.42 [2H, d, *J*=8.7, H-2/H-6' (B)], 7.14 [2H, d, *J*=8.7, H-3/H-5' (B)].

Epicatechin-(4β→8)-catechin (PB1) (**6**)*:* ESI-MS $[M+Na^+]^+$ *m/z* 1021.5; $[M-H^+]^-$ *m/z* 997.5; $[\alpha]_D^{20^o} = +2.2^o$ (methanol; *c* 0.002); ^{1}H NMR (300 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.33 (10xOAc, m), 2.56 [1H, dd, *J*=16.8, 9.3, H-4β (F)], 3.21 [1H, dd, *J*=16.8, 6.6, H-4α (F)], 4.42 [1H, s, H-4 (C)], 4.33 [1H, d, *J*=9.9, H-2 (F)], 5.05 [1H, ddd, *J*=9.9, 9.3, 6.6, H-3 (F)], 5.15 [1H, m, H-3 (C)], 5.45 [1H, s, H-2 (C)], 5.99 [1H, d, *J*=2.1, H-6 (A)], 6.29 [1H, d, *J*=2.1, H-8 (A)], 6.68 [1H, s, H-6 (D)], 7.16 [1H, d, *J*=8.4, H-5 (B)], 7.25 [1H, dd, *J*=8.4, 1.8, H-6' (B)], 6.88 [1H, d, *J*=1.8, H-2' (B)], 6.95 [1H, d, *J*=8.4, H-5' (E)].

Epicatechin-(4β→8)-epicatechin (PB2) (**7**)*:* ESI-MS $[M+Na^+]^+$ *m/z* 1021.5; $[M-H^+]^-$ *m/z* 997.5; $[\alpha]_D^{20^o} = +4.5^o$ (methanol; *c* 0.002); ^{1}H NMR (500 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.33 (10xOAc, m), 2.79-2.97 [1H, m, H-4β (F)/H-4α (F)], 4.42 [1H, s, H-4 (C)], 4.51 [1H, s, H-2 (F)], 5.07 [1H, m, H-3 (F)], 5.13 [1H, m, H-3 (C)], 5.54 [1H, s, H-2 (C)], 5.95 [1H, d, *J*=1.5, H-6 (A)], 6.19 [1H, d, *J*=1.5, H-8 (A)], 6.62 [1H, s, H-6 (D)], 6.85 [1H, dd, *J*=8.5, 2.0, H-6' (E)], 6.98 [1H, d, *J*=2.0, H-2' (E)], 6.99 [1H, d, *J*=8.5, H-5' (E)], 7.14 [1H, d, *J*=8.5, H-5' (B)], 7.32 [1H, d, *J*=2.0, H-2' (B)].

Epicatechin-(4β→8)-epigallocatechin (**8**): ESI-MS $[M+Na^+]^+$ *m/z* 1079.4; $[M-H^+]^-$ *m/z* 997.5; $[\alpha]_D^{20^o} = +22^o$ (methanol; *c* 0.002); ^{1}H NMR (300 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.33 (11xOAc, m), 2.90 [1H, m, H-4β (F)/H-4α (F)], 4.47 [1H, m, H-4 (C)], 4.51 [1H, s, H-2 (F)], 5.10 [1H, m, H-3 (F)], 5.14 [1H, m, H-3 (C)], 5.57 [1H, s, H-2 (C)], 6.06 [1H, d, *J*=2.4, H-6 (A)], 6.25 [1H, d, *J*=2.4, H-8 (A)], 6.65 [1H, s, H-6 (D)], 7.36 [1H, d, *J*=1.8, H-2' (B)], 7.17 [1H, d, *J*=8.4, H-5' (B)], 7.25 [1H, dd, *J*=8.4, 1.8, H-6' (B)], 6.89 [1H, s, H-2/H-6' (E)].

4´-O-Methyl-epiafzelechin **(9a)**: ESI-MS *m/z* 437.5 $[M+Na]^+$; CD (MeOH): $[\Theta]_{240}$=+13,000 $[\Theta]_{280}$=-8,800; $[\alpha]_D^{20^o} = +22^o$ (methanol; *c* 0.002); ^{1}H NMR (300 MHz; $CDCl_3$; δ in ppm; *J* in Hz): δ 1.25-2.30 (3xOAc, m), 2.87 [1H, dd, *J*=17.7, 2.1, H-4β (C)], 2.98 [1H, dd, *J*=17.7, 4.2, H-4α (C)], 5.11 [1H, s, *J*=<1, H-2 (C)], 5.39 [1H, m, *J*=<1, H-3 (C)], 6.57 [1H, d, *J*=2.1, H-6 (A)], 6.67 [1H, d, *J*=2.1, H-8 (A)], 7.20 [1H, d, *J*=8.4, H-5' (B)], 7.27 [1H, dd, *J*=8.4, 1.8, H-6' (B)], 7.36 [1H, d, *J*=1.8, H-2' (B)].

HPLC Characterization

Chemicals and reagents

All reagents and solvents were analytical or HPLC grade, including the ethyl acetate and trifluoroacetic acid (TFA) (Merck, Germany). Ultra-pure water obtained using a Milli-Q® UF-Plus apparatus (Millipore, USA) was used in all experiments.

Instrumentation and chromatographic conditions

The analyses were carried out using a HPLC system (Gilson, USA) consisting of a solvent delivery pump (Model 321), a variable wavelength UV/VIS detector (Model 156), a manual injection valve (Rheodyne®, USA) with a 20 μL loop, degasser (Model 184), and a thermostatted column compartment (Model 831). Data collection and analyses were performed using UniPoint™ LC System Software (Gilson, France). A gradient elution was performed on a Phenomenex® Gemini RP C-18 column (250 mm x 4.6 mm) (Phenomenex International, USA), 5 μm particle size, Phenomenex® SecurityGuard™ (RP C-18 cartridge) (20 mm x 4.6 mm). The mobile phase consisted of water (0.05% TFA) as solvent A and acetonitrile (0.05% TFA) as solvent B, and both were degassed and filtered through a 0.45 μm pore-size filter (Millipore, USA). Separations were effected by a linear gradient as follows: 0 min 13% B; 10 min 17% B; 16 min 19.35% B; 20 min 22.65% B; 23 min 29.81% B; 25 min 65% B; 28 min 13% B; and 32 min 13% B. The mobile-phase flow rate was 0.8 mL min^{-1} and the injection volume was 100 μL. The chromatographic runs were carried out at 28 °C. UV detection was performed at 210 nm.

For the determination of peak purity, the Varian ProStar module (Varian, USA) with ProStar 210 Solvent Delivery and a ProStar 335 HPLC-DAD was used.

Sample preparation and purification

An accurately weighed portion of 50 mg of the CE was dissolved in 500 μL water, mixed in a tube shaker, and extracted with 500 μL ethyl acetate, in a microtiter shaker at 1800 rpm (IKA®, MS1 Minishaker) for 3 min (n=6). The tubes were then placed in a refrigerated microcentrifuge (Eppendorf®, Centrifuge 5415R), at 4,000 rpm, for the total separation of the phases, for 4 min at 5 °C. The ethyl acetate phase was separated. After evaporation of solvents, and drying under air flow, the residue was reconstituted to 10 mL with

methanol:water (1:1; v/v) (Solution test–SS). The sample was filtered through a 0.5 μm membrane filter (Millipore, USA). The sample injection volume was 100 μL.

Acknowledgments

The authors are grateful for financial support from Capes, FINEP and CNPq. We gratefully acknowledge the recording of NMR spectra by Dr. Ivânia Terezinha Albrecht Schuquel (Department of Chemistry, State University of Maringá). Dr. Heinrich Luftmann (Institute of Organic Chemistry, Münster, Germany) recorded the ESI-mass. Our gratitude to Dr. Janet W. Reid for revising the English text.

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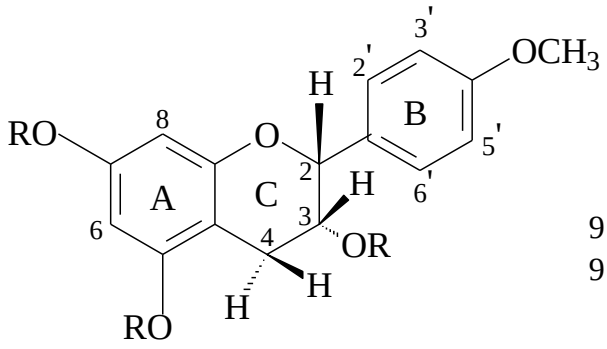
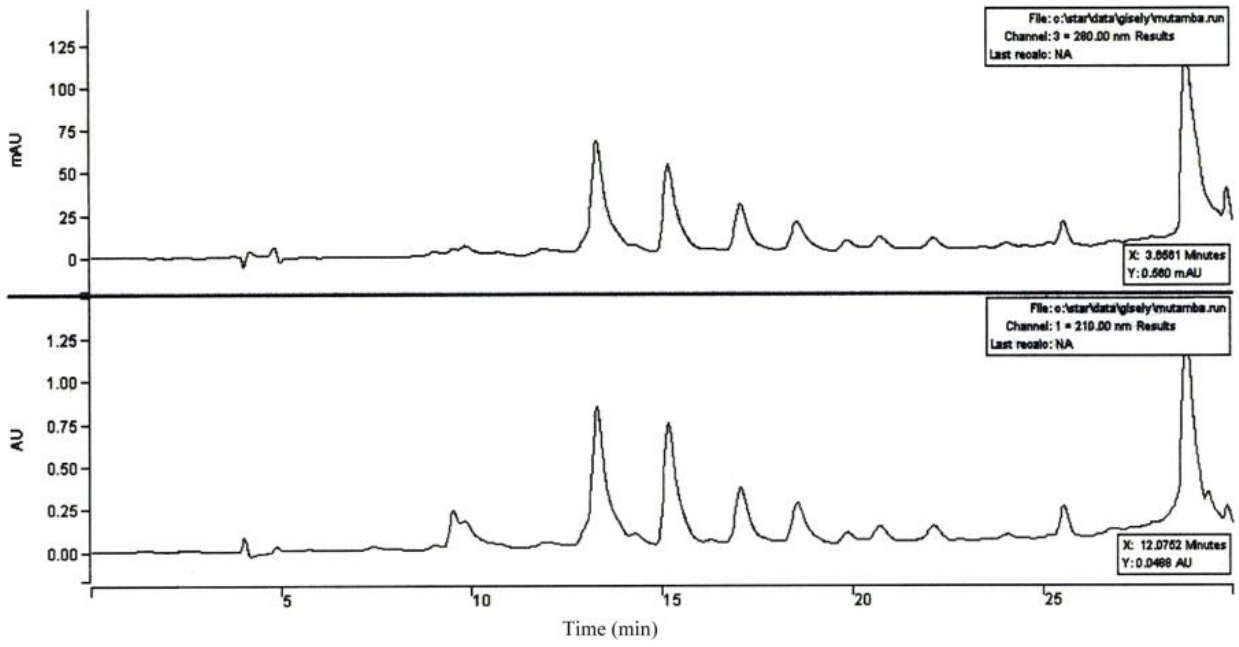


