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Validação de método espectrofotométrico para quantificação de flavonoides totais de plantas medicinais da Amazônia e otimização do processo extrativo para potencial aplicação como conservante natural

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapá para obtenção do Título de Mestre em Ciências Farmacêuticas.

Orientador: Prof. Dr. Gabriel Araújo da Silva

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"Em seu coração o homem planeja o seu caminho, mas o Senhor determina seus passos. - Provérbios 16:9"

"A sabedoria oferece proteção, como o faz o dinheiro, mas a vantagem do conhecimento é esta: a sabedoria preserva a vida de quem a possui. - Eclesiastes 7:12."

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RESUMO

No Amapá, região norte do Brasil, o uso de espécies vegetais para fins terapêuticos é muito comum entre a população local, muitas vezes a partir de soluções alcoólicas ou hidroalcoólicas, como as tinturas, que são geralmente comercializadas por farmácias de manipulação local, para uso popular. No entanto, a aplicação de extratos vegetais em alimentos apresenta um elevado potencial a ser explorado, visto que há uma crescente demanda pela substituição de aditivos alimentares sintéticos por naturais. Uma vez que os aditivos sintéticos estão associados a doenças degenerativas, tais como câncer. Com base nisso, o trabalho determinou através da ICH de 27 de outubro de 2014 e RDC Nº 166, de 24 de julho de 2017, os parâmetros de validação de metodologia analítica para quantificação de flavonoides totais de 9 tinturas comercializadas pelo Instituto de Pesquisa Científica e Tecnológica do Amapá, dando condições para avaliar a qualidade dos produtos por meio da confirmação da atividade farmacológica. O método espectrofotométrico apresentou-se sensível (especificidade) quanto ao aparelho utilizado, linear, preciso, exato e robusto. A linearidade do método foi determinada de forma comparativa entre os padrões rutina, que obteve coeficiente de correlação linear igual a R^2= 0,999 e quercetina, R^2= 0,9932 em concentrações variando de 0,5 a 40 µg/mL. Duas espécies com melhores resultados quanto as quantificações de flavonoides totais foram selecionadas, *Dalbergia subcymosa* Ducke e *Croton cajucara* L., para estudo dos seus potencias de aplicação como aditivos alimentares, visando a inibição da oxidação lipídica em alimentos. Para isso, os extratos dessas plantas foram produzidos, fazendo-se necessária a caracterização quantitativa em condições de extração otimizadas, com a variação de dois fatores: concentração de solvente (% Etanol), variando em: 100%; 70% e 40% e proporção droga vegetal:solvente, massa de soluto (SM), variando em 10: 1 (0,1g); 20: 1 (0,05g) e 40: 1 (0,025g), em busca de metabólitos secundários, como fenóis, confirmação da presença de flavonoides e análise da presença de marcador químico por cromatografia líquida de alta eficiência. Após a caracterização química, as espécies foram avaliadas frente à análise do poder de redução do ferro, sequestro do radical livre DPPH· e atividade antioxidante total para determinação da atividade

antioxidante, utilizando o antioxidante sintético butil-hidroxitolueno (BHT) como padrão. Em todos os testes realizados a espécie *C.c*. apresentou melhor resultado na condição de otimização de 0,05g SM e 70% Etanol enquanto que a espécie *D.s.* apresentou melhor resultado em 40% e 100% Etanol. A aplicação em alimento rico em lipídeos foi realizada na gema do ovo através do teste de substâncias reativas ao ácido tiobarbitúrico (TBARS), em que ambas as espécies apresentaram resultados promissores nas condições de extração de 0,05g e 70%Etanol, porém a *Dalbergia* demonstrou potencial de inibição lipídica igual ao controle positivo, BHT, apresentando-se assim como uma ótima alternativa natural ao antioxidante sintético.

Palavras-chave: Conservantes alimentares, antioxidantes naturais, *Dalbergia subcymosa* Ducke *e Croton cajucara* L.

ABSTRACT

In Amapá, northern region of Brazil, the use of plant species for therapeutic purposes is very common among the local population, often from alcoholic or hydroalcoholic solutions, such as dyes, which are usually marketed by local manipulation pharmacies for use. However, the application of plant extracts in foods shows a higher potential to be explored, as there is a growing demand for the replacement of synthetic food additives with natural ones, since synthetic additives are associated with degenerative diseases sush as cancer. Based on this, the work determined through the ICH of October 27, 2014 and RDC Nº 166 of july 24, 2017, the validation parameters of analytical methodology for quantification of total flavonoids of 9 dyes, marketed by the Institute of Scientific Research of Amapá, giving conditions to evaluate the quality of those products by confirming the phamacological activity. The spectrophotometric method was sensitive (specifity) regarding the apparatus used, linear, precise, accurate and robust. The linearity of the mathod was determined comparatively between the rutin standards, which obtained a linear corralation coefficient of $R^2 = 0.999$ and quercetin $R^2 = 0.9932$ at concentrations ranging from 0.5 to 40 µg / mL. Two species with the best results for total flavonoid quantification were selected, *Dalbergia subcymosa* Ducke and *Croton cajucara* L., to study their application potential as food additives, aiming at inhibiting lipid oxidation in foods. For this, the extracts of these plants were produced, requiring quantitative characterization under optimized extraction conditions, with the variation of two factors: solvent concentration (% Ethanol), varying in: 100%; 70% and 40% and plant drug: solvent ratio, solute mass (SM), ranging from 10: 1 (0.1g); 20: 1 (0.05g) and 40: 1 (0.025g), searching for secondary metabolites such as phenols, confirmation of the presence of flavonoids and analysis of the presence of chemical marker by high performance liquid chromatography. After chemical characterization, the species were evaluated against the analysis of iron reduction power, DPPH · free radical scavenging and total antioxidant activity to determine antioxidant activity, using the synthetic antioxidant butyl hydroxytoluene (BHT) as standard. In all tests performed *C.c.*L. presented better results in the optimization condition of 0,05g SM e 70% Ethanol while *D.s.D.* presented better results in 40% and 100% Ethanol. The

application in food rich of lipid was performed in the egg yolk through the Thiobarbituric acid reactive substance test (TBARS), in which both species showed promising results in the extraction conditions of 0.05g and 70% Ethanol, but *Dalbergia.* demonstrated lipid inhibition potential equal to the positive control, BHT, thus presenting itself as a great natural alternative to synthetic antioxidant.

Keywords: Foodpreservatives, natural antioxidants, *Dalbergiasubcymosa*Ducke *e* *Crotoncajucara*L.

1 INTRODUÇÃO

Na Amazônia oriental, o uso de produtos provenientes de plantas medicinais é uma prática frequente da população local. A diversidade biológica é característica da região norte do Brasil e constitui-se entre os maiores reservatórios de diversidade genética das mais diferentes espécies herbáceas encontradas em sistemas evoluídos e nos mais diferentes graus de complexidade. Muitas dessas plantas são ricas em compostos secundários, ou seja, substâncias biologicamente ativas, como compostos fenólicos e flavonoides. Atribuindo às espécies atividade farmacológica (Cruz, 2010; A. A. Oliveira et al., 2013).

Entretanto, a atividade farmacológica amplamente conhecida pela população por vezes não é confirmada por metodologia analítica. O desenvolvimento e validação de metodologia analítica para a determinação da qualidade dos produtos finais é extremamente importante, principalmente quando relacionado a produtos farmacêuticos, visando a quantificação e confirmação dos compostos majoritários responsáveis pela atividade farmacológica das espécies (Brasil, 2010 & ICH, 2014).

No Amapá, o Instituto de Pesquisas Científicas e Tecnológicas do Amapá (IEPA) realiza estudos com plantas regionais para o desenvolvimento de fitoterápicos e fitocosméticos, que são oferecidos a população a baixo custo. Dentre os produtos produzidos no instituto, destacam-se as tinturas, que são extratos alcoólicos de cascas e/ou folhas das plantas estudadas.

Com o interesse de atribuir outra aplicabilidade aos extratos das plantas estudadas pelo IEPA, essa pesquisa propõe a validação de método

espectrofotométrico para quantificação de flavonoides totais em 9 extratos doados pelo IEPA, com efeito na análise comparativa entre as curvas de calibração dos padrões rutina e quercetina e a posterior investigação do potencial de aplicação de duas espécies que estão entre as melhores resultados, *D. subcymosa* D. e *C. cajucara* L., como aditivos naturais em alimentos.

O uso de matéria-prima de origem vegetal para a obtenção de extratos é uma atividade de grande interesse para a indústria de alimentos. Nos grandes mercados consumidores mundiais podem-se encontrar produtos que já incorporam matéria-prima vegetal em suas formulações. Oferecer extratos purificados na composição desses produtos é um meio de prolongar a vida útil do alimento, agregar maior valor a este, trazendo benefícios a saúde do consumidor, que hoje com a maior conscientização acerca dos fenômenos ambientais busca por alternativas mais saudáveis para inserir à sua alimentação (Akcan, Estévez, & Serdaroğlu, 2017).

No presente trabalho, a formulação do aditivo vegetal será realizada a partir de um planejamento fatorial que visa gerar condições de otimização do processo extrativo variando dois fatores, concentração de solvente (% Etanol): 100%; 70% e 40% e proporção droga vegetal: solvente, massa de soluto (SM): 10: 1 (0,1g); 20: 1 (0,05g) e 40: 1 (0,025g). A aplicação dos extratos vegetais em condições otimizadas será realizada em alimento rico em lipídeos, em homogenato de ovo comercial através do teste de substâncias reativas ao ácido tiobarbitúrico (TBARS), segundo o método proposto por Esterbauer e Cheeseman, 1990., para avaliação da estabilidade lipídica do alimento frente à comparação estatística com um antioxidante sintético, que neste caso será o butil-hidroxitolueno (BHT).

A partir dos resultados obtidos, a pesquisa visa promover a valorização de uma matéria-prima regional, agregando valor a espécies regionais, oportunizando o desenvolvimento de diversos outros trabalhos para aprimorar a busca por novos resultados, gerando a perspectiva de um consumo mais consciente e saudável.

2 REFERENCIAL TEÓRICO

2.1 EXTRATOS VEGETAIS

Extratos são preparações concentradas de diversas consistências possíveis obtidas a partir de matérias-primas vegetais secas, que passaram ou não por tratamento prévio (inativação enzimática, moagem, etc.) e preparado por processos envolvendo um solvente. Isso implica basicamente em duas etapas no processo de fabricação: a separação dos compostos específicos de um meio complexo (a droga, ou parte da planta utilizada, como raiz, caule, folha) com a utilização de um solvente; e a concentração, por eliminação mais ou menos completa dos solventes (Yu et al., 2017).

Nos grandes mercados consumidores mundiais pode-se encontrar com frequência os mais diversos produtos possíveis com aditivos de origem vegetal. Bebidas carbonatadas formuladas com especiarias e sucos de frutas (limão/gengibre, hortelã-pimenta ou *Mentha piperita*, cereja/zimbro, etc.), águas minerais vitaminadas com vitamina C natural (e extrato de acerola), chocolate com óleo essencial de laranja e extrato de tília, infusões com rooibos (*Aspalathus linearis* Burman F.), geléias ricas em isoflavonas, em carotenos, ketchup com licopeno, entre outras alternativas para a aplicação dessas substâncias naturais (M. V. Albuquerque, 2012).

Os extratos vegetais também são amplamente utilizados pela população mundial, por suas propriedades e fins terapêuticos. No Brasil, mais precisamente na região amazônica, o uso de infusões e extratos vegetais é de cunho cultural dos

nortistas. Em sua constituição, apresentam compostos orgânicos (antioxidantes e antimicrobianos naturais) com a capacidade de inibir a oxidação lipídica e proliferação microbianas. Com base nisso, diversos estudos têm sido realizados para avaliação destes no combate a oxidação de alimentos (Caleja et al., 2016, 2015; Mahajan, Bhat, & Kumar, 2016; Yu et al., 2017)

Leão et al.(2017) afirmam que os aditivos naturais se destacam quando comparados aos sintéticos, pois apresentam vantagens principalmente quanto a eficiência que é melhor, ou muitas vezes superior sendo, portanto, prováveis substitutos com viabilidade em sua utilização em alimento, visto que os ingredientes naturais com propriedades antioxidantes e antimicrobianas também podem ter benefícios para a saúde na prevenção de diversas doenças relacionadas ao estresse oxidativo/nitroxidativo, como câncer, doenças cardiovasculares, aterosclerose, distúrbios neurológicos, hipertensão ou diabetes mellitus (Carocho & Ferreira, 2013).

2.1.1 *Dalbergia subcymosa* Ducke e *Croton cajucara* L.

O bioma amazônico é caracterizado por alta umidade e temperatura durante todo o ano, abrangendo a floresta tropical. Os limites na região Norte, no estado de Amapá, exibem uma área de transição consistindo no planalto da Guiana, que enriquece muito mais a diversidade biológica e geográfica desta área. Tratando-se de espécies ocorrentes na região amazônica, quando utilizadas em pesquisas, exploram geralmente atividades biológicas como subsídio para que possa ser usufruído pela sociedade a fim de sanar ou até aprimorar algum elemento vital (S. Alves, 2008).

Estudo realizado por Correia et al. (2008) detectaram atividade inibitória em plantas nativas da região amazônica, dentre elas a *Dalbergia subcymosa* Ducke, espécie nativa do Amapá e do estado do Pará. A espécie é integrante da família Leguminosae, tem por nome popular, verônica e se dá na forma de arbusto, conforme identificação registrada por Lima et al. (2015).

