UNIVERSIDADE ESTADUAL PAULISTA

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AVALIAÇÃO DOS EFEITOS *IN VITRO* DE ESPÉCIES VEGETAIS COMO POTENCIAIS ATIVOS DESPIGMENTANTES

JÉSSICA ELEONORA PEDROSO SANCHES SILVEIRA

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de PG em Biologia Geral e Aplicada

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Prof. Dra. Vanessa Alves Arruda

“Hay hombres que luchan un dia y son buenos.

Hay otros que luchan un año y son mejores.

Hay quienes luchan muchos años y son muy buenos.

Pero hay los que luchan toda la vida:

esos son los imprescindibles.”

Bertolt Brecht

Dedico este trabalho

À minha família:

Meu pai Hélio, minha mãe Nelma,

Minha irmã Pâmela e meu irmão Hélio César

"Paz e harmonia - esta é a verdadeira riqueza de uma família."

(Benjamin Franklin)

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"Se você deseja um ano de prosperidade, cultive grãos
Se você deseja 10 anos de prosperidade, cultive árvores
Mas se você quer 100 anos de prosperidade, cultive gente"
Ditado Chinês

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"Aprender é a única coisa de que a mente nunca se cansa,
nunca tem medo e nunca se arrepende."
Leonardo da Vinci

Resumo

O acúmulo anormal da melanina em partes específicas do corpo como manchas (melasma, sardas, efélides, manchas senis etc) tem se tornado um problema estético. Em adição, o crescente interesse por terapias complementares gera demanda para tratamentos despigmentantes, utilizando-se de fontes naturais, especialmente de origem vegetal, considerados seguros e eficazes. Na melanogênese estão envolvidos uma série de mediadores, dentre os quais se destacam as citocinas; como a interleucina 1 alfa (IL-1 α) e o fator de necrose tumoral alfa (TNF-α); o hormônio melanócito estimulante alfa (α-MSH) e a tirosinase. Neste estudo, o efeito de 12 extratos vegetais foi investigado a fim de se encontrar agentes com potencial ação e uso como despigmentante. Como triagem, os métodos de lipoperoxidação e DPPH foram adotados para avaliar o potencial antioxidante e o ensaio anti-tirosinase *in vitro* foi importante na seleção das plantas para a avaliação em cultura de células, na qual os extratos de *Rheum rhaponticum* L. e *Coccoloba uvifera* L., ambas da família Polygonaceae, foram escolhidos. Os melanócitos em cultura celular foram submetidos ou não à radiação ultravioleta e estudou-se a atividade da tirosinase e a produção de IL-1 α, TNF-α e α-MSH nestas condições. Ambos os extratos escolhidos apresentaram efeitos inibidores da atividade da tirosinase, tanto nos grupos expostos ou não à radiação solar, diminuição da produção do hormônio α-MSH, nas maiores concentrações estudadas nos grupos também expostos ou não à radiação ultravioleta, diminuição da produção de IL-1α em células expostas ou não à luz solar e também diminuição da produção de TNF-α pelos melanócitos expostos à radiação ultravioleta. Os extratos estudados mostraram-se eficazes como fortes candidatos para aplicação cosmética como agentes clareadores da pele.

Abstract

The accumulation of an abnormal melanin amount in different specific parts of the skin as more pigmented patches (melasma, freckles, ephelide, senile lentigines etc.) might become an esthetic problem yet. Moreover, a move towards complementary therapies created a demand for a natural, safe, and efficacious depigmenting treatment, particularly from plant source. Melanogenesis is under control of several endogenous mediators, among them are cytokines; such as interleukin 1 alpha (IL-1α) and Tumor Necrosis Factor alpha (TNF-α); the melanocyte stimulating hormone alpha (α-MSH) and tyrosinase. In this study, the effect of 12 plant extracts was investigated in order to find potential depigmentating agents. As a screening, lipoperoxidation and DPPH method were adopted to evaluate the antioxidant potential and the tyrosinase *in vitro* assay was important in plant selection for cell culture assessment, in which *Rheum rhaponticum* L. and *Coccoloba uvifera* L., plants from Polygonaceae family, were chosen. Melanocytes in cell culture were submmited or not under solar radiation and the tyrosinase activity and the production of IL-1 α, TNF-α and α-MSH under these conditions were studied. Both of the chosen extracts showed inhibitory effects of tyrosinase activity, in the groups that were or not exposed to UV radiation, decrease of α-MSH production, regarding the highest concentration of the exposed and non-exposed to UV radiation groups, decrease of IL-1α production in the cells which were or not submitted to solar light and also decrease of TNF-α production by the melanocytes which were exposed to UV. The extracts showed their effectiveness as strong candidates for cosmetic application as whitening agents.

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1. Introdução

O sistema de pigmentação da pele humana está baseado em dois tipos celulares, melanócitos e queratinócitos, os quais interagem como uma unidade funcional denominada unidade melanina-epidermal (Fitzpatrick e cols., 1979), cuja atividade funcional é o fator determinante da coloração da pele (Romero-Graillet e cols., 1997).

Nos mamíferos, a melanina é responsável pela coloração da pele, cabelos e olhos, sendo que na espécie humana, possui papel fundamental na pigmentação da pele e dos cabelos. A melanina é produzida por um processo fisiológico denominado melanogênese, tendo a função de proteção da pele dos prejuízos induzidos pela radiação solar via absorção da luz ultravioleta e remoção das espécies reativas de oxigênio (Nerya e cols., 2003).

A melanogênese é um processo fisiológico resultante da síntese dos pigmentos de melanina, os quais têm importante função protetora contra a fotocarcinogênese cutânea (Baurin e cols, 2002).

Nos humanos, o estímulo da pigmentação cutânea sobre o nível constitutivo basal, comumente chamado de bronzeado, é fisiologicamente estimulado pela radiação ultravioleta (UV) da luz solar. O escurecimento da pele induzido pela luz solar envolve um aumento do número de melanócitos, assim como um estímulo na síntese da melanina e da transferência de melanina dos melanócitos aos queratinócitos (Buscá e cols., 2000).

Distúrbios na quantidade e distribuição da melanina podem causar uma série de doenças relacionadas à hipopigmentação ou à hiperpigmentação. O acúmulo anormal de melanina é responsável por diversos processos de hiperpigmentação como melasma, sardas e melanomas malignos, além de representar, para a espécie humana, um sério problema estético, o qual gera uma enorme demanda de produtos cosméticos.

O principal estímulo para a pigmentação da pele *in vivo* é a radiação ultravioleta, a qual aumenta a melanização dos melanócitos (Rosen e cols., 1987). Para estimular a melanogênese, a radiação ultravioleta pode agir diretamente sobre os melanócitos ou indiretamente por meio da liberação de fatores derivados dos queratinócitos ou de outras células que circundam os melanócitos (Friedmann & Gilchrest, 1987; Bos & Kapsenberg, 1993; Gilchrest e cols., 1996), tais como fator de crescimento dos fibroblastos, endotelina 1

(ET-1), hormônio adrenocorticotrópico (ACTH), hormônio melanócito estimulante alfa (α-MSH), prostaglandinas, histamina e óxido nítrico (NO).

Os melanócitos são células da pele especializadas cuja origem embrionária se dá na crista neural, os quais são responsáveis pela produção de melanina. A síntese da melanina ocorre em organelas intracelulares especializadas denominadas melanossomos (Buscá e cols., 2000), os quais contêm enzimas específicas necessárias à produção deste pigmento. Entre elas, as mais bem caracterizadas são tirosinase, proteína relacionada a tirosinase 1 e dopacromo tautomerase (DCT). A tirosinase catalisa as duas reações da formação de melanina, a hidroxilação da tirosina, resultando em 3,4-dihidroxifenilalanina (DOPA) e a oxidação da DOPA em Dopaquinona. Nos mamíferos, dois tipos principais de melaninas são produzidos, as eumelaninas e as feomelaninas.

As eumelaninas são polímeros de alto peso molecular com complexa estrutura química e possuem coloração marrom à preta. Estão presentes nos seguintes grupos étnicos: caucasianos brancos, mongolóides e negros.

As feomelaninas possuem estrutura química semelhante às eumelaninas e sua coloração vai do amarelo ao vermelho, estando presente nos caucasianos brancos (Barel, 2003).

Dentre os fatores secretores dos queratinócitos, os quais induzem a formação dos melanócitos, estão as prostaglandinas E2, α-MSH (Hormônio Melanócito Estimulante alfa), ACTH (hormônio adrenocorticotrófico), endotelina-1 e NO (óxido nítrico). O α-MSH, o ACTH e a PGE2 ativam a via dos melanócitos, enquanto que o NO ativa os eventos de sinalização dependentes do GMP (Guanosina monofosfato) cíclico (Buscá e cols, 2000).

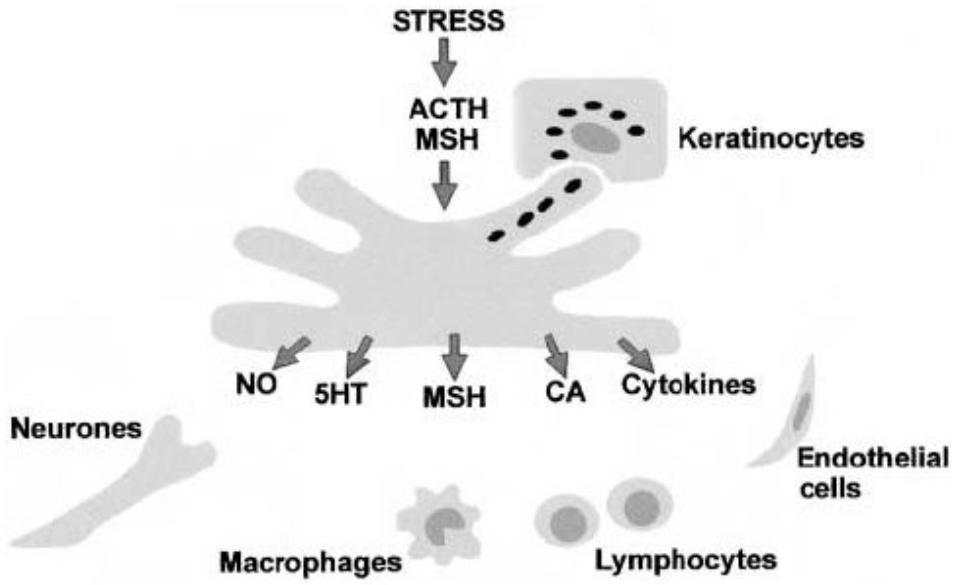

Figura 1 – A melanogênese é resultante de vários processos reguladores, envolvendo os efeitos diretos da radiação UV sobre os melanócitos e efeitos indiretos através da liberação de fatores derivados dos queratinócitos. Os melanócitos produzem substâncias, tais como as citocinas e α-MSH (hormônio melanócito estimulante alfa), com a finalidade de regular diversos tipos celulares na epiderme em resposta ao estresse ambiental (Tsatmali e cols; 2002).

O α-MSH é produzido, em conjunto com vários outros peptídeos, por clivagem proteolítica de um grande precursor, a pro-opiomelanocortina (POMC). O principal sítio de produção deste hormônio é a hipófise, entretanto, também ocorre síntese em locais extrahipofisários incluindo a pele (Thody e cols; 1983 e Wakamatsu e cols; 1997). Considerando que os queratinócitos epidermais são as maiores fontes destes peptídeos, a radiação UV é responsável por elevar os níveis de α-MSH na pele e a resposta dos melanócitos a este peptídeo (Tsatmali e cols; 2002).

O estímulo dos melanócitos com α-MSH aumenta a eumelanogênese em maior grau que a feomelanogênese, o que contribui para a pigmentação cutânea (Tsamali e cols; 2002). O estímulo dos melanócitos ocorre via receptor da melanocortina (MC1R), sendo que mutações com perda de função do gene MCR1 afeta os melanócitos humanos na proteção

contra os danos do DNA causado pela radiação UV, o que pode ocasionar câncer de pele (Lassalle; 2003).

A POMC é uma proteína precursora que além de ser produzida em alguns tecidos não-hipofisários, é também originada nos queratinócitos. Quando produzida em um nível cutâneo, os peptídeos derivados da POMC (exemplo ACTH, α-MSH e endotelina-1) têm função importante na fisiologia da pele, incluindo imunomodulação e respostas locais ao estresse, sendo que as diferentes células da pele podem expressar de diferentes maneiras os genes da POMC (Mazurkiewicz e cols; 2000).

Nos melanócitos há produção de uma série de citocinas e fatores de crescimento, aos quais eles também reagem e, portanto podem ser considerados tipos celulares imunocompetentes com o potencial de modular sua resposta frente a diferentes condições. Estudos demonstram que os melanócitos tanto podem agir no estímulo da produção como responder ao estímulo de IL-1α (interleucina 1 alfa) e IL-6 (interleucina 6), visto que a exposição à radiação ultravioleta estimula a síntese de IL-1α (Tsamali e cols; 2002).

A exposição do tecido cutâneo ao estresse (radiação ultravioleta) aumenta a produção do hormônio α-MSH, juntamente com a liberação de citocinas proinflamatórias (TNF-α e IL-1α), as quais normalmente regulam a expressão de moléculas de adesão de células às células adjacentes. O TNF-α (fator de necrose tumoral alfa) está envolvido em várias reações imunes celulares e inflamatórias através da ativação do receptor correspondente, tendo sua atividade modulada por vários estímulos cutâneos, no qual o mais importante é a radiação ultravioleta (Slominski e cols.; 2004).

Enquanto o comportamento dos melanócitos é influenciado por mediadores inflamatórios, as células adjacentes contribuem para o processo através da ativação de citocinas próinflamatórias como a IL-1α e o TNF-α. A molécula de adesão intercelular 1 (ICAM-1), um importante regulador das interações célula-alvo imunes, é normalmente expressa em níveis muito baixos em culturas normais de melanócitos. Estes níveis podem ter aumento significativo de maneira dose-dependente com elevação dos níveis de TNF-α e IL-1 α (Lee e cols.; 2002).

A tirosinase catalisa duas distintas reações de conversão da tirosina em Dopa (atividade tirosina hidroxilase) e a oxidação da Dopa resultante em dopaquinona (atividade dopa oxidase). A enzima oxida fenóis e difenóis usando um mecanismo catalítico que

depende da presença de cobre no sítio ativo. A partir da produção da dopaquinona, uma série de reações enzimáticas e não-enzimáticas ocorrem para produzir compostos dihidroxiindólicos. Os pigmentos de melanina, eumelanina e feomelanina, são produzidos por oxidação e polimerização destes compostos indólicos (Mayer; 1987; Sánchez-Ferrer e cols.; 1995; Van Gelder e cols.; 1997; Ito e cols.; 2000; Ito; 2003). No Sistema Nervoso Central esta enzima participa da síntese de dopamina que é precursora direta de outras catecolaminas importantes como a noradrenalina e adrenalina, sendo recentemente implicada em inúmeras doenças neurodegenerativas, especialmente a Doença de Parkinson (Asanuma e cols.; 2003).

A tirosinase (oxidase polifenólica EC 1.14.18.1) possui o papel primordial de participar da biossíntese da melanina e tem recebido especial atenção nos últimos anos como uma ferramenta indispensável para o desenvolvimento de uma grande variedade de pesquisas e estudos (Seo e cols.; 2003), visto o grande espectro de uso e potencialidades dos agentes inibidores e indutores desta enzima para as indústrias farmacêutica, cosmética e de alimentos. Esta enzima possui uma ampla distribuição nos animais, insetos, vegetais e fungos, onde ocorre em várias isoformas (Yokochi e cols.; 2003 e Jaenicke & Decker; 2003).

A tirosinase é a enzima que desencadeia a melanogênese, iniciando a cascata de reações que converte tirosina ao biopolímero melanina. Duas proteínas relacionadas à tirosinase (TRPs) são conhecidas, TRP-1, a mais abundante glicoproteína dos melanócitos, e a TRP-2. As TRPs possuem funções catalíticas e reguladoras na melanogênese (Seiberg e cols.; 2000). A produção de TRP-1 possui função reguladora na desestabilização da tirosinase, levando à diminuição da produção de melanina (Seiberg e cols.; 2000).

Na epiderme humana, diversos mecanismos enzimáticos e não-enzimáticos estão disponíveis para controlar o acúmulo de espécies reativas de oxigênio, sendo a tirosinase um dos mais importantes sistemas para a eliminação destas espécies, já que esta enzima é capaz de utilizar superóxido para produzir melanina (Perluigi e cols.; 2003; Friedmann & Gilchrest; 1987), de modo que compostos antioxidantes, capazes de inibir a produção de espécies reativas de oxigênio podem reduzir a hiperpigmentação ou prevenir a melanogênese.

Assim sendo, o desevolvimento de agentes clareadores da pele no tratamento de hiperpigmentação induzida pela luz ultravioleta ou por condições médicas como melasma e melanodermia pós-inflamatória é uma importante área de pesquisa (Choi e cols.; 2005). A atividade biológica de várias plantas tem sido estudada com o propósito de uso em cosméticos (Mizuno & Tanaka; 1986). Além disso, fontes como plantas têm sido avaliadas para o desenvolvimento de antioxidantes naturais que podem estar envolvidos com produtos para cuidados anti-idade e anti-rugas (Anes & Saul; 1987). Muitos componentes endógenos de plantas foram relatados como agentes que retardam o processo de oxidação no seu ambiente natural e em produtos nos quais estes compostos foram adicionados (Pratt; 1994).

2. Objetivos

A melanina tem importante função na proteção da pele contra diversos efeitos prejudiciais da radiação solar ultravioleta, entretanto, o seu acúmulo anormal gera um problema estético. Os agentes tópicos disponíveis para o tratamento da hiperpigmentação incluem os inibidores da tirosinase, retinóides, hidroquinonas e agentes citotóxicos aos melanócitos. Infelizmente, os resultados destes tratamentos, às vezes, não são satisfatórios, e se faz necessário buscar terapias mais eficazes, mais seguras e menos irritantes. Em adição, o crescente interesse por terapias complementares gera demanda para tratamentos despigmentantes, utilizando-se de fontes naturais, especialmente de origem vegetal, considerados seguros e eficazes.

Dentre os mediadores responsáveis pela formação da melanina estão as citocinas como a interleucina 1 alfa (IL-1 α) e o fator de necrose tumoral alfa (TNF-α), o hormônio melanócito estimulante alfa (α-MSH) e a tirosinase. Além das formações de espécies reativas de oxigênio que contribuem para este processo.

Considerando as informações apresentadas, o presente estudo visou investigar extratos vegetais como potenciais ativos despigmentantes, especialmente através dos efeitos de inibição da enzima tirosinase e antioxidante *in vitro*, pelo método DPPH e de peroxidação lipídica e também selecionar as espécies vegetais mais promissoras nestes estudos iniciais para se avaliar seus efeitos sobre a atividade da tirosinase e a produção do hormônio melanócito estimulante alfa, da interleucina 1 alfa e do fator de necrose tumoral alfa em culturas celulares (melanócitos), comparando grupos celulares expostos e não expostos à radiação ultravioleta.

