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Verciane Schneider Cezarotto

Vaccinium ashei Reade: DA ANÁLISE FITOQUÍMICA DAS FOLHAS AO DESENVOLVIMENTO DE NANOPARTÍCULAS E AVALIAÇÃO DAS ATIVIDADES ANTIOXIDANTE E ANTIDEPRESSIVA

Santa Maria, RS
2017

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Farmacêuticas, Área de concentração em Desenvolvimento e Avaliação de Produtos Farmacêuticos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Doutora em Ciências Farmacêuticas.**

Orientadora: Prof.ª Dr.ª Letícia Cruz

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E-mail: verciane@uri.edu.br

Verciane Schneider Cezarotto

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Aprovado em 1° de dezembro de 2017:

Letícia

Letícia Cruz, Profa. Dra. (UFSM)

(Presidente/Orientadora)

Andrea Hermann Betti

Andresa Betti, Profa. Dra. (FEEVALE) Por parecer

Fabiane Moreira Farias, Profa. Dra. (UNIPAMPA)

Sara Marchesan de Oliveira

Sara Marchesan de Oliveira, Profa. Dra. (UFSM)

Scheila Rezende Schaffazick, Profa. Dra. (UFSM)

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“Aos nossos filhos, frutos do amor verdadeiro, repleto de compreensão e cumplicidade, nossa continuidade.”

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RESUMO

Vaccinium ashei READE: DA ANÁLISE FITOQUÍMICA DAS FOLHAS AO DESENVOLVIMENTO DE NANOPARTÍCULAS E AVALIAÇÃO DAS ATIVIDADES ANTIOXIDANTE E ANTIDEPRESSIVA

AUTORA: Verciane Schneider Cezarotto
ORIENTADORA: Profa. Dra. Letícia Cruz

O cultivo e comercialização do *Vaccinium ashei* Reade, conhecido como mirtilo, tem se tornado cada vez mais popular devido às propriedades nutricionais e farmacológicas vinculadas ao seu consumo. Apesar do interesse comercial estar voltado aos frutos, estudos tem demonstrado que as folhas, as quais apresentam elevados teores de compostos fenólicos, tem sido relacionadas a diversas atividades biológicas. Sendo assim, o presente trabalho avaliou, primeiramente, a influência do período de coleta e da cultivar sobre o perfil de compostos fenólicos, bem como a atividade antioxidante de folhas de *V. ashei*. A segunda etapa concentrou-se na avaliação da atividade antidepressiva do extrato hidroalcoólico de folhas de *V. ashei* da cultivar Climax (coleta de dezembro/2013) (EHV1) através da avaliação da administração aguda (10, 25 e 50 mg/Kg, v.o.) sobre o tempo de imobilidade dos animais pelo Teste de Natação Forçada (TNF) e Testes de Suspensão pela Cauda (TSC). Além disso, o efeito da administração crônica (50 mg/Kg, v.o.) foi avaliado empregando-se o Teste de Estresse Crônico Moderado e Imprevisível (ECMI). Por fim, foram desenvolvidas nanoestruturas usando Eudragit® RS100 pelo método nanoprecipitação contendo extrato hidroalcoólico de folhas de *V. ashei* da cultivar Climax (coleta de março/2014) (EHV2). As nanopartículas (NPEHV) foram caracterizadas e avaliadas quanto aos efeitos antioxidantes. Além disso, a atividade antidepressiva (1, 2,5, 5, 10 e 25 mg/Kg, v.o.) e o envolvimento de neurotransmissores monoaminérgicos foram avaliados *in vivo*. Os resultados demonstraram que o perfil qualitativo das folhas de *V. ashei* apresentou similaridade na composição, contudo a proporção de cada composto fenólico, bem como a atividade antioxidante foi influenciada pela cultivar e pelo período de coleta. Além disso, a administração aguda de EHV1 (50 mg/Kg, v.o.) reduziu significativamente o tempo de imobilidade dos animais no TNF e TSC, sem alterar a atividade locomotora no teste de campo aberto. O tratamento crônico de EHV1 (50 mg/Kg, v.o.) também foi capaz de reverter o comportamento do tipo depressivo e o tempo de imobilidade dos animais no TNF, sem alterar o peso corporal, parâmetros hematológicos e concentração de glicogênio hepático e cerebral dos animais submetidos ao regime de estresse. Estes resultados não foram observados após a administração aguda de EHV2 (50 mg/Kg, v.o.). Contudo, a nanoencapsulação de EHV2 foi capaz de promover os efeitos antioxidantes e a atividade antidepressiva, envolvendo neurotransmissores dopaminérgicos e noradrenérgicos. As NPEHV apresentaram características físico-químicas compatíveis com os nanosistemas. Sendo assim, os resultados indicam um potencial terapêutico com a possibilidade de desenvolvimento de uma nova formulação com alto valor agregado para as folhas de *V. ashei*, consideradas subprodutos do cultivo dos frutos.

Palavras-chave: Mirtilo. Composição fenólica. Estresse oxidativo. Depressão. Nanotecnologia.

ABSTRACT

Vaccinium ashei READE: FROM THE LEAVES PHYTOCHEMICAL ANALYSIS TO THE NANOPARTICLES DEVELOPMENT AND ANTIOXIDANT AND ANTIDEPRESSANT ACTIVITIES EVALUATIONS

AUTHOR: VERCIANE SCHNEIDER CEZAROTTO
ADVISOR: PROFA. DRA. LETÍCIA CRUZ

The *Vaccinium ashei* Reade, known as blueberry, production and commercialization has become each time more popular due to its nutritional and pharmacological properties related to its consumption. Despite having a commercial interest focus on the fruits, studies have demonstrated that the leaves present high contents of phenolic compounds. This way, the current work was firstly evaluated the influence of the harvest period and cultivar on phenolic compounds profile, as well as the *V. ashei* leaves antioxidant activity. The second stage focused on the antidepressant activity evaluation of the hydroalcoholic extract from the *V. ashei* leaves belonging to the Climax cultivar (December/2013 collection) (HEV1). Through the evaluation of acute administration (10, 25 and 50 mg/Kg, p.o.), the animals immobility time was evaluated using forced swimming test (FST) and tail suspension test (TST). Besides, the effect of the chronic administration (50 mg/Kg, p.o.) was evaluated applying the unpredictable and moderate chronic stress test (UMCS). Lastly, it was developed nanostructures using Eudragit RS100 by the nanoprecipitation method with *V. ashei* leaves hydroalcoholic belonging to the Climax cultivar (March/2014 collection) (EHV2). The nanoparticles (NPEHV) were characterized and evaluated regarding the antioxidant effects. Furthermore, the antidepressant activity (1, 2.5, 5, 10 and 25 mg/Kg, p.o.) and the monoaminergic neurotransmitters involvement were *in vivo* evaluated. The results showed that the qualitative profile of the *V. ashei* leaves presented similarity in its composition; however, the proportion of each phenolic compound, as well as the antioxidant activity was influenced by the cultivar and collection period. Besides, the EHV1 acute administration (50 mg/Kg, p.o.) significantly reduced the immobility time of the animals during the FST and TST, without altering the locomotor acitivity in the open field test. The EHV1 chronic treatment (50 mg/Kg, p.o.) was also able to reverse the depressive-like behavior and the immobility time during the FST, without changing the body weight, haematological parameters and concentration of hepatic and cerebral glycogen of the animals submitted to the stress regimen. These results were not observed for the EHV2 acute administration (50 mg/Kg, p.o.). Nevertheless, the EHV2 nanoencapsulation was able to promote the antioxidant effects and antidepressant activity, involving dopaminergic and noradrenergic neurotrasmitters. The NPHEV presented physicochemical characteristics compatible with drug delivery nanosystems. In conclusion, the results indicate the therapeutic potential and a new formulation for *V. ashei* leaves, considered byproducts of fruits cultivation.

Key words: Blueberry. Phenolic compounds. Oxidative stress.. Depression. Nanotecnology.

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CLAE-UV/DAD	Cromatografia líquida de Alta Eficiência com Detecção por Arranjo de Diodo
cv.	Cultivar
DPPH	2,2-difenil-1-picrilhidrazila
ECMI	Estresse Crônico Moderado e Imprevisível
EE	Eficiência de encapsulamento
EHV1	Extrato hidroalcoólico de *Vaccinium ashei* Reade (cv. clímax; coleta de dezembro/2013)
EHV2	Extrato hidroalcoólico de *Vaccinium ashei* Reade (cv. clímax; coleta de março/2014)
EROS	Espécies reativas do oxigênio
GABA	Ácido gabaaminobutírico
MAO	Monoaminoxidases
NPEHV	Nanopartículas contendo extrato hidroalcoólico de *Vaccinium ashei* Reade (cv. clímax; coleta de março/2014)
ORAC	Capacidade de Remoção do Radical Oxigênio
PACA	poli(cianoacrilatos de alquila)
PCL	poli-Ɛ-caprolactona
PLA	poli(ácido lático)
PLGA	poli(D,L-lactídeo-co-glicolídeo)
SNC	Sistema Nervosos Central
TNF	Teste de Natação Forçada
TSC	Teste de suspensão pela cauda
v.o.	Via oral

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1 INTRODUÇÃO

1 INTRODUÇÃO

Plantas medicinais representam uma fonte natural para a busca de novos produtos destinados ao tratamento de diversas doenças (ARMENDÁRIZ-BARRAGÁN et al., 2016). Por este motivo, o seu uso e os benefícios para a saúde humana tem despertado interesse científico ao longo dos anos (BONIFÁCIO et al., 2014; HAMINIUK et al., 2012; NILE; PARK, 2014).

Dentro destas plantas com interesse biológico, destaca-se o *Vaccinium ashei* Reade (*V. ashei*), planta frutífera da família das Ericaceae, também conhecida por mirtilo ou *blueberry* (GOLDMEYER et al., 2014; SOUSA; PEIXOTO; TOLEDO, 1995), reconhecida em todo o mundo devido ao alto teor de compostos bioativos e aos benefícios para a saúde decorrentes de seu consumo (FERLEMI; LAMARI, 2016; WU et al., 2017).

Estudos com os frutos demonstraram várias atividades biológicas, tais como antioxidante (CASTREJÓN et al., 2008; DULEBOHN et al., 2008; RODRIGUES et al., 2011; SELLAPPAN; AKOH; KREWER, 2002), neuroprotetora (GIACALONE et al., 2011; JOSEPH; SHUKITT-HALE; CASADESUS, 2005), anti-inflamatória (JOSEPH; EDIRISINGHE; BURTON-FREEMAN, 2014), antimicrobiana (CAILLET et al., 2012; LACOMBE et al., 2012), hipoglicemiante (GRACE et al., 2009) e antinociceptiva (RAMIREZ et al., 2010).

Contudo, apesar de o interesse comercial deste gênero estar direcionado unicamente para os frutos, as folhas têm demonstrado elevados teores de compostos fenólicos (LI et al., 2013), cuja presença tem sido relacionada às propriedades antioxidante (EHLENFELDT; PRIOR, 2001; PILJAC-ZEGARAC; BELSCAK; PILJAC, 2009), antileucêmica (SKUPIEŃ et al., 2006), anti-hipertensiva (SAKAIDA et al., 2007), hipolipidêmica (LI; LI; GENG, 2011; NAGAO et al., 2008; YUJI et al., 2013) e na prevenção da aterosclerose (BASU et al., 2010) e do câncer (MECHIKOVA et al., 2010).

Além disso, dentre estes compostos fenólicos, destaca-se a presença do ácido clorogênico e da rutina (MATSUO et al., 2010), os quais tem demonstrado potencial antidepressivo em modelos animais (DONATO et al., 2015; DU et al., 2014; GUAN; LIU, 2016; MA et al., 2015; SHEWALE; PATIL; HIRAY, 2012), atividade ainda não explorada para esta planta.

Contudo, para que as plantas medicinais apresentem eficácia terapêutica é necessário que os compostos bioativos estejam disponíveis para exercer suas funções biológicas (BONIFÁCIO et al., 2014) e uma simples extração nem sempre pode ser utilizada como produto final (ZORZI et al., 2015).

Os extratos preparados a partir de plantas medicinais caracterizam-se por ser uma mistura complexa e estudos mostram que o emprego terapêutico ainda é restrito em função desta complexidade na composição, além da toxicidade, os quais podem afetar a atividade biológica (ARMENDÁRIZ-BARRAGÁN et al., 2016).

Além disso, alguns destes compostos possuem baixa biodisponibilidade em função de sua solubilidade ou tamanho molecular inadequado, o que dificulta a absorção e/ou a passagem através das membranas biológicas após administração oral ou tópica. Há de se considerar ainda que, devido à baixa concentração de muitos compostos bioativos, elevadas concentrações do extrato são necessárias para a obtenção do efeito desejado (BONIFÁCIO et al., 2014; COIMBRA et al., 2011; GUPTA; PRASAD; AGGARWAL, 2010; KESARWANI; GUPTA, 2013; SHOJI; NAKASHIMA, 2004). Somado a isso, os extratos vegetais apresentam compostos com diferentes características no que se refere à estabilidade, como por exemplo, os compostos fenólicos (incluindo o ácido clorogênico, antocianinas e o ácido gálico) que são biologicamente instáveis e susceptíveis à degradação e à oxidação (HAN et al., 2015).

Neste sentido, o desenvolvimento de nanopartículas poliméricas contendo compostos de origem natural tem sido foco de muitas pesquisas mostrando-se uma estratégia promissora no tratamento de muitas doenças (AJAZUDDIN; SARAF, 2010; BITENCOURT et al., 2016, 2017a; BONIFÁCIO et al., 2014; RASHID et al., 2017; VINCEKOVIC et al., 2017).

Estes nanossistemas a base de polímeros biocompatíveis como o Eudragit® RS 100 são capazes de controlar a liberação dos constituintes ativos em concentração suficiente, durante todo o período de tratamento (BONIFÁCIO et al., 2014). Além disso, apresentam como principais vantagens o aumento da solubilidade e da biodisponibilidade, redução da toxicidade, aumento do índice terapêutico, melhoria da estabilidade e proteção frente à degradação química e física (AJAZUDDIN; SARAF, 2010; ARMENDÁRIZ-BARRAGÁN et al., 2016; BITENCOURT et al., 2016; BONIFÁCIO et al., 2014; COIMBRA et al., 2011; HAN et al., 2015; KESARWANI; GUPTA, 2013; KHOEE; YAGHOOBIAN, 2009; VINCEKOVIC et al., 2017), em comparação com as preparações livres (BRIGGER; DUBERNET; COUVREÛ, 2002).

Considerando as dificuldades que os extratos vegetais podem apresentar relacionadas à instabilidade e biodisponibilidade e as potencialidades das nanopartículas poliméricas como efetivos sistemas de liberação, a encapsulação de extratos vegetais torna-se uma alternativa promissora para a obtenção de um medicamento final que possa assegurar a qualidade, segurança e eficácia terapêutica (ZORZI et al., 2015).

Cabe ressaltar que, até o momento, não há relatos na literatura sobre a preparação de nanoparticulas poliméricas contendo o extrato de *V. ashei*, assim como também não foram encontrados registros na literatura sobre a avaliação da atividade antidepressiva dos extratos isolados e associados à nanopartículas poliméricas, destacando o caráter inédito deste trabalho.

Sendo assim, o objetivo deste trabalho foi realizar análise fitoquímica dos extratos de folhas de *V. ashei*, bem como preparar e caracterizar nanopartículas poliméricas de Eudragit® RS100 contendo o extrato hidroalcoólico das mesmas. Em seguida, as atividades antioxidante e antidepressiva do extrato isolado e associado a nanopartículas poliméricas de Eudragit® RS100 foram avaliadas.

2 OBJETIVOS

2 OBJETIVOS

2.1 OBJETIVO GERAL

Realizar análise fitoquímica em extratos de folhas de *V. ashei* e avaliar as atividades antioxidante e antidepressiva do extrato isolado e associado a nanopartículas poliméricas de Eudragit® RS100.

2.2 OBJETIVOS ESPECÍFICOS

- Quantificar os compostos fenólicos totais e flavonoides totais nos extratos das folhas de diferentes cultivares de *V. ashei* coletadas em meses diferentes;

- Determinar o perfil de compostos fenólicos por CLAE-UV-DAD (Cromatografia Líquida de Alta Eficiência com Detecção por Arranjo de Diodo) nos extratos das folhas de diferentes cultivares *V. ashei* coletadas em meses diferentes;

- Preparar nanopartículas poliméricas de Eudragit® RS100 contendo extratos das folhas de *V. ashei*;

- Caracterizar as nanopartículas poliméricas quanto ao teor de metabólitos secundários, eficiência de encapsulamento, diâmetro médio de partícula, índice de polidispersão, potencial zeta e pH;

- Avaliar a atividade antioxidante dos extratos de diferentes cultivares de *V. ashei* coletadas em meses diferentes;

- Investigar a atividade antidepressiva do extrato das folhas de *V. ashei*;

- Avaliar a atividade antioxidante e antidepressiva das nanopartículas poliméricas contendo o extrato das folhas de *V. ashei*.

3 REVISÃO DA LITERATURA

3 REVISÃO DA LITERATURA

3.1 PLANTAS MEDICINAIS

Ao longo dos tempos, os seres humanos sempre confiaram na Natureza para atender às suas necessidades básicas, inclusive na busca por medicamentos no tratamento de diversas doenças. As plantas medicinais, em particular, formaram a base da medicina tradicional, com os primeiros registros que datam de 2600 aC, documentando o uso de aproximadamente 1000 substâncias derivadas de plantas da Mesopotâmia, incluindo óleos de *Cedrus* (Cedro) e *Cupressus sempevirens* (cipreste), *Glycyrrhiza glabra* (alcaçuz), espécies de *Commiphora* (mirra) e *Papaver somniferum* (papoula), os quais são empregadas ainda hoje (CRAGG; NEWMAN, 2013).

No entanto, a busca dos componentes ativos presentes nas plantas medicinais só começou no século XIX, levando à concepção dos primeiros fármacos com as características atuais. Friedrich Serturner, em 1806, foi pioneiro quando isolou a alcaloide morfina da papoula: um evento que provocou uma busca contínua de outros medicamentos derivados de plantas. Em 1824, Pierre-Jean isolou a codeína, um agente antitussivo e, em 1848, George Merck Fraz a papaverina, um alcaloide anti-espasmódico, ambos isolados da mesma planta. Outros exemplos importantes de fármacos isolados de plantas medicinais incluem a atropina isolada de *Atropa belladonna* por Meinin 1831; a cafeína obtida por Runge em 1820 a partir de *Coffea arábica*; a digoxina isolada por Claude-Adolphe Nativelle em 1869 da *Digitalis lanata*; e o curare isolado por Win-stersteiner e Dutcher em 1943 de *Chondrodendron tomentosum* (DUTRA et al., 2016).

Contudo, o marco histórico no desenvolvimento global da indústria farmacêutica foi a descoberta de salicina (analgésica e antipirética) a partir da *Salix alba* por Rafaele Piria, em 1832. Em 1839, foi realizada a primeira modificação estrutural da salicina, produzindo ácido alicílico para o tratamento de artrites reumatóides. Do ácido salicílico, Felix Hoffman sintetizou a aspirina (ácido acetilsalicílico), em 1897. Assim, nasceu a famosa indústria farmacêutica da Bayer na Alemanha, bem como a primeiro patente na área dos fármacos (DUTRA et al., 2016).

Atualmente, estima-se que cerca de 30% dos medicamentos terapêuticos disponíveis são derivados de fontes naturais, principalmente de plantas e microorganismos. Em algumas áreas terapêuticas, por exemplo, na oncologia, a quantidade de medicamentos derivados de plantas atinge 60% (CALIXTO, 2000; CRAGG; NEWMAN, 2013; NEWMAN; CRAGG, 2012).

Desta forma, as plantas medicinais têm representado historicamente uma fonte rica para a descoberta de fármacos bem-sucedidos e, ainda hoje tem um importante papel na identificação

de novos marcadores farmacológicos apesar de seus conhecidos desafios, uma vez que produzem inúmeros metabólitos secundários altamente especializados para exercer funções biológicas e que ainda, estão longe de serem investigados exaustivamente (ATANASOV et al., 2015).

Neste contexto, destaca-se o *Vaccinium ashei* Reade, planta frutífera que tem despertado interesse científico devido aos benefícios para a saúde decorrentes de seu consumo (FERLEMI; LAMARI, 2016; WU et al., 2017), além das propriedades sensoriais e ao alto valor nutricional (CHU et al., 2017; WU et al., 2017).

3.2 *Vaccinium ashei* READE

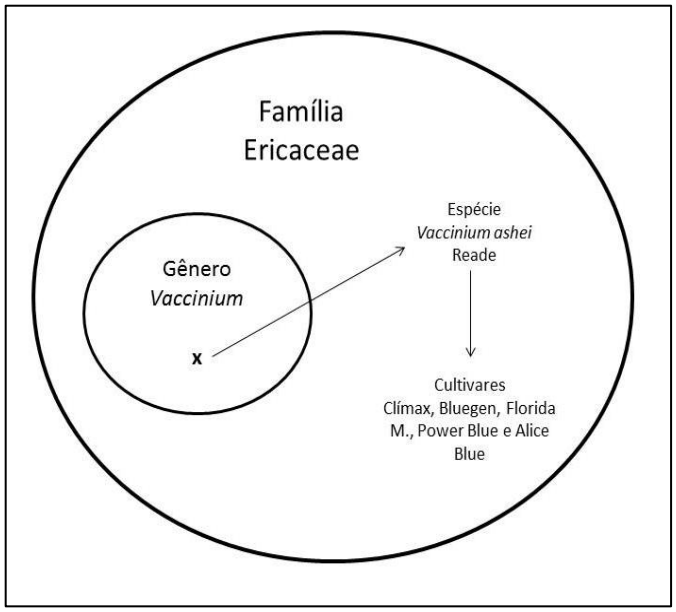
O *Vaccinium ashei* Reade, pertence à família das Ericaceae e ao gênero *Vaccinium* spp. e inclui-se no grupo de pequenas frutas como a amora (*Rubus fruticosus* sp.), morango (*Fragaria vesca* L.) e framboesa (*Rubus idaeus* L.) (FACHINELLO, 2008; SOUSA; PEIXOTO; TOLEDO, 1995). A relação taxonômica entre família, gênero, espécie e cultivares, está demonstrado na Figura 1 para um melhor entendimento.

O gênero *Vaccinium* spp. é amplamente cultivado ao redor do mundo e inclui cerca de 450 espécies, sendo que as principais espécies com expressão comercial são divididas em três grupos rabbiteye (*V. ashei*), highbush (*V. corymbosum*) e lowbush (*V. angustifolium*) classificados de acordo com o genótipo, crescimento e tipo de fruto produzido (CHU et al., 2017; FACHINELLO, 2008).

O grupo highbush (espécie *V. corymbosum* L.) apresenta arbustos altos e adapta-se geralmente em locais com maior altitude. A necessidade de frio hibernal (abaixo de 7,2 ºC) destas plantas está geralmente entre 200 a 850 horas (PAGOT, 2006; RIEGER, 2006).