Segundo identificação proposta por Maciel et al. (2002), *Croton cajucara* L, pertencente à família Euforbiaceae, é uma planta nativa da região amazônica, mais precisamente da região leste e central, comumente encontrada nos estados do Pará e Amapá. É uma planta arbustiva, que é conhecida popularmente pela população nativa da região como sacaca.

Essas espécies apresentam inúmeros benefícios à saúde humana, sendo utilizadas para tratamento de patologias, devido a presença de seus compostos secundários. O Instituto de Pesquisa Científica e Tecnológica do Estado do Amapá (IEPA) realiza estudos e comercializa os extratos dessas espécies na forma de tinturas para o uso da população local. Esses e outros produtos fabricados com espécies nativas exploram a atividade biológica dos compostos extraídos das matrizes vegetais (metabólitos secundários) para fins medicinais.

Todavia, na literatura revisada são poucos os trabalhos científicos publicados relacionados às atividades biológicas ou efeito terapêutico dos extratos destas espécies. Sendo o uso terapêutico baseado principalmente em informações etnofarmacólogicas, ou seja, uso popular. Assim, as espécies *Dalbergia subcymosa* Ducke e *Croton cajucara* L. são potenciais fontes de compostos químicos com atividades antioxidantes e antimicrobianas, para possível aplicação na conservação de alimentos.

2.1.2 Metabólitos Secundários

As plantas acumulam em seus tecidos numerosos metabólitos secundários que não são necessários para a sua fisiologia, mas que contribuem para protegê-las contra agentes patogênicos, predadores e outros fatores de estresse. Os metabólitos secundários são caracterizados por serem de natureza complexa, possuir baixos valores em relação à massa molecular e significativa atividade biológica. A exemplo desses compostos tem-se: flavonoides, taninos e fenólicos simples (R. J. Pereira & Cardoso, 2012).

A importância quanto ao estudo e exploração dessas espécies químicas naturais (metabólitos secundários) está baseada no fato de que, apresentam como característica de interesse comercial as atividades antioxidante e antimicrobiana. Por serem capazes de inibir a ação de espécies reativas como os radicais livres responsáveis por propagar reações que geram integrantes indesejáveis que degradam os alimentos. Ou, inibir a proliferação microbiana no alimento (Ferreira & Oliveira, 2016; Zeng, Zhang, Gao, Jia, & He, 2012).

2.1.3 Compostos fenólicos

Vários efeitos benéficos à saúde têm sido atribuídos aos compostos fenólicos presentes nas frutas, vegetais, chás e vinhos. Estudos epidemiológicos, clínicos e *in vitro* mostram múltiplos efeitos biológicos relacionados aos compostos fenólicos da dieta, tais como: atividades antioxidante, antiinflamatória, antimicrobiana e anticarcinogênica (Abe, Vieira, Mota, Lajolo, & Genovese, 2007).

Segundo o Instituto Angelo; Jorge (2007), os compostos fenólicos são originados do metabolismo secundário das plantas, sendo essenciais para o seu crescimento e reprodução, além disso, se formam em condições de estresse como, infecções, ferimentos, radiações UV, dentre outros. A principal atividade biológica esperada em extratos ricos em compostos fenólicos é a antioxidante.

Compostos antioxidantes podem ser divididos em duas classes: os que possuem atividade enzimática e os não-enzimáticos. Na primeira, estão os compostos capazes de bloquear a iniciação da oxidação, ou seja, as enzimas que removem as espécies reativas ao oxigênio. Na segunda classe, estão moléculas que interagem com as espécies radicalares e são consumidas durante a reação. Nesta classificação, incluem-se os antioxidantes naturais e sintéticos, como os compostos fenólicos (Degáspari, Waszczynskyj, & Santos, 2004; Halliwell, 2009).

Compostos fenólicos são incluídos na categoria de interruptores de radicais livres, sendo muito eficientes na prevenção da autoxidação. Este mecanismo de ação dos antioxidantes, presentes em extratos de plantas, possui um papel importante na redução da oxidação lipídica em tecidos, vegetal e animal, pois quando incorporado na alimentação humana não conserva apenas a qualidade do alimento, mas também reduz o risco de desenvolvimento de patologias, como arteriosclerose e câncer. A diversidade estrutural dos compostos fenólicos deve-se à grande variedade de combinações que acontece na natureza e os compostos resultantes são chamados de polifenóis, dentre eles destacam-se os flavonóides, os fenólicos simples ou ácidos fenólicos, os taninos e os tocoferóis como os mais comuns antioxidantes fenólicos de fonte natural (Angelo & Jorge, 2007).

Para quantificação desses compostos, costuma-se empregar o reagente de *Folin-ciocalteu*. Esta metodologia foi inicialmente desenvolvida por Singleton e Rossi (1965) e posteriormente modificada por vários pesquisadores. Esse reagente consiste em uma solução de íons poliméricos formados a partir de ácidos fosfomolibdicos e fosfotungsticos que oxidam os fenolatos, reduzindo-os a ácidos, formando um complexo de cor azul Mo-W que pode ser lido em espectrofotômetro a um comprimento de onda de 740 nm (Augusto, Martin, Junior, & Vieira, 2015).

2.2 VALIDAÇÃO DE METODOLOGIA ESPECTROFOTOMÉTRICA

A quantificação de ativos é geralmente realizada por meio de Cromatografia Líquida de Alta Eficiência (CLAE), determinação Eletroquímica ou por espectrofotometria na região do UV-VIS. Entre as diferentes técnicas aplicadas, apesar da cromatografia apresentar maior precisão nos resultados, a espectrofotometria com detecção (UV) além de ser uma técnica de fácil execução, possui um menor custo, mostrando-se eficaz na detecção de compostos e no controle de qualidade de matéria-prima e produtos farmacêuticos (Antônio, Melo, Junior, Eduardo, & Miranda, 2017).

O desenvolvimento de uma metodologia analítica com o emprego de técnica espectrofotométrica, representa uma alternativa para laboratórios que não possuem equipamentos sofisticados, como cromatógrafo, para tal procedimento. Devido a inexistência em literatura de uma metodologia mais simples e menos onerosa para quantificação de flavonoides totais nas tinturas das espécies vegetais analisadas, o presente trabalho propõe o desenvolvimento e validação de um método analítico, através da espectrofotometria de absorção na região do ultravioleta. O método

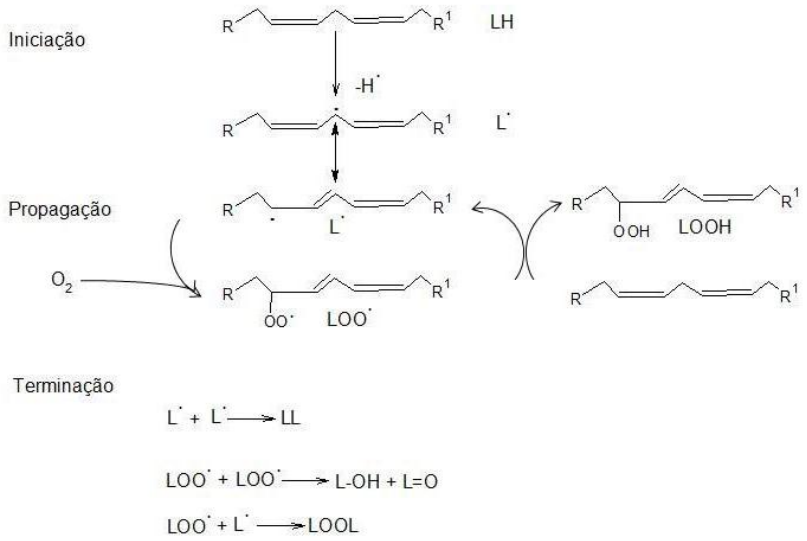
proposto será comparado estatisticamente com a legislação, RDC Nº 166, de 24 de julho de 2017, no intuito de disponibilizar uma nova alternativa de análise quantitativa que possibilite assegurar o controle de qualidade das tinturas (Rubim, Laporta, & Bandeira, 2012).

2.3 OXIDAÇÃO LIPÍDICA EM ALIMENTOS (RANCIFICAÇÃO)

A oxidação lipídica ou processo de rancificação é responsável por promover transformações profundas nas características do alimento (Toniolo, 2012). Essas alterações atribuem sabores e odores desagradáveis a um determinado produto, e, por conseguinte acabam tornando-o inadequado para o consumo, visto que modifica a qualidade nutricional a partir da formação de compostos potencialmente tóxicos, conforme expresso por Ramalho; Jorge (2006).

O processo de peroxidação lipídica é induzido para o efeito pro-oxidante de metais de transição. Este processo configura-se em uma vasta evidência após a ocorrência de reações de íons metálicos com H_2O_2 (peróxido de hidrogênio) no citossol e em membranas biológicas, visto que os últimos são o alvo principal do dano oxidativo. Em outras palavras, por um mecanismo catalítico, os metais de transição produzem a peroxidação lipídica por meio da estimulação da capacidade oxidativa de H_2O_2, promovendo processos mediados por espécies reativas de oxigênio (radicais livres), por outro mecanismo, eles se ligam a fosfolipídeos carregados que alteram as propriedades físicas da bicamada lipídica nas membranas celulares, mesmo mecanismo esperado nas amostras de alimentos (Repetto, Semprine, & Boveris, 2012).

Figura 1 - Representação das etapas da peroxidação lipídica: LH, captura de H em ácidos graxos poliinsaturados; L•, radical lipídico; LOO•, radical peroxil; LOOH, hidroperóxidos lipídicos; O_2, oxigênio atmosférico; LL, LOOL, L-OH e L=O, produtos secundários da oxidação lipídica.



Fonte: Autoral.

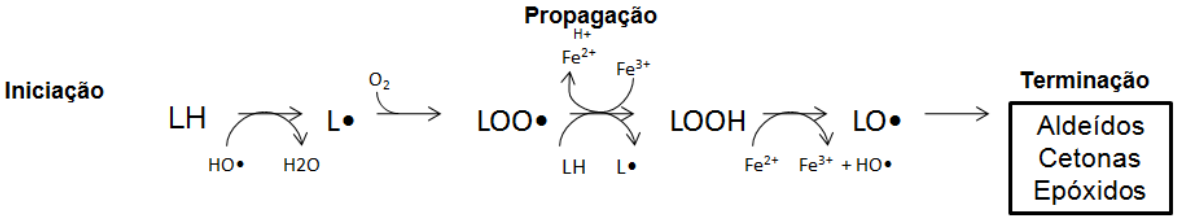
Na primeira fase (Figura 1) ocorre a formação de radicais lipídicos (L•) oriundos dos ácidos graxos poliinsaturados (LH), a partir da retirada de hidrogênio do grupo metileno da molécula. Conhecida também como a etapa de indução da rancificação ou iniciação, dado pelo fato de ainda ser baixa a concentração de produtos da oxidação, a percepção do aumento da concentração de radicais livres, que ainda não são identificadas alterações nas características organolépticas do produto (Toniolo, 2012).

Na presença do oxigênio atmosférico (O_2) esses radicais livres reagem rapidamente formando radicais peróxidos (LOO•), que por sua natureza reativa interagem com os ácidos graxos poliinsaturados e geram os radicais hidroperóxidos (LOOH) (que se degradam por conta de sua instabilidade química, gerando acetonas e aldeídos que são interferentes no sabor e odor), além de outras espécies reativas, que suscitam reações em cadeia, por ser um processo autocatalítico conhecido também como a etapa de autoxidação, ocorrendo a propagação do dano (Ramalho & Jorge, 2006; Toniolo, 2012).

Por fim, na etapa de terminação ocorre a formação de compostos complexados (LL; LOOL; L-OH e L=O), que são os produtos secundários da oxidação lipídica, acontece o decréscimo do consumo de oxigênio e aumento na concentração de peróxidos (Ramalho & Jorge, 2006; Toniolo, 2012).

Os hidroperóxidos lipídicos, na presença ou ausência de íons metálicos catalíticos, produzem uma grande variedade de produtos. Apesar dos hidroperóxidos serem compostos não voláteis e inodoros, são relativamente instáveis, e se decompõem espontaneamente ou em reações catalisadas para formar compostos aromáticos voláteis, como cetonas e aldeídos (Figura 2), conhecidos por *off-flavours,* que se desenvolvem durante a oxidação lipídica e servem de indicador para o alimento que já não é mais comestível. Os *off-flavours* são produtos secundários da peroxidação lipídica e podem ser usados para avaliar o grau de peroxidação em um sistema. Temos como exemplo o malondialdeído (MDA), que é um dos principais produtos de decomposição dos hidroperóxidos de ácidos graxos poliinsaturados, formado durante o processo oxidativo (Gueraud et al., 2010).

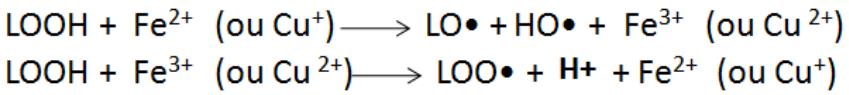
Figura 2 - Representação geral da peroxidação lipídica: LH, captura de H em ácidos graxos poliinsaturados; LO•, radical alcoxil; LOO•, radical peroxil; LOOH, hidroperóxidos.



Fonte: Autoral.

