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OBTENÇÃO DE PROANTOCIANIDINAS DO BARBATIMÃO E AVALIAÇÃO DA TOXICIDADE SOBRE CÉLULAS DE TUMOR MAMÁRIO HUMANO MDA-MB-435 E MCF-7

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Dissertação apresentada como parte dos requisitos para Obtenção do título de Mestre em Ciências Farmacêuticas da Universidade Federal de Alfenas. Área de concentração: Obtenção, identificação e avaliação de compostos bioativos.
Orientadora: Prof. Dra. Cibele Marli Cação Paiva Gouvêa

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A Banca examinadora abaixo-assinada, aprova a Dissertação apresentada como parte dos requisitos para Obtenção do título de Mestre em Ciências Farmacêuticas da Universidade Federal de Alfenas. Área de concentração: Obtenção, identificação e avaliação de compostos bioativos.

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Dedico ao meu pai, à minha mãe e ao meu irmão que sempre me apoiaram e verdadeiramente torceram por mim em todos os momentos para a concretização desse sonho.

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RESUMO

O objetivo deste trabalho foi isolar proantocianidinas de folhas de barbatimão e avaliar a atividade citotóxica para células de tumor mamário humano, MCF-7 (ER+) e MDA-MB-435 (ER-). As proantocianidinas foram isoladas da fração aquosa de folhas de barbatimão, utilizando-se cromatografia em coluna de Sephadex LH-20 e metanol (10-100%) e acetona (70%) como eluentes. A presença de proantocianidinas, nas frações obtidas, foi monitorada por cromatografia de camada delgada e esta revelou que a fração eluída com metanol 100% (usada nos experimentos) apresentou o maior teor de proantocianidinas. A despolimerização ácida permitiu quantificar as procianidinas na amostra e a análise por HPLC revelou a presença de ácido gálico, procianidina dimérica B1 e (-)-epicatequina-3-*O*-galato. A fração apresentou atividade antioxidante, sendo que a capacidade antioxidante total foi de 470,28 ± 14,36 mg ácido ascórbico/g de fração. A atividade seqüestrante de radicais livres DPPH 50% foi de 12 µg/mL e a atividade inibidora máxima da oxidação de proteínas foi obtida com 50 µg/mL de fração. A fração apresentou atividade citotóxica para células de tumor mamário MCF-7 e MDA-MB-435. O tratamento das células com a fração aumentou a atividade redutora de MTT, o que indica aumento da viabilidade celular. Entretanto, a análise morfológica revelou intensa vacuolização citoplasmática dependente da concentração da fração. Os vacúolos acumularam MTT, indicando tratarem-se de vacúolos autofágicos. Outras alterações morfológicas observadas com o tratamento foram a perda da morfologia celular típica, condensação da cromatina (principalmente formação de núcleo picnótico), alterações na membrana plasmática e após 48 h de tratamento houve marcante diminuição do tamanho das células. Não foi observado o padrão de DNA escada característico de apoptose nos diversos tratamentos. Os resultados parecem indicar que a fração isolada pode induzir autofagia nos dois tipos celulares estudados. A autofagia pode potencializar os efeitos antitumorais de quimioterápicos, o que pode ser útil na terapia contra o câncer e as folhas de barbatimão parecem ser uma valiosa fonte de substâncias bioativas.

Palavras-chave: Neoplasias da Mama. Proantocianidinas. *Stryphnodendron adstringens*.

ABSTRACT

The aim of the present study was to isolate proanthocyanidins from barbatimão leaves and evaluate its cytotoxic activity to MCF-7 (ER+) e MDA-MB-435 (ER-) cells. The proanthocyanidins were isolated from the aqueous fraction of barbatimão leaves using Sephadex LH-20 column chromatography and methanol (10-100%) and acetone (70%) as eluents. The proanthocyanidins presence in the obtained fractions was monitored by thin layer chromatography and it revealed that the methanol 100% eluted fraction (used in the experiments) exhibited the highest content of proanthocyanidins. The acid depolimerization allowed quantifying the procyanidins in the sample and the HPLC analysis revealed the presence of gallic acid, procyanidin dimmer B1 and (-)-epicatechin-3-*O*-gallate. The fraction exhibited antioxidant activity, being the total antioxidant capacity 470.28 ± 14.36 mg ascorbic acid/g of fraction. The 50%DPPH free radical scavenging activity was obtained with 12 µg/ml and the maximum protein oxidation inhibitory activity was achieved with 50 µg/ml fraction. The fraction was cytotoxic to breast cancer MCF-7 and MDA-MB-435 cells. Cell fraction treatment enhanced the reducing MTT activity that indicates increased cell viability. However, the morphological analysis showed intense cytoplasmic vacuolization in a dose-dependent manner. The vacuoles accumulated MTT, which seems to be consistent with autophagic vacuoles. Other morphological changes observed after cell treatment include cell lost of typical morphology, chromatin condensation (mainly pyknosis), membrane blebbing and after 48 h-treatment there was prominently cell shrinkage. No DNA-laddering formation, typical of apoptosis, was obtained after cell treatment. The results seem to indicate that the isolated fraction could induce autophagy in both studied breast cancer cell lines. Autophagy could enhance the effects of antitumor chemotherapy that can be useful in anti-cancer therapy and barbatimão leaf seems to be a valuable source of bioactive substances.

Key words: Breast neoplasia. Proanthocyanidins. *Stryphnodendron adstringens*.

SUMÁRIO

1 REVISÃO DE LITERATURA

1.1 Barbatimão: caracterização e propriedades medicinais

Stryphnodendron adstringens (Martius) Coville (Fabaceae), conhecida popularmente como barbatimão, barbatimão-verdadeiro, barba-de-timão, chorãozinho-roxo e casca-da-virgindade, é uma árvore comum no cerrado, com ampla distribuição geográfica, ocorrendo em vários estados, desde o Pará até São Paulo, incluindo Minas Gerais. Apresenta altas densidades em várias localidades do Brasil central e é encontrada mais freqüentemente em fitofisionomias do cerrado típico, campo-sujo e cerradão (FELFILI et al., 1993; 1994; FELFILI; SILVA JÚNIOR, 1993; BORGES FILHO; FELFILI, 2003).

A planta é uma espécie perenifólia, com pico de floração, produção de folhas novas e queda de folhas entre julho e outubro. Apresenta inflorescências com número variável de flores pequenas de cor marrom, hermafroditas, com longevidade de apenas um dia. As folhas são compostas, bipinadas, com seis a oito pares de folíolos por pina. O fruto é um legume séssil, grosso e carnoso, linear-oblongo com cerca de 10 cm de comprimento (CORRÊA, 1984). O caule atinge de 20 a 30 cm de diâmetro e de 4 a 5 m de altura (FELFILI; SILVA JÚNIOR, 1993).

O barbatimão é importante fonte de compostos fenólicos, sendo que a casca desta planta possui pelo menos 20% de taninos (MELLO; DE PETEREIT; NAHRSTEDT, 1996a). Santos et al. (2002) analisaram o extrato acetônico de folhas e casca de *S. adstringens* e verificaram a presença de flavonóis, ácidos fenólicos, ésteres de ácido gálico, taninos e outros compostos fenólicos complexados a glicosídios e ao ácido gálico. *S. adstringens* possui prodelfinidinas 3-flavonóis, prorobinetidinas e procianidinas diméricas (MELLO; DE PETEREIT; NAHRSTEDT, 1996a; 1996b; 1999).