3. Triagem

Com o objetivo de selecionar plantas com potencialidades de atividade biológica de interesse para o projeto, foi realizada revisão bibliográfica com base em estudos etnofarmacológicos (priorizando-se espécies utilizadas tópica e popularmente para problemas de pele) e quimiotaxonômicos (considerando-se espécies vegetais que possuem em sua composição grupos químicos com atividade sobre a pigmentação como estilbenos, hidroquinonas ou espécies de gêneros botânicos já descritos na literatura como agentes despigmentantes, especialmente inibidores da tirosinase). A partir desta revisão foram selecionadas 12 espécies vegetais (Tabela 1) para a realização do presente estudo. Esta seleção obedeceu dois critérios: qualidade das informações etnofarmacológicas e quimiotaxonômicas e disponibilidade de obtenção do material vegetal.

As plantas foram adquiridas através da empresa Chemyunion Química Ltda. dos fornecedores Quimer (*Coccoloba uvifera* L., *Eugenia crenata* Vell., *Eugenia uniflora* L., *Myrcia sphaerocarpa* DC, *Polygala senega* L., *Polygonum acre* H. B. & K., *Rheum officinale* Baill., *Rheum rhaponticum* L., *Syzigyum jambolanum* DC e *Vitex agnus-castus* L.) e Santosflora (*Jacaranda caroba* Hort. e *Tabebuia avellanedae* Lorentz), os quais são responsáveis pela identificação taxonômica e depósito de exsicata.

O método de extração utilizado para todas as plantas citadas acima também foi realizado conforme metodologia utilizada pelo Departamento de Pesquisa e Desenvolvimento da Chemyunion Química Ltda, usando-se extração metanólica a quente por 4h sob agitação e refluxo, seguida de filtração e concentração do extrato em rotaevaporador. Todos os extratos obtidos foram armazenados em vidro âmbar.

Na etapa de triagem das espécies vegetais foram realizados testes *in vitro* para determinação da atividade antioxidante: método DPPH, 1,1-difenil-2-picrilhidrazina, (Blois, 1958 e Brand-Williams e cols.; 1995), e método de peroxidação lipídica (Stocks e cols.; 1974 e Fee & Tietelbaun; 1972) e da atividade antitirosinase: método antitirosinase *in vitro* conforme descrito por Kim e cols.; 2003.

Tabela 1 – Plantas escolhidas para a avaliação do efeito despigmentante.

Nome científico/família	**Nome popular**	**Partes utilizadas**
Coccoloba uvifera L. (Polygonaceae)	Uva de mato	Raiz
Eugenia crenata Vell. (Myrtaceae)	Cambuí	Folha e talo
Eugenia uniflora L. (Myrtaceae)	Pitanga	Folha e talo
Jacaranda caroba Lem. (Bignoniaceae)	Carobinha	Folíolos
Myrcia sphaerocarpa DC (Myrtaceae)	Pedra-ume-caá	Folha
Polygala senega L. (Polygalaceae)	Polígala	Raiz
Polygonum acre H.B. & K. (Polygonaceae)	Erva de bicho	Talo e folhas
Rheum officinale Baill. (Polygonaceae)	Ruibarbo chinês	Rizoma
Rheum rhaponticum L. (Polygonaceae)	Ruibarbo europeu	Rizoma
Syzygium jambolanum DC (Myrtaceae)	Jambolão	Talo e folhas
Tabebuia avellanedae Lorentz (Bignoniaceae)	Ipê roxo	Folíolos
Vitex agnus-castus L. (Lamiaceae)	Agno casto	Fruto

2.1. Método do DPPH

O método DPPH foi realizado para confirmar o potencial das plantas escolhidas. As ações dos extratos foram analisadas quanto ao potencial das amostras captarem o radical

livre DPPH (1,1-difenil-2-picrilhidrazina). Os compostos testes foram preparados através de uma diluição seriada nas concentrações de $1,9x10^{-4}$ a 100 mg/mL (metanol).

100µL de cada diluição foram adicionados em duplicata à placa de 96 poços. A seguir, 50 µL de uma solução metanólica de DPPH 0,4mM foi adicionada por poço. Após 5 minutos de incubação à temperatura ambiente, ao abrigo da luz, a redução do radical livre DPPH foi mensurada pela leitura da absorbância em 517 nm, contra um branco específico (metanol) em cada avaliação, formado somente pelas amostras nas suas respectivas diluições.

Como controle foi utilizado 50µL de solução metanólica de DPPH mais 100µL de metanol. Os resultados foram expressos em porcentagem de inibição do radical DPPH, calculado segundo a equação:

% de Inibição = [(Absorbância do controle - absorbância da amostra)/absorbância do controle] X 100

A determinação da IC_{50}, ou seja, concentração da amostra que causa 50% de inibição da concentração inicial de DPPH foi obtida por regressão linear dos pontos plotados graficamente. Para a plotagem dos pontos, foram utilizados os valores das médias obtidas de triplicatas realizadas para cada um dos testes (Anexo 1).

Os dados obtidos com os compostos foram comparados com Trolox, um tocoferol usado como controle positivo na avaliação da atividade antioxidante (Friaa & Brault, 2006).

2.2. Peroxidação lipídica

A avaliação da atividade antioxidante foi realizada através do método de peroxidação lipídica a qual se baseia no princípio de que o ácido tricloroacético precipita as proteínas das amostras de cérebro (de ratos Wistar machos, cujas cabeças foram doadas pelos alunos do Laboratório de Fitomedicamentos do Instituto de Biociências da UNESP, campus de Rubião Junior em Botucatu que estudam colite), enquanto que o reativo tiobarbitúrico reage com o malonildialdeído (MDA), liberado pela lipoperoxidação causada pelo ferro e ácido ascórbico, gerando uma cor cuja intensidade é determinada no espectrofotômetro. Quanto maior a intensidade da cor, maior a concentração de MDA. Um

composto será antioxidante se inibir a lipoperoxidação causada pelo ferro e ácido ascórbico, reduzindo os valores de MDA. Os compostos testes foram preparados através de uma diluição seriada nas concentrações de 6,25 a 400mg/mL (DMSO 20%).

As amostras teste foram preparadas em eppendorf de 2mL, onde se adicionou 50 µL da solução de Ferro-Ácido Ascórbico 100 µM, 1mL de membrana de cérebro diluída, 50 µL do composto teste, reativo tiobarbitúrico (Sigma T-5500), sob agitação e banho-maria em tempos determinados. Após centrifugação, o sobrenadante foi colocado em duplicata em microplaca de 96 poços. A leitura foi realizada a 532nm (espectrofotômetro Bio Tek^{TM} Power Wave 340), contra brancos específicos e os respectivos controles.

A concentração inibitória 50% (IC_{50}) foi determinada com base na fórmula 1-ABS teste/ABS controle máximo através de regressão linear dos pontos plotados graficamente.

Os dados obtidos com os compostos foram comparados com a Quercetina, usada como controle positivo na avaliação da atividade antioxidante, e com o Ácido Kójico, agente com ação clareadora, usado em preparações cosméticas no tratamento tópico de discromias.

2.3. Atividade antitirosinase

Para a avaliação da atividade antitirosinase, os compostos testes foram preparados através de uma diluição seriada nas concentrações de 50 a 3200 mg/mL (DMSO 20%).

A tirosinase é um polifenol oxidase, sendo um heterotetrâmero constituído por 2 cadeias leves e 2 cadeias pesadas, os quais, por sua vez, formam 3 domínios. Possui atividades importantes como de monofenalase e difenalase, conforme o substrato no qual atue. Quando age na tirosina, apresenta atividade de monofenalase, produzindo DOPA; quando age na DOPA, apresenta atividade de difenalase, formando Dopaquinona. A Dopaquinona, numa seqüência de outras reações forma dopacromo, que pode ser lido a 492 nm no espectrofotômetro.

60µL de cada diluição foram adicionados em duplicata à placa de 96 poços. A seguir, 30 µL de enzima tirosinase foi adicionada por poço. Após 5 minutos de incubação à temperatura ambiente, adicionou-se 80µL de substrato L-tirosina, a atividade antitirosinase

foi mensurada através de 10 leituras cinéticas da absorbância em 492 nm, contra um branco específico (DMSO 20%) em cada avaliação, formado somente pelas amostras nas suas respectivas diluições e os valores foram expressos em concentração (de dopacromo).

Como controle foi utilizado ácido kójico, preparado da mesma forma que as amostras teste. Os resultados foram determinados com base na fórmula 1-Concentração teste/Concentração do controle máximo através de regressão linear dos pontos plotados graficamente.

4. Resultados preliminares da triagem e seleção de espécies para estudos

No método de DPPH, todas as plantas apresentaram algum potencial antioxidante através da captação de radicais livres nas concentrações mais altas estudadas, sendo que os extratos de *Eugenia uniflora* L., *Eugenia crenata* Vell., *Coccoloba uvifera* L., *Syzigyum jambolanum* DC, *Rheum rhaponticum* L. e *Rheum officinale* Baill. mostraram-se mais potentes que os demais (Tabela 2 e Anexos).

No método de peroxidação lipídica, os extratos que apresentaram melhor atividade antioxidante foram os mesmos que mostraram-se potentes na avaliação realizada pelo método de captação de radical livre: *Rheum officinale* Baill., *Eugenia crenata* Vell., *Syzigym jambolanum* DC, *Rheum rhaponticum* L, *Eugenia uniflora* L. e *Coccoloba uvifera* L (Anexos).

Os resultados dos efeitos dos extratos vegetais sobre a atividade da tirosinase (Tabela 3 e Anexos) foram essenciais para a seleção das espécies vegetais para os estudos posteriores, visto que as espécies mais potentes na inibição da atividade da tirosinase foram *Rheum rhaponticum* L. e *Coccoloba uvifera* L. Estes resultados em combinação com aqueles descritos para a atividade antioxidante permitiram a escolha destas espécies para os estudos de seus efeitos sobre as culturas de melanócitos estimuladas com irradiação ultravioleta, cujos resultados se encontram nos manuscritos apresentados na próxima seção.

Tabela 2 - Concentração inibitória 50% da atividade antioxidante

Amostras estudadas	**Concentração inibitória 50% (IC_{50})**
Quercetina	0,53
Rheum officinale Baill. (Polygonaceae)	5,93
Eugenia crenata Vell. (Myrtaceae)	7,17
Syzigyum jambolanum DC (Myrtaceae)	8,70
Rheum rhaponticum L. (Polygonaceae)	8,92
Eugenia uniflora L. (Myrtaceae)	9,24
Coccoloba uvifera L. (Polygonaceae)	9,84
Jacaranda caroba Hort. (Bignoniaceae)	13,63
Myrcia sphaerocarpa DC (Myrtaceae)	18,95
Polygonum acre H.B. & K. (Polygonaceae)	23,50
Vitex agnus-castus L. (Lamiaceae)	51,85
Ácido kójico	103,18
Tabebuia avellanedae Lorentz (Bignoniaceae)	108,34
Polygala senega L. (Polygalaceae)	270,44

Tabela 3 – Concentração inibitória 50% da atividade antitirosinase

Amostras estudadas	**Concentração inibitória 50% (IC_{50})**
Ácido kójico	0,02
Rheum rhaponticum L. (Polygonaceae)	0,06
Coccoloba uvifera L. (Polygonaceae)	0,09
Eugenia crenata Vell. (Myrtaceae)	0,89
Rheum officinale Baill. (Polygonaceae)	2,18
Syzigyum jambolanum DC (Myrtaceae)	2,42
Eugenia uniflora L. (Myrtaceae)	3,45
Polygonum acre H.B. & K. (Polygonaceae)	12,88
Myrcia sphaerocarpa DC (Myrtaceae)	41,86
Tabebuia avellanedae Lorentz (Bignoniaceae)	107,97
Vitex agnus-castus L. (Lamiaceae)	173,12
Polygala senega L. (Polygalaceae)	$1,42 \times 10^{6}$
Jacaranda caroba Hort. (Bignoniaceae)	$2,46 \times 10^{173}$

5. Artigo científico sobre *Rheum rhaponticum* L.

O artigo foi escrito de acordo com as normas de publicação da Revista Científica *Experimental Dermatology* e submetido em dezembro de 2007.

A methanolic *Rheum rhaponticum* L. root extract inhibits IL-1, TNF-α and α-MSH production and tyrosinase activity in melanocyte submitted to solar-simulated radiation

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Abstract

Pigmentation of human skin is closely involved in protection against environmental stresses, in particular exposure to ultraviolet (UV) radiation. However, the accumulation of melanin in the skin can cause some problems as melasma, solar lentigines and actinic keratoses. Ultraviolet radiation exposure can generate the production of many mediators that stimulate the melanin synthesis, contributing for the pigmentation increasing. Among these mediators, IL-1 (Interleukin 1), TNF-α (tumor necrosis factor alpha), α-MSH (melanocyte stimulating hormone alpha) and the tyrosinase are important factors related to skin pigmentation. In this research, we have examined the photoprotective effect of the methanolic *Rheum rhaponticum* L. root extract (RRE) in melanocytes using a solar simulator as the source of UV radiation (SSR). We found that RRE has a protective effect against SSR-induced damage, resulting in the decrease of IL-1 and TNF-α levels. In addition, the tyrosinase activity and α-MSH production were also normalized, contributing for the maintenance of the cell homeostasis.

Key words: *Rheum rhaponticum* L., cytokines, α-MSH, tyrosinase activity.

Introduction

Melanin pigmentation of the skin in mammals was shown to result from the close interaction between the epidermal melanocytes and the keratinocytes [(1)]. Melanocytes are key components of the skin pigmentary system through their ability to produce melanin. These cells are found at many locations throughout the body. In the skin they are associated with the hair follicle and in some mammals, including humans, are also found in the basal layer of the interfollicular epidermis. Mature melanocytes form long dendritic processes that ramify among the neighboring keratinocytes. In this way, each melanocyte makes contact with around 30–40 keratinocytes and this constitutes the epidermal–melanin unit. This association enables the melanocyte to transfer melanin into the keratinocytes, where it determines skin color and helps in protecting against the damaging effects of UVR[(2)].

In human epidermis, the process of UV stimulation is composed of three major steps; the first step is the proliferation of melanocytes, followed by the synthesis and activation of tyrosinase and finally the transfer of melanosomes to keratinocytes. During the first two steps, a complex network exists in the epidermis for secreting and responding to autocrine and paracrine cytokines by keratinocytes and melanocytes. Corresponding receptors, which are also regulated in their expression by various cytokines, participate in the complex process in which there is cross-talk in signaling between cytokines to support the enhanced proliferation of melanocytes. These paracrine mediators include α-melanocyte-stimulating hormone among others. The secretion of α-melanocyte-stimulating hormone has been reported to be triggered by primary inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α, which are released by UVB-exposed cells [(3)].

Melanogenesis is target for a variety of hormones and other biological modifiers. UV light stimulates the secretion of alpha-melanocyte stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH), prostaglandin E2, endothelin-1 (ET-1) and nitric oxide, which induces melanogenesis process [4; 5]. On the other hand, cytokines, such interleukin-1 (IL-1), IL-6, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and transforming growth factor-beta (TGF-β) are produced by melanocytes and keratinocytes to compensate the exacerbated production of melanin, mainly by the decrease of expression and release of tyrosinase [6; 7; 8; 9].

Rheum rhaponticum L. (Polygonaceae family) commonly known as Sibiric Rhubarb is a plant originates from Central Asia. This plant, in the 17th century was introduced into Europe and has been cutivated since then in Europe, United States, East Asia and Latin America [10]. The standardized extract from roots of this plant consists mainly of rhaponticin and desoryrhaponticin and small amounts of the aglycones *trans*-rhapontigenin and desoxythapontigenin, secondary metabolites have a hydroxystilbene backbone and are structurally related to resveratrol[11]. Indeed, after the administration of *Rheum rhaponticum* L. extract in rats, two resveratrol (piceatannol and rhapontigenin glucosides), as well as resveratrol, rhapontigenin and other stilbenes derivatives were identified as mean metabolites[12]. Hydroxystilbenes are plant polyphenols exerting a number of health-promoting effects, including antioxidant activity[13] and inhibition of the tyrosinase activity [14, 15], while resveratrol is a potent natural metabolites useful as whitening agent [16,17].

Many plant extracts and their active principles have been described and utilized as cosmetic agents, in particular, as skin lightening products. The growing demand for depigmenting agents is being perceived, and effective compounds from natural sources, acting as tyrosinase inhibitors or blocking others melanogenic pathways, have been reported [15, 18, 19, 20].

In this way, we evaluated the effects of a dry extract of the root of *Rheum rhaponticum* L. (RRE) in human melanocytes culture under solar-simulated radiation. Considering the down-regulating activity of some cytokines on melanogenesis process, we first evaluated the effects of RRE on the release of IL-1α and TNF-α in cell supernatants. We found that RRE decrease IL-1α and TNF-α levels submitted to UV radiation. Our data also demonstrate a decrease in the levels of tyrosinase. Additional studies were performed in order to reveal the possible effects of RRE on α-MSH production after UV radiation and, corroborating the hypopigmenting effect of RRE, the levels of these hormones were found reduced after cell treatment.

Materials and Methods

Plant Material

Dry extract of *Rheum rhaponticum* L. (RRE) was manufactured and provided by Chemyunion Química Ltda (Sorocaba, SP, Brazil). Crude plants (root), obtained for Brazilian suitable suppliers, were sliced into small pieces and extracted with methanol. The extract was concentrated in a rotary evaporator (Buchi RE 111 Buchi Laboratoriums-Tecnick AG,Flawil, Switzerland) in order to remove the solvent. The dry extract obtained was dissolved in dimethyl sulfoxide 20% (DMSO) for the assessment of antioxidant and antityrosinase activities. For the determination of radical scavenging activity, the solvent used was methanol. In addition, for evaluation of the melanocyte cultures, the extract was dissolved in culture medium and diluted into appropriate concentrations immediately before use. The final concentration of the dried extract was 31%.

Antioxidant activity (*In vitro* Lipid peroxidation)

Trichloroacetic acid precipitates proteins of rats brain while the tiobarbituric reactive reacts with malonyldialdehyde (MDA), released by the lipoperoxidation caused by iron and ascorbic acid, producing a color whose intensity is determinated by spectrophotometer. The increase of intensity is a result of the increase of MDA concentration. An element will be considered antioxidant if it is capable to inhibit the lipoperoxidation caused by the reagents, reducing the MDA values. The test elements were prepared through a serial dilution in 400 to 6,25 mg/mL doses (DMSO 20%).

For the test samples, it was added 50 μL of Iron-Ascorbic Acid 100 μM solution, 1 mL of brain homogenate, 50 μL of the plant extract and tiobarbituric. After centrifugation, the supernatant was paced in duplicate in 96-well microplate. The measurements were performed in spectrometer (Bio Tek™ Power Wave 340). Lipid peroxide inhibitory activity was expressed as IC_{50}.

In vitro Tyrosinase Assay

Antityrosinase effect was assessed through enzymatic assay. Potassium phosphate buffer (50mM) at pH 6.5, tyrosinase (333 units/mL) and test compounds dissolved in DMSO 20% were inserted into 96-well plates. After 5 min at room temperature, L-tyrosine was added.

The diphenolase activity of tyrosinase was measured spectrophotometrically by following the increase in absorbance at 475nm by production of dopachrome from L-DOPA. The reaction was done at 37° C.