Os hidroperóxidos lipídicos são instáveis na presença de metais de transição, tais como ferro ou cobre (Figura 3) formando, também, radicais alcoxil (LO•) e peroxil (LOO•). Os iões metálicos de transição Fe^{2+} e Cu^{+} estimulam a peroxidação lipídica, contribuindo também para a rancidez em alimentos, devido a formação do processo de clivagem redutiva de hidroperóxidos de lipídios endógenos de fosfolipídios de membrana para os radicais alcoxilo e peroxilo correspondentes em um processo que é conhecido como peroxidação lipídica dependente de radicais peroxilo. Os mecanismos dessas duas reações parecem envolver a formação de complexos de Fe(II)-Fe(III) ou Fe(II)-O_2-Fe(III) com taxas máximas de formação de radicais HO• em uma razão Fe(II)/Fe(III) igual a 1 (um), Cu^{2+} e Cu^{+} são conhecidos por sua capacidade de decompor hidroperóxidos orgânicos para formar radicais orgânicos alcoxil e peroxil (Repetto et al., 2012).

Figura 3 - Reação de Fenton: LOOH, hidroperóxidos; Fe^{2+}, Fe^{3+}, metais de transição; LO•, radical alcoxil; HO•, radical hidroxila;LOO•, radical peroxil.



Fonte: Autoral.

Os radicais alcoxil podem sequestrar átomos de hidrogênio de outros ácidos graxos poliinsaturados e hidroperóxidos lipídicos, formando outros radicais lipídicos (L•) e radicais LOO•, respectivamente. Assim, contribuem também para a propagação da peroxidação lipídica (LOUREIRO, MASCIO & MEDEIROS, 2002).

A oxidação lipídica é considerada uma das principais causas da perda de qualidade nos alimentos. Com o intuito de retardá-la, faz-se necessário o uso de substâncias com poder antioxidante. Devido à crescente preocupação com a saúde, há uma forte tendência de uso de compostos oriundos de fontes naturais em substituição dos antioxidantes sintéticos, amplamente utilizados (Augusto et al., 2015).

2.3 CONSERVANTES ALIMENTARES

As indústrias de engenharia e tecnologia de alimentos intensificaram a utilização de agentes químicos para conservação, coloração e aromatização dos produtos alimentícios ao longo dos anos. Com a conscientização a acerca de aspectos nutricionais e ambientais, as mudanças nos hábitos alimentares exigiram mudanças na fabricação e preparo desses produtos, para manter a integridade e

um bom aspecto do alimento através de aditivos alimentares, com o objetivo de atrair mais consumidores (Antunes & Araújo, 2000).

No processo de aplicação destes, é feito o uso de conservantes sintéticos, no entanto estudos toxicológicos têm demonstrado a possibilidade destes antioxidantes apresentarem efeito carcinogênico em experimentos com animais. Apesar de apresentar estabilidade e eficiência quanto ao seu uso no processo produtivo, esse fato é um viés da base para que os órgãos regulamentadores, a exemplo da ANVISA e Ministério da Saúde, estabeleçam valores máximos de aplicação, como se observa para o BHT que no Brasil o seu limite de adição é de 100 mg/g de alimento (Ramalho & Jorge, 2006).

No que tange a utilização do BHT (antioxidante sintético), que tem uso permitido no Brasil, em alimentos ricos em óleos e gorduras como manteiga, carnes, cereais, bolos, biscoitos, cerveja, salgadinhos, batatas desidratadas, gomas de mascar e cosméticos, é importante destacar que relatórios internacionais baniram há alguns anos esses tipos de aditivos, visto que apresentam relação com o desenvolvimento de doenças graves. Esse fato é observado, também, em países da Europa, nos Estados Unidos e Canadá que apresentam uma proibição sólida, visando a manutenção e preservação da saúde e bem-estar da sociedade consumidora (M. V. Albuquerque, 2012).

Segundo CAMPBELL (2000), o antioxidante é um composto redutor, facilmente oxidado, que evita que outras substâncias se oxidem. Podem atuar de diferentes maneiras, impedindo a formação de espécies reativas de oxigênio, inibindo as reações em cadeia com o ferro e o cobre, ou evitando danos oxidativos por meio da interceptação de radicais livres, impedindo assim que os mesmos

reajam com lipídios, aminoácidos, duplas ligações de ácidos graxos poli-insaturados (Guimares et al., 2010).

Para retardar ou inibir a oxidação dos lipídios constituintes dos alimentos é necessário diminuir a incidência de todos os fatores que a favorecem, mantendo o mínimo dos níveis de energia, isolando do contato com o oxigênio, que são responsáveis pelo desencadeamento do processo de formação de espécies reativas. Porém, quando essas medidas não são viáveis ou eficazes, há a adição de agentes antioxidantes, em pequenas quantidades, atuam interferindo nos processos de oxidação de lipídios inibindo a ação dos radicais livres que dão origem ao processo de rancificação (Ramalho & Jorge, 2006).

Com base nisso, os compostos antioxidantes apresentam a função de retardar o aparecimento dos fenômenos relativos a oxidação dos lipídios, afim de manter intactas suas características sensoriais e estabelecer uma segurança quanto ao seu consumo. Esses aditivos não devem causar efeitos fisiológicos negativos ou produzir cores, odores nem sabores anômalos daqueles esperados pelo consumidor dos alimentos (Andreo & Jorge, 2006).

Ramalho; Jorge (2006) complementaram essa ideia afirmando que os antioxidantes em alimentos devem apresentar eficácia em baixas concentrações (0,001 a 0,01%); compatibilidade com o alimento (ou seja, resistentes inclusive aos tratamentos a que seja submetido o alimento) e fácil aplicação e estabilidade quanto ao período determinado de validade.

Desta forma, uma característica encontrada na composição dos extratos vegetais é ressaltada, em que os metabólitos secundários obtidos apresentam a características de substâncias antioxidantes, possuindo uma ligação muito forte

com a aplicação na indústria alimentícia. Pois, além de apresentar benefícios ao organismo humano, esses antioxidantes naturais apresentam potencial para substituir os antioxidantes sintéticos, que se tratando especificamente em inibir a oxidação lipídica do alimento, pode garantir a sua conservação com uma segurança maior e, como em alguns casos já registrados, sendo mais eficientes e efetivos que os conservantes sintéticos (Vizzoto, M.; Krolow, A. C.; Weber, 2010).

Portanto, em se tratando de conservantes naturais à partir de espécies vegetais, após o processo de extração se faz necessário a verificação da atividade antioxidante dos compostos extraídos. Esta verificação pode se dar pelo uso de metodologias para quantificação e avaliação da capacidade antioxidante e antimicrobiana dos compostos obtidos. Cabe ressaltar que a atividade dos extratos produzidos não depende somente da presença e concentração de metabólitos secundários que apresentam atividade comprovada, como os compostos fenólicos, mas também da metodologia de extração aplicada (Hernández-Sala, Ramírez-Villa, Veloz-Rendón, Riviera-Hernandéz, & Al., 2009).

3 OBJETIVOS

2.1 OBJETIVO GERAL

Validar metodologia analítica para quantificação de flavonoides totais de nove extratos vegetais da Amazônia e avaliar o potencial antioxidante de dois extratos hidroalcólicos vegetais (*Dalbergia subcymosa* Ducke e *Croton cajucara* L.) na estabilidade oxidativa de alimentos.

2.2 OBJETIVOS ESPECÍFICOS

- Validar metodologia analítica, identificando e quantificando as classes de compostos bioativos e marcadores químicos;
- Determinar a atividade antioxidante dos extratos;
- Avaliar os efeitos dos extratos na estabilidade oxidativa de alimentos.

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Validation of a spectrophotometric method for the determination of chemical markers in medicinal plants of the Amazon

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SUMMARY Dyes prepared from medicinal plants are alcoholic or hydroalcoholic solutions synthesized from vegetable raw materials or chemical substances, usually marketed for popular use. In Amapá, due to the lack of specialized equipment, the dyes produced by some local pharmacies do not go through methods related to the quantification of markers of plant species, neither other quality control tools. Therefore, aiming at the validation of an analytical method for the standardization of pharmacy tinctures of the Institute of Scientific Research and Technology of Amapá (IEPA), the present work determined through the ICH of October 27, 1994 and RDC Nº 166, of July 24 of 2017, the linearity, specificity, precision, accuracy and robustness for the quantification of total flavonoids, giving conditions to evaluate the quality of the product. As a result, the method presented high specificity at 420nm, being linear, precise, accurate and robust in different equipment and variations of the AlCl3

concentration. The validation parameters conferred to the method high reliability for determination of total flavonoids as a tool to monitor the quality of these dyes.

Keywords: Quality Control, Parameters, Standardization, Plant Extracts.

ABSTRACT As tinturas preparadas a partir de plantas medicinais são soluções alcoólicas ou hidroalcoólicas sintetizadas a partir de matérias-primas vegetais ou de substâncias químicas, geralmente comercializadas para uso popular. No Amapá, ainda por falta de equipamentos especializados, as tinturas produzidas por algumas farmácias de manipulação locais, não passam por métodos relacionados à quantificação de marcadores das espécies vegetais, tampouco outras ferramentas de controle de qualidade. Assim, objetivando a validação de método analítico para a padronização das tinturas da farmácia do Instituto de Pesquisa Científica e Tecnologia do Amapá (IEPA), o presente trabalho determinou através da ICH de 27 de outubro de 1994 e RDC Nº 166, de 24 de Julho de 2017, a linearidade, especificidade, precisão, exatidão e robustez para a quantificação de flavonoides totais, dando condições para avaliar a qualidade do produto. Como resultado, o método apresentou alta especificidade em 420nm, sendo linear, preciso, exato e robusto em diferentes equipamentos e variações da concentração de $AlCl_3$. Os parâmetros de validação conferiram ao método alta confiabilidade para determinação de flavonoides totais como ferramenta de monitoramento da qualidade dessas tinturas.

Palavras-chave: Controle de Qualidade, Parâmetros, Padronização, Extratos Vegetais.

1.INTRODUCTION

In the eastern Amazon, the use of products derived from medicinal plants is a frequent practice of the local population. Biological diversity is characteristic of the northern region of Brazil and is one of the largest reservoirs of genetic diversity of the most different herbaceous

species found in evolved systems and in the most different degrees of complexity. Many of these plants are rich in secondary compounds, i.e. biologically active substances, such as phenolic compounds (Cruz, 2010; A. A. Oliveira et al., 2013).

Among these phenolic compounds, flavonoids represent one of the most important and diversified groups. This class of secondary metabolites is widely distributed in the plant kingdom and because of its facility of establishing chemical relationships with other compounds, are directly associated with food ingredients and benefits to human health. Thus, it has been noted that there has been a growing interest in flavonoid research, because of its health benefits reported in several epidemiological studies (S. Alves, 2008; Borgo, Xavier, Moura, Richter, & Suyenaga, 2010; M. Junior et al., 2000; Kumar & Pandey, 2013; Sanguigni, Manco, Sorge, Gnessi, & Francomano, 2017; Shen et al., 2018).

These chemical compounds are considered the active principles or chemical markers of many herbal medicines, which are preparations obtained from medicinal plants, ie exclusively derived from plant drugs. The most common pharmaceutical form of phytotherapics are dyes, which are alcoholic or hydroalcoholic preparations resulting from the extraction of plant materials or the dilution of the respective extracts. The use of these drugs is very common among the population of the state of Amapá, both in the form of home preparations and consumption of these in the form of tinctures marketed in private and government pharmacies, which daily attend a large flow of local consumers (ANVISA, 2017).

The quality control of these products must be carried out according to the legislation. Different analytical methods are used and described for the most diverse types of samples and are used for the detection and quantification of active principles, adulterants or degradation products in pharmaceutical formulations. Among these methods, the

spectrophotometric and chromatographic methods deserve special mention. According to the National Agency of Sanitary Surveillance (ANVISA), the standardization of natural extracts consists of the

determination by analytical method of the active principles or chemical markers and the validation must guarantee, through experimental studies, that the method meets the requirements of the analytical applications , assuring the reliability of the results for the category that the analysis fits (ANVISA 2017, PNPF; Junior et al. 2017).

Some of the species that are commonly used by the local population are endogenous to the Amazon and the border region between the state of Amapá and Pará. Due to their singularities and pharmacological activities, the dyes of Arrabidaeachica V. popularly known as "Pariri" (Lot nº 14104), the Ptychopetalumolacoides B., popularly known as "Muirapuama"(Lot nº 14051), *Licaniamacrophylla*B., "Anauerá" (Lot nº 14145), *Ourateahexasperma*Bail, "Barbatimão"(Lot nº 14071), *Vataireaguianensis*Aublet, "Faveira"(Lot nº 14082), *Tabebuia*sp., "Pau D'arco"(Lot nº 14162), *Crotoncajucara*L., "Sacaca"(Lot nº 14033), *Calophyllumbrasilliense*Cambess, "Jacareúba"(Lot nº 14119), *Dalbergiasubcymosa* D., "Verônica" (Lot nº 14083) were acquired from the Center of Natural Products of the Institute of Scientific and Technological Research of Amapá for the accomplishment of this study.

According to Lima(2014), the herbal medicines produced by the Nucleus of Natural Products of the Institute of Scientific and Technological Research of Amapá (IEPA) still, due to lack of specialized equipment, do not have standardization. In this manner, because it is also a government pharmacy, the dyes produced at the institute were used to evaluate the quantification of flavonoids, knowing that the pharmaceutical products of these species need the development and validation of an analytical method for quality control (ANVISA, 2017; ICH, 2005).

In this scenario, the objective of the present study was the validation of the analytical spectrophotometric method for the standardization of tinctures of the chosen species, which

are frequently consumed by the local population. The following parameters were determined: linearity, specificity, precision, accuracy, and robustness of the quantification method of total flavonoids in accordance with legal regulations.