O grupo lowbush (espécie *V. angustifolia*) apresenta arbustos de pequeno porte (menos de meio metro de altura), necessita de mais frio para seu melhor desenvolvimento, tempo superior a mil horas de frio por ano e produz frutos muito macios, de tamanho pequeno e baixa acidez (PAGOT, 2006; RIEGER, 2006).

Figura 1 – Relação taxonômica entre cultivar, espécie, gênero e família



Fonte: Adaptado de Booth et al. (2012).

As plantas do grupo rabbiteye (espécie *V. ashei*), utilizadas neste estudo, são plantas que podem atingir até 10 metros de altura e adaptam-se às regiões pouco frias. São consideradas pelos geneticistas como as que oferecem as maiores possibilidades para o melhoramento, pois são tolerantes a uma variação maior de pH do solo e a altas temperaturas. Além disso, apresentam certa resistência à seca e baixa necessidade de frio (PAGOT, 2006; RIEGER, 2006). Para a maior parte das regiões de clima frio do Sul do Brasil, a espécie *V. ashei* é a mais promissora (FACHINELLO, 2008).

Dentro destes grupos existe ainda um grande número de cultivares (cv.), as quais resultaram de aprimoramentos genéticos com a finalidade de obter frutos de melhor qualidade comercial (características físicas, químicas e sensoriais) (RASEIRA; ANTUNES, 2004).

Os frutos de *V. ashei* Reade (Figura 2), apresentam-se de cor azul-escura, de formato achatado, recobertos de cera, com sabor doce-ácido e muitas sementes de pequeno tamanho. O tamanho varia de 1 a 2,5 cm de diâmetro e 1,5 a 4 g de peso, dependendo da cultivar (FACHINELLO, 2008).

Estudos com os frutos deste gênero têm demonstrado capacidade de capturar radicais livres (CASTREJÓN et al., 2008; DULEBOHN et al., 2008; RODRIGUES et al., 2011; SELLAPPAN; AKOH; KREWER, 2002), atividade neuroprotetora (GIACALONE et al., 2011; JOSEPH; SHUKITT-HALE; CASADESUS, 2005), anti-inflamatória (JOSEPH; EDIRISINGHE; BURTON-FREEMAN, 2014), antimicrobiana (CAILLET et al., 2012;

LACOMBE et al., 2012), antinociceptiva (RAMIREZ et al., 2010), hipoglicemiante (GRACE et al., 2009) e antidiabéticas através da proteção das células β pancreáticas do estresse oxidativo induzido pela glicose e melhora da sensibilidade à insulina (DEFURIA et al., 2009; JOHNSON et al., 2016; SÁNCHEZ-VILLAVICENCIO et al., 2017). Além disso, o consumo dos frutos foi relacionado à proteção óssea (SHEN et al., 2012), melhora de desordens oftálmicas (CALÒ; MARABINI, 2014), infecções no trato urinário (JEPSON; CRAIG, 2007), redução dos fatores de riscos associados às doenças cardiovasculares (BASU et al., 2010; DE PASCUAL-TERESA; MORENO; GARCÍA-VIGUERA, 2010; JOHNSON et al., 2016; STULL et al., 2010) e câncer (NETO, 2007; SEERAM, 2008).

Mais recentemente (SHI et al., 2017) relatou o emprego dos frutos para o tratamento da obesidade e suas comorbidades relacionadas, como o Diabetes tipo 2 e inflamações crônicas através da redução do estresse oxidativo, regulação do metabolismo da glicose, melhora no perfil lipídico e redução nos níveis de citocinas inflamatórias em modelos animais e ensaios clínicos preliminares.

Figura 2 – Frutos de *Vaccinium ashei* Reade

Fonte: Da autora.

Tais bioatividades têm sido associadas principalmente aos compostos fenólicos, tais como antocianinas, ácidos fenólicos e proantocianidinas presentes nos frutos (DE PASCUAL-TERESA; MORENO; GARCÍA-VIGUERA, 2010; GU et al., 2002; OU; HAMPSCH-WOODILL; PRIOR, 2001; PRIOR et al., 2001; WU et al., 2017). Além destes compostos, foram identificados uma variedade de vitaminas (A, B, C, K, ácido fólico), minerais (potássio,

magnésio, cálcio, fósforo, ferro, manganês), açúcares, pectina e os ácidos cítrico, málico e tartárico (SILVEIRA; VARGAS; ROSA, 2007; SPAGOLLA et al., 2009).

Dentre estas atividades a capacidade antioxidante tornou-se uma característica de qualidade desejável e essencial dos frutos do gênero *Vaccinium* sp. e um preditor para seleção de cultivares (WU et al., 2017).

As folhas de *V. ashei* Reade (Figura 3) formam-se nos nós dos ramos, com inserção alternada, variando na forma elipse estreita ou ovaladas. Podem atingir 75 mm e podem apresentar pelos na página inferior. O número de folhas por ramo (de 10 a 30) depende das cultivares e do vigor do ramo em que se formaram (FONSECA; OLIVEIRA, 2007).

As folhas, consideradas subprodutos do cultivo dos frutos (DENG et al., 2014; FERLEMI; LAMARI, 2016; ZHU et al., 2013), tem sido estudadas desde 1927 devido à ampla utilização em preparações medicinais para pacientes diabéticos (ALLEN, 1927). Um estudo realizado por (WATSON, 1928) demonstrou que a ingestão de 0,3 g/dia de folhas na forma de infusão, durante 3 meses, exerceu efeitos benéficos em 16 voluntários com *Diabetes mellitus*.

Figura 3 – Folhas de *Vaccinium ashei* Reade

Fonte: Da autora.

Contudo, atualmente o uso tradicional frente a várias doenças, como resfriado comum, inflamação, diabetes e disfunção ocular parece estar esquecido. No entanto, o interesse científico em relação à composição química e às propriedades farmacológicas das folhas deste gênero têm crescido nos últimos anos, considerando-as como uma fonte alternativa de compostos bioativos (FERLEMI; LAMARI, 2016).

Dentre os principais compostos identificados nas folhas pode-se destacar a presença de ácidos fenólicos e ésteres, flavonóis, antocianinas e procianidinas (FERLEMI; LAMARI, 2016; MATSUO et al., 2010; NACZK et al., 2006), os quais têm sido relacionados às atividades antioxidante (EHLENFELDT; PRIOR, 2001; PERVIN; HASNAT; LIM, 2013; PILJAC-ZEGARAC; BELSCAK; PILJAC, 2009), antileucêmica (SKUPIEŃ et al., 2006), anti-hipertensiva (SAKAIDA et al., 2007), hipolipidêmica (LI; LI; GENG, 2011; NAGAO et al., 2008; YUJI et al., 2013), antimicrobiana (PERVIN; HASNAT; LIM, 2013), atividade protetora frente à catarata ocular induzida por selenite e dano oxidativo induzido por selenite no cérebro e fígado de ratos neonatais (FERLEMI et al., 2015, 2016) e na prevenção do câncer (MECHIKOVA et al., 2010).

As folhas do gênero *Vaccinium* spp. tem demonstrado atividade hipoglicemiante em ratos diabéticos, bem como em humanos com diabetes tipo II (CIGNARELLA et al., 1996; MARTINEAU et al., 2006). Além disso, as proantocianidinas com um grau de polimerização de 8 a 9 a partir de folhas de *Vaccinium* spp. demostraram potencial para suprimir a expressão de RNA subgenômico do vírus da hepatite C (TAKESHITA et al., 2009).

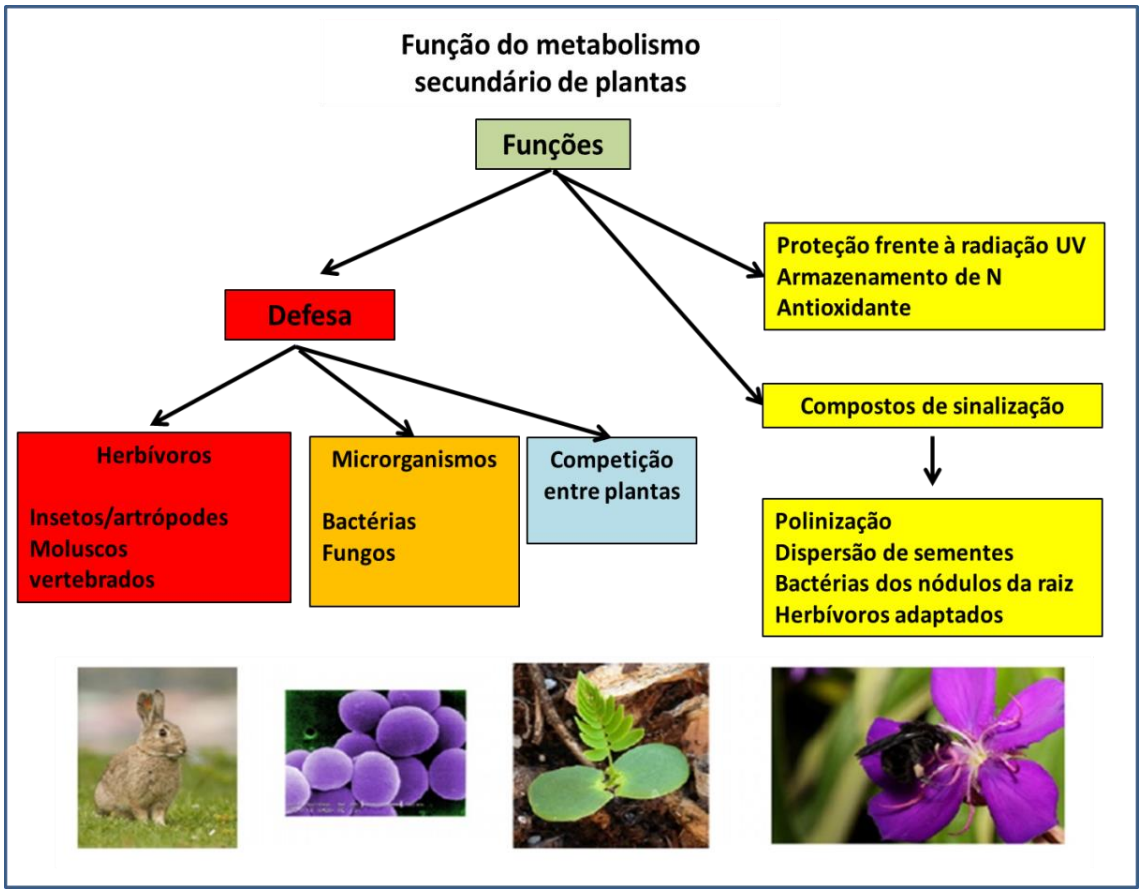
Em folhas de *V. ashei* Reade foram identificadas a presença de flavan-3-ols e proantocianidinas como compostos majoritários, além da presença de glicosídeos de flavonols e ácido clorogênico (MATSUO et al., 2010), o qual tem demonstrado propriedades antioxidantes, anti-inflamatórias, cardioprotetoras e neuroprotetoras (FERLEMI; LAMARI, 2016).

3.3 FATORES QUE INFLUENCIAM O METABOLISMO VEGETAL

O metabolismo vegetal pode ser dividido em metabolismo basal e metabolismo especializado. O primeiro refere-se à produção de metabólitos de distribuição ubiquitária e inclui todos os compostos essenciais para o crescimento e desenvolvimento das plantas, isto é, os metabólitos primários. O metabolismo especializado, por sua vez, inclui todos os compostos que envolvem as inter-relações com o meio ambiente (metabólitos secundários) (SIMÕES et al., 2017).

Os metabólitos secundários de plantas, embora não tenham nenhum efeito imediato na sobrevivência das plantas, permitem que as mesmas se comuniquem e reajam para superar ameaças iminentes do meio (VERMA; SHUKLA, 2015). Neste sentido, desempenham diversas funções na planta (Figura 4), tais como proteção frente à radiação ultravioleta e defesa frente à herbivoria e microrganismos. Além disso, podem agir como compostos de sinalização, atraindo insetos para a polinização e dispersão de sementes (WINK, 2016).

Figura 4 – Funções do metabolismo secundário de plantas



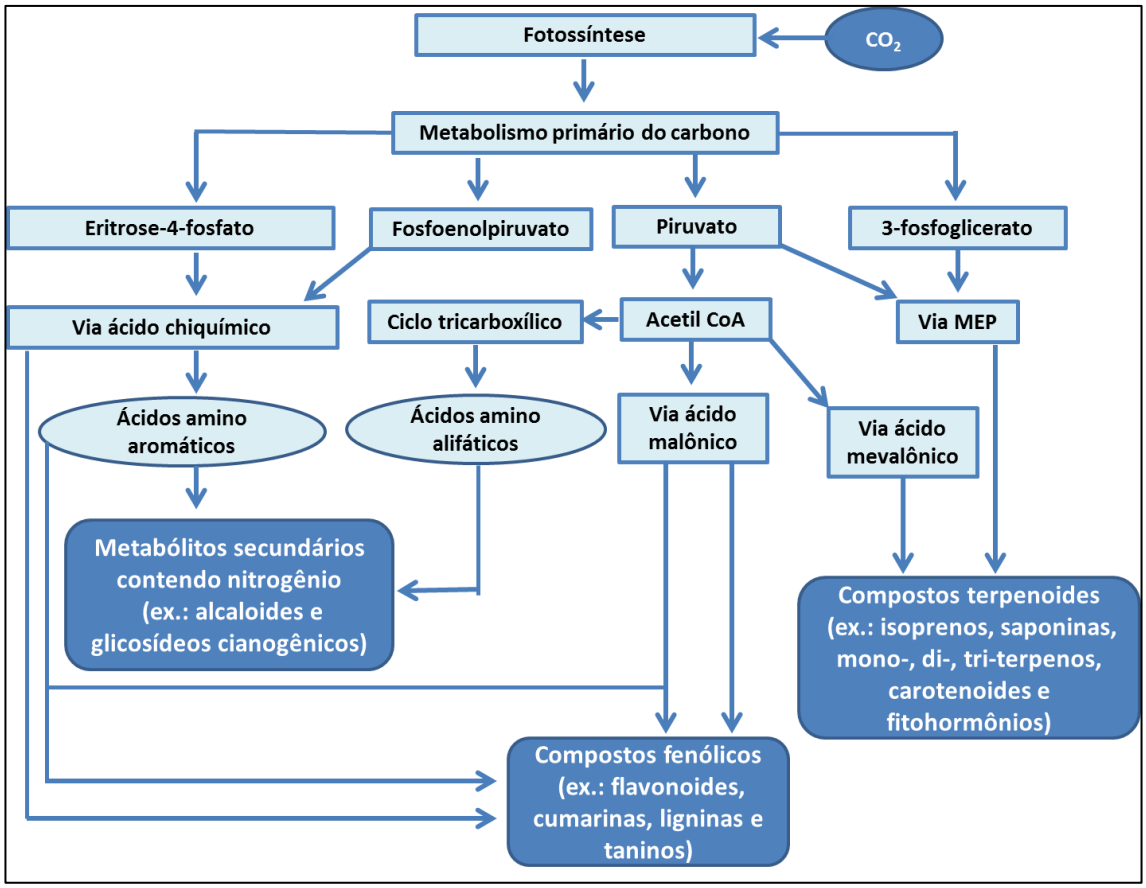
Fonte: Adaptado de Wink (2016). UV: ultravioleta; N: nitrogênio.

Com base na via biossintética, podem ser classificados em três grupos principais: terpenos (ou isoprenóides), compostos fenólicos (fenilpropanoides e flavonoides) e compostos que contém nitrogênio (alcaloides, glucosinolatos e glicosídeos cianogênicos) (Figura 5) (VERMA; SHUKLA, 2015).

Até o presente momento, mais de 200.000 estruturas químicas diferentes são conhecidas (WINK, 2016), dentre as quais muitas são amplamente reconhecidas por seus efeitos nutracêuticos e benéficos para a saúde (TIWARI; CUMMINS, 2013).

Neste sentido, considerando que os metabólitos secundários representam a interface da planta com o meio ambiente, a síntese e regulação destes compostos é frequentemente influenciada por diversos fatores, os quais provocam variações no conteúdo total e/ou nas proporções relativas dos mesmos (GOBBO-NETO; LOPES, 2007).

Figura 5 – Rota biossintética das plantas



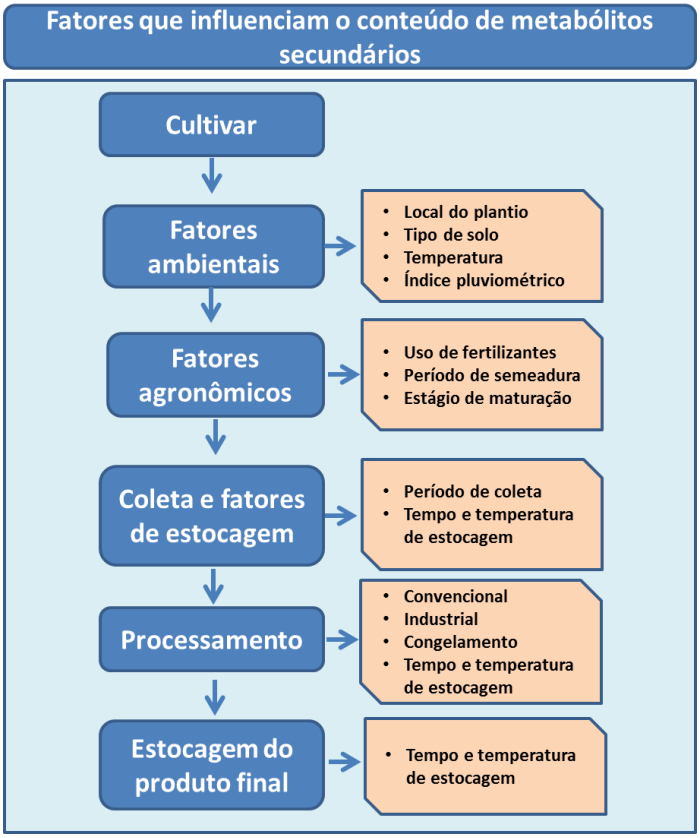
Fonte: Adaptado de Verma; Shukla (2015). MEP: metileritritolfosfato.

Dentre uma ampla variedade de fatores que regulam a biogênese dos metabólitos secundários de plantas, pode-se destacar os fatores genéticos, ontogenéticos (isto é, a sequência de eventos envolvidos no desenvolvimento) e morfogenéticos (presença de tecidos especializados), além dos efeitos bióticos e abióticos (VERMA; SHUKLA, 2015).

Os efeitos abióticos incluem todos os efeitos físicos que regem o habitat, como a disponibilidade hídrica, radiação solar, temperatura, variação sazonal, composição do solo (salinidade, emprego de fertilizantes, nutrientes, hormônios do crescimento) e local do plantio. Os efeitos bióticos incluem as interações da planta com microorganismos como fungos, vírus, bactérias, nematoides, que causam estresse na planta (PAVARINI et al., 2012; VERMA; SHUKLA, 2015).

Além destes fatores, de acordo com Tiwari; Cummins (2013), o conteúdo destes compostos também pode variar de acordo com as condições da pré e pós-coleta, como estágio da maturação e condições de processamentos e estocagem (Figura 6).

Figura 6 – Fatores que influencia o conteúdo de metabólitos secundários



Fonte: Adaptado de Tiwari; Cummins (2013).

Esta variação pode ser vista em diferentes classes de metabólitos secundários e diferentes atividades farmacológicas (SONI; BRAR; GAUTTAM, 2015).

Sendo assim, considerando que as plantas demostram sazonalidade na produção de metabólitos secundários e a variabilidade resultante pode influenciar na bioatividade das mesmas (SCOGNAMIGLIO et al., 2014), conhecer os fatores que induzem a estas variações parece ser um dos pontos cruciais durante o desenvolvimento de produtos à base de plantas (MICHEL et al., 2017; PAVARINI et al., 2012; SCOGNAMIGLIO et al., 2014).

Parece claro que, definir, por exemplo, condições e períodos de cultivo e/ou coleta que assegure atividade farmacológica, perfil fitoquímico e rendimento dos materiais vegetais a níveis desejáveis permite, inclusive traçar estratégias alternativas para aumentar a produtividade destes compostos a partir de plantas (MICHEL et al., 2017).

3.4 NANOPARTÍCULAS POLIMÉRICAS

A investigação de sistemas nanoestruturados para o tratamento, diagnóstico e prevenção de doenças tem sido foco de várias pesquisas nos últimos anos, especialmente na última década, fornecendo oportunidades promissoras no campo da pesquisa de doenças antes consideradas intratáveis (LIU; FENG, 2015).

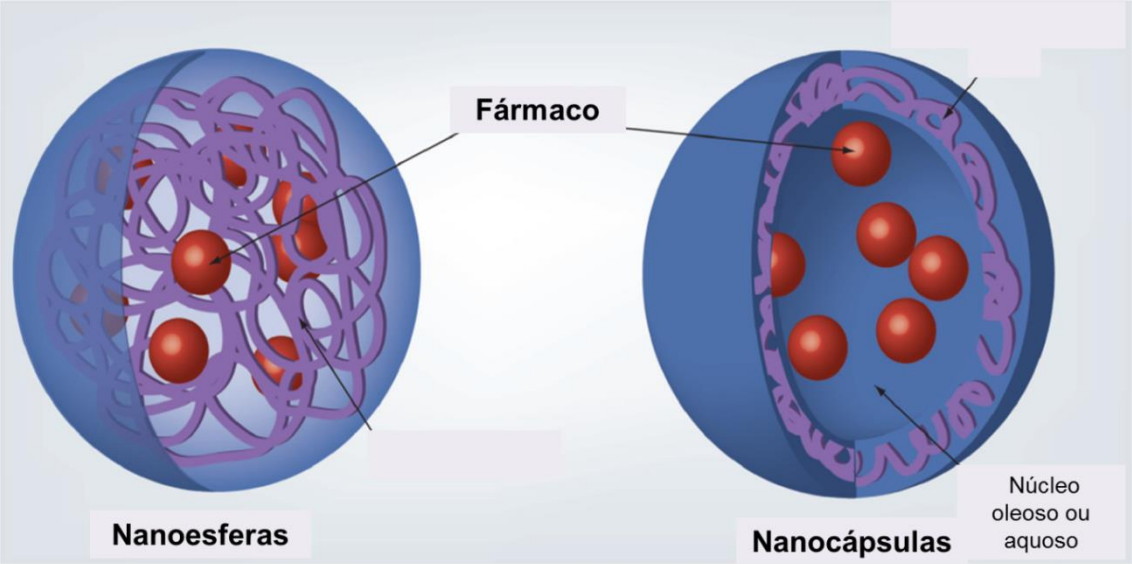
Tais sistemas têm sido utilizados na administração, liberação e vetorização de fármacos em sítios específicos de ação, devido a sua principal vantagem, o aumento do índice terapêutico (GARCIA-ORUE et al., 2017; MILOVANOVIC et al., 2017).

Dentre os principais nanocarreadores pode-se destacar os lipossomas, as ciclodextrinas, as nanopartículas lipídicas e as nanopartículas poliméricas, os quais apresentam diferentes características entre si (SOUZA, 2012).