Cell culture and treatment protocol

Human epidermal melanocytes (Cryopreserved HEM, Cat. 104-05n), melanocyte growth medium (Cat. 135-500) and trypsin/EDTA solution (Subculture Reagent Kit, Cat. 090K) were purchased from Cell Applications, Inc™ San Diego, CA. Cells were subcultured in 25 cm^2 flasks (Corning Inc, New York, NY) at 37°C in 5% CO_2 humidified incubator, and expanded for at least five passages. The medium was changed twice a week. At 80-90% confluent, cells were trypsinized and seeded into 24- well culture plates (Nunc, Roskilde, DM). Twenty-four hours after melanocytes seeding, cells were washed with PBS and irradiated with ultraviolet radiation in a dose of 300mJ/cm^2(Seiberg et al, 2000). Immediately after irradiation, cells were incubated with doses of 4.9, 2.4, 1.2 and 0.6 mg/mL of RRE (*Rheum rhaponticum* L. extract) dissolved in culture medium. After 48 h of treatment, cell-free supernatants and cell lysate were collected and assays were performed using commercial kits. Each experiment was conducted in triplicate of three independent experiments. Selection of these doses was based on previous results of cytotoxicity assays (data not shown).

Ultraviolet radiation

A Multiport solar UVA and UVB simulator (Model 601; Solar Light Co., Philadelphia, PA) was used for the UV irradiation. The simulator was equipped with a 150W Xenon lamp, with a liquid filter and 1-mm Schott WG 320 filter, emitting a continuous spectrum of radiation beginning at 290 nm through the infrared spectrum and maximally peaking at 400 nm. The lamp was housed in a black plastic tube with six apertures, 1-cm in diameter. The apparatus was calibrated before each use. The UV irradiation time lasted for 1 min to reach a dose of 300mJ/cm^2 .

Quantification of IL-1α and TNF-α Levels

IL-1α and TNF-α were quantified by using a commercially available ELISA kit (DuoSet, R&D Systems, Minneapolis, MN). Cytokine determinations were done according to R&D sandwich ELISA protocol. Briefly, anti-human IL-1α and TNF-α capture antibody were coated onto a 96-well microplate (Nunc) overnight at room temperature. A blocking step was performed for 1 h at room temperature (RT). After washing, the recombinant standards and samples were added to the coated plates and incubated for 2 h at RT. The plate was washed and then incubated with detection antibody for 1 h. The IL-1α and TNF-α binding was colored by streptavidin-horseradish peroxidase, and the optical density was read using a microplate reader at 450 nm, after stopping the reaction using 2 N H_2SO_4. Cytokine titers were expressed as pg per mL, calculated by reference to standard curves constructed with known amounts of recombinant cytokines.

Tyrosinase Activity in Melanocyte Culture

Tyrosinase activity was evaluated through immunoprecipitation assay kit of protein tyrosine kinase (USBiological, Swampscott, MA). Phosphopeptide standard and cell lysate samples were added to a pre-coated streptavidin plate at 37°C for 30 minutes. After washing, blocking buffer solution was added and incubated at 37°C for additional 30 minutes. The block solution was discarded and anti-phosphotyrosine HRP (horseradish peroxidase) was incubated for 1 hour at room temperature. Substrate solution was added during 15 minutes and the enzyme reaction was stopped by an acid solution. Optical

density was read using a microplate reader at 450 nm. Tyrosine kinase activity was compared with known standards and expressed as pg per mL.

Quantification of α-Melanocyte Stimulating Hormone (α-MSH)

α-MSH was quantified by using a commercially available enzymatic immunoassay kit (Phoenix Pharmaceuticals, Inc., Belmont, CA). α-MSH peptide standard, cell lysate samples, primary antiserum and biotinylated peptide were added to each well of a pre-coated immunoplate for 2 hours. After washing, streptavidin-horseradish peroxidase was added and incubated for 1 h, followed further washes. After substrate solution was added, incubation was continued by 1 hour, 2N HCl was added to stop the enzyme reaction, and the optical density was read using a microplate reader at 450 nm. α-MSH titers were expressed as pg per mL, calculated by reference to standard curves constructed with known amounts of recombinant peptides.

Statistical Analysis

For statistical analysis o the cell culture experiments, a parametric method, the one-way analysis of variance (ANOVA) followed by the Tukey test, was used to compare data among all groups. Statistical significance was considered when $P < 0.05$, $P<0.01$ or $P<0.001$.

Results

Antioxidant and antityrosinase effects of the methanolic *Rheum rhaponticum* L. root extract

The methanolic *Rheum rhaponticum* L. root extract presented antioxidant and antityrosinase activities in the *in vitro* assays. Quercetin, as it is known as a potent antioxidant, was used as reference product in the antioxidant effect (Quercetin IC_{50} - 0,53µg/mL; *Rheum rhaponticum* L. extract IC_{50} – 8,92 µg/mL). In comparison to the reference product which was used, the *Rheum rhaponticum* L. extract antioxidant assay results demonstrated that the plant extract has an antioxidant potential. Regarding the antityrosinase assay, Kojic Acid, widely used in whitening products, was the reference product and the results of *Rheum rhaponticum* L. root extract was quite similar to the reference (Kojic Acid IC_{50} - 0,02µg/mL; *Rheum rhaponticum* L. extract IC_{50} - 0,06µg/mL). Besides the antioxidant potential, *Rheum rhaponticum* L. demonstrates *in vitro* effectiveness in antityrosinase assay.

Rheum rhaponticum L. extract inhibits IL-1 levels in melanocytes

Cytokines are also known to down-regulate transcription factors crucial to regulate melanocyte proliferation and to decrease melanossome transfer to neighboring keratinocytes [6, 7, 8, 9]. With the purpose to investigate possible immunostimulatory effects of RRE, we assayed the production of IL-1 in melanocyte culture supernatants (figure 1). In basal conditions, without SSR, a decrease in the levels of cytokines was observed in the cells which were treated with RRE. This behavior was similar to that observed in the groups that were treated with kojic acid. In the groups only submitted to UV radiation, increase of 1,73 fold was observed to IL-1α production in relation to controls. However, in

the groups with RRE treatment, there was a decrease in the citokyne production in relation to the control exposed to UV radiation and the results were comparable to that observed in the cells in basal condition.

Rheum rhaponticum L. extract decreases TNF-α levels in melanocytes induced by UV light

TNF-α, along with a myriad of other cytokines, is modulated in the skin by diverse stimuli, most importantly UVR and it also regulates the expression of POMC (pro-opiomelanocortin) peptides and MC1-R (melanocortin receptor 1) in melanocytes [(12)].This cytokine is present in both the epidermis and dermis of normal skin [(15)].

With the same purpose of testing IL-1 levels, TNF-α levels were also assessed in melanocytes. Although the fact that RRE presented no effect in the groups in basal conditions (without SSR), a decrease of TNF-α occurred in the groups which were exposed to UV radiation, leading to a basal condition (figure 2).

Tyrosinase Activity

Tyrosinase is the enzyme that catalyses the rate-limiting step of melanin synthesis[(8)] and in the present study, the effect of a dry extract of *Rheum rhaponticum* L. (RRE) was evaluated on tyrosinase activity in melanocytes cell culture submitted to UV radiation and incubated with different doses of RRE during 48h. Our results demonstrated a reduction in tyrosinase activity, in basal conditions. As we expected, the UV radiation (UVR) produced a rise of 1.51 fold in enzymatic activity, which was prevented by the treatment of cells with all tested doses of RRE (figure 3).

Quantification of α-Melanocyte Stimulating Hormone (α-MSH)

Another important observation obtained in this study was that the incubation of human melanocytes with 4.9, 2.4 and 1.2mg/mL of RRE led to a reduction in the release of α-MSH (figure 4), which is known to be one of the triggering factors for tyrosinase activation. The reduced effects on α-MSH release were elicited by the two highest doses (4.9 and 2.4mg/mL) tested leading to decreases in the levels of this parameter, in relation to control basal. Considering the groups which were exposed to UV radiation, the decrease in α-MSH levels was observed to all tested doses.

Discussion

Melanin plays an important role in protecting human skin from the harmful effects of UV sun radiation, however its abnormal accumulation in different specific parts of the skin as more pigmented patches, might become an aesthetic problem [(6)]. UV irradiation of the skin results in important damages, such as erythema, swelling, photoaging and skin cancer. One of the modern aims of dermatology is to find substances that can act as photoprotective agents and can also ameliorate the skin injuries. In this study, we have analysed some of the mechanisms in which RRE exerts this protective effect. Tyrosinase inhibition is the most common approach to achieve skin hypopigmentation as this enzyme catalyses the rate-limiting step of melanin synthesis [(6)]. Tyrosinase inhibition is the most common approach to achieve skin hypopigmentation as this enzyme catalyses the rate-limiting step of melanin synthesis [(6)]. Intracellular H_2O_2, generated in response to these cytokines induces a transcient reduction of tyrosinase and other melanogenic protein activities, through the down-regulation of transcriptional controls of tyrosinase

expression[8]. In this way, at first, *in vitro* antioxidant and antityrosinase effects were observed, demonstrating that *Rheum rhaponticum* L. could be involved in the process of helping the skin healthy condition. In order to confirm our finds, we tested the tyrosinase activity in melanocytes culture and as we expected, the results were similar to those from *in vitro*.

Moreover, a number of biological effectors can reduce normal or hyperpigmentation in mammals by various means that cover melanogenesis biochemistry. Cytokines such IL-1, IL-6, TNF-α, IFN-γ and TGF-β belong to these factors and could be release with th.e purpose to the counterbalance the excessive production of positive regulators of melanogenesis [6]. Cytokines are also known to down-regulate transcription factors crucial to regulate melanocyte proliferation and to decrease melanossome transfer to neighboring keratinocytes [6,7,8,9].

Indeed, using human melanocytes culture, we have shown that RRE down-regulates the production of IL-1α and TNF-α.

As alpha-melanocyte stimulating hormone (α-MSH) is produced and released by keratinocytes and melanocytes and it is involved in regulating melanogenesis and melanocyte formation [21], another parameter that was also observed in cell cultures was α-MSH production. The release of this hormone was diminished regarding the highest concentrations of RRE. Both in basal conditions and the groups submitted to UV radiation, the highest doses of RRE were effective in reducing α-MSH. And considering the groups that received UV radiation, even the lowest dose led to a decrease of α-MSH production.

Several lines of evidence indicate the relationship between free radicals and cytokines either because ROS can be the mediatiors of some of the biological effects of cytokines or because free radicals can stimulate the secretion of

different citokynes. In fact, exposure of sensitive cells to cytotoxic concentrations of TNF-α is associated with the accumulation of malonyl dialdehyde, a marker of lipid peroxidation, and the toxicity is prevented by the presence of oxyradical scavengers [22]. The hydroxyl radical is one of the most reactive radicals generated from biologic molecules and can damage living cells [22].Some plant extracts have the ability to scavenge hydroxyl radicals and may protect cellular lipids against free radical reactions [23].

The production of IL-1, TNF-α, and α-MSH is closely related to the UV radiation stimuli and these processes can be interlinked to contribute for the skin damage. Moreover, the production of ROS contributes for accelerating the external consequences of this hazard, as the skin is one of the tissues that suffer the most. In our experiments, *Rheum rhaponticum* L. extract markedly inhibited tyrosinase activity *in vitro* and melanocytes cultures. Indeed, *Rheum rhaponticum* L. extract decreased IL-1, TNF-α and α-MSH production in melanocytes submitted to solar stimulation. Since *Rheum rhaponticum* L. is a plant with several hydroxystilbenes actives as antioxidant and antityrosinase activity, its is possible that these secondary metabolites are related to inhibitory effects detected in this work. In this way, *Rheum rhaponticum* L. can be used as an auxiliary agent in pharmaceutical and or cosmetic preparations for restablishing the skin normal condition after stresses, such as UV radiation.

Several hypopigmenting agents from natural source have been developed and utilized to ameliorate various cutaneous hyperpigmentary disorders and complexion

discolorations [20, 24, 25, 26]. In the same line, the findings presented in this study corroborate with this new depigmenting category, once demonstrated another compound able to correct dysfunctions in melanogenesis metabolism.

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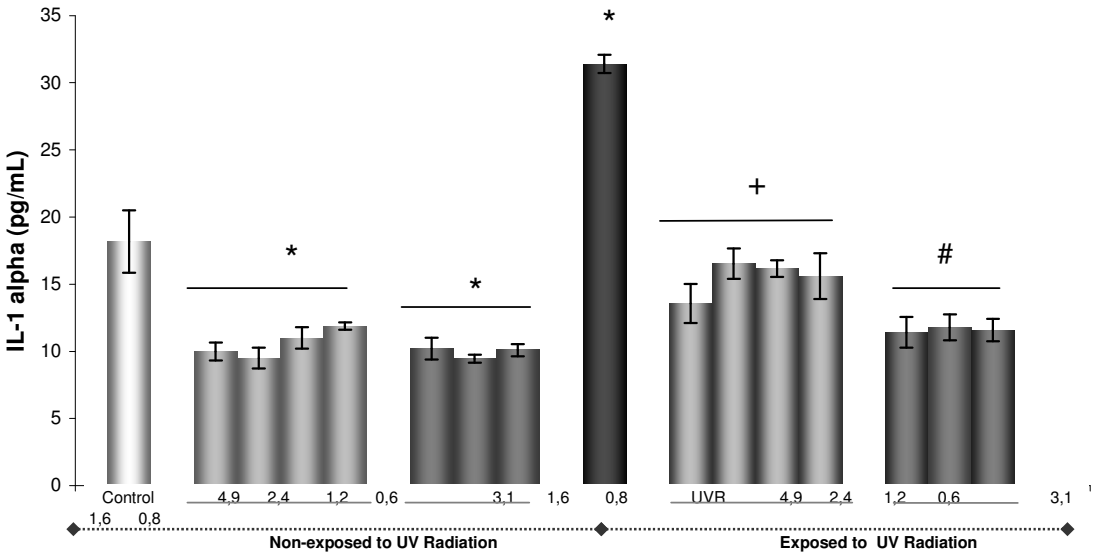

Fig. 1. Interleucin-1 (IL-1) production by human melanocytes treated with *Rheum rhaponticum* L. extract (RRE) and Kojic Acid (AK) with and without solar-simulated radiation (SSR). Cells were treated for 48 hours and IL-1 levels were measured in culture supernatants. The data are presented as mean ± SD of three individual experiments, performed in triplicate. * $P < 0.001$ in relation to control; # $P < 0.001$ in relation to the control exposed to UV radiation; + $P < 0.001$ in relation to SSR control and $P < 0.001$ in relation to all tested concentrations of kojic acid (AK) exposed to UV radiation.

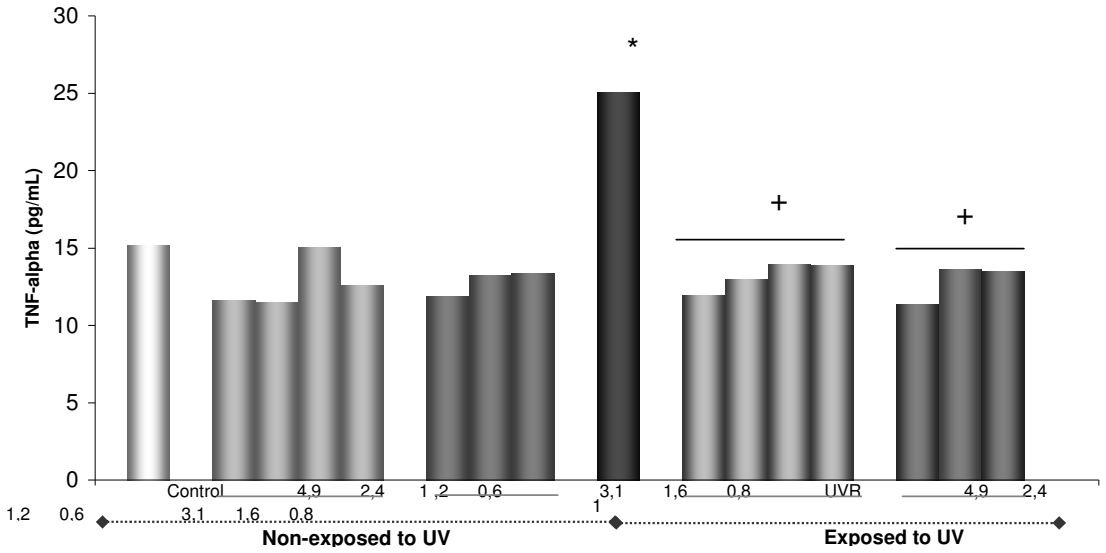

Fig 2. *Rheum rhaponticum* L. extract (RRE) inhibits Tumor Necrosis Factor α (TNF-α) induced by solar-simulated radiation (SSR). * P < 0.001 in relation to control; + P<0.01 in relation to control treated with UV radiation.

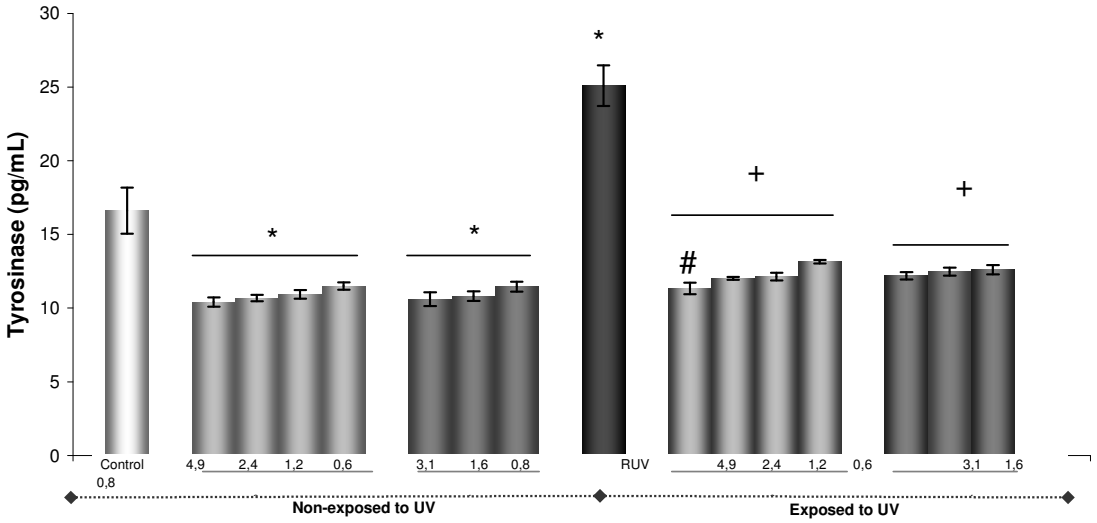

Fig. 3. The effect of the extract of *Rheum rhaponticum* L.(RRE) on tyrosinase activity. Human melanocytes were treated with RRE for 48h and tyrosinase activity was measured in culture lysate. The data are presented as mean ± SD of three individual experiments, performed in triplicate. * P<0.001 in relation to control; + P<0.001 in relation to control exposed to SSR; # P<0.01 in relation to RRE 0.0006 exposed to UV radiation

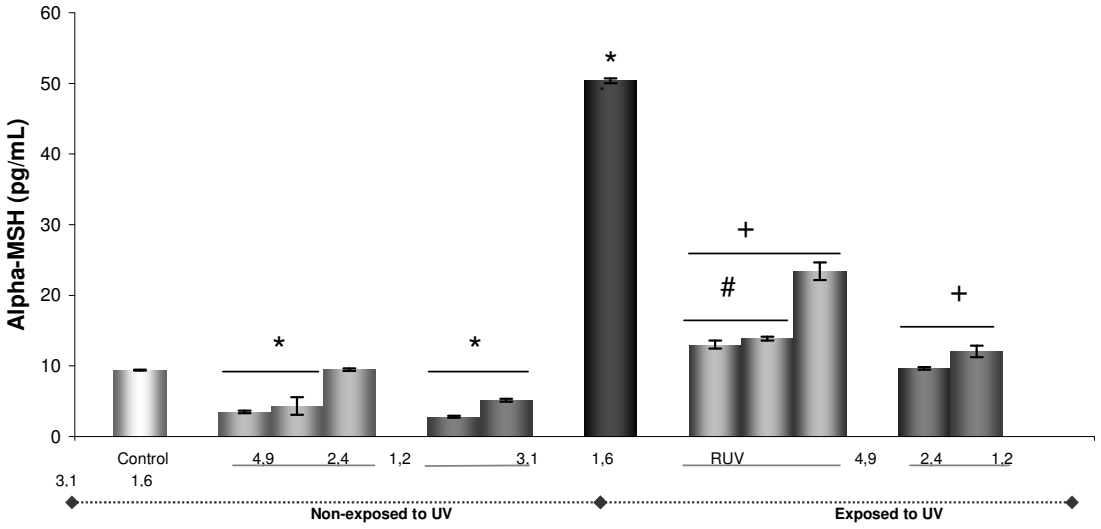

Fig. 4. The effect of the extract of *Rheum rhaponticum* L. (RRE) on alpha-Melanocyte Stimulating Hormone (α-MSH) production. Human melanocytes were treated with RRE for 48h and α-MSH levels were measured in culture lysate. The data are presented as mean ± SD of three individual experiments, performed in triplicate. * P<0,001 in relation to control; + P<0.001 in relation to control exposed to UV radiation; # P<0.001 in relation to RRE 0.0012 exposed to UV radiation and P<0.01 in relation to all tested concentrations of Kojic Acid (AK) exposed to UV radiation. (ANOVA, Tukey).

