UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

PROPRIEDADES FUNGICIDA E ANTIOXIDANTE DE EXTRATOS VEGETAIS

Leandro Kenji Takao

SÃO CARLOS
2015

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Tese apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais, para obtenção do título de doutor em Ecologia e Recursos Naturais.

Orientação: Profa. Dra. Sonia Cristina Juliano Gualtieri

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2015

Ficha catalográfica elaborada pelo DePT da Biblioteca Comunitária/UFSCar

T136pf

Takao, Leandro Kenji.
Propriedades fungicida e antioxidante de extratos vegetais / Leandro Kenji Takao. -- São Carlos : UFSCar, 2015.
94 f.

Tese (Doutorado) -- Universidade Federal de São Carlos, 2015.

1. Cerrados. 2. *Alternaria alternata*. 3. DPPH. 4. *Psidium laruotteanum*. 5. Ultrassom. I. Título.

CDD: 574.5247 (20ª)

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ecologia e Recursos Naturais

Folha de Aprovação

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Profa. Dra. Silmara Cristina Fanti
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Dedico este trabalho aos amigos que
mais me incentivaram, José Pedro,
Maristela e Rosa

AGRADECIMENTOS

À profa. Sonia Cristina Juliano Gualtieri pela paciência, orientação e confiança.

À Maristela Imatomi pelo incentivo, orientações e companheirismo. Serei eternamente grato.

Aos professores da Universidad de Cádiz, Miguel Palma, Ascension Torres Martines, Francisco Antonio Macías e José Maria Gonzalez Molinillo, por seus ensinamentos sobre química e pela hospitalidade. Agradeço imensamente à profa. Rosa Varela Montoya pela incrível atenção, orientação e carinho.

Muito obrigado ao José Pedro Nepomuceno Ribeiro e ao Reginaldo Sadao Matsumoto pela amizade e incentivo científico sem os quais não teria começado esta jornada.

À Widiastuti Setyaningsih e ao Irfan Saputro pela amizade durante o meu período em Cádiz e pelos agradáveis momentos ao lado do UPLC.

Às companheiras de laboratório da Botânica/UFSCar, Maria Augusta Machado, Viviane de Cássia Pereira, Luciana Jatobá, Simoni Anese e Flávia Pontes, pela companhia de todos os dias e imensa ajuda nos trabalhos.

A todos os meus amigos que fizeram desse período algo sempre mais tranquilo.

À minha família pela compreensão, eterno apoio e carinho.

Ao Programa de Pós-Graduação em Ecologia e Recursos Naturais e todo o corpo de funcionários, especialmente ao João e à Roseli.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Pessoal de Nível Superior (CAPES), pelas bolsas concedidas.

Enfim, agradeço a todos que de alguma forma me ajudaram a aprender.

RESUMO

O cerrado possui a flora mais rica entre as savanas tropicais, sendo um dos pontos quentes para conservação da biodiversidade no mundo. No entanto, o potencial químico de suas espécies vegetais é ainda subutilizado, enquanto o desmatamento e a perda de biodiversidade são questões de grande relevância. No cerrado, as plantas estão expostas a pressões ambientais (competição, patógenos, seca, radiação UV nociva, etc) que podem estimular a produção de metabólitos secundários como defesa. Estes compostos geralmente podem ser explorados devido suas propriedades biológicas. O objetivo deste estudo foi avaliar as propriedades fungicidas e antioxidante de plantas do cerrado. Primeiro uma triagem foi realizada com extratos etanólicos de folhas de 28 espécies vegetais. Não houve nenhuma atividade contra *Alternaria alternata*, um fungo filamentoso que causa uma das doenças mais severas em tangerinas (mancha marrom de Alternaria). No entanto, os mesmos extratos apresentaram um potencial antioxidante elevado associado com o conteúdo fenólico. Outras investigações foram feitas usando extrações por infusão enfocando a família Myrtaceae, uma das famílias mais abundantes de plantas no cerrado. A maioria das espécies apresentou alta atividade antioxidante e alto conteúdo fenólico. Por fim, a extração de piceido, um composto com alta atividade antioxidante, foi otimizada de raízes de *Psidium laruotteanum*. Uma metodologia de superfície de resposta foi aplicada para avaliar a influência do solvente, pH, amplitude, ciclo, temperatura e razão solvente-sólido usando uma extração de ultrasonido. Uma alta quantidade de piceido foi extraída e a raiz de *Psidium laruotteanum* se mostrou como sendo a fonte mais concentrada deste composto relatada até hoje. Estes resultados apontam as espécies de plantas do cerrado como uma importante fonte de compostos bioativos, enfatizando a necessidade de preservar e estudar sua biodiversidade.

Palavras-Chave: antioxidante, cerrado, DPPH, fenóis, piceid, ultrasom.

ABSTRACT

The Brazilian savanna holds the richest flora among the tropical savannas, being one of the hot spots for conservation of the biodiversity in the world. However, the chemical potential of its plant species is still underused while deforestation and loss of biodiversity are main issues. In the Brazilian savanna, plants are exposed to environmental pressures (competition, pathogens, drought, injurious UV radiation, etc) that may stimulate the production of secondary metabolites as a defense. These compounds can generally be explored due to their biological properties. The aim of this study was to assess the fungicidal and antioxidant properties of plants from the Brazilian savanna. First a screening was conducted with ethanolic leaf extracts of 28 plant species. There was no activity against *Alternaria alternata*, a filamentous fungi that causes one of the most severe diseases in tangerines (Alternaria brown spot disease). However, the same extracts presented a high antioxidant potential associated with the phenolic content. Other investigations were made using infusion extractions focusing on the Myrtaceae family, one of the most abundant 'families of plants in the Brazilian savanna. Most species also presented high antioxidant activity and phenolic content. At last, the extraction of piceid, a compound with high antioxidant activity, was optimized from roots of *Psidium laruotteanum*. A response surface methodology was applied to assess the influence of solvent, pH, amplitude, cycle, temperature and solvent-solid ratio using an ultrasound extraction. A high amount of piceid was extracted and *Psidium laruotteanum* root was found to be the most concentrated source of this compound reported up to date. These results point the plant species from the Brazilian savanna as an important source of bioactive compounds, emphasizing the need to preserve and study its biodiversity.

Keywords: Brazilian savanna, DPPH, piceid, phenolics, ultrasound.

ESTRUTURA DA TESE

O primeiro capítulo “Screening of leaf extracts of cerrado species against *Alternaria alternata* from Ponkan tangerines” traz uma triagem de extratos etanólicos de folhas de 28 espécies do cerrado em relação à atividade fungicida sobre *Alternaria alternata*, causador de uma das doenças mais severas em tangerinas. No entanto, nenhum extrato apresentou efeito inibitório. Dessa forma, estudos sobre a atividade fungicida não foram mais aprofundados.

O segundo capítulo “High antioxidant activity and phenolic content of ethanolic leaf extracts of plants from the Brazilian savanna” analisa os mesmos extratos do primeiro capítulo mas com relação à atividade antioxidante, relacionando-a com o conteúdo fenólico dos extratos. Esse artigo foi submetido ao Brazilian Journal of Biology.

O terceiro capítulo “Antioxidant activity and phenolic content of leaf infusions of Myrtaceae species from cerrado (Brazilian savanna)” foca na família Myrtaceae e aborda a extração das folhas através do método de infusão, testando em seguida a atividade antioxidante. Esse artigo foi aceito para publicação pelo Brazilian Journal of Biology e a carta de aceite está anexada no início do referido capítulo.

O último capítulo “Optimized Ultrasound-Assisted Extraction of *Psidium laruotteanum* roots: a concentrated source of piceid from the Brazilian savanna” apresenta a otimização de extração de piceido, um estilbeno com potentes propriedades antioxidantes e medicinais. A raiz de *Psidium laruotteanum* foi analisada após verificarmos alta atividade antioxidante nas folhas dessa espécie e decidirmos ampliar o estudo para outros órgãos. Esse artigo será submetido ao Journal of Agricultural and Food Chemistry na seção de química analítica.

SUMÁRIO

INTRODUÇÃO GERAL

As plantas estão frequentemente sob pressão de fatores bióticos e abióticos (REJEB et al., 2014). Elas desenvolveram uma variedade de compostos oriundos do metabolismo secundário como mecanismo de defesa contra a herbivoria, patógenos, competição, radiação UV-B, seca, entre outros. Esses metabólitos podem ser classificados em três principais grupos, fenólicos, terpenos e os compostos nitrogenados (e.g. alcalóides) (TAIZ e ZEIGER, 2010). A grande riqueza de metabólitos secundários faz das espécies vegetais uma das principais fontes naturais de substâncias químicas bioativas.

Compostos fenólicos

Os compostos fenólicos são os metabólitos sencundários mais abundantes nas espécies vegetais (DAI e MUMPER, 2010). Eles são caracterizados basicamente por possuir um anel aromático com pelo menos um grupo hidroxila ligado (Figura 1) (CROTEAU et al., 2000). Com mais de 8000 compostos já descritos pela literatura, os fenóis variam desde moléculas simples como os ácidos fenólicos (e.g. ácido gálico) a compostos altamente polimerizados como os taninos (e.g. ácido tânico) (Figura 2) (DAI e MUMPER, 2010). Baseando-se na sua estrutura química, pode-se classificar os compostos fenólicos em diferentes grupos, incluindo flavonóides, lignanas, ácidos fenólicos, estilbenos, ligninas e cumarinas (MANACH et al., 2004; VAN DUYNHOVEN et al., 2010).

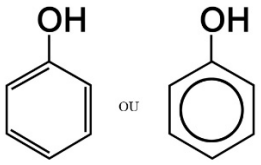

Figura 1. Estrutura química básica de um composto fenólico, com um grupo hidroxila ligado diretamente ao anel aromático.

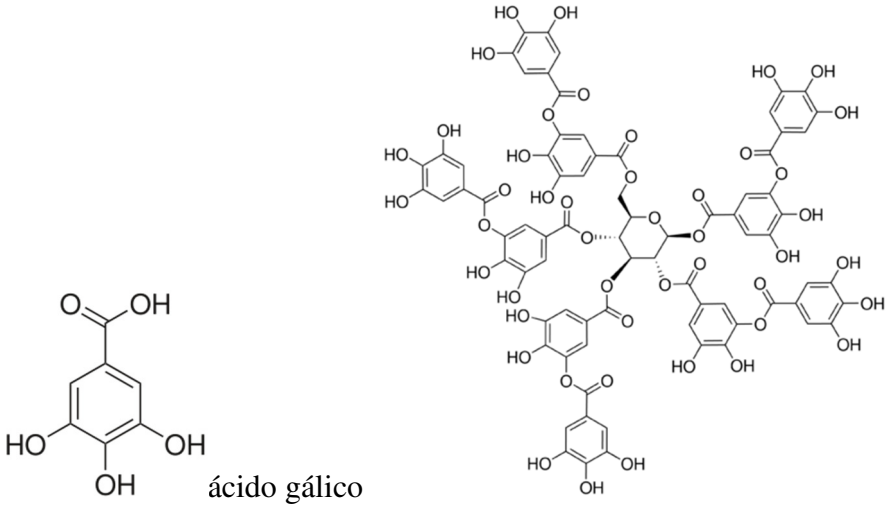

Figura 2. Compostos fenólicos com estrutura simples (ácido gálico) e mais complexa (ácido tânico).

Rotas de biossíntese

Os compostos fenólicos são originados a partir de duas rotas do metabolismo vegetal: do ácido chiquímico, do ácido malônico e da combinação de ambas (Figura 3). A rota do ácido chiquímico é responsável pela síntese da maioria dos compostos fenólicos. A partir de precursores de carboidratos simples resultantes da glicólise (i.e. ácido fosfoenolpirúvico) e da rota da pentose fostato (i.e. eritrose-4-fosfato) é formado o ácido chiquímico. A rota do ácido malônico, por sua vez, produz uma quantidade menos significativa de compostos fenólicos. O ácido malônico é formado a partir da acetilcoenzima A (TAIZ e ZEIGER, 2010).

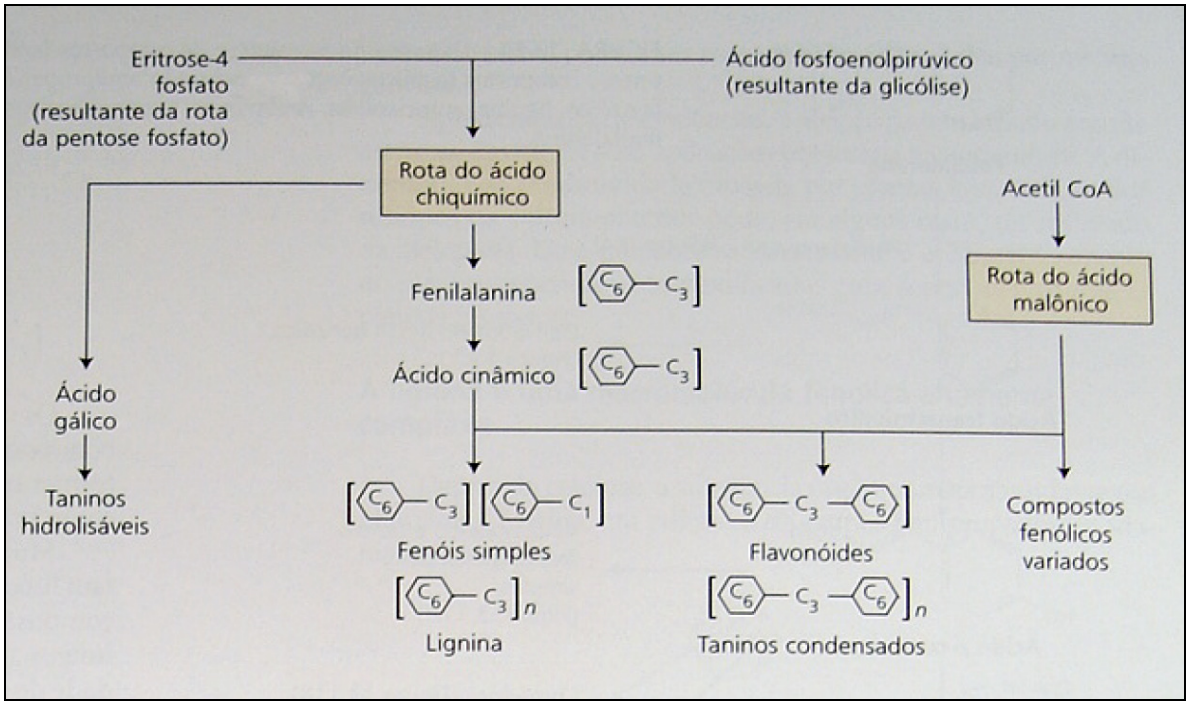

Figura 3. Compostos fenólicos de origem vegetal são sintetizados pelas rotas do ácido chiquímico e do ácido malônico (TAIZ e ZEIGER, 2010).

Armazenamento

Os compostos fenólicos podem estar presentes em todas as partes das plantas, incluindo folhas, frutos, flores, raízes e caules (HARBORNE e WILLIAMS, 1971; ZHOU et al., 2011; HAMINIUK et al., 2012; KUO et al., 2013; HASSAAN et al., 2014). Eles são geralmente encontrados na forma glicosilada, em vez de compostos livres (VERMERRIS e NICHOLSON, 2006). O armazenamento desses compostos pode ocorrer nos vacúolos celulares, similarmente os outros metabólitos secundários. Ao sofrer um estímulo, o composto pode ser liberado do vacúolo (WINK, 1997) e/ou sofrer glicólise, podendo exercer maior atividade.