	R
9	H
9**a**	Ac

Compound 9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Channel: 3 = 280.00 nm Results
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mAU
125
100
75
50
25
0
X: 3.5581 Minutes
Y: 0.580 mAU
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AU
1.25
1.00
0.75
0.50
0.25
0.00
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Y: 0.0488 AU
5
10
15
20
25
Time (min)

Figure 1

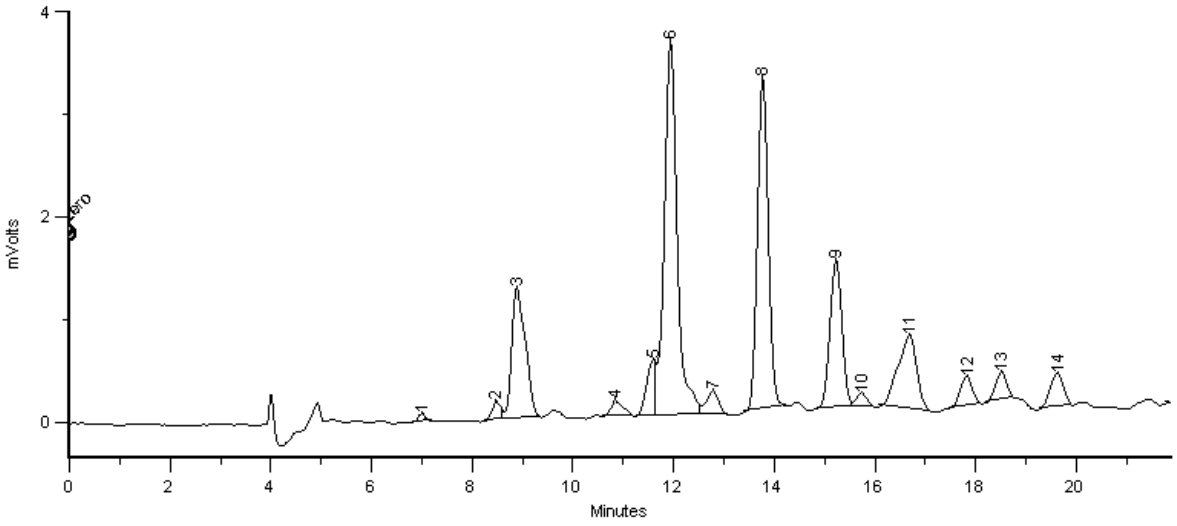


Figure 2

Table 1

Peak	RSD of RT (%)	RSD of area (%)	Retention Factor (*k*)	Separation Factor (α)	Peak Resolution	Theoretical Plates	Peak asymmetry
1	2.72	4.54	1.76	1.76	4.170	9329.15	**2.07**
2	1.95	4.91	2.13	1.21	0.72	7320.87	**0.68**
3	2.97	1.72	2.22	1.04	2.85	4447.61	**1.88**
4	2.47	4.60	2.70	1.22	1.53	7706.01	**1.86**
5	2.03	4.28	2.92	1.08	0.56	19335.53	**0.33**
6	2.25	3.75	2.99	1.02	1.16	12357.21	**1.01**
7	2.14	4.94	3.19	1.07	1.38	10462.69	**1.09**
8	2.01	1.62	3.44	1.08	1.93	19282.86	**1.12**
9	2.15	2.11	3.79	1.10	0.88	18695.05	**1.12**
10	1.59	3.27	3.93	1.04	1.31	20501.45	**1.55**
11	1.47	1.28	4.16	1.10	1.37	8643.47	**0.59**
12	1.75	3.64	4.44	1.07	1.14	22725.97	**1.19**
13	1.72	4.37	4.62	1.04	1.83	30473.78	**1.00**
14	1.57	4.84	4.92	1.06	4.91	22312.92	**1.14**
Reference ICH[40]	**-**	**-**	**>2**	**>1**	**>2**	**>2000**	**≤2**

Figure 1 - Chromatograms of *G. ulmifolia* with different extract UV detection methods at 210 and 280 nm.

Figure 2 - Standardized chromatographic fingerprint. Column=Phenomenex® Gemini RP C-18; flow rate=0.8 mL min^{-1}; detection=210 nm; temperature=28 °C.

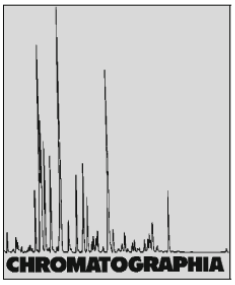
Table 1 - System suitability test results (n=12).

CAPÍTULO III

RP-LC-UV DETERMINATION OF PROANTHOCYANIDINS IN *GUAZUMA ULMIFOLIA*

(ARTIGO PUBLICADO NO PERIÓDICO CHROMATOGRAPHIA)

RP-LC-UV Determination of Proanthocyanidins in *Guazuma ulmifolia*



Gisely C. Lopes, Marcos L. Bruschi, João Carlos Palazzo de Mello✉

Programa de Pós-graduação em Ciências Farmacêuticas, Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá, Av. Colombo, 5790, Maringá, PR CEP 87.020-900, Brazil; E-Mail: mello@uem.br

Received: 17 September 2008 / Revised: 6 December 2008 / Accepted: 7 January 2009

Abstract

A simple, reproducible, and efficient liquid chromatographic method was developed with UV detection. Water (0.05% TFA):acetonitrile (0.05% TFA) was used as the mobile phase in a gradient system for the determination of procyanidin B2 (PB2) and epicatechin (EC) in the bark of *Guazuma ulmifolia* Lam. The analysis was performed using a Phenomenex Gemini RP C_{18} column (5 μm) as stationary phase, at 30 °C, with a flow rate of 0.8 mL min^{-1}, at a wavelength of 210 nm for detection and determination. The main validation parameters of the method were also determined. Calibration curves were found to be linear, with ranges of 20.00–150.00 (PB2) and 10.00–110.00 μg mL^{-1} (EC). The correlation coefficients of linear regression analysis were between 0.9981 and 0.9988, and the detection limits were between 2.89 and 2.54 μg mL^{-1}. The contents of PB2 and EC were successfully determined, with satisfactory reproducibility and recovery. Recoveries of the PB2 and EC were 103.00 and 104.01%, respectively. The method was successfully applied to the determination of procyanidins in the bark of *G. ulmifolia*.

Keywords

Column liquid chromatography
Quality control and validation method
Proanthocyanidins
Guazuma ulmifolia

Introduction

Proanthocyanidins are oligomers or natural polymers of flavan-3-ols. They are the most abundant polyphenols in plants after lignins, and may represent up to 50% in many kinds of bark [1]. They can be divided into procyanidins and prodelphinidins. Procyanidins are composed of oligomers and polymers, consisting of catechin and/or epicatechin units linked mainly through C4 → C8 and/or C4 → C6 bonds (B-type). The flavan-3-ol units can be doubly linked by a C4 → C8 bond and/or an additional ether bond from O7 → C2 (A-type) [2]. Procyanidins have different pharmacological activities, including antioxidant [3], anti-ulcer [4], anti-inflammatory [5], antitumoral [6], and immunomodulatory [7]. These activities largely depend on their structure, particularly their degree of polymerization [8]. Dimeric procyanidin B2 is composed of two molecules of the flavan-3-ol epicatechin linked by a 4 → 8 bond, is widely distributed in plants, and is present in grape seeds, apples, cacao beans, guaraná (*Paullinia cupana*), and pine (*Pinus maritima*) bark extract (Pycnogenol). Recently, procyanidin B2 was detected in human plasma after the consumption of flavanol-rich chocolate [9]. Several studies have shown that procyanidin B2 has several pharmacological properties that benefit human health, such as antioxidant activity [10], anti-tumor effect [11], in vitro reduction of the oxidation of human low-density lipoprotein [12], and promoting hair growth [13, 14]. Therefore, many studies have been carried out to

Separation Analysis Applied to Pharmaceutical Sciences in Brazil.

Original
DOI: 10.1365/s10337-009-0990-6

Published online: 07 February 2009

evaluate the percutaneous penetration [15], oral absorption, and bioavailability of proanthocyanidins [16, 17]. In this context, several methods have been reported for the determination of proanthocyanidins in plant extracts [18–21]. Although these methods can separate procyanidins well, there are several limitations that complicate their routine use. The development and validation of an efficient analytical method is an integral part of the quality control of the source material, in order to guarantee the safety and effectiveness of the resulting compound [22, 23].

Guazuma ulmifolia Lam. (Sterculiaceae), popularly known as "Mutamba", is a tropical-American plant found from Mexico to southern South America. In the popular medicine of several Latin-American countries, it is used for the treatment of burns, diarrhea, inflammations, and alopecia. Polysaccharides, epicatechin and procyanidin oligomers, such as procyanidins B2 and B5, three trimers [procyanidin C1; epicatechin-(4β → 6)-epicatechin-(4β → 8)-epicatechin; epicatechin-(4β → 8)-epicatechin-(4β → 6)-epicatechin] and one tetramer [15, 24] have been isolated and identified from its extract. The anti-diabetic properties [25, 26], hypotensive and vasorelaxant activity [27, 28], anti-ulcer [24, 29, 30], anti-bacterial [31, 32], and antiviral activity [33] from the bark, aerial parts, fruits, crude extract, and fractions were attributed to the presence of proanthocyanidins.

Here, we report a sensitive liquid chromatographic (LC) determination method with UV detection, for the separation and quantitative analysis of procyanidin B2 and epicatechin, the marker components of *G. ulmifolia,* to provide a scientific basis for quality control of extracts from its bark. At this time, this plant was not investigated at the point of LC. The method was validated by regulation RE 899/2003 of the National Health Surveillance Agency, Brazil [34] and the ICH guidelines [35]. The following validation characteristics were assessed: specificity, linearity, limit of detection (LOD) and quantitation (LOQ), accuracy, precision, and sample stability and robustness.

Experimental

Plant Material

Bark of *G. ulmifolia* Lam., Sterculiaceae, was collected in August 2005 in the city of Jataizinho, state of Paraná, Brazil (S 23°18′26.1″; W 050°58′19.4″; 377 m altitude; Garmin v.2.24). The species was identified by Prof. Dr. Cássia Mônica Sakuragui. Voucher specimens are deposited in the herbarium of the Department of Biology of the State University of Maringá under number HUEM 12.051.

Chemicals and Reagents

All reagents and solvents were analytical and LC grades, including ethyl-acetate and trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany). Ultra-pure water obtained using a Milli-Q UF-Plus apparatus (Millipore, Bedford, USA) with conductivity of 18 mΩ was used in all experiments. Epicatechin (Sigma, USA) and procyanidin B2 (isolated and certified by the spectroscopic methods at the Pharmacognosy Laboratory of Maringá State University) of the highest grade (purity > 99.0%) were used as external standards.