2. MATERIALS AND METHODS

2.1. SAMPLES

The herbal products containing *Arrabidaeachica*, *Ptychopetalumolacoides*Benth, *Licaniamacrophylla*B.,*Ourateahexasperma*Bail, *Vataireaguianensis*Aublet, *Tabebuia*sp., *Crotoncajucara*L., *Calophyllumbrasilliense*Cambess, *Dalbergiasubcymosa*D., were purchased in October 2014 at the Institute of Scientific and Technological Research of Amapá (IEPA), in hermetically sealed commercial packages and labeled in amber glass bottles. The commercial samples obtained were sent to the Laboratory of Organic Chemistry and Biochemistry at the State University of Amapá (UEAP) for analysis.

2.2. VALIDATION OF SPECTROPHOTOMETRIC METHOD

The proposed method was validated according to RDC No. 166 of July 24, 2017 of ANVISA (National Agency for Sanitary Surveillance), which regulates the validation of analytical and bioanalytical methods in Brazil, and ICH recommendations of October 27, 1994 (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use).

After the analysis, the results were brought to statistical treatment through the software Graphpad® and Statistica®, to vary the concentration of AlCl3 in the samples.

2.2.1. Specificity

The whites of the samples were zeroed and then subjected to spectrophotometer (BIOSPECTRO SP-22) absorbance readings at 420nm to determine the specificity of the method.

2.2.2. Linearity

The linearity was determined by obtaining two analytical curves, in which the flavonoids rutin (quercetin-3-rhamnosyl-glucoside) and its aglyconequercetin were used as the standard for the validation of the analytical method for quantification of flavonoids and guarantee of the response of the standardization method of samples. Concentrations of 0.5; 1.0; 2.0; 2.5; 5.0; 10; 15; 20; 25; 30 μg / mL for quercetin and 2.5; 5.0; 10; 15; 20; 25; 30; 40 μg / mL for rutin were determined.

2.2.3. Precision

Repeatability (intraday precision) was evaluated by means of spectrophotometric measurements of the solutions at different concentration levels, considering the linear interval of the method, in triplicate. Intermediate precision (inter-day precision) was evaluated similarly to repeatability, however, spectrophotometric measurements were performed on different days with different analysts.

2.2.4. Accuracy

The accuracy was expressed by obtaining the mean of the absorbance values of the samples, to analyze the agreement between the value found and the value accepted by the legislation (95%).

2.2.5. Robustness

It was determined by variations between different apparatus, and variation of solvent concentration (AlCl 3).

2.3. DETERMINATION OF THE TOTAL FLAVONOID CONTENT

The total flavonoid content was determined according to the described by Wannes et al. (2010) using the aluminum chloride that reacts with the flavonoids of the sample generating a chromophore, which is evaluated at 420 nm in a spectrophotometer (BIOPROSS-SP22). The calibration curve was performed with rutin and quercetin for comparative analysis. The result was expressed in mg of standard equivalents / g of extract.

3. RESULTS

The presence in plants of compounds which, when administered in the form of tea, bottles, dyes, powders, or as pure isolated substance, or in solid or liquid semi-solid pharmaceutical forms, have the property of provoking beneficial reactions to the organism, resulting in the recovery of health is called active principle. Compounds that perform therapeutic action, either in isolation or a set of substances that act synergistically (Ding et al., 2015).

For the analytical development of a new method or the application of a traditional method in a new sample or matrix, mainly samples of biological origin if necessary demonstrate the ability to select the method, between compounds with related structures that may be present in these matrices or until even in pharmaceutical forms. This should be confirmed by obtaining positive results (preferably relative to the known reference material) in samples containing the drug, compared to negative results obtained with samples not

containing it, but structurally similar compounds for determining the specificity of the proposed method (E. J. A. G. Junior et al., 2017).

In this work, the determination of the specificity was determined by eliminating the absorbance values obtained only with the diluted sample, that is, the whites of the samples, subjecting them to the absorbance readings in a spectrophotometer (BIOSPECTRO SP-22) at 420nm as a guarantee of the insulation of the interfering compounds. The absorbance readings were performed at this wavelength because it is referenced in the standard for the identification of the chromophore formed between flavonoids and $AlCl_3$.

After obtaining specificity data. A comparative analysis was performed between the calibration curves of the flavonoids rutin and quercetin to verify the best response. The study was performed at concentrations of 0.5; 1.0; 2.0; 2.5; 5.0; 10; 15; 20; 25; 30 μg / mL for quercetin and 2.5; 5.0; 10; 15; 20; 25; 30; 40 μg / mL for rutin and is shown in Figures 1 and 2, referring to the graphs of the calibration curves for the two standards and their waste dispersion measurements, plotted in STATISTICA® software for Windows.Through the construction of the curve, it was possible to determine the linear range of detection that obeys the Law of Beer depends on the compound analyzed and the type of detector used. The linearity was determined by the correlation coefficient (R), obtained by the graphs related to the equipment response (UV / VIS detector) as a function of the analyte concentrations, through the analytical curves. In both analyzed analytical curves, the linearity in the working range was superior to that recommended in the current legislation (> 0.99).

The accuracy of the method, or repeatability, expresses the fidelity obtained under the same operating conditions, applied in a short time interval. This was determined by repeatability inter and intra-day. In the inter-day precision when evaluating the variability among analysts, relative standard deviations were obtained lower than those recommended

in RDC No. 166, July 24, 2017, and ICH of October 27, 1994. As well as in intra-day precision when evaluating the variability between analyzes by the same analyst the results were satisfactory, as presented in Table 1.

The robustness of an analytical method is the measure of its ability to resist small and deliberate variations in analytical parameters. It indicates its confidence during normal use and was determined through the parameters related to different apparatus, and variation of AlCl3 concentration, as can be seen in Table 2.

A response surface graph was generated, according to Figure 3, to guarantee the response of the method refers to the variation parameter of the wavelength reading.

The purpose of the Response Surface (RS) graph is to produce an optimized and robust mathematical equation, validating for the next Control phase the few vital factors (x) that are highly influential in the process result (y).

The graph shown in Figure 3, obtained through Statistic® software, shows the response of the method for wavelength variation and presented a positive response for this parameter, ranging from 415-425nm. Indicating the critical point of absorbance at 425.3125nm.

The paretos's charge, shown in Figure 4, was generated to determine the difference in $AlCl_3$e concentration and wavelength, also expressing the p-value (statistical significance).

Accuracy refers to how close the value of a measure is to its proposed value, the agreement between the value found and the value accepted as true or as a reference. According to RDC No. 166, dated July 24, 2017, and ICH dated October 27, 1994, it should not be less than 95%, as described in Table 3.

After the validation and certification of the reliability parameters, the method was applied to the quantification of total flavonoids in the samples, as evidenced in Table 4. However, the method was not able to quantify flavonoids in two of the studied species, *Tabebuia sp. ePtychopetalumolacoides*B..

4. DISCUSSION

For the analytical development of a new method or the application of a traditional method in a new sample or matrix, mainly samples of biological origin it is necessary to demonstrate the selection ability of the same. This should be confirmed by obtaining positive results (preferably relative to the known reference material) in samples containing the drug, compared to negative results obtained with samples not containing the drug, but structurally similar compounds for determining the specificity of the proposed method (Ding et al., 2015; E. J. A. G. Junior et al., 2017).

Through the comparative study of the calibration curves of the flavonoids rutin and quercetin, it was possible to observe that the absorption of the rutin excerpt the absorption of quercetin. Absorbance readings were performed at 420nm, referenced in the standard for identification of chromophore formed between flavonoids and the AlCl3. This fact can be evidenced when we observed the curve at the same concentration point for the two flavonoids, for example in 30 μg of the standards / mL we observed a mean response of 0.550 absorptions for the rutin and 1,800 for quercetin.

In the UV range that quantifies the flavonoids through absorption, quercetin, which is a flavonoidaglicone, in the same concentration absorbs more than the rutin, since the mass of rutin is corresponding to aglycone and sugars. This fact has relevance for the quantification of flavonoids in the species studied because the metabolite of interest is not identified in the

plant species as aglycone, on the contrary, it is found mainly in association with sugars. Thus, the routine is more efficient as a parameter of identification of the actual flavonoid content in plant species, due to its glycosylated form.

The construction of the calibration curve made it possible to determine the linearity of the method, and the absorbances were plotted in a scatter plot for the visualization of the calibration curve and statistically the correlation coefficient (R2 = 0.9999), the angular coefficient = 0.0189) and intersection on the Y-axis (b = -0.007).

By determining the method's precision parameter, it was possible to verify that the repeatability decreases the method variance, which revealed a coefficient of variance with different analysts on the same day of 0.16%, lower than the maximum value required (Table 1). Intermediate precision, which evaluates the variation of tests performed in days and with different analysts, showed the same value of Relative Standard Deviation (RSD) in the third point of the analyst 01 and in the first point of the analyst 02, indicating that there is no significant difference between the deviations (Table 1).

Another parameter to be analyzed for the validation of the methodology was the robustness of the method, which was robust to the analyzed variabilities (different apparatus and different AlCl3 concentrations), except for the higher concentration of AlCl3 (1.5%). The lack of robustness at this concentration is due to the poor solubility of AlCl3 in methanol (Table 2). For the identification of a possible optimal point of robustness, a response surface graph was plotted in the Statistic® software for Windows, finding the region of maximum yield located at the top left (point referring to 0,679855; Figure 3). The response of the method to wavelength variation was obtained through the response surface graph, presenting a positive response for this parameter, ranging from 415-425nm and indicating the critical point of absorbance at 425.3125nm.

The histogram, shown in figure 4, generated for AlCl3 concentration difference, did not obtain a satisfactory answer. According to the indication of the wavelength critical point for absorbance reading, the Matlab® software was able to detect the critical point of AlCl3 concentration in the samples, 1.0474%, indicating that up to this point the method is robust. This aspect is best represented by the graph referring to Figure 4, which shows the critical point of the aluminum chloride concentration variation analysis equivalent to 1.0474% at p = 0.05, establishing that the result was significant since don't exceeds 5%.

The accuracy of the method obtained results of the percentages 98.91; 100.02 and 101.91% for low, medium and high concentrations, respectively. Thus, the analyzes for the proposed spectrophotometric quantification are in accordance with the legislation, presenting reliability of results according to the legislation.

After the validation of the method according to the ICH of October 27, 1994, quantification of flavonoids in plant species was performed. With this, it was possible to identify that the species LicaniamacrophyllaBenth. (Chrysobalanaceae), popularly known as "anauerá" or "anuera", used by the Amazonian communities in the treatment of amoebic parasitoses and dysenteric disorders. presented a positive result for flavonoids, agreeing with the literature. However, Ramos et al. (2014), when performing the phytochemical screening of anauerá peel, evidenced a negative result for flavonoids (Medeiros et al., 2012).

It has also been observed that A. chica, which is a scandic shrub traditionally indicated to treat inflammation symptoms and skin conditions whose ethanolic extract are chemically investigated and tested against yeast and dermatophyte fungi. The species obtained a positive response, waking up with identifications found in the literature. However, there are no records of quantification of the chemical compound (M. Alves et al., 2010; Takemura et al., 1995; Vicentino & Menezes, 2007).

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Figura 1: **A**-calibration curve of rutina (2,5-40µg/mL); **B**- calibration curve of quercetina (0,5-25µg/mL).

Figura 2: **A**-calibration curve of rutin Waste (2,5-40µg/mL); **B**- calibration curve of quercetinWates (0,5-25µg/mL).

Figura 3: Graph method Response Surface variables difference AlC3 concentrations by absorption (23).

Figura 4: Response methodto AlCl3 concentration variations and wavelengths.

Tabela 1: Precision parameters presented as repeatability inter and intra-day.

	Precisão Inter-dia (Analista 01)			Precisão Inter-dia (Analista 02)			Precisão intra-dia		
Concentração (µg/mL)	Dia 01	Dia 02	DPR (%)	Dia 01	Dia 02	DPR (%)	Analista 01	Analista 02	DPR (%)
10	0.350	0.330	**0.14**	0.326	0.354	**0.19**	0.332	0.354	**0.15**
20	0.697	0.691	**0.02**	0.653	0.718	**0.22**	0.670	0.718	**0.16**
30	1.048	0.963	**0.19**	0.974	1.062	**0.20**	0.976	1.062	**0.19**

Tabela 2: Robustness parameter for diferent apparatus and concentration of reagents.

Espectrofotômetro				
Concentração (µg/mL)	Ap. 01		Ap. 02	DPR (%)
10	0.330		0.332	**0.02**
20	0.691		0.670	**0.10**
30	0.963		0.976	**0.04**
	Conc. $AlCl_3$(%)			
	0,5	1	1,5	DPR(%)
10	0,495	0,475	0,549	**16,78**
20	0,475	0,466	0,473	**2,43**
30	0,487	0,489	0,391	**2,07**

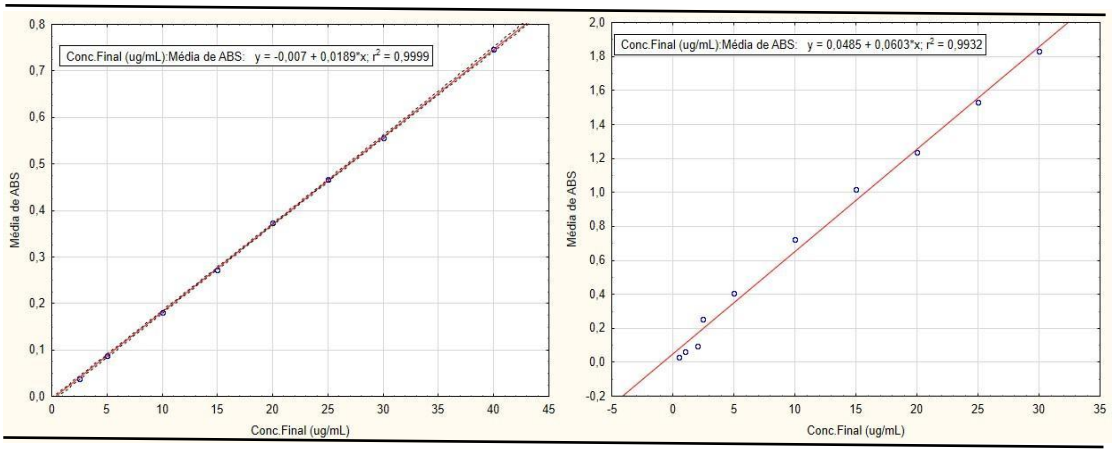
Tabela 3: Accuracy parameters, obtaining the mean of absorbance of the sample values, accuracy and RSD (%).