Atividade biológicas

Muitos estudos mostram o potencial dos compostos fenólicos de origem vegetal em relação a diferentes atividades biológicas, como bactericida (BISIGNANO et al., 1999; MEDINA et al., 2006; ROMERO et al., 2007), inseticida (PAVELA, 2011; SINGH et al., 2014) e fungicida (MARSTON et al., 1988; AHANJAN et al., 2007; SILVA et al., 2007).

Ainda, os compostos fenólicos têm atraído grande atenção sobretudo pela sua capacidade antioxidante e propriedades medicinais relacionadas. A capacidade redutora da hidroxila ligada ao anel aromático dos fenóis é a principal responsável pela propriedade antioxidante; o hidrogênio deste grupo pode ser doado para neutralizar radicais livres reativos (BREWER, 2011), bloqueando a cadeia de reações oxidativas e evitando danos às células (TACHAKITTIRUNGROD et al., 2007).

Os radicais livres e outras moléculas reativas são importantes reguladores de processos fisiológicos (NATHAN e DING, 2010). No entanto, níveis elevados dessas moléculas reativas podem causar danos a moléculas biológicas e afetar o balanço de oxido-redução celular (DOWLING e SIMMONS, 2009). Esse estresse oxidativo está envolvido com vários processos patológicos (YOSHIHARA et al., 2010). O consumo a longo prazo de antioxidantes de origem natural apresenta efeitos contra tais patologias, por exemplo contra o cancer (YANG et al., 2001), doenças neurodegenerativas (LETENNEUR et al., 2007) e cardiovasculares (NARDINI et al., 2007) e diabetes (RIZVI et al., 2005).

Estímulo da produção de fenóis nas plantas

A produção de compostos fenólicos pode ser influenciada por diversos fatores bióticos, como a competição (DUMAY et al., 2004), a herbivoria (HANLEY et al., 2007) e patógenos (MANACH et al., 2004). Além disso, a produção desses metabólitos é estimulada como mecanismo de defesa a fatores ambientais, como a deficiência nutricional (CHISHAKI

e HORIGUCHI, 1997) e hídrica (HURA et al., 2007). Outro importante fator no aumento da produção de fenóis é a radiação ultravioleta danosa (MANACH et al., 2004). Principalmente os flavonóides podem absorver e/ou dissipar a radiação UV-B (280-320 nm), evitando danos aos tecidos, sem alterar a radiação fotossinteticamente ativa (TATTINI et al., 2004).

Cerrado

O cerrado é um dos *hotspots* para a conservação da biodiversidade e apresenta a flora mais rica dentre as savanas tropicais (MYERS et al., 2000; KLINK e MACHADO, 2005). Apesar disso, o potencial químico das espécies ainda é subutilizado enquanto a perda de biodiversidade e o desmatamento são assuntos de grande relevância. O cerrado apresenta um solo pobre (HARIDASAN, 2008) com uma maior competição por nutrientes entre as espécies vegetais. As plantas ainda enfretam um maior deficit de água durante a estação seca, que ocorre de abril a setembro (HARIDASAN, 2001; GOTTSBERGER e SILBERBAUER-GOTTSBERGER, 2006). Além disso, em biomas como o cerrado *sensu stricto*, onde a vegetação é mais aberta, a incidência de radiação solar e radiação UV-B é maior. Com essas pressões ambientais espera-se que as espécies vegetais apresentem uma produção de compostos fenólicos aumentada como mecanismo de defesa.

Assim, considerando que as espécies vegetais possuem mecanimos químicos de defesa para superar pressões bióticas e abióticas, e que diversos compostos fenólicos (e.g. flavonóides) têm sido descritos em espécies do cerrado (RODRIGUES, 2007; LUIZ-FERREIRA et al., 2008; COQUEIRO et al., 2013), tivemos os seguintes objetivos:

Objetivo Geral:

- Buscar espécies vegetais do cerrado e compostos com potenciais atividades fungicida e antioxidante.

Objetivos específicos:

1. Avaliar a atividade fungicida de extratos foliares etanólicos de 28 diferentes espécies de plantas do cerrado contra o fungo *Alternaria alternata*, causador da pior doença em tangerinas (a macha marrom de Alternaria).
2. Verificar a atividade antioxidante de extratos foliares etanólicos de 28 diferentes espécies de plantas do cerrado e quantificar os compostos fenólicos.
3. Estudar a atividade antioxidante de infusões foliares de espécies da família Myrtaceae do cerrado, avaliando o conteúdo fenólico correspondente.
4. Otimizar a extração de piceido, um estilbeno com propriedades medicinais, a partir de raízes de *Psidium laruotteanum*.

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CAPÍTULO 1

Screening of leaf extracts of cerrado species against *Alternaria alternata* from Ponkan tangerines

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Screening of leaf extracts of cerrado species against *Alternaria alternata* from Ponkan tangerines

Takao, LK.; Machado, MA.; Imatomi, M.; Gualtieri, SCJ.

RESUMO

Alternaria alternata é o fungo causador de uma das doenças mais severas em tangerinas, a mancha marrom de Alternaria. Essa doença é de difícil controle e causa diversos prejuízos ao cultivo de tangerinas, principalmente poncã e tangor Murcott. Atualmente não há um fungicida específico para esta doença. Dessa forma, estudamos extratos etanólicos de folhas de 28 espécies do cerrado com o intuito de selecionar aquelas com potencial concentração de compostos fungicidas contra conídios de *Alternaria alternata*. O método referência para teste de susceptibilidade de fungos filamentosos da *Clinical and Laboratory Standards Institute* (documento M38-A) foi utilizado. No entanto, nenhum extrato apresentou efeito fungicida nas concentrações testadas (1000-7.8 µg/mL). Já o fungicida piraclostrobina, usado como controle, inibiu a atividade metabólica de conídios de *Alternaria alternata* a partir da concentração de 7.8 µg/mL.

Palavras-chave: citros, Brasil, mandarina, natural fungicide.

ABSTRACT

Alternaria alternata is a fungi that causes of the most severe diseases in tangerines, Alternaria brown spot. This disease is of difficult control and causes many losses to the production of tangerines, mainly Ponkan and Murcott tangor. Currently, there is not a specific fungicide for this disease. Thus, we studied ethanolic leaf extracts of 28 plant species from the Brazilian savanna aiming to select those with potential concentration of fungicidal compounds against conidia of *Alternaria alternata*. The reference method for antifungal susceptibility testing of filamentous fungi of the Clinical and Laboratory Standards Institute (document M38-A) was used. However, none of the extracts presented fungicidal effect in the tested concentrations (1000-7.8 µg/mL). The fungicide pyraclostrobin, used as control, inhibited the metabolic activity of *Alternaria alternata* conidia from the concentration of 7.8 µg/mL.

Keywords: Brazil, citros, fungicida natural, mandarin.

1. INTRODUCTION

Brazil is one of the largest producers of tangerines in the world. In 2012, its production reached 959,672 tonnes in 51,841 hectares (FAO, 2012). In the State of Sao Paulo, Ponkan tangerine (*Citrus reticulata* Blanco) and Murcott tangor (*Citrus sinensis* (L.) *Osbeck* × *Citrus reticulata* Blanco) are the main varieties (IEA, 2014). However, they are highly susceptible to Alternaria brown spot disease (ABS), one of the most severe diseases in tangerines (PACHECO et al., 2012). ABS is caused by *Alternaria alternata* (FR.) Keissler, a filamentous fungi that can damage leaves, twigs and fruits (TIMMER et al., 2003).

Currently in Brazil, there is not a specific commercial fungicide to control ABS in citrus (AGROFIT, 2014) and several applications of foliar fungicides can be necessary to obtain fruits with good quality (TIMMER et al., 2003). The difficulty and increasing costs to control and manage it force producers to substitute tangerines with other crops (STUART et al., 2009). As an alternative, studies have shown the possibility to select plant extracts in the search for new bioactive compounds. For example, CARVALHO et al. (2011) showed that methanolic extracts of *Anadenanthera colubrina* inhibited *Alternaria alternata.* Then, two bioactive compounds against this fungi were isolated, β-sitosterol and β-sitosteryl linoleate (CAMPOS et al., 2014).

Thus, the present study aimed to test ethanolic leaf extracts from 28 plant species from the Brazilian savanna against the fungi *Alternaria alternata*.

2. MATERIAL AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO) and ethanol (Synth, Brazil) were of analytical grade.

Other materials were: RPMI-1640 medium (Sigma-Aldrich, Brazil), MOPS buffer (3-[N-morpholino] propanesulfonic acid), resazurin sodium salt, sodium hydroxide, (Vetec, Brazil), potato dextrose agar (PDA) (Himedia, India) and fungicide comet® with active ingredient pyraclostrobin at 250 g/L (BASF, Brazil).

Plant materials

Leaves of twenty eight cerrado species with no signs of diseases (from at least five individuals) were collected in a cerrado (Brazilian savanna) sensu stricto area of the Universidade Federal de São Carlos (21º58'5"S and 47º53'12"W), São Carlos, São Paulo, Brazil, on April/2014. They were dried at 40ºC in a forced circulation chamber and ground in an electric mill.

Voucher specimens of the studied species were deposited at the Herbarium of the Universidade Federal de São Carlos: *Anacardium humile* A.St.-Hil. (8700), *Bauhinia rufa* (Bong) Steud. (8701), *Blepharocalyx salicifolius* (Kunth) O.Berg (8702), *Byrsonima coccolobifolia* Kunth (8703), *Campomanesia pubescens* (Mart. ex DC.) O.Berg (8704), *Davilla ellitica* A.St.-Hil. (8705), *Drimys brasiliensis* Miers (8706), *Erythroxylum suberosum* A.St.-Hil. (8707), *Gochnatia pulchra* Cabrera (8708), *Kielmeyera variabilis* Mart. & Zucc. (8709), *Leandra aurea* (Cham.) Cogn. (8710), *Miconia albicans* (Sw.) Steud. (8711), *Miconia ferruginata* DC. (8712), *Miconia ligustroides* (DC.) Naudin (8713), *Myrcia bella* Cambess. (8714), *Myrcia lingua* (O.Berg.) Mattos (8715), *Myrcia tomentosa* (Aubl.) DC. (8716), *Piptocarpha rotundifolia* (Less.) Baker (8717), *Pradosia brevipes* (Pierre) T.D.Penn. (8718), *Psidium cinereum* Mart. ex. DC. (8719), *Psidium laruotteanum* Cambess. (8720), *Roupada montana* Aubl. (8721), *Schefflera vinosa* (Cham. & Chultdl.) (8722), *Solanum lycocarpum* A.St.-Hil. (8723), *Tocoyena formosa* (Cham. & Schltdl.) K.Schum. (8724), *Virola sebifera* Aubl. (8725), *Vochysia tucanorum* Mart. (8726) and *Xylopia aromatica* Mart. (8727).

Extracts preparation

Extracts (10% concentration: weight/volume) were prepared with powdered dry leaves and ethanol. After stored at 6ºC for 24 hours, they were filtered through a Büchner funnel-filter paper (3 µm) and dried under reduced pressure at 38ºC (IKA® RV10 rotary evaporator).

Antifungal susceptibility test

This test was performed according to reference method for antifungal susceptibility testing of filamentous fungi (document M38-A) (CLSI, 2002), with modifications.

Broth medium

Broth medium was prepared as described: 10.4 g of RPMI-1640 medium (with glutamine and without bicarbonate) and 34.53 g of MOPS buffer were dissolved in distilled water (900 mL), adjusting pH to 7.0 at 25ºC with 0.1 mol/L sodium hydroxide. Distilled water was added to a final volume of 1 L and the resultant medium was filter sterilized.

Microorganism and inoculum

Strains of *Alternaria alternata* (LRS 182/10) isolated from Ponkan tangerines were obtained from the mycology collection "Dra. Victoria Rossetti" (UPDS/APTA/SAA), Sorocaba, Sao Paulo, Brazil. Preserved stocks (Castellani method) were cultured onto PDA at 25ºC under 12h photoperiod for 21 days. Then, 6 mm discs with mycelial growth were cultured on PDA plates under constant illumination at 27ºC for 21 days to promote sporulation. An aqueous solution of Tween 80 (10 mL; 1%: v/v) was added to the plates to collect conidia. This conidial suspension was filtered through cheesecloth, homogenized for 15s in vortex and diluted to 8-12 $\times 10^4$ conidia/mL (Figure 1).

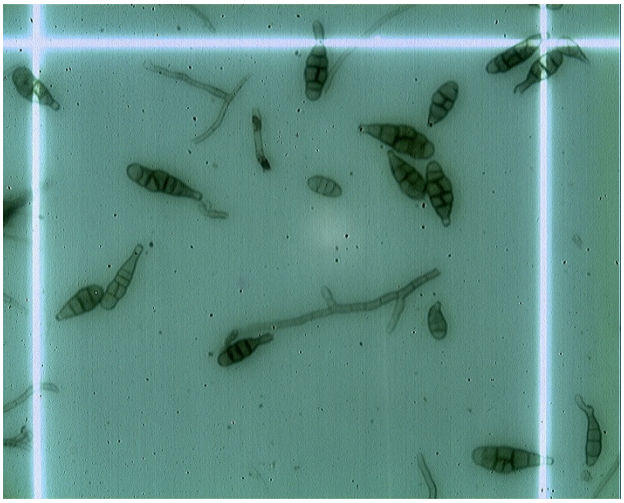

Figure 1. Conidia of *Alternaria alternata* produced after 21 days at 27ºC under constant illumination on PDA medium.

Preparation of samples

Solutions of the plants' extracts and pyraclostrobin (using fungicide comet®) were prepared at 4000 µg/mL in RPMI-1640 medium with 5% of DMSO. Firstly, the extracts and comet were dissolved in DMSO and then completed with q.s. medium.

Preparation of microplates

Tests were performed in microdilution plates of 96 wells. Broth medium (0.1 mL) was added in each well and sample solutions (0.1 mL) were serially diluted two-fold. Subsequently, wells received inocula (0.1 mL) resulting in a final concentration of 4-6 × 10^4 conidia/mL, 1000-7.8 µg/mL of plant extracts and 1000-0.03 µg/mL of pyraclostrobin. The microplates were incubated in BOD chambers at 25ºC for 3 days under 12h photoperiod. All growth and sterility controls were evaluated.

Determination of minimum inhibitory concentration (MIC)

Visual readings were performed using resazurin dye as indicator. The color change from blue to pink indicates metabolic activity (reduction of resazurin). Each well received 30 µL of sterilized solution of resazurin (100 µg/mL of distilled water) (PALOMINO et al.,

2002). After 12 hours of incubation at 25ºC in the dark, MIC endpoints were defined as the lowest concentration that remained blue (no growth) or changed to slightly purple (partial growth inhibition).

Data analysis

The *in vitro* assays presented three repetitions and the highest MIC within these was considered the final endpoint. There was no statistical analysis because this is a qualitative test.

3. RESULTS

The results are summarized in the Figure 2. It is presented just one figure because the effects of all extracts, including their replicas, were the same. The conidia of *Alternaria alternata* presented normal growth in the RPMI medium (growth control) and in the presence of DMSO (solvent control). The medium and the solvent were also sterile, so they had no influence in the results. The fungicide pyraclostrobin presented a MIC of 7.8 µg/mL. However, none of the 28 plant extracts presented inhibitory activity against the conidia of *Alternaria alternata* at the tested concentrations (1000-7.8 µg/mL).