Instrumentation and Chromatographic Conditions

The analyzes were carried out using an LC system (Gilson, USA) consisting of a solvent delivery pump (Model 321), a variable wavelength UV–Vis detector (Model 156), a manual injection valve (Rheodyne, USA) with a 20 µL loop, degasser (Model 184), and thermostatted column compartment (Model 831). Data collection and analyses were performed using UniPoint LC System Software (Gilson, Villiers-le-Bel, France). A gradient elution was performed on a Phenomenex Gemini RP C-18 column (250 × 4.6 mm) (Phenomenex International, USA), 5 µm particle size, Phenomenex SecurityGuard (RP C-18 cartridge) (20 × 4.6 mm). The mobile phase consisted of water (0.05% TFA) as solvent A and acetonitrile (0.05% TFA) as solvent B, and both were degassed and filtered through a 0.45 µm pore size filter (Millipore, Bedford, USA). Separations were effected by a linear gradient as follows: 0 min 13% B, 10 min 17% B, 16 min 19.35% B, 20 min 22.65% B, 23 min 29.81% B, 25 min 65% B, 28 min 13% B, and 32 min 13% B. The mobile phase flow rate was 0.8 mL min^{-1} and the injection volume was 100 µL. The chromatographic runs were carried out at 28 °C. UV detection was performed at 210 nm.

For the determination of peak purity, the Varian ProStar module (Varian, Palo Alto, CA, USA) with ProStar 210 Solvent Delivery and a ProStar 335 LC-DAD was used.

The statistical analyses of the data were performed by means of STATISTICA 7.0 software (Statsoft Inc., Tulsa, OK, USA).

Sample Preparation and Purification

Air-dried stem bark (900 g) was exhaustively extracted with Me_2CO–H_2O (7:3; 9 L) by turbo-extraction (Ultra-Turrax UTC115KT; 20 min; $t \leq 40$ °C), and the combined extracts were filtered and evaporated under reduced pressure to 1.0 L and freeze-dried (120 g, CE). An accurately weighed portion of 50 mg of the CE was dissolved in 500 µL water, mixed in a tube shaker, and extracted with 500 µL ethyl-acetate, in a microtiter shaker at 1,800 rpm (IKA, MS1 Minishaker) for 3 min ($n = 6$). Later, the tubes were placed in a refrigerated microcentrifuge (Eppendorf, Centrifuge 5415R), at 4,000 rpm, for the total separation of the phases, for 4 min at 5 °C. The ethyl-acetate phase was separated. After evaporation of solvents, and drying under air flow, the residue was reconstituted to 10 mL with methanol:water (1:1; *v*/*v*) (Solution test–SS). The sample was filtered through a 0.5 µm membrane filter (Millipore, Bedford, USA). The sample injection volume was 100 µL.

Preparation of Standard Solutions

An epicatechin reference standard stock solution of 400 μg mL^{-1} was prepared in methanol:water (1:1; *v*/*v*). Calibration standard solutions at five levels were prepared by serially diluting the stock solution to concentrations of 10.00, 40.00, 70.00, 100.00, and 120.00 μg mL^{-1}. The same mode was prepared for the procyanidin B2 standard. A procyanidin B2 stock solution of 250 μg mL^{-1} was prepared in methanol:water (1:1; *v*/*v*). Calibration standard solutions at seven levels were prepared by serially diluting the stock solution to concentrations of 20.00, 40.00, 50.00, 70.00, 90.00, 120.00, and 150.00 μg mL^{-1}. The samples were filtered through a 0.5 μm membrane (Millipore, Bedford, USA) prior to injection. Each analysis was repeated five times, and the calibration curves were fitted by linear regression.

Method Validation

Specificity

The specificity, defined as the ability of the method to measure the analyte accurately and specifically in the presence of components in the sample matrix, was determined by analysis of chromatograms of the standard solution and the sample solutions (SS). The Varian Prostar system (DAD detector) described above was employed.

Linearity

The linearity between peak area and concentration was analyzed using three calibration curves obtained on three different days with standard solutions of procyanidin B2 and epicatechin at seven and five different concentrations each, respectively, ranging from 20.00 to 150.00 μg mL^{-1} for procyanidin B2 and 10–110 μg mL^{-1} for epicatechin. The linearity of the SS was available separately; the concentration range was selected during the release studies. The data for peak area versus drug concentration were treated by linear regression analysis.

Sensitivity

The LOD and the LOQ were determined from the calibration curves of the epicatechin and procyanidin B2 standards. LOD was calculated according to the expression 3 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve. LOQ was established by using the expression 10 σ/S [35].

Accuracy

The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the procyanidin B2 and epicatechin standards to the sample, before the extraction process, at three different levels, three solutions each in triplicate (18, 39, and 65%). The percent recovery was determined by comparing the results of the analyses of the fortified samples, with the nominal value by means of Student's *t* test and analysis of variance (ANOVA).

Precision

The precision was estimated at two different levels: inter-day precision and intra-day precision. The test of intermediate precision (inter-day precision) was carried out using three samples in three different levels, and each sample was injected three times. The intra-day precision of the method was determined after injecting three samples (SS), during three consecutive days (three times each). The areas of the peaks corresponding to the compounds of interest were determined at 210 nm. Analysis of variance was used for estimating the total variability of the analytical method. Precision was expressed as the relative standard deviations (% RSD) of the concentrations of epicatechin and procyanidin B2 [34, 35].

Robustness

Three sample solutions were prepared and analyzed under the established conditions and by changing the wavelength parameter from 210 to 208 nm [34].

Sample Stability

The stability was demonstrated by analysis of the sample (SS) after it remained in a solution of methanol:water (1:1, *v*/*v*) for 24 h at a temperature of 32 ± 3 °C under natural light.

Results and Discussion

Optimization of the Chromatographic Conditions

An RP-LC method developed for *G. ulmifolia* can be conveniently employed for routine quality control. The chromatographic separations were performed on a C18 analytical column.

The mobile phase consisted of a mixture of water (TFA 0.05%) and acetonitrile (TFA 0.05%). The effects of the composition and pH of the mobile phase on the retention time of the extract and standard solutions were investigated. The particular parameters examined were the percentage of acetonitrile, the purification of the extract, and the pH values of the mobile phase component.

The elimination of high-molecular-weight phenolic compounds from the plant extract is critically important, because of the interaction that these compounds show with the stationary phase. This interaction can seriously damage the analytical column, leading to interference with the chromatographic process. Currently, the most widely employed sample preparation methodologies are solid-phase extraction and liquid–liquid extraction. Therefore, in this study, the extraction of proanthocyanidin compounds from *G. ulmifolia* was optimized by using a mixture of water:ethyl acetate. The utilization of the simple one-step liquid–liquid extraction method should completely extract the target constituents from the matrix. The extraction efficiency was evaluated by LC, and the results demonstrated the reliability of the process.

The small structural differences between the substances, with the presence or absence of hydroxyl or methoxyl, contribute to the obtaining of good

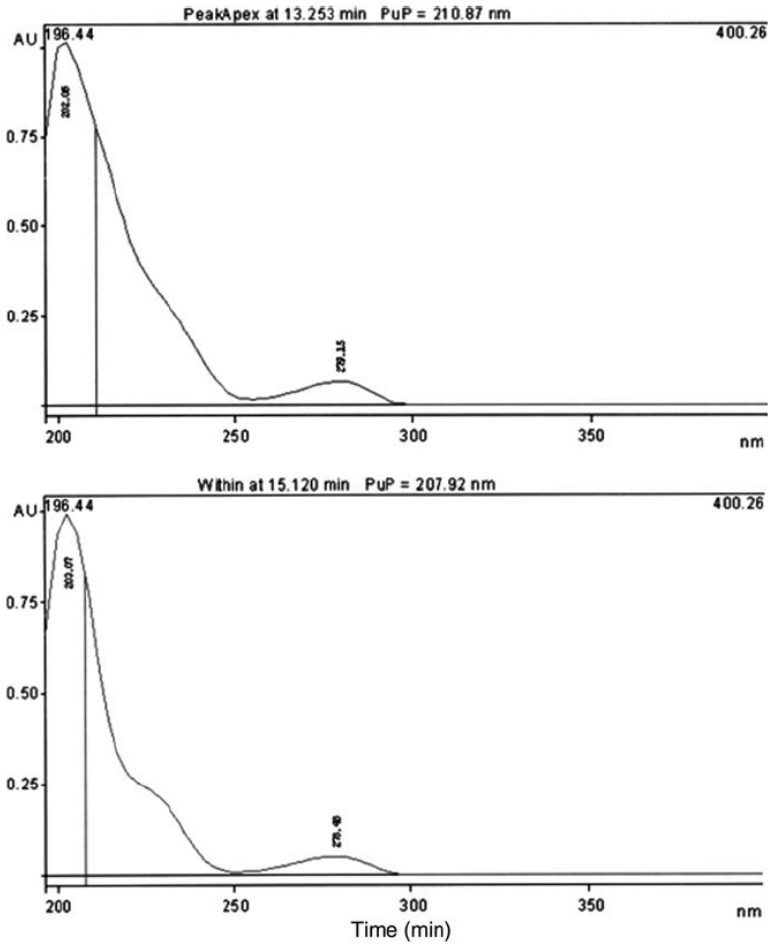


Fig. 1. Diode array spectrum, 200–400 nm, of solution test of the barks of *Guazuma ulmifolia*; the major peaks: 13.25 min, procyanidin B2 and 15.12 min, epicatechin

separation between substances; these are distributed in the column according to their polarity and the eluotropic strength of the eluent system [36]. However, in extracts rich in proanthocyanidins, the order of elution in the column depends on the degree of polymerization of the molecule, with a significant correlation between the retention time and the molar mass of the substance [37]. The retention of the analytes is strongly influenced by the percentage of acid in the mobile phase. Use of the C18 column allowed us to explore a pH range between 1.0 and 4.0. In developing the method, the addition of 0.05–0.1% TFA, which suppresses the ionization of phenol groups, to all the solvent systems led to better separation of the polyphenol compounds [38, 39]. Therefore, use of TFA is essential for complete separation.

Different ratios of methanol–water and acetonitrile–water were tried for analysis, and the mobile phase was modified with two different solvent systems pumped at different flow rates. Flow rate is critical because it affects the peak symmetry parameters. The optimization of flow rate is critical, since the extent of longitudinal broadening is inversely related to the flow rate of the mobile phase. With either high or low flow rates, an ideal Gaussian curve of the peak is not obtained, because the peak symmetry parameters are affected, i.e., the asymmetry factor deviates from unity [36]. After testing several acidic extraction producers (data not shown), we determined that acetonitrile:TFA 0.05% and water:TFA 0.05% showed the best results, enabling the fastest separation (32 min) and best resolution. Acetonitrile was used as the organic solvent because it provided better sensitivity and lower background noise than methanol.

Evaluation of the solution test of *G. ulmifolia* by LC with photodiode-array detection was indispensable to define the quantification parameters. Through this resource, the UV spectra of the two major peaks with retention times of 13.25 and 15.12 min could be observed. Comparing the spectra obtained, we observed that these substances show two bands (A and B) that are very similar to the proanthocyanidins, with maximum absorption (λ_{max}) in the same range of wavelength, between 210 and 280 nm. The same profile was found in procyanidin B2 and epicatechin (Fig. 1).