O extrato bruto obtido por decocção ou infusão da casca e das folhas é utilizado, popularmente, para o tratamento de leucorréia, diarréia, inflamações e como cicatrizante (SANTOS; TORRES; LEONART, 1987; PANIZZA et al., 1988; MACEDO; FERREIRA, 2004). *S. adstringens* apresenta ainda diversas outras atividades demonstradas. O extrato aquoso de barbatimão apresentou atividade antiinflamatória, analgésica e protetora da mucosa gástrica (BERSANI-AMADO et al., 1996; LIMA; MARTINS; DE SOUZA, 1998; AUDI et al., 1999; MARTINS; LIMA; RAO, 2002; LOPES et al., 2005).

O extrato metanólico da casca de *S. adstringens* apresentou toxicidade para mitocôndria isolada de fígado de rato, promovendo o desacoplamento, a inibição do transporte de elétrons e da ATP sintase, em concentrações superiores a 125 µg/mL (REBECCA et al., 2003). O extrato acetônico de casca de barbatimão inibiu a replicação de poliovírus e herpesvírus bovino e a síntese de antígenos virais (FELIPE et al., 2006). O extrato bruto de barbatimão ainda inibiu o crescimento de *Herpetomonas samuelpessoai* (HOLETZ et al., 2005) e de *Candida albicans* (ISHIDA et al., 2006). Bersani-Amado et al. (2007) observaram efeito antinociceptivo *in vivo* do extrato bruto de barbatimão e suas frações aquosa e acetato de etila. A administração do extrato metanólico de casca de barbatimão para camundongos, por 7 dias, não apresentou toxicidade. Contudo, a administração prolongada (30 dias) de 800 e 1600 mg/kg para ratos foi tóxica, induzindo diminuição da massa corporal, aumento de glicose e aspartato aminotransferase séricas, além de involução do timo (REBECCA et al., 2002).

1.2 Taninos: proantocianidinas

Os taninos são compostos fenólicos solúveis em água, podem precipitar alcalóides e proteínas e segundo a estrutura química são classificados em três grupos: hidrolisáveis, complexos ou parcialmente hidrolisáveis e condensados ou não-hidrolisáveis (COS et al., 2004; KOLECKAR et al., 2008).

Os taninos hidrolisáveis consistem de ésteres de glicose ou outro poliol com ácido gálico (galotaninos) ou ácido elágico (elagitaninos), sendo os últimos mais freqüentes. Os taninos complexos apresentam uma unidade 3-flavanol que se liga (ligação glicosídica) a um galotanino ou elagitanino. Os taninos condensados são oligômeros e polímeros compostos por unidades fenólicas 3-flavanol e como não se apresentam glicosilados, não são prontamente hidrolisáveis. Contudo, o aquecimento dos taninos condensados em meio alcoólico-ácido produz pigmentos vermelhos (antocianinas) e por isso são denominados proantocianidinas (FIGURA 1) (COS et al., 2004; KOLECKAR et al., 2008).

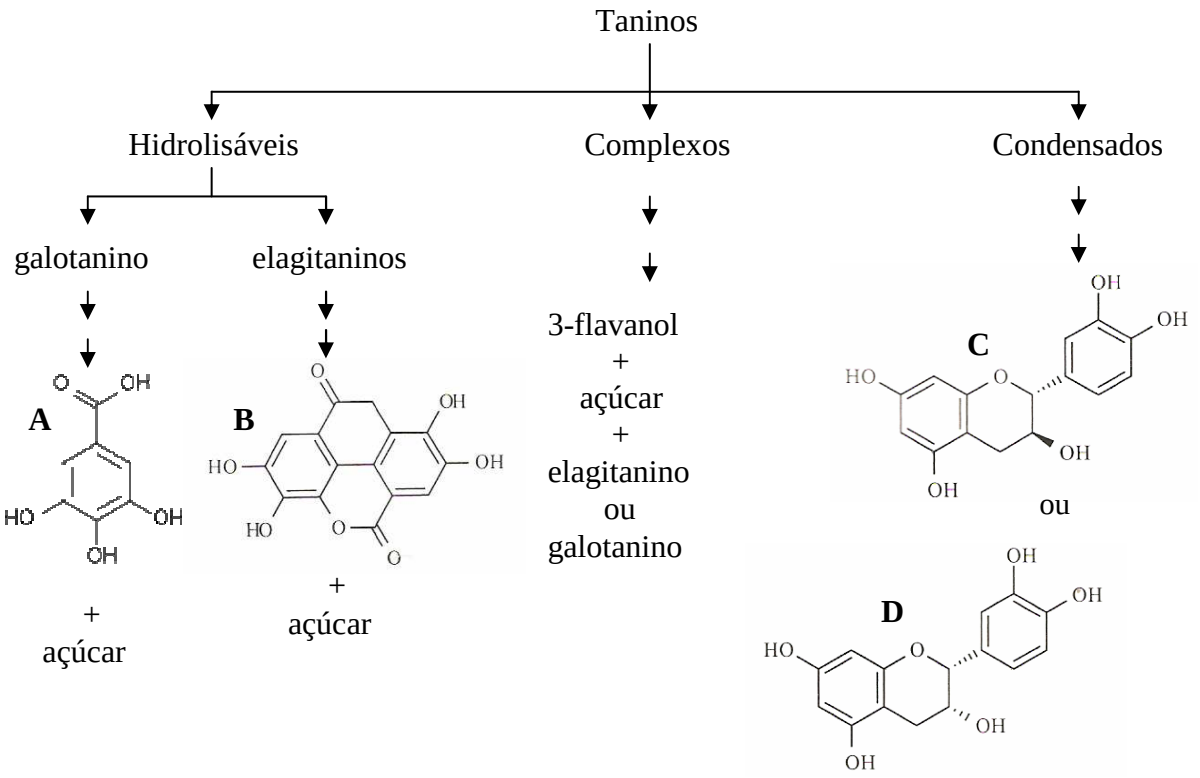

Figura 1- Classificação dos taninos. **A**, ácido gálico; **B**, ácido elágico; **C**, (+)-catequina; **D**, (-)-epicatequina. Fonte: adaptado de Cos et al. (2004).

As proantocianidinas originam-se por ligação, principalmente, tipo β entre o C4 do anel C de uma unidade nucleofílica flavanil e o C8 (C4→ C8) ou C6 (C4 → C6) do anel A de outra unidade flavanil, formando compostos oligoméricos ou poliméricos compostos de subunidades 3-flavanol, principalmente, (+)-catequinas e (-)-epicatequinas (FIGURA 2). Os taninos condensados representam um grupo com grande diversidade estrutural devido à variação no padrão de hidroxilação, estereoquímica dos três centros quirais, localização e tipo de ligação entre os monômeros, além da possibilidade de glicosilação e esterificação com o ácido gálico. São classificados de acordo com o padrão de hidroxilação, sendo os mais abundantes as procianidinas. As procianidinas do tipo B (diméricas) e C (triméricas) apresentam ligação simples entre os monômeros, enquanto as procianidinas A (diméricas) possuem ligação éter adicional entre o C2 de uma unidade e a hidroxila do C7 e/ou C5 de outra unidade (FIGURA 2) (COS et al., 2004; KOLECKAR et al., 2008).