Concentração (µg/mL)	Absorbância (nm±DP)	Exatidão (%)	DPR (%)
10	0,321±0,003	98,91	0,88
20	0,654±0,004	100,02	0,64
30	1,003±0,009	101,91	0,89

Tabela **4**: Content of total flavonoids of the species in milligrams (mg) of quercetin per gram (g) of extract.

Extrato	**Flavonoides Totais (mg de rutina/g±SD)**	**Flavonoides Totais (mg de quercetina/g±SD)**
Calophyllum brasiliense	8.07±0.15	0.80±0,08
Dalbergia Subcymosa DUCKE	7.20±0.13	2.22±0,43
Vatairea guianensis	6.38±0.80	3.13±0.08
Arrabidaea Chica	6.22±0.07	2.66±0.07
Croton cajucara	6.00±0.83	2.02±0.44
Ouratea hexasperma	2.61±0.14	4.03±0.13
Tabebuia sp.	N.D.	N.D.
Ptychopetalum olacoides	N.D.	N.D.
Licania macrophylla	9.76 ± 0.25	2.14±0.03

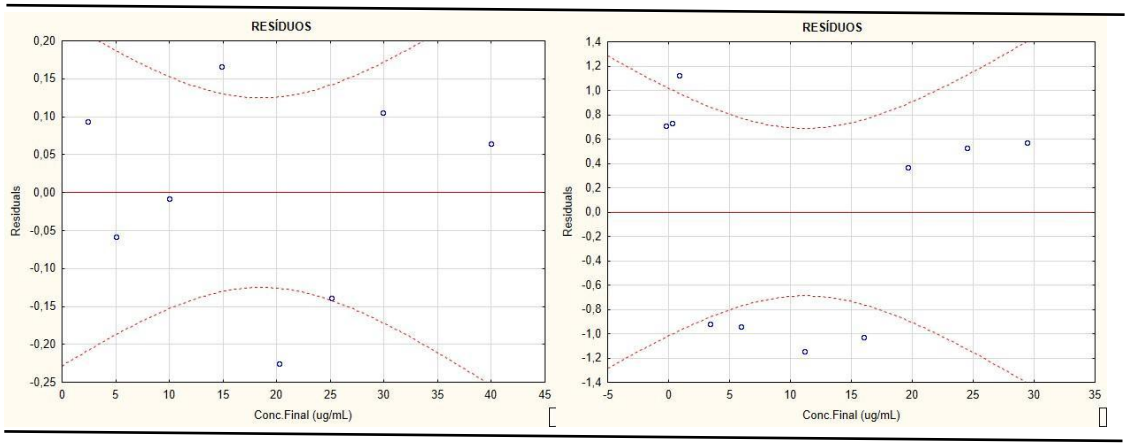
Figure caption



B

A

Figura 4: **A**- Curva de calibração rutina(2,5-40µg/mL). O gráfico da linearidade, obtido através do software STATISTICA®, fornece a resposta do coeficiente de correlação linear (r2) através da média das absorbâncias (ABS) do padrão rutina; **B**- Curva de calibração quercetina (0,5-25µg/mL). O gráfico da linearidade, obtido através do software Statistic®, fornece a resposta do coeficiente de correlação linear (r2) através da média das absorbâncias (ABS) do padrão quercetina.



B

A

Figura 5:**A-** Resíduos da curva de calibração rutina(2,5-40 µg/mL). Através do gráfico de resíduos, obtido através do softwareStatistic®, pode-se observar a dispersão dos dados da amostra de rutina; **B-** Resíduos da curva de calibração quercetina (0,5-30µg/mL). Através do gráfico de resíduos, obtido através do software STATISTICA® pode-se observar a dispersão dos dados da amostra de rutina.

Figura 5: **A-** Resíduos da curva de calibração rutina(2,5-40 µg/mL). Através do gráfico de resíduos, obtido através do softwareStatistic®, pode-se observar a dispersão dos dados da amostra de rutina; **B-** Resíduos da curva de calibração quercetina (0,5-30µg/mL). Através do gráfico de resíduos, obtido através do software STATISTICA® pode-se observar a dispersão dos dados da amostra de rutina.

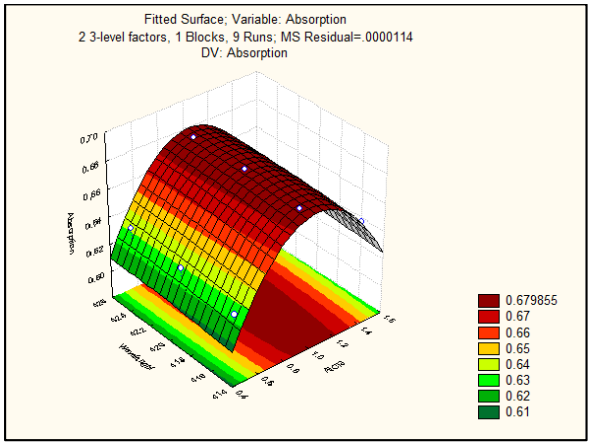


Figura 6: Gráfico da Superfície de Resposta do método, variáveis: diferença de concentrações de AlC_3 e absorbância (23).

Figura 6: Gráfico de Superfície de Resposta do método, variáveis: diferença de concentrações de AIC_3 e absorbância (23).

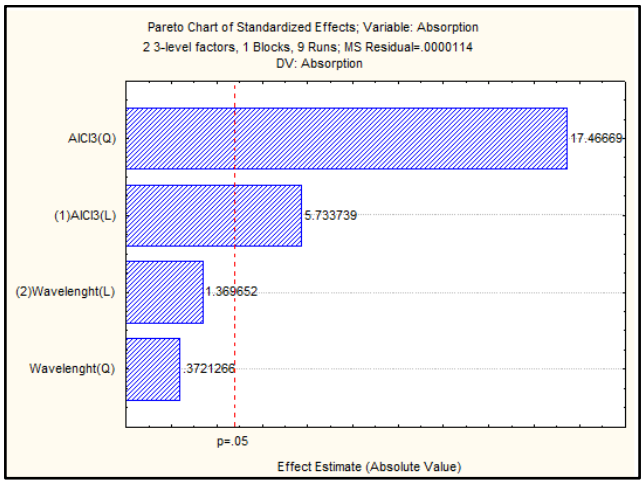


Figura 7: Resposta do método às variações de concentração de $AlCl_3$ e comprimentos de onda.

Figura 7: Resposta do método às variações de concentração de $AlCl_3$ e comprimentos de onda.

4 ARTIGO 2

Submetido para publicação na revista Industrial Crops and Products.

Optimization of phenolic extracts, flavonoids and antioxidant activity of Amazonian medicinal plants for use as a food preservative

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Abstract

The increasing search for natural alternatives in the various sectors of the industry, mainly food, has aroused interest in scientific research that evaluates the feasibility of applying new natural additives in products of commercial interest as an alternative to synthetic additives. The present work aimed at the extraction optimization of compounds with antioxidant activity of native plant species of the Amazon, *Dalbergia Subcymosa* D. and *Croton cajucara* L., through the quantification of total phenolics and total flavonoids contents. For the evaluation of the antioxidant activity of the extracts tests of total antioxidant activity, DPPH• free radical scavenging and reduction power of the iron ions were evaluated. Extracts with better extraction conditions were analyzed by UPLC-DAD and oxidative stability in commercial egg homogenate, which evaluates thiobarbituric acid reactive substances (TBARS). The extracts presented antioxidant activity similar to the standard (synthetic antioxidant, hydroxytoluenebutylated) and simple phenolic compounds were the major compounds identified in the extracts of the two species. The results were taken to statistical analysis, obtaining optimization response by analysis of variance (ANOVA) and response surface method (RSM).

Keywords: response surface method; *Dalbergia Subcymosa* D.; *Croton cajucara* L.

1.Introdution

In the Amazon, the use of products derived from medicinal plants is a frequent practice of the local population. Biological diversity is characteristic of northern Brazil and is one of the largest reservoirs of genetic diversity. Many of these plants are rich in secondary compounds, ie biologically active substances, such as phenolic compounds. Among the several species used in the Amazon for therapeutic purposes, we used *Dalbergia subcymosa* Ducke and *Croton cajucara* L., usually known as Verônica and Sacaca, respectively (Cruz, 2010; A. A. Oliveira et al., 2013).

The consumption of polyphenolic-rich foods has been postulated to contribute to a reduced risk of chronic diseases, cardiovascular disease, neurodegenerative, carcinogenic and Alzheimer, an area extensively studied. However, another focus is the application of vegetable extracts as a preservative in foods to replace synthetic food additives (Akcan et al., 2017; Caleja et al., 2016, 2015; Carocho et al., 2015).

Antioxidants are indispensable in the food industry to prolong the shelf life of foods, and the unsafe use of synthetic antioxidants such as BHA (hydroxyanisolebutylate) and BHT (hydroxytoluenebutylate) is unavoidable. With this, there is a growing interest in the substitution of synthetic antioxidants, though partial by natural antioxidants, often in the form of extracts, which are safe and act at least similar to synthetic ones. Research carried out in the middle of 2009 to 2018 aims to substitute these synthetic additives for vegetable extracts that have this activity in foods (Mariutti and Bragagnolo, 2009; Pereira, 2009; Toniolo, 2012; Caleja et al., 2016, Carocho et al., 2016; Carocho et al., 2015; Cheng et al., 2017).

Currently, attention is focused on natural matrices due to its bioactive compounds and high applicability potential, making use in the process of several techniques for extraction of these compounds. In addition to the extraction technique, the extraction efficiency is directly affected by several factors, among which the type and quantity of solvent are one of the most important. These factors combined with chemical characterization are essential for the exploitation of these matrices, attributing them to scientific and industrial applicability (Albuquerque et al., 2017; Can-Cauich et al., 2019).

Thus, the present work designed to evaluate by means of statistical methodology the effects of two factors, amount of vegetal drug in solvent (SM) and concentration in percent of the solvent used (% EtOH), in the optimization of the polyphenols extraction process and the activity antioxidant of the species *Dalbergia subcymosa* Ducke and *Croton cajucara* L.,

to identify the best point aiming natural alternative to the synthetic antioxidant popularly used in the food industry, hydroxytoluenebutylated (BHT), with effect on the lipid stability of food according to thiobarbituric acid reactive substances methodology (TBARS).

2. Methods and materials

2.1. *Botanical Material*

The leaves of the species *Croton cajucara* L. of the popular name "sacaca", and the barks of *Dalbergia subcymosa* D., of the popular name "verônica", were collected in the lowland area of the municipality of Porto Grande in the state of Amapá, in a single time of the year (2017).

2.2. *Extraction technique*

The identification of the botanical material was carried out by the Institute of Scientific and Technological Research of Amapá (IEPA), then cleaned and dried in the shade and at room temperature. The barks of the species *Dalbergia subcymosa* D. and the leaves of *Croton cajucar*a L. were cut into smaller pieces, ground in a knife mill and extracted with ethanol at different concentrations (100%, 70% and 40% w/v). The extraction of the components was based on the maceration process and performed in the three proportions of the drug:solvent (1:10, 1:20 and 1:40 w/v). The samples were then subjected to gravimetric analysis to determine the optimal extraction time.

2.3. *Gravimetric analysis*

Gravimetry was performed by transferring 1mL of each sample to crucibles followed by heating for 24h at 105ºC for the period of 1-7 days, according to the observation of minimum variation of relative standard deviation (SD).

2.4. Experimental design

The solvent chosen for the extraction was ethanol (C_2H_6O), an experimental design was adopted based on the effects of the response of each test performed in relation to the variation of the adopted factors (table 1). The combined effect of the variables was studied using a factorial experimental design, with two factors and three levels (23), the controlled variables (the factors) were: the sample mass in solvent (SM), in the following proportions: 10: 1 (-1), 20: 1 (0) and 40: 1 (1) and ethanol content (% EtOH), 40% (- 1); 70% (0) and 100% (1). Other factors were kept constant, such as the particle size of the samples, the temperature, and shelter of the light.

2.5 Total Flavonoids content

The total flavonoid content was determined according to the methodology described by Wannes et al. (2010) using 5% aluminum chloride ($AlCl_3$). The extract samples were prepared at a concentration of 50 μg/ml. To the sample, solutions were added 2.5 mL of the $AlCl_3$ solution and taken out of the light for 30 minutes to observe the formation of the yellowish-colored chromophore. Afterward, the samples were taken to read absorbances at 420 nm in a spectrophotometer (BIOPROSS-SP22). The calibration curve was performed with quercetin (5-30 mg/L). The result was expressed in mg of quercetin standard equivalents per g of extract. The analyses were performed in triplicate.