Based on previous work [38, 40] and on data for the UV spectra, all proanthocyanidins were found to have maximum absorbances at 210 nm and from 275 to 280 nm. Figure 2 shows the chromatograms of a sample solution at 210 and 280 nm. Comparing the absorbances at the two wavelengths, the absorbances at 210 nm were higher than those at 280 nm for procyanidin B2 and epicatechin, in the system described. Thus, chromatograms recorded at 210 nm showed considerable improvement in signal-to-noise ratio. Typical chromatograms obtained from the samples are shown in Fig. 3. There were no peaks due to other, minor coextracted materials interfering with procyanidin B2 and epicatechin. The retention times for procyanidin B2 and epicatechin were 12.39 and 14.82 min, respectively. Procyanidin B2 and epicatechin have UV maxima at 210 and 208 nm, respectively. In this study, the detection wavelengths were set at 210 nm.

Figure 3 shows the chromatogram for all compounds, indicating no interference between two analytes. Good separation is seen as the retention times of procyanidin B2 and epicatechin, using the chromatographic conditions described above. The purity of the chromatographic peaks

obtained for procyanidin B2 and epicatechin was evaluated using a DAD detector. The resolution of the peaks of procyanidin B2 and epicatechin in the sample, after analysis of the chromatograms by means of a photodiode-array detector, indicated the specificity of the method. The UV spectra of the compounds (procyanidin B2 and epicatechin) confirmed the absence of impurities, because these did not change between the beginning and end of elution.

Linearity and Calibration

Based on $1/x$ weighted linear regression analysis, the responses for both standards in related concentration ranges were linear. The calibration equations were $Y = 818.21x - 2177.9$ ($n = 7$, $r^2 = 0.9981$) for procyanidin B2 and $Y = 885.51x + 953.56$ ($n = 5$, $r^2 = 0.9988$) for epicatechin. The RSD of the slopes were $\leq 5\%$ for both analytes ($n = 5$). Table 1 shows the back-fit calculations for curve data for the standards used in the validation runs, as well as the precision and accuracy of the back-fit calculations.

Sensitivity

The LOD and quantitation assess the sensitivity of the method, and are calculated from the peak-to-noise ratios. The LOD, defined as the lowest concentration of procyanidin B2 and epicatechin in *G. ulmifolia* that can be detected but not necessarily quantified under the stated experimental conditions, was 2.89 and 2.54 μg mL^{-1}, respectively. The LOQ, defined as the lowest concentration of procyanidin B2 and epicatechin that can be determined with acceptable precision and accuracy, was 8.78 and 7.70 μg mL^{-1}, respectively.

Accuracy

Accuracy was determined by analyzing a sample of known concentration and comparing the measured value with the true value, using the method of standard addition. Table 2 summarizes the results for accuracy, expressed as percentage of recovery and RSD for both approaches. The method showed good accuracy.

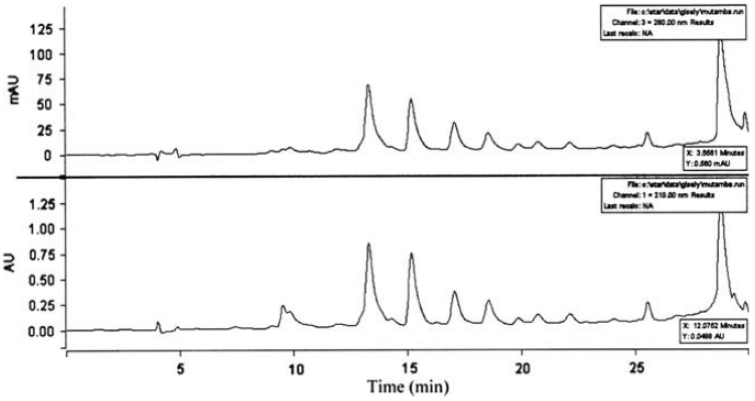


Fig. 2. The chromatograms of solution test of the barks the *Guazuma ulmifolia* at 210 and 280 nm

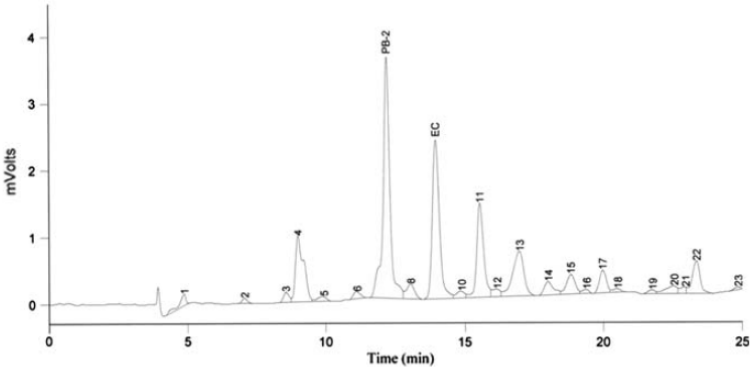


Fig. 3. Typical chromatograms obtained from SS of *Guazuma ulmifolia*. Procyanidin B2 retention time in 12.39 min and epicatechin in 14.82 min

Table 1. Curve parameter summary and back-calculated calibration curve concentrations for procyanidin B2 and epicatechin

	Procyanidin B2	Epicatechin
Linear range (μg mL^{-1})	20–150	10–120
Detection limit (μg mL^{-1})	2.89	2.54
Quantitation limit (μg mL^{-1})	8.78	7.70
Regression data[a]		
n	7	5
Slope (*b*)	818.21	885.50
Standard deviation of the slope	8,15	8.66
Relative standard deviation of the slope	0.996	0.978
Intercept (*a*)	−2177.95	953.56
Standard deviation of the intercept	718.56	682.32
Correlation coefficient (r^2)	0.9981	0.9988
Standard error of regression	0.047	0.039

[a] $y = ax + b$, where x is the concentration of two the compound and y is the peak area

Precision

The results obtained from these analyses are listed in Tables 3 and 4, as mean recovery (%). The data showed that there were no significant differences between the assay results, either within-day

Table 2. Recoveries of procyanidin B2 and epicatechin in samples

Proantocyanidins	Samples (%)	Concentration		Recovery (%) ± RSD	Mean recovery (%) ± RSD
		Added (μg mL^{-1})	Found (μg mL^{-1})		
Procyanidin B2	18	22.62	23.42	103.54 ± 4.38	103.00 ± 5.17
	39	49.43	49.78	100.71 ± 4.59	
	65	84.04	91.12	108.43 ± 4.52	
Epicatechin	18	18.04	17.03	94.42 ± 5.14	104.01 ± 7.43
	39	36.09	38.56	106.84 ± 1.20	
	65	61.35	67.69	110.33 ± 2.73	

Table 3. Inter-day repeatability for analysis of procyanidin B2 and epicatechin in sample solutions

Compound	Theorical concentration of the SS (μg mL^{-1})	Intra-day (μg mL^{-1}) mean ± RSD (%) [$n = 3$]
Procyanidin B2	780	74.74 ± 1.00
	1,300	131.70 ± 0.34
	2,160	210.26 ± 0. 20
Epicatechin	780	48.58 ± 1.68
	1,300	81.50 ± 0.80
	2,160	126.20 ± 0.57

Table 4. Intra-day repeatability for analysis of procyanidin B2 and epicatechin in sample solutions

Compound	Day of the analysis	Inter-day (μg mL^{-1}) mean ± RSD (%) [$n = 9$]
Procyanidin B2	Day 1	130.95 ± 1.97
	Day 2	125.81 ± 3.36
	Day 3	128.76 ± 2.42
Epicatechin	Day 1	86.13 ± 2.79
	Day 2	84.90 ± 1.09
	Day 3	85.55 ± 1.73

Table 5. Evaluation of experimental robustness and responses obtained

No. exp.	nm	Flow rate (mL min^{-1})	Concentration (μg mL^{-1}) [$n = 5$]	
			Procyanidin B2	Epicatechin
1	210	0.8	109.41 ± 2.82	77.46 ± 0.69
2	208	0.8	104.30 ± 1.54	78.78 ± 0.51

or between days, indicating that the precision of the proposed method was good (RSD less than 5%).

Robustness and Stability

Robustness is defined as the capability of an analytical procedure to remain unaffected by small but deliberate changes in the method parameters [35]. To ensure that the LC method is insensitive to minor changes in the experimental conditions, it is important to demonstrate the robustness of the method. When the wavelength was changed by 2 nm, from 210 to 208 nm, this alteration caused no significant change in the resolution of the procyanidin B2 and epicatechin. The experimental domain of the selected variables is reported in Table 5. The ranges examined were small deviations from the method settings, and corresponding responses in the peak area ratio considered were observed.

The stability of the sample is a parameter that must be evaluated during the development of the analytical methods. The importance of this assay is due to innumerable external factors (temperature, luminosity, relative humidity) and internal factors (reactions to hydrolysis, oxidation, and condensation) that could affect the precision of the method. Tannins are substances that are relatively unstable under changes in temperature and luminosity [39]. Therefore, to establish the conditions of stability of the SS is of great importance for the determination of PB2 and EC in the dried extract of *G. ulmifolia*. The results obtained in the study of the solution (both zero time and the sample solution after 24 h) indicated that the solutions were stable for 24 h, because during this time the contents did not decrease below the minimum percentage (94% for procyanidin B2). The data were assessed by Student's t test and ANOVA, and showed no significant differences ($p = 0.05\%$). The concentration was 109.87 ± 2.76 and 103.37 ± 4.65 for PB2, and 75.53 ± 3.21 and 75.17 μg mL^{-1} ± 1.60% for epicatechin, at time zero and after 24 h, respectively. Through this test it was possible to establish that the substances analyzed show good stability under the conditions used, for a 24-h period. Thus, studies of the stability over prolonged periods must be carried out in order to confirm the stability shown in the present assay.

Conclusions

The present paper describes a rapid and robust LC assay for separation and quantitative analysis of proanthocyanidins in *G. ulmifolia*. Efficient removal of interfering substances (condensed tannins of high-molecular-weight) was achieved by means of a liquid–liquid extraction. The clean-up of samples was very simple and demonstrated good efficiency. After the optimization of chromatographic conditions for the separation of procyanidins B2 and epicatechin, the method, found to be specific and suitable for routine analysis because of its simplicity, sensitivity, accuracy, and reproducibility, can be

conveniently used for the analysis of procyanidin B2 and epicatechin in *G. ulmifolia* extract. The extract showed good stability under the conditions used.

Acknowledgments

The authors thank the Brazilian funding agencies CNPq (Conselho Nacional de Pesquisa) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). Our gratitude to Admir Arantes, Lucas Sica de Toledo and Cláudio Roberto Novello for technical support.