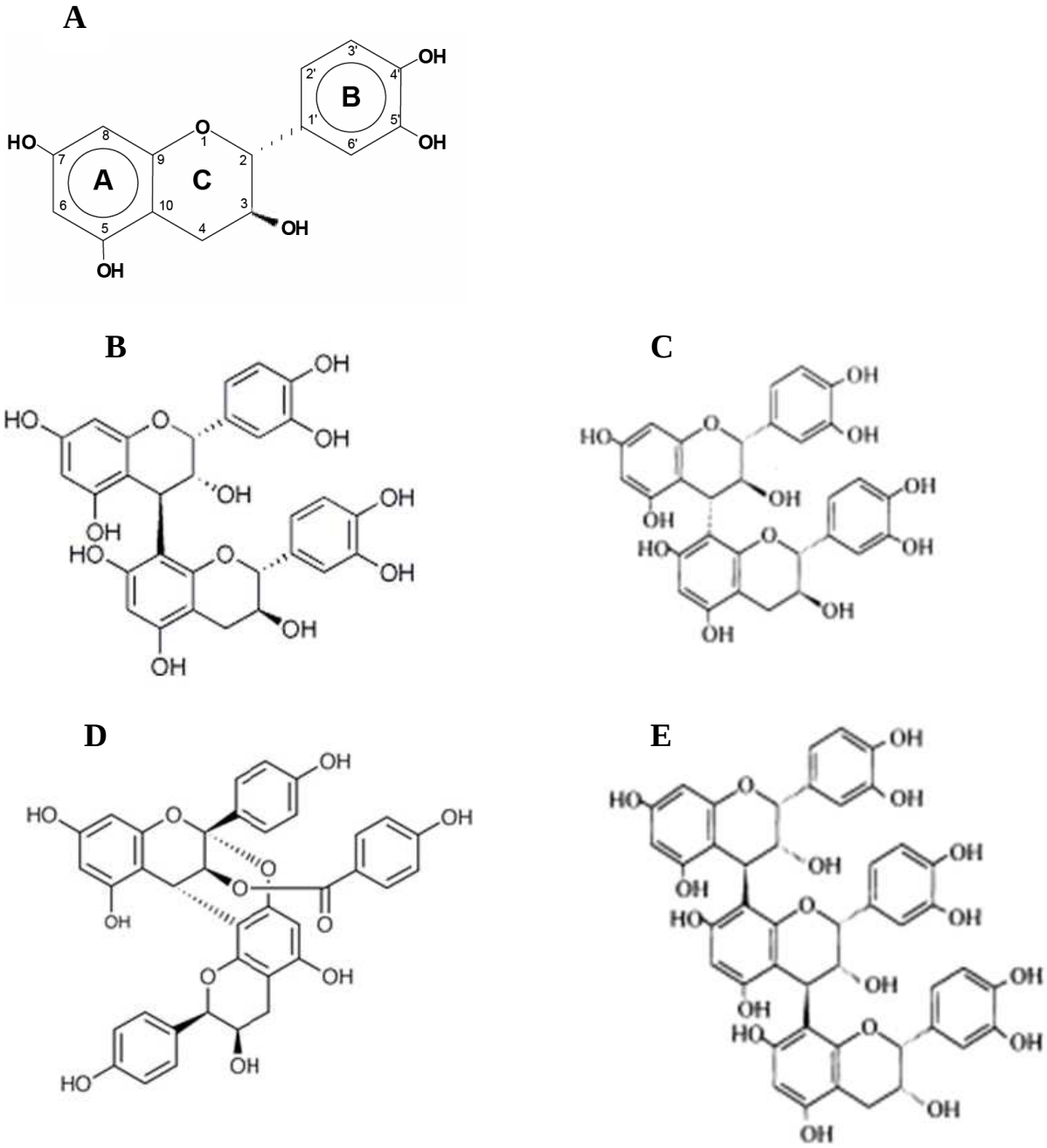

Figura 2- Fórmulas estruturais da catequina e procianidinas. **A**, catequina; **B**, procianidina B_1 (epicatequina-4β→8-catequina); **C**, procianidina B_3 (catequina-4α→8-catequina); **D**, procianidina A_2 (epicatequina-4β→8, 2β→7-epicatequina; **E** procianidina C_1 (epicatequina-4β→8-epicatequina-4β→8-epicatequina). Fonte: modificado de Cos et al. (2004).

1.3 Tumor mamário: incidência e fatores de risco

O câncer de mama é o segundo tipo de câncer mais freqüente no mundo e o primeiro entre as mulheres. É relativamente raro antes dos 35 anos de idade, mas acima desta faixa etária sua incidência cresce rápida e progressivamente. As estatísticas indicam o aumento de sua freqüência tanto nos países desenvolvidos quanto nos países em desenvolvimento. Segundo a Organização Mundial da Saúde, nas décadas de 60 e 70 registraram-se aumento de 10 vezes nas taxas de incidência, ajustadas por idade, nos diversos continentes (INCA, 2008). No Brasil, o câncer de mama é o prevalente entre as mulheres. O Ministério da Saúde, por meio do Instituto Nacional do Câncer estimou 49.400 novos casos de câncer de mama no Brasil, em 2008 (INCA, 2008). No Estado de Minas Gerais o câncer de mama também é o mais freqüente entre as mulheres, sendo a estimativa para 2008, de 4.280 novos casos de câncer de mama por 100.000 mulheres e na capital de 860 (INCA, 2008).

Está bem estabelecido que fatores de risco relacionados à vida reprodutiva da mulher, tais como: menarca precoce, idade da primeira gestação a termo acima dos 30 anos, anticoncepcionais orais, menopausa tardia e terapia de reposição hormonal estão relacionados com o desenvolvimento do câncer de mama. Além desses, a idade continua sendo um dos mais importantes fatores de risco. As taxas de incidência aumentam rapidamente até os 50 anos, e posteriormente ocorre de forma mais lenta, o que tem sido atribuído à menopausa.

Alguns estudos apontam para dois tipos principais de câncer de mama: o primeiro tipo, mais freqüente na pré-menopausa, é caracterizado por ser metastático agressivo e não expressar o receptor de estrógeno (ER-); o segundo, com maior freqüência na pós-menopausa, está associado com características indolentes, principalmente, por expressar o receptor de estrógeno (ER+). As variações morfológicas tumorais também estão relacionadas ao receptor de estrógeno, como por exemplo, os carcinomas medulares não expressam receptor de estrógeno, enquanto os carcinomas tubulares e lobulares expressam o receptor de estrógeno.

Os carcinomas medulares estão também associados às mutações no gene *BRCA1*, localizado no cromossomo 17 e são mais freqüentes em populações de baixo risco, como as japonesas. Por outro lado, os carcinomas tubulares e lobulares têm associação com as mutações do gene *BRCA2*, localizado no cromossomo 13 e são mais comuns em populações de alto risco, como nos Estados Unidos (JO et al., 2005; INCA, 2008). Outras mutações genéticas, como as que ocorrem no gene *p53* parecem ser desencadeadoras da doença, surgindo com maior freqüência em alguns grupos étnicos (VALLE, 1999). O gene *HER2* é

um receptor dos fatores de crescimento epidérmico com propriedades oncogenéticas e mutações com ganho de função aparecem em 20 a 30% dos casos de câncer de mama (DOWSET et al., 2000).

Apesar de ser considerado um câncer de relativamente bom prognóstico, as taxas de mortalidade por câncer de mama continuam elevadas no Brasil, muito provavelmente porque a doença ainda é diagnosticada em estádios avançados (INCA, 2008).

1.4 Células MCF-7 e MDA-MB 435

Linhagens celulares têm sido utilizadas para estudos da biologia de carcinoma mamário humano, bem como para a avaliação da atividade antitumoral de diversos compostos (DIMRI; BAND; BAND, 2005). Dentre as linhagens celulares humanas utilizadas destacam-se as células MCF-7 e MDA-MB-435 (FIGURA 3).