6. Artigo científico sobre *Coccoloba uvifera* L.

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Inhibition of the IL-1, TNF-α and α-MSH production and tyrosinase activity by *Coccoloba uvifera* L. in UV-stimulated melanocytes

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Summary

Pigmentary disorders are caused by various factors, including inflammation, imbalance of hormones and genetic disorder. Excessive exposure to ultraviolet radiation (UVR) causes not only post-inflammatory pigmentation, but can also lead to skin cancer. In melanocytes, UVR stimulates many mediators, such as interleukin 1 α, tumor necrosis factor α, melanocyte stimulating hormone α and tyrosinase. The effect of UVR in melanocytes and the surrounding cells is the object of several studies; however, natural agents that can protect skin must be investigated in order to replace the synthetic ones which result in lots of side effects and to bring other options to cosmetic and pharmaceutical fields. Our results demonstrated that *Coccoloba uvifera* L. (Polygonaceae family) was efficient in protecting the increase production of pro-inflammatory cytokines and melanocyte stimulating hormone α, as well as its showed inhibitory tyrosinase activity in melanocytes culture submitted to ultraviolet radiation.

Key words: melanocyte, tyrosinase, α-MSH, IL-1α, TNF-α

Introduction

There are a number of environmental and intrinsic factors that regulate the cutaneous physiological system. Among these factors, Slominski and Wortsman, 2000 refers that most prominent environmental factor affecting the skin is the solar radiation, particularly within the UVA and UVB wavelengths. Many of the effects of UV on human skin are indirectly mediated by up-regulation of various growth factors and cytokines, some of which work as paracrine or autocrine regulators of melanocytes (Halaban et al, 1988; Imokawa et al, 1992; Wakamatsu et al, 1997). Melanocyte stimulating hormone alpha (α-

MSH) and pro-inflammatory cytokines such as interleukin 1 alpha (IL-1α) and tumor necrosis factor alpha (TNF-α) synthesized by keratinocytes and melanocytes are important mediators of response to skin to UV-radiation.

α-MSH is a melanocortin peptide which is produced together with several other peptides by the proteolitical cleavage of the large precursor protein pro-opiomelanocortin (POMC) and the keratinocytes and melanocytes secrete α-MSH in response to ultraviolet radiation (Chakraborty et al, 1996). Melanocortin peptides exert their effects through melanocortin receptors (MCRs). On binding the MC1-R, α-MSH activates adenylate cyclase which, in turn, causes an increase in the intracellular cAMP. This increase results, via protein kinase A (PKA), in the activation of tyrosinase, the rate-limiting enzyme in the melanin pathway (Tsamali et al, 2002).

Cytokines, such as IL-1α and TNF-α, are highly produced in the surrounding cells in response to several stimuli. They should exist in supernatants of cultures or in the epidermis at concentrations sufficient to stimulate melanocytes and have the potential to activate melanocytes at physiological concentrations in vitro. (Imokawa, 2004). Interleukin-1 alpha (IL-1α), IL-6, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and transforming growth factor-beta (TGF-β) are produced by melanocytes and keratinocytes to compensate the exacerbated production of melanin, mainly by the release of tyrosinase (Solano, 2006; Slominski, 2004; Briganti, 2003; Swope, 1991).

The highest incidence of cancer is found in the skin, but endogenous pigmentation is associated with markedly reduced risk and agents that enhance skin pigmentation have the potential to reduce both photodamage and skin cancer incidence (Brown, 2001).

Ultraviolet (UV) radiation causes sunburn reactions and immunosuppression, among other effects and it is considered to be an important environmental hazard for humans (Kripke, 1990). At cellular levels, UV radiation triggers cytokine production (Takashima and Bergstresser, 1996) such as TNF-α, IL-1α and IL-6 and these cytokines

are thought to play pathogenic roles in the development of UV induced cutaneous inflammation (Takashima and Bergstresser, 1996). The effect of the cytokines on POMC gene expression is mediated by the tyrosine phosphorylation cascade (Katahira et al, 1998), contributing for enhancing the skin pigmentation.

On the other hand, abnormal melanogenesis can cause many disorders and aesthetic problems, such as postiinflammatory pigmentation and melasma (Taylor, 2002). Postinflammatory hyperpigmentation can be considered the default pathophysiologic response to cutaneous injury. This response is thought to be predicated to the labile response of melanocytes to irritation or inflammation (Grimes and Stockon, 1988). Moreover, melasma occurs due to hormonal factors, ultraviolet radiation and also the lability of melanocytes (Taylor, 2002). For these reasons, new discovery on agents that can affect melanogenesis are required.

Many plant extracts and their active principles have been described and utilized as cosmetic agents, in particular, as skin lightening products. The growing demand for depigmenting agents is being perceived and effective compounds from natural sources, acting as tyrosinase inhibitors or blocking others melanogenic pathways, have been reported (Maeda, 1996; Kim, 2002; Simonot, 2002; Choi, 2005).

Coccoloba uvifera L. commonly known as Jamaican Kino and sea grape is a plant from Polygonaceae family originates from Jamaica and Tropical South America (McGookin and Heibron, 1925). This botanical family includes several active plants with antioxidant and inhibitory tyrosine hydroxystilbenes compounds (Cuedent et al., 2000; Kim et al., 2002; Aggarwal et al., 2004; Raal et al., 2007).

In this way, *Coccoloba uvifera* L. dry extract was investigated on UV-stimulated melanocytes in view of to evaluate its effects on the production of interleukin 1alpha (IL-1α), tumor necrosis factor alpha (TNF-α), melanocyte stimulating hormone alpha (α-MSH) and tyrosinase activity.

Results

Coccoloba uvifera L. presented antioxidant potential

The antioxidant potencial was assessed using the lipoperoxidation method. The effect of the plant extract was compared to quercetin, a potent well-known antioxidant agent. The IC_{50} (inhibition concentration 50) of methanolic *Coccoloba uvifera* L. root extract (CUE) was 9,84µg/mL, while IC_{50} of Quercetin was 0,53µg/mL.

Coccoloba uvifera L. extract inhibits IL-1 levels in melanocytes

Cytokines are also known to down-regulate transcription factors crucial to regulate melanocyte proliferation and to decrease melanossome transfer to neighboring keratinocytes (Solano et al, 2006; Solominski et al, 2004; Briganti et al, 2003; Swope et al, 1991). With the purpose to investigate possible immunostimulatory effects of CUE, we assayed the production of IL-1 in melanocyte culture supernatants (figure 1). In basal conditions, without UV radiation exposure, a decrease in the levels of cytokines was observed in the cells which were treated with the plant extract and this behavior was similar to that observed in the groups that were treated with kojic acid. In the groups only submitted to UV radiation, increase of 1,73 fold was observed to IL-1α production in relation to control. However, in the groups with CUE treatment, there was a decrease in the cytokine production in relation to the control exposed to UV radiation and the results were comparable to that observed in the cells in basal condition.

Coccoloba uvifera extract inhibits TNF-α levels in melanocytes induced by UV light

TNF-α, along with a myriad of other cytokines, is modulated in the skin by diverse stimuli, most importantly UVR and it also regulates the expression of POMC (pro-opiomelanocortin) peptides and MC1-R (melanocortin receptor 1) in melanocytes (Bergamini et al, 2004).This cytokine is present in both the epidermis and dermis of normal skin (Solominski et al, 2004).

With the same purpose of testing IL-1α levels, TNF-α levels were also assessed in melanocytes. Although the fact that CUE presented no effect in the groups in basal conditions, a decrease of TNF-α occurred in the groups which were exposed to UV radiation, leading to a basal condition (figure 2). The plant extract effect was very similar to kojic acid behavior.

Decrease in the intracellular levels of tyrosinase

Tyrosinase is the enzyme that catalyses the melanin synthesis and it was evaluated, in the present study, the effect of a dry extract of *Coccoloba uvifera* L. (CUE) on tyrosinase activity in melanocytes cell culture submitted to UV radiation and incubated with different doses of CUE during 48h. Our results demonstrated a reduction in tyrosinase activity, in basal conditions. As we expected, the UV radiation (UVR) produced a sharp rise of 1,51 fold in enzymatic activity, which was prevented by the treatment of cells with all doses of testes of CUE (figure 3).

The results observed in cell culture were similar to those of the enzymatic assay, in which the plant extract activity was compared to kojic acid, a potent inhibitor of tyrosinase

activity, and both effects presented almost the same results (*Coccoloba uvifera* L. IC_{50}: 0,09µg/mL; Kojic Acid IC_{50}: 0,02µg/mL).

Quantification of α-Melanocyte Stimulating Hormone (α-MSH)

α-MSH which is known to be one of the triggering factors for tyrosinase activation and the release of this factor was assessed under the effect of *Cocoloba uvifera* extract. The reduced effects on α-MSH release were elicited by the two highest doses (19.5 and 9.8mg/mL) tested leading to decreases in the levels of this parameter, in relation to control and the same behavior was observed regarding the groups with were exposed to UV radiation.

Discussion

Epidermal and dermal hyperpigmentation can be dependent on either increased numbers of melanocytes or activity of melanogenic enzymes. Upon exposure of skin to UV radiation, melanogenesis is initiated with the first step of amino acid tyrosine oxidation by tyrosinase, producing DOPA which is then metabolized to further formation of melanin (Ifuku, 2003).

Ultraviolet light, tanning, photoaging, drugs and chemicals, as much, hormonal influences and chronic inflammation, could increase the production of the triggering factors to skin melanization such as alpha-melanocyte stimulating hormone (α-MSH) release (Briganti et al., 2003).

Alpha-melanocyte stimulating hormone (α-MSH) is produced and released by keratinocytes and melanocytes and it is involved in regulating melanogenesis and

melanocyte formation (Costin, 2007). After the binding to a melanocyte-specific receptor, α-MSH activates the adenylate cyclase to G-protein pathway, culminating in the up-regulation of tyrosinase, which leads to melanin synthesis (Lin, 2007).

Several pharmacologic and cosmetic agents inhibit tyrosinase or other melanogenic pathway targets (Briganti et al., 2003), leading to skin color reestablishment. As unusual pigmentation caused by medical conditions such as melasma, postinflammatory melanoderma and solar lentigo or by UV radiation is undesirable (Solominski et al., 2000), the development of agents that act in different ways is essential. In this way, we examined in the present study the effects of a *Coccoloba uvifera* L. extract (CUE) on tyrosinase activity both *in vitro* and in melanocytes culture submitted or not to UV radiation and incubated with different doses of CUE. Our results demonstrated a reduction in tyrosinase activity in both tests and considering the cell cultures, in basal conditions, with the doses of 19.6×10^{-3}, 9.8×10^{-3}, 4.9×10^{-3} and 2.4×10^{-3} mg/mL, there is a decrease up to 0.61, 0.68, 0.70 and 0.72 fold in relation to control, (figure 3). As we expected, the UV radiation (UVR) produced a sharp rise of 1,51 fold in enzymatic activity, which was prevented by the treatment of cells with all tested doses of CUE.

Even in melanocytes their actions are not confined to melanogenesis and evidence is emerging that α-MSH affects several aspects of melanocyte behavior. There is increasing evidence that melanocytes may have a number of functions. Melanocytes are capable of secreting a wide range of signaling molecules and it has been suggested that they could function as regulatory cells in maintaining epidermal homeostasis. Their ability to respond to regulatory peptides such as α-MSH might be an integral part of such a function. (Ito et al., 2000).

Besides the pigmentation stimulation, α-MSH has been shown to have a variety of other functions, including modulation of the inflammatory system and the immune response (Ito et al., 2000).

Therefore, another important observation obtained with this study was that the incubation of human melanocytes with 19.5 x 10^{-3}, 9.8 x 10^{-3} and 4.9 x 10^{-3}mg/mL of CUE led to a reduction in the release of α-MSH (figure 4). The reduced effects on the α-MSH release were elecited by the highest tested doses (19.8 and 9.8mg/mL) leading respectively to 0.47 and 0.59mg/mL fold decreases in the level of this parameter, in relation to basal condition. Concurrently, reduced levels were found in the cultures submitted to UV radiation and treated also with the highest doses of CUE (19.8 x 10^{-3} and 9.8 x 10^{-3}mg/mL).

In the other side, a number of biological effectors can reduce normal or hyperpigmentation in mammals by various means that cover melanogenesis biochemistry. Cytokines such IL-1α, IL-6, TNF-α, IFN-γ and TGF-β belong to these factors and could be release with the purpose to the counterbalance the excessive production of positive regulators of melanogenesis (Solano, 2006). In addition to interleukin 1 α and 6, tumor necrosis factor alpha (TNF-α) is able to decrease pigmentation by acting also on tyrosinase activity (Solano et al., 2006). Intracellular H_2O_2, generated in response to these cytokines induces a transcient reduction of tyrosinase and other melanogenic protein activities, through the down-regulation of transcriptional controls of tyrosinase expression (Briganti, 2003). Cytokines are also known to down-regulate transcription factors crucial to regulate melanocyte proliferation and to decrease melanossome transfer to neighboring keratinocytes (Solano, 2006; Slominski, 2004; Briganti, 2003; Swope, 1991).

With the purpose to investigate possible immunostiulatory effects of CUE, the production of IL-1 and TNF-α were also measured in the melanocytes supernadants. Regarding basal conditions, only the groups in which IL-1 production was assessed, there was a decrease of this cytokine release. However both IL-1 and TNF-α productions decreased in the groups stimulated by UV radiation.

To speculate the mechanisms through which *Coccoloba uvifera* extract acts, we focus in its botanical family. Plants that belong from Poligonacea family had shown its activity as antioxidants and tyrosinase inhibitors due to the presence of hydroxyestilbenes (Cuendet et al., 2000; Tida, Hase, Shimomura et al., 1995 and Kim et al., 2002). As *C. uvifera* belongs to the same family of these plants that already been studied, it can present the same compounds. Thus, the botanical family of *C. uvifera* extract could be one of the possible explanation for the results obtained in this work.

As melanocytes are not simply melanin-producing cells and their actions are not confined to melanogenesis, it is important to evaluate the mechanisms involved in melanocytes response to UV radiation to discover agents that can help the treatment of UV-related diseases.

The skin response to UV radiation is a complex process that includes many factors. For this reason, agents that contribute for reestablishing the normal skin condition have to comprise as many mediators activities as possible. Moreover, their efficacy is closely related to the quantity of processes that they can affect. Therefore, the knowledge about cytokines production, α-MSH release and tyrosinase activity is crucial in order to investigate agents that act in melanocytes function.

In our experiments, *Coccoloba uvifera* L. extract inhibited tyrosinase activity *in vitro* and melanocytes cultures. Indeed, *Coccoloba uvifera* L. extract inhibited IL-1, TNF-α and α-MSH production in melanocytes submitted to solar stimulation. In this way, this plant can be used as an auxiliary agent in pharmaceutical and or cosmetic preparations for restablishing the skin normal condition after stresses, such as UV radiation.

Material and Methods

Plant Material

Dry extract of *Coccoloba uvifera* L. (CUE) was manufactured and provided by Chemyunion Química Ltda (Sorocaba, SP, Brazil). Crude plants, obtained for Brazilian suitable suppliers, were sliced into small pieces and extracted with methanol. The extract was concentrated in a rotary evaporator (Buchi RE 111 Buchi Laboratoriums-Tecnick AG,Flawil, Switzerland) in order to remove the solvent. Extract was dissolved in culture medium and diluted into appropriate concentrations immediately before use.

Cell culture and treatment protocol

Human epidermal melanocytes (Cryopreserved HEM, Cat. 104-05n), melanocyte growth medium (Cat. 135-500) and trypsin/EDTA solution (Subculture Reagent Kit, Cat. 090K) were purchased from Cell Applications, Inc™ San Diego, CA. Cells were subcultured in 25 cm^2 flasks (Corning Inc, New York, NY) at 37°C in 5% CO_2 humidified incubator, and expanded for at least five passages. The medium was changed twice a week. At 80-90% confluent, cells were trypsinized and seeded into 24- well culture plates (Nunc, Roskilde, DM). Twenty-four hours after melanocytes seeding, cells were washed with PBS and irradiated with ultraviolet radiation in a dose of 300mJ/cm^2. Immediately after irradiation, cells were incubated with doses of 19.5, 9.8, 4.9 and 2.4 mg/mL of CUE (*Coccoloba uvifera* L. extract) dissolved in culture medium. After 48 h of treatment, cell-free supernatants and cell lysate were collected and assays were performed using commercial kits. Each experiment was conducted in triplicate of three independent

experiments. Selection of these doses was based on previous results of cytotoxicity assays (data not shown).

Antioxidant activity (*In vitro* Lipoperoxidation)

Trichloroacetic acid precipitates proteins of rats brain while the tiobarbituric reactive reacts with malonyldialdehyde (MDA), released by the lipoperoxidation caused by iron and ascorbic acid, producing a color whose intensity is determinated by spectrophotometer. The most is the intensity; the most is the MDA concentration. An element will be considered antioxidant if it is capable to inhibit the lipoperoxidation caused by the reagents, reducing the MDA values. The test elements were prepared through a serial dilution in 400 to 6,25 mg/mL doses (DMSO 20%).

For the test samples, it was added 50 μL of Iron-Ascorbic Acid 100 μM solution, 1 mL of brain homogenate, 50 μL of the plant extract and tiobarbituric. After centrifugation, the supernatant was paced in duplicate in 96-well microplate. The measurements were performed in spectrometer (Bio Tek™ Power Wave 340).

In vitro Tyrosinase Assay

Antityrosinase effect was assessed through enzymatic assay. Potassium phosphate buffer (50mM) at pH 6.5, tyrosinase (333units/mL) and test compounds dissolved in DMSO 20% were inserted into 96-well plates. After 5 min at room temperature, L-tyrosine was added.

The diphenolase activity of tyrosinase was measured spectrophotometrically by following the increase in absorbance at 475nm by production of dopachrome from L-DOPA. The reaction was done at 37° C.