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CAPÍTULO IV

PRELIMINARY CHEMICAL STABILITY OF DRIED EXTRACT FROM *GUAZUMA ULMIFOLIA* LAM. (STERCULIACEAE)

(ARTIGO SUBMETIDO AO PERIÓDICO INTERNATIONAL JOURNAL OF PHARMACEUTICS)

Preliminary assessment of the chemical stability of dried extract from *Guazuma ulmifolia* Lam. (Sterculiaceae)

Gisely C. Lopes[a], Renata Longhini[a], Paulo Victor P. Santos[b], Marcos Luciano Bruschi[a], João Carlos P. Mello[aε]

[a]*Programa de Pós-Graduação em Ciências Farmacêuticas, Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá, Av. Colombo, 5790, CEP 87.020-900, Maringá, PR, Brazil*

[b]*Acadêmico de Farmácia, Universidade Estadual de Maringá, Av. Colombo, 5790, CEP 87.020-900, Maringá, PR, Brazil*

ABSTRACT

We report the results of a preliminary estimation of the stability of the dried extract from bark of *Guazuma ulmifolia* Lam. ("Mutamba"), with and without added colloidal silicon dioxide (CSD). The physical and chemical properties and the compatibility of CSD in the extract were evaluated for 21 days of storage under stress conditions of temperature (45±2 °C) and humidity (75±5%). Thermal analysis (TG) was supplemented using selective high-performance liquid chromatography (HPLC) for determination of stability of the characteristic constituents (chemical markers), namely procyanidin B2 (PB2) and epicatechin (EP). The results showed that PB2 is an appropriate compound to use as a chemical marker in quality control of dried extracts of *G. ulmifolia*. The stress study showed that there was no significant difference between the two formulations. However, considering the TG data and

[ε] Corresponding author. Tel.: + 55 4432614816; fax: + 55 4432614999
e-mail address: mello@uem.br (J. C. P. de Mello).

the high temperatures involved, the results suggest that CSD increases the stability of the dried extract of *G. ulmifolia*.

Keywords: Guazuma ulmifolia, thermal analysis, HPLC, stability study, dried extract

1. Introduction

The reasons for the determination of stability of pharmaceuticals are based on concern for public health. The World Health Organization (WHO) defines the stability of drugs and medicines as the ability of a pharmaceutical product to maintain its chemical, physical, microbiological, and biopharmaceutical properties within specified limits throughout the duration of the product (WHO, 1996).

Several studies have reported on the stability of drugs and medicines (Araújo et al., 2003; Tomassetti et al., 2005; Silva-Junior et al., 2009). To the best of our knowledge, few stability studies of vegetable extracts have appeared (Isacchi et al., 2007; Liu and Murphy, 2007; Pureesatien et al., 2008). Measuring the chemical stability of the extracts is challenging because of their chemical complexity, which may include thousands of different compounds. Moreover, the presence of enzymes such as glycosidases, esterases, or oxidases plays an important role in the breakdown of secondary plant metabolites.

Assessment of the chemical stability of plant extracts, many of which are promising candidates for phytomedicines, plays an important role in the process of new-drug development. A variety of environmental conditions, such as light, heat, humidity, and the freeze/thaw cycle, can significantly affect the chemical stability of drugs during storage and handling. Identification of stability-affecting factors facilitates the selection of packaging material and the definition of storage and handling conditions (Gafner and Bergeron, 2005).

Guazuma ulmifolia Lam. (Sterculiaceae), popularly known as “Mutamba”, is a tropical American plant found from Mexico to southern South America. In the popular medicine of several Latin-American countries, it is used for the treatment of burns, diarrhea, inflammations, and alopecia. Polysaccharides, epicatechin (EP), and procyanidin oligomers, such as procyanidins B2 (PB2) and B5, three trimers [procyanidin C1; epicatechin-(4β→6)-

epicatechin-(4β→8)-epicatechin; epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin], and one tetramer (Hör et al., 1996; Rocha et al., 2007) have been isolated and identified from its extract. The anti-diabetic properties (Allarcon-Aguilara et al., 1998; Alonso-Castro and Salazar-Olivo, 2008), hypotensive and vasorelaxant activity (Caballero-George et al., 2002; Magosa et al., 2008), anti-ulcer (Hör et al., 1995; Berenger et al., 2007), anti-bacterial activities (Camporese et al., 2003; Navarro et al., 2003), and antiviral activity (Felipe et al., 2006) of the bark, aerial parts, fruits, crude extract, and fractions have been attributed to the presence of proanthocyanidins.

However, there are no studies on the stability of the constituents of *G. ulmifolia* dried extracts. The determination of proanthocyanidins in bark of *G. ulmifolia* was carried out using the HPLC method, and it was observed that PB2 and EP compounds can be used as chemical markers for routine quality-control analysis (Lopes et al., 2009).

The aim of the present study was to evaluate the chemical stability of the dried extract from bark of *Guazuma ulmifolia* Lam. (Sterculiaceae), with and without an added pharmaceutical excipient.

2. Material and methods

2.1. Plant Material

Bark of *Guazuma ulmifolia* Lam., Sterculiaceae, was collected in August 2005 in the city of Jataizinho, state of Paraná, Brazil (S 23°18'26.1"; W 050°58'19.4"; 377 m altitude; Garmin v.2.24). The species was identified by Prof. Dr. Cássia Mônica Sakuragui. Voucher specimens are deposited in the herbarium of the Department of Biology of the State University of Maringá under number HUEM 12.051.

2.2. Chemicals and reagents

All reagents and solvents were of analytical and HPLC grade, including ethyl acetate and trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany). Ultra-pure water obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, USA) with conductivity of 18.2 mS was used in all experiments. Epicatechin (EP) (Sigma, USA) and procyanidin B2 (PB2) (isolated and certified by spectroscopic methods at the Pharmacognosy Laboratory of Maringá State University) of the highest grade (purity>99.0%) were used as standards. Colloidal silicon dioxide (CSD) was purchased from Degussa (Essen, Germany). All other solvents and chemicals were analytical grade.

2.3. Herbal preparations

Air-dried stem bark (900 g) was exhaustively extracted with 9.0 L of Me_2CO-H_2O (7:3) by turbo-extraction (Ultra-Turrax® model UTC115KT; IKA; USA) for 20 min at temperature ≤40 °C. The extractive dispersion was filtered and evaporated under reduced pressure to 1.0 L and freeze-dried (Christ model Alpha 1-2, Germany), yielding 120 g of crude extract (CE). One gram of CE was dissolved in a mixture of 10 mL water and 400 mg of CSD was added. This mixture (CEA) was freeze-dried under the same conditions described for CE.

2.4. Preliminary stability study

CE and CEA were evaluated for thermal stability under accelerated conditions for 21 days. Samples of the CE and CEA were weighed (200 mg) and packaged in opaque white

polyethylene flasks with a capacity of 10 g. The CE and CEA samples were stored in a climate chamber (BINDER, model KBF 240, USA) with a constant relative humidity of 75±5% and maintained at 45±2 °C, without direct light. Samples were analyzed at the initial time (t_0) and 2, 7, 14, and 21 days after exposure to the atmospheric conditions described above.

2.5. HPLC analysis

Accurately weighed 50 mg of CE and 50 mg of CEA were dissolved in 500 μL water, mixed in a tube shaker, and extracted with 500 μL of ethyl acetate, in a microtiter shaker at 1800 rpm (Minishaker, model MS1, IKA®, USA) for 3 min (n=9). Tubes were placed in a refrigerated microcentrifuge (model 5415R, Eppendorf®, USA) at 4000 rpm, for 4 min at 5 °C, for the total separation of the phases. The ethyl-acetate phase was separated. After evaporation of the solvent and drying under air flow, the residue was reconstituted to 10 mL with methanol:water (1:1; v/v) (test solution–SS). The sample was filtered through a 0.5 μm membrane filter (Millipore, Bedford, USA). The sample injection volume was 100 μL.

The analyses were carried out using a HPLC system (Gilson, USA) consisting of a solvent delivery pump (Model 321), a variable wavelength UV/VIS detector (Model 156), a manual injection valve (Rheodyne®, USA) with a 20 μL loop, degasser (Model 184), and a thermostatted column compartment (Model 831). Data collection and analyses were performed using UniPoint™ LC System software (Gilson, Villiers-le-Bel, France). A gradient was eluted on a Phenomenex® Gemini C-18 column (250 mm x 4.6 mm) (Phenomenex International, USA), 5 μm particle size, Phenomenex® SecurityGuard™ (C-18 cartridge) (20 mm x 4.6 mm). The mobile phase consisted of water (0.05% TFA) as solvent A and acetonitrile (0.05% TFA) as solvent B, and both were degassed and filtered through a 0.45 μm

pore size filter (Millipore, Bedford, USA). Separations were effected by a linear gradient as follows: 0 min 13% B; 10 min 17% B; 16 min 18.35% B; 20 min 22.65% B; 23 min 29.81% B; 25 min 65% B; followed by a 7 min re-equilibration time. The mobile-phase flow rate was 0.8 mL/min and the injection volume was 100 μL . The chromatographic runs were carried out at 28 °C. UV detection was performed at 210 nm.

The purity of peaks was checked by a Diode Array Detector coupled to a Varian ProStar module (Varian, Palo Alto, CA, USA) with ProStar 210 Solvent Delivery and a ProStar 335 HPLC-DAD, comparing the UV spectra of each peak with those of authentic reference samples.

An EP reference standard stock solution of 400 μg/mL was prepared in methanol:water (1:1; v/v). Calibration standard solutions at five levels were prepared by serially diluting the stock solution to concentrations of 10.00; 40.00; 70.00; 100.00; and 120.00 μg/mL. The PB2 standard was prepared in the same way. A PB2 stock solution of 250 μg/mL was prepared in methanol:water (1:1; v/v). Calibration standard solutions at seven levels were prepared by serially diluting the stock solution to concentrations of 20.00; 40.00; 50.00; 70.00; 90.00; 120.00; and 150.00 μg/mL. The samples were filtered through a 0.5 μm membrane (Millipore, Bedford, USA) prior to injection. Each analysis was repeated five times, and the calibration curves were fitted by linear regression.

2.6. Thermogravimetric (TG) analysis

A Simultaneous Thermal Analysis (STA) system (NETZSCH, model STA 409 PG/4/G Luxx, USA), was used for recording the TG thermograms of the CE and CEA. About 10 mg sample was weighed accurately using an STA balance. The weighed sample was heated in a closed aluminum pan at a programmed rate of 10 °C/min in a temperature range

from 30 to 500 °C under a nitrogen flow of 50 mL/min. An empty aluminum pan was used as a reference.

2.7. Total tannins

The percentage of total tannins in CE and CEA at t_0 and day 21 was evaluated using the Folin–Ciocalteau reagent and following a method adapted from the British Pharmacopoeia (British Pharmacopoeia, 2008). Samples of 100 mg and 166 mg of CE and CEA, respectively, were employed. Each analysis was repeated three times.

2.8. Statistical analysis

Experimental data were analyzed by one-way ANOVA, and the statistical significance of means was determined by the LSD and Tukey's HSD tests. The Dunnett test was employed to compare contents on different days of the analyses. Differences were considered significant at $P<0.05$.

3. Results and discussion

The stability of the constituents in the extract of *G. ulmifolia* is important because the pharmacological properties depend on the chemical viability of the extract.

Proanthocyanidins are especially unstable condensed tannins. Their stability is affected by several factors such as pH, storage, temperature, chemical structure, concentration, light, oxygen, solvents, and the presence of enzymes, flavonoids, proteins, and

metallic ions (Castañeda-Ovando et al., 2009). A compatibility study of excipients is essential to develop a stable pharmaceutical dose form, especially when the active agent is unstable.

Two dried extracts of *G. ulmifolia*, prepared by different techniques (with or without CSD), were evaluated for the stability of their main components (markers): PB2 and EP.