As nanopartículas poliméricas, cujo termo inclui as nanocápsulas e as nanoesferas (Figura 7), são sistemas carreadores que apresentam diâmetro inferior a 1 µm, embora a faixa geralmente obtida encontre-se entre 100-500 nm (QUINTANAR-GUERRERO et al., 1998; SCHAFFAZICK et al., 2003; VAUTHIER; BOUCHEMAL, 2009).

As nanocápsulas caracterizam-se por serem sistemas vesiculares constituídos por um invólucro polimérico disposto ao redor de um núcleo oleoso ou aquoso, em que o fármaco está confinado na forma líquida, sólida ou como uma dispersão molecular (ANTON; BENOIT; SAULNIER, 2008; LETCHFORD; BURT, 2007; MORA-HUERTAS; FESSI; ELAISSARI, 2010; QUINTANAR-GUERRERO et al., 1998; RADTCHENKO; SUKHORUKOV; MÖHWALD, 2002). As nanoesferas, por sua vez, são formadas por uma matriz polimérica em que o fármaco fica retido ou adsorvido (MORA-HUERTAS; FESSI; ELAISSARI, 2010; SCHAFFAZICK et al., 2003).

Figura 7 – Representação esquemática de nanocápsulas e nanoesferas poliméricas



Fonte: Adaptado de Bei; Meng; Youan (2010).

Estes sistemas têm sido desenvolvidos visando inúmeras aplicações terapêuticas, como liberação de fármacos no sítio de ação específico e/ou liberação controlada, visando o aumento do índice terapêutico e diminuição dos efeitos adversos. Além disso, são capazes de proteger moléculas instáveis frente à hidrólise enzimática, decomposição química, imunológica e fotodecomposição, sendo planejados, principalmente, para administração parenteral, oral ou oftálmica (ALONSO, 2004; CRUZ et al., 2006; JAAKOLA et al., 2004; OURIQUE et al., 2008; SANTOS et al., 2014; SCHAFFAZICK et al., 2002; SOPPIMATH et al., 2001).

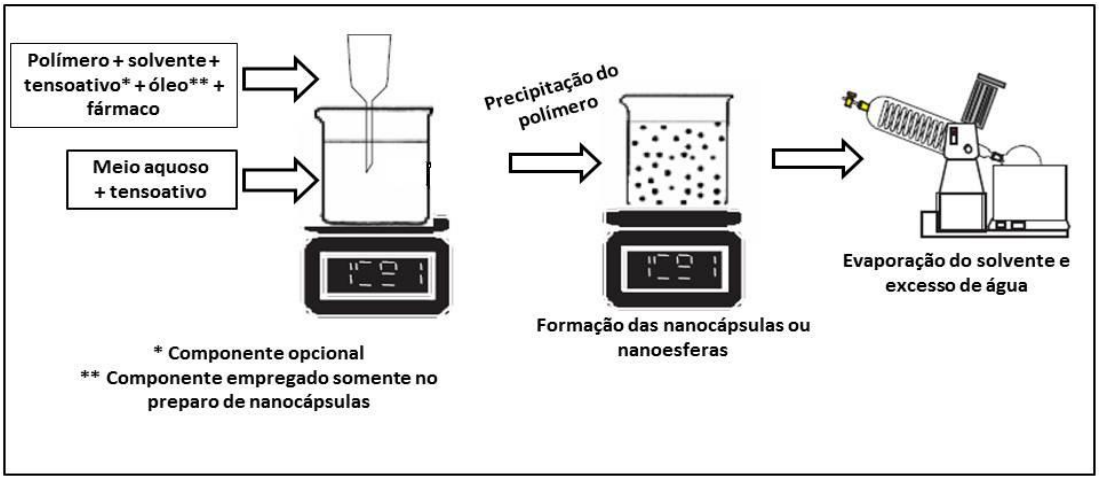
Dependendo das características físico-químicas do fármaco é possível escolher o método para alcançar sua eficiente associação ao nanocarreador. Tais métodos podem ser, de uma forma geral, classificados em métodos baseados na polimerização *in situ* de monômeros dispersos ou na precipitação de polímeros pré-formados (GUTERRES; SCHAFFAZICK; POHLMANN, 2007).

Nanopartículas poliméricas produzidas a partir de polímeros pré-formados têm sido amplamente utilizadas para incorporar, principalmente, princípios ativos lipofílicos por ser uma abordagem simples, rápida, econômica, facilmente controlável, reprodutível, de maior rendimento e que faz emprego de solventes não tóxicos (CHORNY et al., 2002; FESSI et al., 1989; LEGRAND et al., 2007).

Estes métodos podem ser realizados por emulsificação-evaporação do solvente, por deslocamento do solvente, por *salting-out* ou por emulsificação-difusão do solvente (QUINTANAR-GUERRERO et al., 1998; SOUTO; SEVERINO; SANTANA, 2012).

O método de preparação de nanopartículas por deslocamento do solvente, também denominado de nanoprecipitação, utilizado neste trabalho, consiste na precipitação ou deposição interfacial de um polímero pré-formado (QUINTANAR-GUERRERO et al., 1998; SOUTO; SEVERINO; SANTANA, 2012). De maneira geral, esta técnica requer um solvente orgânico miscível com a água (acetona ou etanol), contendo o fármaco a encapsular, um polímero e um tensoativo opcional (de baixa hidrofilia), o qual é vertido sobre uma fase aquosa, adicionada de um tensoativo (de alta hidrofilia), sob moderada agitação (Figura 8) (GUTERRES; SCHAFFAZICK; POHLMANN, 2007).

Figura 8 – Método de preparação de nanopartículas poliméricas por deslocamento do solvente



Fonte: Adaptado de Schaffazick et al. (2003).

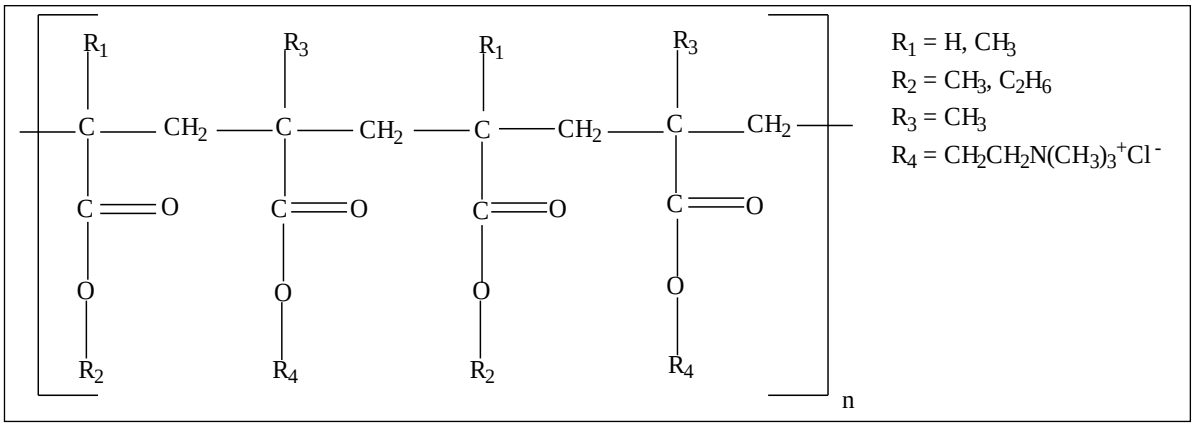
A nanoprecipitação ocorre pela rápida dessolvatação do polímero, uma vez que o mesmo precipita, envolvendo e aprisionando a substância ativa. O mecanismo de formação das nanoesferas, denominado de Efeito Marangoni, é devido a turbulências interfaciais geradas durante o deslocamento do solvente, resultante de fenômenos complexos e cumulativos como fluxo, difusão e variação na tensão superficial (BILATI; ALLÉMANN; DOELKER, 2005; QUINTANAR-GUERRERO et al., 1998).

Os polímeros normalmente usados por esta técnica são poliésteres biodegradáveis como a poli-Ɛ-caprolactona (PCL), poli(ácido lático) (PLA), poli(D,L-lactídeo-co-glicolídeo) (PLGA) e poli(cianoacrilatos de alquila) (PACA). Além destes, podem ser empregados os poli(metacrilatos), conhecidos comercialmente como Eudragits® (MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Eudragit® compreende uma série de co-polímeros biocompatíveis derivados do ácido metacrílico com metacrilato de metila, acrilato de etila, metacrilato de butila, cloridrato de metacrilato de trimetilamônio ou metacrilato de dimetilamino de etila, que exibem diversas funções como melhora da aparência e caracteristicas organolépticas, estabilizadora, protetora e moduladora da liberação (entérica e/ou sustentada) e podem ser empregados em formulações destinadas às vias oral, bucal, tópica, vaginal e retal (VILLANOVA; ORÉFICE; CUNHA, 2010).

O Eudragit® RS100 (Figura 9), de interesse neste estudo, é muito utilizado no desenvolvimento de formas de dosagem de liberação controlada e sustentada (DILLEN et al., 2006), com a finalidade de melhorar a permeabilidade intestinal (LOVEYMI et al., 2012a) e as características físico-químicas do fármaco (ADIBKIA et al., 2011).

Figura 9 – Estrutura molecular do Eudragit® RS100



Fonte: Adaptado de Domingues; Guterres (2008).

O Eudragit® RS100 contém grupamentos de amônio quaternário na faixa de 4,5 a 6,8% (JANA et al., 2014; PIGNATELLO et al., 2002, 2006), os quais fornecem carga positiva à superfície do polímero, o que permite uma interação com fármacos carregados negativamente ou superfícies celulares dos tecidos-alvo, aumentando a absorção celular do complexo fármaco-polímero (ADIBKIA et al., 2011; BODMEIER; CHEN, 1989; DILLEN et al., 2006; LOVEYMI et al., 2012a).

Além disso, o Eudragit® RS100 é insolúvel em pH fisiológico e apresenta capacidade de intumescimento, os quais proporcionam um excelente material para a dispersão de substâncias ativas (JANA et al., 2014; PIGNATELLO et al., 2002, 2006).

A literatura cita o emprego de Eudragit® RS100 em associação com fármacos anti-hipertensivos (JANA et al., 2014), antimicrobianos (DILLEN et al., 2006; LOVEYMI et al.,

2012b) e anti-inflamatórios (ADIBKIA et al., 2011; BODMEIER; CHEN, 1989; PIGNATELLO et al., 2002). Não foram encontrados relatos do emprego de Eudragit® RS100 para nanoencapsulação de extratos vegetais.

3.4.1 Extratos vegetais em nanoparticulas poliméricas

O uso de plantas medicinais tem crescido muito em todo o mundo devido aos seus efeitos terapêuticos pronunciados e baixos efeitos adversos quando comparados com a medicina moderna (KESARWANI; GUPTA, 2013).

No campo da pesquisa, tem-se observado uma mudança no interesse de compostos sintéticos, de elevada afinidade, potência e seletividade, por compostos naturais (ou de base natural), de baixa afinidade e potência intermediária que podem atuar em múltiplos alvos (HARVEY et al., 2010).

Antigamente, o farmacóforo, molécula responsável pela atividade farmacológica, era isolado e modificado quimicamente com a finalidade de aumentar sua potência e especificidade. Atualmente, o interesse está justamente direcionado para o amplo espectro de atividades do extrato bruto (BALUNAS; KINGHORN, 2005; COIMBRA et al., 2011; HARVEY et al., 2010). Uma vez que, do ponto de vista farmacológico, vários compostos podem contribuir para a ação do extrato e tratamentos como isolamento e/ou purificação podem promover a perda parcial ou total da atividade biológica devido à remoção de substâncias quimicamente relacionadas que contribuem para a atividade dos componentes principais (BHATTACHARYA; GHOSH, 2008; KESARWANI; GUPTA, 2013).

Além disso, tem-se considerado o sinergismo existente entre os compostos, a perda de atividade após o isolamento, a instabilidade química e dificuldade observadas na purificação dos compostos (GERTSCH, 2011; KESARWANI; GUPTA, 2013; PFERSCHY-WENZIG; BAUER, 2015; ZORZI et al., 2015).

Contudo, estudos envolvendo extratos de plantas têm demonstrado resultados *in vivo* insuficientes, mesmo após a observação de ensaios satisfatórios *in vitro* (KESARWANI; GUPTA, 2013). Tais resultados podem ser atribuídos ao fato de que a maioria dos compostos naturais tem estabilidade limitada como, por exemplo, os compostos fenólicos que são susceptíveis à oxidação (COIMBRA et al., 2011; HAN et al., 2015; LIU; FENG, 2015). Além disso, podem apresentar baixa biodisponibilidade devido à solubilidade ou permeabilidade celular inadequada, rápida eliminação ou extensiva metabolização (COIMBRA et al., 2011; LIU; FENG, 2015; ZORZI et al., 2015). Por fim, devido muitas vezes à baixa concentração de alguns compostos bioativos, elevadas concentrações do extrato são necessárias para obter o

efeito terapêutico desejado (BONIFÁCIO et al., 2014; COIMBRA et al., 2011; GUPTA; PRASAD; AGGARWAL, 2010; KESARWANI; GUPTA, 2013; SHOJI; NAKASHIMA, 2004).

Neste sentido, acredita-se que a encapsulação de fitocompostos em nanossistemas, como nanopartículas poliméricas, lipossomas, fitossomas, nanoemulsões, microesferas, transferossomas e etossomas, seja capaz de superar estas desvantagens, protegendo e mantendo estes compostos estáveis ou ainda, melhorando sua biodisponibilidade (AJAZUDDIN; SARAF, 2010; CAMPOS et al., 2015; KESARWANI; GUPTA, 2013; LIU; FENG, 2015).

Dentro destes sistemas, o emprego de nanopartículas poliméricas tem se mostrado promissor (AJAZUDDIN; SARAF, 2010; BITENCOURT et al., 2016; BONIFÁCIO et al., 2014), como pode ser observado na Tabela 1. Estes nanossistemas a base de polímeros biodegradáveis e biocompatíveis são capazes de controlar a liberação dos constituintes ativos direcionando-os ao local de ação desejado (BONIFÁCIO et al., 2014).

Tabela 1 – Exemplos representativos de extratos de plantas associados à nanopartículas poliméricas

Planta	Extrato	Atividade avaliada	Sistema	Referencias
Syzygium cumini	Extrato aquoso das sementes	Atividade antioxidante, antifúngica e no tratamento do Diabetes *mellitus*	Nanopartículas de PCL, Span® 80 e Tween® 80	(BITENCOURT et al., 2016, 2017a, 2017b)
Momordica charantia	Extrato aquoso dos frutos	Atividade antimicrobiana	Nanopartículas de prata	(RASHID et al., 2017)
Ziziphus jujuba	Extrato hidroalcoólico de frutos (40-100%)	Atividade antioxidante	Nanopartículas de quitosana	(HAN et al., 2015)
Achyroclines satureoides	Extrato hidroalcoólico (80%) das inflorescências	Atividade antioxidante tópica	Nanopartículas de PCL, Span® 80, Pluronic® F-68	(CARVALHO et al., 2008)
Salvia officinalis	Extrato hidroalcoolico (70%) de folhas	Atividade antioxidante	Nanopartículas de PLGA	(KHALIL; ABDU, 2013)
Picrorhiza kurroa	Extrato hidroalcoolico (80%) de raízes e rizomas	Atividade hepatoprotetora	Nanopartículas de PLA, Pluronic® F-68	(JIA et al., 2015)
Chá branco	Extrato aquoso de folhas	Atividade antioxidante	Nanopartículas de PCL, alginato, Pluronic® F-127	(SANNA et al., 2015)
Centella asiática	Extrato aquoso de folhas	Atividade protetora da pele	Nanopartículas de gelatina	(KWON et al., 2012)
Phytolacca decandra	Extrato etanólico de raízes	Atividade antineoplásica	Nanopartículas de PLGA	(DAS et al., 2012)
Curcuta chinensis	Extrato etanólico de sementes	Hepatotoxicidade	Nanopartículas de Pluronic® F-68	(YEN et al., 2008)

Fonte: adaptado de Zorzi et al. (2015).

Além disso, as nanopartículas poliméricas apresentam como principais vantagens o aumento da solubilidade e da biodisponibilidade, redução da toxicidade, aumento do índice terapêutico, melhoria da estabilidade e proteção frente à degradação química e física em

comparação com as preparações livres (AJAZUDDIN; SARAF, 2010; BITENCOURT et al., 2016; BRIGGER; DUBERNET; COUVREÛ, 2002; COIMBRA et al., 2011; HAN et al., 2015; KESARWANI; GUPTA, 2013; KHOEE; YAGHOOBIAN, 2009).

3.5 ATIVIDADE ANTIOXIDANTE

Os radicais livres são produzidos como uma parte normal do metabolismo celular, pela enzima xantina oxidase, peroxissomos, em processos inflamatórios, fagocitoses, isquemia e exercício físico. Além disso, a exposição a fatores externos como o tabagismo, poluição ambiental, radiação, drogas, pesticidas e solventes industriais podem favorecer a produção de radicais livres (CAROCHO; FERREIRA, 2013; LU et al., 2010; PRIOR, 2015).

Como definição, radicais livres são átomos, moléculas ou íons com elétrons desemparelhados na última camada, altamente instáveis e ativos com outras moléculas. Os mesmos podem derivar do oxigênio, nitrogênio e enxofre, gerando, assim: espécies reativas de oxigênio (ERO's) como o ânion superoxido ($O_2^{-\cdot}$), radical hidroperoxil ($HO_2^{\cdot}$), radical hidroxil ($^{\cdot}OH$), óxido nitrico (NO), peróxido de hidrogênio (H_2O_2), oxigênio singleto ($^1O_2$), ácido hipocloro (HOCl) e peroxinitrito ($ONOO^-$); espécies reativas de nitrogênio (ERN's), como o $ONOO^-$ e; espécies reativas de enxofre (ERE's) facilmente formadas pela reação de ERO's com tiols (LU et al., 2010).

Quando há um desequilíbrio entre a produção de ERO's e a defesa antioxidante, ocorre o processo denominado estresse oxidativo (RAJENDRAN et al., 2014; WOJTUNIK-KULESZA et al., 2016).

Durante as últimas décadas, tem-se proposto que o estresse oxidativo é o principal responsável por diferentes condições fisiopatológicas como o câncer, diabetes, doenças neurodegenerativas, cardiovasculares e falhas na função imunológica e endócrina (RAJENDRAN et al., 2014; WOJTUNIK-KULESZA et al., 2016).

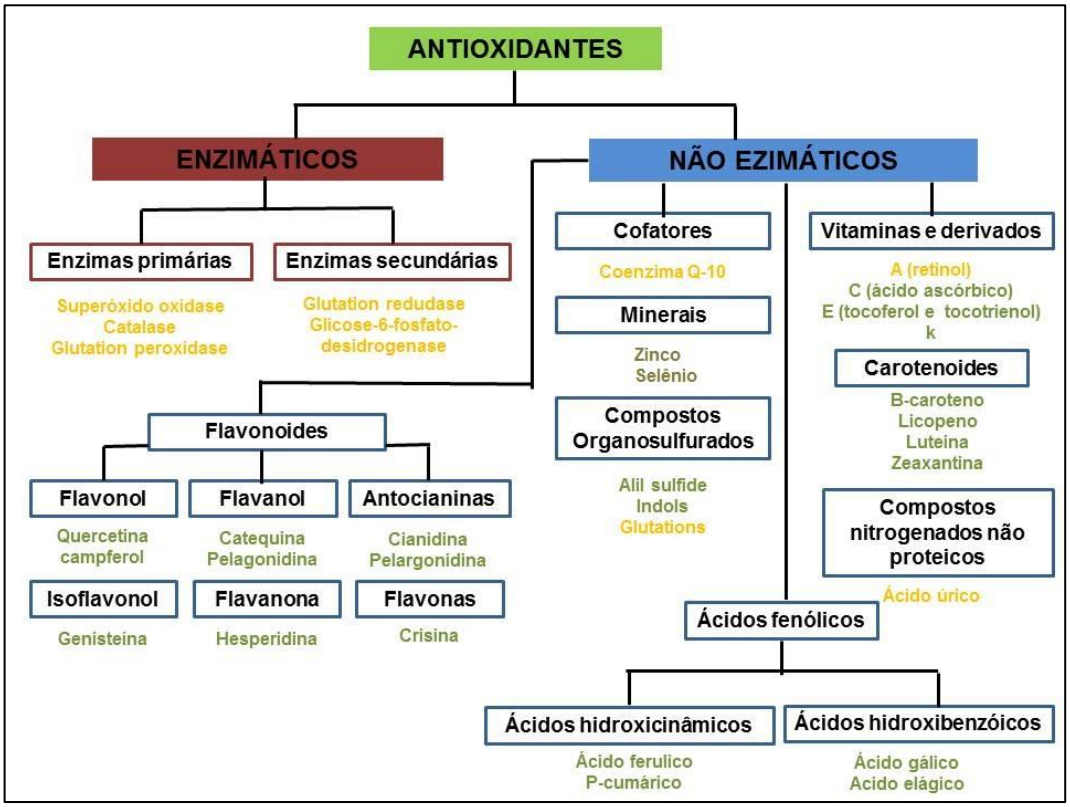
Para combater os danos provocados pelo estresse oxidativo, o organismo dispõe de um sistema de defesa antioxidante. Assim, antioxidante é definido como "qualquer substância que atrasa, previne ou remove o dano oxidativo para uma molécula-alvo" (HALLIWELL; GUTTERIDGE, 2007).

O sistema de defesa antioxidante do organismo compreende uma variada gama de substâncias endógenas e exógenas que atuam em diferentes níveis. De modo geral, este sistema é dividido em antioxidantes enzimáticos e não enzimáticos (Figura 10). Os antioxidantes enzimáticos são divididos em defesa enzimática primária e secundária. Os antioxidantes

enzimáticos primários são formados por enzimas que impedem a formação ou neutralizam os radicais livres (glutationa peroxidase, catalase, superóxido dismutase). Os antioxidantes enzimáticos secundários, não neutralizam os radicais livres diretamente, mas têm um papel de apoio aos outros antioxidantes endógenos (glutationa redutase e glucose-6-fosfato desidrogenase) (CAROCHO; FERREIRA, 2013).

Os antioxidantes não enzimáticos endógenos, por sua vez, incluem a vitamina A (retinol), co-fatores enzimáticos (coenzima Q10), compostos nitrogenados (ácido úrico) e peptídeos (glutation). Apesar da sua eficiência notável, o sistema antioxidante endógeno não é suficiente para manter a concentração de radicais livres em níveis baixos, havendo a necessidade de uma fonte exógena de antioxidantes, como flavonoides, ácidos fenólicos e vitaminas (CAROCHO; FERREIRA, 2013).

Figura 10 – Sistema de defesa antioxidante



Fonte: adaptado de Carocho; Ferreira (2013). Antioxidantes exógenos (em verde); Antioxidantes endógenos (em amarelo).