Ultraviolet radiation

A Multiport solar UVA and UVB simulator (Model 601; Solar Light Co., Philadelphia, PA) was used for the UV irradiation. The simulator was equipped with a 150W Xenon lamp, with a liquid filter and 1-mm Schott WG 320 filter, emitting a continuous spectrum of radiation beginning at 290 nm through the infrared spectrum and maximally peaking at 400 nm. The lamp was housed in a black plastic tube with six apertures, 1-cm in diameter. The apparatus was calibrated before each use. The UV irradiation time lasted for 1 min to reach a dose of 300mJ/cm^2 (Seiberg e cols., 2000).

Quantification of IL-1α and TNF-α Levels

IL-1α and TNF-α were quantified by using a commercially available ELISA kit (DuoSet, R&D Systems, Minneapolis, MN). Cytokine determinations were done according to R&D sandwich ELISA protocol. Briefly, anti-human IL-1α and TNF-α capture antibody were coated onto a 96-well microplate (Nunc) overnight at room temperature. A blocking step was performed for 1 h at room temperature (RT). After washing, the recombinant standards and samples were added to the coated plates and incubated for 2 h at RT. The plate was washed and then incubated with detection antibody for 1 h. The IL-1α and TNF-α binding was colored by streptavidin-horseradish peroxidase, and the optical density was read using a microplate reader at 450 nm, after stopping the reaction using 2 N H_2SO_4.

Cytokine titers were expressed as pg per mL, calculated by reference to standard curves constructed with known amounts of recombinant cytokines.

Tyrosinase Activity

Tyrosinase activity was evaluated through immunoprecipitation assay kit of protein tyrosine kinase (USBiological, Swampscott, MA). Phosphopeptide standard and cell lysate samples were added to a pre-coated streptavidin plate at 37°C for 30 minutes. After washing, blocking buffer solution was added and incubated at 37°C for additional 30 minutes. The block solution was discarded and anti-phosphotyrosine HRP (horseradish peroxidase) was incubated for 1 hour at room temperature. Substrate solution was added during 15 minutes and the enzyme reaction was stopped by an acid solution. Optical density was read using a microplate reader at 450 nm. Tyrosine kinase activity was compared with known standards and expressed as pg per mL.

Quantification of α-Melanocyte Stimulating Hormone (α-MSH)

α-MSH was quantified by using a commercially available enzymatic immunoassay kit (Phoenix Pharmaceuticals, Inc., Belmont, CA). α-MSH peptide standard, cell lysate samples, primary antiserum and biotinylated peptide were added to each well of a pre-coated immunoplate for 2 hours. After washing, streptavidin-horseradish peroxidase was added and incubated for 1 h, followed further washes. After substrate solution was added, incubation was continued by 1 hour, 2N HCl was added to stop the enzyme reaction, and the optical density was read using a microplate reader at 450 nm. α-MSH titers were

expressed as pg per mL, calculated by reference to standard curves constructed with known amounts of recombinant peptides.

Statistical Analysis

For statistical analysis, a parametric method, the one-way analysis of variance (ANOVA) followed by the Tukey test, was used to compare data among all groups. Statistical significance was considered when $P < 0.05$, $P<0.01$ or $P<0.001$.

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Figure legends

Fig. 1. Interleucin-1 (IL-1) production by human melanocytes treated with *Coccoloba uvifera* L. extract (CUE) and Kojic Acid (AK), exposed and non-exposed to ultraviolet radiation. * P<0,001 in relation to control; + P<0,001 in relation to control exposed to ultraviolet radiation; P<0,01 in relation to all tested concentrations of kojic acid and # P<0,05 in relation to the other concentrations of CUE.

Fig. 2. *Coccoloba uvifera* L. extract (CUE) inhibits Tumor Necrosis Factor α (TNF-α) induced by solar radiation. * P<0,001 in relation to control; + P<0,01 in relation to control exposed to ultraviolet radiation

Fig. 3. The effect of the extract of *Coccoloba uvifera* L. (CUE) on tyrosinase activity. * P<0,001 in relation to control; # P<0,001 in relation to control exposed to ultraviolet radiation; + P<0, in relation to all tested concentrations of kojic acid which were exposed to UV radiation and in relation to CUE in the concentration 0,0024 exposed to UV radiation; §P<0,05 in relation to kojic acid in the concentrations 0,1563 and 0,0781 which were exposed to UV; ∆P<0,05 in relation to kojic acid in the concentration 0,0781 exposed to UV radiation.

Fig. 4. The effect of the extract of *Coccoloba uvifera* L. (CUE) on alpha-Melanocyte Stimulating Hormone (α-MSH) production. Human * P<0,001 in relation to control; + P<0,001 001 in relation to control exposed to ultraviolet radiation; # P<0,001 in relation to CUE in the concentrations of 0,0098 and 0,0049 exposed to UV radiation and to all tested concentrations of kojic acid exposed to UV radiation; § P<0,001 in relation to CUE 0,0049 exposed to UVR and to all concentrations of kojic acid groups which were exposed with UVR; ∆P<0,05 in relation to all concentrations of kojic acid groups which were exposed with UVR.

Figure 1

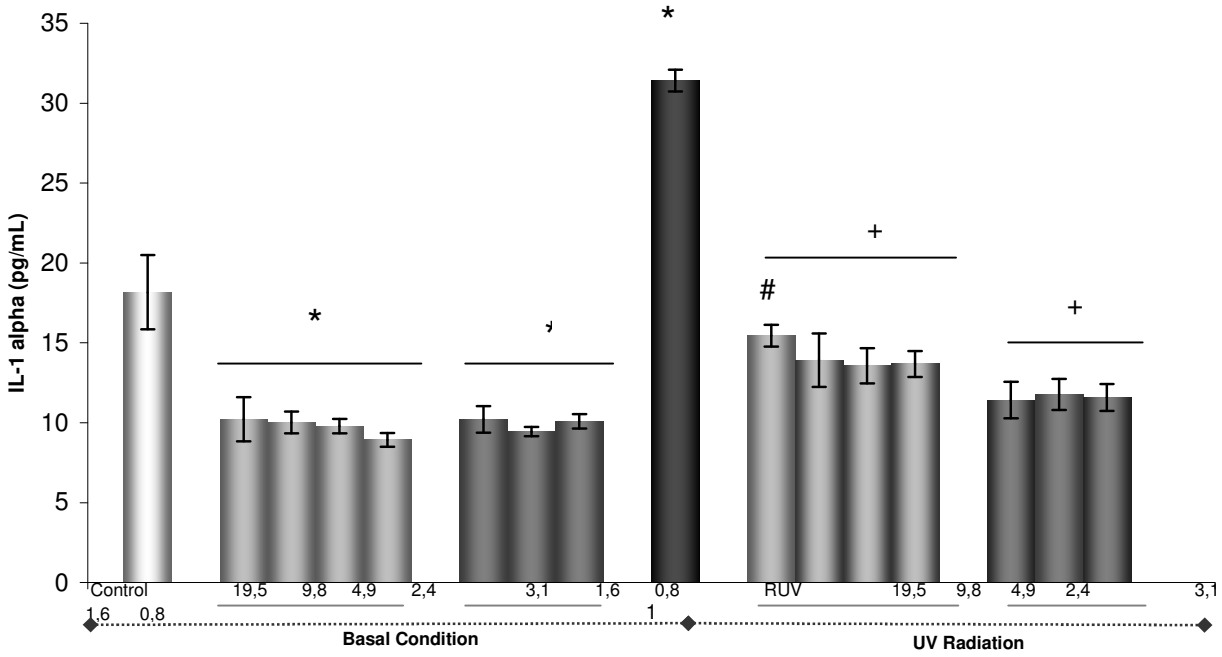


Figure 2

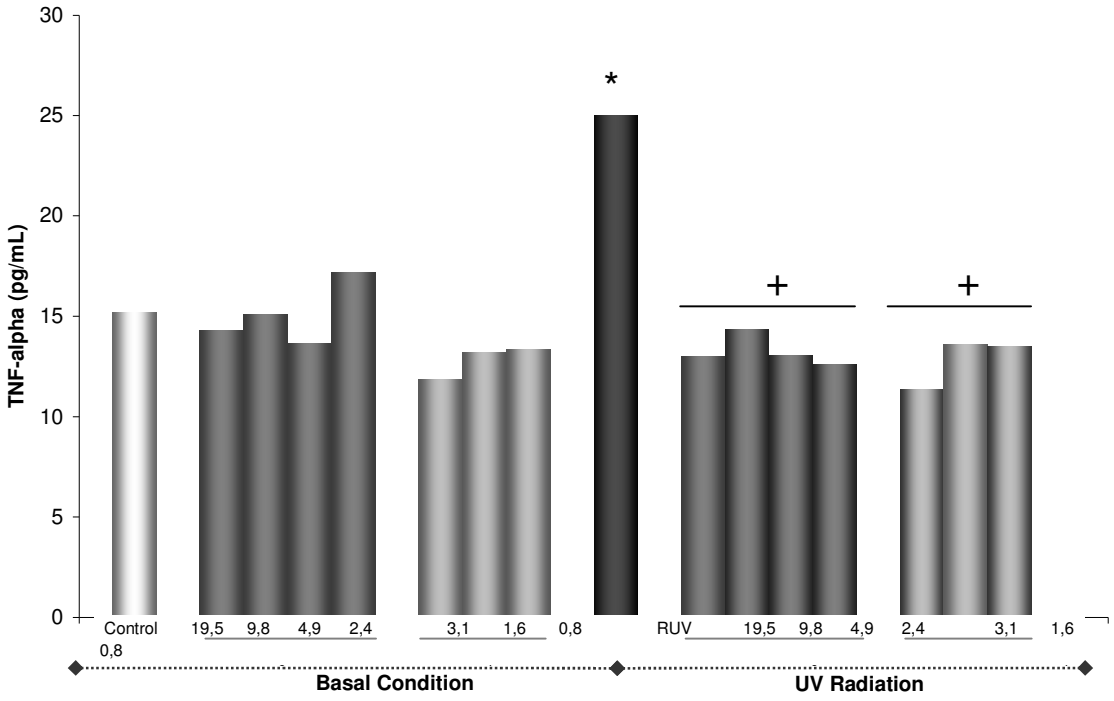

30
25
20
15
10
5
0
TNF-alpha (pg/mL)
*
+
+
Control
19,5
9,8
4,9
2,4
3,1
1,6
0,8
0,8
RUV
19,5
9,8
4,9
2,4
3,1
1,6
Basal Condition
UV Radiation

Figure 3

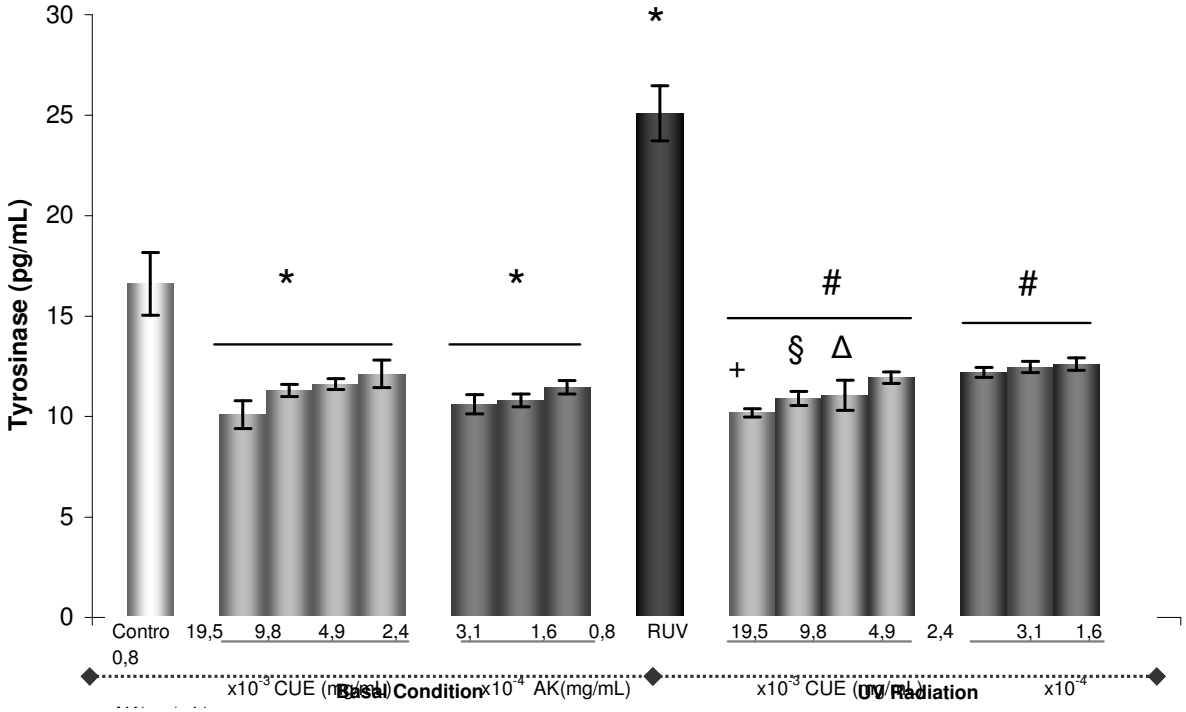
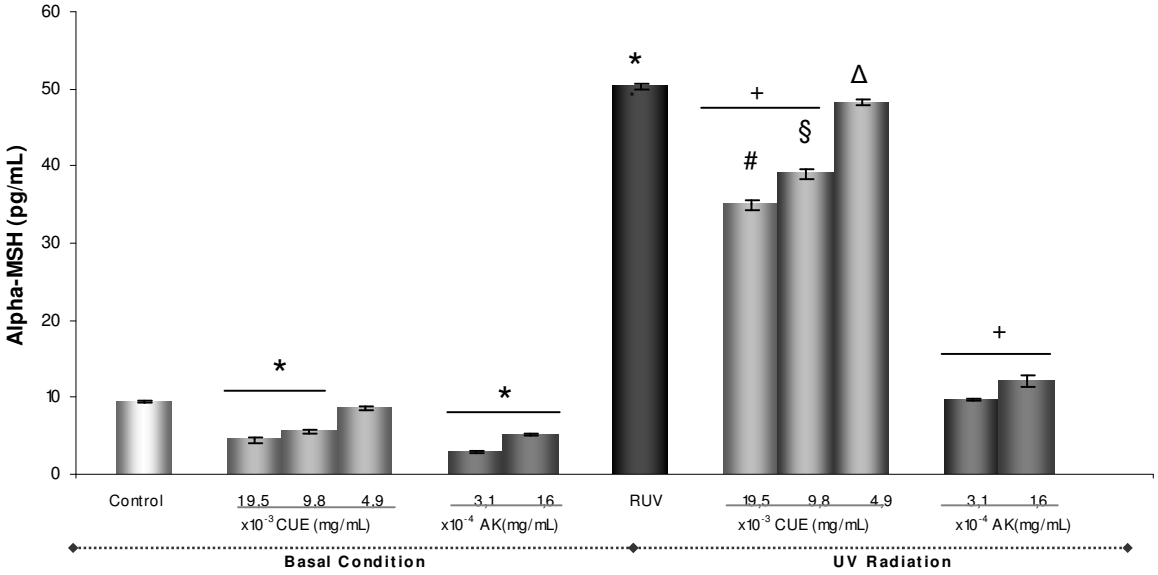

Tyrosinase (pg/mL)
30
25
20
15
10
5
0
Contro
0,8
19,5
9,8
4,9
2,4
3,1
1,6
0,8
RUV
19,5
9,8
4,9
2,4
3,1
1,6
x10-3 CUE (mg/mL)
x10-4 AK(mg/mL)
Basal Condition
UV Radiation

Figure 4


Alpha-MSH (pg/mL)
60
50
40
30
20
10
0
Control
19,5
9,8
4,9
3,1
1,6
RUV
19,5
9,8
4,9
3,1
1,6
x10-3 CUE (mg/mL)
x10-4 AK(mg/mL)
Basal Condition
UV Radiation

6. Discussão e Conclusão

O desenvolvimento de agentes despigmentantes eficazes é uma importante área em crescimento na pesquisa do tratamento da hiperpigmentação induzida pela radiação UV ou por condições dermatológicas como melasma, melanodermias pós-inflamatórias e lentigo solar.

Vários produtos já existentes no mercado atuam através da inibição da enzima tirosinase. Apesar disto, buscam-se novos ativos despigmentantes mais eficazes; e a identificação e elucidação do mecanismo de ação destes ativos é objetivo importante para seu desenvolvimento.

Embora a hipopigmentação possa ser conseguida através de agentes físicos, biológicos e tratamentos físicos (Jacques & McAuliffe, 1991), neste estudo, o objetivo foi pesquisar agentes de fonte vegetal que possam ser usados como ativos despigmentantes. Com este propósito, extratos vegetais foram investigados a fim de verificar o potencial destas plantas como agentes clareadores da pele.

Neste estudo, verificamos o efeito antioxidante de 12 extratos vegetais, através dos métodos de captação de radicais livres por DPPH e de lipoperoxidação e selecionamos as 2 espécies vegetais que apresentaram os melhores resultados: *Rheum rhaponticum* L. e *Coccoloba uvifera* L. para estudar seus efeitos na produção de citocinas IL-1α (interleucina 1 alfa), TNF-α (fator de necrose tumoral alfa), hormônio melanócito estimulante alfa (α-MSH) e na atividade da enzima tirosinase em cultura de melanócitos submetidos a irradiação ultravioleta.

Na epiderme humana, diversos mecanismos enzimáticos e não-enzimáticos estão disponíveis para controlar o acúmulo de espécies reativas de oxigênio sendo que a

tirosinase é capaz de utilizar superóxido para produzir melanina (Perluigi e cols., 2003; Friedmann e Gilchrest, 1987), de modo que compostos antioxidantes, capazes de inibir a produção de espécies reativas de oxigênio podem reduzir a hiperpigmentação ou prevenir a melanogênese.

O estímulo dos melanócitos com α-MSH aumenta a eumelanogênese em maior grau que a feomelanogênese, o que contribui para a pigmentação cutânea, sendo que aumenta também a atividade da tirosinase (Ito e cols., 2000).

Os melanócitos podem também atuar como reguladores das respostas imunes da pele através da produção de uma séria de citocinas, incluindo IL-1α (interleucina 1alfa), IL-6 (interleucina 6), IL-3 (interleucina 3) e TNF-α (fator de necrose tumoral alfa) (Köck et al. 1991,1992).

Baseando-se nestes mecanismos, pode-se observar os importantes efeitos dos extratos de *Rheum rhaponticum* L. e *Coccoloba uvifera* L. como agentes clareadores da pele, comparando os resultados observados com aqueles obtidos pela substância referência, o ácido kójico.

A diminuição de interleucina 1α foi observada tanto em condições basais, onde não houve nenhum estímulo na cultura celular, quanto nas células estimuladas com radiação ultravioleta onde esta diminuição foi mais pronunciada.

Quanto aos resultados referentes ao TNF-α, houve diminuição significativa nos níveis desta citocina, praticamente retornando à condição basal, nas células onde houve estimula da radiação ultravioleta. Em condições basais não houve alterações significativas.

Quanto ao método de avaliação da atividade da tirosinase, ambos extratos apresentaram diminuição significativa; tanto nas células estimuladas quanto nas células não estimuladas por radiação ultravioleta.