The quantification of these markers in the samples in the preliminary test of stability was carried out using external standards. The analytical curves for PB2 and EP are shown in Fig. 1.

Fig.1

No degradation in the CE and CEA samples under stress conditions was observed. No changes in the chromatographic profile occurred during the period of analysis (Fig. 2).

Fig. 2

The peak purity test confirmed that the PB2 and EP peaks remained homogeneous and pure throughout the stress test (data analyzed under DAD). The UV spectra of the compounds (PB2 and EP) did not change between the beginning and end of elution, confirming the absence of impurities.

The preliminary chemical stability of the CE and CEA dried-extract formulations was determined according to the concentration of PB2 and EP at a storage temperature of 45 °C and 75% humidity for 21 days. The final concentration was expressed as μg/mL of PB2 and EP in the dried extract (Table 1).

Table 1.

Fig. 3 shows the mean values of the PB2 and EP in the CE and CEA samples for each day of storage analyzed.

Fig. 3

The PB2 content remained constant after 21 days of storage, in both the CE and CEA. The EP in the CEA showed a significant change in concentration from t_0 to day 21. However, no significant change in the concentration of EP was observed in the CE stored under the same conditions.

Fig. 4

Figure 4 shows the influence of the presence of the excipient in the dried extract. Apparently, the physical and chemical properties of the CSD can significantly accelerate the increase of EP in the CEA after 21 days. In relation to the concentration of PB2, there was no difference between the CE and CEA during the 21 days of analysis.

Proanthocyanidins are commonly composed of monomers of catechin and/or epicatechin with linkages of 4→6 and/or 4→8. Besides these, other monomers are common: gallocatechin, epigallocatechin, robinetinidin, and fisetinidin (Lopes et al., 2008). Proanthocyanidins differ structurally according to the number of hydroxyl groups present in aromatic rings and the stereochemistry of the asymmetric carbon of the heterocyclic nucleus. The presence of *O*-methylation, *O*-glycosylation, and *O*-galoilation increase the structural complexity (De Bruyne et al., 1999).

PB2 is a dimeric proanthocyanidin with chemical linkage of the type 4β→8. Fletcher et al. (1977) showed by NMR studies of the procyanidin peracetate that linkages 4→6 and

4→8 are found in two energetically protected conformations. Therefore, the linkage between epicatechin monomers forming the PB2 may be physically more stable.

The significant change in concentration of EP in the CEA probably occurred by physical interaction of oligomers and/or polymers of condensed tannins in the extract and CSD. This excipient has a large surface area and a high polarity of silanol groups present on its surface, which leads to adsorption of water and formation of hydrogen bonds (Gore and Banker, 1979), facilitated by its hygroscopic property (Kibbe, 2000). Therefore, CSD is commonly used as a desiccant agent to protect hygroscopic chemicals and drugs from atmospheric moisture (Gore and Banker, 1979). Thus, this excipient is an excellent candidate adjuvant for the stabilization of plant extracts.

Extracts rich in phenolic substances are conguent with this assumption because they are rich in hydroxyl, capable of hydrogen interactions by bonds. Döner et al. (1993) evaluated the bonding between polyvinylpolypyrrolidone (PVP) and different classes of flavonoids. The bonding increases with the number of hydroxyl groups present in the flavonoid nucleus. Compounds that contain 7- and 4'-hydroxyl groups bond most effectively. Thus, the same principle can be extrapolated to the CEA. The increase in the concentration of EP in the CEA may result from an interaction by hydrogen bonding between oligomers and/or polymers of the condensed tannins and the silanol hydroxyl group of CSD.

Gore and Banker (1979) observed that silica has the ability to form a monolayer adsorption of water vapor, suggesting that polar water molecules are adsorbed at specific sites on the silica surface. Oligomers and polymers of condensed tannins may show the same pattern of connection to the CSD. Bonding of these substances with CSD would weaken the bonds within the compound, releasing monomeric substances. This would explain the statistical difference found at day 21.

However, the analyses of the total tannin content of CE and CEA at time t_0 and day 21 after the stress tests showed no significant differences. The results for CE were 26.11%±0.51 (RSD% 1.97) and 26.57%±0.78 (RSD% 2.96), and for CEA were 24.98%±1.22 (RSD% 4.88) and 25.37%±0.71 (RSD% 2.78) at times t_0 and day 21, respectively.

These results suggest that the physical interactions occurred in the extract CEA produced no alterations in the content of proanthocyanidin. However, they show that the quality control of extracts containing high content of phenolic compounds must be accomplished using dimeric compounds, which are more physically stable.

Figure 5 and Table 2 show the data for the TG of the CE and CEA. Three losses in mass were observed during the preliminary stability study. The first mass loss refers to the loss of water adsorbed on the surface, the second loss is from the fusion with decomposition of substances in the extract, and the third loss is from the breakdown and decomposition of substances.

Fig. 5

Table 2

The analysis of TG indicated no trend in the behavior of CE and CEA, i.e., during the days of analysis, the total mass underwent no significant increase or decrease.

The analysis of these data leads us to suppose that CSD conferred some stability, because the CEA lost significantly less total mass. It can be assumed that the CEA was protected from heat to some extent by the CSD, influencing the process of degradation, with smaller percentages of loss of mass from the fusion of chemicals. Thus, we can say that the

CSD limited the access of water to the extract and/or prevented its degradation (Moura et al., 1996; Vasconcelos et al., 2005).

Over the 21 days of the study there was no significant difference between the two extracts, but considering the TG data and the high temperatures involved, the data suggest that over the long term the CSD would be a good protector for the plant extracts of *G. ulmifolia*. However, further studies should be performed to confirm these results.

4. Conclusion

This study is the first to assess the individual PB2 and EP degradation in dried extract from *G. ulmifolia*, with and without CSD. The results indicated that the packaging in opaque white polyethylene is moisture-resistant. PB2 is an appropriate compound to use as a chemical marker in quality control of the dried extract of *G. ulmifolia*. The stress test showed that the content of total tannins was unchanged. Therefore, in this 21-day screening study, proanthocyanidins in the dried extract of the *G. ulmifolia* showed good compatibility with CSD under stress conditions.

The stability analysis should be continued according to the stability protocol. The proposed chemical stability and storage conditions need to be confirmed with long-term data.

Acknowledgments

The authors thank the Brazilian funding agencies CNPq (Conselho Nacional de Pesquisa) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). Our gratitude to Prof. Dr. Antonio Medina Neto and Prof. Dr. Jurandir Hillmann Rohling for technical support with TG.

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Fig. 1. Calibration curve for the procyanidin B2 (PB2) standard (A) and the epicatechin (EP) standard (B) obtained by HPLC.

Fig. 2. Typical HPLC chromatograms of stress test samples of the crude extract (CE) and the crude extract + colloidal silicon dioxide (CEA) of *Guazuma ulmifolia*. (A) CE at time zero; (B) CE at day 21; (C) CEA at time zero; (D) CEA at day 21.

Fig. 3. The stability of the crude extract (CE) and the crude extract + colloidal silicon dioxide (CEA) of *Guazuma ulmifolia* at 45 °C and 75% relative humidity.

Fig. 4. Influence of colloidal silicon dioxide on the dried-extract formulations (CE and CEA) of *Guazuma ulmifolia*. (A) procyanidin B2 (PB2) and (B) epicatechin (EP)

Fig. 5. Thermogravimetry curves for the crude extract (- -) and the crude extract + colloidal silicon dioxide (—) of *Guazuma ulmifolia* at time zero.

Table 1. Stability of the constituents procyanidin B2 (PB2) and epicatechin (EP) of the dried extract of *Guazuma ulmifolia* [Mean ± S.D. (R.S.D. %)].

Table 2. Thermogravimetry parameters of the crude extract (CE) and the crude extract + colloidal silicon dioxide (CEA) of *Guazuma ulmifolia*.

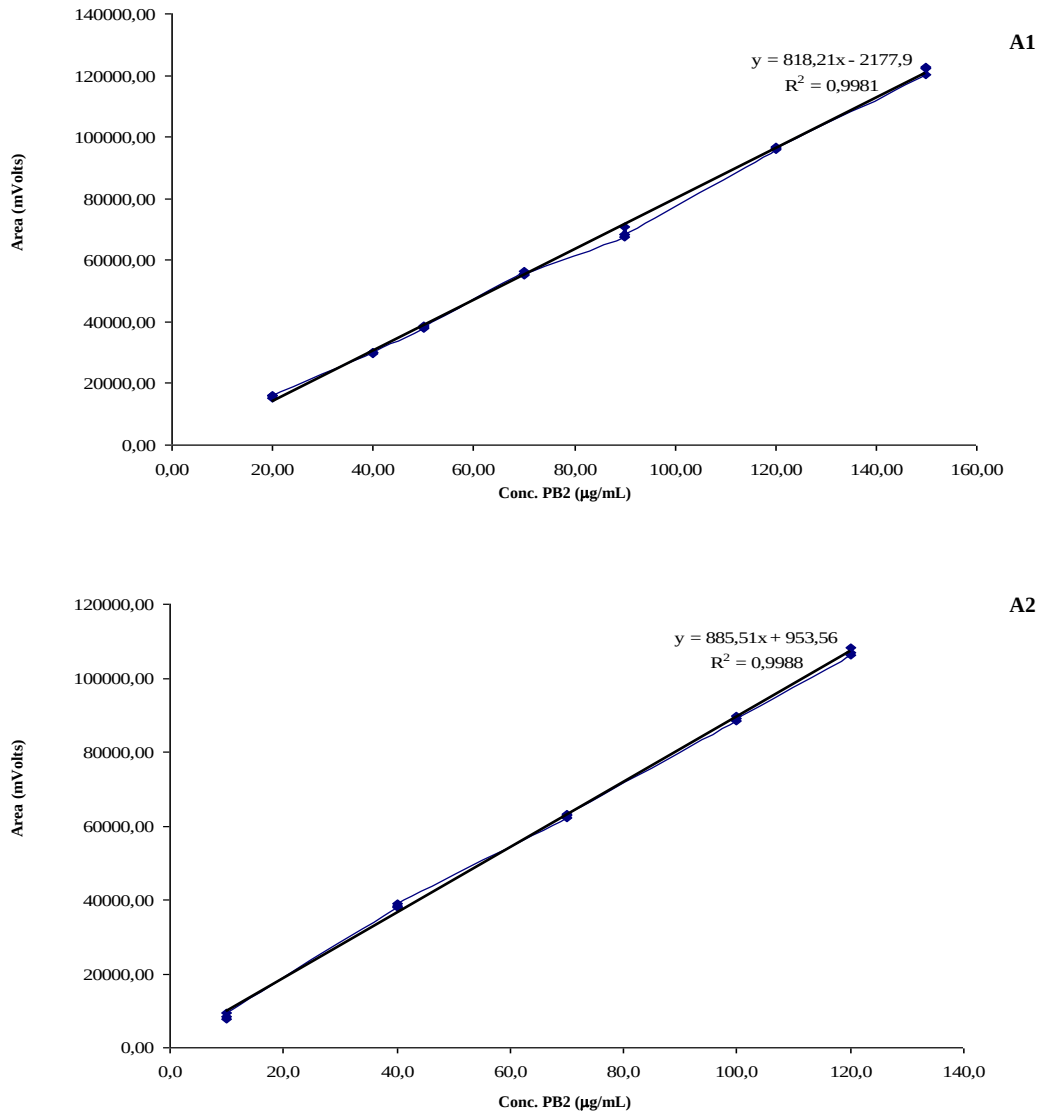
A1
y = 818,21x - 2177,9
R2 = 0,9981
Area (mVolts)
Conc. PB2 (µg/mL)
A2
y = 885,51x + 953,56
R2 = 0,9988
Area (mVolts)
Conc. PB2 (µg/mL)