Os antioxidantes naturais descritos como de maior importância para a terapêutica são as vitaminas E e C, a rutina e demais compostos fenólicos, tendo em vista que os mesmos são capazes de neutralizar os radicais livres e inibir a peroxidação lipídica (SHEN et al., 2016; WOJTUNIK-KULESZA et al., 2016).

Neste contexto, considerando que o estresse oxidativo pode ser caracterizado pela incapacidade de antioxidantes endógenos de impedir os danos oxidativos sobre os alvos biológicos, originado a partir do aumento na produção de ERO/ERN ou um decréscimo na atividade antioxidante, pesquisas sugerem que uma dieta rica em compostos antioxidantes é inversamente proporcional ao risco de desenvolver certas patologias associadas ao estresse oxidativo (LÓPEZ-ALARCÓN; DENICOLA, 2013).

Sendo assim, com o aumento do interesse em compostos bioativos que atuem como antioxidantes, numerosos métodos têm sido desenvolvidos para determinar a atividade antioxidante destes compostos (PRIOR, 2015).

De acordo com López-Alarcón; Denicola (2013), os ensaios baseiam-se em diversas estratégias destinadas a avaliar o consumo de radicais livres estáveis por compostos antioxidantes; a capacidade de antioxidantes para reduzir os íons cúpricos ou férricos; a capacidade de antioxidantes em proteger uma molécula alvo exposta a uma fonte de radicais livres; ou a capacidade de antioxidantes em inibir a oxidação de lipoproteínas de baixa densidade (LDL).

Todas estas metodologias têm suas vantagens e desvantagens e não há um método simples e universal que forneça resultados inequívocos. Desta forma, indica-se utilizar mais de um método durante a pesquisa (ALVES et al., 2010; CAROCHO; FERREIRA, 2013). Dentre estes métodos, a capacidade de remoção do radical oxigênio (ORAC) e o de sequestro do radical livre 2,2-difenil-1-picrilhidrazila (DPPH) são citados como sendo os mais utilizados para determinar a capacidade de antioxidantes de compostos de origem natural (KAMEYA et al., 2014).

3.6 ENSAIOS PARA AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE

3.6.1 Sequestro do radical livre 2,2-difenil-1-picril-hidrazila (DPPH•)

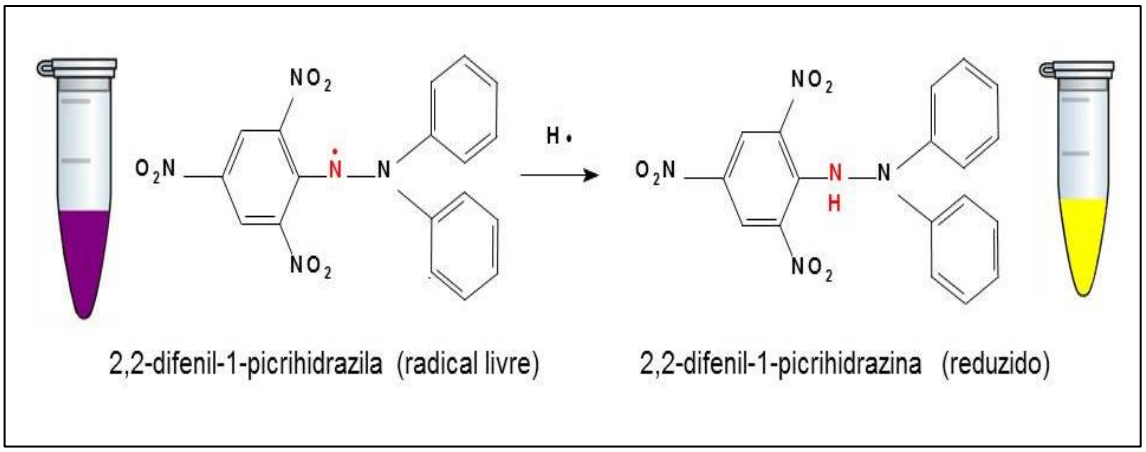
O método do sequestro do radical livre DPPH• é amplamente utilizado para a determinação da capacidade antioxidante de compostos de origem natural e tem sido considerado como o principal mecanismo de ação dos antioxidantes fenólicos (FARHOOSH et al., 2016).

Do ponto de vista metodológico, trata-se de um procedimento simples, rápido, preciso e reprodutível que tem como princípio a medida da capacidade antioxidante de uma

determinada substância em sequestrar o radical DPPH• (Figura 11), reduzindo-o a hidrazina (ALVES et al., 2010; LU et al., 2010).

Quando uma determinada substância que age como doador de átomos de hidrogênio é adicionada a uma solução de DPPH•, a hidrazina é obtida com mudança simultânea na coloração de violeta a amarelo pálido (ALVES et al., 2010).

Figura 11 – Modelo para avaliação da atividade antioxidante utilizando DPPH•



Fonte: Adaptado de Molyneux (2004).

De acordo com Sharma e Bhat (2009) uma série de protocolos tem sido proposta para este ensaio, os quais diferem na concentração de DPPH• (22,5-250 µM), tempo de incubação (5 min-1h), solvente da reação (etanol, metanol) e o pH da mistura de reação. Tais parâmetros resultam em variação entre os resultados, demonstrando a exigência de um ensaio padrão a fim de comparar e validar os resultados de diferentes laboratórios.

3.6.2 Capacidade de remoção do radical oxigênio (ORAC)

Dentre as metodologias desenvolvidas para estimar a capacidade antioxidante, esta tem sido uma das mais utilizadas (LÓPEZ-ALARCÓN; DENICOLA, 2013).

Neste ensaio, o radical peroxil gerado pela reação do AAPH [diidrocloreto de 2,2'-azobis (2-amidino-propano)] com o oxigênio atmosférico, reage com a fluoresceína para formar um produto não fluorescente, que pode ser quantificado por espectrofotometria (ALVES et al., 2010; BENTAYEB et al., 2014).

Desta forma, a atividade antioxidante é avaliada através da inibição da oxidação, induzida pelo radical peroxil, por transferência de átomos de hidrogênio e é determinada através

da diferença entre a área sob a curva (ASC) da amostra subtraída pela área sob a curva do branco, medida pelo decaimento da fluorescência com a adição da substância antioxidante no decorrer do tempo. Os resultados são comparados com os de um antioxidante de referência, geralmente Trolox (ALVES et al., 2010; BENTAYEB et al., 2014)

Dentre as vantagens deste método, pode-se destacar o radical peroxil como fonte de radical livre, tendo em vista que o mesmo é o mais prevalente na biologia humana. Além disso, ORAC pode ser empregado para diversas matrizes (PRIOR, 2015). Porém, como desvantagens pode-se destacar a ocorrência de reações secundárias ou reações associadas com os mecanismos de reparo (BISBY; BROOKE; NAVARATNAM, 2008).

3.7 ATIVIDADE ANTIDEPRESSIVA

A depressão é um grave distúrbio psiquiátrico que afeta aproximadamente 17% das pessoas (FARAHANI et al., 2015). Está previsto para, até 2020, ser a segunda doença mais diagnosticada, em todo o mundo (WORLD HEALTH ORGANIZATION, 2017) e, em função dos seus sintomas específicos, é considerada uma das principais causas de incapacidade humana com um alto fator de morte devido aos riscos de suicídios (PYTKA et al., 2015).

O termo depressão pode descrever tanto um estado transitório de humor experienciado por praticamente todos os individuos em algum momento da vida, como também, uma síndrome clínica ou biocomportamental cujos sintomas envolvem alterações de humor, alteração das funções neurodegenarativas (como distúrbios do sono e apetite), da cognição (como culpa e sentimentos de inutilidade) e psicomotoras (como agitação ou letargia) (FAVA; KENDLER, 2000).

A depressão não é uma doença de causa única, mas a combinação de fatores genéticos, bioquímicos, ambientais e psicológicos (BIENVENU; DAVYDOW; KENDLER, 2011).

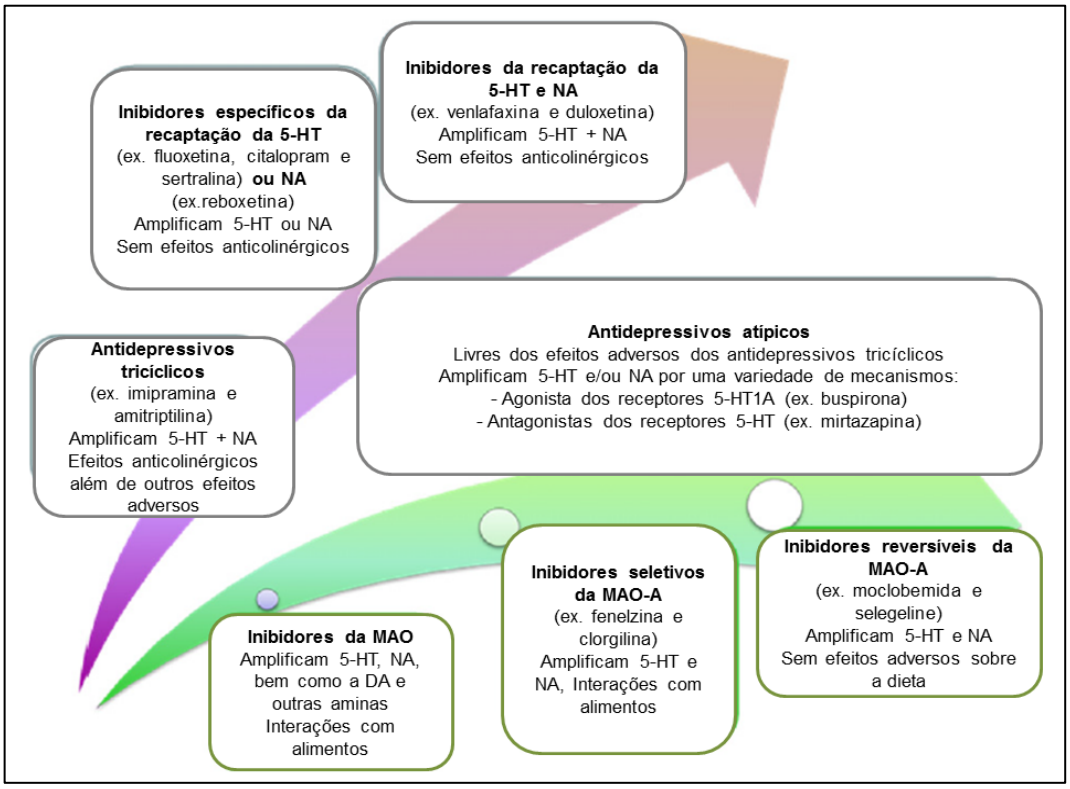
Desde 1965, a teoria monoaminérgica da depressão foi considerada a principal e única causa da doença. Esta hipótese supôs que a depressão estava relacionada com *déficits* de serotonina (5-hidroxitriptamina, 5-HT) e noradrenalina (norepinefrina, NA) em diferentes partes do cérebro. No entanto, ao longo dos anos, essa teoria evoluiu, e surgiram muitas novas abordagens. Os cientistas notaram a forte relação entre o estado de depressão e as mudanças em vários sistemas não apenas serotonérgicos e noradrenérgicos, mas também dopaminérgicos (PYTKA et al., 2015).

No entanto, embora vários antidepressivos estejam disponíveis há décadas, a maioria não é completamente eficaz, além de apresentarem muitos efeitos adversos, como alteração do sono e apetite, alterações gastrintestinais (diarréia ou obstipação intestinal), retenção urinária,

alergias de pele, sudorese, diminuição da libido ou retardo da ejaculação, aumento ou diminuição de peso, náusea, tontura, tremores, os quais comprometem a qualidade de vida dos pacientes (GONG et al., 2014; MILLAN, 2006). Assim, devido à heterogenidade da resposta clínica e à suceptibilidade aos efeitos adversos, estima-se que 50% dos pacientes são inadequadamente tratados pelas intervenções disponíveis (AKIL et al., 2017; BRUNELLO et al., 2002; MACHADO et al., 2008).

A Figura 12 apresenta, de maneira resumida, as maneiras pelas quais as cinco principais classes mais recentes de fármacos antidepressivos apresentaram um refinamento dos mecanismos de ação com relação às classes de antidepressivos clássicos, os antidepressivos tricíclicos e os inibidores da monoamina oxidase (IMAO).

Figura 12 – Desenvolvimento de fármacos antidepressivos



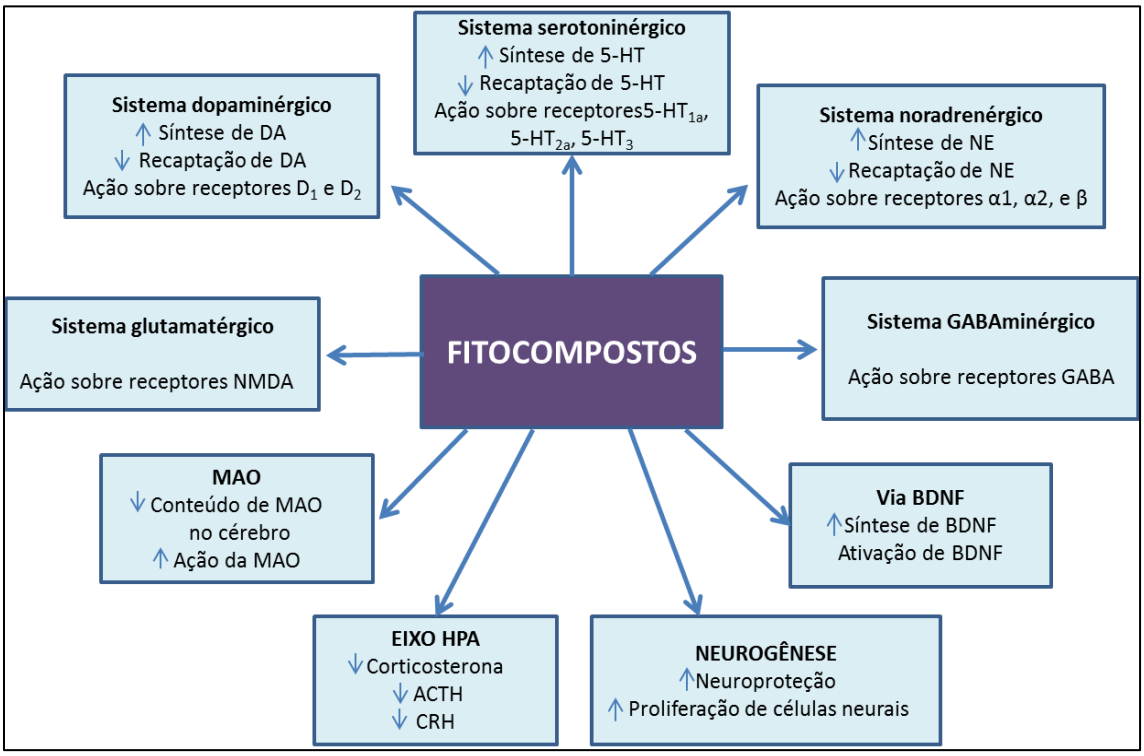
Fonte: Adaptado de Willner; Scheel-Kruger; Belzung (2013). 5-HT: serotonina; NA: noradrenalina; DA: dopamina; MAO: monoaminoaxidases.

Pode-se observar que o desenvolvimento de novos antidepressivos tem demonstrado poucas evidências em termos de melhora na eficácia quando comparados aos antidepresssivos mais antigos. Estes novos fármacos tem mostrado menor incidência de efeitos adversos indesejados, contudo não exploram novos mecanismos de ação, obtendo baixas taxas de

resposta e início de resposta ainda muito lento (WILLNER; SCHEEL-KRÜGER; BELZUNG, 2013).

Neste sentido, explorar os mecanismos neurológicos da atividade antidepressiva de agentes derivados de plantas pode ter um papel crucial no desenvolvimento de novas drogas naturais para o manejo da depressão (FARAHANI et al., 2015) como demonstrado na Figura 12, que explora os diferentes mecanismos de ação pelos quais as plantas medicinais podem exercer seu efeito antidepressivo.

Figura 13 – Diferentes mecanismos de ação pela qual as plantas demonstram seus efeitos antidepressivos



Fonte: Adaptado de Pathak; Agrawal; Dhir (2013) 5-HT: serotonina; ACTH: hormônio adrenocorticotrófico; CRH: hormônio liberador de corticotrofina; DA: dopamina; Fator neurotrófico derivado do cérebro (*BDNF*); GABA: ácido γ-aminobutírico; HPA: hipotálamo- hipófise-adrenal; MAO: monoamino oxidase; NMDA: *N*-metil-D-aspartato.

Como exemplo de plantas, com atividade antidepressiva já bem estabelecida, pode-se citar o caso da erva de São João (*Hypericum perforatum* L.) (NÖLDNER; SCHÖTZ, 2002), considerado uma alternativa efetiva para os antidepressivos de origem sintética para o tratamento de depressão moderada a leve (GUAN; LIU, 2016). Além disso, a literatura relata atividade antidepressiva para extratos de *Valeriana glechomifolia* (MÜLLER et al., 2012) e *Valerians wallichii* (SAH; MATHELA; CHOPRA, 2011), extrato metanólicos de *Byrsonima*

crassifólia (HERRERA-RUIZ et al., 2011) e extratos metanólicos de flores de *Hibiscus rosa-sinensis* Linn (SHEWALE; PATIL; HIRAY, 2012), entre outras.

Dentre os compostos presentes nestas plantas, os compostos fenólicos, parecem ser os responsáveis pela atividade antidepressiva, uma vez que apresentam efeito protetor em diferentes distúrbios neurológicos e mentais, demonstrado atividade antidepressiva em modelos animais de depressão. Além disso, parecem modular a neurotransmissão monoaminérgica no cérebro (PATHAK; AGRAWAL; DHIR, 2013). Dentre estes compostos com potencial antidepressivo, destaca-se os ácidos clorogênicos e ferulico e os flavonoides rutina, quercetina, resveratrol e as proantocianidinas (PATHAK; AGRAWAL; DHIR, 2013).

O mecanismo de ação antidepressiva do ácido clorogênico não está claro, mas a hipótese é que o mesmo possa atuar através da via opioidérgica (PARK et al., 2010). Além disso (WU et al., 2016), relataram que o ácido clorogênico é capaz de atravessar a barreira hematoencefálica para exibir sua proteção neuronal e promovendo a liberação de serotonina através da expressão de sinapsina I.

Com relação aos flavonoides, mais do que um grupo farmacofórico parecem estar relacionados aos efeitos antidepressivos. Em modelos animais de depressão, os mesmos parecem modular a liberação dos neurotransmissores 5-HT, NA e DA, além do ácido 5-hidroxiindolacético (PATHAK; AGRAWAL; DHIR, 2013). Extratos ricos em rutina que tem apresentado atividade antidepressiva, por exemplo, tem demonstrado que a mesma é essencial para a atividade uma vez que pode aumenta direta ou indiretamente a biodisponibilidade dos outros constituintes necessários para esta atividade biológica (NÖLDNER; SCHÖTZ, 2002). Além disso, Machado et al. (2008) e Du et al. (2014) relataram o envolvimento dos sistemas serotoninérgico, noradrenérgicos e dopaminérgico na ação antidepressiva da rutina.

Ademais, flavonoides de várias classes têm sido efetivos na depressão e melhora dos sintomas da doença de Parkinson por inibirem a enzima MAO (monoaminoxidase) A ou B (GONG et al., 2014; JÄGER; SAABY, 2011).

3.7.1 Testes e modelos animais de depressão

Existem dois testes comumente usados para avaliar a atividade do tipo antidepressiva, o Teste de Natação Forçada (TNF) e Teste de Suspensão pela Cauda (TSC). Ambos são baseados na observação de que quando os roedores são submetidos a uma situação inescapável, após as tentativas iniciais de escapar, os animais adotam rapidamente a postura imóvel. Acredita-se que essa mudança de comportamento, ou seja, a imobilidade possa ser interpretada

como desespero comportamental (CHENU, 2005; CRYAN; MOMBEREAU; VASSOUT, 2005).

Tendo em vista que os antidepressivos clinicamente eficazes diminuem significativamente o tempo de imobilidade no TNF e TSC em roedores (PYTKA et al., 2015), estes testes representam uma valiosa ferramenta na busca de alternativas terapêuticas para a depressão, no estudo dos aspectos neurobiológicos da depressão, bem como os mecanismos de ação pelos quais exercem seus efeitos (GONG et al., 2014; KRISHNAN; NESTLER, 2008).

O TNF, descrito originalmente por Porsolt et al (1977; 1978) consiste em colocar os animais em um cilindro inescapável contendo água (PORSOLT; BERTIN; JALFRE, 1977; PORSOLT et al., 1978). A imobilidade dos animais é interpretada como uma estratégia de enfrentamento de estresse passivo ou comportamento do tipo depressivo (CRYAN; VALENTINO; LUCKI, 2005; DEUSSING, 2006). Tratamentos clínicos efetivos para a depressão foram previamente detectados por TNF como antidepressivos tricíclicos, inibidores da monoaminoaxidase e antidepressivos atípicos. Além disso, TNF é capaz de distinguir drogas que não são antidepressivas, por exemplo, drogas com efeito ansiolítico como é o caso de benzodiazepínicos que não são ativos no TNF (CRYAN; VALENTINO; LUCKI, 2005).

O segundo teste muito utilizado, o TSC, foi proposto por Steru et al. (1985) e consiste em suspender os animais pela cauda por um tempo determinado e observar o tempo de imobilidade. O TSC é um dos modelos mais tradicionais para o estudo da depressão em animais, por apresentar alto valor preditivo devido à resposta aos medicamentos antidepressivos existentes, além de ser simples, barato e permitir a automatização. (DEUSSING, 2006; DUARTE et al., 2008). O TSC tem muitas vantagens sobre o TNF, como a falta de efeitos hipotérmicos, a capacidade de testar animais com *déficit* motor e o aumento da sensibilidade a uma maior variedade de compostos antidepressivos (CARR; LUCKI, 2011). Uma grande desvantagem é que a sua aplicação restringe-se a utilização de camundongos (DEUSSING, 2006; DUARTE et al., 2008).

Embora humanos e roedores apresentem diferenças marcantes na anatomia encefálica, diversos circuitos que regem respostas comportamentais e fisiológicas, estão conservados entre estas espécies (CRYAN; VALENTINO; LUCKI, 2005). Além disso, estes testes têm se mostrado altamente sensíveis para compostos com atividade antidepressiva, além de serem simples, rápidos e reprodutíveis (DEUSSING, 2006; GONG et al., 2014; KRISHNAN; NESTLER, 2008). Como desvantagens destes ensaios, é que nem TSC, nem TNF refletem o início lento da ação de antidepressivos como é observado em pacientes deprimidos (DEUSSING, 2006).

Além destes testes, acredita-se que o modelo de Estresse Crônico Moderado e Imprevisível (ECMI) seja provavelmente o modelo que melhor traduza os sintomas da depressão (WILLNER, 1997, 2017). Durante o teste, os animais são expostos a uma variedade de agentes estressores por um período de tempo determinado. A sequência relativamente imprevisível dos agentes estressores induz mudanças no estado hedônico dos animais, redução da atividade no campo aberto e interação social, além de outras alterações comportamentais que lembram o progresso da depressão clínica em seres humanos (KATZ; ROTH; CARROLL, 1981; MAO et al., 2009; TÕNISSAAR et al., 2008; WILLNER, 1997, 2005; WILLNER et al., 1987).