Na avaliação da produção do hormônio melanócito estimulante α (α-MSH), apesar de resultados semelhantes, principalmente em condições basais, onde somente nas maiores concentrações do extrato foi observada diminuição estatisticamente significativa, o extrato de *Rheum rhaponticum* apresentou melhores resultados para a cultura de células expostas à radiação ultravioleta, visto que foi observada uma menor produção de hormônio.

Em comparação com o ácido kójico, os resultados obtidos com os extratos estudados foram semelhantes em todas as análises realizadas, mostrando-se eficazes na hipopigmentação em função dos efeitos apresentados.

Assim sendo, pode-se concluir que as plantas *Coccoloba uvifera* L. e *Rheum rhaponticum* L. por interferirem na produção dos mediadores da melanogênese, seja diminuindo a produção dos mesmos ou retornando à condição basal após o estímulo da radiação ultravioleta, podem ser considerados potenciais agentes despigmentantes.

Em adição, outras espécies inicialmente analisadas apresentaram importantes efeitos antioxidantes e inibidores da atividade da tirosinase, os quais também podem ser espécies potenciais para a realização de novos estudos tendo-se em vista a obtenção de novos produtos úteis no controle do processo de pigmentação da pele, tais como *Eugenia crenata* Vell., *Eugenia uniflora* L., *Rheum officinale* Baill. e *Syzigyum jambolanum* DC.

7. Referências Bibliográficas

As referências bibliográficas específicas de cada artigo não foram inseridas neste item.

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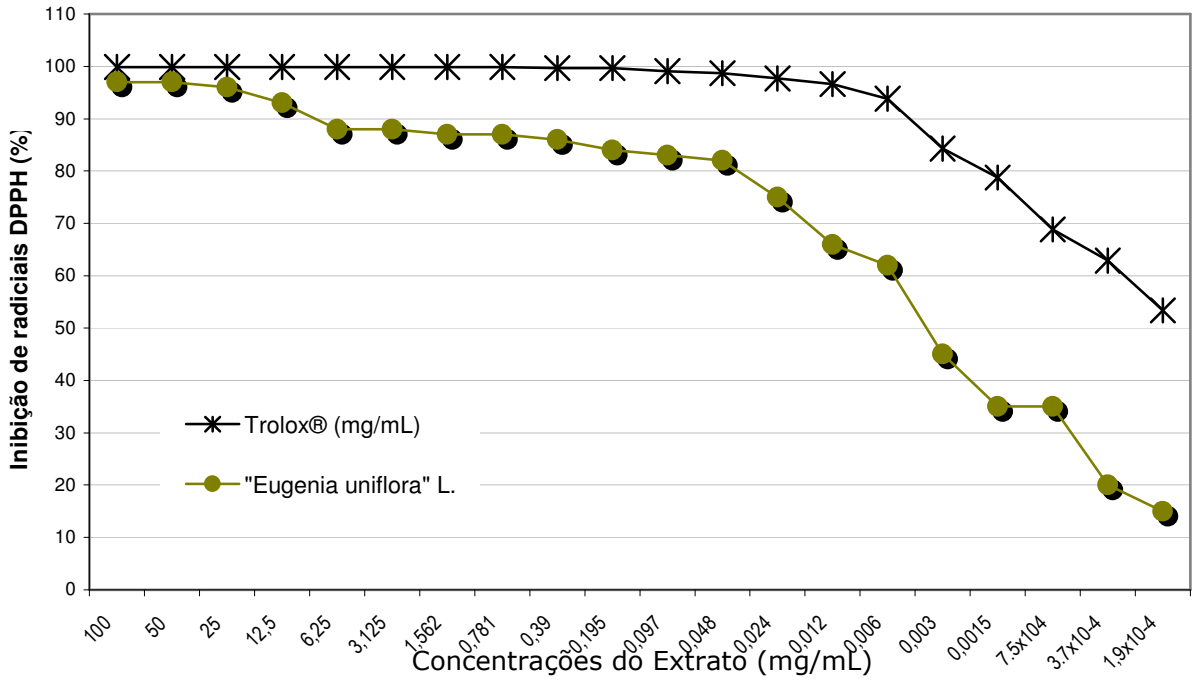
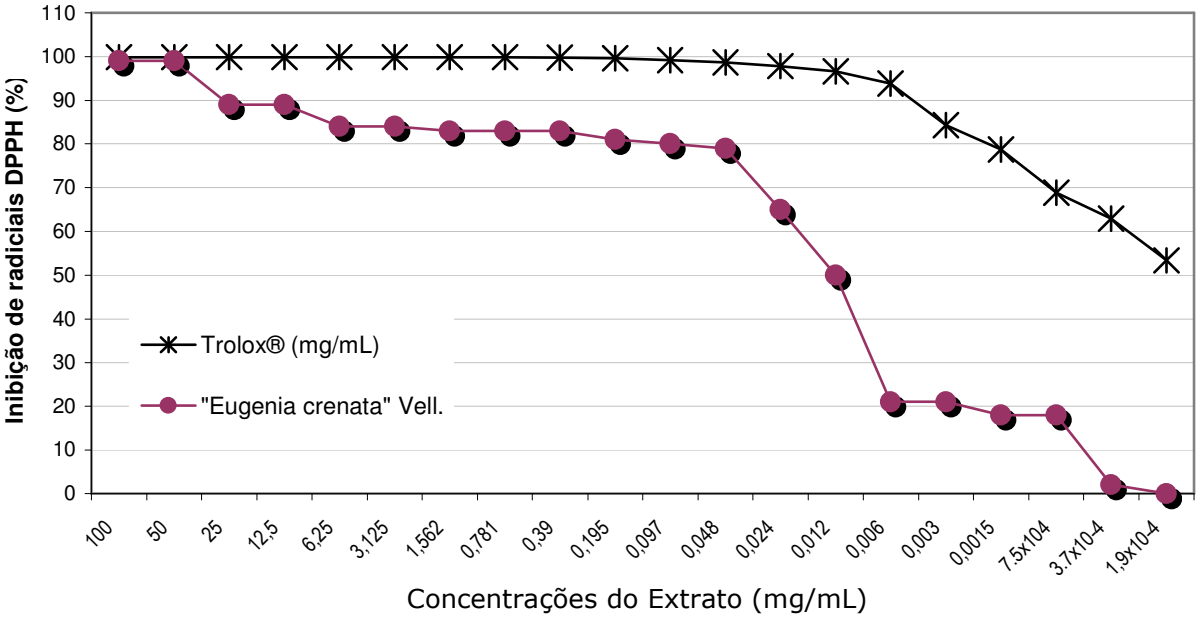
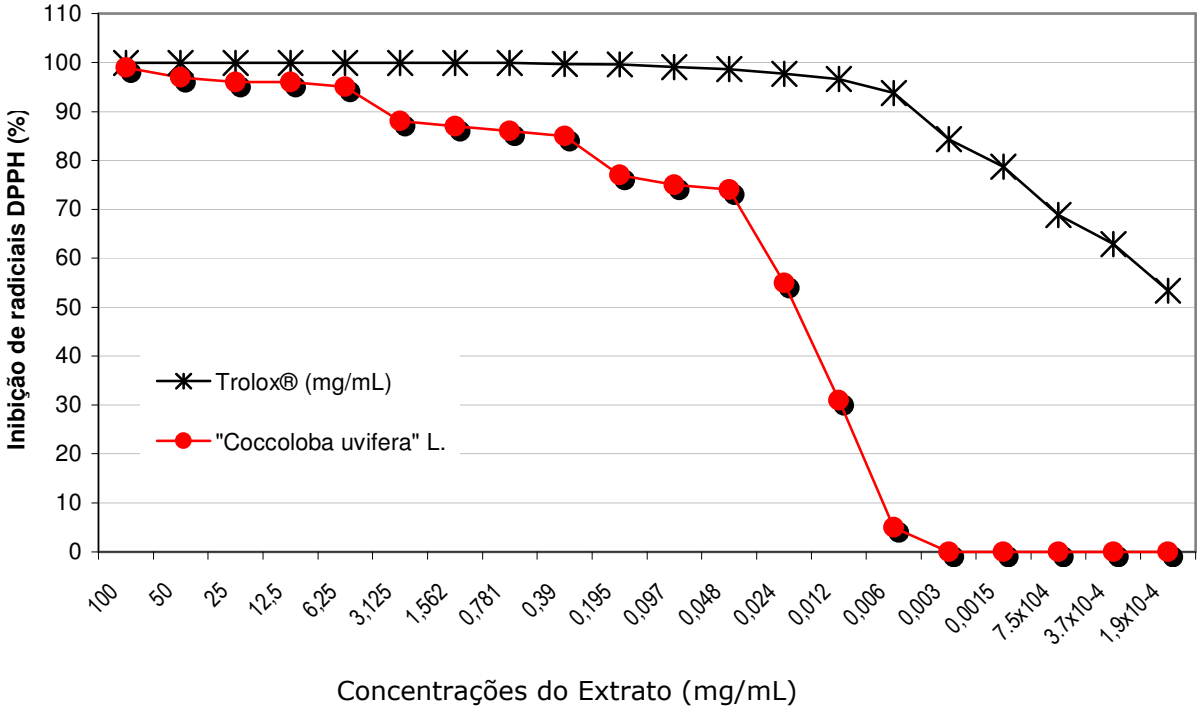
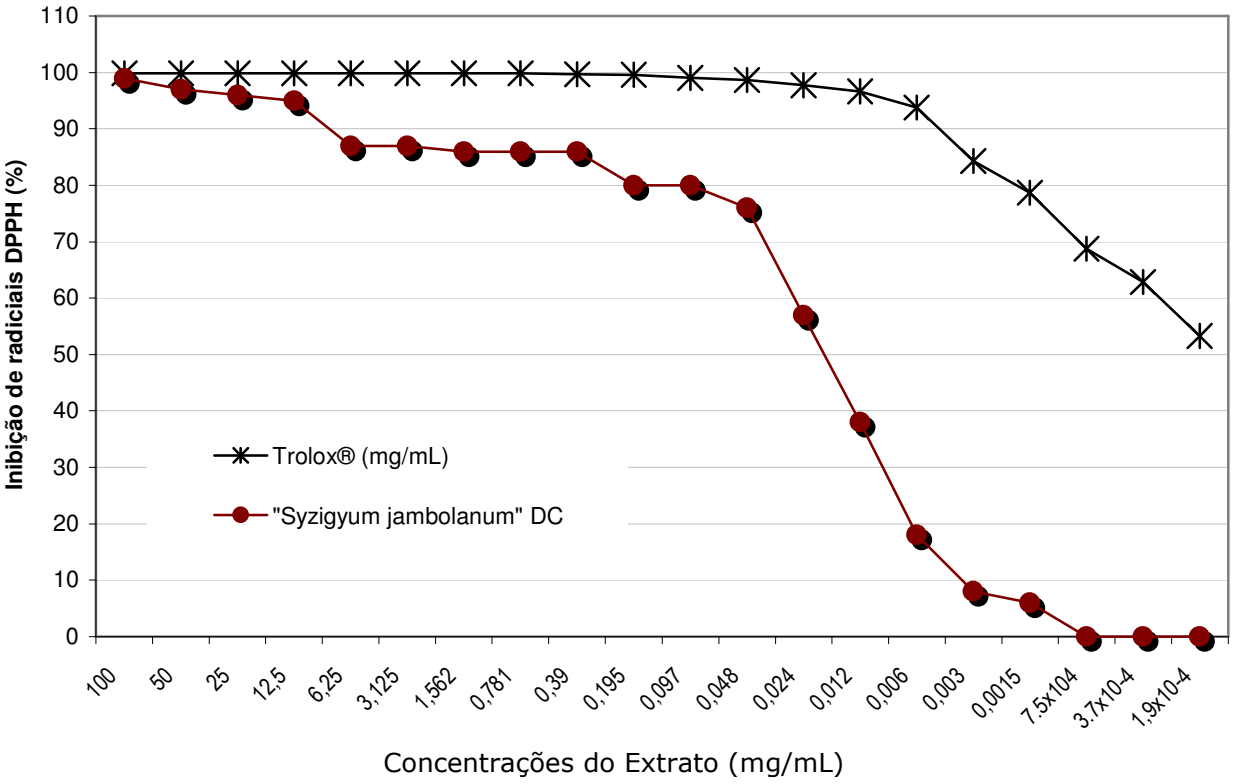
8. Anexos

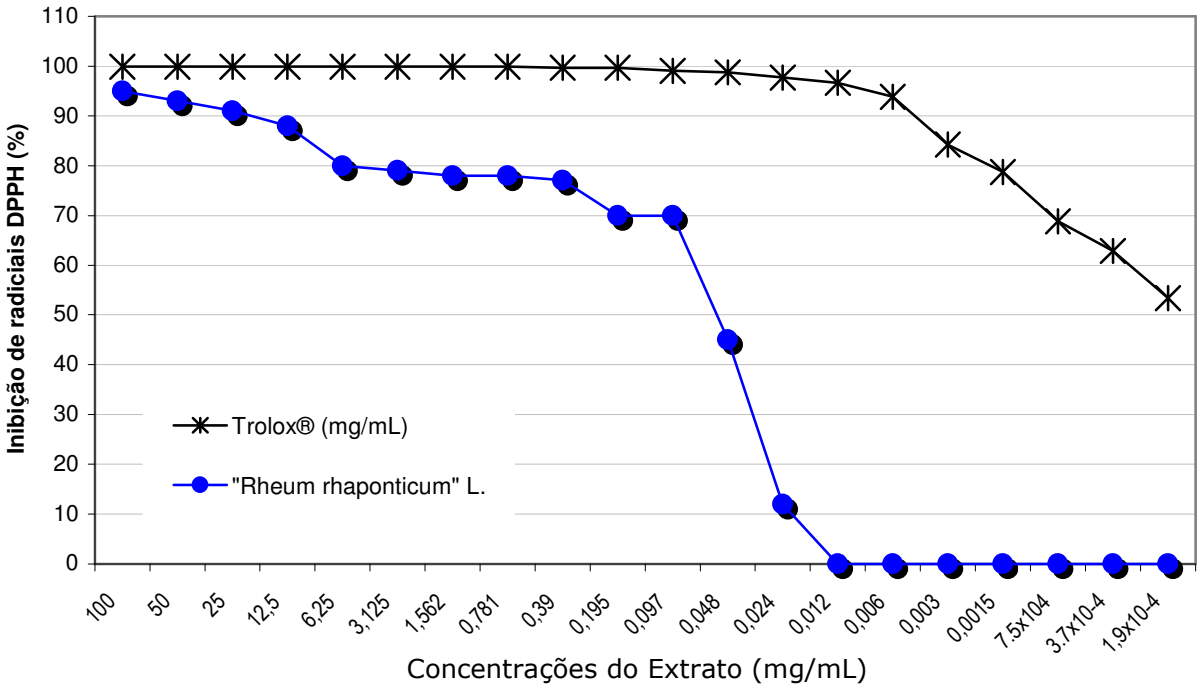
Gráficos referentes à avaliação da atividade antioxidante através da captação de radicais-livres pelo método DPPH

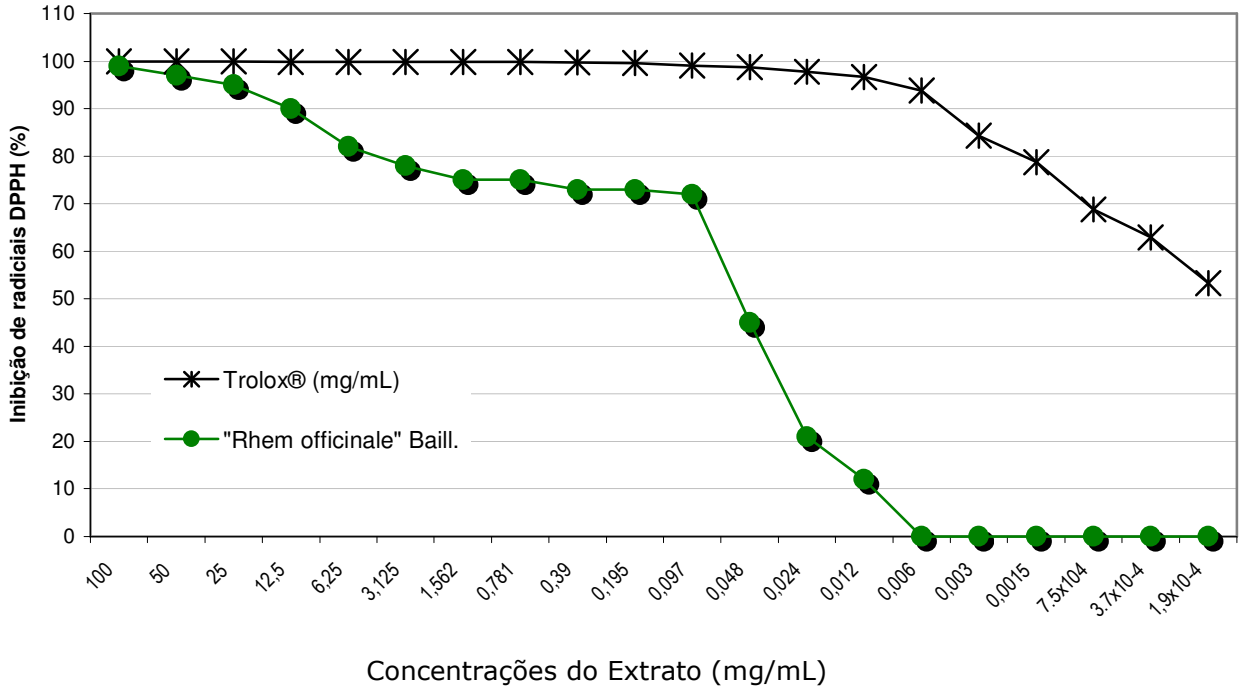


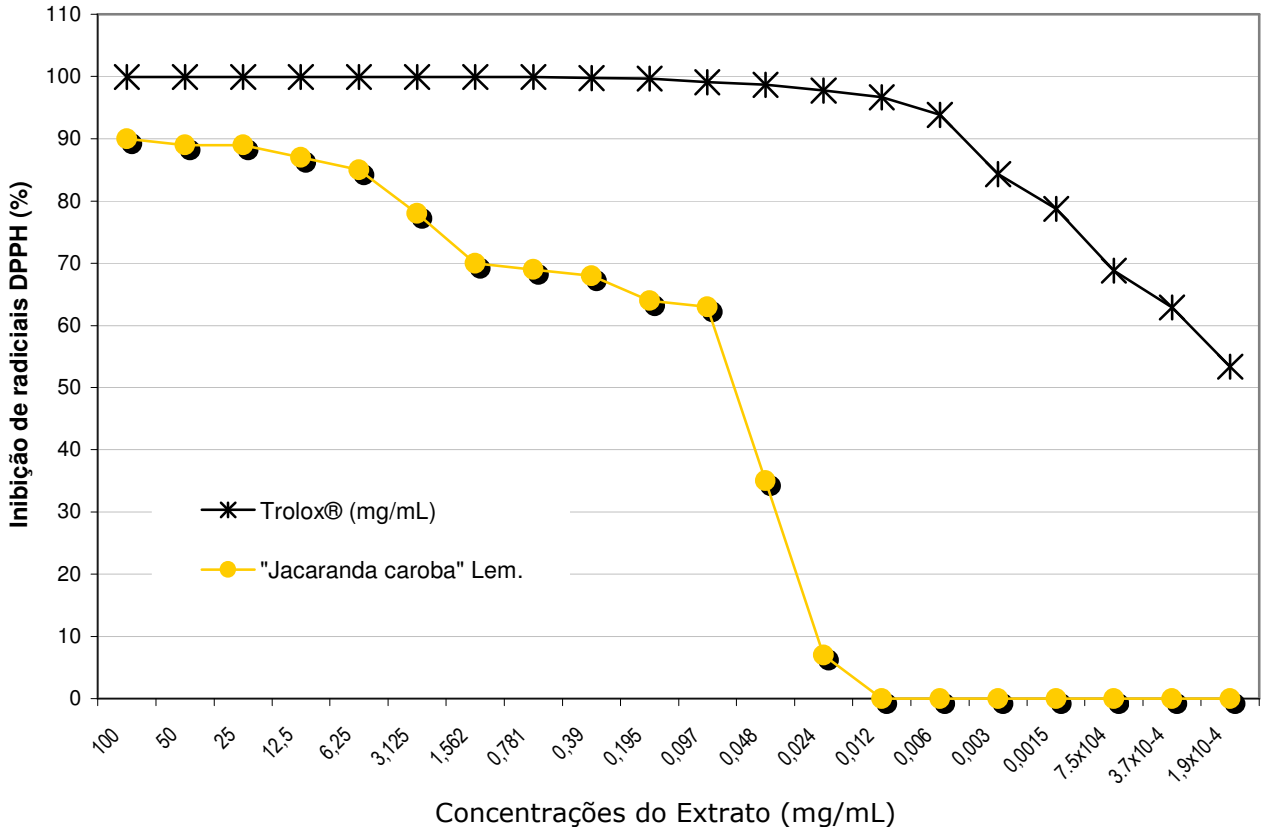

Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Eugenia crenata" Vell.
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)