A **B**

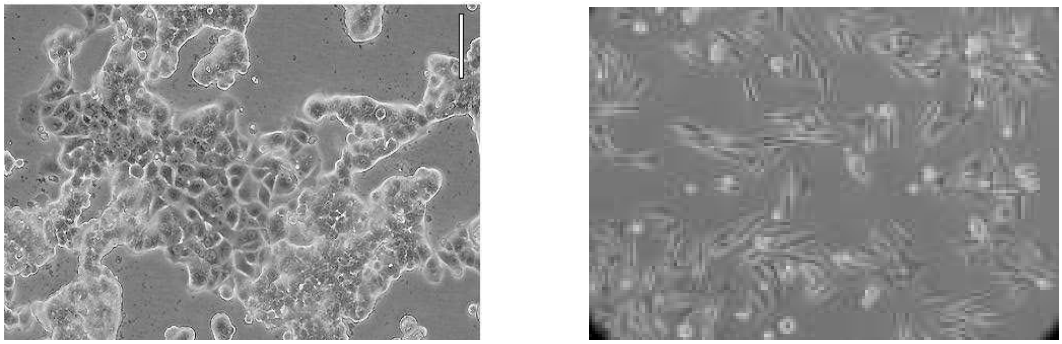

Figura 3- Células de adenocarcinoma mamário humano. A, MCF-7; B, MDA-MB-435. Fonte: American Type Culture Colection (http://www.atcc.org/CulturesandProducts/CellBiology/CellLinesandHybridomas/tabid/169/Default.aspx)

As células MCF-7 expressam o receptor de estrógeno (ER+) e a análise citogenética revelou aneuploidia (2n= 66-87), a presença de um grande cromossomo submetacêntrico e três grandes subtelocêntricos em 80% das metáfases, nulissomia do cromossomo 20 e dissomia do X. Estas células mantêm várias características do epitélio mamário diferenciado, incluindo a capacidade de processar o estradiol por meio de receptores de estrógeno

citoplasmáticos. Contém o oncogene Tx-4, seu crescimento é inibido por TNFα e a secreção de IGFBP pode ser modulada pelo tratamento com anti-estrogênios.

As células MDA-MB-435 são independentes de estrógeno, pois não expressam o receptor de estrógeno (ER-), expressam os receptores para EGF e TGFα e o oncogene WNT7B. A análise citogenética dessas células revelou aneuploidia, com duplicação cromossômica (2n= 52-68), ausência dos cromossomos 8 e 15, rearranjos cromossômicos e trissomia de autossomos.

As linhagens celulares de carcinoma mamário humano, MCF-7 e MDA-MB-435, apresentam potencial proliferativo diferente, bem como diferentes respostas a agentes quimioterápicos e antitumorais potenciais obtidos de plantas, devido à suas características genéticas.

1.5 Efeito de proantocianidinas sobre células de tumor mamário

A prospecção da atividade biológica de compostos com atividade antitumoral potencial pode ser avaliada utilizando-se culturas celulares. As culturas celulares oferecem ainda a possibilidade de estudos sobre o mecanismo de ação de drogas e compostos, o que é dificultado em modelos animais e humanos.

A formação de radicais livres e de espécies reativas de oxigênio no organismo está envolvida em processos fisiológicos. O organismo dispõe de um sistema antioxidante composto por substâncias endógenas e vindas da dieta, tais como os compostos fenólicos. A produção excessiva de radicais livres e de espécies reativas de oxigênio ou falhas no sistema antioxidante podem causar o estresse oxidativo, com conseqüente lesão de biomoléculas (lipídios, DNA, açúcares e proteínas). As lesões no DNA podem desencadear diversas patologias dentre elas o câncer e doenças degenerativa, além de outras (GOUVÊA, 2004).

As proantocianidinas são antioxidantes naturais e apresentam amplo espectro de atividade biológica. Podem exercer efeito protetor no organismo contra a oxidação de proteínas e lipídeos minimizando os efeitos deletérios de espécies reativas e os riscos de doenças como o câncer. O tamanho e composição das proantocianidinas parecem estar relacionados à sua atividade antioxidante, bem com a complexação ao ácido gálico na posição 3, aumenta a atividade antioxidante. O mecanismo de ação antioxidante das proantocianidinas

inclui a atividade seqüestrante de radicais livres e a inibição enzimática (ARIGA, 2004; COS et al., 2004).

Além da atividade antioxidante os compostos fenólicos exercem outras atividades que podem estar relacionadas à capacidade de seqüestrarem radicais livres. Estas atividades são mediadas por interação a receptores e incluem atividade antiproliferativa, de regulação do ciclo celular e indução de apoptose, constituindo-se importantes na prevenção e bloqueio do crescimento de células tumorais (WILLIAMS; SPENCER; RICE-EVANS, 2004).

As proantocianidinas de extrato de uva mostraram efeito citotóxico para células MCF-7, além de outras linhagens tumorais (YE et al., 1999; FARIA et al., 2006). As proantocianidinas de uva foram também citotóxicas para outras linhagens de células de tumor mamário, tais como 4T1 e MDA-MB-468, induzindo diminuição da proliferação e viabilidade celular, bem como induziu apoptose das células 4T1, provavelmente por alterar a relação Bax/Bcl-2, em favor da apoptose (MANTENA; BALIGA; KATIYAR, 2006).

Kozikowski et al. (2003) demonstraram que as procianidinas de cacau e sintéticas (com estrutura molecular idêntica à da procianidina de ocorrência natural) inibiram várias linhagens de células tumorais mamárias humanas: MCF-7, MDA-MB-231, MDA-MB-435 e SKBR-3. Utilizando a linhagem MDA-MB-231 foi possível estabelecer que a inibição celular foi provocada pela paralisação do ciclo celular na fase G0/G1 e a morte celular foi mais consistente com necrose do que apoptose.

A proantocianidina galato, prodelfinidina B-2 3,3'-di-O-galato (PB233'OG), apresenta atividade antioxidante e antiviral e Kuo et al. (2004) demonstraram que este composto também apresenta atividade anti-proliferativa de células MCF-7. A PB233'OG induziu apoptose das células sem mediação de p53 e p21/WAF1. Os autores sugeriram que a apoptose foi induzida pelo sistema apoptótico Fas/Fas ligante.

Em outro estudo, Ramljak et al. (2005) demonstraram que procianidina pentamérica natural de cacau induziu paralisação do ciclo celular na fase G0/G1 das linhagens celulares de carcinoma mamário MDA-MB-231, MDA-MB-436, MDA-MB-468, SKBR-3 e MCF-7 e de células 184A1N4 e 184B5 imortalizadas por benzo(a)pireno. Entretanto, culturas primárias de células epiteliais mamárias normais e MCF-10A espontaneamente imortalizadas foram resistentes ao tratamento. Os autores verificaram que a procianidina pentamérica induziu despolarização da membrana mitocondrial das células MDA-MB-231, mas não das células MCF-10A. As demais células apresentaram resultados variáveis. A despolarização das células MDA MB-231, revelou desfosforilação específica, sem alteração da expressão de proteína moduladoras da fase G1 como Cdc2, p53 e outras. A desfosforilação da p53 pela procianidina

pentamérica foi também confirmada nas células MDA-MB-468. Contudo, nestas células o tratamento induziu diminuição da expressão e fosforilação da p53. Este trabalho demonstrou que as células de carcinoma mamário humano são seletivamente susceptíveis aos efeitos citotóxicos da procianidina pentamérica. Em outro trabalho, taninos condensados obtidos de *Acacia catechu* inibiram o crescimento de células MCF-7, em baixa concentração, provavelmente por inibição da biossíntese de lipídeos (ZHANG et al., 2008)

Além das proantocianidinas, os flavonóides especialmente, epicatequina galato e epigalocatequina galato apresentam atividade antitumoral, atuam modulando o ciclo celular e podem potencializar o efeito de quimioterápicos (VALCIC et al., 1996; SEERAM; ZHANG; NAIR, 2003; CHISHOLM; BRAY; ROSENGREN, 2004; FARABEGOLI et al., 2007; HSIEH; WU, 2008).