2.6. Total Phenolic Content

The total phenolicscontent was determined by the Folin-Ciocalteau method. In that an aliquot (0.5 mL) of the solution of the appropriately diluted extract was added to 2.5 mL of Folin-Ciocalteau reagent (0.2 N) after the addition was allowed a period of 5 min, 2mL of 7.5% sodium carbonate (Na_2CO_3) solution was added. The sample was incubated at room temperature (25ºC) for 2h. The absorbance was observed at 760 nm in a spectrophotometer (BIOPROSS-SP22) and compared to the calibration curve of gallic acid (50-200 mg/L). The results were expressed in mg equivalent of gallic acid per gram of extract. The analyses were performed in triplicate (Singleton, Orthofer, & Raventós, 1999).

2.7. DPPH• free radical scavenging

The DPPH free radical scavenging assay was performed in comparison with a standard curve BHT (hydroxytoluenebutylated). Aliquots of 0.1 ml of the sample at 12.5 μg/ml were transferred to test tubes, then 3.9 ml were added to the DPPH• solution (60 μM). The solutions were taken out of the light for 30 min and after that period the absorbance readings were taken at 512 nm in a spectrophotometer (BIOPROSS-SP22), using only DPPH • (60 μM) as white. Each sample was analyzed in triplicate and the results were expressed as IC 50 of free radical sequestration (Brand-Williams, Cuvelier, & Berset, 1995).

2.8. Total Antioxidant Capacity by the Phosphomolybdenum Complex Reduction power

The antioxidant activity was determined according to the method of Prieto et al. (1999). An aliquot of 0.1 mL of properly diluted extract solution at 200 μg/mL was added to 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95ºC for 90 minutes. The samples were brought to room temperature until cooling, and the absorbance of each solution read in a spectrophotometer (BIOPROSS-SP22) at 695 nm. The results were compared with the calibration curve of ascorbic acid and BHT and expressed as μmol equivalent of ascorbic

acid or BHT per gram of extract (g E_{AG}/g or E_{BHT}/g). The analyses were performed in triplicate.

2.9. Ferric Reduction Power

The analyzes of the reduction power of the extracts were carried out according to a methodology described by Santos et al. (2007). From the sample extracts, dilutions were obtained at a concentration of 100 μg/mL for analysis. As solutions were used without 1% ferrocyanide, 10% trichloroacetic acid, 0.1% $FeCl_3$ and then 0.2 M phosphate (pH 6.6). An aliquot of 1 ml of each drop was transferred to the effect of a solution of 2.5 ml of the quick solution and 2.5 ml of the ferrocyanide solution.

The reaction medium was heated in a water bath for 20 min at 450 ° C. After heating, 2.5 ml of 10% trichloroacetic acid was added. For the new test tubes, 2.5 ml of the previously obtained solution, 2.5 ml of distilled water and 0.5 ml of FeCl3 were transferred. The final solutions were taken to read absorbances at 700 nm in a spectrophotometer (BIOPROSS-SP22). The analyses were performed in triplicate and as standard (100%) for the test BHT was used.

2.10. Chromatographic analysis of the best extract

The extracts of the bark of the Veronica and of the leaves of sacaca were analyzed at a concentration of 5mg/mL in methanol in the best condition of response to the adopted experimental model. Chromatographic analysis was performed using a UPLCShimadzu system, equipped with a vacuum degasser, automatic sampler, one quaternary pump and a diode arrangement detector (DAD). Chromatographic separations were performed on a Shimadzu C-18 reverse phase column (100 × 4.6 mm, 2.1μm particle size) at 30ºC using formic acid (1% v/v) (A) and acetonitrile (B) as mobile phases at 0.2 mL/min and sample volumes of 2μL. The analysis was performed in 30 minutes with gradient system initiated at

5% B with ramp up to 95% B to a fingerprint analyses (García-villalba, Carlos, Tomás-barberán, & Rocha-guzmán, 2017).

2.11. Inhibition of lipoperoxidation induced by AAPH

Thiobarbituric acid reactive substances (SRAT) is a methodology described by Esterbauer and Cheeseman (1990), performed to quantify the level of lipid peroxidation in a lipid-rich substrate. The test is performed by preparing a pool of 4 commercial egg yolks (lipid-rich substrate) with 10 g of the previously homogenized buds, solubilized in 90 ml of 20 mM phosphate buffered saline (PBS) solution at pH 7.32, resulting in a 10% homogenate.

An aliquot of 1.75 mL of the homogenate was used to constitute the reaction medium (MR) plus 0.05 mL sample extract and standard BHT, both with 25, 50 or 100 μg / mL concentration. Lipid peroxidation was induced by the addition of 0.2 mL of 2,2'-azobis-2-amidinopropane solution (AAPH, 0.12 M). This reaction medium was incubated for 30 min at 37 ° C and, after cooling, received 4mL of the 0.7% thiobarbituric acid solution (TBA) solubilized in 15% trichloroacetic acid (TCA). The solution was again incubated in a water bath at 100 ° C for 45 minutes. After cooling, the samples received 3mL of butanol P.A. to isolate the species that reacted with the TBA / TCA solution (0.73%), isolated in a centrifuge at 6000 rpm for 5 minutes to perform the phase separation. The samples were taken to the spectrophotometer to read their absorbances at 532 nm in spectrophotometer (BIOPROSS-SP22), with results expressed in equivalent monoaldehydes formed, and statistical evaluation calculated in ANOVA in GrapghpadPrism7 software (V. B. Oliveira et al., 2015).

2.12. Data evaluation

The results obtained for optimization of extraction were treated by STATISTICA® software for Windows for surface response tests (RSM), paretocharc, desirability and predicted and observed values (López et al., 2018).

3. Results and discussions

The inherent characteristics of natural matrices have gained notoriety mainly in relation to the extraction of compounds of interest such as phenolics and flavonoids, the type of solvents and the susceptibility of these compounds to undergo degradation when subjected to external factors such as the type of solvent, pH, temperature, and even the amount of plant drug for the production of a matrix. For these and other factors it is that the selection of a suitable extraction method and ideal conditions to conduct the extraction gained particular relevance (Brito et al., 2012; Cásedas, Les, Gómez-Serranillos, Smith, & López, 2017; Chemat et al., 2017; Khadhraoui et al., 2018; López et al., 2018). Regarding these aspects, efforts have been made to develop and compare extraction processes with a view to better extraction yields for the application of these vegetable matrices to foods, for example (Guimares et al., 2010).

Therefore, the present study was conducted by selecting two factors for the extraction of compounds with antioxidant activity, amount of applied drug mass and concentration (%) of the solvent used (ethanol).

The solvent chosen for extraction was ethanol (C_2H_6O), because many biologically important polar compounds were extracted with hydroalcoholic solution (ethanolic), aiming also to reduce the interference of this in the application of the extract. As a result, the composition of this type of hydroalcoholic solution was studied for the extraction of compounds with antioxidant activity.

The extracts were subjected to the tests to identify the compounds of interest by spectrophotometry and determination of antioxidant activity and the application was performed to evaluate the lipid stability of food according to the methodology of the

substances reactive to thiobarbituric acid (TBARS), these substances are products of process degradation lipid oxidation.

The conditions of established variables of mass proportions of applied plant drug and percentage (%) of ethanol were evaluated for each test through graphs of response to the method. The factors adopted were submitted to a Box Behken type factorial analysis with 2numberoffactors (independent variable); SM (solutemass) and% EtOH block generation, 9 cases experiment sand 3 number of blocks randomly.

The Pareto graph, obtained through analysis of variance ANOVA, was used to analyze which factor adopted had the highest significance in the optimized value obtained for each sample of extract at 5% of significance ($p < 0.05$). The results observed in the pareto graph are confirmed through the method response surface (RSM), which corresponds to an effective form of the statistical method useful for mathematical modeling, obtaining predictive models as a function of the independent variables and the response variables, enabling a visual and quantitative response of the results of the factors against the dependent variable (Statistica, 2017).

The critical values were obtained through the test's desirability response profile, which establishes a relationship between the predicted responses in one or more dependent variables and the convenience of the responses, indicating which levels of the predictor variables produce the most desirable predicted responses in the dependent variables (Statistica, 2017).

A fourth test was applied to evaluate the predicted and observed values, demonstrating the efficiency of the optimization model adopted against the results extracted from the experiments performed.

The samples of sacaca (*Croton cajucara* L.) presented higher statistical significance compared to the Pareto plot for the two factors, SM (Solute Mass) and% EtOH (percentage

of ethanol in both axes (linear and quadratic). % EtOH showed greater significant influence to the optimization model adopted for the quantification tests of phenolics and total flavonoids and determination of the antioxidant activity of the extracts against the BHT (synthetic antioxidant) standard by determining the total antioxidant activity (phosphomolybdenum reagent reduction test), free radical sequestration (FRS) DPPH • and iron reduction power The results in relation to the quantitatively adopted factorial design and graphical representations are shown in Table 2 and Figure 1.

Veronica samples presented different values and proportions for the best extraction condition in each test, both the analytical quantifications and the analyzes of the antioxidant potential. On the other hand, it was observed a constancy in the samples of bag at the points of 0.05g of vegetal mass and 70% of concentration of the solvent (ethanol), for this reason the conditions of better extracts were adopted for the analysis for both plant species, *Dalbergia Subycimosa* D. *e Croton cajucara* L.

The identification of the compounds was performed by comparing the retention time and UV-VIS spectrum and quantification were performed at 280nm for single phenolic with standard gallic acid (0.1-100μg/ml) and at 340nm for quercetin standard flavonoids (0.5-500μg/ml). The phytochemical analysis of the extracts of verônica and sacaca by UPLC-DAD showed the presence of simple phenolic compounds identified between 0.5 and 1min of retention time for the sample of verônica and 0.5 to 0.75min of the sample of sacaca as major compounds (Figure 3 and 4).

Table 3 for the identified compounds indicates each observed signal, retention time, as well as maximum UV absorption and concentration in gallic acid or quercetin equivalents. It is important to note that the range corresponding to the retention time of 0.5

to 15min indicates simple phenolic compounds and the range of 15 to 30min compounds of the flavonoid class for the two species.

To carried out the quantification of phenolic compounds were performed two calibration curves, one used gallic acid ($0.0032x+134.56$; $R^2=0.9999$) and other with quercetin ($0.0047x+256.79$; $R^2=0.9999$), result showed in table 2.

In order to identify the effective behavior of the extracts with the best response to the optimization model, inhibition of lipid oxidation, the test for evaluation of thiobarbituric acid reactive substances (TBARS) was applied, evaluating the effects of addition and experimental factors of the antioxidant activity of the extract and the extracts of the saccharide against the antioxidant commercially used in food, hydroxytoluenebutylated (BHT) to identify the degree of inhibition on the commercial egg homogenate.

Lipid oxidation promotes changes in the food, these changes attribute unpleasant flavors and odors to the product making it unsuitable for consumption, since it modifies the nutritional quality from the formation of potentially toxic compounds. This process is called lipid peroxidation, which is an autocatalytic reaction and induces the pro-oxidative effect of transition metals, generating lipid hydroperoxides that constitute a vast evidence, after the occurrence, of reactions of metal ions with H_2O_2 (peroxide of hydrogen), producing lipid peroxidation and enabling processes mediated by reactive oxygen species (free radicals). Lipid hydroperoxides, in the presence or absence of catalytic metal ions, produce a wide variety of products that decompose to form volatile aromatic compounds, such as ketones and aldehydes, known as *off-flavors*. These compounds develop during lipid oxidation and serve as an indicator for food that is no longer edible. *Off-flavors* are by-products of decomposition of the lipid peroxidation reaction and can be used to assess the degree of peroxidation in a system. We have as example malondialdehyde (MDA), which is considered

the main compound reactive to the acid-barbituric acid. (Gueraud et al., 2010; Repetto et al., 2012).

The experiment used the best extracts obtained, one for each species, based on the optimum result of extraction of the sacaca, which in the majority of the tests indicated significance in SM: 0,05g; % EtOH: 70% and of the Veronica, which showed a concentration of 40% ethanol in the DPPH free radical sequestration assay and quantification of total flavonoids. As the extract extracts in 40% of ethanol presented significance in this proportion for the tests of quantification of total phenolics and reduction power of iron ions, they were also evaluated in the conditions of 0.05g of SM and 40% EtOH. Samples and standard (BHT) were analyzed at three different concentrations 25μg/mL; 100 μg/mL and 250 μg/mL, to verify the best results and the lipid stability was determined through thiobarbituric acid reactive substances by reading the absorbances in a spectrophotometer(Lima Veeck et al., 2015).

The data were submitted to analysis of variance (ANOVA) for comparisons between the means of the lipoperoxidation indices and the Tukey test. The differences were considered statistically significant when $P < 0.05$ (figure 4).

After the test, it was possible to observe that the *Dalbergia subcymosa* D. samples. at concentrations of 100 and 250 μg/mL, presented a high significance in the optimization conditions of 0.05g SM and 70% EtOH within the limit of 5% significance (c, $p < 0.001$) and at concentrations of 25 μg/mL, 100 μg/mL and 250 μg/mL for the optimization conditions of 0.05g SM and 40% EtOH (c, $p < 0.001$) relative to the negative control, reactive medium only with AAPH. The samples of *Croton cajucara* L., at the three established concentrations, presented responses within the limit of significance, $p < 0.005$, for optimization conditions of 0.05g SM and 70% EtOH vs. negative control, as evidenced in figure 4.