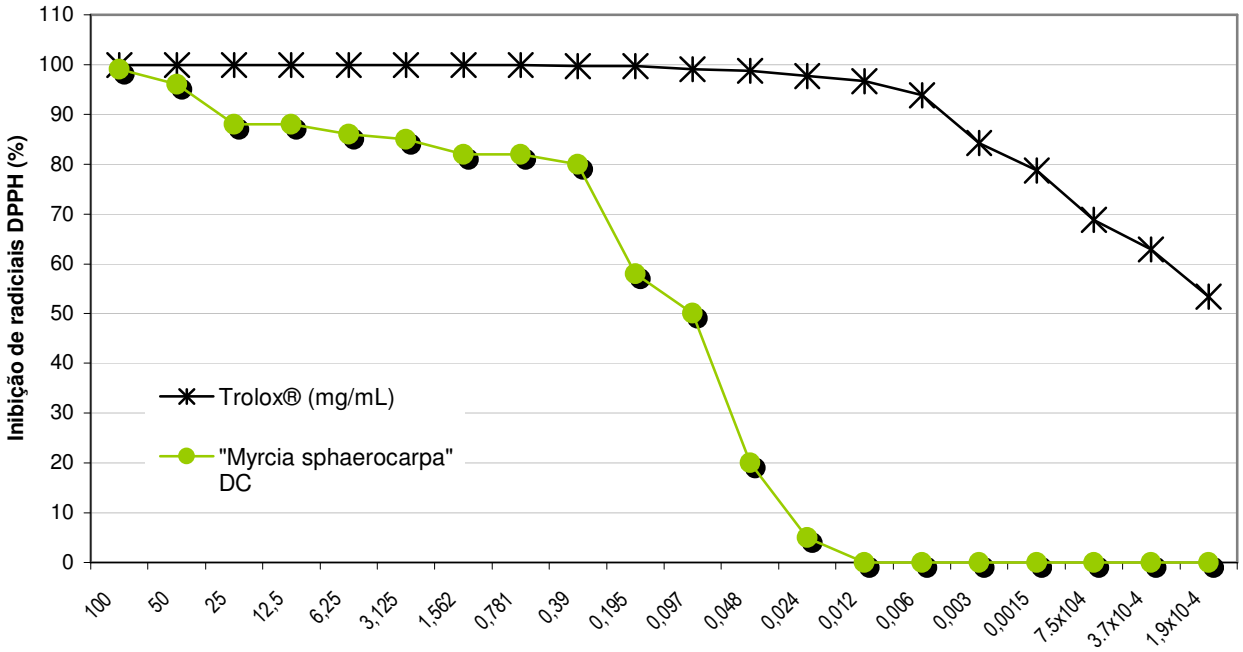

Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Coccoloba uvifera" L.
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)

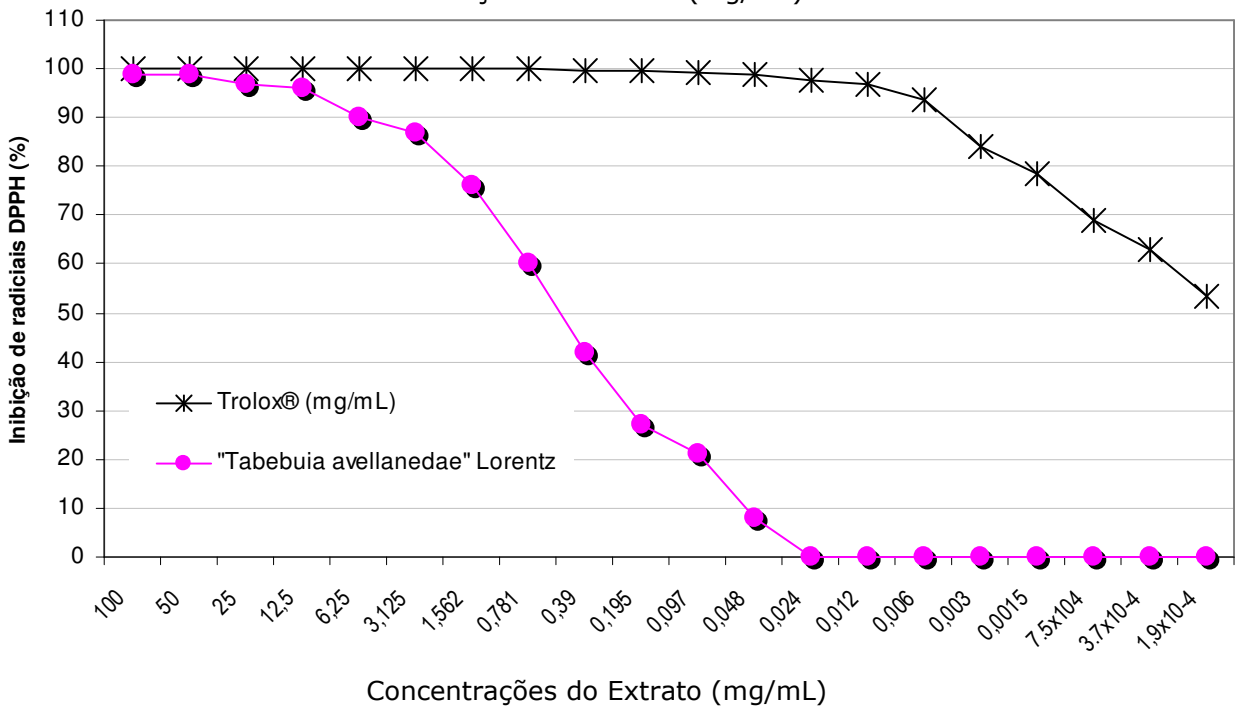

Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Syzigyum jambolanum" DC
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)

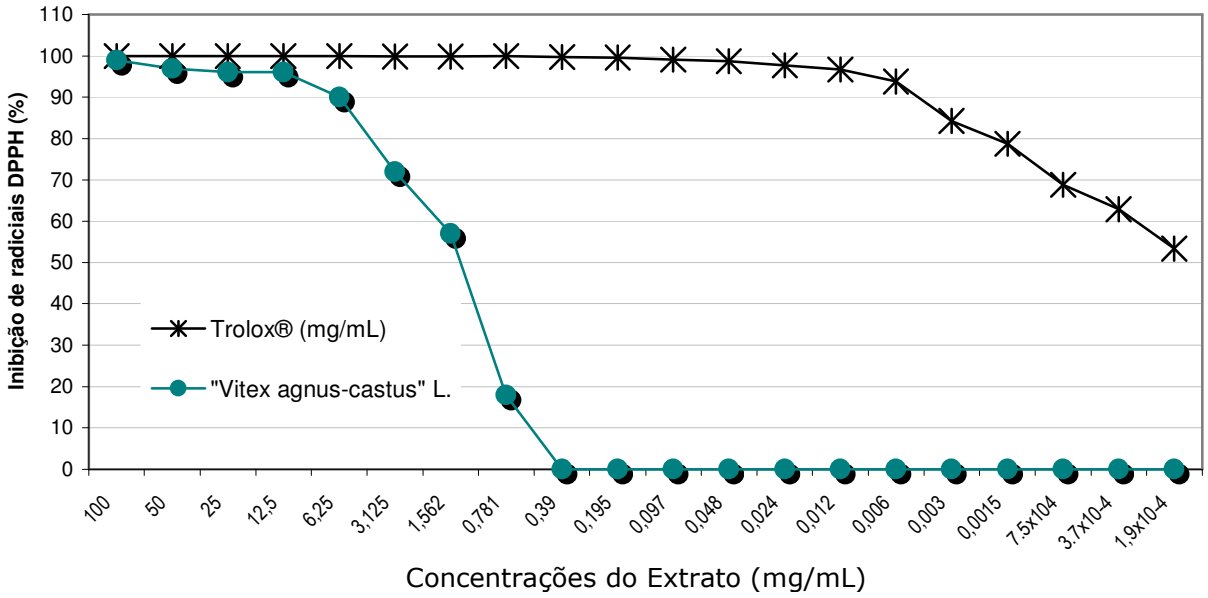

Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Rheum rhaponticum" L.
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)

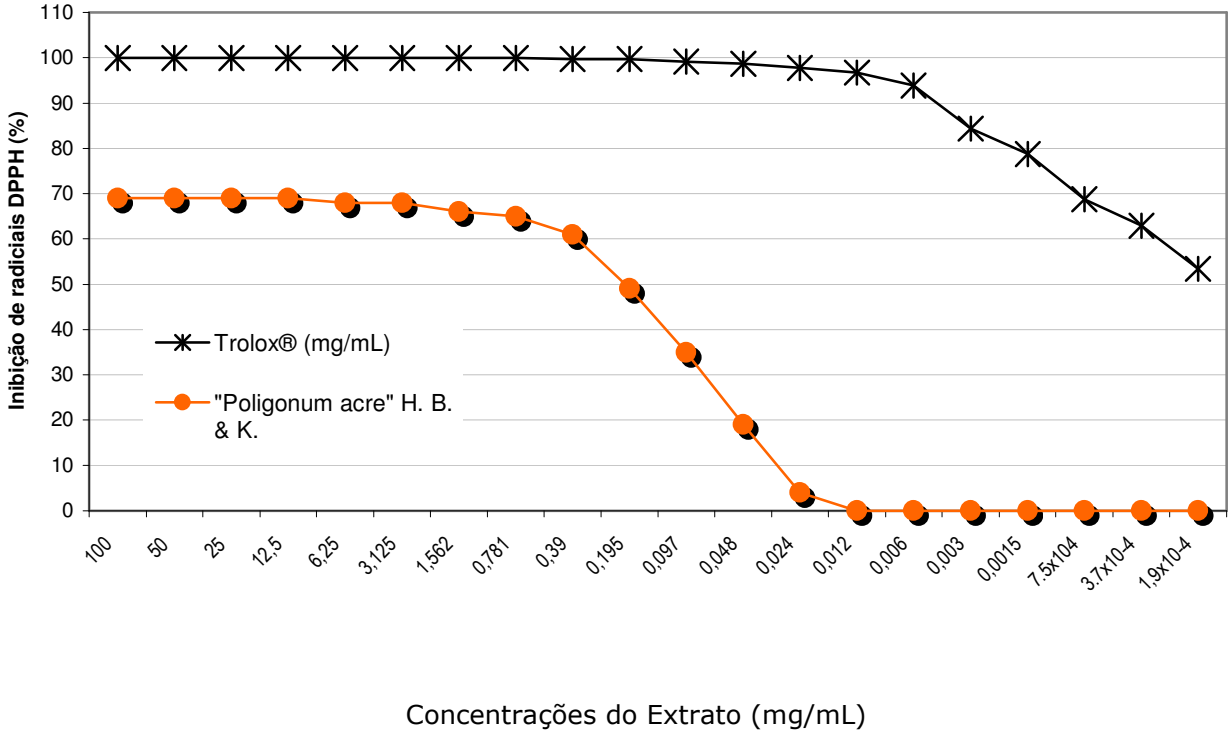

Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Rhem officinale" Baill.
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)

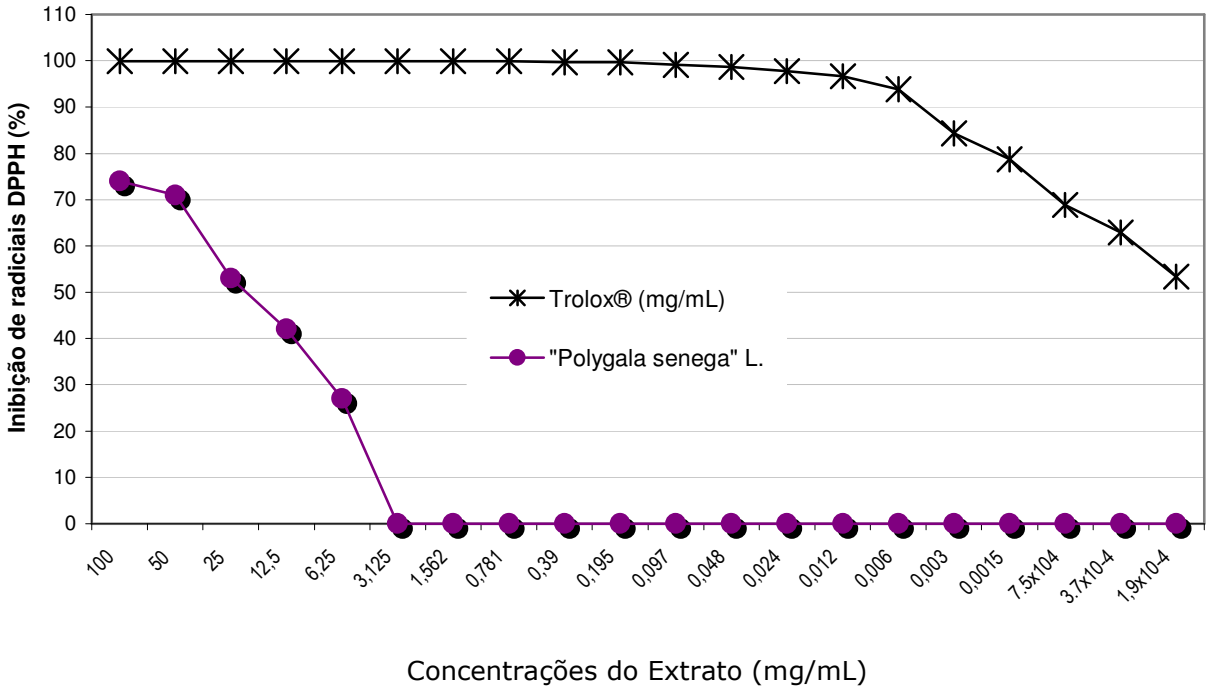

Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Jacaranda caroba" Lem.
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)


Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Myrcia sphaerocarpa" DC
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)


Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Tabebuia avellanedae" Lorentz
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)


Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Vitex agnus-castus" L.
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)


Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Poligonum acre" H. B. & K.
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)


Inibição de radiciais DPPH (%)
110
100
90
80
70
60
50
40
30
20
10
0
Trolox® (mg/mL)
"Polygala senega" L.
100
50
25
12,5
6,25
3,125
1,562
0,781
0,39
0,195
0,097
0,048
0,024
0,012
0,006
0,003
0,0015
7,5x104
3,7x10-4
1,9x10-4
Concentrações do Extrato (mg/mL)

Dados brutos referentes ao Ensaio de Peroxidação Lipídica

Amostra	Nome científico	[sol.estoque]	[na reação]	Abs 1	Abs 2	Média Abs
Branco 1	Branco 1	-	-	0,033	0,031	0,0320
Branco 2	Branco 2	-	-	0,032	0,033	0,0325
10	10	-	-	0,034	0,037	0,0355
20	20	-	-	0,592	0,6	0,5960
40	40	-	-	0,554	0,541	0,5475
60	60	-	-	0,122	0,119	0,1205
80	80	-	-	0,183	0,182	0,1825
100	100	-	-	0,303	0,291	0,2970
120	120	-	-	0,33	0,343	0,3365
140	140	-	-	0,488	0,472	0,4800
C1	C1	-	-	0,52	0,547	0,5335
C2	C2	-	-	0,577	0,578	0,5775
Cmax	Cmax	-	-	0,693	0,689	0,6910
Quercetina	Quercetina	2,50	0,1136	0,489	0,501	0,4950
		5,00	0,2273	0,446	0,432	0,4390
		10,00	0,4545	0,338	0,336	0,3370
		20,00	0,9091	0,246	0,242	0,2440
		40,00	1,8182	0,155	0,157	0,1560
Ruibarbo chinês	*Rheum officinale*Baill.	6,25	0,2841	0,539	0,514	0,5265
		12,5	0,5682	0,51	0,498	0,5040
		25	1,1364	0,458	0,44	0,4490
		50	2,2727	0,434	0,389	0,4115
		100	4,5455	0,324	0,315	0,3195
		200	9,0909	0,239	0,218	0,2285
Pitanga	*Eugenia uniflora* L.	6,25	0,2841	0,579	0,581	0,5800
		12,5	0,5682	0,562	0,546	0,5540
		25	1,1364	0,547	0,542	0,5445
		50	2,2727	0,526	0,509	0,5175
		100	4,5455	0,439	0,424	0,4315
		200	9,0909	0,336	0,372	0,3540
		400	18,1818	0,031	0,03	0,0305
Ruibarbo europeu	*Rheum rhaponticum*L.	6,25	0,2841	0,584	0,611	0,5975
		12,50	0,5682	0,579	0,54	0,5595
		25,0	1,1364	0,547	0,536	0,5415
		50	2,2727	0,498	0,503	0,5005
		100	4,5455	0,428	0,424	0,4260
		200	9,0909	0,303	0,287	0,2950
		400	18,1818	0,092	0,086	0,0890