O ECMI como modelo de depressão induzida foi desenvolvido para simular o desenvolvimento e o progresso da depressão clínica em seres humanos, bem como para avaliar a eficácia de compostos candidatos através de testes comportamentais, como o teste de preferência por sacarose e o teste de nado forçado (TNF) como desfecho do estudo (DANG et al., 2009; MAO et al., 2009; TÕNISSAAR et al., 2008; ZHOU et al., 2007).

O estresse é caracterizado como um conjunto de respostas adaptativas (físicas, mentais e emocionais) iniciado por uma ameaça ambiental, ou seja, um estado de alarme que promove uma série de alterações endócrinas com vistas à autopreservação. A resposta ao estresse ativa o eixo hipotálamo-hipófise-adrenal (HPA), com consequente aumento da secreção de hormônios glicocorticoides, a partir do córtex adrenal, e de catecolaminas, a partir da medula adrenal e nervos simpáticos os quais, por sua vez, fornecem um *feedback* para o cérebro, influenciando estruturas neurais que controlam a emoção e cognição (NESTLER et al., 2002; RODRIGUES; LEDOUX; SAPOLSKY, 2009).

Acredita-se que a exposição ao estresse ou a eventos/condições traumáticas tem um forte impacto sobre a manifestação de depressão sugerindo que pacientes deprimidos devam ter prejuízos em estratégias de lidar com situações aversivas (DEUSSING, 2006; KESSLER, 1997; LEE; OGLE; SAPOLSKY, 2002), os quais que podem ser revertidos por tratamento com antidepressivos (DEUSSING, 2006; KESSLER, 1997; LEE; OGLE; SAPOLSKY, 2002).

4 CAPÍTULO 1

Influência do período de coleta e da cultivar sob a composição fenólica e propriedades antioxidantes de folhas de *Vaccinium ashei* Reade

CAPÍTULO 1

Influência do período de coleta e da cultivar sob a composição fenólica e propriedades antioxidantes de folhas de *Vaccinium ashei* Reade

1.1 INTRODUÇÃO

O presente capítulo compreendeu a avaliação da influencia de diferentes cultivares e períodos de coleta sobre a composição fenólica e atividade antioxidante dos extratos hidroalcoolicos de folhas de *Vaccinium ashei* Reade.

Foi avaliado o conteúdo de fenólicos totais, flavonoides totais e o perfil cromatográfico por HPLC-UV/DAD de cinco cultivares diferentes (Aliceblue, Powderblue, Climax, Bluegem e FloridaM) de folhas de *Vaccinium ashei* Reade coletadas nos meses de dezembro/2013 e março/2014.

Adicionalmente, foi avaliada a atividade antioxidante destes extratos empregando-se dois métodos *in vitro*: método de sequestro do radical 2,2-difenil-1-picrilhidrazil (DPPH) e o método de sequestro do radical peroxil (ORAC).

Estes ensaios foram realizados na URI – Universidade Regional Integrada do Alto Uruguai e das Missões, Campus de Frederico Westphalen.

4.1 PUBLICAÇÃO 1 - INFLUENCE OF HARVEST SEASON AND CULTIVAR ON THE VARIATION OF PHENOLIC COMPOUNDS COMPOSITION AND ANTIOXIDANT PROPERTIES IN *Vaccinium ashei* LEAVES

molecules MDPI

Article

Influence of Harvest Season and Cultivar on the Variation of Phenolic Compounds Composition and Antioxidant Properties in *Vaccinium ashei* Leaves

Verciane Schneider Cezarotto [1,2], Sandro Rogério Giacomelli [3], Maria Helena Vendruscolo [2], Angélica Signor Vestena [2], Caroll Schneider Cezarotto [2], Ritiel Corrêa da Cruz [1], Luana Haselein Maurer [4], Luana Mota Ferreira [1], Tatiana Emanuelli [4] and Letícia Cruz [1,*]

1 Departamento de Farmácia Industrial, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil; verciane@uri.edu.br (V.S.C.); ritieldc@gmail.com (R.C.C.); luanamotaferreirra@gmail.com (L.M.F.)

2 Departamento de Ciências da Saúde, Curso de Farmácia, Universidade Regional Integrada do Alto Uruguai e das Missões, Frederico Westphalen, RS 98400-000, Brazil; maria.vendruscolo@gmail.com (M.H.V.); angelica_signor@hotmail.com (A.S.V.); k_rollcezarotto@hotmail.com (C.S.C.)

3 Departamento de Ciências Exatas e da Terra, Curso de Química, Universidade Regional Integrada do Alto Uruguai e das Missões, Frederico Westphalen, RS 98400-000, Brazil; srgiacomelli@uri.edu.br

4 Departamento de Tecnologia e Ciência dos Alimentos, Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria RS 97105-900, Brazil; luanahmaurer@gmail.com (L.H.M.); tatiemanuelli@gmail.com (T.E.)

* Correspondence: leticiacruz@smail.ufsm.br; Tel.: +55-55-3220-9373

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Abstract: The effect of variation of harvest season and cultivar on the total phenolic content (TPC), total flavonoid content (TFC), HPLC-UV/DAD profile and antioxidant properties in *Vaccinium ashei* (Rabbiteye blueberry) leaves grown in Brazil was evaluated. The cultivars collected in December and March were Aliceblue, Powderblue, Climax, Bluegem and FloridaM. It was observed that leaves from March had the highest TPC values (222 ± 1 mg gallic acid equivalents/g to Aliceblue cultivar) and highest TFC values (49.8 ± 0.8 and 48.7 ± 0.7 μg rutin/g to Clímax and Powderblue cultivars, respectively). The chromatographic profile was quantitatively similar, however, the proportions of each compound were influenced by cultivar and harvest season. Chlorogenic acid and rutin were the main identified phenolic compounds, but chlorogenic acid was the most abundant in both harvest seasons. Antioxidant capacities values ranged from 5.80 ± 0.04 to 105 ± 2 μg/mL (DPPH) and 178 ± 5 to 431 ± 8 mmol Trolox/100 g (ORAC). The cultivar Bluegem by March had the highest values in both assays. The results indicate that the blueberry leaves from different cultivars and harvest seasons have different phenolic compounds content and different antioxidant capacities. In addition, the antioxidant properties demonstrated a high correlation with rutin content.

Keywords: harvest season; cultivars; phenolics; antioxidant properties; blueberry leaves; *Vaccinium ashei*

1. Introduction

Vaccinium genus (blueberry, family Ericaceae), is known around the world due to its several beneficial effects for human health due to its potential activities against a wide range of degenerative diseases such as inflammatory reactions, oxidative stress induced by aging and cardiovascular problems [1–4]. Commercial interest is mainly focused on the blueberry fruits, which are consumed fresh or processed, and the blueberry leaves are considered a waste or byproduct which so far were

discarded, what could be a mistake [5–8]. As the total phenolic compound concentration in leaves is three times higher than those observed in the fruits [9–12] there is a growing scientific interest in them.

Berry phenolics comprise a wide variety of secondary metabolites divided into phenolic acids (such as hydroxybenzoic and hydroxycinnamic acids), flavonoids (flavonol, flavanol and anthocyanin) and condensed (proanthocyanidin) and hydrolysable tannins [12]. Regarding these compounds, several studies have demonstrated numerous benefits related to their high consumption, for example, to prevent oxidative damage originated by reactive oxygen species [13–15].

However, the phenolic contents and their related pharmacological activities are often dependent on pre- and post-harvest factors such as species (intraspecies and interspecies differences), environmental characteristics (climatic conditions, humidity and brightness), agronomic features (soil, water supply, use of fertilizers or manure), ripeness, harvesting, transportation method, storage, drying process and extraction methods [7,15–18]. Hence, the plant chemical composition is influenced by the seasons and may present variability through the months, leading to different final bioproducts with variable composition and pharmacological properties [17]. Due to this, it is essential to evaluate the optimal cultivation time for the highest amount of active compounds and maximum biological activity [19].

Many reports have suggested an antioxidant potential of blueberry leaves [6,9,10,20], but there is no studies about how the harvest season or cultivars influence the activity of blueberry leaves [4,5,7,8,15]. Further, to the best of our knowledge, no reports were found about the variability of phenolic compounds in the blueberry leaves regarding the harvest season for rabbiteye blueberry growing in Brazil. The rabitteye blueberry group is the main cultivar in Brazil because of its tolerance to heat, low demand in the cold season, early flowering and prolonged period between flowering and maturation [21].

Therefore, considering the variations of phenolic composition and consequently the biological activity differences among the different cultivars, the harvest period and the limited information on the phenolic composition of rabbiteye blueberry leaves produced in Brazil, the main objectives of this study were: (1) to evaluate the total phenolic and flavonoids content of blueberry leaves of five different varieties (Clímax, Bluegem, Aliceblue, Powderblue and FloridaM) collected during two different harvest times: December and March; (2) to assess in details the metabolite profile of the phenolic compounds by HPLC-UV/DAD; (3) to determine the antioxidant properties in terms of DPPH and ORAC; and (4) to correlate the variation of the phenolic composition and antioxidant properties with the different times of harvest and the blueberry leaves variety. The results in this study could help decide which is the best cultivar and harvest period to benefit from the maximum pharmacological effects of the blueberry leaves.

2. Results and Discussion

2.1. Influence of the Cultivar and Harvest Season on Phenolic Compounds Contents

The TPC and TFC quantification in rabbiteye blueberry leaves from different harvest seasons and cultivars is shown in Table 1. The leaves collected in March presented the highest TPC (ranging from 154 ± 1 to 222 ± 1 mg GAE/g) and TFC contents (ranged to 49.8 ± 0.8 to 38.3 ± 0.8 μg rutin/g) (Table 1) confirming that the leaves' phytochemical composition varies according to the stages of the plant growth [15] and the plant maturity status is reflected the physiological, biochemical and structural processes of the plant tissue [5].

There are many reports about TPC and TFC of blueberry leaves and fruits, what makes it possible to notice that the *Vaccinium* genotype and the harvest season are factors that exert a great influence on the content of these antioxidant compounds. Zhu et al., observed that for the aqueous extract of blueberry leaves (*Vaccinium ashei*) cultivated in China, in different seasons, the highest TFC (114.21 ± 0.03 mg rutin equivalent/g extract) was reported in May and the highest TPC (425.2 ± 0.2 mg gallic acid equivalent/g extract) in November [8]. Similarly, Li et al., reported that

rabbiteye blueberry (*Vaccinium ashei*; cv. Brightwell) from Nanjing (China), harvested in July had the highest TPC (339 ± 3 mg GAE/g DW) and TFC (198 ± 2 mg of quercetin/g DW) in the extract of leaves in comparison with fruits and pomace [6]. Routray and Orsat observed that highbush blueberry (*Vaccinium corymbosum*) leaves had a high amount of total phenolics in October (for Nelson 152 ± 3 and for Elliot 156 ± 2 mg GAE/g dry matter) [15].

Table 1. TPC and TFC rabbiteye blueberry leaves from different harvest seasons and cultivars.

Cultivars	TPC (mg/g) [1]		TFC (μg/g) [2]	
	December	March	December	March
Bluegem	75.4 ± 0.6 [a]	170 ± 2 [a]	18.9 ± 0.2 [a]	45.2 ± 0.5 [a]
Powderblue	79 ± 1 [b]	154 ± 1 [b]	24.3 ± 0.3 [b]	48.7 ± 0.7 [b]
Clímax	133.6 ± 0.4 [c]	185 ± 1 [c]	32.3 ± 0.2 [c]	49.8 ± 0.8 [b]
FloridaM	93.1 ± 0.5 [d]	166 ± 1 [d]	21.3 ± 0.2[d]	38.3 ± 0.8 [c]
Aliceblue	110 ± 2 [e]	222 ± 1 [e]	19.5 ± 0.3 [a]	39.1 ± 0.6 [d]
Means ± SD	98 ± 21	179 ± 26	23 ± 5	44 ± 5

Two-way ANOVA followed by Student Newmann-Keuls; Values are expressed as mean ± SEM ($n = 3$). Different letters in the same column indicate significant differences ($p < 0.05$). [1] TPC is expressed as milligrams of gallic acid (GAE) per gram of dry weight (DW). [2] TFC is expressed as micrograms of rutin per gram of dry weight (DW).

According to Venskutonis and co-workers, during seasonal development, blueberry plants first concentrate the secondary metabolites in the fruits, but later those metabolites are concentrated in the leaves vegetative portion [7]. This observation corroborates the results obtained in this study. On the other hand, these data are not in agreement with Percival and Mackenzie's report. In that study during harvest, blueberry (*Vaccinium angustifolium*) leaves presented a higher total phenolic content in green leaf tissues at harvest than those observed two weeks after the collection period [22].

In Brazil, the flowering of blueberry fruits starts in August and ends between early September and the end of October. The blueberry fruit maturation starts during the second half of December until January and the harvest period lasts 37 days [21]. Leaves from December (late spring and early summer) are from the end of fruit ripening and leaves from March (late summer and early autumn) correspond to the phenological stage of the plants when pruning occurs.

Considering the effect of cultivars on TPC and TFC values, the Clímax and Aliceblue cultivars showed higher TPC levels for both collections (133.6 ± 0.4 to 222 ± 1 mg GAE/g). Regarding the TFC quantification, the Clímax from December showed (32.3 ± 0.2 μg/g) and Bluegem (45.2 ± 0.5 μg/g), Powderblue (48.7 ± 0.7 μg/g). The Clímax (49.8 ± 0.8 μg/g) from March presented the highest compound contents. Ehlenfeldt and Prior previously reported TPC values from fruits and leaves of 87 *Vaccinium corymbosum* cultivars from late July [9]. In the leaves, phenolics values were higher than in the fruit, ranging from 23.6 GAE/g of fresh weight (cv. Reka) to 77.4 GAE/g of fresh weight (cv. Little Giant), with a mean of 44.80 GAE/g of fresh weight. In dried blueberry leaves (*Vaccinium corymbosum*; cv. Bluecrop), collected at the beginning of August, Skupien et al., reported a TPC of 111.5 mg% [23].

2.2. Phenolic Compounds Identification by HPLC

A typical HPLC chromatogram of solution and percentages of phenolic compounds in blueberry leaves extracts from different harvest months and cultivars are shown in Figures 1 and 2, respectively. All blueberry leaves extracts had similar phenolic composition, however quantitative differences were observed depending on the cultivar and collection month (Table 2).

Three phenolic compounds were identified: chlorogenic acid (retention time - tR = 19.3 min, peak 1), rutin (tR = 30 min, peak 2) and quercetin (tR = 38 min, peak 3), comparing the retention time and UV spectra with 11 commercial standards (Figure 1). The quantification of chlorogenic acid, rutin and quercetin by HPLC-UV/DAD was based on reference standard calibration curves. Calibration

curve for chlorogenic acid: y = 168508x − 52225 (r = 0.9980); rutin: y = 72823x − 1900.7 (r = 0.9982); and quercetin: y = 187893x − 163671 (r = 0.9984).

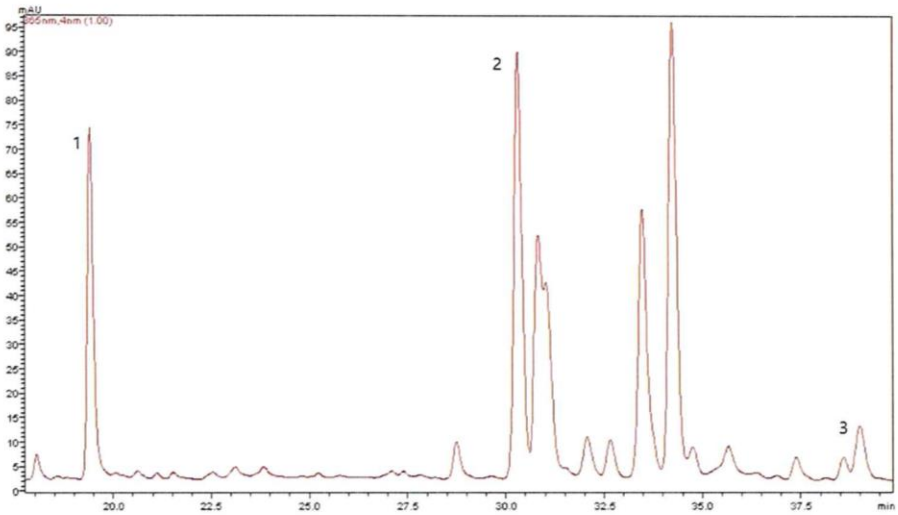

Figure 1. Typical HPLC chromatogram of rabbiteye blueberry leaves, injected volume 40 μL, at λ = 365 nm. Peaks: 1: chlorogenic acid; 2: rutin; 3: quercetin

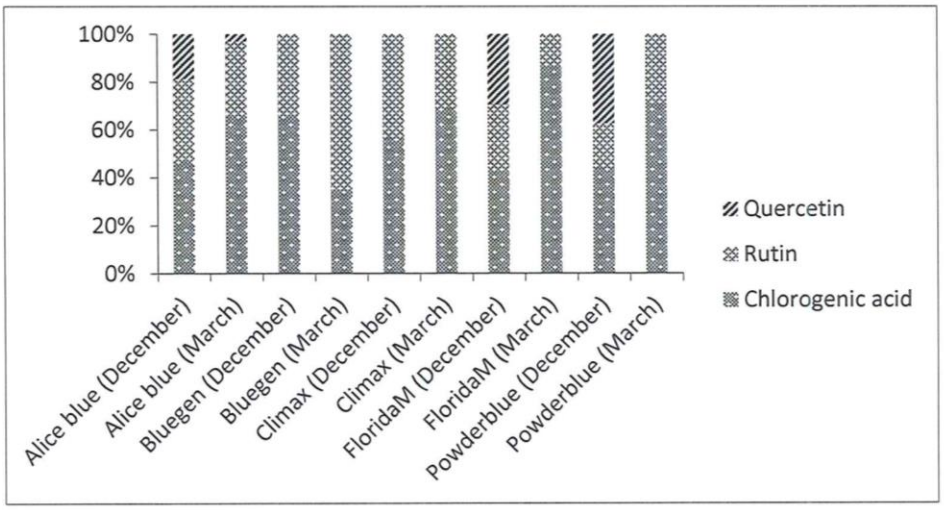

Figure 2. Percentages of the phenolic compounds chlorogenic acid, rutin and quercetin of rabbiteye blueberry leaves from different harvesting months and cultivars by HPLC-UV/DAD.

Chlorogenic acids (3-*O*-caffeoylquinic acids) were the most prevalent phenolic compound in blueberry leaf extracts (2.03 ± 0.03 to 21.28 ± 0.05 mg/g DW; corresponding to 32.2 and 87%, respectively) (Table 2 and Figure 2), which was consistent with Ferlemi et al. [12] who reported that the leaves are one the richest sources of chlorogenic acids and previous leaves analysis from 38 rabbiteye blueberry, 37 northern highbush blueberry and 29 southern highbush blueberry leaves collected in China in October showed substantial differences from each other. The eight chlorogenic acids were detected and this compound was the most abundant phenolic compounds in leaves of all cultivars [4].

In present study, the content of rutin ranged from 2.59 ± 0.04 to 15.8 ± 0.1 mg/g DW (corresponding to 13 and 64.8%, respectively). Quercetin was not detected in the chromatograms of some cultivars (0.83 ± 0.02 to 11.9 ± 0.2 mg/g DW)

In a study performed by Ferlemi et al. (2015) *Vaccinium corymbosum* leaf extract (cv. Bluecrop and Patriot) demonstrated by LC-ESI/MS and HPLC-DAD five major polyphenols (chlorogenic acid, rutin, hyperoside, isoquercetin and quercetin aglycone) which were able to protect the affected tissues (cortex, liver, from the overdose of selenite) and enhanced the antioxidant state of the least perturbed tissues [24].

Chlorogenic acid is an ester of caffeic acid and quinic acid, while rutin (quercetin-3-*O*-rutinoside) is a quercetin glycoside. These compounds have a well-established antioxidant activity [25,26]. The chlorogenic acid antioxidant activity is attributed to the catechol phenyl ring and the double bond together with the catechol group serving as a site for the attack of free radicals [27]. Besides, chlorogenic acids have activity against hepatocellular carcinomas and fibroblastic sarcomas, as well as anti-inflammatory, cardioprotective, cardioprotective, and neuroprotective properties [4,12]. In turn, rutin has demonstrated a number of pharmacological properties, including cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activities [28] suggesting the pharmacological potential of blueberry leaves.

Table 2 shows the effect of harvest season on chlorogenic acid values. It is possible to observe that the Powderblue and Aliceblue cultivars from March showed higher chlorogenic acid content (21.28 ± 0.05 and 18.21 ± 0.05 mg/g of DW, respectively). Besides, FloridaM, Climax and Aliceblue cultivars from December showed higher chlorogenic acid content (17.34 ± 0.05, 15.87 ± 0.03 and 15.58 ± 0.05 mg/g of DW, respectively). The Bluegem cultivar from March showed higher rutin content (15.84 ± 0.13 mg/g of DW). Quercetin was identified in Aliceblue, Powderblue and FloridaM cultivars and the highest concentrations were observed in the December collection.

This variation was also observed by Zhu et al. where the presence of the chlorogenic acid (34 ± 2, 8.8 ± 0.2 and 14.07 ± 0.02 mg/g), caffeic acid (0.5 ± 0.1, 0.09 ± 0.01 and 0.13 + 0.01 mg/g), rutin (7 ± 1, 2.30 ± 0.03 and 2.6 ± 0.4 mg/g), hyperoside (3 ± 1, 1.1 ± 0.2 and 4.8 ± 0.7 mg/g), galuteolin (27 ± 2) and quercitrin (4.2 ± 0.4, 1.33 ± 0.03 and 2.10 ± 0.07 mg/g) was detected in aqueous extracts of blueberry leaves from different seasons, specifically for the samples from the months of May, September and November, respectively [8].

Table 2. Phenolic compounds blueberry contents of rabbiteye leaves from different harvesting months and cultivars by HPLC-UV/DAD [1].

Cultivars/ Compounds	Clímax		Aliceblue		Bluegem		Powderblue		FloridaM	
	December	March	December	March	December	March	December	March	December	March
Chlorogenic acid	15.87 ± 0.03 [a]	9.8 ± 0.01 [b]	15.58 ± 0.05 [a]	18.21 ± 0.05 [c]	7.41 ± 0.02 [d]	2.03 ± 0.03 [e]	8.76 ± 0.01 [f]	21.28 ± 0.05 [g]	17.3 ± 0.3 [h]	14.11 ± 0.07 [i]
Rutin	12.13 ± 0.02 [a]	4.38 ± 0.01 [b]	11.3 ± 0.1 [c]	8.64 ± 0.09 [d]	3.73 ± 0.08 [e]	15.8 ± 0.1 [f]	3.6 ± 0.1 [g]	8.9 ± 0.2 [d]	11.42 ± 0.02 [c]	2.59 ± 0.04 [g]
Quercetin	N.D.	N.D.	6.20 ± 0.09 [a]	0.83 ± 0.02 [b]	N.D.	N.D.	7.4 ± 0.2 [c]	N.D.	11.9 ± 0.2 [d]	N.D.