4. CONCLUSION

Thus, through the optimized results obtained it was concluded that *Dalbergia subycimosa* D. and *Croton cajucara* L. studied showed positive responses to the quantification of phenolic compounds and total flavonoids and were confirmed by the chromatographic analysis. The responses in relation to the antioxidant activity of the extracts compared to the BHT standard showed greater activity for the *Croton cajucara* L. species under conditions of 0.05g of plant drug: solvent and 70% ethanol, with significant results also for conditions of 0.05g of vegetable drug: solvent and 40% of ethanol, which was confirmed by thiobarbituric acid reactive substances (TBARS) test. *Dalbergia subycimosa* D. samples also presented significant results under the same conditions, but *Croton cajucara* L. extracts responded better to the optimization model adopted.

For future studies, other factors, besides those adopted in this research, should be considered in order to provide a more robust response for the optimized extraction of the compounds, since the antioxidant activity of the extracts was proven through the tests carried out in this research.

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Table 1. Factorial planning and determination of the study variables for optimization of the two main variables involved (vegetable drug ratio: solvent (SM) and percentage of ethanol (% EtOH), extraction solvent used). The first column shows the number of standard runs, column two the number of replicates, the fourth column indicates the experimental blocks and the last two the results of the factorial planning for each study factor according to the experimental model adopted, with two factors and three levels (23).

VariableCodedvalues				
Standard run	**Replicate**	**Block**	**SM**	**%EtOH**
17	2	3	0,025000	100,0000
4	1	2	0,025000	70,0000
5	1	2	0,10000	100,0000
10	2	1	0,05000	100,0000
15	2	2	0,05000	40,0000
16	2	3	0,10000	40,0000
11	2	1	0,10000	70,0000
24	3	2	0,05000	40,0000
6	1	2	0,05000	40,0000
18	2	3	0,05000	70,0000
26	3	3	0,025000	100,0000
2	1	1	0,10000	70,0000
22	3	2	0,025000	70,0000
25	3	3	0,10000	40,0000
1	1	1	0,05000	100,0000
7	1	3	0,10000	40,0000
20	3	1	0,10000	70,0000
9	1	3	0,05000	70,0000
12	2	1	0,025000	40,0000

13	2	2	0,025000	70,0000
8	1	3	0,025000	100,0000
19	3	1	0,05000	100,0000
3	1	1	0,025000	40,0000
14	2	2	0,10000	100,0000
27	3	3	0,05000	70,0000
23	3	2	0,10000	100,0000
21	3	1	0,025000	40,0000

Table 2. Factors responses to the mathematical optimization model adopted.

Analysing before Box-Behnken factorial design: 3**(2-0) full factorial design, 3 blocks, 9 runs + 2 replications										
Critical values for analitic quantifications and antioxidant activity determination (~SM/%EtOH)										
Effect estimates	PhV	PhS	FV	FS	TAAV	TAAS	FRSV	FRSHS	FeRV	FeRS
	0,1 / 70	0,0625 / 55	0,0625 / 40	0,0625 / 70	0,08125 / 100	0,0625 / 70	0,08125 / 40	0,1 / 100	0,025 / 85	0,025 / 40
P (SM / %EtOH)	0,178250 / 0,227351	0,000138 / 0,000003	0,079343 / 0,000000	0,000004 / 0,000000	0,036812 / 0,000068	0,000035 / 0,000177	0,104168 / 0,053129	0,035810 / 0,072384	0,210320 / 0,000207	0,000210 / 0,000000
R	0,37283	0,83389	0,87836	0,86668	0,58757	0,72765	0,26981	0,30874	0,52949	0.99441

- The first line shows the best effects estimates obtained through the desirability analysis (figure 1) of the method for each factor (vegetable drug: solvent ratio (SM) and percentage of ethanol (% EtOH)) for each plant species (*Dalbergiasubcymosa*DUCKE (V) and *Croton Cajucara* L. (S)) in the quantification tests of total phenolics and flavonoids and in the determination of the antioxidant activity of the samples by the tests of total antioxidant activity (TAA); Free radical Sequestration DPPH • (FRS) and reduction power of iron ions (FeR). The second line shows the values of p within the limit of 5% of significance ($p < 0.05$) for each sample in each test. The last line describes the correlation of the factors adopted with the test performed, according to a descriptive statistical model, for each plant species.

Table 3. Description of the chromatographic analysis performed using an UPLC-DAD system, column C-18, at 30 ° C using formic acid (1% v / v) (A) and acetonitrile (B) as mobile phases at 0, 2mL/min and sample volumes of 2µL for 30 minutes with gradient system initiated at 5% B with ramp up to 95% B, at 280nm for phenolics and 340nm for flavonoids.

***DalbergiaSubcymosa* DUCKE**				***CrotonCajucara* L.**			
Picos	T.R. (min)	Conc. (µg/ml)	UV-λmax (nm)	Picos	T.R. (min)	Conc. (µg/ml)	UV-λmax (nm)
1	0.807	51.88	265 (EqGallicacid)	1	0,3253	51.72	274 (EqGallicacid)
2	3.094	468.38	289/477 (EqGallicacid)	2	0,3439	5.35	335/271 (EqGallicacid)
3	20.756	119.86	332/286 (Eqquercetin)	3	0,6506	3.53	256/349 (EqGallicacid)
4	21.960	138.94	330/662 (Eqquercetin)	4	2,2437	6.08	265/229/346 (EqGallicacid)
5	23.236	74.76	331/491 (Eqquercetin)	5	2,5851	2.27	254/353/232 (EqGallicacid)
6	30.737	6.77	331/491 (Eqquercetin)	6	2,8810	4.58	254/337 (EqGallicacid)
				7	2,9610	2.94	265/240/342 (EqGallicacid)
				8	3,4130	3.63	254/352 (EqGallicacid)
				9	3,5356	4.98	259/346 (EqGallicacid)
				10	7,5966	0.39	246/349 (EqGallicacid)
				11	10,872	2.59	267/347 (Eqquercetin)
				12	11,297		267/347 (Eqquercetin)

- The first column shows the peaks visualized in the chromatograms (figure 4) obtained by reading each species (*DalbergiaSubcymosa* DUCKE and *Croton Cajucara* L.). The second column shows the retention times at

which the peaks were observed (T.R.). The third column shows the area on the curve (A.C.) and the last column, the maximum UV absorption of each observed peak (UV λmax).

Figure caption

Figura 8: Answers of the vegetal species in front of the adopted experimental design. The Pareto chart graphs for the quantification of total phenolics showed a significance in the quadratic axis for the proportional factor drug vegetable: solvent (SM), with no significance in percentage of ethanol (% EtOH) for samples of D. subcymosa D. and the two factors presented significance, within the limit of significance of 5% ($p < 0.05$), for the samples of C. CajucaraL. The total flavonoid test, only the linear response of the% EtOH factor was significant for the samples of D. subcymosa D. and the two factors were significant for the samples of C. Cajucara L. The total antioxidant activity showed a significant linear response for the factor of % EtOH and SM for D. Subcymosa DUCKE and all factors in the linear and quadratic axis were significant for C. CajucaraL. The free radical sequestration assay DPPH did not present significance for any factor in the samples of D. subcymosaDUCKE by the Pareto graph and was significant only for SM in C. CajucaraL C CajucaraL. presented significance in all factors and axes in the test of Reduction Power of the iron ions being more expressive in % EtOH and D. SubcymosaDUCKE showed significance in the linear response to the% EtOH factor. The response surfaces follow below the Pareto graph with the% EtOH factor on the "x" axis and SM on the "z" axis. The predicted and observed values are shown in the scatter plots below the response surface.

Figura 9. Desirability of the experimental optimization model for the two factors adopted (drug vegetable ratio: solvent (SM) and percentage of ethanol (% EtOH)) in each test. Quantification of total phenolics: DalbergiaSubcymosa DUCKE- 0.1g SM and 70% EtOH, Croton Cajucara L.- 0.0625g and 55% EtOH. Quantification of total flavonoids: D. Subcymosa D.- 0.0625g SM and 40% EtOH, C. Cajucara L.- 0.0625g SM and 70% EtOH. Total Antioxidant activity: D. SubcymosaD.- 0.08125g SM and 100% EtOH, C. Cajucara L- 0.0625g SM and 70% EtOH. Radical Free Sequestration DPPH •: D. SubcymosaD.- 0.08125g SM and 40% EtOH, C. CajucaraL.- 0.1g SM and 100% EtOH. Reducing Power: D. SubcymosaD.- 0.025g SM and 85% EtOH.

Figura 10. UPLC-DAD chromatograms of *Dalbergiasubcymosa* DUCKE and *Croton Cajucara*L.. At 280nm for flavonoids in 0,1 at 15min retencion time and 360 nm for flavonols in 15 at 30 min of retencion time.

Figura 11. Lipidic oxidation induced by AAPH in egg yolk homogenate, transcribed by the thiobarbituric acid reactive species formation index (SRAT nM), having D. subcymosa (25 μg / mL, 100 μg / mL and 250 μg / mL) and C. Cajucara L. (25 μg / mL, 100 μg / mL and 250 μg / mL) as samples using BHT (25 μg / mL, 100 μg / mL and 250 μg / mL) as standard.

Figura 1

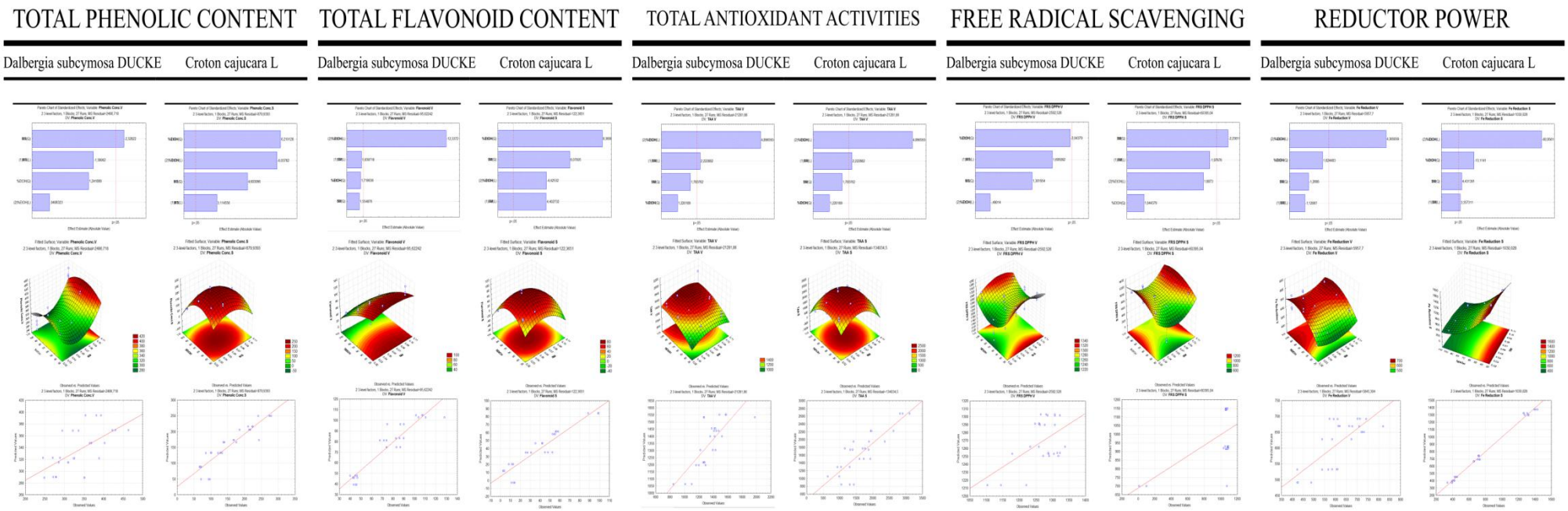
TOTAL PHENOLIC CONTENT
Dalbergia subcymosa DUCKE
Croton cajucara L
TOTAL FLAVONOID CONTENT
Dalbergia subcymosa DUCKE
Croton cajucara L
TOTAL ANTIOXIDANT ACTIVITIES
Dalbergia subcymosa DUCKE
Croton cajucara L
FREE RADICAL SCAVENGING
Dalbergia subcymosa DUCKE
Croton cajucara L
REDUCTOR POWER
Dalbergia subcymosa DUCKE
Croton cajucara L