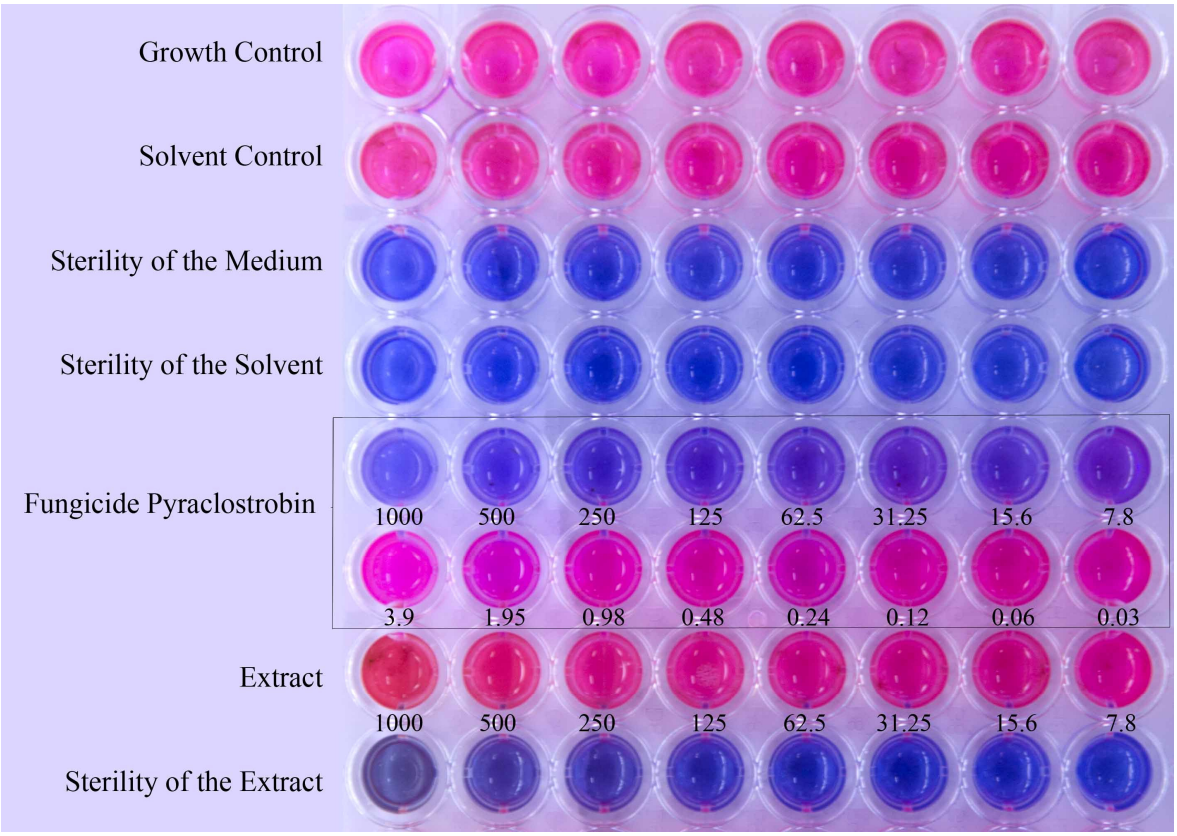

Figure 2. Fungicidal activity against *Alternaria alternata* conidia in RPMI medium using resazurin dye as indicator. Pink color: normal growth, presence of metabolic activity. Blue/Purple: inhibition of growth, low or no metabolic activity. Numbers are the concentrations of the fungicide and extracts in µg/mL. The concentration of conidia in each well was 4-6 × 10^4 conidia/mL.

4. DISCUSSION

Although pyraclostrobin showed inhibitory activity against *Alternaria alternata* in our study, some researchers have already shown resistant varieties isolated from other crops, such as pistachio (AVENOT et al., 2008). This fungi also causes diseases in melon and SENHOR et al. (2009) assessed the efficiency of three other fungicides, thiabendazole, azoxystrobin and imazalil. *In vitro* inhibition was verified on the mycelial growth, sporulation and germination, but there was no effect *in vivo*.

Finding alternative sources of compounds to control *Alternaria alternata* is also difficult. For example, among the methanolic plant extracts from 105 species analyzed by CARVALHO et al. (2011) at 444 µg/mL, just three were active against the conidia of *Alternaria alternata*. In our study a higher maximum concentration of 1000 µg/mL was used but no fungicidal activity was observed. Additional concentrations were not considered due to dilution difficulties. Thus, in case future investigations are conducted with these species, extractions with preliminary steps of separation should be examined in order to increase the concentration of possible bioactive compounds.

In conclusion, none of the ethanolic leaf extracts from the studied plant species of the Brazilian savanna inhibited *Alternaria alternata*, the causal agent of brown spot disease. However, studies on this issue should be encouraged as it still causes many losses to the production of tangerines and other crops worldwide, and the indiscriminate use of fungicides may present human health concerns.

ACKNOWLEDGEMENTS

To CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for providing the research for the first author and for funding the Research Productivity Grant for the last author. We also thank Dr. Eduardo Feichtenberger and *Instituto Biológico* (UPDS/APTA, Sorocaba, São Paulo) for donating the *Alternaria alternata* strains (LRS 182/10).

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CAPÍTULO 2

High antioxidant activity and phenolic content of ethanolic leaf extracts of plants from the Brazilian savanna

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RESUMO

Compostos fenólicos têm sido explorados devido às suas propriedades antioxidantes e benefícios à saúde humana. Na savana brasileira (cerrado), espécies vegetais são expostas a pressões ambientais que podem estimular a produção desses compostos. Dessa forma, as folhas de 28 espécies vegetais foram investigadas com relação à atividade antioxidante e ao conteúdo de fenóis. A atividade antioxidante foi avaliada através do índice de atividade antioxidante (AAI) pelo método DPPH e o conteúdo de fenóis totais (TPC) pelo ensaio de Folin–Ciocalteu. A maioria das espécies apresentaram altos AAI associados com altos TPC. *Leandra aurea* apresentou a atividade antioxidante mais forte (AAI = 8.792, IC_{50} = 3.50 $\mu g \cdot mL^{-1}$) e teve um TPC de 423.07 mg de ácido gálico/g de extrato etanólico seco. Os extratos de outras vinte e uma espécies mostraram valores de AAI acima de 2.0 (atividade antioxidante muito forte): *Miconia albicans*, *Byrsonima coccolobifolia*, *Miconia ligustroides*, *Campomanesia pubescens*, *Davilla elliptica*, *Mycia bella*, *Tocoyena formosa*, *Blepharocalyx salicifolius, Myrcia tomentosa, Erythroxylum suberosum, Virola sebifera, Miconia ferruginata, Kielmeyera variabilis, Psidium cinereum, Myrcia lingua, Drimys brasiliensis, Pradosia brevipes, Psidium laruotteanum, Anacardium humile, Schefflera vinosa* e *Roupala montana.* Assim, os extratos de espécies vegetais do cerrado foram potentes antioxidantes e podem ser fontes the compostos benéficos naturais.

Palavras-chave: cerrado, estresse ambiental, fenóis, sequestro de radical livre.

ABSTRACT

Phenolic compounds have been explored due to their antioxidant properties and benefits to human health. In the Brazilian savanna, plant species are exposed to environmental pressures that may stimulate the production of these compounds. Thus, the leaves of 28 plant species were investigated in relation to their antioxidant potential and phenolic content. The antioxidant activity was assessed using the antioxidant activity index (AAI) by the DPPH method and the total phenolic content (TPC) by the Folin–Ciocalteu assay. Most species presented high AAI associated with high TPC. *Leandra aurea* was the most potent antioxidant (AAI = 8.792, IC_{50} = 3.50 $\mu g \cdot mL^{-1}$) and showed a TPC of 423.07 mg of gallic acid/g of dry ethanolic extract. The extracts of other twenty one species had AAI values above 2.0 (very strong antioxidant activity): *Miconia albicans*, *Byrsonima coccolobifolia*, *Miconia ligustroides*, *Campomanesia pubescens*, *Davilla elliptica*, *Mycia bella*, *Tocoyena formosa*, *Blepharocalyx salicifolius, Myrcia tomentosa, Erythroxylum suberosum, Virola sebifera, Miconia ferruginata, Kielmeyera variabilis, Psidium cinereum, Myrcia lingua, Drimys brasiliensis, Pradosia brevipes, Psidium laruotteanum, Anacardium humile, Schefflera vinosa* and *Roupala montana.* Thus, extracts of the Brazilian savanna species were potent antioxidants and may be sources of natural beneficial compounds.

Keywords: cerrado, environmental stress, free radical scavenging, phenolics.

1. INTRODUCTION

Phenolics are chemical compounds known to present several benefits for human health, mainly related to their antioxidant properties (PANDEY and RIZVI, 2009). Studies suggest that long term consumption of natural antioxidants present potential against cancer (YANG et al., 2001), neurodegenerative (LETENNEUR et al., 2007) and cardiovascular diseases (NARDINI et al., 2007), diabetes (RIZVI et al., 2005) and aging (JOSEPH et al., 2005). Phenolics are generally characterized as possessing an aromatic ring with at least one hydroxyl group attached (CROTEAU et al., 2000). The reducing power of the aromatic hydroxyl group is the main responsible for the antioxidant properties; the hydrogen of this group can be donated and neutralize reactive free radicals (BREWER, 2011), blocking oxidative chain reactions and avoiding damages to cells (TACHAKITTIRUNGROD et al., 2007).

In plants, phenolics are the most abundant secondary metabolites (DAI and MUMPER, 2010). They can be classified in different groups, including flavonoids, lignans, phenolic acids, stilbenes, lignins, coumarins, cinnamic and benzoic acids (MANACH et al., 2004; VAN DUYNHOVEN et al., 2010). All of them originate from either the shikimic acid or the malonate/acetate pathways (CROTEAU et al., 2000), generally in response to environmental pressures. The production of these compounds can be stimulated as a defense against competition (DUMAY et al., 2004), herbivory (HANLEY et al., 2007), pathogens (MANACH et al., 2004), nutrient deficiency (CHISHAKI and HORIGUCHI, 1997), drought (HURA et al., 2007; PEREIRA et al., 2014) and injurious ultraviolet radiation (MANACH et al., 2004).

The Brazilian savanna holds the richest flora among the tropical savannas (KLINK and MACHADO, 2005), being one of the hot spots for conservation of the biodiversity in the world (MYERS et al., 2000). However, the chemical potential of its

plant species related to their benefits to human health is still underused while deforestation and loss of biodiversity are main issues. In the Brazilian savanna, plants grow in poor soils (HARIDASAN, 2008) consequently the competition for nutrients is high. During the dry season, from April to September (GOTTSBERGER and SILBERBAUER-GOTTSBERGER, 2006), plants also face a surface water deficit in the soil (HARIDASAN, 2001). High sunlight incidence is another factor, mainly in biomes presenting a more opened vegetation, such as the cerrado sensu stricto. Thus, it is expected the plant species to present high concentrations of phenolics to overcome these environmental stresses.

In the search for new alternative sources of health beneficial compounds, we assessed the antioxidant activity of ethanolic leaf extracts of 28 plant species from the Brazilian savanna and quantified their total phenolics.

2. MATERIAL AND METHODS

2.1. Chemicals

Ethanol, methanol and acetone of analytical grade were purchased from Synth (Brazil). Additional chemicals were: anhydrous sodium carbonate (Synth, Brazil), DPPH (2,2-diphenyl-1-picrylhydrazyl) and quercetin (Sigma Aldrich, Brazil), Folin-Ciocalteu reagent (Haloquimica, Brazil) and gallic acid (Vetec, Brazil).

2.2. Plant materials

Leaves of 28 species with no signs of diseases were collected in the cerrado sensu stricto (Brazilian savanna) of the UFSCar campus in São Carlos (21º58'5"S and 47º53'12"W, São Paulo, Brazil) on April/2014. Individuals (at least five) with higher exposition to sunlight were preferred for collection. All leaves were dried in a forced circulation chamber at 40ºC and powdered in an electric mill. Preserved specimens were

stored at the Herbarium of the UFSCar within 18 families and the following numbers: I) Anacardiaceae: *Anacardium humile* A.St.-Hil. (8700); II) Annonaceae: *Xylopia aromatica* Mart. (8727); III) Araliaceae: *Schefflera vinosa* (Cham. & Schltdl.) (8722); IV) Asteraceae: *Gochnatia pulchra* Cabrera (8708) and *Piptocarpha rotundifolia* (Less.) Baker (8717); V) Callophilaceae: *Kielmeyera variabilis* Mart. & Zucc. (8709); VI) Dilleniaceae: *Davilla ellitica* A.St.-Hil. (8705); VII) Erythroxylaceae: *Erythroxylum suberosum* A.St.-Hil. (8707); VIII) Fabaceae: *Bauhinia rufa* (Bong) Steud. (8701); IX) Malpighiaceae: *Byrsonima coccolobifolia* Kunth (8703); X) Melastomataceae: *Leandra aurea* (Cham.) Cogn. (8710), *Miconia albicans* (Sw.) Steud. (8711), *Miconia ferruginata* DC. (8712) and *Miconia ligustroides* (DC.) Naudin (8713); XI) Myristicaceae: *Virola sebifera* Aubl. (8725); XII) Myrtaceae: *Blepharocalyx salicifolius* (Kunth) O.Berg (8702), *Campomanesia pubescens* (Mart. ex DC.) O.Berg (8704), *Myrcia bella* Cambess. (8714), *Myrcia lingua* (O.Berg.) Mattos (8715), *Myrcia tomentosa* (Aubl.) DC. (8716), *Psidium cinereum* Mart. ex. DC. (8719) and *Psidium laruotteanum* Cambess. (8720); XIII) Proteaceae: *Roupada montana* Aubl. (8721); XIV) Rubiaceae: *Tocoyena formosa* (Cham. & Schltdl.) K.Schum. (8724); XV) Sapotaceae: *Pradosia brevipes* (Pierre) T.D.Penn. (8718); XVI) Solanaceae: *Solanum lycocarpum* A.St.-Hil. (8723); XVII) Vochysiaceae: *Vochysia tucanorum* Mart. (8726); XVIII) Winteraceae: *Drimys brasiliensis* Miers (8706).

2.3. Ethanolic extraction

Powdered dry leaves and ethanol (10%: weight/volume) were mixed and stored at 6ºC for 24 hours. Then, they were ultrasound extracted for 15 min and filtered through filter paper (3 µm). The filtrates were dried under reduced pressure at 38ºC (IKA® RV10 rotary evaporator).

2.4. Antioxidant activity assessment

The antioxidant activity index (AAI) by the DPPH free radical scavenging effect was used to assess the antioxidant properties of the samples (SCHERER and GODOY, 2009). Extracts diluted in methanol (0.05 mL) at six different concentrations were added to 1.95 mL of 0.08 mM DPPH methanolic solution. The absorbances of the samples were measured at 517 nm (Femto spectrophotometer, model 800XI, Sao Paulo, Brazil), after 90 minutes incubation at room temperature in the dark. Gallic acid and quercetin were used as positive witnesses and methanol as blank.

The antioxidant activity index was calculated as: AAI = (final concentration of DPPH in the reaction) / IC_{50}, where the final concentration of the reaction was 30.75 $\mu g \cdot mL^{-1}$. The concentration providing 50% inhibition (IC_{50}) was calculated by the linear regression equation between the extract's concentration and scavenging capacity. This scavenging capacity was calculated as: $I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 = absorbance of the blank, and Abs_1 = absorbance of the tested sample. SCHERER and GODOY (2009) established the following criteria for AAI: poor activity < 0.5 < moderate < 1.0 < strong < 2.0 < very strong.