Two way ANOVA followed by Student Newmann-Keuls; Values are expressed as mean ± SEM (n = 3). Different letters in the same line indicate significant differences ($p < 0.05$).[1] Expressed as milligram per gram of dry weight (DW). N.D.: not detected

Grace and co-workers showed that UV light stimulates the production of foliar chlorogenic acid content in plants [29]. Fully exposed leaves produced higher levels of chlorogenic acid, whereas in shaded leaves it similar chlorogenic acid contents were found between seasons. Besides, one of the abiotic stresses which affects temperate plants is the low temperature, so in autumn, an increase in the content of a range of cryoprotective substances with the purpose of maximize their cold tolerance it can be seen [30]. Considering this, it is possible to suggest that the plant from March used in this study was already accumulating the metabolites to be prepared for the winter. After this period, the rutin,

quercetin and chlorogenic acid concentration in the leaves decreases the development of the flowers in spring and resource allocation shifting from defense to reproduction [19].

2.3. Influence of Cultivar and Harvest Season on Extracts Antioxidant Activity

The antioxidant properties of the blueberry leaves extracts from different harvest months and cultivars by the DPPH (2,2-diphenyl-2-picrylhydrazyl) free radical capture method and the oxygen radicals removal ability method (ORAC) are shown in Table 3. The DPPH and ORAC methods are common analyses used to evaluate the antioxidant activities of medicinal plants. To the best of our knowledge, no report on the antioxidant activity of rabbiteye blueberry as a result of different harvesting months and cultivars from Brazil exists.

Table 3. Rabbiteye blueberry leaves antioxidant properties considering different harvesting months and cultivars by DPPH and ORAC methods

Cultivar	IC_{50} for DPPH (μg/mL)		ORAC Values (mmol Trolox/100 g)	
	December	March	December	March
Bluegem	105 ± 2^{a}	5.80 ± 0.04^{a}	211 ± 6^{a}	431 ± 8^{a}
Powderblue	60.1 ± 0.2^{b}	12.1 ± 0.6^{b}	374 ± 2^{b}	202 ± 9^{b}
Clímax	25 ± 2^{c}	12.39 ± 0.02^{b}	341 ± 5^{c}	283 ± 6^{c}
Florida M	25.6 ± 0.1^{c}	16.0 ± 0.2^{c}	178 ± 5^{d}	181 ± 9^{d}
Aliceblue	24.5 ± 0.2^{c}	12.8 ± 0.2^{b}	245 ± 5^{e}	338 ± 4^{e}
Means ± SD	48 ± 35	11 ± 4	270 ± 84	287 ± 102.01

Two way ANOVA followed by Student Newmann-Keuls; Values are expressed as mean ± SEM ($n = 3$). Different letters in the same column indicate significant differences ($p < 0.05$).

According to the results expressed in Table 3, the values ranged from 5.80 ± 0.04 to 105 ± 2 μg/mL (DPPH) and 178 ± 5 to 431 ± 8 mmol Trolox/100 g (ORAC). In general, the cultivars from March showed higher average antioxidant properties than the ones from the December collection, regardless of the test employed. Concerning the influence of the cultivar type, the Bluegem from March presented the highest antioxidant properties in both assays (5.80 ± 0.04 μg/mL DPPH and 431 ± 8 mmol Trolox/100 g ORAC).

The results demonstrated that there was a variation in the antioxidant activity depending on the cultivar and the collection period, regardless the method used in the evaluation. As previously exposed, changes in environmental conditions during the different seasons and genetic predisposition can explain these variations [15]. For the species *Vaccinium corymbosum*, Ehlenfeldt and Prior reported values of about 490.4 μmol Trolox/g (ORAC) for hydroalcoholic extracts of leaves from different cultivars [9] and Pervin, Hasnat and Lim reported values of about 0.12 ± 0.003 mg/mL by DPPH assay [20].

Moreover, in studies with blueberry fruits, the total antioxidants activities in six different varieties varied about 2.6 times according to the ORAC assay, and 2 times by the peroxyl radical scavenging capacity (PSC) assay [31]. The same was observed by Cardeñosa and co-workers, where the genotype influenced the antioxidant capacity and the content of the three groups of phenolics in blueberry fruits [32]. In accordance of Sarkar and co-workers, the genotype versus environment interactions are most critical in the in vitro anti-diabetic-relevant functionalities of blueberry bioactives [33].

The correlation between the phenolic compounds (total phenolic, flavonoid content, chlorogenic acid and rutin contents) with antioxidant activity (DPPH and ORAC) of blueberry leaves from December and March is shown in Table 4.

According to Table 4, rabbiteye blueberry leaves from December showed the highest correlation coefficient for interaction between DPPH and chlorogenic acid (0.99), which describes a strong positive correlation ($0.8 < r < 1$). On the other hand, interactions between DPPH and total phenolics (0.76) and the interaction between ORAC and total flavonoids (0.69) presented a moderate positive correlation

(0.5 < r < 0.8). Despite the high overall phenolics and flavonoids total content, no correlation was found for interactions between phenolics, flavonoids total content and chlorogenic acid versus DPPH or ORAC to blueberry leaves from March (Table 4).

Table 4. Correlation between the total phenolic content (TPC), total flavonoid content (TFC), chlorogenic acid content, rutin content and antioxidant capacities by free radical capture method DPPH (2,2-diphenyl-2-picrilhidrazil) and the ability method removing oxygen radicals (ORAC).

	DPPH		ORAC	
	December	March	December	March
TPC	0.76	0.43 [a]	0.25	0.40
TFC	0.30	0.08	0.69	0.05
Chlorogenic acid	0.99	0.65 [a]	0.24 [a]	0.69 [a]
Rutin	0.98	0.83	0.21 [a]	0.80

[a] showed negative correlation

In contrast, rabbiteye blueberry leaves from March showed a strong positive correlation (0.8 < r < 1) between rutin and DPPH (0.83) and between rutin and ORAC (0.80). The same was observed between DPPH and chlorogenic acid (0.98) to rabbiteye blueberry leaves from December. This correlation helps to understand the contribution of rutin to the antioxidant capacity of rabbiteye blueberry leaves. In accordance with Yang and co-workers [34], rutin play an important role in terms of antioxidant capacity against numerous in vitro antioxidant systems and this capacity depends on its concentration.

In conclusion, in view of the different phenolic compounds contents and the antioxidant properties identified in the leaves, this study contributes to better understand the influence of different cultivars and harvest seasons, as well as, it extends the blueberry aplications not limiting them only to its fruits. The results demonstrated that the Bluegem variety harvested in March is the most promising, and considering the high cost associated with growing blueberry fruits, the use of their leaves can be considered advantageous in this aspect. The byproducts derived from leaves could be used as infusions being a coadjuvant treatment for many conditions where oxidative stress is involved. Moreover, these byproducts can be viewed as intermediate products to produce final pharmaceutical and nutraceutical dosage forms.

3. Materials and Methods

3.1. Rabbiteye Blueberry Leaves

The rabbiteye blueberry leaves samples (*Vaccinium ashei*) were directly collected from the producer at the orchard in Golden Valley in the city of Erechim (Rio Grande do Sul State, Brazil) in December 2013 and March 2014 (coordinates 27°38'3"S and 52°16'26"W). The following cultivars: Alice Blue, Flórida M, Bluegem, Clímax and Powderblue were used. Dried voucher specimens are preserved in the Herbarium of the Department of Botany at the Federal University of Rio Grande do Sul (UFRGS, Porto Alegre, RS, Brazil) under the registeration numbers ICN 186811, ICN 186812, ICN 186813, ICN 186814, ICN 186815, respectively. The extract preparations were carried out with dried leaves obtained under the following conditions: the leaves were dried in an oven (40 °C) and grounded in a knife mill (80 μm).

3.2. Reagents and Standards

Only chemicals of analytical grade were used. Methanol, ethanol, ascorbic acid, gallic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Folin-Ciocalteau phenol reagent (2 mol/L), aluminum chloride, sodium carbonate, DPPH radical (1,1-diphenyl-2-picrylhydrazyl), coumarin, 4-hydroxycoumarin, catechin, quercetin, rutin, chrysin, kaempferol and rosmarinic and caffeic acids were acquired from Sigma Aldrich Chemical Co. (St. Louis,

MO, USA). Fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and (AAPH) 2,2′-azobis-2-amidinopropane were obtained from Aldrich (Milwaukee, WI, USA). For the ORAC assessment, a fluorescein stock solution (407 μmol/L) was prepared in a potassium phosphate buffer (75 mmol/L; pH 7.4) and kept at 4 °C in the dark. The work solution of fluorescein (81 nmol/L) was freshly prepared after dilution with phosphate buffer.

3.3. Extracts Preparation

A total of 60 g of the powdered material of each cultivars were extracted three times (three aliquots of 400 mL) by maceration using water-ethanol (1:1, *v*/*v*) for 72 h at room temperature [35]. The blueberry hydroethanolic leaf extracts were concentrated under low pressure at 50 °C and dried by lyophilization. The extracts were stored in amber glass at 10 °C to further analysis.

3.4. Total Flavonoids Content (TFC)

Total flavonoid contents of different extracts were quantified by the method described by Woisky and co-workers [36] based on the flavonoid-aluminum complex formation. An aliquot of hydroethanolic leaf extract (0.5 mL) diluted in methanol (1 mg/mL) was mixed with aluminum chloride solution (0.5 mL, 2%; *w*/*v*) and methanol (2.5 mL) and incubated for 30 min at room temperature. Absorbance was measured at 420 nm using a UV-Vis spectrophotometer (Perkin Elmer, series 200, North Billerica, MA, USA. This way, the TFC was determined by interpolating the absorbance of the samples against a calibration curve ($y = 0.0072\ x + 0.0311$, $r = 0.9983$) constructed with rutin standard solution (5 to 150 μg/mL) and expressed as μg of rutin (rutin equivalents) per g of dry weight (DW).

3.5. Total Polyphenolic Content (TPC)

The total phenolic content quantification was performed by the Folin-Ciocalteau method [37]. Briefly, Folin-Ciocalteau reagent (0.5 mL; 2 mol/L) was added to hydroethanolic leaf extracts (1 mL) diluted in methanol (0.15 mg/mL) and this mixture was left standing for 5 min before the addition of 20% Na_2CO_3 (2 mL). The solution was then resting for 10 min before measurement at 730 nm in the UV-Vis spectrophotometer (Perkin Elmer, series 200). The total phenolic content was expressed in milligrams equivalent of gallic acid (GAE) per g of dry weigh (DW). The equation obtained for the calibration curve of gallic acid in the range of 100–1000 μg/mL was $y = 0.0014\ x + 0.088$ ($r = 0.9975$).

3.6. Polyphenolics Qualitative Identification by HPLC-UV-DAD

High performance liquid chromatography (HPLC-UV-DAD) was performed with a Prominence Auto-Sampler (SIL-20A) equipped with Shimadzu LC-20AT (Shimadzu, Kyoto, Japan) pumps connected to a DGU-20A5 degasser and a CBM-20A integrator. A SPD-M20A UV-Vis DAD and LC Solution 1.22 SP1 software were used. Analyses were carried out using a Phenomenex C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles. Injection volume was 40 μL and the gradient elution was conducted according to the slightly modified method [38]. The UV absorption spectra was recorded in the 200–400 nm range. Each hydroethanolic leave extract was individually screened for the presence of the following polyphenolic compounds: gallic, chlorogenic and caffeic acids, coumarin, 4-hydroxycoumarin, catechin, quercetin, rutin, chrysin, kaempferol and rosmarinic acid. The compound identification was performed by comparing their HPLC retention times and UV absorption spectra with the respective commercial standards. Standard stock methanolic solutions were prepared in the concentration range of 2.5–60.0 μg/mL. Quantification was carried out by integrating the peaks using external standard method at 327 nm wavelength for chlorogenic acids and 365 nm for quercetin and rutin. Chromatographic operations were carried out at room temperature and in triplicate.

3.7. *Antioxidant Activity Analysis*

3.7.1. DPPH assay

The DPPH assay was used to measure radical scavenging activity [39]. Different levels (250, 125, 50, 25, 10 and 5 μg/mL) of samples were prepared in ethanol. The DPPH ethanol solution (1 mL; 0.3 mM) was added to 2.5 mL of sample solutions at room temperature. After 30 min of incubation, the decrease in absorbance at 518 nm was evaluated. A blank solution was prepared with ethanol (1.0 mL) plus plant extract solution (2.5 mL). As negative control, a DPPH solution (1.0 mL; 0.3 mM) plus ethanol (2.5 mL) was used. The positive controls were the ascorbic acid standard solutions. The inhibition percentage the DPPH solution absorbance was calculated using: 100 − {[(Abssample − Absblank) × 100]/Abscontrol}. The results were expressed as concentration of the extract required to scavenge 50% DPPH free radicals (IC_{50}) in μg/mL.

3.7.2. ORAC Assay

The oxygen radical absorbance capacity (ORAC) assay was performed according to Ou, Hampsch-Woodill and Prior with modifications [40]. An aliquot of fluorescein solution (150 μL) was added to diluted extract or Trolox standards (25 μL, 0–96 μmol/L) prepared in phosphate buffer in a black 96-well plate and incubated at 37 °C for 10 min. The reaction was initiated with 25 μL of the peroxyl radical generator AAPH (152 mmol/L) prepared before its use. The fluorescence was measured (λexc = 485 nm and λem = 528 nm) every minute for 90 min using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA) maintained at 37 °C. Standards and samples were prepared in triplicate. Results for ORAC were determined by graphical regression analysis and expressed as mM of Trolox equivalents per 100 g of extract.

3.8. *Statistical Analysis*

Results were expressed as mean ± standard error mean (SEM). Student's t-test was used for comparison between two means and two -way analysis of variance (ANOVA) followed by student Newmann-Keuls was used for comparison of more than two means. A difference was considered statistically significant when $p < 0.05$. The IC_{50} values were calculated from linear regression analysis. The Pearson correlation analysis between antioxidant activity and total phenolic and flavonoids, chlorogenic acid and rutin content was performed.

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Sample Availability: Not available.

5 CAPÍTULO 2

Avaliação do efeito antidepressivo de extratos hidroalcoólicos de folhas de *Vaccinium ashei* Reade livres e nanoencapsulados

CAPÍTULO 2

Avaliação do efeito antidepressivo de extratos hidroalcoólicos de folhas de *Vaccinium ashei* Reade livres e nanoencapsulados

2.1 INTRODUÇÃO

O capítulo anterior (Capítulo 1) contemplou a análise fitoquímica dos extratos de diferentes cultivares de *Vaccinium ashei* Reade. Com base nos resultados obtidos, pode-se observar a presença de elevados teores de compostos fenólicos os quais, segundo a literatura, estão relacionados com a prevenção e tratamento de diversas propriedades terapêuticas. Dentre estes compostos fenólicos destaca-se a presença de ácido clorogênico e rutina os quais, em estudos prévios demonstraram redução no tempo de imobilidade em modelos animais clássicos para a atividade do tipo antidepressiva, sem alteração na atividade locomotora (GUAN; LIU, 2016).

A partir disso, considerou-se a hipótese de que os extratos de *Vaccinium ashei* Reade pudessem apresentar resultados promissores neste sentido. Desta forma, considerando que a cultivar Clímax é a mais produzida no Brasil, a mesma foi selecionada para dar continuidade aos estudos.

Primeiramente, conforme descrito no manuscrito 1, intitulado “Acute and chronic antidepressant-like effects of hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) leaves” foram realizados testes de atividade do tipo antidepressiva em modelos agudos e crônicos nos extratos hidroalcóolicos da cultivar Clímax, coleta de dezembro de 2013.

A partir dos resultados positivos obtidos para este extrato reproduziu-se o modelo agudo da atividade antidepressiva nos extratos hidroalcóolicos da cultivar Clímax, coleta de março de 2014. Contudo, este extrato não demonstrou atividade do tipo antidepressiva o que fomentou a possibilidade de desenvolvimento de nanopartículas poliméricas contendo o extrato associado às mesmas, o que resultou no manuscrito 2, intitulado “Nanoencapsulation increases the antidepressant-like and antioxidant effects of Rabbiteye Blueberry (*Vaccinium ashei*) leaves extract”.

As nanoestruturas foram produzidas no Laboratório de Tecnologia Farmacêutica da UFSM e os ensaios farmacológicos na URI – Universidade Regional Integrada do Alto Uruguai e das Missões, Campus de Frederico Westphalen.

5.1 MANUSCRITO 1 - ACUTE AND CHRONIC ANTIDEPRESSANT-LIKE EFFECTS OF HYDROALCOHOLIC EXTRACT OF RABBITEYE BLUEBERRY (*VACCINIUM ASHEI* READE) LEAVES

Verciane Schneider Cezarotto [a,b], Rafaela Dal Pupo Pizzi [b], Adriel dos Santos [b], Karen Freitas Santos [b], Carlos Eduardo Blanco Linares [b], Ana Cristina Stein [b], Leticia Cruz [a,*]

[a] Departamento de Farmácia Industrial, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
[b] Departamento de Ciências da Saúde, Curso de Farmácia, Universidade Regional Integrada do Alto Uruguai e das Missões, Frederico Westphalen, RS 98400-000, Brazil

*Corresponding author:
Letícia Cruz, Departamento de Farmácia Industrial, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal de Santa Maria, Santa Maria, 97105-900, Brazil.
Phone: +55 55 32209373. Fax: +55 55 32208149.
E-mail: leticiacruz@smail.ufsm.br

E-mail addresses:
verciane@uri.edu.br (V. S. Cezarotto), rafaelapizzi@yahoo.com.br (R D. Pizzi), adriel@uri.edu.br (A. dos Santos), karen@uri.edu.br (K. F. Santos), clinares@uri.edu.br (C. E. B. Linares), anacristina@uri.edu.br (A. C. Stein), leticiacruz@smail.ufsm.br (L. Cruz).

Abstract

Ethnopharmacological relevance: Several recent studies have reported that rabbiteye blueberry (*Vaccinium ashei* Reade) leave have functional activities. However, the hydroalcoholic extract effects on acute and chronic depression animal models have not been evaluated until now.

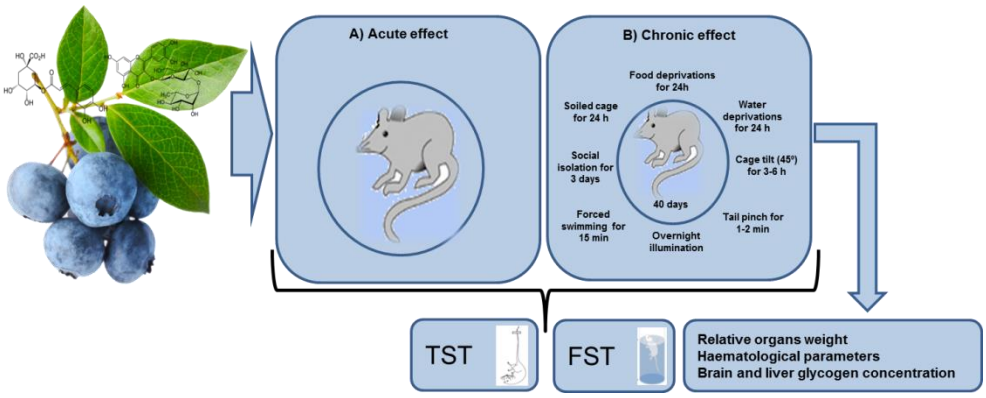
Aim of the study: This study investigated whether the hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) leaves (HEV) exhibits antidepressant-like effect on acute and chronic depression animal models.

Materials and Methods: The effect of HEV (10, 25 and 50 mg/kg, p.o) on the immobility time was assessed in the forced swimming test (FST) and tail suspension test (TST), and locomotor activity was evaluated in the open field test (OFT). Besides, it was evaluated the chronic antidepressant-like effect in rats exposed to unpredictable chronic mild stress (UCMS) model. UCMS rats were orally administered with HEV (50 mg/kg, p.o) daily within the UCMS procedure. Additionally, the relative organs weight, hematological parameters and glycogen concentration in brain and liver UCMS were evaluated.

Results: The results demonstrated that the HEV (50 mg/kg, p.o) acute administration significantly reduced the immobility time in both the FST and TST without altering the locomotor activity in the open field test (OFT). Chronic treatment with HEV (50 mg/kg, p.o) reversed the depressive-like behavior and the immobility time in the FST showing a significant decrease, without significantly changing relative organs weight, hematological parameters and glycogen concentration in brain and liver in rats UCMS.

Conclusion: The results indicate that HEV produced significant antidepressant-like effects, suggesting its therapeutical potential in the management of depression and other related disorders.

Graphical abstract:



Key Words:

Vaccinium ashei Reade, antidepressant-like, forced swimming test, tail suspension test, unpredictable chronic mild stress

1. Introduction

Depression is one of the most prevalent psychiatric disorders with a substantial lifetime risk and high personal and socio-economic burden (Millan, 2006; García-gonzález et al., 2017). Depression can be characterized by profound dysregulation of mood associated with other abnormalities, such as cognitive dysfunction, sleep and appetite disturbance, and fatigue (Fitzgerald, 2013). According to World Health Organization, the number of people living with depression increased by more than 18% between 2005 and 2015. Prevalence rates vary by age, peaking in older adulthood (above 7.5% among females aged 55-74 years, and above 5.5% among males) (WHO, 2017).

Early evidence suggested that noradrenaline, serotonin, and dopamine signaling has been implicated in the aetiology of depression (Krishnan and Nestler, 2008). These neurotransmitters play important roles in mediating behavioral activity induced by antidepressant drugs (Den Boer et al., 2000; Guan and Liu, 2016; Palucha-Poniewiera et al., 2017).

Although there are more than 30 antidepressants available (Fabbri et al., 2016), many display limited efficacy, a pronounced delay to onset of action, and undesirable adverse effects such as cardiotoxicity, hypertensive crisis, sexual dysfunction, cognitive déficits, body weight change and sleep disturbances (Masand and Gupta, 2002; Adell et al., 2005; Schechter et al., 2005; Millan, 2006; Ashok Kumar et al., 2014; Kivrak et al., 2014). In addition, treatment resistance has been observed in 30% of patients (Al-Harbi, 2012) and complete remission in only 50% of patients (Nestler et al., 2002; Fabbri et al., 2016).

Evidence has shown that genetic factors play a critical role in determining differences in treatment outcomes with antidepressants (Crisafulli et al., 2014) and changing classes of antidepressants is not associated with a significant advantage in terms of response or remission (Souery et al., 2011). Thus, it becomes urgent to develop news antidepressant drugs from natural source safer and more effective that could improve conventional antidepressant therapies (Guan and Liu, 2016).