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ARTIGO 1- Antioxidant Properties of Proanthocyanidins Isolated from *Stryphnodendron adstringens* Leaves

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SUMMARY. We isolated proanthocyanidins from barbatimão leaves and evaluated their antioxidant properties. Proanthocyanidins were isolated from the aqueous fraction from barbatimão leaves, using Sephadex chromatography. The TLC allowed identifying that 100% methanol eluted the fraction with the highest content of proanthocyanidins. This fraction seems to contain dimeric proanthocyanidin, procyanidin B1 and epicatechin 3-*O*-gallate. The extract content of proanthocyanidins was 242.17 ± 14.27 mg of proanthocyanidins / g of extract. The total antioxidant capacity was 470.28 ± 14.36 mg ascorbic acid/g of proanthocyanidin. These compounds also exhibited DPPH free radical scavenging activity (SA), being the SA_{50} 12 µg/ml. The reducing power of barbatimão leaves proanthocyanidins increased with concentration increasing and the maximum activity was obtained with 100 µg/ml of proanthocyanidins. The isolated proanthocyanidins presented a dose-dependent protein oxidation inhibitory activity, and 50 µg/ml of proanthocyanidins produced maximum inhibition. In conclusion barbatimão leaves can be rich source of proanthocyanidins that act as antioxidants, which may be highly relevant to the maintenance of normal health and disease management in humans.

KEY WORDS: Proanthocyanidins, *Stryphnodendron adstringens*, antioxidant

INTRODUCTION

Stryphnodendron adstringens (Martius), Coville (Fabaceae) grows in open fields and savannah regions in Brazil, where it is popularly known as "barbatimão"[1]. The stem bark has been empirically used as wound healing, astringent, antimicrobial, antifungal, antidiarrheal and hypoglycemic agent[2,3].

This plant is a rich source of tannins (10-37%)[4], mainly proanthocyanidins or condensed tannins. It is also known as oligomeric proanthocyanidins, pycno-genols or leukocyanidins, oligomers or polymers of flavan-3-ols. The proanthocyanidins that exclusively consist of epicatechin units are designated procyanidins, the most abundant type of proanthocyanidins in plants. The less common proanthocyanidins containing epigallocatechin subunits are called prodelphinidin[5]. Mello *et al*.[6,7] isolated and identified several flavan-3-ols, prodelphinidins and prorobinetinidins from an ethyl-acetate extract of barbatimão stem bark. These polyphenols are composed of monomeric flavan-3-ol units [(+)-catechin and/or (-)-epicatechin.

Several biological effects are attributed to proanthocyanidins, namely, antibacterial, antiviral, anticarcinogenic, anti-inflammatory, and antiallergic activity[8,9]. Moreover, a special interest has been devoted to their antioxidant activity since proanthocyanidins can exert a protective action in the organism against protein and lipid oxidation, reducing the risk of chronic diseases such as coronary heart disease and certain types of cancer[10,11]. In healthy individuals, the production of oxidative species is balanced with the antioxidant defense system. Oxidative stress is defined as disturbances in the balance in favor the oxidative species leading to potential biomolecules damage[12,13].

The aim of the present work was to isolate the most abundant fraction of proanthocyanidins of barbatimão leaves and evaluate its antioxidant properties. There is no published work on the barbatimão leaf proanthocyanidins isolation and properties.

MATERIALS AND METHODS

Extraction and fractionation of plant material

Leaves of *Stryphnodendron adstringens* (Martius), Coville was collected in Alfenas (Minas Gerais State, Brazil) in Julho/2007. A voucher specimen is deposited at the Herbarium of the Universidade Federal de Alfenas (Brazil). The dryed leaves were ground and the proanthocyanidins were extracted according to Foo & Lu[14], with modifications. To obtain proanthocyanidin polymers representative of those present in intact plant tissues, extreme care

was taken to prevent exposure of the preparations to heat, light, and air, and the dry plant material was homogenized three times, during 5 days each, with acetone/water (7:3), an efficient solvent system for extracting proanthocyanidin polymers. The combined extracts were concentrated by low-pressure evaporation at 30 °C to eliminate acetone, and the concentrated aqueous solution was extracted again with ethyl acetate (1:1, 5 times), to remove lipids and fat-soluble pigments. The remaining aqueous solution was lyophilized, and 2 mg of freeze-dried product was dissolved in 10% aqueous methanol and fractioned on a 40 cm x 2 cm i.d. Sephadex LH-20 column (Pharmacia, Uppsala, Sweden). After the column was rinsed with 100 ml of water (discarded), fractions were collected by increasing the methanol content of the eluent from 0 to 100% (v/v) in increments of 10% (100 ml each) followed by 2 x 100 ml fractions of acetone-water (70:30, v/v). The proanthocyanidin composition of the fractions was monitored using thin-layer chromatography (TLC) system.

TLC

Analytical TLC was performed according to Rosch *et al*.[15] on 20 cm x 20 cm silica gel (Merck) using methanol-toluen-acetic acid (3:3:1, v/v/v). The spots were visualized with vanillin (5%) and HCl (10%).

Quantitative analysis of proanthocyanidins

The content of proanthocyanidins of the water fraction was determined photometrically after acid depolymerization to the corresponding anthocyanidins[16]. Triplicate samples were run for each set.

Total antioxidant capacity

The total antioxidant capacity of the proanthocyanidins isolated (2.0 mg/ml) was determined by the phophomolibdeniun method[17], based on the spectrofotometrically determination of Mo^{+4} to Mo^{+5} reduction and ascorbic acid was used as standard. Triplicate samples were run for each set.

DPPH free radical scavenging assay

The hydrogen atoms or electrons donation ability of the proanthocyanidin fraction (0.5-200.0 µg/ml) was measured from the bleaching of purple colored methanol solution of DPPH according to Yen & Wu[18]. The scavenging activity was determined according to the equation:

DPPH scavenging activity (%) = $100 - [(\frac{Ac - At}{Ac}) \times 100]$

Where, Ac is the absorbance of the control (without proanthocyanidins) and At is the absorbance of the different solutions of proanthocyanidins. Triplicate samples were run for each set.

Reducing power

The ferric reducing potential of the proanthocyanidins (10.0-500.0 µg/ml) was determined according to the method of Oyaizu[19]. Ascorbic acid was used as standard and higher absorbance of the reaction mixture indicated greater reducing power. Triplicate samples were run for each set.

Protein oxidation assay

The effects of proanthocyanidins (10.0-500.0 µg/ml) on protein oxidation were carried out according to the method of Lenz *et al*.[20] Triplicate samples were run for each set.

Statistical analysis

Data were analyzed using ANOVA with post hoc analysis by Tukey test, when $p<0.05$. Results are expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

The extensive investigations with the proanthocyanidins have identified various molecular targets that can potentially be used for the prevention or treatment of cancers and other diseases caused by the oxidative stress[5]. Natural and synthetic antioxidant compounds can exert a number of effects in vivo; e.g., promoting increased synthesis of endogenous antioxidant defenses or themselves acting directly as antioxidants. The feasibility of an extract or compound exerting antioxidant effects can be evaluated by in vitro tests that investigate how the putative antioxidant can or cannot react with relevant free radicals[21]. In the present work we have demonstrated, for the first time that the proanthocyanidins isolated from barbatimão leaves present antioxidant properties.

The TLC allowed identifying that the fraction with the highest content of proanthocyanidins was isolated with 100% methanol. This fraction seems to contain dimeric proanthocyanidin, procyanidin B1 and epicatechin 3-*O*-gallate.

The content of proanthocyanidins isolated from the aqueous fraction of barbatimão leaves demonstrated a concentration of 242.17 ± 14.27 mg of proanthocyanidins/g of extract. The content obtained in the present work was superior to that obtained by others[22-24], indicating that the barbatimão leaf is a rich source of proanthocyanidins.