Figure 1

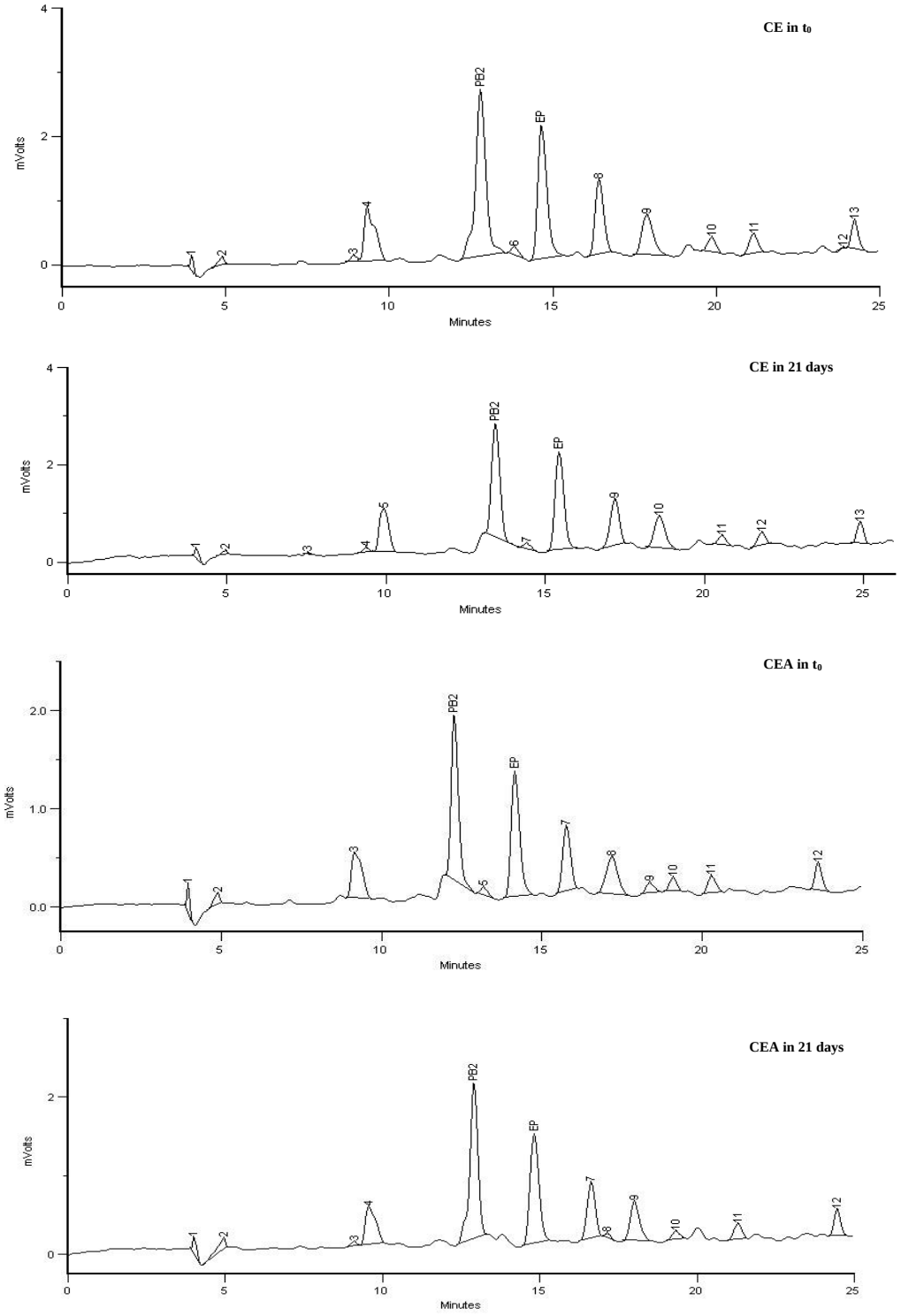
CE in t0
PB2
EP
mVolts
Minutes
CE in 21 days
PB2
EP
mVolts
Minutes
CEA in t0
PB2
EP
mVolts
Minutes
CEA in 21 days
PB2
EP
mVolts
Minutes

Figure 2

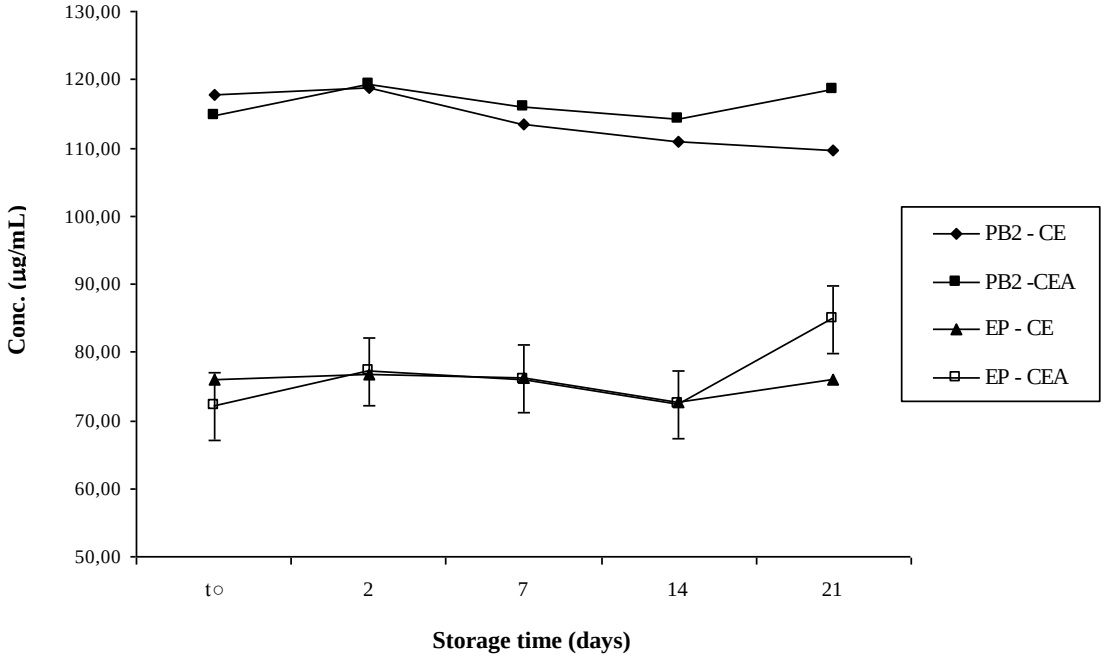
130,00
120,00
110,00
100,00
90,00
80,00
70,00
60,00
50,00
Conc. (µg/mL)
to
2
7
14
21
Storage time (days)
PB2 - CE
PB2 -CEA
EP - CE
EP - CEA

Figure 3

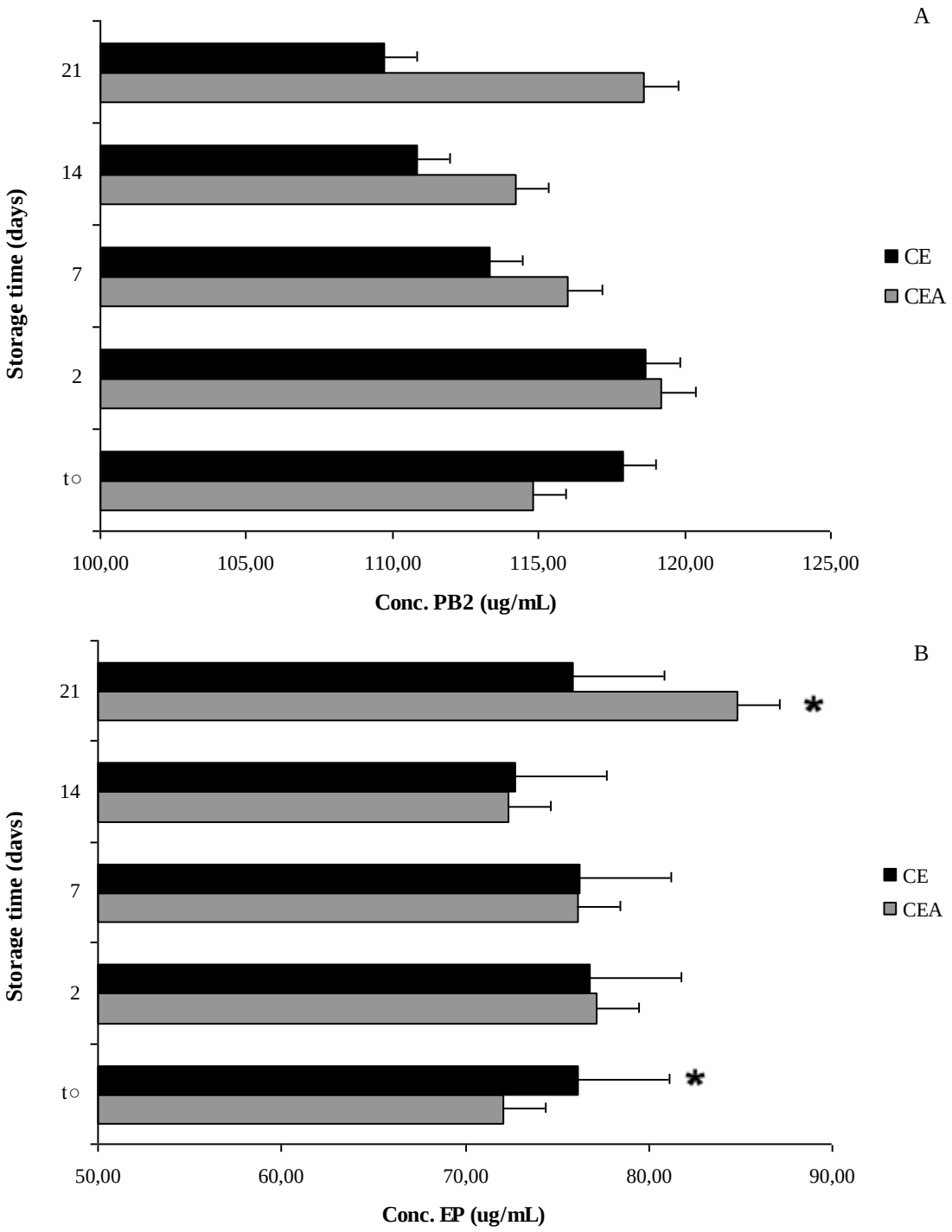
A
21
14
7
2
t○
Storage time (days)
100,00
105,00
110,00
115,00
120,00
125,00
Conc. PB2 (ug/mL)
CE
CEA
B
21
14
7
2
t○
Storage time (davs)
*
*
50,00
60,00
70,00
80,00
90,00
Conc. EP (ug/mL)
CE
CEA