According to the literature, a large number of natural products evaluated in a variety of animal models have shown psychotherapeutic potential demonstrating a growing interest in this research field (Gu et al., 2012; Ashok Kumar et al., 2014; Liu et al., 2014; Mannan et al., 2015; Xing et al., 2015; Lin et al., 2016; Shen et al., 2016; Wang et al., 2016; Xu et al., 2016). Among the natural products, blueberries can be highlighted due to their therapeutic potential. Rabbiteye blueberry (*Vaccinium ashei* Reade) is a blueberry species of native from the southeastern United States (Wang et al., 2011) and is the main cultivar in Brazil (Antunes et al., 2008). Although the commercial interest is directed to the fruits, the leaves have shown several activities, such as strong oxygen radical absorbance capacity (Ehlenfeldt and Prior, 2001; Piljac-Zegarac et al., 2009), antileukemic activity against sensitive HL60 cells *in vitro* (Skupien et al., 2006), hypotensive effects (Sakaida et al., 2007), hypolipidemic effects (Li et al, 2011; Nagao et al., 2008; Yuji et al., 2013), and the prevention of atherosclerosis (Basu et al., 2010), and cancer (Mechikova et al., 2010). However, blueberry leaves' traditional used for diabetes, inflammation, common cold and ocular disturbance treatment they are nowadays seldom (Ferlemi and Lamari, 2016).

Phytochemical studies demonstrated that the main constituints of rabbiteye blueberry leaves are phenolics compounds (Matsuo et al., 2010; Venskutonis et al., 2016; Vyas et al., 2013; Cezarotto et al., 2017), which have shown antidepressive potential (Shewale et al., 2012; Du et al., 2014; Donato et al., 2015; Ma et al., 2015; Guan and Liu, 2016; Li et al., 2016). Considering this, we hypothesized about the antidepressant potential of the rabbiteye blueberry (*Vaccinium ashei* Reade) leaves.

In the present study, forced swimming test (FST), tail suspension test (TST) and open-field test (OFT) were used to investigate the antidepressant-like effect of acute administration of the hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) leaves; in the sequence, a chronic unpredictable mild stress (UCMS) model was used which helps the understanding of the neurobiological consequences of chronic stress and the reversal and repair of those effects by chronic antidepressant treatment (Nestler et al., 2002; Willner, 2005; Willner, 2017).

2. Materials and methods

2.1 Plant material

Leaves of rabbiteye blueberry (*Vaccinium ashei* Reade; cv. clímax) were collected from the producer in the city of Erechim (Rio Grande do Sul, Brazil) in December 2013 (coordinates 27°38'3"S and 52º 16'26"W). Dried *voucher* specimen was deposited at herbarium of the Federal University of Rio Grande do Sul (UFRGS, Porto Alegre, RS) under the registration number ICN 186814. The leaves were dried in an oven (40 °C) and finely ground (80 µm).

2.2 Extract preparation

Rabbiteye blueberry (*Vaccinium ashei* Reade) leaves hydroalcoholic extract (HEV) was obtained from dried and powdered plant material (60 g) extracted three times (three aliquots of 400 mL) by maceration using water:ethanol (1:1, v/v) for a period of 72 h at room temperature (Cezarotto et al., 2017). The HEV was, filtrated, concentrated under low pressure at 50 °C to eliminate the solvent and dried by freeze-drying. The extract was stored in amber glass at 10 °C to further analysis.

2.3 Animals

Behavioral tests were carried-out with adult male Wistar rats (60 days old, 200–300 g) and adult male Balb-C mice (30 days old, 25–35 g) purchased from the vivarium at Universidade Regional do Médio e Alto Uruguai e das Missões, Campus Frederico Westphalen (Brazil). The animals were housed by five rats or five mice in plastic cages (rats, 42 cm x 27 cm x 16 cm; mice 17 cm x 28 cm x 13 cm) and were kept under a 12 h light/dark cycle (lights on at 7 a.m.) at constant temperature of (22 ± 2 ºC) and humidity (60% RH), with free access to standard certified rodent diet and tap water.

All experimental protocols were approved by The Animal Care Local Ethical Committee (CEUA URI-FW; protocol 004/2015) and performed according to Brazilian law (Brazil, 2008), which are in

compliance with the International guiding principles for biomedical research involving animals (CIOMS, 1985).

2.4 Drugs and reagents

For the behavioral experiments, the following drugs were used: fluoxetine hydrochloride and imipramine hydrochloride from Galena® (Porto Alegre, Brazil). Polysorbate 80 was from Merck (Darmstadt, DE). All other reagents were of analytical grade and were purchased from local supplier.

2.5 Acute Antidepressant-like effects

2.5.1 Forced swimming test (FST) in mice

The FST is widely used for detecting antidepressant activity and was carried out according to Porsolt et al. (1977) with minor modifications. Briefly, mice were individually placed in an inescapable acrylic cylinder (25 cm tall; 10 cm diameter) filled with water (23 ± 1 °C). The duration of immobility (in seconds) was scored for 6 min after 1 h administration. Immobility time was recorded when the mouse remained floating motionless or making only the movements necessary to keep its head above water. HEV were suspended in saline with 2% of polysorbate 80. Different groups of mice were acutely treated, per os (10 mL/Kg), with HEV (10, 25 or 50 mg/kg); imipramine (20 mg/kg) and 2% polysorbate 80 solution in saline (vehicle control group).

2.5.2 Forced swimming test (FST) in rats

The FST was conducted according to the method of Porsolt et al. (1978) with minor modifications. Briefly, rats were placed in a plexiglass cylinder separately (40 cm tall, 18 cm diameter) filled with water (23 ± 1 °C). The rats were submitted to a first session of swimming for 15 min. At the end of the swimming exposition, the animals were removed from the water and gently dried. The treatments were administered 5 min, 19 and 23 h after the first swimming exposition. One hour after the last injection (24 h after the first swimming session), the animals were submitted to a second swimming exposure (5 min), and their immobility time was measured. Distinct groups of rats were treated, per os (1 mL/1kg), with HEV 150 mg/kg (3 administrations of 50 mg/kg); imipramine 60 mg/kg (3 administrations of 20 mg/kg); fluoxetine 90 mg/kg (3 administrations of 30 mg/kg); and 2% polysorbate 80 solution in saline (vehicle control group).

2.5.3*Tail suspension test (TST)*

The TST was conducted according to Steru et al. (1985) with minor modifications. Animals were suspended by tail 60 cm above the floor using adhesive tape (1 cm from the tip of the end) in a dim light room. Immobility time was recorded (in seconds) by a blind observer to treatment during 6 min, and mice were considered immobile when they hung passively and completely motionless. Distinct groups of mice were acutely treated, per os (10 mL/Kg), with HEV (10, 25 or 50 mg/kg); fluoxetine (30 mg/kg) and vehicle control group with 2% polysorbate 80 solution in saline.

2.5.4 Locomotor activity

The spontaneous locomotor activity was performed in the open-field (OFT). Thus, forty-five minutes after the administration, mice were individually placed in a transparent acrylic box (40 x 30 x 30 cm) with the floor divided into 24 equal squares. Animals were habituated to arena for 5 min and then, the number of crossings was recorded during a period of 6 min (Stein et al., 2012).

2.6 Chronic antidepressant-like effects

2.6.1 Unpredictable Chronic Mild Stress (UCMS) procedure

A UCMS procedure was adopted as described by Willner (1987; 2005) with slight modifications. The UCMS protocol consisted of various stressors: (1) food deprivation for 24 h, (2) water deprivations for 24 h, (3) cage tilt (45º) for 3-6 h, (4) tail pinch for 1 to 2 min, (5) overnight illumination, (6) forced swimming for 15 min, (7) social isolation for three days, and (8) soiled cage for 24 h. These stressors lasted for forty days and each cage of animals received one stress per day individually. From the twentieth day of the experiment, four groups (G1-G4) received the different treatments. The G1 group, non stressed, and G2 group, stressed, were treated with vehicle control (2% polysorbate 80 solution in saline). Groups G3 and G4, both stressed, were treated with fluoxetine (20 mg/kg) and HEV (50 mg/kg) respectively (Fig.1). After the UCMS procedure end, all rats were submitted to FST (conditions describe in 2.5.2).

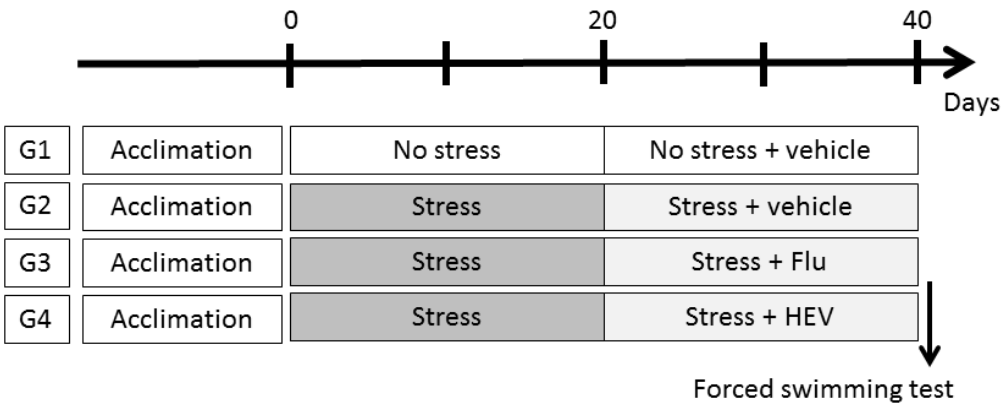


Fig. 1. Schematic representation of the experimental procedure

2.6.2 Body weight gain

Rats body weight was measured every five days throughout the UCMS procedure. Deltas body weights (ΔP) were calculated considering the difference between two adjacent weights using the following equations:

$\Delta P_{(n)}$= Body weight$_{(x)}$ - Body weight$_{(y)}$

2.6.3 Relative organs weight

After the end of UCMS procedure and behavioral testing, all rats were anesthetized with thiopental sodium (50 mg/kg, i.p.). The organs (adrenal glands, kidneys, spleen, liver, heart and brain) were immediately removed, weighted and stored. The relative organ weight (mg/g) was calculated considering the body mouse weight by using the following equation:

Relative weight organ (mg/g) = organ weight/ body rats weight

2.6.4 Blood sampling

At the end of the experiment, blood was collected by intra-cardiac puncture and transferred to polypropylene tubes containing EDTA as earlier reported by Malomo et al. (2002). Homogenized blood samples were analyzed immediately after the experiments completion.

2.6.5 Haematological analysis

Haematological parameters were estimated using automated haematological analyzer ABX Micros 60 Veterinary (HORIBA). The haemoglobin concentration (Hb), red blood cell count (RBC), white blood cell count (WBC), and platelet count (PLC) were thus determined. Differential leukocyte count was done with Giemsa-stained blood smerars. Lymphocytes, neutrophils, eosinophils, monocytes and basophils were identified and counted based on the morphology.

2.6.6 Measurement of glycogen concentration in brain and liver

After the end of UCMS procedure and behavioral testing (at 40° day) the liver and brain from rats that received HEV UCMS (50 mg/Kg), fluoxetine UCMS (20 mg/Kg), Vehicle UCMS and Vehicle groups were removed. The glycogen was isolated from the tissues as described by Krisman (1962) and the results expressed as mg of glycogen/g of tissue (Frederico et al., 2012).

2.7 Statistical analysis

Student’s t-test was used for comparison between two means and a one or two-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test depending on the experimental design was used for comparison of more than two means. A difference was considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Effects of acute HEV treatment on the immobility time in the FST, TST and locomotion activity

The acute effects of HEV on the immobility time in the FST and TST are shown in Fig. 2. After acute treatment, a one-way ANOVA revealed a significant effect of the treatment in both the TST [$F(4,44) = 7.01$, $P<0.001$] (1A) and FST [$F(4,50) = 8.148$, $P<0.001$] (1B) in mice and [$F(3,28) = 24.564$, $P<0.001$] (1C) in rats. Further post-hoc analysis revealed a significant decrease in the immobility time in reaction by the administration of HEV at the doses of 10, 25 and 50 mg/kg and imipramine at 20 mg/kg in the FST [$P<0.05$, $P<0.05$, $P<0.01$, $P<0.001$, respectively] and by the HEV administration at 50 mg/kg and fluoxetine at 30 mg/kg in the TST [$P<0.05$ and $P<0.001$, respectively], indicating that HEV exerts antidepressant-like effects in mice.

Furthermore, a significant decrease in the immobility time in rats was observed by the three HEV administrations at 50 mg/kg, fluoxetine at 30 mg/kg and imipramine at 30 mg/kg in the FST in rats [$P<0.001$, $P<0.001$, $P<0.001$, respectively].

To verify that the changes in immobility time in the FST and TST were not attributed to non-specific side-effects, animals treated with HEV were tested in the spontaneous locomotor activity. Treatment with HEV at 10 mg/kg, the lowest effective dose, did not exhibit significant effect ($P=0.825$) in the general locomotor activity in mice (Fig. 3) when compared to the vehicle group.

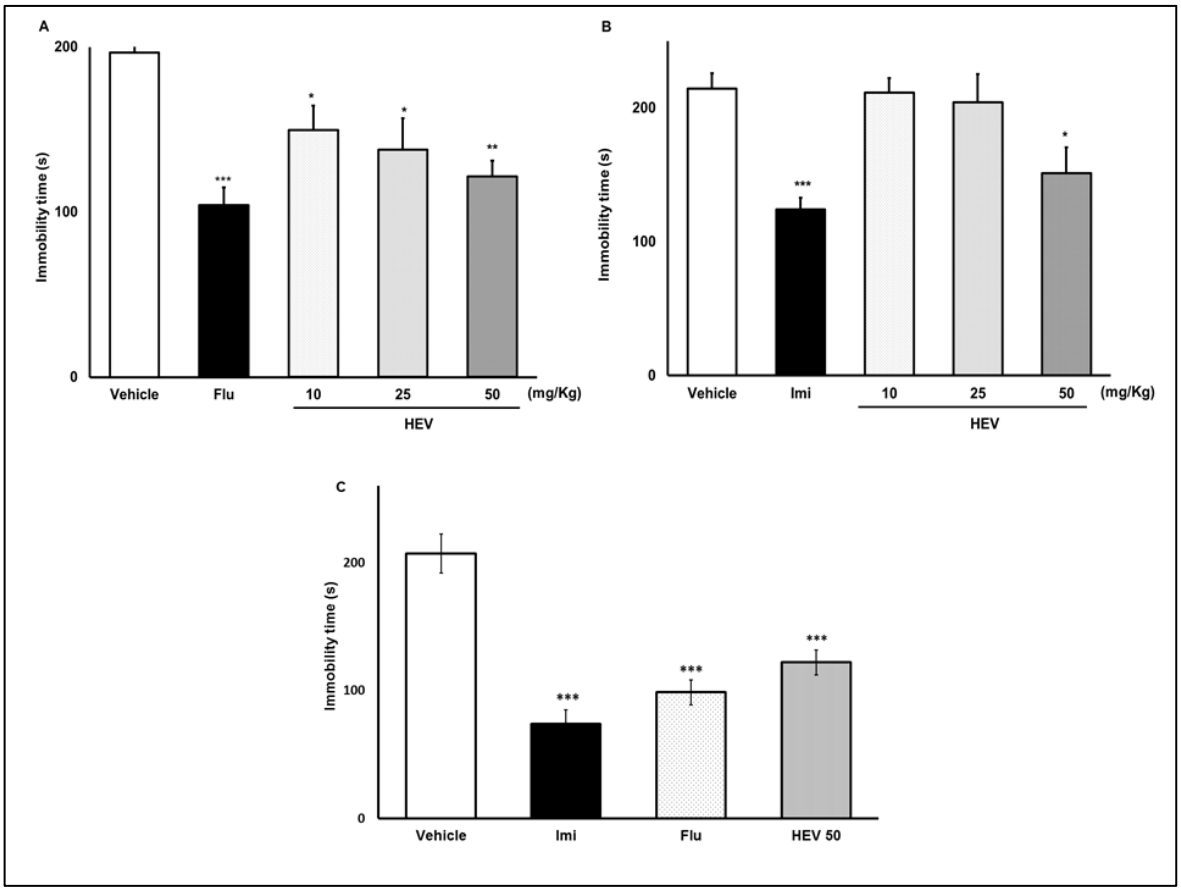


Fig. 2. Effects of hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) (HEV) after acute treatment on immobility TST and FST in mice and rats. Effect of10, 25 and 50 mg/Kg HEV and 30 mg/kg fluoxetine administration in mice TST (A); Effect of 10, 25 and 50 mg/Kg HEV and 20 mg/kg imipramine hydrochloride in mice FST(B); and effect three administration of 50 mg/Kg HEV, 20 mg/kg imipramine hydrochloride and 20 mg/kg fluoxetine in rats FST (C). Values are expressed as mean ± SEM (n=7-10 mice/rats/groups). One-way ANOVA followed by Student-Newman-Keuls comparisons: ***P<0.001, **P<0.01 and *P<0.05 compared to the respective control group (Vehicle).

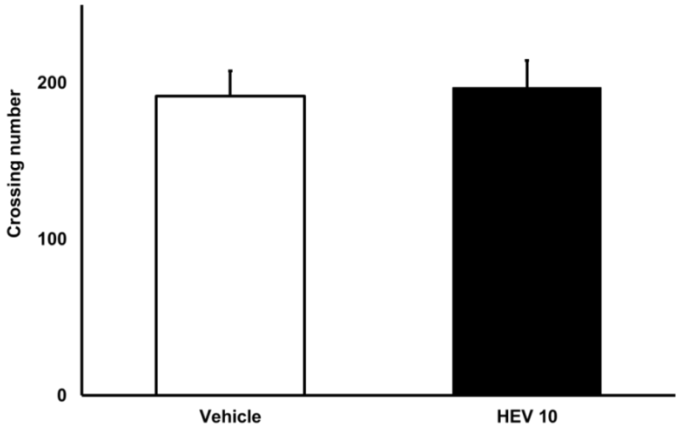


Fig. 3. Effects of hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) (HEV) after acute treatment on spontaneous locomotor activity performed in the open-field. The crossing number was measured in mice receiving 2% polysorbate 80 solution in saline (Vehicle) or 10 mg/Kg HEV. Student t test (P=0.825); values are expressed as mean ± SEM (n=10-12).

3.3. Effects of chronic HEV treatment on the forced swimming test in UCMS rats

The HEV chronic effects on the immobility time in the rats FST in UCMS rats are shown in Fig. 4. After chronic treatment (20 days, 1 x day), a one-way ANOVA revealed a significant effect of the treatment in the FST [F(3,27) = 34.474, P<0.001].

Post-hoc analysis revealed a significant decrease in the immobility time in reaction by the administration of HEV at 50 mg/kg and fluoxetine at 20 mg/kg in the FST [P<0.001 and P<0.001, when compared with both vehicle and vehicle UCMS, respectively].

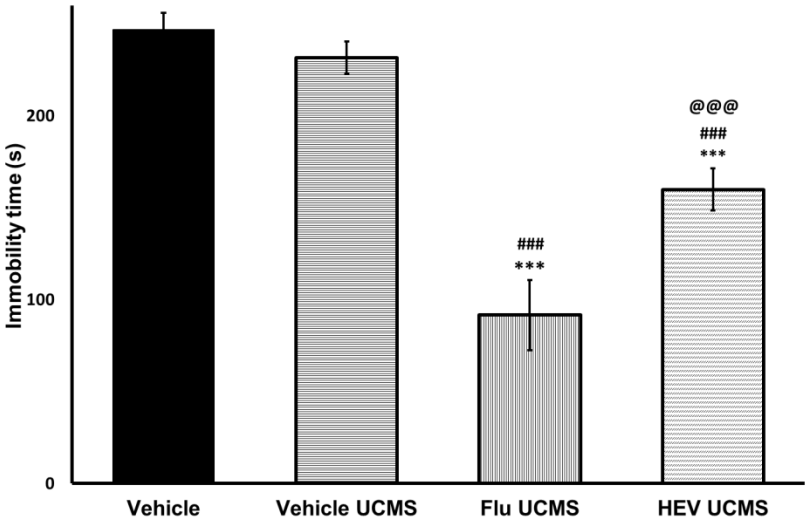


Fig. 4. Effects of hidroalcoolic extract of rabbiteye blueberry (*Vacciniumashei* Reade) (HEV) on immobility FST in UCMS rats. After 20 days of repeated treatment, the immobility time was measured in non-stressed rats receiving 2% polysorbate 80 solution in saline (Vehicle) and stressed rats receiving 2% polysorbate 80 solution in saline (Vehicle UCMS), 50 mg/Kg HEV (HEV UCMS) or 20 mg/kg fluoxetine (Flu UCMS).One way ANOVA followed by student Newmann-Keuls; values are expressed as mean ± SEM (n=7-10). Significant statistic difference ###P < 0.001 when compared with Vehicle group; ***P<0.001 when compared with Vehicle UCMS group; and @@@P<0.001 when compared with Flu UCMS group.

3.4. Effects of HEV treatment on the body weight gain in UCMS rats

The body weight gain in rats measured during UCMS procedure is shown in Fig. 5. The body weight gain under UCMS initially (ΔP1) was significantly reduced in rats compared with non-stressed control (P<0.001), possibly due to the effects of the stress regimen. This difference was not observed for ΔP2 and ΔP3. When treatments started at 20° day (ΔP4), fluoxetine at 20 mg/kg significantly reduced the body weight in rats under UCMS. HEV at 50 mg/kg appeared retard the decrease the body weight [two-way ANOVA, $F_{treatment}(3,279)= 40.649$, $p<0.001$; $F_{delta}(6,279)= 82.752$, $p<0.001$;$F_{interaction}(18,279)= 10.342$, $p<0.001$].

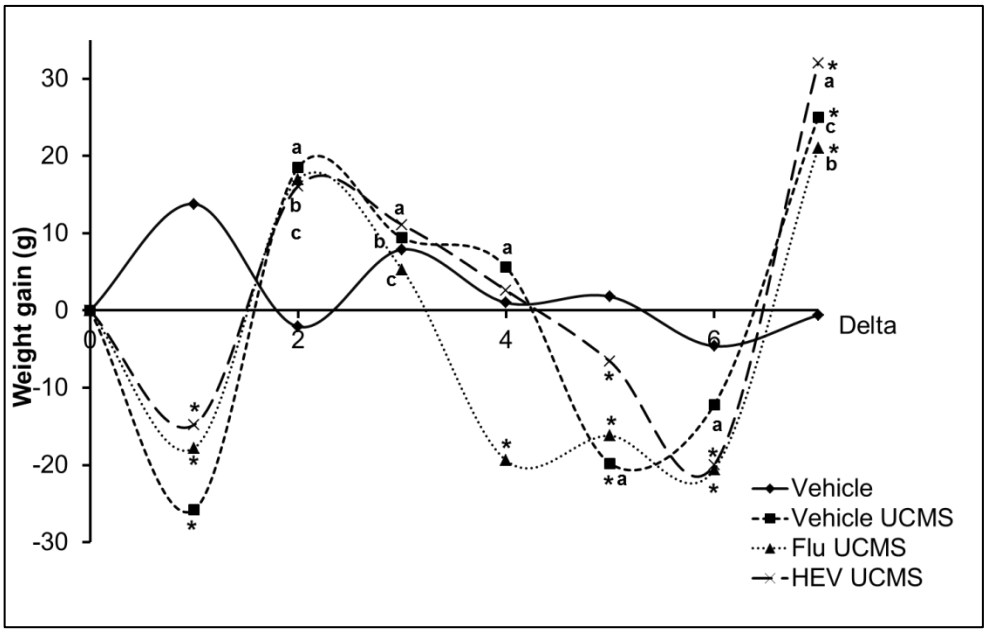


Fig. 5. Effects of hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) (HEV) treatment on body weight in rats under UCMS. Stressed rats received 50 mg/Kg HEV (HEV UCMS) and 20 mg/kg fluoxetine (Flu UCMS). None stressed and stressed vehicle groups received 2% polysorbate 80 solution in saline (Vehicle and Vehicle UCMS).Two-way ANOVA; values are expressed as mean ± SEM (n=10). Significant statistic difference ***P<0.001 when compared with Vehicle group. Letters (a, b e c) represent the differences (P<0.001) within the treatments (Vehicle UCMS, Flu UCMS and HEV UCMS, respectively) in relation to the first delta.