The proanthocyanidins isolated presented antioxidant activity. The total antioxidant capacity was 470.28 ± 14.36 mg ascorbic acid/g of proanthocyanidin. These compounds also exhibited DPPH free radical scavenging activity (SA), being the SA_{50} 12 µg/ml (Fig. 1). We obtained a maximum SA with 100 µg/ml of proanthocyanidin, and the maximum SA for ascorbic acid was obtained with 12.5 µg/ml. However, the proanthocyanidin from barbatimão leaves were more active than that from *Prunus*[25].

One of the mechanisms of action involved in the antioxidant activity is the ability of donating H^+ (a single electron) to free radical specie[26,27]. It has been reported that a great number of flavan-3-ols and proanthocyanidins present radical scavenging activity and it was found that the introduction of a gallic acid function at position 3 increases significantly the radical scavenging activity, while glycosylation of the position 3 decreases the scavenging ability[10]. The results suggests the human use of the proanthocyanidins from barbatimão leaves, as free radicals seem to be involved in the majority or in all of human diseases onset and progression[28]. Barbatimão leaves proanthocyanidins could be an auxiliary to prevent the DNA, lipids and protein lesion caused by free radicals.

There is some literature evidence that the reducing power of a compound is related to its electron transfer ability and may therefore serve as a significant indicator of its potential antioxidant activity[29]. The reducing power of barbatimão leaves proanthocyanidins increased with concentration increasing and the maximum activity was obtained with 100 µg/ml of proanthocyanidins (Fig. 2). Although the isolated proanthocyanidins presented a lesser reducing power than ascorbic acid, it exhibited potent reducing ability, which may account to its antioxidant activity. The isolated proanthocyanidins showed a higher reducing power than that reported to some plant extracts[22,23].

The carbonyl derivatives are formed by the free radical attack to the amino-acyl residues of proteins or by the reaction with oxidants from lipid and carbohydrates degradation. The oxidative protein modification brings structural alterations and consequent enzyme inactivation[13]. The isolated proanthocyanidins presented a protein oxidation inhibitory activity, which increased with the concentration increasing. The maximum protein oxidation inhibition was obtained with 50 µg/ml of proanthocyanidins (Fig. 3).

The inhibitory activity of proanthocyanidins on the protein oxidation, observed in the present study may be related to a proanthocyanidin free radical scavenging activity. Protein oxidation inhibitory activity of proanthocyanidins was also demonstrated by Senthilmohan *et al*.[30]. They conducted a 12 week clinical trial with Enzogenol®, a commercially available proanthocyanidin-rich flavonoid extract derived from the bark of *Pinus radiate*, and demonstrated that the diary ingestion of 480 mg Enzogenol® reduced by 51% the protein carbonyl concentration after 6 and 12 weeks of supplementation by older human subjects. In the present work we obtained a 48,87 % decreasing of protein carbonyl formation with 50 μg/ml barbatimão proanthocyanidins.

CONCLUSIONS

In conclusion the oligomeric proanthocyanidins isolated from barbatimão leaves present antioxidant activity, which may indicate its use as an auxiliary to prevent some diseases caused by the oxidative stress.

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Figure legends

Fig. 1. DPPH free radical sacavenging activity. Proant, proanthocyanidins from the leaves of *S. adstringens*; AA, ascorbic acid. The results are the mean ± SD of 3 independent experiments in triplicate.

Fig. 2. Reducing power. Proant, proanthocyanidins from the leaves of *S. adstringens*; AA, ascorbic acid. The results are the mean ± SD of 3 independent experiments in triplicate.

Fig. 3. Protein oxidation inhibition activity of proanthocyanidins from the leaves of *S. adstringens*. The results are the mean ± SD of 3 independent experiments in triplicate.

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ARTIGO 2 -Biological & Pharmaceutical Bulletin

Regular Article

Pharmacognosy

Low Concentration of Procyanidin rich fraction from *Stryphnodendron adstringens* (Barbatimão) Leaves Inhibits Growth of MCF-7 and MDA-MB-435 Cells

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The cytotoxic effect of a procyanidin rich fraction isolated from *Stryphnodendron adstringens* (barbatimão) leaves was evaluated in two human breast cancer cell lines, the estrogen-dependent ER (+) MCF-7 and the estrogen-independent ER (-) MDA-MB-435 cells. HPLC analysis revealed that this fraction is composed by gallic acid, proacyanidin dimer B1 (main component) and (-)-epicatechin-3-*O*-gallate. Fraction, at concentrations higher than 5 μg, induced cell death an increased the capacity of these cells to reduce MTT. This was concurrent with the appearance of an intense intracytoplasmic vacuolization, which seems to be consistent with autophagic vacuoles. The accumulation of the formazan product in these vacuoles could justify the enhancements of MTT reduction. Other morphological changes observed after cell treatment include cell lost of typical morphology, chromatin condensation (mainly pyknosis), membrane blebbing and after 48 h-treatment there was prominently cell shrinkage. No DNA-laddering formation, typical of apoptosis, was obtained after treatment of the cells with barbatimão fraction. These results led to the suggestion that the barbatimão fraction could induce autophagy in both breast cancer cell lines. In view of cancer therapy, autophagy could be important to enhance the effects of anti-cancer therapies and barbatimão leaf would be a valuable resource of bioactive substances.

Key words: Procyanidin B1, barbatimão, condensed tannins, breast carcinoma, cancer

Stryphnodendron adstringens (Martius) Coville (Fabaceae) is a native tree that grows in open fields and savannah regions in Brazil, where it is popularly known as "barbatimão".[1)] The stem bark has been empirically used as wound healing, astringent, antimicrobial, antifungal, antidiarrheal and hypoglycemic agent.[2,3)] Barbatimão is rich source of proanthocyanidins, including procyanidins, prodelphinidins and prorobinetinidins.[4-6)] This class of phenolic compounds has attracted great attention due to their wide range of biological activities and they can potentially be used for the prevention or treatment of cancers of various organs.[7-9)] It has been reported that proanthocyanidins from grape seed inhibited proliferation and induced apoptosis of MCF-7 and MDA-MB-468, while enhanced normal cells growth and viability.[10,11)] Pentameric proanthocyanidins from *Theobroma cacao* selectively inhibited growth of several breast cancer cells lineage through G_1-modulatory effect[12)] and more recently proanthocyanidins from catechu has been proven to significantly inhibit MCF-7 cells growth.[13)]

In this study we demonstrated, for the first time, that the fraction containing procyanidin dimmer B1 isolated from barbatimão leaves inhibited MCF-7 and MDA-MB-435 breast carcinoma cells lineage growth. Breast carcinoma is one of the most common malignant tumors worldwide, and is the first malignancy in women. Therefore, we chose two phenotypically different human breast carcinoma cell lines as model to explore the effects of procyanidin rich barbatimão leaf fraction on cancer cell growth in order to assess its value in cancer prevention and therapy.