2.5. Total phenolics determination

The total phenolic content (TPC) of the extracts was calculated using the Folin-Ciocalteu method (GEORGE et al., 2005 - modified). Extracts were diluted in acetone and 0.05 mL was mixed with 4.95 mL of distilled water. Aliquots of 0.5 mL of these solutions were added to 2.5 mL of Folin-Ciocalteu reagent (pre-diluted, 10 times, with distilled water). After standing for 2 min at room temperature, 2 mL of aqueous Na_2CO_3 solution (75 g/L) were added. The mixture was incubated for 15 min at 50ºC and cooled in ice water bath. The absorbances were measured at 760 nm. A calibration curve was prepared with gallic acid solutions (1 to 8 $\mu g \cdot mL^{-1}$) and the TPC were expressed as mg of gallic

acid equivalents (GAE)/g of dry ethanolic extract, according to the calibration curve: $y = 0.110x + 0.007$ ($r^2 = 0.999$ and $p < 0.05$).

2.6. Data analysis

The assays were carried out in triplicate and the AAI and TPC data were compared by ANOVA and Tukey test ($\alpha = 0.05$). A correlation test was also performed between the AAI and TPC values. The analyses were carried out using PAST software – version 2.5 (HAMMER et al., 2001).

3. RESULTS AND DISCUSSION

The ethanolic extractions showed yields from 4.7 to 21.35%. All samples were tested in their linear range of scavenging capacity of DPPH, presenting excellent correlations with the concentrations (r^2 between 0.95 and 0.99, $p < 0.05$) (as shown in Table 1). High coefficients of determination are essential for a better estimation of the IC_{50} and AAI.

The antioxidant activity index (AAI) proposed by SCHERER and GODOY (2009) was used to enable comparisons with other studies. Several plant species leaves had high antioxidant activity (as shown in Table 1). Twenty two out of 28 species had very strong activity (AAI > 2.0; $IC_{50} < 15$ µg.mL^{-1}). *Leandra aurea* was the most potent extract, reaching an IC_{50} value of just 3.50 µg.mL^{-1} (AAI = 8.792), considering a concentration of 30.75 µg·mL^{-1} of DPPH in the reaction. The following 21 species showed a range of AAI from 7.992 (IC_{50} = 3.85 µg·mL^{-1}) of *M. albicans* to 2.128 (IC_{50} = 14.46 µg.mL^{-1}) of *R. montana*. Moreover, four species had strong activity (1.0 < AAI < 2.0), *B. rufa*, *S. lycocarpum*, *G. pulchra* and *X. aromatica* with IC_{50} of 17.43, 20.71, 23.18 and 23.59 µg·mL^{-1}, respectively. *M. albicans* had an activity much higher than previously reported by PIERONI et al. (2011) (methanolic extract, AAI = 0.53). Also using methanolic extracts, BONACORSI et al. (2011) had reported lower AAI (1.34) for *D. elliptica* and similar AAI (2.36) for *A. humile*.

The antioxidant activity of plants from the Brazilian savanna is still high and promising when compared to traditional ayurvedic medicinal plants used for cognitive disorders, evaluated by MATHEW and SUBRAMANIAN (2014). They reported the phenolic compounds and respective antioxidant and anti-cholinesterase activities as responsible for the beneficial properties of methanolic extracts from *Nardostachys jatamansi* (rhizome, AAI = 0.20), *Punica granatum* (fruit, AAI = 1.12), *Nelumbo nucifera* (flower, AAI = 1.35), *Emblica officinalis* (fruit, AAI = 8.71) and *Terminalia chebula* (fruit, 7.17).

Our extracts presented high TPC, between 60.04 and 492.19 mg of GAE/g of dry ethanolic extract. *B. coccolobifolia* had the highest phenolic content, followed by *M. ligustroides* (469.19 mg of GAE) and *L. aurea* (423.07 mg of GAE). Higher antioxidant capacities were associated with higher total phenolic contents ($r^2 = 0.814$, $p < 0.05$) (see Figure 1), suggesting these as the responsible for the free radical quenching effect. Some phenolic compounds had already been described for leaves of *M. albicans* (quercetin, quercetin-3-*O*-glucoside, rutin), *D. elliptica* (quercetin-3-*O*-alpha-L-arabinopyranoside, quercetin-3-*O*-*β*-D-galactopyranoside) (RODRIGUES, 2007), *A. humile* (amentoflavone, (+)-catechin, methyl gallate, quercetin-3-*O*-*β*-D-galactopyranoside) (LUIZ-FERREIRA et al., 2008) and *K. variabilis* (quercitrin, podocarpusflavone A, quercetin-3-O-β-glucoside, quercetin-3-O-β-galactoside) (COQUEIRO et al., 2013).

Table 1. Antioxidant activity index (AAI) and total phenolic content (TPC) of ethanolic leaf extracts of 28 plant species from the Brazilian savanna. IC_{50} ($\mu g \cdot mL^{-1}$): concentration providing 50% inhibition of the free radical DPPH; SD: standard deviation. Different letters: significant difference inside each column ($p < 0.05$). r^2: coefficient of determination of the antioxidant assay. Final concentration of DPPH in the reaction: 30.75 $\mu g \cdot mL^{-1}$. TPC in mg of gallic acid/g of dry ethanolic extract. *: dry weight basis.

Sample	Family	Mean r^2	Mean IC_{50}	Mean AAI ± SD		Mean TPC ± SD		Extraction Yield (%)*
Gallic acid	-	0.99	1.09	28.29±1.04	a	-		-
Quercetin	-	0.99	1.74	17.67±0.20	b	-		-
Leandra aurea	Melastomataceae	0.99	3.50	8.79±0.17	c	423.07±4.51	bc	9.30
Miconia albicans	Melastomataceae	0.99	3.85	7.99±0.23	d	350.94±31.56	de	20.35
Byrsonima coccolobifolia	Malpighiaceae	0.99	4.02	7.65±0.15	de	492.19±72.32	a	18.07
Miconia ligustroides	Melastomataceae	0.99	4.05	7.60±0.13	d	469.65±6.89	ab	13.70
Campomanesia pubescens	Myrtaceae	0.97	4.32	7.13±0.21	e	387.00±18.03	cd	15.67
Davilla elliptica	Dilleniaceae	0.99	4.94	6.23±0.22	f	268.29±21.30	fgh	12.14
Myrcia bella	Myrtaceae	0.99	5.04	6.10±0.23	fg	260.77±43.00	fgh	21.25
Tocoyena formosa	Rubiaceae	0.99	5.29	5.81±0.03	g	305.85±4.51	ef	6.80
Blepharocalyx salicifolius	Myrtaceae	0.98	5.86	5.25±0.17	h	269.79±9.02	fgh	15.94
Myrcia tomentosa	Myrtaceae	0.99	6.00	5.13±0.10	h	316.37±6.89	ef	11.64
Erythroxylum suberosum	Erythroxylaceae	0.99	7.33	4.19±0.01	ij	261.01±15.03	fgh	10.90
Virola sebifera	Myristicaceae	0.99	7.79	3.95±0.06	jk	166.84±7.56	jklm	7.09
Miconia ferruginata	Melastomataceae	0.98	8.48	3.63±0.12	jk	273.03±9.02	fg	8.85
Kielmeyera variabilis	Calophillaceae	0.99	9.13	3.37±0.09	kl	209.91±6.01	hijk	15.64
Psidium cinereum	Myrtaceae	0.99	9.35	3.29±0.05	kl	240.97±7.56	ghi	9.61
Myrcia lingua	Myrtaceae	0.99	9.46	3.25±0.10	kl	212.92±15.90	ghij	11.74
Drimys brasiliensis	Winteraceae	0.99	9.48	3.24±0.08	kl	272.03±16.55	fgh	12.50
Pradosia brevipes	Sapotaceae	0.99	9.80	3.14±0.07	l	236.96±3.01	ghi	21.35
Psidium laruotteanum	Myrtaceae	0.99	9.95	3.09±0.12	l	190.88±12.15	ijkl	16.41
Anacardium humile	Anacardiaceae	0.99	12.04	2.56±0.10	m	162.01±10.04	jklm	6.57
Schefflera vinosa	Araliaceae	0.99	14.00	2.20±0.13	mn	135.57±14.46	lmn	19.36
Roupala montana	Proteaceae	0.99	14.46	2.13±0.06	no	142.78±2.75	lmn	6.29
Bauhinia rufa	Fabaceae	0.99	17.43	1.77±0.06	op	239.55±12.62	ghi	8.07
Solanum lycocarpum	Solanaceae	0.99	20.71	1.49±0.01	pq	116.47±2.27	mno	4.70
Gochnatia pulchra	Asteraceae	0.99	23.18	1.33±0.11	q	133.30±1.80	lmn	9.35
Xylopia aromatica	Annonaceae	0.98	23.59	1.30±0.04	q	90.92±0.90	no	8.74
Vochysia tucanorum	Vochysiaceae	0.96	62.96	0.49±0.02	r	60.04±0.69	o	3.95
Piptocarpha rotundifolia	Asteraceae	0.97	68.52	0.45±0.02	r	150.05±0.90	klmn	8.40

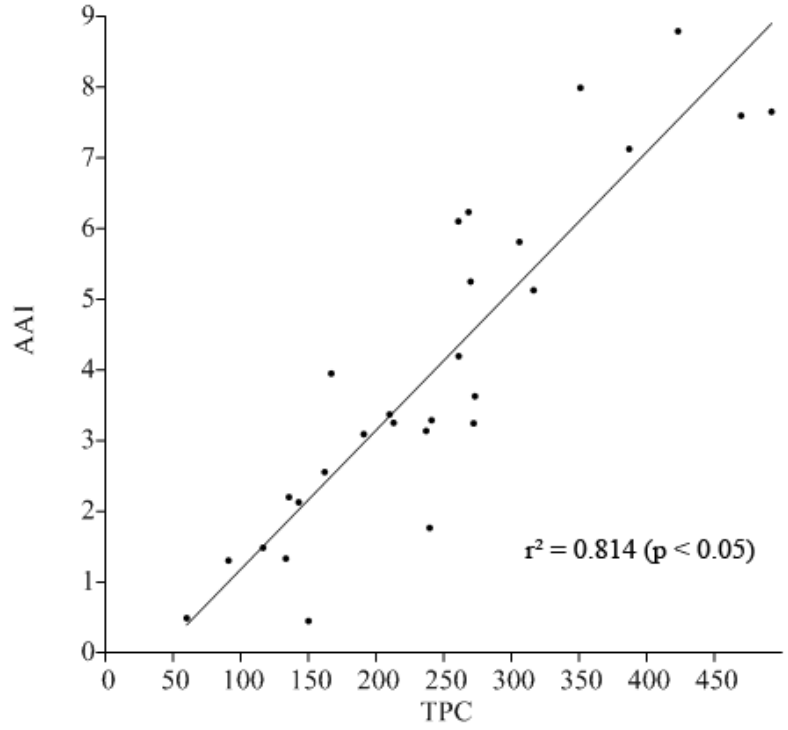

Figure 1. Correlation between antioxidant activity index (AAI) and total phenolic content (TPC, in mg of gallic acid/g of dry ethanolic extract) of ethanolic leaf extracts of 28 plant species from the Brazilian savanna.

The effectiveness as antioxidant can change between different phenolics (ISMAIL et al., 2004) present in the extract. This property is related to the number of active groups (O-H) and their position in the compounds (BENDARY et al., 2013). The *ortho* position is the more active, followed by *para* and then *meta* position (BENDARY et al., 2013). The hydrogen of the active group is donated and neutralizes the free radical DPPH (SOUSA et al., 2007). For example, flavonoids (e.g. quercetin, kaempferol, myricetin) generally present more hydroxyl groups than ferulic acids and, consequently, higher antioxidant potential (ISMAIL et al., 2004). The chemical structure of polyphenols is also relevant to the bioavailability, the portion of the compound that reaches the systemic circulation and the specific sites where it is bioactive (PORRINI and RISO, 2008). Phenolics generally

present glycoside forms and this can increase or decrease the bioavailability (D'ARCHIVIO et al., 2010). Thus, the medicinal properties among different types of phenolics can considerably differ (PANDEY and RIZVI, 2009) and their identification is an important step for the exploration of plant extracts.

In conclusion, 26 plant species from the Brazilian savanna displayed high antioxidant activities associated with high total phenolic contents, being promising sources of health beneficial compounds. As far as we know, this is the first assessment of the antioxidant potential and total phenolics of the majority of the studied species. Thus, it contributes valuable knowledge about the bioactive properties of plant leaves. However, further studies are necessary to confirm if these properties are actually a result of environmental stresses imposed to plants in the Brazilian savanna and what classes of phenolics are produced.

ACKNOWLEDGEMENTS

To the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) for funding this research. We also thank José Luis García Galindo and Tatiane Regina Albarici for helping with laboratory methodologies.

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CAPÍTULO 3

Antioxidant activity and phenolic content of leaf infusions of Myrtaceae species from cerrado (Brazilian savanna)

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São Carlos, 26 de maio de 2014.

AP-B-C**008/14**

Ref: **BJB-033/14**

Título: **Antioxidant activity and phenolic content of Myrtaceae leaf infusions from cerrado (Brazilian savanna)**

Prezados Autores: **Takao, L.K.; Imatomi, M.; Gualtieri, S.C.J.**

Pela presente, vimos informar-lhes que o manuscrito acima mencionado foi aceito no **Brazilian Journal of Biology**, está previsto para publicação no **Volume 76 Número 1 (Fevereiro de 2016)**.
Sem mais, despedimo-nos.

Atenciosamente

Profa. Dra. Takako Matsumura Tundisi
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***Observação:** Houve uma mudança no título seguindo instrução de um dos avaliadores. No entanto, na carta de aceite consta o nome original do arquivo de submissão do artigo.

Antioxidant activity and phenolic content of leaf infusions of Myrtaceae species from cerrado (Brazilian savanna)

Takao, LK.; Imatomi, M.; Gualtieri, SCJ.

RESUMO

Há um considerável interesse na descoberta de novos antioxidantes de origem vegetal. Muitos estudos enfatizaram a atividade antioxidante de espécies pertencentes à família Myrtaceae. No entanto, há poucos relatos sobre espécies de cerrado. Neste estudo, a atividade antioxidante e o conteúdo fenólico de 12 espécies nativas de Myrtaceae do cerrado foram avaliados (*Blepharocalyx salicifolius*, *Eugenia bimarginata*, *Eugenia dysenterica*, *Eugenia klotzschiana*, *Hexachlamys edulis*, *Myrcia bella*, *Myrcia lingua*, *Myrcia splendens*, *Myrcia tomentosa*, *Psidium australe*, *Psidium cinereum* e *Psidium laruotteanum*). O potencial antioxidante foi estimado através do índice de atividade antioxidante (AAI) pelo método do DPPH e o conteúdo fenólico total (TPC) pelo ensaio de Folin-Ciocaulteu. Houve uma alta correlação entre os valores de TPC e AAI. *P. laruotteanum* teve o maior TPC (576,56 mg de equivalente em ácido gálico por g de extrato) e foi o antioxidante mais potente (AAI = 7,97, IC_{50} = 3,86 $\mu g.mL^{-1}$), com atividade próxima da quercetina pura (IC_{50} = 2,99 $\mu g.mL^{-1}$). Os extratos de nove espécies apresentaram IC_{50} de 6,24 a 8,75 $\mu g.mL^{-1}$. Além disso, a maioria das espécies teve valores de TPC e AAI similares ou maiores que *Camellia sinensis*, cujo chá é comumente consumido e apresenta fortes propriedades antioxidantes. Os resultados mostraram que as espécies de Myrtaceae de cerrado analisadas apresentam conteúdos fenólicos e atividades antioxidantes elevadas. Dessa forma, elas são uma fonte potencial de novos antioxidantes.