Figure 4

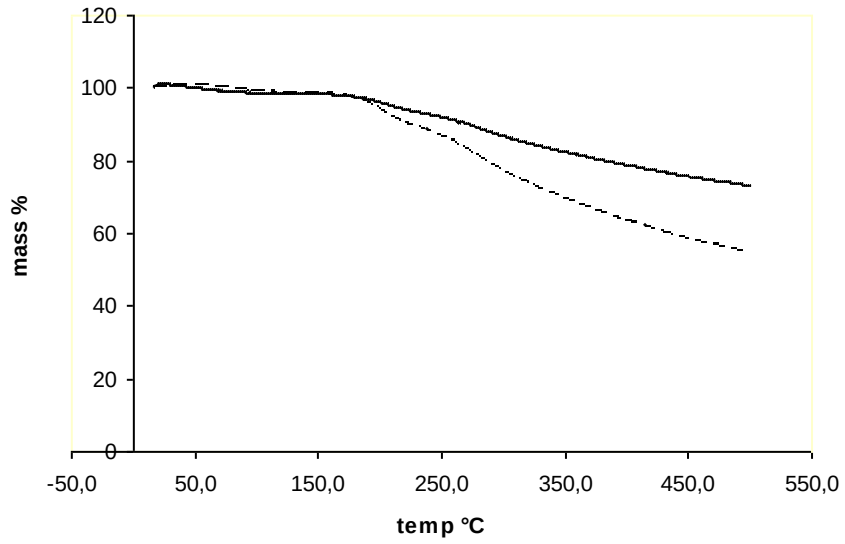
120
100
80
60
40
20
0
mass %
-50,0
50,0
150,0
250,0
350,0
450,0
550,0
temp °C

Figure 5

Table 1

Days of storage	CE (μg ml^{-1})		CEA (μg ml^{-1})	
	PB2	EP	PB2	EP
Time zero	117.86 ± 4.54 (3.85)	76.11 ± 1.44 (1.90)	114.80 ± 2.71 (2.36)	72.09 ± 2.32 (3.22)
2	118.66 ± 6.00 (5.06)	76.78 ± 3.47 (4.51)	119.18 ± 1.25 (1.05)	77.18 ± 3.40 (4.40)
7	113.31± 5.19 (4.58)	76.24 ± 2.44 (3.20)	116.02 ± 5.10 (4.40)	76.10 ± 1.44 (1.89)
14	110.86 ± 10.47 (9.45)	72.74 ± 1.25 (1.72)	114.22 ± 4.84 (4.23)	72.35 ± 2.44 (3.38)
21	109.73 ± 9.88 (9.01)	75.88 ± 6.94 (9.15)	118.61 ± 7.79 (6.56)	84.81 ± 2.38 (2.81)

Table 2 Thermogravimetry parameters on the CE and CEA

Sample	Mass loss (%)	Days of storage				
		t_0	2	7	14	21
CE	1	3.68	4.44	4.51	4.85	4.90
	2	9.70	8.44	8.98	8.38	8.27
	3	32.46	33.06	32.29	31.79	30.75
	Total mass loss (%)	45.87	46.22	46.81	45.45	44.85
CEA	1	2.31	3.54	3.11	2.07	2.36
	2	4.80	4.15	5.13	4.85	4.97
	3	19.79	19.39	20.89	18.98	19.21
	Total mass loss (%)	27.33	27.60	29.47	26.03	26.93

CAPÍTULO V

5.1 DISCUSSÃO GERAL

O interesse na elucidação química de extratos vegetais ricos em compostos fenólicos fundamenta-se na significativa influncia que estas substâncias apresentam sobre a nutrição e a saúde humana. Na literatura encontramos diversos trabalhos que contemplam o estudo químico de drogas vegetais que apresentam taninos e flavonóides como substâncias majoritárias. No entanto, um número reduzido dessas espécies são tipicamente brasileiras.

O principal objetivo deste trabalho foi o estudo químico do extrato acetônico obtidos a partir das cascas de *Guazuma ulmifolia* Lam., e o desenvolvimento e validação de método analítico, que permitisse a análise e quantificação de substâncias presentes nesses extratos. Podendo, ainda, este método ser aplicado ao controle de qualidade de extratos semipurificados e/ou formas farmacêuticas.

O extrato seco, desde que preparado adequadamente, apresenta inúmeras vantagens frente à forma líquida convencional, tais como, menor espaço necessário para o armazenamento do produto, maior concentração, estabilidade e facilidade de padronização dos princípios ativos presentes, o que aumenta de certa forma, o valor agregado do produto (BOTT, 2008).

Na tecnologia fitofarmacêutica a melhor estabilidade, maior facilidade de manipulação e dosagem mais precisa apresentada pelos extratos secos de plantas medicinais são as principais características responsáveis pelo aumento no interesse pelo seu uso como forma intermediária para o preparo de medicamentos (SENNA *et al*., 1997).

Neste trabalho, os resultados obtidos com o estudo cromatográfico foram compatíveis com os relatos da literatura. Procianidinas haviam sido previamente isoladas de *G. ulmifolia* (HÖR *et al*., 1996 e ROCHA, 2005), demonstrando que os métodos empregados para a produção dos extratos e o isolamentos de substâncias são adequados para os fins propostos.

A cromatografia líquida de alta eficiência (CLAE) é a técnica mais utilizada para análise de fármacos. A combinação desta técnica cromatográfica com a detecção no ultravioleta fornece um método preciso, exato e robusto para a análise quantitativa de substâncias nas amostras. Além disso, é aplicável para o monitoramento da estabilidade de fármacos e medicamentos, com a possibilidade de quantificação de produtos de degradação (WATSON, 2005).

O desenvolvimento do perfil químico do extrato bruto por CLAE, forneceu resolução e seletividade entre os picos o que possibilitou a quantificação de duas substâncias majoritárias no extrato: procianidina B2 (PB2) e epicatequina (EP).

A escolha do método analítico depende do objetivo da análise, ou seja, qualitativa, semi-quantitativa ou quantitativa. Além disso, outros fatores devem ser considerados, entre eles: a complexidade da amostra, sua pureza e teor. A aplicação do trabalho proposto demonstrou ganho de tempo e dinheiro por parte do analista que venha a realizar a etapa de purificação do extrato para a análise cromatográfica. O trabalho contribuiu ainda para a diminuição da carga de resíduos gerados na purificação da amostra. O uso da micro-extração foi fundamental para esse processo. Por meio das análises por CLAE, foi possível avaliar estatisticamente a repetibilidade da etapa de purificação do extrato utilizando o método da micro-extração. Constatou-se que durante todo o trabalho as áreas de PB2 e EP não apresentaram diferenças significativas ($p<0,05$) demonstrando a exatidão do método de extração.

A validação de um método analítico é o processo pelo qual se estabelecem, por estudos laboratoriais, quais características de desempenho do método cumprem os requisitos para a aplicação analítica a que se destina. Assim, a validação é imprescindível para assegurar a credibilidade do método (ERMER, 2001). O método desenvolvido para o extrato de *G.*

ulmifolia, mostrou-se linear, sensível, seletivo e robusto na análise de PB2 e EP no extrato bruto na faixa estabelecida.

Para o desenvolvimento de um novo medicamento, o controle de qualidade deve acompanhar todo o processo de produção, desde a qualidade da droga vegetal até sua transformação em um produto acabado à disposição do consumidor. Seu objetivo é garantir que o usuário receba um medicamento com qualidade, seguro e eficaz. O controle de qualidade inclui o desenvolvimento e validação de métodos analíticos sensíveis, específicos e confiáveis, além do estudo da estabilidade (WATSON, 2005).

O estudo de estabilidade preliminar consiste na realização do teste na fase inicial do desenvolvimento do produto, utilizado diferentes formulações e com duração reduzida. Neste estudo, são empregadas condições extremas de temperatura e umidade com o objetivo de acelerar possíveis reações entre seus componentes e o surgimento de sinais que devem ser observados e analisados conforme as características específicas de cada tipo de produto. Devido às condições em que é conduzido, este estudo não tem por finalidade estimar a vida útil do produto, mas sim auxiliar na triagem de excipientes (BRASIL-ANVISA, 2003).

O estudo preliminar da estabilidade objetiva portanto, identificar fatores degradantes para as substâncias em questão. Fornecendo assim as primeiras informações necessárias sobre a estabilidade, as quais serão posteriormente úteis para a realização de um estudo acelerado (ICH, 1996).

Os resultados do teste preliminar de estabilidade comparativo entre o extrato seco e o extrato seco com dióxido de silício coloidal, evidenciaram que a PB2 é substância mais adequada como substância química de referência para a análise da qualidade desses extratos. Demonstrou ainda, através da análise térmica dos extratos, que o dióxido de silício coloidal confere melhoria na estabilidade do extrato, pois, de certa forma, protegeu o extrato do calor, influenciando no processo de degradação, conferindo porcentagens menores de perda de

massa para a fusão das substâncias químicas. Assim, pode-se dizer que o dióxido de silício coloidal limita o acesso de água ao extrato.

5.2. CONCLUSÕES

- O estudo químico de extratos semipurificados das cascas de "mutamba" demonstrou que as formas de extração e fracionamento utilizadas foram adequadas ao isolamento de taninos condensados. Foram isoladas e identificadas as substâncias: *ent*-catequina, epicatequina, *ent*-galocatequina, epigalocatequina, epiafzelequina-(4β→8)-epicatequina, epicatequina-(4β→8)-catequina (PB1), epicatequina-(4β→8)-epicatequina (PB2) e epicatequina-(4β→8)-epigalocatequina.
- Foi isolada e identificada a substância inédita, 4´-*O*-metil-epiafzelequina. Esse fato denota o potencial que a droga vegetal possui e pode contribuir para o conhecimento químico e taxonômico do gênero e da família botânica;
- A otimização da purificação da amostra para CLAE através da micro extração em tubo Eppendorf®, mostrou-se um método preciso e econômico.
- O sistema cromatográfico desenvolvido utilizou como fase móvel ótima para a separação de proantocianidinas [procianidina B2 (PB2) e epicatequina (EP)], acetonitrila:ácido trifluoroacético 0,05% e água:ácido trifluoroacético 0,05% em sistema gradiente, com fluxo de 0,8 mL/min e detecção a 210 nm.
- O método proposto por CLAE demonstrou ser rápido, específico, preciso, exato e reprodutível para a determinação de PB2 e EP nos extratos estudados.
- O estudo preliminar da estabilidade indicou a PB2 como substância química de referência para o controle de qualidade do extrato.
- A análise térmica sugere que o dióxido de silício coloidal confere melhoria na estabilidade do extrato, nas condições de estudo.

5.3. PERSPECTIVAS

- Avaliação da estabilidade acelerada dos extratos;
- Estudo pré-formulação (loção capilar);
- Avaliação da atividade promotora de crescimento de pêlos em camundongos geneticamente modificados;
- Avaliação da estabilidade acelerada de forma farmacêutica tópica (loção capilar)

5.4. Referências bibliográficas

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