MATERIALS AND METHODS

Extraction and Fractionation of Plant Material Leaves of *Stryphnodendron adstringens* (Martius) Coville was collected in Alfenas (Minas Gerais State, Brazil) in Julho/2007. A voucher specimen (N. 196) is deposited at the Herbarium of the Universidade Federal de Alfenas (Brazil). The dryed leaves were ground and the proanthocyanidins were extracted by three times homogenation with acetone/water (7:3), during 5 days each. The combined extracts were concentrated by low-pressure evaporation at 30° C to eliminate acetone, and the concentrated aqueous solution was extracted with ethyl acetate (1:1), for 5 times, to remove lipids and fat-soluble pigments. The remaining aqueous solution was lyophilized, and 2 mg of freeze-dried product was dissolved in 10% aqueous methanol and fractioned on a 40 cm x 2 cm i.d. Sephadex LH-20 column (Pharmacia, Uppsala, Sweden). The elution rate was 0.5 ml/min and fractions of 50 ml each were collected by increasing the

methanol content of the eluent from 0 to 100% (v/v) in increments of 10% (100 ml each) followed by 2 x 100 ml fractions of acetone-water (70:30, v/v). The proanthocyanidin content of the fractions was monitored using thin-layer chromatography (TLC) system.[14)]

TLC Chromatographic fractions were analyzed on 20 cm x 20 cm silica gel plates (Merck) using methanol-toluen-acetic acid (3:3:1, v/v/v). The spots were visualized with vanillin (5%) and HCl (10%)[15)] The fraction eluted with 100% methanol showed the highest content of proanthocyanidins. It was used in the experiments and referred as "barbatimão fraction".

***n*-Butanol-HCl hydrolysis** This reaction was performed with a sample of barbatimão fraction (1 mg) in a capped tube containing 5% HCl in *n*-butanol (2 ml). The mixture was heated in a boiling water bath for 1 h. The resulting cyanidin indicated the presence of procyanidin.[14)]

Degradation with Phloroglucinol A sample of barbatimão fraction (2 mg) was mixed with phloroglucinol (2 mg) and 1 ml of 1% concentrated HCl in ethanol was added. After standing for 1 h at ambient temperature the reaction products were examined by HPLC.[14)]

High Performance Liquid Chromatography The HPLC analysis were performed on a Shimadzu high performance liquid chromatography LC-20AD system equipped with a diode array detector, vacuum degasser, quaternary pump, auto-sampler and thermostated column compartment. After filtration on Millipore filter paper (0.22 μm) 20 μl solution (barbatimão fraction after phloroglucinol degradation) were injected on a Shim-pack Shimadzu RP-18 column (4.5 μm pore size, 4.6 mm i.d. x150 mm) and eluted with a flow rate of 0.5 ml/min with column temperature set at 30^{o} C. The following solvents were used: A [acetic acid/water (2/98 v/v)] and B [acetic acid/acetonitrile/water (2/20/78 v/v)]. The gradient applied was 100 to 80% A for 20 min and then 100% B for 60 min followed by washing and reconditioning for 10 min. The products were detected by absorption at 280 nm.[14)]

Identification Gallic acid, (-)-epicatechin-3-*O*-gallate and procyanidin dimer B1 were identified by comparison with authentic standards at 280 nm.

Cell Culture The two human breast cancer cell lines used, ER (+) MCF-7 (CR117) and ER (-) MDA-MB-435 (CR119), were provided by the Rio de Janeiro Cell Bank (Brazil). Cells were grown as monolayers and maintained in RPMI-1640 medium supplemented with 20% heat-inactivated FBS, 2mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, at 37^{o} C in a humidified atmosphere with 5% CO_2.

Cell Treatment Viable cells were counted using Trypan blue excluding method in a hematocytometer. The exponential growing MCF-7 and MDA-MB-435 cells were obtained

by plating 1 $\times 10^5$cells/ml in 96-well microtiter plates, followed by 24 h incubation. After that the cells were treated with six different amounts (5, 10, 20, 40, 80 and 160 µg) of barbatimão fraction. Cyclophosphamide (CP; Sigma) was used as positive control (550 µg for MCF-7; 50 µg and 110 µg for MDA-MB-435 cells). The barbatimão fraction was dissolved in sterilized water, added to cell cultures and the plates were incubated for another 24 or 48 h, at 37^o C.

MTT Assay After cell treatment, 10 µL of MTT (5 mg/ml) were added and cells incubated for further 4 h. Formazan products were solubilized with isopropanol and the absorbance was measured at 570 nm. The percentage of MTT reduction was calculated comparing the absorbance of treated cells to that of untreated control cells.[16)]

Cell Morphologic Analysis Cells growing exponentially in coverslips were treated as described above. After treatment cells were fixed with 4% buffered paraformaldehyde and stained with hematoxylin-eosin. Slides were mounted in Entellan and observed under a light microscope. Typical apoptotic nuclear condensation was used as the morphological marker of apoptosis.[17)]

Determination of Apoptosis by DNA Ladder Measurement The DNA of the cells was isolated using the SDS/Proteinase K/RNase A extraction method. The presence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments that are approximately 180–200 bp multiples and necrosis by the appearance of a smear on the agarose gel.[18)]

Statistical Analysis Data were analyzed using ANOVA with post hoc analysis by Tukey test, when $p<0.05$. Results are expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

The extensive investigations with the proanthocyanidins have identified various molecular targets that can potentially be used for the prevention or treatment of cancers.[7,8)] In the present study we obtained, for the first time, a fraction rich in procyanidin from brabatimão leaves which is cytotoxic for breast carcinoma cells.

The TLC allowed identifying that the fraction with the highest content of proanthocyanidins was isolated with 100% methanol (barbatimão fraction). This fraction was shown to have procyanidin by the production of cyanidin on heating in highly acidic medium. We next reacted the barbatimão fraction with phloroglucinol. This reaction involved cleavage of the interflavonoid bonds of procyanidins to generate carbocations at C-4 of the flavan extender units that were then captured by the nucleophilic phloroglucinol to give flavan-

phloroglucinol addition products and releasing the botton or terminal flavan-3-ol and these products were analyzed by HPLC. The HPLC analysis allowed identifying three major components of the barbatimão fraction: gallic acid (1), procyanidin dimmer B1 [2, epicatechin-(4β→8)-catechin; the main component] and (-)-epicatechin-3-*O*-gallate (3; Fig. 1).

The effect of the barbatimão fraction on cell viability was investigated by the widely used MTT assay (Fig. 2). The barbatimão fraction increased the MTT reducing capacity of MCF-7 and MDA-MB-435 cells in a dose-dependent manner. No significant enhancements in MTT reducing activity were observed in MCF-7 cells after treatments with low concentrations (5 and 10 µg) for 24 and 48 h, but higher concentrations than those increased cell capacity to reduce MTT. In MCF-7 cells, this capacity reached maximum values of 168.07 ± 9.27 % and 192.67 ± 9.57 % for treatment with 40 µg fraction respectively, after 24 and 48 h. In MDA-MB-435 cells no enhancement in MTT reducing activity was observed with 5 µg, but fraction concentrations higher than that increased MTT reducing capacity, as observed for MCF-7 cells. Maximum values of 135.89 ± 13.21 % and 129.98 ± 12.98 % were registered for treatments with 80 µg (24 h) and 40 µg (48 h). These results led us to investigate the cause of this intense formazan production in cells. A direct reduction of MTT by the extract was excluded as the cause of this enhanced formazan production since the barbatimão fraction showed no capacity to reduce this tetrazolium salt at any concentration used (data not shown). However, microscopic observation of cells showed a profound cell morphology change and an intense cytoplasmic vacuolization, mainly in cells treated with 40 µg barbatimão fraction, which was not detected in untreated control cells. The results of the morphological analysis do not correlated with the viability increasing indicated by the MTT assay. Small vacuoles that occupied almost all the cytoplasm and remained uncolored after staining with hematoxilin-eosin were visualized in cells treated with barbatimão fraction and at a lesser extent with cyclophosphamide (CP). Other morphological changes observed after fraction treatment include cell lost of typical morphology, assuming a round (MCF-7 and MDA-MB-435) or a very thin spindle shape (MDA-MB-435), chromatin condensation (mainly pyknosis) and membrane blebbing. It was also observed some swelled cells after 24 h of treatment, which may indicate a perturbation in membrane fluidity, and after 48 h-treatment there was prominently cell shrinkage. The barbatimão fraction used in the present work is composed of flavan-3-ols derivative. It has been reported that a great number of flavan-3-ols and proanthocyanidins with an introduction of a gallic acid function at position 3 have a

significantly increased biological activity, while glycosylation of the position 3 decreases the activity.[7,19,20)]

A 24 h-treatment with 5 µg barbatimão fraction was able to induce the appearance of vacuoles. In spite of the intense intracellular vacuolization, the viability estimated by the MTT reduction of MCF-7 and MDA-MB-435 remained very high (>100%). Based on the knowledge that MTT is also reduced in intracellular vesicles and that these vesicles accumulate the reduced formazan product[21)] we hypothesized that the enhancements of formazan detected after cell treatment, could be due to an accumulation of the MTT reduced product in the vacuoles induced by the compounds present in the barbatimão fraction. To prove this, we selected the highest concentration of the fraction and, after treatment, cells were incubated with MTT for 4 h and immediately observed by light microscopy (Fig. 5). As expected, an intense accumulation of violet-colored formazan within the cytoplasmic vacuoles was observed, which could explain the enhancements in MTT reduction detected.