Figura 2

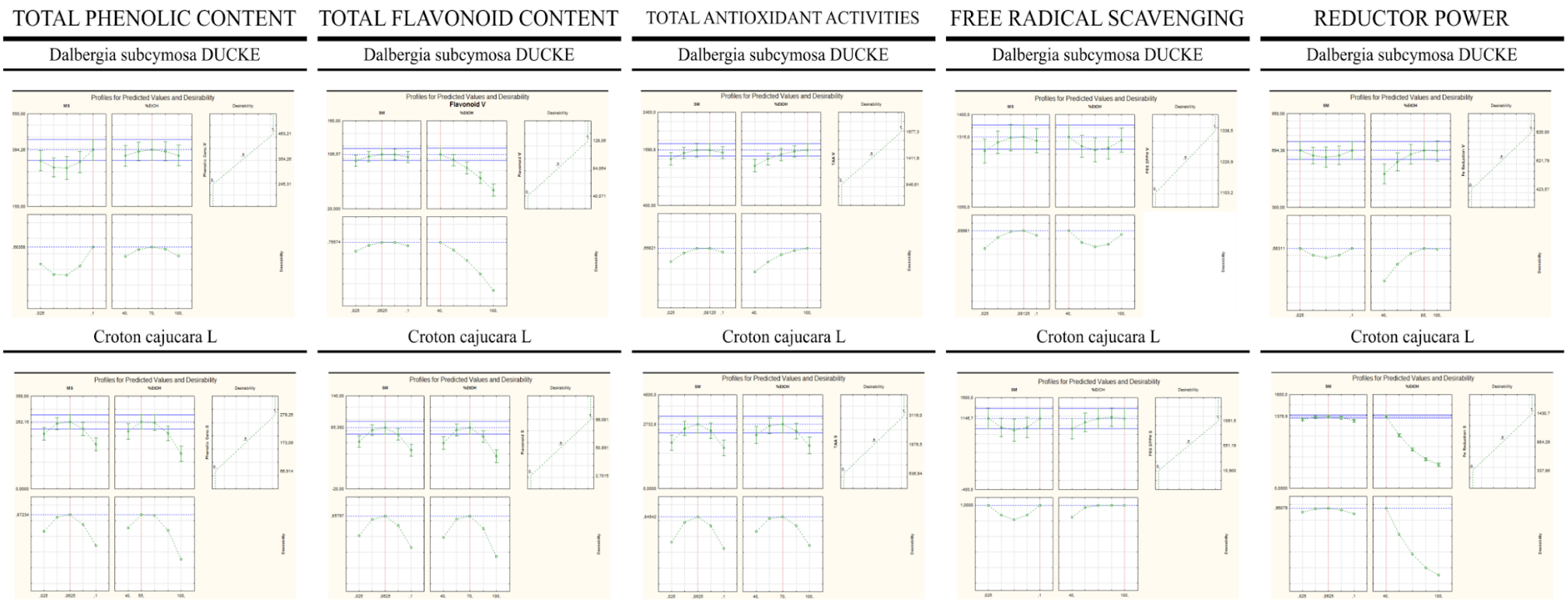
TOTAL PHENOLIC CONTENT
TOTAL FLAVONOID CONTENT
TOTAL ANTIOXIDANT ACTIVITIES
FREE RADICAL SCAVENGING
REDUCTOR POWER
Dalbergia subcymosa DUCKE
Croton cajucara L
Profiles for Predicted Values and Desirability

Figura 3

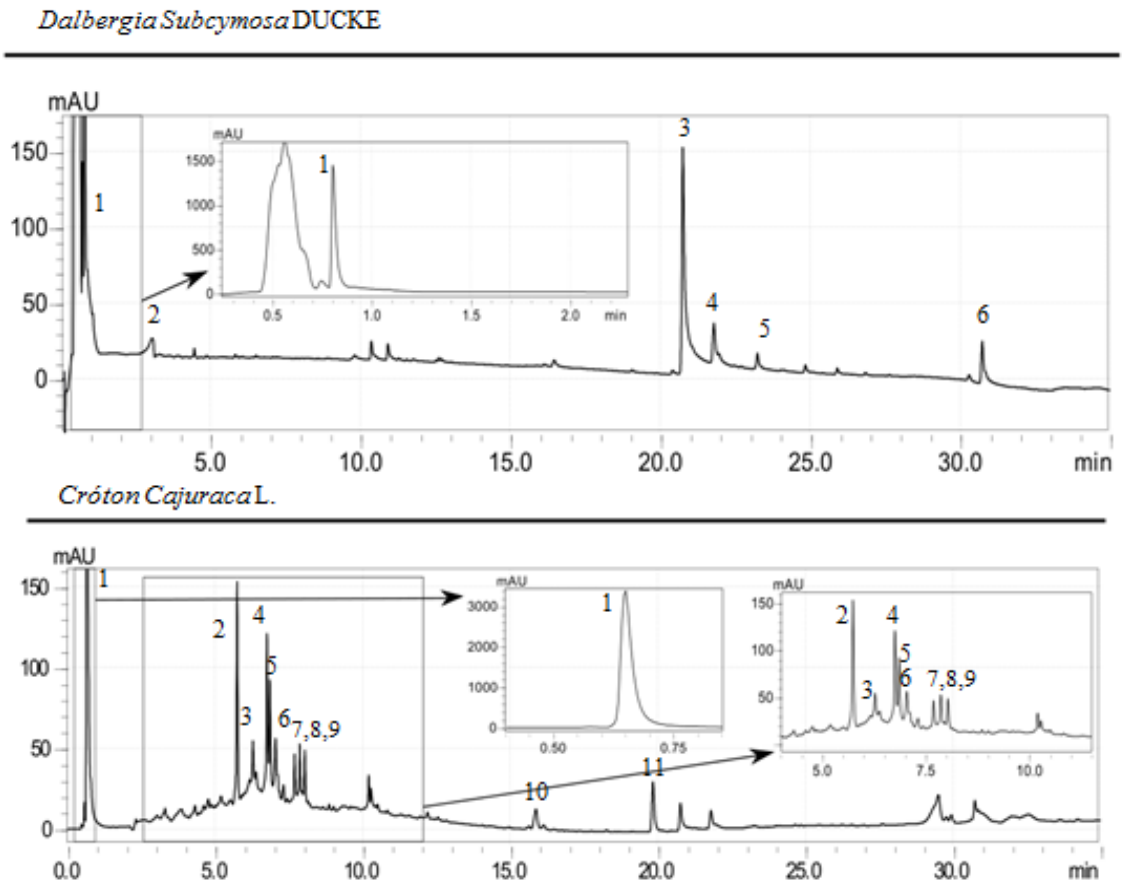
Dalbergia Subcymosa DUCKE
mAU
150
100
50
0
1
2
3
4
5
6
5.0
10.0
15.0
20.0
25.0
30.0
min
Cróton Cajuraca L.
mAU
150
100
50
0
1
2
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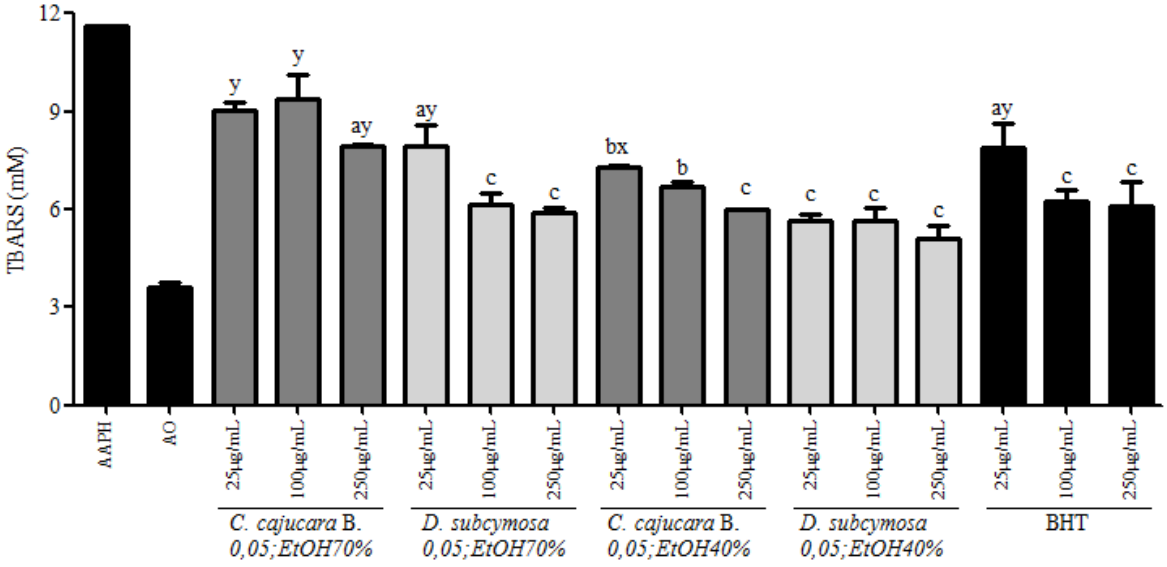
Figura 4



a p<0,05; b p<0,005; c p<0,001 - comparado com AAPH
x p<0,05; y p<0,005; z p<0,001 - comparado com AO

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5 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Dessa forma, através dos resultados obtidos pôde-se concluir que as espécies de *Dalbergia Subcymosa* D. e *Croton cajucara* L. estudadas apresentaram respostas positivas frente flavonóides totais, observando-se uma resposta mais robusta frente ao padrão rutina. Resultados estes que foram confirmados por análise cromatográfica em UPLC-DAD. A otimização do processo extrativo das espécies comparativamente ao padrão BHT, mostrou melhor condição de extração para as amostras de *Croton cajucara* L.. No entanto, as duas espécies vegetais apresentaram-se viáves para aplicação em alimentos como alternativa ao antioxidante sintético BHT, baseado no teste das substâncias reativas ao ácido tiobarbitúrico (TBARS). Nas condições otimizadas, a D.Subcymosa revelou-se como um ótimo substituinte ao BHT com resultados de inibição proporcionalmente iguais. Com estudo visa mostrar que é possível o desenvolvimento e aplicação de uma alternativa natural a um antioxidante sintético popularmente utilizado no Brasil tanto para alimentos quanto para cosméticos, gerando um conservante natural ECO friendly e saudável, através de espécies vegetais da região amazônica. Viabilizando, assim, o desenvolvimento tecnológico do estado, atendendo a demandas ambientais, possibilitando o incentivo no desenvolvimento de novos produtos regionais e com potencial de relevância para as atividades biotecnológicas nacionais, considerando se tratar de uma área emergente e com ótimas condições de expansão. Para estudos futuros, pretende-se realizar o desenvolvimento do produto final à partir das matérias-primas regionais analisadas.

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ANEXO I

Revista 1 : Acta Amazônica

INSTRUCTIONS TO AUTHORS

It is expected that manuscripts submitted to Acta Amazonica are prepared according to the Instructions to Authors (guidelines). Thus, please make sure your manuscript follows these guidelines before uploading your submission. Manuscripts that do not adhere to the Journal´s instructions are returned to authors.

1. Maximum size of an individual file should be 2 MB.

2. A letter submitting the manuscript (cover letter) should state that:

a) The research data are original and accurate; b) all the authors participated substantially and are prepared to assume public responsibility for its content; c) the contribution presented to this journal has not previously been published, nor has it been submitted for publication elsewhere, entirely or in part. Upload the cover letter upon submission.

3. The manuscripts must be written in English. The veracity of the information contained in the manuscript is exclusive responsibility of authors.

4. Maximum length for articles and reviews is 30 pages (or 7500 words, disregarding the title page), ten pages (2500 words) for short communications, and five pages for other contributions.

5. Manuscripts properly formatted according to the "Instructions to authors" are sent to Associate Editors for preevaluation. In this first judgment it is taken into consideration the scientific relevance and intelligibility of the manuscript, and its scope within the Amazonian context. In this phase, contributions outside of the scope of the Journal or of little scientific value are declined. Manuscripts approved in the first judgment are sent to scientific referees for evaluation, at least two reviewers; experts from institutions other than those of the authors.

6. Acceptance of manuscripts will be based on the scientific content and the correct formatting according to the Journal guidelines.

7. Manuscripts requiring corrections will be returned to the authors for revision. The revised version needs to be uploaded in the Journal system in TWO weeks' time. A response letter is required to be returned with the revised version. In this letter, please detail the modifications made to the manuscript. Recommendations not incorporated into the revised version, if any, need to be responded. The entire process is online, and can be followed at the Journal Web site, http://mc04.manuscriptcentral.com/aa-scielo.

8. Follow these instructions to prepare and upload the manuscript:

a. Authorship and manuscript information (Title page, please use the word format): This page must contain the manuscript title, authorship (last name in uppercase letter), full institutional address of the authors, and email of the corresponding author. Do NOT abbreviate names of institutions. Use an asterisk (*) to indicate the corresponding author. Only the e-mail of the corresponding author is required in the title page of the manuscript. Upon submission, upload this file selecting the option: "Title page".

b. Main body of the text (main document , please use the word format). The text of the manuscript should follow this order: Title, Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgements , References, Figure legends, and Tables. It is also required to include a “Titulo, Resumo and Palavras-chave” in Portuguese OR Spanish. Upon submission upload this file as "Main document".

c. Figures. Up to seven figures are permitted for articles. Each figure MUST be uploaded as a separate file. Figures

should be in the graphic format (JPG or TIFF) and of high quality and resolution (300 dpi). Use 600 dpi for bitmap illustration. Upload each of these files selecting the option: "Figure".

d. Tables. Five tables are permitted for articles. Use single spacing and the table function for typing tables. Please insert the Tables at the end of the text of the manuscript (main document), after the "Figure legends".

9. Short communications are to be written separating the topics (Introduction, Materials and Methods, Results, Discussion and Conclusions) in paragraphs, but

without including their titles. They also have to include all the sections of the full article (e.g.: Title, authorship, affiliation, electronic address, Abstract, Keywords, Acknowledgements, References). Three figures and two tables are permitted. Upload the "title page", "main document", figures and tables as described previously (Item 8).

10. Full name of authors and their institutional addresses and e-mails must be registered in the Journal system.

11. IMPORTANT NOTE: Manuscripts not properly formatted according to the "Instructions to Authors" are NOT accepted for publication.

FORMAT AND STYLE

12. The manuscript is to be prepared with a text editor (e.g. doc or docx), typed using "Times New Roman" 12- point font. It should be double-spaced with 3-cm margins; pages and lines consecutively numbered. For tables see Item 8d.

13. Title. Adjust to the left and capitalize the first letter of the sentence. Avoid using scientific names.

14. Abstract. It should have up to 250 words (150 for short communications). Initiate the Abstract with a couple of lines (rationale), and after that clearly state the objectives. The Abstract must succinctly contain the methodology, results and conclusions, emphasizing important aspects of the study. It should be intelligible for itself. Scientific names of species and other Latin terms should be in italic. Avoid acronyms, but if they are required give their meaning. Do not use references in this section.

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