Palavra-chave: sequestro de radical livre.

ABSTRACT

There is considerable interest in identifying new antioxidants from plant materials. Several studies have emphasized the antioxidant activity of species belonging to the Myrtaceae family. However, there are few reports on these species from the cerrado (Brazilian savanna). In this study, the antioxidant activity and phenolic content of 12 native Myrtaceae species from the cerrado were evaluated (*Blepharocalyx salicifolius*, *Eugenia bimarginata*, *Eugenia dysenterica*, *Eugenia klotzschiana*, *Hexachlamys edulis*, *Myrcia bella*, *Myrcia lingua*, *Myrcia splendens*, *Myrcia tomentosa*, *Psidium australe*, *Psidium cinereum*, and *Psidium laruotteanum*). Antioxidant potential was assessed using the antioxidant activity index (AAI) by the DPPH method and total phenolic content (TPC) by the Folin–Ciocalteu assay. There was a high correlation between TPC and AAI values. *Psidium laruotteanum* showed the highest TPC (576.56 mg GAE/g extract) and was the most potent antioxidant (AAI = 7.97, IC_{50} = 3.86 $\mu g \cdot mL^{-1}$), with activity close to that of pure quercetin (IC_{50} = 2.99 $\mu g \cdot mL^{-1}$). The extracts of nine species showed IC_{50} of 6.24–8.75 $\mu g \cdot mL^{-1}$. Most species showed TPC and AAI values similar to or higher than those for *Camellia sinensis*, a commonly consumed tea with strong antioxidant properties. The results reveal that the analyzed Myrtaceae species from the cerrado possess high phenolic contents and antioxidant activities. Thus, they are a potential source of new natural antioxidants.

Keyword: free radical scavenging.

1. INTRODUCTION

Free radicals and other small reactive molecules have emerged as important regulators of many physiological and pathological processes (NATHAN and DING, 2010). Increased levels of these short-lived reactive molecules can cause oxidative damage to biological macromolecules and disrupt the cellular reduction–oxidation (redox) balance (DOWLING and SIMMONS, 2009). Oxidative stress caused by the accumulation of free radicals in the body is involved in various pathological processes including cardiovascular diseases, cancer, neurodegenerative disorders, and aging (YOSHIHARA et al., 2010).

An antioxidant is a compound that can delay or inhibit the oxidation of lipids or other molecules by blocking the initiation or propagation of oxidative chain reactions, which prevents or repairs the damage done to the cells (TACHAKITTIRUNGROD et al., 2007). The consumption of natural antioxidants presents potential health benefits (YOSHIHARA et al., 2010). Thus, there is considerable interest in finding new antioxidants from plant materials. Antioxidant compounds from plants, particularly polyphenols, can inhibit the propagation of free radical reactions and protect the human body from diseases (PERRON and BRUMAGHIM, 2009; LIZCANO et al., 2010).

Several studies have emphasized the antioxidant activity of species belonging to the Myrtaceae family such as *Feijoa sellowiana* (WESTON, 2010), *Psidium guajava* (TACHAKITTIRUNGROD et al., 2007), and *Eucalyptus rostrata* (OKAMURA et al., 1993). Furthermore, many members of this family are used in folk medicine, mainly as antidiarrheal, antimicrobial, cleansing, antirheumatic, anti-inflammatory, and cholesterol-lowering agents (STEFANELLO et al., 2011). However, there are few reports on the antioxidant activity of Myrtaceae species from the cerrado (Brazilian savanna), although phytochemical studies reveal the presence of quercetin and kaempferol, which are compounds considered to be potent antioxidants (IMATOMI et al., 2013). Therefore, in the

present study, we aimed to evaluate the antioxidant activity and quantify polyphenols in leaf infusions of 12 Myrtaceae species from the cerrado.

2. MATERIAL AND METHODS

2.1. Plant materials

Leaves of 12 Myrtaceae species with no signs of herbivory or disease (from at least three individuals per species) were collected in the cerrado (Brazilian savanna) sensu stricto area of the Universidade Federal de São Carlos (21°58′5″S and 47°53′12″W), São Carlos, São Paulo, Brazil, on July 15, 2013.

Voucher specimens were deposited at the Herbarium of the Universidade Federal de São Carlos. The 12 species included *Blepharocalyx salicifolius* (Kunth) O. Berg (8308), *Eugenia bimarginata* O. Berg (8310), *Eugenia dysenterica* DC. (8545), *Eugenia klotzschiana* O. Berg (8311), *Hexachlamys edulis* (O. Berg) Kausel & D. Legrand (8546), *Myrcia bella* Cambess. (8314), *Myrcia lingua* (O. Berg) Mattos (8315), *Myrcia splendens* DC. (8317), *Myrcia tomentosa* DC. (8318), *Psidium australe* Cambess. (8319), *Psidium cinereum* Mart. (8320), and *Psidium laruotteanum* Cambess. (8321). After collection, the leaves were dried at 40°C for 48 h and ground in an electric mill.

Green tea is commonly used for its antioxidant properties (NAMAL SENANAYAKE, 2013). Thus, a commercial green tea (dry leaves and stalks of *Camellia sinensis* (L.) Kuntze) was used for comparison with Myrtaceae species, Yamamatoyama (Midori Indústria de Chá Ltda., lot number: 242).

2.2. Chemicals

The reagents used in the experiment were DPPH (2,2-diphenyl-1-picrylhydrazyl) and quercetin from Sigma Aldrich, Folin–Ciocalteu reagent from Haloquímica, gallic acid from Vetec, anhydrous sodium carbonate, and methanol from Synth.

2.3. Infusion extraction

The extraction was performed with 20 g of powdered dry leaves of Myrtaceae or green tea and 200 mL of distilled water for 10 min in a thermostatic water bath at 95°C. The extracts were filtered through a filter paper (pore size = 3 µm) in a Büchner funnel and lyophilized (Terroni Enterprise I lyophilizer). The yields were calculated on dry weight basis of the plant material.

2.4. Total phenolics determination assay

The total phenolic content (TPC) in the extracts was determined by a modified Folin–Ciocalteu method (GEORGE et al., 2005). Extracts were diluted in distilled water (0.4 mg/5 mL) and 0.5 mL of the diluted solutions or distilled water (blank) were each mixed with 2.5 mL of Folin–Ciocalteu reagent (1/10, pre-diluted with distilled water). After allowing the diluted extracts to stand for 2 min at room temperature, 2 mL of aqueous Na_2CO_3 solution (75 g/L) was added to each of them. The mixture was vortexed, incubated for 15 min at 50°C, and cooled in an ice water bath. Sample absorbances were measured at 760 nm. A calibration curve was prepared with gallic acid (1–8 $\mu g \cdot mL^{-1}$). TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract according to the calibration curve, $y = 0.133x + 0.001$ ($r^2 = 0.999$), where y denotes absorbance and x denotes gallic acid concentration in mg/L.

2.5. Antioxidant activity assay

The antioxidant properties of the samples were assessed by the DPPH method (SCHERER and GODOY, 2009). Methanolic solutions of the extracts (0.05 mL) at six different concentrations were added to 1.95 mL of DPPH methanolic solution at 0.08 mM. Methanol PA and 2 commercial antioxidants, gallic acid and quercetin, were used as negative and positive controls, respectively. After 90 min of incubation in the dark at room temperature, the absorbances of the samples were measured at 517 nm (Femto

spectrophotometer, model 800XI). Antioxidant activity index (AAI) was calculated as follows: AAI = (final concentration of DPPH in the reaction)/IC_{50}, where the final concentration of the reaction was 30.75 $\mu g \cdot mL^{-1}$. The concentration for 50% inhibition (IC_{50}) was calculated by the linear regression equation between the extract concentration and the corresponding scavenging effect. The scavenging effect was calculated as follows: $I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 indicates absorbance of the negative control, and Abs_1 is the absorbance with the tested extract at different concentrations. SCHERER and GODOY (2009) established the following criteria of AAI values for plant extracts: poor activity < 0.5 < moderate < 1.0 < strong < 2.0 < very strong.

2.6. Data analysis

Assays were performed in triplicates. The data were compared by ANOVA and Tukey test (α = 0.05). Correlations between TPC and AAI values were calculated. Statistical analyses were performed with PAST software, version 2.5 (HAMMER et al., 2001).

3. RESULTS

Extraction yields between 10.4% and 29.2% were obtained after the infusion of powdered dry leaves in distilled water and lyophilization (Table 1). There was a high correlation between phenolic contents and antioxidant activities of these extracts. The determination coefficient (r^2) between them was 0.767 (Figure 1).

The phenolic contents of these extracts were in the range of 287.98–576.56 mg GAE/g extract (Table 1). *P. laruotteanum*, *B. salicifolius*, *E. klotzschiana*, *E. dysenterica*, *H. edulis*, and *M. tomentosa* showed a significantly higher TPC than that found in green tea. TPC of these species ranged from 412.10 to 576.56 mg GAE/g extract, whereas the value of green tea was 259.53 mg GAE/g extract. The highest TPC was found in *P.*

laruotteanum, which was more than twice the amount obtained for green tea. Phenolics also represented at least one fourth of the extract weights from the remaining five species (287.98–324.72 mg GAE/g extract).

In addition, all infusion extracts presented very strong AAIs (>2) according to the criteria established by SCHERER and GODOY (2009) for plant extracts (Table 1). The IC_{50} values were lower than 13 $\mu g \cdot mL^{-1}$, considering a final concentration of DPPH in the reaction of 30.75 $\mu g \cdot mL^{-1}$. P. laruotteanum was also the most potent antioxidant (AAI = 7.97) with activity close to that of quercetin (AAI = 10.35), corresponding to one third of that of pure gallic acid (AAI = 23.23). Nine species statistically displayed the same antioxidant potential as that of green tea (AAI = 4.13), which was equivalent to half the activity of quercetin.

Table 1. Antioxidant activity index (AAI) and total phenolic content (TPC) of leaf infusions from 12 Myrtaceae species. Gallic acid and quercetin: reference antioxidants. Green tea: reference antioxidant infusion. r^2: determination coefficient of free radical scavenging effect on the concentration of the substance/extract I, II, and III (three repetitions). IC_{50}: concentration for 50% inhibition. SD: standard deviation. Different letters: significant difference ($p < 0.05$). GAE: gallic acid equivalents. *: dry weight basis. -: not evaluated.

DPPH: 30.75 µg·mL^{-1}	r^2			Mean IC_{50} (µg·mL^{-1})	Mean AAI ± SD	Mean TPC ± SD (mg GAE/g dry extract)	Extraction yield (%)*
	I	II	III				
Gallic acid	0.996	0.998	0.997	1.33	23.23 ± 1.14 a	-	-
Quercetin	0.994	0.998	0.993	2.99	10.35 ± 0.92 b	-	-
Psidium laruotteanum	0.997	0.997	0.998	3.86	7.97 ± 0.36 c	576.56 ± 21.82 a	22.6
Blepharocalyx salicifolius	0.998	0.995	0.997	6.24	4.95 ± 0.39 d	431.70 ± 10.63 bc	23.2
Eugenia klotzschiana	0.996	0.996	0.961	6.40	4.81 ± 0.26 de	461.50 ± 12.38 b	25.3
Eugenia dysenterica	0.990	0.991	0.996	6.83	4.52 ± 0.37 de	412.10 ± 17.20 b	25.6
Hexachlamys edulis	0.991	0.989	0.997	6.93	4.44 ± 0.23 de	422.10 ± 44.16 bcd	10.4
Psidium australe	0.987	0.990	0.994	7.10	4.35 ± 0.30 de	359.26 ± 11.91 bcdef	29.2
Green tea	0.984	0.982	0.982	7.45	4.13 ± 0.01 de	259.53 ± 4.30 f	16.2
Myrcia tomentosa	0.988	0.993	0.999	7.46	4.13 ± 0.17 de	463.69 ± 54.42 b	18.0
Myrcia bella	0.995	0.988	0.998	7.73	3.99 ± 0.24 de	312.99 ± 8.60 ef	19.4
Myrcia lingua	0.984	0.971	0.988	7.80	3.95 ± 0.16 de	318.93 ± 47.69 def	25.8
Psidium cinereum	0.993	0.970	0.993	8.75	3.52 ± 0.21 ef	324.72 ± 3.28 def	22.6
Myrcia splendens	0.996	0.994	0.995	12.48	2.47 ± 0.14 f	287.98 ± 55.66 f	19.4
Eugenia bimarginata	0.945	0.992	0.996	12.83	2.40 ± 0.05 f	318.93 ± 57.43 def	24.3

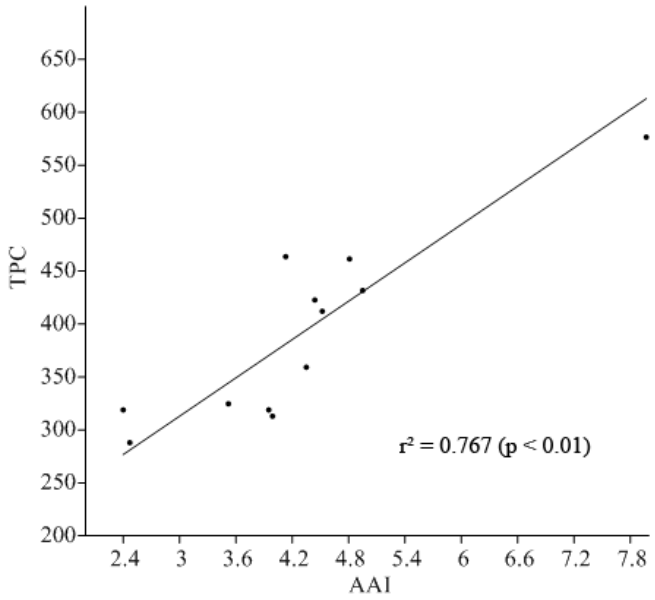

Figure 1. Linear correlation between total phenolic content (TPC in mg of gallic acid equivalents/g dry extract) and antioxidant activity indexes (AAI) of leaf infusions from 12 Myrtaceae species.

4. DISCUSSION

Extract´s yield and chemical composition are determined by the extraction method (DAI and MUMPER, 2010). Water infusion is a simple, fast, cheap, and non-toxic procedure to extract phenolic compounds efficiently because of their water polarity. Therefore, this method was used in our study, resulting in high extraction yields of phenolics.

Phenolics are the most abundant secondary metabolites in plants (DAI and MUMPER, 2010). Myrtaceae species have the ability to accumulate phenolics (SALVADOR et al., 2011). These organic compounds are important defense antioxidants (PIETTA, 2000), which are more potent than Vitamin C and E and carotenoids (RICE-EVANS et al., 1996). Some authors reported phenolic contents in leaves of other Myrtaceae species. COUTINHO et al. (2008) found a TPC of 7.2–21.2 mg GAE/g extract

in *Campomanesia adamantium*. SALVADOR et al. (2011) reported high values for *Eugenia chlorophylla*, *Eugenia pyriformis*, *Myrcia laruotteana*, and *Myrcia obtecta* (343.7–429.3 mg GAE/g extract). In these studies, the extraction methods were different. They used organic solvents such as hexane, chloroform, methanol, and ethanol. However, some of our TPC values were still higher than these results.