It is important to point out that untreated MCF-7 and MDA-MB-435 cells are typical adherent cells (Figs. 3 and 4). After treatment with barbatimão fraction almost all the cells had detached from the microplate and were floating, which do not correlated the viability assay. In the CP-treated samples dead floating cells were also observed at a lesser extent than in fraction-treated samples, and the adherent cells seemed to be intact. This observation suggested that the viability assessed using the MTT assay do not corresponded to the morphological features of the fraction-treated cells. Increases of the cellular MTT-reducing activity in the presence of growth inhibition have also been reported by others[17,22,23)] and represent a pitfall in the MTT viability assay. This false negative result would have lead to the conclusion that our fraction did not affect cell growth. The antiproliferative effect of the fraction would have been missed if only the MTT assay was used. Despite the great use of MTT assay, the cellular mechanism of MTT reduction is not yet completely understood. It was demonstrated that MTT can be reduced in intracellular vesicles, many of which were identified as endosomes and lisossomes, with autophagy being suggested as a possible mechanism for the accumulation of the MTT formazan product in these vesicles[21)]. Autophagy is defined as a process in which protein and organelles are degraded by lysosomal proteases.[24,25)]

CP an anticancer drug has been reported to be apoptotic[26,27)] so we next investigated whether typical biochemical apoptotic features appeared as cell death proceeded. We assessed DNA ladder formation using a DNA fragmentation assay (Fig. 6 and 7). When MCF-7 cells were treated with CP, there was little effect, with at most 20% cell death and no DNA ladder

was detected, even after 48 h of treatment. To our surprise, when cells were treated with barbatimão fraction we still did not detect a typical low-molecular-weight DNA-ladder, in spite of the morphological changes observed. The same was obtained for MDA-MB-435. Although DNA-ladder formation is a well-known apoptotic feature, it was not seen in all of the treated cells analyzed in the present study. Taken our results together it seems that morphologically defined apoptotic and non-apoptotic cell death occurred in the MCF-7 and MDA-MB-435 cell samples treated with barbatimão fraction.

It has been reported that MCF-7 cells are deficient of functional caspase-3 owing to a deletion within exon 3 of the *CASP-3* gene and functional caspase-3 is essential to induce internucleossomal DNA laddering formation during apoptosis.[28)] Kugawa *et al.*,[29)] also found the presence of the characteristic apoptotic cell morphology, but no DNA-ladder formation in MCF-7 cells treated with CP. These authors concluded that CP induced apoptosis without its classical biochemical features. However, the MDA-MB-435 cells are not reported to be *CASP-3* gene-defective and did not exhibit DNA fragmentation, which suggests that cell death other than apoptosis could be also occurring.

Morphological, biochemical and molecular observations revealed that active self-destruction of cells is not confined to apoptosis but cells may use different pathways to commit suicide.[24,25)] It was demonstrated that tamoxifen in low doses trigger autophagy of MCF-7 cells[30)] and it also seems the case of the treatment with prenylated flavones.[17)] The findings of the present study suggest that a subfraction of dying cells may undergo autophagic cell death with an apoptotic nuclear morphology. Therefore, in a given cell type several pathways leading to active death may co-exist. Further differences between autophagic cell death and current models of apoptosis include DNA fragmentation. In this study no DNA-laddering formation was observed, suggesting that autophagy might possibly being occurring after barbatimão fraction treatment. In the present study pyknotic nuclei, which constitute the predominant type of nuclear alteration, indicate that relative little DNA cleavage may result in dramatic changes in nuclear and cell morphology and lead cells to commit suicide. A possible perturbation of the cell membrane fluidity could be also induced by the barbatimão fraction-treatment, although necrosis was not clearly observed.

We showed that the enhancements in MTT reduction observed in barbatimão fraction-treated cells were accompanied by an intense vacuolization of the cytoplasm and that the MTT formazan product was accumulated in these cytoplasmic vacuoles. This accumulation could explain the observed increases in MTT reduction. This vacuolization observed in both MCF-7 and MDA-MB-435 cells that could be due to the induction of autophagic vacuoles

added to the high viability of cells, led us to think that we might have encountered compounds with the capacity to induce autophagy of breast adenocarcinoma cells. In view of cancer therapy autophagy could be important when apoptosis is deregulated and its induction could enhance the effects of anti-cancer therapies. Studies are in progress in our laboratory to identify molecularly the type(s) of cell death induced by barbatimão fraction.

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Figure legends

Fig. 1. HPLC-DAD (absorbance, 280 nm) plot of barbatimão fraction phloroglucinol degradation products. Peaks: 1, gallic acid; 2, procyanidin dimer B1; 3, (-)-epicatechin-3-*O*-gallate.

Fig. 2. Effect of barbatimão fraction on the cell viability estimated by the MTT reducing capacity of MCF-7 (A) and MDA-MB-435 (B) cells. Cells were treated with 550 μg cyclophosphamide (CP) and with 5, 10, 20, 40, 80 and 160 μg fraction, for 24 and 48 h. Results are the mean ± SD of three independent experiments.

Fig. 3. Morphological alterations induced by the barbatimão fraction on MCF-7 cells. Light microscopy of stained untreated control MCF-7 cells and treated with 550 μg cyclophosphamide and with 5 and 160 μg fraction, for 24 and 48 h. Scale bar = 1 μm.

Fig. 4. Morphological alterations induced by the barbatimão fraction on MDA-MB-435 cells. Light microscopy of stained untreated control MCF-7 cells and treated with 110 μg cyclophosphamide and with 5 and 160 μg fraction, for 24 and 48 h. Scale bar = 1 μm.

Fig. 5. Accumulation of MTT formazan product in the cytoplasmic vacuoles of MCF-7 (A) and MDA-MB-435 (B) cells induced by barbatimão fraction-treatment for 48 h. Light microscopy. Scale bar = 1 μm.

Fig. 6. Gel Electrophoresis of DNA extracted from MCF-7 cells treated with barbatimão fraction. 1, 100 bp ladder marker; 2, Control; 3, 550 μg cyclophosphamide; fraction: 4, 5 μg; 5, 10 μg; 6, 20 μg; 7, 40 μg; 8, 80 μg; 9, 160 μg. These data are the representative of three independent experiments.

Fig. 7. Gel Electrophoresis of DNA extracted from MDA-MB-435 cells treated with barbatimão fraction. 1, 100 bp ladder marker; 2, Control; cyclophosphamide: 3, 50 μg; 4, 110 μg; fraction: 5, 5 μg; 6, 10 μg; 7, 20 μg; 8, 40 μg; 9, 80 μg; 10, 160 μg. These data are the representative of three independent experiments.

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