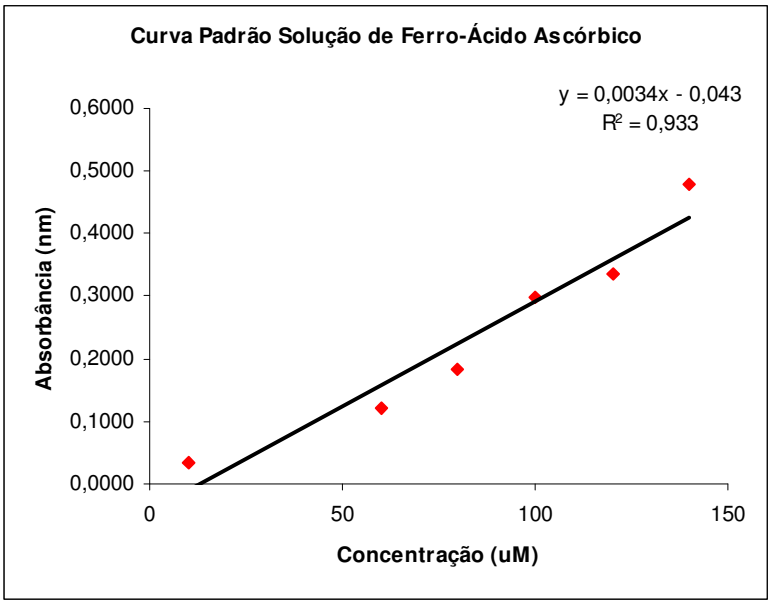
Amostra	Nome científico	[sol.estoque]	[na reação]	Abs 1	Abs 2	Média Abs
Pedra-ume-caá	*Myrcia sphaerocarpa*DC	6,25	0,2841	0,594	0,595	0,5945
		12,50	0,5682	0,605	0,578	0,5915
		25,0	1,1364	0,551	0,557	0,5540
		50	2,2727	0,554	0,558	0,5560
		100	4,5455	0,579	0,554	0,5665
		200	9,0909	0,457	0,458	0,4575
		400	18,1818	0,34	0,328	0,3340
Cambui	*Eugenia crenata*Vell.	6,25	0,2841	0,599	0,585	0,5920
		12,50	0,5682	0,584	0,573	0,5785
		25,0	1,1364	0,542	0,547	0,5445
		50	2,2727	0,468	0,471	0,4695
		100	4,5455	0,34	0,337	0,3385
		200	9,0909	0,158	0,16	0,1590
		400	18,1818	0,046	0,047	0,0465
Jambolão	*Syzigyum jambolanum*DC	6,25	0,2841	0,587	0,551	0,5690
		12,50	0,5682	0,591	0,634	0,6125
		25,0	1,1364	0,555	0,527	0,5410
		50	2,2727	0,509	0,492	0,5005
		100	4,5455	0,4	0,395	0,3975
		200	9,0909	0,262	0,256	0,2590
		400	18,1818	0,1	0,097	0,0985
Erva de bicho	*Polygonum acre*H.B. & K.	6,25	0,2841	0,536	0,528	0,5320
		12,50	0,5682	0,515	0,523	0,5190
		25,0	1,1364	0,521	0,531	0,5260
		50	2,2727	0,496	0,52	0,5080
		100	4,5455	0,489	0,48	0,4845
		200	9,0909	0,451	0,453	0,4520
		400	18,1818	0,394	0,388	0,3910
Agno casto	*Vitex agnu- castus* L.	6,25	0,2841	0,555	0,557	0,5560
		12,50	0,5682	0,573	0,541	0,5570
		25,0	1,1364	0,56	0,561	0,5605
		50	2,2727	0,542	0,541	0,5415
		100	4,5455	0,538	0,552	0,5450
		200	9,0909	0,497	0,504	0,5005
		400	18,1818	0,486	0,497	0,4915

Amostra	Nome científico	[sol.estoque]	[na reação]	Abs 1	Abs 2	Média Abs
Polígala	*Polygala senega*L.	6,25	0,2841	0,511	0,508	0,5095
		12,50	0,5682	0,517	0,513	0,5150
		25,0	1,1364	0,522	0,51	0,5160
		50	2,2727	0,52	0,495	0,5075
		100	4,5455	0,516	0,515	0,5155
		200	9,0909	0,511	0,506	0,5085
		400	18,1818	0,506	0,497	0,5015
Uva de mato	*Coccoloba uvifera*L.	6,25	0,2841	0,618	0,605	0,6115
		12,50	0,5682	0,568	0,611	0,5895
		25,0	1,1364	0,569	0,573	0,5710
		50	2,2727	0,54	0,541	0,5405
		100	4,5455	0,397	0,405	0,4010
		200	9,0909	0,207	0,205	0,2060
		400	18,1818	0,144	0,138	0,1410
Carobinha	*Jacaranda caroba* Lem.	6,25	0,2841	0,712	0,712	0,7120
		12,50	0,5682	0,633	0,615	0,6240
		25,0	1,1364	0,618	0,617	0,6175
		50	2,2727	0,609	0,607	0,6080
		100	4,5455	0,594	0,614	0,6040
		200	9,0909	0,587	0,589	0,5880
		400	18,1818	0,55	0,564	0,5570
Ácido kójico	Ácido kójico	6,25	0,2841	0,656	0,624	0,6400
		12,50	0,5682	0,581	0,568	0,5745
		25,0	1,1364	0,621	0,609	0,6150
		50	2,2727	0,656	0,654	0,6550
		100	4,5455	0,639	0,631	0,6350
		200	9,0909	0,617	0,62	0,6185
		400	18,1818	0,607	0,596	0,6015
Ipê roxo	*Tabebuia avellanedae* Lorentz	6,25	0,2841	0,67	0,624	0,6470
		12,50	0,5682	0,629	0,616	0,6225
		25,0	1,1364	0,61	0,62	0,6150
		50	2,2727	0,588	0,653	0,6205
		100	4,5455	0,596	0,603	0,5995
		200	9,0909	0,586	0,595	0,5905
		400	18,1818	0,574	0,573	0,5735

Amostra	Nome científico	% Inib 1	% Inib 2	% Inib t	equação da reta	R2	IC_{50}
Quercetina	Quercetina	29%	27%	28%	y=0,4466x+0,2616	0,9545	0,53
		36%	37%	36%			
		51%	51%	51%			
		65%	65%	65%			
		78%	77%	77%			
Ruibarbo chinês	*Rheum officinale* Baill.	17%	12%	15%	y=0,053x+0,1857	0,9421	5,93
		22%	15%	19%			
		30%	25%	28%			
		33%	34%	34%			
		50%	46%	48%			
		63%	63%	63%			
Pitanga	*Eugenia uniflora* L.	11%	1%	6%	y=0,0476x+0,06	0,9897	9,24
		14%	7%	11%			
		16%	8%	12%			
		19%	13%	16%			
		33%	28%	30%			
		48%	37%	43%			
		95%	95%	95%			
Ruibarbo europeu	*Rheum rhaponticum* L.	10%	6%	8%	y=0,0423x+0,1225	0,9865	8,92
		11%	17%	14%			
		16%	18%	17%			
		24%	23%	23%			
		34%	35%	35%			
		53%	56%	55%			
		86%	87%	86%			
Pedra-ume-caá	*Myrcia sphaerocarpa* DC	9%	9%	9%	y=0,0218x+0,0868	0,9614	18,95
		7%	11%	9%			
		15%	15%	15%			
		15%	15%	15%			
		11%	15%	13%			
		30%	30%	30%			
		48%	50%	49%			
Cambui	*Eugenia crenata* Vell.	8%	10%	9%	y=0,0485x+0,1524	0,9069	7,17
		10%	12%	11%			
		17%	16%	16%			
		28%	28%	28%			
		48%	48%	48%			
		76%	75%	76%			
		93%	93%	93%			

Amostra	Nome científico	% Inib 1	% Inib 2	% Inib t	equação da reta	R2	IC_{50}
Jambolão	*Syzigyum jambolanum*DC	10%	16%	13%	y=0,043x+0,1261	0,9517	8,70
		9%	3%	6%			
		15%	19%	17%			
		22%	25%	23%			
		39%	40%	39%			
		60%	61%	60%			
		85%	85%	85%			
Erva de bicho	*Polygonum acre* H.B. & K.	23%	23%	23%	y=0,0112x+0,2368	0,9855	23,50
		26%	24%	25%			
		25%	23%	24%			
		28%	25%	26%			
		29%	30%	30%			
		35%	34%	35%			
		43%	44%	43%			
Agno casto	*Vitex agnus-castus* L.	20%	19%	20%	y=0,0059x+0,1941	0,8737	51,85
		17%	21%	19%			
		19%	19%	19%			
		22%	21%	22%			
		22%	20%	21%			
		28%	27%	28%			
		30%	28%	29%			
Polígala	*Polygala senega*L.	26%	26%	26%	y=0,0009x+0,2566	0,5702	270,44
		25%	26%	25%			
		25%	26%	25%			
		25%	28%	27%			
		26%	25%	25%			
		26%	27%	26%			
		27%	28%	27%			
Uva de mato	*Coccoloba uvifera* L.	9%	11%	10%	y=0,0378x+0,1282	0,9584	9,84
		16%	10%	13%			
		16%	15%	16%			
		20%	20%	20%			
		41%	40%	41%			
		69%	70%	70%			
		79%	80%	79%			
Carobinha	*Jacaranda caroba* Lem.	-5%	-5%	-5%	y=0,03053x+0,0839	0,9827	13,63
		7%	9%	8%			
		9%	9%	9%			
		10%	10%	10%			
		12%	9%	11%			
		13%	13%	13%			
		19%	17%	18%			

Amostra	Nome científico	% Inib 1	% Inib 2	% Inib t	equação da reta	R2	IC_{50}
Ácido kójico	Ácido kójico	3%	8%	6%	y=0,0045x+0,0357	0,8998	103,18
		14%	16%	15%			
		8%	10%	9%			
		3%	4%	3%			
		6%	7%	6%			
		9%	9%	9%			
		10%	12%	11%			
Ipê roxo	*Tabebuia avellanedae* Lorentz	1%	8%	5%	y=0,0038x+0,0883	0,9041	108,34
		7%	9%	8%			
		10%	9%	9%			
		13%	4%	8%			
		12%	11%	12%			
		14%	12%	13%			
		15%	15%	15%			



Dados brutos referentes ao Ensaio Antitirosinase *in vitro*

Amostras	Nome científico	[sol.estoque]	[na reação]	[DopaCr]1	[DopaCr]2	Média
BL	BL	----	----	----	----	----
65,78	65,78	----	----	58,614273	57,87917	58,25
95,67	95,67	----	----	99,255014	92,42905	95,84
148	148	----	----	151,34237	157,1182	154,23
222	222	----	----	233,46397	237,9796	235,72
333	333	----	----	328,92246	317,8959	323,41
Controle	Controle	----	----	35,405995	35,82605	35,62
		----	----	311,38498	297,208	304,30
Ácido kójico	Ácido kójico	17,5	2,625	328,39738	337,5337	332,97
		35	5,25	280,30059	262,238	271,27
		70	10,5	216,13652	211,8309	213,98
		140	21	149,97718	161,3188	155,65
		280	42	72,056172	84,13288	78,09
Ruibarbo chinês	*Rheum officinale* Baill.	50	7,5	308,0245	321,6764	314,85
		100	15	287,96667	301,5136	294,74
		200	30	280,4056	284,1861	282,30
		400	60	286,91652	253,9419	270,43
		800	120	262,23804	283,451	272,84
		1600	240	206,0551	217,9218	211,99
		3200	480	157,43323	170,9801	164,21
Pitanga	*Eugenia uniflora* L.	50	7,5	327,59596	315,6045	321,60
		100	15	316,57682	303,9372	310,26
		200	30	334,61796	346,3933	340,51
		400	60	280,38649	265,0461	272,72
		800	120	213,73148	225,7229	219,73
		1600	240	219,99727	223,8864	221,94
		3200	480	224,3185	236,634	230,48
Cambui	*Eugenia crenata* Vell.	50	7,5	328,89233	321,6543	325,27
		100	15	306,09783	309,6628	307,88
		200	30	281,89892	315,9286	298,91
		400	60	257,05184	283,6274	270,34
		800	120	274,01267	252,4065	263,21
		1600	240	182,40254	215,46	198,93
		3200	480	184,88725	129,2514	157,07

Amostras	Nome científico	[sol.estoque]	[na reação]	[DopaCr]1	[DopaCr]2	Média
Jambolão	*Syzigyum jambolanum* DC	50	7,5	341,09981	342,9363	342,02
		100	15	331,05295	334,5099	332,78
		200	30	298,53567	296,159	297,35
		400	60	267,53083	296,159	281,84
		800	120	284,2756	259,1044	271,69
		1600	240	280,60255	279,8463	280,22
		3200	480	156,90727	171,1673	164,04
Pedra-ume-caá	*Myrcia sphaerocarpa*DC	50	7,5	362,70597	381,6114	372,16
		100	15	349,20212	392,1984	370,70
		200	30	336,45449	384,8523	360,65
		400	60	303,28903	317,7652	310,53
		800	120	285,89606	276,0653	280,98
		1600	240	277,90178	344,4488	311,18
		3200	480	262,8855	277,0375	269,96
Agno casto	*Vitex agnus-castus* L.	50	7,5	356,10487	353,5278	354,82
		100	15	323,09405	358,5592	340,83
		200	30	357,94562	359,9091	358,93
		400	60	336,47018	331,9297	334,20
		800	120	307,26358	318,3081	312,79
		1600	240	294,01017	300,7596	297,38
		3200	480	262,59467	266,0307	264,31
Erva de bicho	*Polygonum acre* H.B. & K.	50	7,5	361,62712	362,118	361,87
		100	15	350,45989	354,8777	352,67
		200	30	358,68192	355,2458	356,96
		400	60	359,90909	361,0135	360,46
		800	120	324,44393	326,162	325,30
		1600	240	276,46166	279,1614	277,81
		3200	480	226,14778	219,8892	223,02
Polígala	*Polygala senega* L.	50	7,5	372,30348	381,9981	377,15
		100	15	391,93817	385,4342	388,69
		200	30	374,26695	380,0346	377,15
		400	60	361,87256	358,6819	360,28
		800	120	365,92221	378,3166	372,12
		1600	240	345,4285	363,2224	354,33
		3200	480	325,42567	320,0261	322,73
Ruibarbo europeu	*Rheum rhaponticum* L.	50	7,5	338,0655	347,5147	342,79
		100	15	311,43595	312,6631	312,05
		200	30	259,40403	269,4668	264,44
		400	60	218,78477	214,4897	216,64
		800	120	141,35047	139,5097	140,43
		1600	240	84,28716	88,33681	86,31
		3200	480	52,135359	51,39906	51,77

Amostras	Nome científico	[sol.estoque]	[na reação]	[DopaCr]1	[DopaCr]2	Média
Carobinha	*Jacaranda caroba* Lem.	50	7,5	297,28811	298,2034	297,75
		100	15	310,91541	329,4241	320,17
		200	30	325,86475	327,3902	326,63
		400	60	287,72867	291,9999	289,86
		800	120	256,50792	250,2028	253,36
		1600	240	210,94801	189,8969	200,42
		3200	480	128,06558	123,6926	125,88
Ipê roxo	*Tabebuia avellanedae* Lorentz	50	7,5	303,18649	305,017	304,10
		100	15	295,25419	281,7286	288,49
		200	30	312,84764	290,7796	301,81
		400	60	329,62751	327,1868	328,41
		800	120	288,84733	296,2712	292,56
		1600	240	244,20268	249,2875	246,75
		3200	480	230,37199	228,7449	229,56
Uva de mato	*Coccoloba uvifera* L.	50	7,5	289,4575	274,5082	281,98
		100	15	296,06776	311,2205	303,64
		200	30	304,81363	306,6442	305,73
		400	60	225,89736	237,5924	231,74
		800	120	126,03166	134,5741	130,30
		1600	240	48,437434	55,04769	51,74
		3200	480	10,097955	42,74245	26,42

Amostras	Nome científico	% Inib 1	% Inib 2	% Inib t	equação da reta	R^2	IC_{50}
Ácido kójico	Ácido kójico	0%	-3%	-3%	y=0,279Ln(x)-0,3065	0,9966	0,02
		15%	16%	16%			
		34%	34%	34%			
		54%	52%	52%			
		78%	76%	76%			
Ruibarbo chinês	*Rheum officinale* Baill.	6%	3%	3%	y=0,1069Ln(x)-0,32184	0,9505	2,18
		12%	9%	9%			
		15%	13%	13%			
		13%	16%	16%			
		20%	16%	16%			
		37%	34%	34%			
		52%	49%	49%			
Pitanga	*Eugenia uniflora* L.	-6%	-4%	-4%	y=0,095Ln(x)-0,274	0,748	3,45
		-2%	0%	0%			
		-8%	-10%	-10%			
		9%	12%	12%			
		31%	29%	29%			
		29%	28%	28%			
		28%	26%	26%			
Cambui	*Eugenia crenata* Vell.	-6%	-5%	-5%	y=0,1262Ln(x)-0,3576	0,9122	0,89
		1%	1%	1%			
		9%	3%	3%			
		17%	13%	13%			
		12%	15%	15%			
		41%	36%	36%			
		40%	49%	49%			
Jambolão	*Syzigyum jambolanum* DC	-10%	-11%	-11%	y=0,1107Ln(x)-0,3624	0,7721	2,42
		-7%	-8%	-8%			
		4%	4%	4%			
		14%	9%	9%			
		8%	12%	12%			
		9%	9%	9%			
		49%	47%	47%			

Amostras	Nome científico	% Inib 1	% Inib 2	% Inib t	equação da reta	R^2	IC_{50}
Pedra-ume-caá	*Myrcia sphaerocarpa* DC	-17%	-20%	-20%	y=0,0841Ln(x)-0,395	0,8372	41,86
		-13%	-20%	-20%			
		-9%	-17%	-17%			
		2%	0%	0%			
		8%	9%	9%			
		10%	-1%	-1%			
		15%	13%	13%			
Agno casto	*Vitex agnus-castus* L.	-11%	-13%	-13%	y=0,0664Ln(x)-0,3009	0,8409	173,12
		-1%	-8%	-8%			
		-12%	-14%	-14%			
		-5%	-6%	-6%			
		4%	0%	0%			
		8%	5%	5%			
		18%	16%	16%			
Erva de bicho	*Polygonum acre* H.B.& K.	-13%	-15%	-15%	y=0,0981Ln(x)-0,4284	0,7515	12,88
		-10%	-12%	-12%			
		-12%	-14%	-14%			
		-13%	-15%	-15%			
		-1%	-4%	-4%			
		14%	12%	12%			
		29%	29%	29%			
Polígala	*Polygala senega*L.	-16%	-20%	-20%	y=0,0389Ln(x)-0,3199	0,7092	1,42E+06
		-23%	-24%	-24%			
		-17%	-20%	-20%			
		-13%	-15%	-15%			
		-14%	-18%	-18%			
		-8%	-13%	-13%			
		-2%	-3%	-3%			
Ruibarbo europeu	*Rheum rhaponticum* L.	-6%	-9%	-9%	y=0,2509Ln(x)-0,5088	0,9913	0,06
		3%	1%	1%			
		19%	16%	16%			
		32%	31%	31%			
		56%	55%	55%			
		74%	73%	73%			
		84%	84%	84%			

Amostras	Nome científico	% Inib 1	% Inib 2	% Inib t	equação da reta	R^2	IC50
Carobinha	*Jacaranda caroba* Lem.	-7%	-4%	8%	y=0,0013Ln(x)-0,028	0,957	2,46E+173
		-27%	-32%	1%			
		-194%	-162%	-1%			
		3%	3%	11%			
		17%	21%	22%			
		35%	39%	38%			
		55%	57%	61%			
Ipê roxo	*Tabebuia avellanedae* Lorentz	-18%	-20%	6%	y=0,0502Ln(x)-0,0818	0,5009	107,97
		-40%	-44%	11%			
		-144%	-140%	7%			
		-9%	-8%	-1%			
		2%	-1%	10%			
		22%	18%	24%			
		30%	30%	29%			
Uva de mato	*Coccoloba uvifera* L.	0%	4%	13%	y=0,2294Ln(x)-0,5253	0,8687	0,09
		-21%	-23%	6%			
		-32%	-33%	6%			
		22%	18%	29%			
		57%	57%	60%			
		84%	83%	84%			
		96%	89%	92%			