Phenolic content and antioxidant activity are parameters of quality for tea pertaining to its biological properties (ANESINI et al., 2008). For this reason, several studies have been performed to evaluate these parameters and their functional properties (e.g., anti-inflammatory or anticarcinogenic activity) (YAO et al., 2006; CHAN et al., 2007; ANESINI et al., 2008; NISHIYAMA et al., 2010; SENGER et al., 2010). In our study, green tea was used as another reference (in addition to the pure substances, quercetin, and gallic acid) due to its abundance of flavonoids, including catechins and their derivatives, which may constitute up to 30% of its dry weight (LORENZO et al., 2013). In our study, leaf infusions of Myrtaceae species showed phenolic concentrations similar to or even higher than those of green tea.

AAI by the DPPH method was used because it is considered appropriate for comparing extracts and pure compounds. There is no difference in AAI values when different solutions of DPPH and concentrations of the compounds/extracts are used (SCHERER and GODOY, 2009).

The relation of extraction yield, TPC and antioxidant activity must be taken into account in the analysis of results. One species can display high TPC and AAI values but low yield. Others may display lower antioxidant activity but higher extraction yield. The understanding of the balance between these factors is necessary for further studies and their application. Despite this variation, the studied species displayed high antioxidant

activities and can be considered as promising for future studies. *Psidium laruotteanum* was the most potent, possessing the highest phenolic content and antioxidant activity.

To the best of our knowledge, this is the first comparative survey on the antioxidant potential of Myrtaceae leaf infusions from the cerrado. The focus of antioxidant studies in the Myrtaceae family from Brazil has been on edible fruits (MARIN et al., 2008; RUFINO et al., 2009; RUFINO et al., 2010; PEREIRA et al., 2012) with few reports on leaves (COUTINHO et al., 2008; SALVADOR et al., 2011). Thus, our results contribute valuable knowledge about the bioactive properties of native Myrtaceae species.

Further studies should be conducted to isolate, characterize, and understand the mode of action of these phenolic compounds. Likewise, evaluation of the effect of environmental abiotic factors, such as temperature and humidity, on the production and concentration of these compounds is desirable.

ACKNOWLEDGEMENTS

We are grateful to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for providing a scholarship to the first author and for funding the Research Productivity Grant of the third author.

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CAPÍTULO 4

Optimized Ultrasound-Assisted Extraction of *Psidium laruotteanum* roots: a concentrated source of piceid from the Brazilian savanna

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Optimized Ultrasound-Assisted Extraction of *Psidium laruotteanum* roots: a concentrated source of piceid from the Brazilian savanna

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RESUMO

Psidium laruottenaum é uma Myrtaceae que ocorre como arbusto ou arvoretas. É nativa do cerrado e distribuída pela América Central e do Sul. Neste estudo, o composto benéfico à saúde *trans*-piceido foi descrito pela primeira vez no gênero *Psidium*. A extração assistida por ultrasom de raízes de *P. laruottenaum* foi otimizada por uma metodologia de superfície de resposta. Um delineamento composto central foi usado para avaliar seis variáveis de extração. Extrações ótimas foram obtidas com 62.5% de MeOH em água, a 69.1 °C, amplitude de 70%, ciclo de 0.5 s^{-1}, pH 5.7 e razão solvente-sólido de 40:1, por 10 minutos. O método mostrou excelente recuperação (90-95%) e precisão (CV: 0.69% para repetibilidade e 0.43% para precisão intermediária). Essa espécie aparece como a fonte vegetal mais concentrada de piceido relatada até hoje (2.9% em raízes secas). Além disso, uma extração muito rápida (5 min) pode proporcionar 96% da recuperação de piceido da raiz de *Psidium laruotteanum*.

Keywords: Araçá-cascudo; Estilbeno; Q-ToF-MS; Extração Assistida por Ultrasom; UPLC-PDA.

ABSTRACT

Psidium laruottenaum is a Myrtaceae that occurs as shrubs or small trees. It is native to the Brazilian savanna and distributed in Central and South America. In this study, the health beneficial compound *trans*-piceid was described for the first time in the genus *Psidium*. The ultrasound-assisted extraction from *P. laruottenaum* roots was optimized by a response surface methodology. A central composite design was used to assess six extraction variables. Optimal extractions were obtained with 62.5% MeOH in water at 69.1 °C, amplitude of 70%, cycle of 0.5 s^{-1}, pH 5.7 and solvent-solid ratio of 40:1, for 10 minutes. The method showed excellent recovery (90-95%) and precision (CV: 0.69% for repeatability and 0.43% for intermediate precision). This species appears as the most concentrated plant source of piceid reported to date (2.9% in dry roots). Additionally, a very fast extraction (5 min) can afford for 96% of the recovery of piceid from the roots of *Psidium laruotteanum*.

Keywords: Araçá-cascudo; Q-ToF-MS; Stilbene; Ultrasound-Assisted Extraction; UPLC-PDA.

1. INTRODUCTION

Piceid (reservatrol-3-β-mono-D-glucoside) is a stilbene described as the major form of resveratrol in nature (REGEV-SHOSHANI et al., 2003). Recently, this compound is gaining more attention because an increasing number of studies have shown physiological and pharmaceutical properties in common with resveratrol, including antioxidant, anti-cancer (SU et al., 2013) and anti-inflammatory (LANZILLI et al., 2012). Additionally, after oral ingestion it can be metabolized into resveratrol which is absorbed and becomes available in higher concentrations (ZHOU et al., 2009).

In plants, the glycosylation of resveratrol protects it from enzymatic oxidation (REGEV-SHOSHANI et al., 2003) and piceid is found in higher concentrations. However, most studies still report low concentrations of this compound. Some examples of food sources containing piceid are cocoa and chocolate products (0.35–7.14 $\mu g \cdot g^{-1}$) (HURST et al., 2008), peanut butter (0.067–0.225 $\mu g \cdot g^{-1}$) (IBERN-GOMEZ et al., 2000) tomato skin (0.1–50 $\mu g \cdot g^{-1}$) (RAGAB et al., 2006), hop cone (0.42–11.01 $\mu g \cdot g^{-1}$) (JERKOVIC and COLLIN, 2007), grape berry skin (42.19 $\mu g \cdot g^{-1}$) (ROMERO-PÉREZ et al., 2001) and wine (1.19–22.08 $mg \cdot L^{-1}$) (MORENO-LABANDA et al., 2004). Grapes and wine are widely studied, as resveratrol and its glycosides (e.g. piceid) have a potential role in the "French paradox", the lower incidence of cardiovascular diseases associated with moderate wine consumption (CATALGOL et al., 2012).

The Brazilian savanna presents an enormous plant biodiversity. However, most species are still underexploited, while deforestation and loss of biodiversity are main issues. During preliminary investigations, we noticed piceid as an important compound in *Psidium laruotteanum* roots. This species, known popularly as *araçá-cascudo*, belongs to the same genus of guava (*Psidium guajava*), family Myrtaceae, and occurs as shrubs or small trees. Its fleshy fruits present exotic flavor and are consumed *in natura* or as processed products

(FRANZON et al., 2009). Native to the Brazilian savanna it is also distributed in Bolivia, Colombia, Costa Rica, Guyana, Paraguay, Suriname and Venezuela (SOBRAL et al.; TROPICOS.ORG.). As far as we are concerned, there is just one investigation about the biological properties (phytotoxicity of leaf extracts) (IMATOMI et al., 2013) with no assessment on the chemical composition of this species.

Several techniques, including microwave-assisted extraction (KAUFMANN and CHRISTEN, 2002), supercritical fluid extraction (NAHAR and SARKER, 2012) and accelerated solvent extraction (MOTTALEB and SARKER, 2012) can be developed in order to combine maximum extraction yield of bioactive compounds with short time of extraction and low solvent consumption. For this purpose, ultrasound-assisted extraction is a valuable alternative method. It is simple, efficient, environmental friendly and presents low instrumental requirements (WANG and WELLER, 2006).

Thus, the aim of this study was to assess the potential concentration of piceid in *Psidium laruotteanum* roots and improve its extraction. An ultrasound-assisted method was optimized using a central composite design (CCD) at three levels with response surface methodology (RSM). The influences of six variables, type of solvent, temperature, amplitude, cycle, pH and solvent-solid ratio, on the response were discussed and an optimal condition of extraction was obtained.

2. MATERIALS AND METHODS

Chemicals. Solvents were of HPLC-grade: hexane, methanol, acetic acid and acetonitrile were purchased from Panreac (Barcelona, Spain). Piceid standard was obtained from Sigma Aldrich (St. Louis, MO, USA) and water was purified in a Milli-Q Integral system (Millipore, Billerica, MA, USA).

Plant sample. Roots of *P. laruotteanum* were collected from five individuals in the Brazilian savanna (21º58'5"S and 47º53'12"W), São Carlos, São Paulo, Brazil, on August 2014. A voucher specimen (number: 8720) was deposited at the Herbarium of the Federal University of São Carlos. The material was dried at 35 ºC and reduced to powder in an electric grinder. Fat and wax were removed by washing 25 g of powdered roots with 100 mL of hexane for 15 minutes at 20-30 ºC using a 45 kHz ultrasound bath (USC500T, VWR Collection, Pennsylvania, USA). This process was repeated four times in total. Later the solid was filtered through Whatman filter paper (3 µm) under reduced pressure, dried at 35 ºC and stored at ambient temperature in the dark until extraction. The powdered roots had less than 0.01% of fat and wax.

Ultrasound-assisted extraction (UAE) of piceid. Powdered roots (0.25 g) were extracted in 27 × 80 mm (diameter × height) glass tubes under water bath with controlled temperature (Frigiterm, J.P. Selecta, Barcelona, Spain). An ultrasonic system with a 7 mm diameter probe (model UP200S, 200 W and 24 kHz, Hielscher Ultrasonics GmbH, Teltow, Germany) was placed at 5 mm from the bottom of the tube. A set volume and type of solvent were used under specific UAE conditions, following the design of experiment (DOE). After extraction, the solid phase was removed by centrifugation (J. P. Selecta, Barcelona, Spain) at 4000 rpm for 5 minutes. Then, the supernatant was adjusted to the initial volume of extraction and filtered through nylon filter (0.22 µm) before injection into the UPLC-PDA/MS system.

Determination of piceid. To confirm *trans*-piceid in *Psidium laruotteanum* roots, a 100% MeOH extract was analyzed in an Acquity UPLC H-Class system coupled to quadrupole-time-of-flight mass spectrometer (Q-ToF-MS, Synapt G2, Waters Corp., Milford, MA, USA). Masslynx software - version 4.1 was used to control the equipment. The injection volume was set to 2 µL and the separation was performed on a reverse-phase

C18 column (Acquity UPLC® BEH, 2.1 x 100 mm, 1.7 µm, Waters Corporation, Ireland). The mobile phase consisted of (A) water with 5% formic acid and (B) methanol with 5% formic acid at a flow rate of 0.45 $mL \cdot min^{-1}$. The elution gradient was: 0 min, 0% B; 1 min, 0% B; 3 min, 5% B; 4 min, 10% B; 4.5 min, 10% B; 5 min, 20% B; 7 min, 20% B; 8 min, 30% B; 10 min, 30% B. Subsequently, the column was washed with 100% B for 2 min and equilibrated with 0% B for 2 min. An electrospray source operating in positive ionization mode was used under the following conditions: desolvation gas flow = 500 $L \cdot h^{-1}$, desolvation temperature = 350 ºC, cone gas flow = 10 $L \cdot h^{-1}$, source temperature = 110 ºC, capillary = 3.0 kV, cone voltage = 10 V and trap collision energy = 4 eV. Full-scan mode was used to measure the mass/charge ratios (*m/z*) between 50 and 1200 Da.

For quantification of piceid, extraction samples were re-analyzed on an Acquity UPLC H-Class system coupled to an Acquity UPLC Photodiode Array (PDA) Detector controlled by Empower 3 Chromatography Data Software (Waters Corporation, Milford, MA, USA). Separations were performed in a reverse phase C18 column at 47 °C (Acquity UPLC® BEH, 2.1 x 100 mm, 1.7 µm, Waters Corporation, Ireland). The mobile phase consisted of phase A (water with 2% acetic acid) and phase B (acetonitrile with 2% acetic acid). The flow rate was 0.6 $mL \cdot min^{-1}$ in a 4 minutes gradient as follows: (%B): 0 min, 0%; 1 min, 0%; 1.1 min, 10%; 2 min, 10%; 3 min, 20%; 3.5 min, 60%; 4 min, 100%. After this, the column was washed with 100% B for 3 min and equilibrated with 0% B for 3 min. The Photodiode Array (PDA) Detector was set in the wavelength range of 200-400 nm for the 3D scan with collection data rate at 40 $pts \cdot s^{-1}$ to identify the compound. Whereas, for compound quantification the PDA detector was set at fixed wavelength of 317 nm for 2D scan with collection data rate at 80 $pts \cdot s^{-1}$. These values fit the maximum absorbance of piceid (± 317 nm). The injection volume of the sample was 0.3 µL and piceid was identified by spiking and comparing the retention time and maximum UV absorptions with the standard.

Design of experiment. Response surface methodology was used to establish the optimal condition to extract piceid from *P. laruotteanum* roots. A central composite design (CCD) was developed with six independent variables (x_1, solvent; x_2, temperature; x_3, amplitude; x_4, cycle; x_5, pH; x_6, solvent-solid ratio) at three levels. These levels were normalized and forced to fit values of -1, 0 and 1 so that all variables affected the response more evenly, independently of the unit (BAŞ and BOYACı, 2007). The complete design had 46 different experimental runs (Table 1). The resulting data was fit to a polynomial model to obtain the response surface. The effect of each variable and the interaction effects between variables were also explored. The general function of the CCD is described in the following equation:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} b_i x_i x_j \quad (1)$$

where x_i are the studied variables; y is the extraction yield of piceid; and b are the coefficients obtained by the least square method.

The experimental design, analysis, and surface response were developed using the software STATGRAPHICS Centurion (Trial Version 16.1.18, Statpoint Technologies, Inc., USA). Analysis of Variance (ANOVA) and the Least Significant Difference (LSD) test were used to determine the significance of differences between the means.

Performance of the method. A calibration curve was developed to quantify piceid. Solutions of standard piceid were prepared in triplicate at six concentrations ranging from 0.1 to 1 mg·mL^{-1}. The linear regression equation between the solutions' concentrations and respective peak surface areas was used to quantify piceid in the extractions. Two extractions were done to verify the amount of piceid in the optimal UAE condition.

The method for extraction of piceid from *P. laruotteanum* roots was validated according to procedures described in the ICH Guideline Q2 (R1) (ICH, 2005). The

considered parameters were precision and recovery. Precision was assessed considering repeatability (intra-day) and intermediate precision (extra-day). Repeatability was determined by the analyses of nine extractions under the same conditions on the same day. Intermediate precision was measured by three extractions on each of three consecutive days. Finally, precision was expressed as the Coefficient of Variance (CV) of piceid peak surface area.

The recovery of the method was determined by comparing the response in spiked and non-spiked root samples in duplicate. The standard solution of piceid was spiked at a concentration of 0.6 $mg \cdot mL^{-1}$.

Extraction kinetics. Kinects was analyzed at the UAE optimal condition of piceid from *P. laruotteanum* roots. Six times of extraction were assessed in duplicate: 5, 10, 15, 20, 25 and 30 minutes.

3. RESULTS AND DISCUSSION

Identification of *trans*-piceid. Piceid was confirmed in *Psidium laruotteanum* roots by mass spectra in positive ion mode (Figure 1). The fragmentation of the ion 391 $[M+H]^+$ into the ion 229 $[M+H]^+$ due to loss of 162 Da (glucose) indicated the presence of piceid. Later, the chromatographic peaks of the extraction solutions were just checked by comparing the retention time and UV-vis spectra (λ_{max}: 232.9 and 317.3 nm) with the standard before quantification. To the best of our knowledge, this is the first time *trans*-piceid is described and quantified in the genus *Psidium*.

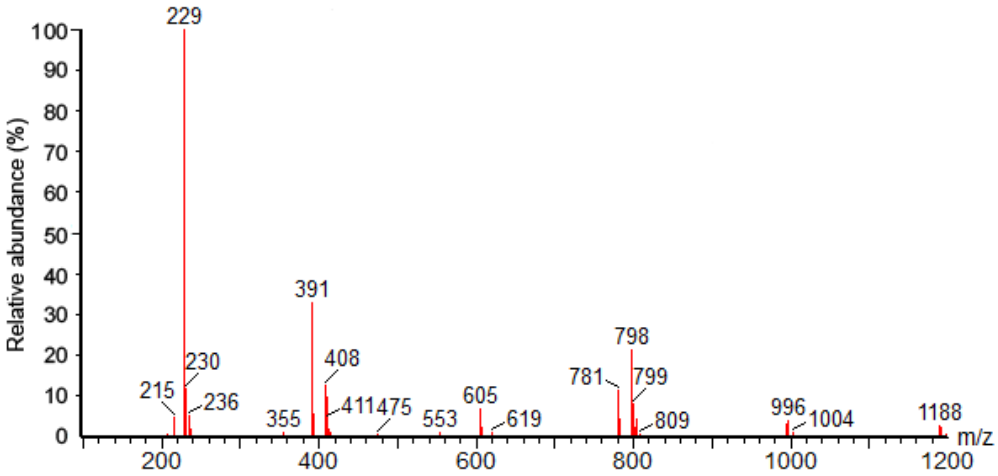

Figure 1. UPLC-MS spectrum of piceid acquired in positive ion mode of the 100% MeOH extract of *Psidium laruotteanum* roots. Retention time: 5.518 minutes.

Development of the UAE method. To optimize the extraction of piceid from *P. laruotteanum* roots, a central composite design was developed (Table 1). The analyzed variables were solvent composition (x_1: 50, 75, 100% methanol in water), temperature (x_2: 30, 50, 70 ºC), amplitude (x_3: 30, 50, 70%), cycle (x_4: 0.3, 0.5, 0.7 s^{-1}), solvent pH (x_5: 3, 5, 7) and solvent-solid ratio (x_6: 20:1, 30:1, 40:1). Relative values to the maximum peak surface area of piceid were used as the responses.

Table 1. Central composite design with 6 variables at 3 levels of the UAE of piceid and the observed responses.

DOE runs	Extraction factors						Relative values to maximum yield (%)
	x_1: Solvent (MeOH %)	x_2: Temperature (^{0} C)	x_3: Amplitude (%)	x_4: Cycle (s^{-1})	x_5: pH	x_6: Solvent-solid ratio	
1	50 (−1)	30 (−1)	30 (−1)	0.7 (+1)	7 (+1)	20 (−1)	80.4
2	100 (+1)	30 (−1)	30 (−1)	0.7 (+1)	7 (+1)	40 (+1)	70.8
3	100 (+1)	70 (+1)	70 (+1)	0.3 (−1)	3 (−1)	40 (+1)	86.8
4	50 (−1)	30 (−1)	30 (−1)	0.3 (−1)	7 (+1)	40 (+1)	84.1
5	50 (−1)	70 (+1)	30 (−1)	0.3 (−1)	7 (+1)	20 (−1)	85.2
6	50 (−1)	70 (+1)	30 (−1)	0.3 (−1)	3 (−1)	40 (+1)	98.5
7	100 (+1)	70 (+1)	30 (−1)	0.7 (+1)	3 (−1)	40 (+1)	84.9

8	50 (−1)	70 (+1)	30 (−1)	0.7 (+1)	3 (−1)	20 (−1)	88.2
9	75 (0)	50 (0)	70 (+1)	0.5 (0)	5 (0)	30 (0)	93.0
10	100 (+1)	30 (−1)	70 (+1)	0.7 (+1)	7 (+1)	20 (−1)	73.7
11	75 (0)	30 (−1)	50 (0)	0.5 (0)	5 (0)	30 (0)	84.0
12	100 (+1)	30 (−1)	70 (+1)	0.3 (−1)	7 (+1)	40 (+1)	73.6
13	75 (0)	50 (0)	30 (−1)	0.5 (0)	5 (0)	30 (0)	92.7
14	75 (0)	50 (0)	50 (0)	0.3 (−1)	5 (0)	30 (0)	90.7
15	75 (0)	50 (0)	50 (0)	0.5 (0)	5 (0)	20 (−1)	88.7
16	50 (−1)	30 (−1)	70 (+1)	0.7 (+1)	3 (−1)	20 (−1)	87.0
17	75 (0)	70 (+1)	50 (0)	0.5 (0)	5 (0)	30 (0)	100.0
18	50 (−1)	70 (+1)	70 (+1)	0.3 (−1)	7 (+1)	40 (+1)	98.5
19	75 (0)	50 (0)	50 (0)	0.5 (0)	3 (−1)	30 (0)	89.1
20	50 (−1)	30 (−1)	70 (+1)	0.3 (−1)	7 (+1)	20 (−1)	83.6
21	100 (+1)	30 (−1)	70 (+1)	0.7 (+1)	3 (−1)	40 (+1)	79.0
22	100 (+1)	70 (+1)	70 (+1)	0.7 (+1)	7 (+1)	40 (+1)	91.3
23	100 (+1)	30 (−1)	30 (−1)	0.3 (−1)	3 (−1)	40 (+1)	67.0
24	100 (+1)	70 (+1)	70 (+1)	0.3 (−1)	7 (+1)	20 (−1)	84.6
25	100 (+1)	70 (+1)	30 (−1)	0.7 (+1)	7 (+1)	20 (−1)	77.1
26	50 (−1)	30 (−1)	30 (−1)	0.3 (−1)	3 (−1)	20 (−1)	80.8
27	75 (0)	50 (0)	50 (0)	0.7 (+1)	5 (0)	30 (0)	91.5
28	75 (0)	50 (0)	50 (0)	0.5 (0)	5 (0)	30 (0)	93.7
29	100 (+1)	30 (−1)	70 (+1)	0.3 (−1)	3 (−1)	20 (−1)	72.5
30	50 (−1)	30 (−1)	70 (+1)	0.7 (+1)	7 (+1)	40 (+1)	90.8
31	50 (−1)	30 (−1)	70 (+1)	0.3 (−1)	3 (−1)	40 (+1)	87.5
32	50 (−1)	70 (+1)	30 (−1)	0.7 (+1)	7 (+1)	40 (+1)	96.7
33	75 (0)	50 (0)	50 (0)	0.5 (0)	5 (0)	40 (+1)	95.4
34	100 (+1)	50 (0)	50 (0)	0.5 (0)	5 (0)	30 (0)	86.2
35	50 (−1)	70 (+1)	70 (+1)	0.7 (+1)	3 (−1)	40 (+1)	98.9
36	100 (+1)	30 (−1)	30 (−1)	0.7 (+1)	3 (−1)	20 (−1)	72.5
37	50 (−1)	50 (0)	50 (0)	0.5 (0)	5 (0)	30 (0)	95.1
38	50 (−1)	70 (+1)	70 (+1)	0.3 (−1)	3 (−1)	20 (−1)	87.5
39	50 (−1)	70 (+1)	70 (+1)	0.7 (+1)	7 (+1)	20 (−1)	88.8
40	50 (−1)	30 (−1)	30 (−1)	0.7 (+1)	3 (−1)	40 (+1)	91.5
41	100 (+1)	30 (−1)	30 (−1)	0.3 (−1)	7 (+1)	20 (−1)	67.8
42	100 (+1)	70 (+1)	30 (−1)	0.3 (−1)	7 (+1)	40 (+1)	87.3
43	100 (+1)	70 (+1)	70 (+1)	0.7 (+1)	3 (−1)	20 (−1)	78.4
44	75 (0)	50 (0)	50 (0)	0.5 (0)	5 (0)	30 (0)	93.8
45	75 (0)	50 (0)	50 (0)	0.5 (0)	7 (+1)	30 (0)	91.2
46	100 (+1)	70 (+1)	30 (−1)	0.3 (−1)	3 (−1)	20 (−1)	84.5

The design had 46 runs with 12 center points and their data were used to obtain a mathematical model. The significance of each effect was determined by ANOVA, comparing the mean square against an estimate of the experimental error. The absolute

values of the estimated effects divided by their standard values are plotted in a Pareto chart (Figure 2). Variables or combination of variables crossed by the vertical line had a significant effect on the response ($p<0.05$).

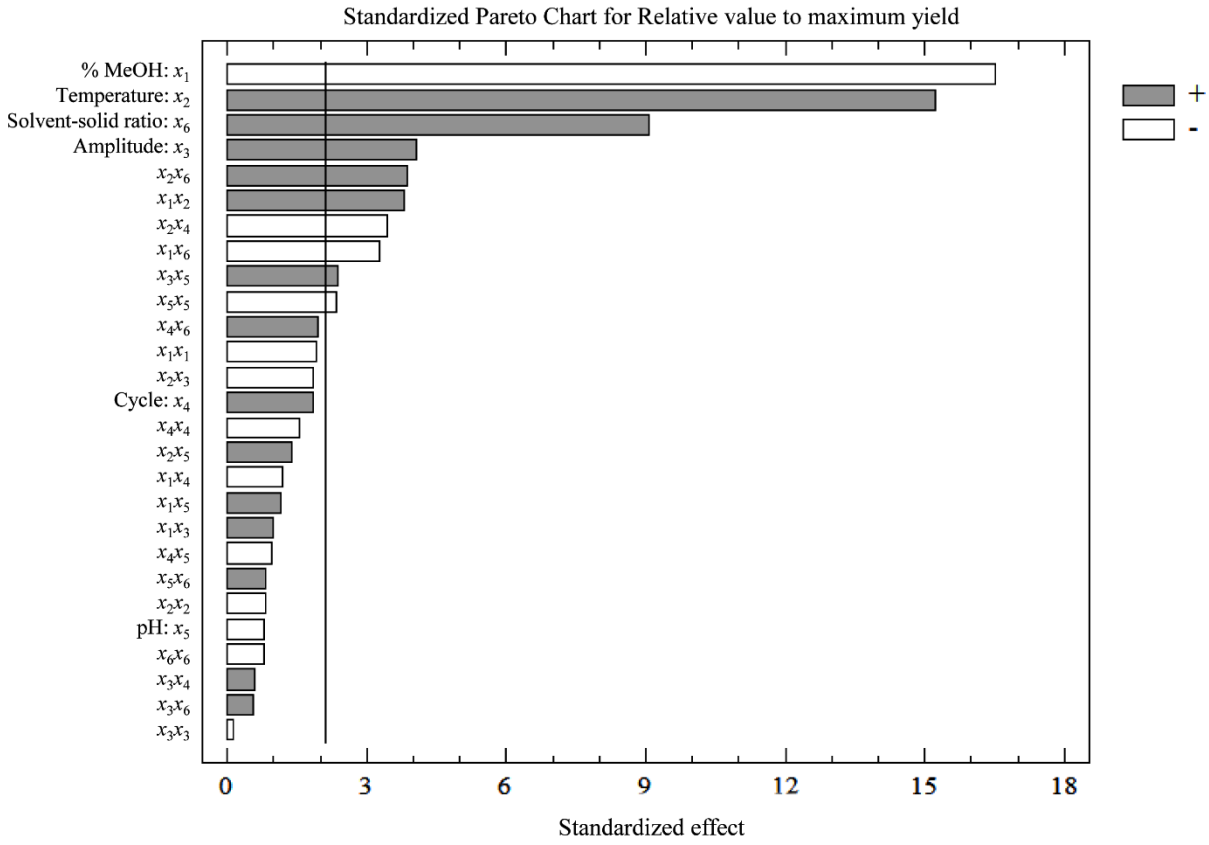

Figure 2. Effects of variables and their interactions on the extraction of piceid from *Psidium laruotteanum* roots.

The most significant variables on the extraction of piceid were solvent (x_1), temperature (x_2), solvent-solid ratio (x_6) and amplitude (x_3). The extraction solvent had a negative effect so a higher recovery was achieved on decreasing the percentage of methanol in water. In contrast, temperature, solvent-solid ratio and amplitude had a positive effect as the recovery was higher on increasing these variables. Studies on *Polygonum cuspidatum* roots have also demonstrated better recovery of piceid when increasing the solvent polarity

and the temperature (BEŇOVÁ et al., 2010; KUO et al., 2013). This probably happens because piceid contains a glucoside that increases its polarity (KUO et al., 2013) and heating favors the extraction by increasing the solubility and diffusion coefficient of solutes (SPIGNO et al., 2007).

Greater responses using higher solvent to solid ratios are explained by the principle of mass transference, where the driving force is the concentration difference between the solid and the liquid (AL-FARSI and LEE, 2008). This ratio could be increased using higher solvent volumes or less solid. However, the reduction of solid could increase the error and increasing the volume affect the viability due to longer procedures to evaporate the solvent and concentrate the solute. Consequently, no higher ratios were investigated. As expected the amplitude also presented a positive effect on the extraction of piceid. The formation and collapse of cavitation bubbles are proportional to this variable. Thus, the higher the amplitude, the higher is the ultrasonic intensity (PINGRET et al., 2013) that breaks the cells of the plant matrix and releases the compounds into the extraction medium (VINATORU et al., 1997).

Additionally, six interactions between variables affected the response and were better analyzed using 3D surface plots. The regression coefficients for the variables and their interactions were calculated. Therefore, the equation for the fitted model was:

$$y = 0.931537 - 0.0544345x_1 + 0.0501971x_2 + 0.0134014x_3 + 0.00611988x_4 - 0.00265226x_5 + 0.0298564x_6 - 0.0239046x_1^2 + 0.0129269x_1x_2 + 0.00335561x_1x_3 - 0.00403281x_1x_4 + 0.00388026x_1x_5 - 0.0111145x_1x_6 - 0.0102286x_2^2 - 0.0063366x_2x_3 - 0.0116993x_2x_4 + 0.00466528x_2x_5 + 0.0131688x_2x_6 - 0.00169071x_3^2 + 0.00200283x_3x_4 + 0.00805471x_3x_5 + 0.00186403x_3x_6 - 0.0193076x_4^2 - 0.00323346x_4x_5 + 0.00662813x_4x_6 - 0.0292788x_5^2 + 0.00283952x_5x_6 - 0.0097754x_6^2 \quad (2)$$

where *y* is piceid yield; x_1, solvent; x_2, temperature; x_3, amplitude; x_4, cycle; x_5, pH; and x_6, solvent-solid ratio.

The *R*-Squared statistic indicated that the model as fitted explained 97.9418% of the variability in the relative value to maximum yield. The standard error of the predicted value showed a standard deviation of the residuals of 0.0192. For this reason, the model could be used to estimate the responses for the optimization of UAE of piceid.

Response surface and optimization of the method. The eq. 2 was used to build 3D surface plots and determine the optimal levels of the variables for the UAE of piceid. While examining a pair of variables the others were kept constant in the central point.

Solvent of extraction had a significant relationship with temperature and solvent-solid ratio (Figure 2). A decrease in MeOH from 100 to 50%, with an increase in temperature until 70 ºC (Figure 3) or in solvent-solid ratio to 40:1 (Figure 4), resulted in higher extraction yields of piceid. Besides favoring the response, lower MeOH % enabled extractions at higher temperatures with no volume variation due to solvent evaporation. No higher temperatures than 70ºC were applied because of solvent evaporation during the extraction. In addition, polyphenolics can oxidize or degrade, decreasing the yield and conformation of the compounds (DAI and MUMPER, 2010).

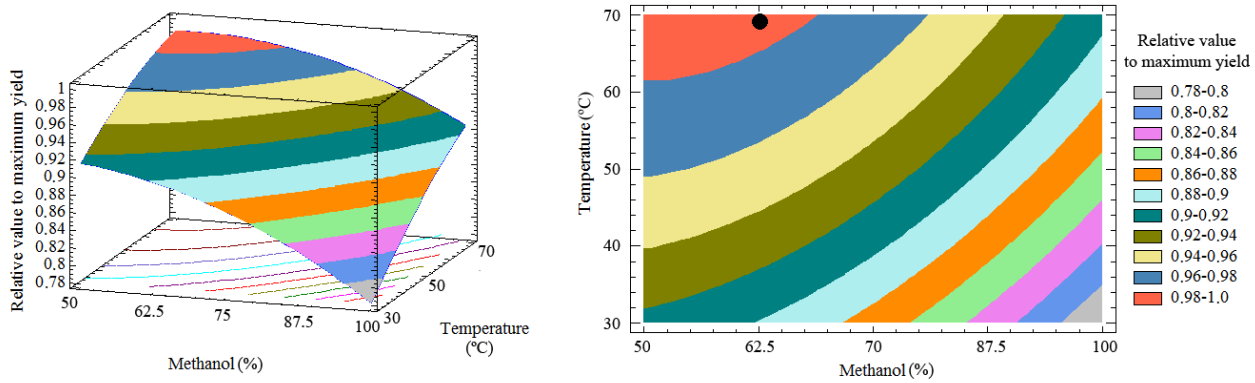

Figure 3. Response surface and contour plots between solvent (MeOH %) and temperature. Black dot: final optimal condition considering all variables.

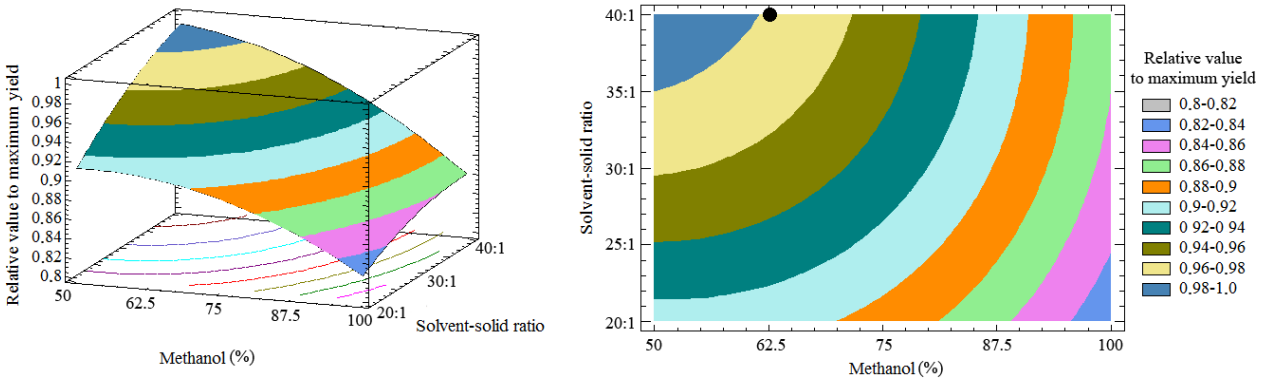

Figure 4. Response surface and contour plots between MeOH % and solvent-solid ratio. Black dot: final optimal condition considering all variables.

Temperature of extraction also had significant relationship with solvent-solid ratio and cycle (Figure 2). Increasing temperature from 30 to 70 ºC, with an increase in solvent-solid ratio from 20:1 to 40:1 (Figure 5) favored the extraction yield of piceid. Temperature had a slightly higher effect to the response than solvent-solid ratio. Increasing temperature until 70ºC at a solvent-solid ratio of 20:1 reaches 95% of response, while an increase of solvent-solid ratio until 40:1 at 30ºC reaches 90% of response. Nevertheless, these variables present a direct correlation where the maximum response is achieved when both variables are

increased. Figure 6 explores the interaction of temperature with ultrasound cycle. Increasing the temperature from 30 to 70 ºC and keeping the cycle in the range of 0.32 and 0.62 resulted in higher responses.

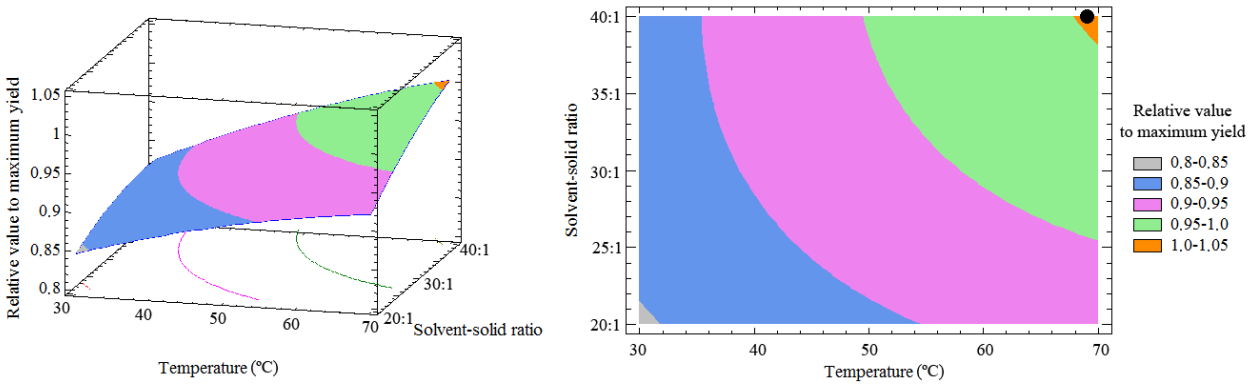

Figure 5. Response surface and contour plots between temperature and solvent-solid ratio. Black dot: final optimal condition considering all variables.

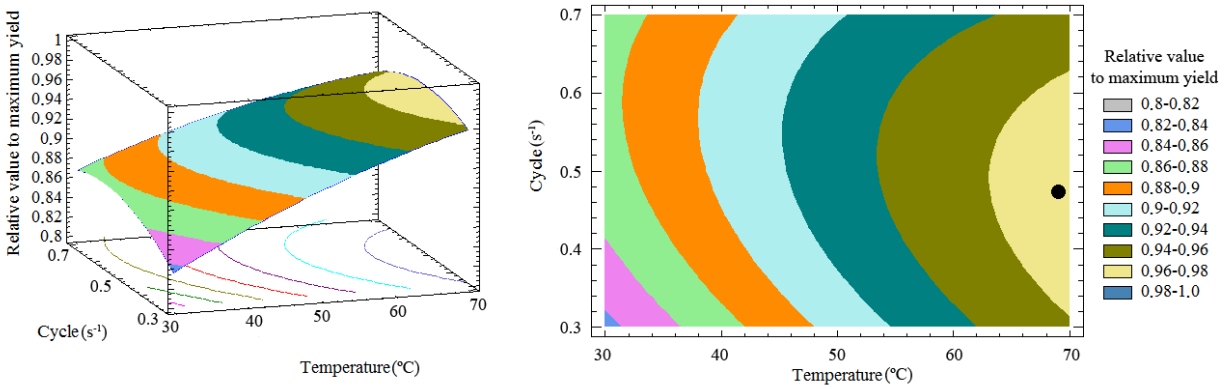

Figure 6. Response surface and contour plots between temperature and cycle. Black dot: final optimal condition considering all variables.

The effect of amplitude interacting with pH of extraction is shown in Figure 7. Higher amplitudes associated with pH between 4.5 and 5.9 resulted in higher recoveries of piceid.

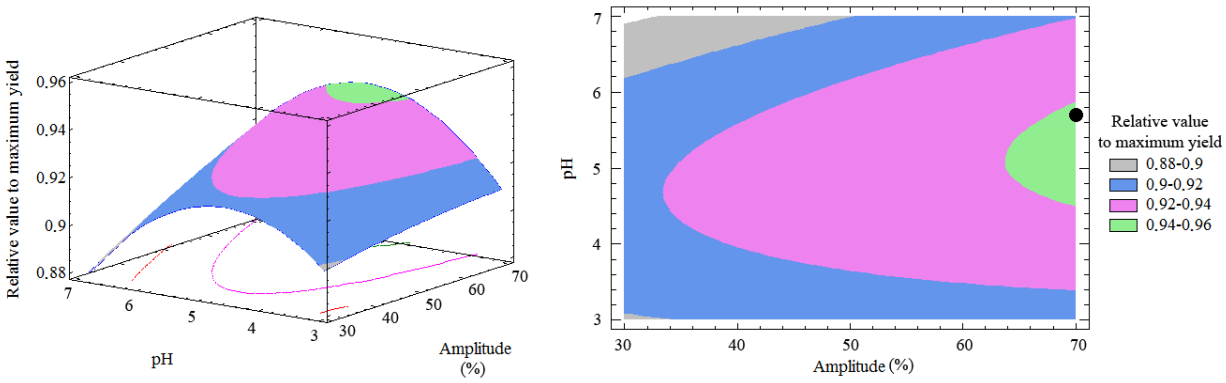

Figure 7. Response surface and contour plots between amplitude and pH. Black dot: final optimal condition considering all variables.

Optimal condition and method validation. The following optimal condition was obtained according to the RSM: ultrasound-assisted extraction using 62.7% of MeOH in water, temperature of 69.1 °C, amplitude of 70%, cycle of 0.47 s^{-1}, pH of 5.7 and a solvent-solid ratio of 40:1. The model equation estimated an extraction yield of 29.13 $mg \cdot g^{-1}$. Thus, two extractions were done in similar conditions (Table 2) and the resulting yield was 29.15±0.17 $mg.g^{-1}$, which fits perfectly the predicted value.

The same conditions were used to validate the method, considering precision and recovery (ICH, 2005). Precision was evaluated by the repeatability (analysis of nine extractions in the same day) and intermediate precision (six extractions in each of three consecutive days). The method was precise with coefficient of variation for repeatability and intermediate precision of just 0.69% and 0.43%, respectively. The recovery of piceid ranged from 90% to 95% and it was estimated by comparing the response in samples spiked and non-spiked with standard piceid. These results are then consistent for piceid extraction using the UAE method.

Table 2. Optimal and used extraction conditions with respective piceid yields.

	MeOH in water (%)	Temperature (ºC)	Amplitude (%)	Cycle (s^{-1})	pH	Solvent-solid ratio	Extraction yield of Piceid ($mg \cdot g^{-1}$)
Optimal condition	62.7	69.1	70	0.47	5.7	40:1	29.13*
Used condition	62.5	69.1	70	0.5	5.7	40:1	29.15±0.17

*: estimated value by the model´s equation.

Extraction kinetics. Time is another key factor in determining the viability of extraction methods. Defining the shorter efficient extraction is essential in order to minimize energy costs and avoid the oxidation of compounds (DAI and MUMPER, 2010). In 10 minutes the maximum amount of piceid was extracted from roots of *P. laruotteanum* using the optimal UAE conditions (Figure 6). However, five minutes seems more valuable considering a difference of just 1.15±0.16 $mg.g^{-1}$ (lower than 4%) in half of the time.

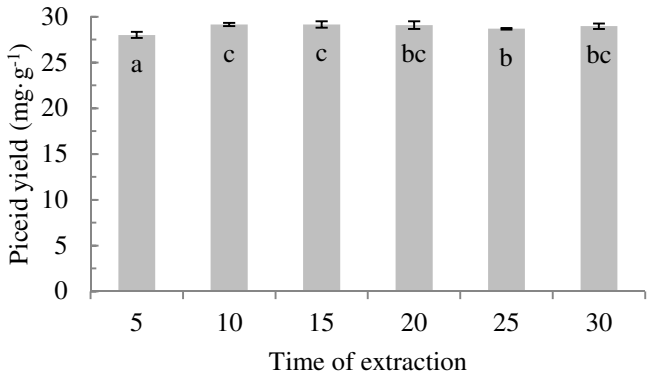

Figure 6. Piceid yield under different times of ultrasound-assisted extraction. Different letters: significant difference ($p < 0.05$).

Many studies have focused on the extraction of piceid from other plant sources, like grape berry skin (ROMERO-PÉREZ et al., 2001), tomato skin (RAGAB et al., 2006), hop

cone (JERKOVIC and COLLIN, 2007), cocoa powder (HURST et al., 2008) but just achieved low concentrations, ranging from 0.042 to 50 $\mu g \cdot g^{-1}$ in longer extraction procedures (around 30 minutes). The most concentrated source of piceid reported so far is *Polygonum cuspidatum* roots. Recently, KUO et al. (2013) obtained 10.77 $mg \cdot g^{-1}$ in optimized ultrasound-assisted extraction. However, our study shows a much higher concentration in roots of *Psidium laruotteanum*, 29.13 $mg \cdot g^{-1,}$ and adds valuable knowledge about the chemical potential of this species. Further investigations should be encouraged to explore the physiology of *P. laruotteanum* and the influence of abiotic and biotic variables on its secondary metabolism to improve the production of compounds with biological activities, such as piceid.

In conclusion, a simple, fast and efficient UAE method was obtained to recover piceid from *Psidium laruotteanum* roots. Solvent, temperature, solvent-solid ratio and amplitude strongly influenced the extraction. Cycle and pH had minor effects but might be considered to achieve a higher recovery due to interactions with other variables. The proposed method showed excellent precision and recovery. Under optimal extraction conditions, the yield of piceid was 29.15 $mg \cdot g^{-1}$ of dry roots. *P. laruotteanum* appears as the most concentrated plant source of the potent beneficial compound *trans*-piceid. Thus, this opens a new commercial extraction opportunity, stimulating the exploration of native species, as a very fast extraction (5 min) could afford for 96% of the recovery of piceid from *Psidium laruotteanum* roots.

ACKNOWLEDGEMENTS

L. K. T. thanks CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the scholarship (process number: 99999.005974/2014-03). The authors also thank Maristela Imatomi for the valuable help with botanical issues and considerations.

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CONCLUSÕES FINAIS

Os extratos etanólicos foliares das vinte e oito espécies de plantas do cerrado não apresentaram efeito fungicida contra *Alternaria alternata*, fungo causador da mancha marrom em tangerinas.

Os extratos etanólicos de vinte e seis espécies de plantas do cerrado apresentaram forte atividade antioxidante devido ao alto conteúdo de compostos fenólicos.

As infusões foliares de espécies da família Myrtaceae mostraram alta atividade antioxidante relacionada à alta concentração de compostos fenólicos.

A extração assistida por ultrassom de *trans*-piceido, um composto fenólico com propriedades antioxidantes e medicinais, foi otimizada. A raiz de *Psidium laruotteanum* (Myrtaceae) apresentou uma concentração de 2,9% em raízes secas, sendo a fonte mais concentrada deste composto relatada até hoje.

Estes resultados apontam as espécies de plantas do cerrado como uma importante fonte de compostos bioativos, enfatizando a necessidade de preservação e estudo de sua biodiversidade.