3.5 Effects of HEV treatment on the relative organ weight in UCMS rats

The Table 1 demonstrates the relative organ weight in rats under UCMS. Fluoxetine at 20 mg/kg treatment significantly increased relative weight in kidneys and brain compared to Vehicle UCMS and Vehicle group (P<0.05) and decrease relative weight in heart compared to Vehicle group (P<0.05). The same was observed to adrenal glands and spleen when compared to vehicle UCMS group (P<0.05). HEV at 50 mg/kg caused an increase in relative weights of spleen and brain compared to vehicle UCMS group (P<0.05) and decrease relative weight in heart compared to Vehicle group (P<0.05).

Table 1. Effects of hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) (HEV) treatment on relative organ weight in rats under UCMS

Organ	Relative Weight (mg/g)			
	Vehicle	Vehicle UCMS	Flu UCMS	HEV UCMS
Adrenal glands	0.015±0.023	0.014±0.002	0.018± 0.003*	0.014±0.005
Kidneys	0.61±0.05	0.62±0.04	0.68±0.03*#	0.64±0.03
Spleen	0.20±0.02*	0.18±0.02	0.21± 0.01*	0.20±0.02*
Liver	3.19±0.21	3.28± 0.19	3.09±0.19	3.24±0.19
Heart	0.33±0.03	0.29±0.02#	0.31±0.03#	0.29±0.02#
Brain	0.47±0.08	0.41±0.05	0.55±0.06*#	0.48±0.04

Stressed rats received 50 mg/Kg HEV (HEV UCMS) and 20 mg/kg fluoxetine (Flu UCMS). Non stressed and stressed vehicle groups received 2% polysorbate 80 solution in saline (Vehicle and Vehicle UCMS). One-way ANOVA; values are expressed as mean ± SEM (n=8-10). Difference from Vehicle UCMS *P<0.05 and from Vehicle #P<0.05

3.6 Effects of HEV treatment on the parameters haematological in UCMS rats

The Table 2 demonstrates the haematological parameters measured in rats under UCMS. An increase was observed in leucocytes from vehicle and fluoxetine stressed groups compared to vehicle (P<0.001). Also, leucocytes from fluoxetine stressed group was higher than HEV stressed group (P<0.001). No differences were observed between vehicle and HEV stressed groups (P>0.05). A decrease in erythrocytes, hemoglobin and hematocrit (P<0.001) and an increase in platelet (P<0.001) was observed in all rats stressed groups compared to vehicle group. Cell differential counting showed an increased lymphocytes cells in fluoxetine stressed group compared to HEV stressed group (P<0.001).

Table 2. The parameters hematological measured in rats after UCMS procedure

Hematological parameters	Vehicle	Vehicle UCMS	Flu UCMS	HEV UCMS
Leucocytes	3.5±0.6	4.7±0.2**	5.1±1.0**, &&&	3.8±0.5
Erythrocytes	8.1±0.3	7.3±0.3***	6.8±0.5***	7.1±0.4***
Hemoglobin (g/dL)	14.5±0.5	13.2±0.6**	12.9±1.0***	12.9±0.8***
Hematocrit	42.2±1.5	37.7±2.1***	35.2±2.8***	37.3±2.3***
Platelet ($\times 10^3$/µL)	314.9± 236.4	588.4±31.9***	556.4±83.4***	603.5±74.8***
Lymphocytes (%)	83.5±9.4	81,3±6.7	88.9±5.3&&&	80.0±4.0
Neutrophils (%)	9.9±2.6	14.5±5.0@@@	5.7±2.5	10.5±3.1
Eosinophiles (%)	1.2±1.0	-	2.1±1.1	2.0±1.2
Monocytes (%)	4.6±2.3	4.8±2.1	2.0±2.5	5.8±3.2
Basophiles (%)	0.9±0.9	-	-	0.6±1.1

Stressed rats received 50 mg/Kg HEV (HEV UCMS) and 20 mg/kg fluoxetine (Flu UCMS). Non stressed and stressed vehicle groups received 2% polysorbate 80 solution in saline (Vehicle and Vehicle UCMS). One-way ANOVA; values are expressed as mean ± SEM (n=8-10). Difference from Vehicle **P<0.01; ***P<0.001;; Difference from Flu UCMS @@@P<0.001; Difference from HEV UCMS &&&P<0.001.

3.7 Effects of HEV treatment on the glycogen in brain and liver in UCMS rats

The Figure 6 demonstrates the glycogen concentration in brain and liver in rats under UCMS. UCMS regiment showed no significant changes in these parameters [F(3,38) = 2.464, P=0.079] and [F(3,38)= 2.764 P=0.056], respectively.

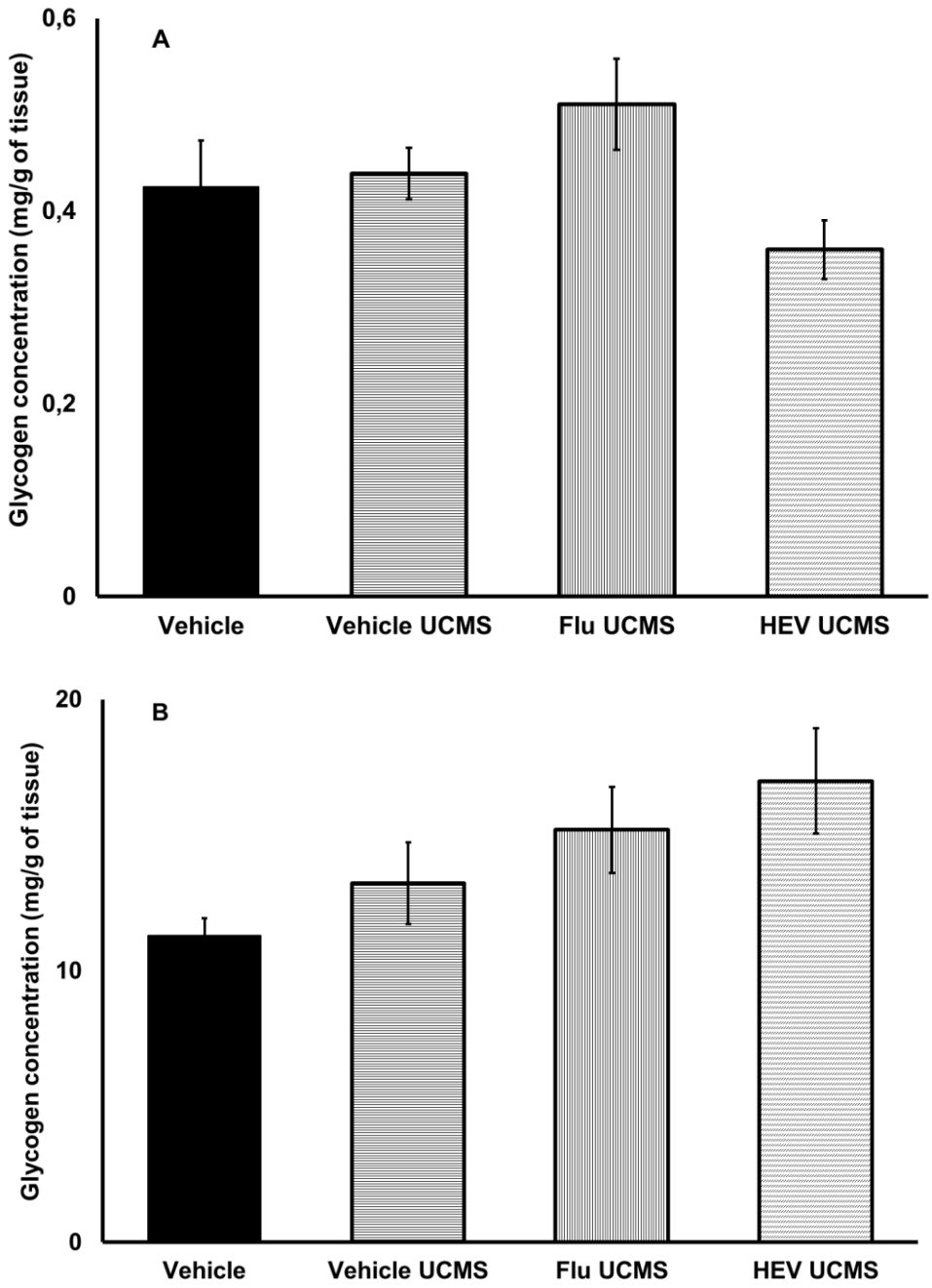


Fig. 6. Effects of hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) (HEV) on the glycogen in brain (A) and liver (B) of the rats receiving 2% polysorbate 80 solution in distilled water (vehicle), and rats UCMS receiving 2% polysorbate 80 solution in saline (vehicle UCMS), 50 mg/Kg HEV (HEV UCMS) and 20 mg/kg fluoxetine (Flu UCMS). One-way ANOVA; values are expressed as mean ± SEM (n=10).

4. Discussion

Despite the wide pharmacological activity attributed to the rabbiteye blueberry (*Vaccinium ashei* Reade) leaves, there are no scientific reports evaluating its pharmacological effects for treating mood disorders.

In the present study, it was found that the hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei* Reade) leaves (HEV) produced significant antidepressant-like effects in TST and FST, without modifying the locomotor activity.

FST and TST have been widely used to screen for antidepressant activity. The behavioral despair model represents a valuable tool in the search for therapeutic alternatives for this pharmacological activity (Krishnam and Nestler, 2008; Gong et al; 2014). The immobility displayed by rodents when subjected to unavoidable stress reflects a state of despair or lowered mood, which are thought to reflect depressive disorders in humans that is reduced by treatment with antidepressant drugs (Shewale et al., 2012). It was observed that HEV (50 mg/Kg) treatment significantly reduced the immobility time in both models.

This dose able to reduce the immobility time in both models is significantly lower than those needed to produce an antidepressant effect of the *Hypericum perforatum* L. (Hypericaceae), medical plants commonly called St. John's wort, which has long been recognized as a treatment for depression (500-1800 mg/die containing 0.96-2.7 mg/die total hypericins) (Butterwech et al., 2003; Kasper et al., 2010; Galeotti, 2017).

Besides that, to avoid the false-positive results, the effect of HEV on locomotor activity was evaluated in OFT. These results showed that treatment with HEV 10 mg/kg did not alter the general locomotor activity.

In our previous study, rabbiteye blueberry (*Vaccinium ashei* Reade) leaves (cv. climax) (Cezarotto et al., 2017), showed high total flavonoids and polyphenolic contents. Besides that, the chromatographic profile identified two phenolic compounds most abundant, chlorogenic acids (CGA, 3-*O*-caffeoylquinic acid) and rutin, important flavonoids, have been reported to possess antidepressant-like effect in studies with animal models (Guan and Liu, 2016). Although we cannot rule out the participation of other components in the antidepressant-like effect of the HEV, this result suggests a significant role for rutin and CGA in the antidepressant properties.

Noldner and Schotz (2002) reported that ethanolic *Hypericum* extract (E1) significantly shortened the time spend immobile in the FST and methanolic *Hypericum* extract (M1) was inactive (both at 300 mg/kg). Analytical characterization using HPLC showed that the inactive extract had a reduced level of the rutin (E1: 3.5%, M1: 0.8%). The authors reported that rutin could therefore directly or indirectly enhance the bioavailability of the other constituents necessary for the biological activity suggesting that rutin is essential for the activity. These evidences have been confirmed by Wurglics and Schubert-Zsilavecz (2006) and Herrera-Ruiz et al. (2011).

In later, Machado and co-workers (2008) reported the involvement of serotonin and noradrenaline systems in the antidepressant-like action of rutin. These authors investigated the antidepressant potential of the ethanolic extract of *Schinus molle* L. and rutin isolated and they found doses of 600-

1000 mg/kg (p.o.) and 0.3 e 3 mg/kg (p.o.), respectively, reduced the immobility time in the FST, but not in the TST. In the present work, the lower effective dose of HEV in TST was 10 mg/kg, p.o. (corresponding to 0.12 mg/Kg of the rutin), and in FST was 50 mg/kg, p.o. (corresponding to 0.61 mg/Kg of the rutin), and this result leads one to believe that the extract had a higher sensitivity for TST. According to Cryan et al. (2005a, 2005b), both TST and FST are good predictive tests of antidepressant-like effect, however, TST presents a greater sensitivity for selective serotonin reuptake inhibitors, such as fluoxetine. Thus, it is suggested that HEV may have a mechanism of action involving serotonin neurotransmission.

Furthermore, extracts prepared using 75% ethanol of *Hemerocallis Citrina*, rich in rutin, significantly decreased the immobility times in the TST (400 mg/kg, p.o.), and increased the serotonine and dopamine levels in the central nervous system proving that the antidepressant-like activity of extract might be related to the serotonergic and dopaminergic systems (Du et al., 2014).

Park et al. (2010) reported the antidepressant activities of the CGA isolated from the *Artemisia capillaris* Thunb as manifested in the FST, TST and rotarod test models of depression CGA (30 mg/kg/day, for 14 days) significantly reduced the immobility period in both TST and FST. The expression of the pituitary gland and hypothalamic POMC mRNA or plasma β-endorphin levels was increased by the extract and CGA, suggesting that the *Artemisia capillaris* Thunb. And CGA increase β-endorphin, which may perform as an important physiological regulator in response to depression.

Posteriorly, Wu et al. (2016) demonstrated that the extract of *Eucommia ulmoides*, rich in CGA, at 200 and 400 mg/kg/day was orally administered for 7 days and showed antidepressant-like effects in the TST in mice. The authors also reported that CGA is able to cross the blood-cerebrospinal fluid barrier to exhibit its neuron protection and promotion of serotonin release through enhancement synapsin I expression. Thus, it is strengthened our hypothesis of that antioxidant-like activity observed in this study by HEV extract may have a mechanism of action promoting serotonin release.

In order to confirm the antidepressant-like effects in mice exerted by HEV, the UCMS procedures were used. In the UCMS model of depression, rats or mice are exposed chronically (greater than 2 weeks) to a constant bombardment of unpredictable micro-stressors (e.g., tilted cage, food or water deprivation, paired caging, continuous light, bed wetting). Stressed animals show depressive-like phenotype in behavioral tests, such as FST (Porsolt et al., 1978; Castagne et al., 2011; Ruan et al., 2014; Filho et al., 2015), that can be restored to normal levels by chronic treatment with antidepressant drugs (Czeh et al., 2016; Willner, 2017; Willner et al., 1987).

In this study, rats exposed to chronic stress exhibited depressive-like behaviors showed by a significant increase in the immobility time in the FST, which was reversed by the chronic administration (twenty days) of HEV at 50 mg/kg, as well as fluoxetine (20 mg/kg). The models that are based on environmental or social stressor are highly relevant in research aiming to understand the underlying pathophysiology of depression (Czeh et al., 2016).

The body weight gain in rats under UCMS was significantly altered which could be due to the direct effect of stress on the food intake behavior of the rats. Since the UCMS paradigm effectively produce a number of chronic stress-related changes, including reduced body weight gain (Ulrich-Lai et al, 2006), these results confirm the stress regimen of this study.

Moreover, fluoxetine (20 mg/kg) treatment decreased significantly body weight gain in accordance with a previous report (Gamaro et al., 2008). HEV (50 mg/Kg) appeared retard the decrease of the body weight gain. The effects of stress on body weight are still controversial and these discrepancies may be due to the variety in animal strains, stress schedule and stimuli intensity (Jiang et al., 2013). Although some studies showed no changes in the body weight (First et al., 2011; Jiang et al., 2013), there are some reports indicating a decrease in the body weight (Liu and Zhou, 2012; Li et al., 2013; Lin et al., 2016; Xu et al., 2016). Body weight gain has been attributed to corticotrophin-releasing hormone (CRH), the main hypothalamic regulator of the pituitary-adrenal axis and has been a major anorexiogenic peptide, whose secretion is stimulated by neuropeptide Y (NPY) (Charmandari et al., 2005).

Since depressed patients often struggle body weight changes (Pytka et al., 2017), no change in body weight for more time by HEV is a positive indicator and represents a protection factor to the applied stress. Moreover, it indicates that chronic HEV treatment does not have significant toxicity, since the body weight changes serve as a crucial indicator of the general health of animals (Hilaly et al., 2004; Betti et al., 2012; Han et al., 2016;). The increase or decrease in body weight of an animal may indicate important physiological changes, such as liver or hormonal, failure to absorb components (Antonelli-Ushirobira et al., 2010) or anorexigenic effect (Betti et al., 2012).

UCMS regiment showed no significant changes in any of the evaluated organs. On the other hand, it was observed a mass gain in the adrenals, kidneys, spleen and brain of stressed rats that received fluoxetine (20 mg/Kg) and mass gain in the spleen and brain to stressed rats receiving HEV (50 mg/Kg).

The adrenal gland is an essential stress-responsive organ that is part of both the hypothalamic-pituitary-adrenal axis and the sympatho-adrenomedullary system (Ulrich-Lai et al., 2006). Stress in animals leads to hyperactivity of adrenals which increases the production of corticotropic hormone and ultimately results in increased weight of adrenals (Kishor et al., 2017). The maintaining weight of adrenal in normal level by HEV is beneficial and might be due to normal production of corticotropic hormone, which was not observed in treatment with fluoxetine.

In accordance with Rai et al. (2003), the increased weight of spleen following treatment might be due to the inhibition of recruitment of lymphocytes to blood from spleen, which is a major storage pool of lymphocytes. However, no change was observed in lymphocytes level.

For any group, no differences in liver weight were observed, which may indicate a possible safety of repeated administration of the treatments (Betti et al., 2012). Increased secretion of stress hormones, which are identified to increase the mRNA levels and metabolic activities in the hepatic cells, could be the reason behind increased weight of liver during stress (Kishor et al., 2017). Thus, these results indicate a possible safety of the extract in relation to the liver, an important marker of toxicity.

To differential blood cells count, results show that leukocytes in vehicle UCMS was increased compared to vehicle group. This result is in agreement with previous studies where different stressors have been associated with significant leukocytes count in rats and mice (Engler et al., 2004; Stefanski and Guner, 2006; Zager et al., 2007; Im et al., 2012). Results also showed that leukocytes in HEV UCMS group did increased like fluoxetine UCMS group compared to vehicle. This show that the HEV treatment

may be prevents leukocytes alterations in UCMS, and this may possible by the chemical profile constitutions of HEV. In the red cells series, results showed decreased erythrocytes in vehicle UCMS, fluoxetine UCMS and HEV UCMS compared to vehicle. These results may be explained by the fact that erythrocytes has been associated like an important part of innate immunity and play an important role in immune responses, eliminating immune complexes, improving phagocytosis, doing the presentation of antigens and activating complements through membrane surface receptors (Zhang et al., 2013).

Hemoglobin and hematocrit were decreased in vehicle UCMS, fluoxetine UCMS and HEV UCMS compared to vehicle. These results show that the pronounced decreased in these parameters possibly caused by the stress and the drugs do not prevent the alterations. In the other hand, platelet was increased in vehicle UCMS, fluoxetine UCMS and HEV UCMS compared to vehicle. Stressful conditions was associated with haematological changes, due to the activation of the hypothalamo-pituitary-adrenocortical (HPA) axis (Hasan et al., 1992; Toumi et al., 2016) thrombocytosis is an example of these changes, which may aim to prepare the organism for potential injury following the stressful experience (Toumi et al., 2016). A study by Zhuang et al. (2016) showed that after induced UCMS, the protein expression of IL-6 and TNF-α significantly increased and it is known that one of the mechanisms that stimulate thrombocytopoesis is by increased levels of IL-6. And in our study, none of the treated groups reversed the increase in platelet count. Also exposed to hematocrit and hemoglobin, this result is possibly caused by the stress and the drugs do not prevent the alterations.

In the differential cells count, lymphocytes in fluoxetine UCMS group were higher than HEV UCMS group. The absence of differences between HEV UCMS group and vehicles groups show that HEV do not produced significant alterations in lymphocytes. To neutrophils count, only vehicle UCMS group showed increased compared fluoxetine UCMS. The absence of differences between HEV UCMS, fluoxetine UCMS and vehicles groups show that HEV and fluoxetine do not produced significant alterations in this cell line. The absence of significant alterations in the differential cells count after HEV treatment are positive since the hematopoietic system is an important index of physiological and pathological status in humans and animals (Betti et al., 2012).

Glycogen is a branched polymer the main function of which is to act as energy and carbon storage in a molecular form readily available to tissues dependent on glucose oxidation. Brain glycogen content is a short-term energy source that supports local and specific neural activities, such as memory formation, sensory and sleep and wake cycles stimulation, as is protective under stress and pathological conditions. In contrast, liver glycogen provides glucose to the rest of the body during fasting periods (Duran and Guinovart, 2015). In this study UCMS regiment showed no significant changes in these parameters.

5. Conclusion

In summary, this study demonstrated for the first time the antidepressant-like effect of HEV leaves using acute and chronic animal models. Additionally, the results support a low toxicity potential of HEV because the repeated administration did not trigger any alteration in the evaluated parameters

(body weight gain, hematological markers and glycogen concentration). Thus, our data reinforce the great biological properties of *Vaccinium ashei* Reade and confirm its folk use as a natural medicine approach. New studies are being conducted in order to better elucidate the antidepressant-like effect of HEV as well as its putative mechanism of action.

Author contributions

V.S.C collected plant and extract preparation; V.S.C, A.C.S. and L.C. designed the study; R.D.P. and A.S. performed the acute and chronic depression animal models; K. F. S. and C. E. B. L. performed hematological parameters and glycogen concentration in brain and liver. All authors participated in the interpretation and analysis of the data and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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5.2 MANUSCRITO 2 - NANOENCAPSULATION PROMOTES THE ANTIDEPRESSANT-LIKE AND ANTIOXIDANT EFFECTS OF RABBITEYE BLUEBERRY (*Vaccinium ashei*) LEAVES EXTRACT

Verciane Schneider Cezarotto [a,b], Eduarda Piovesan Franceschi [b], Ana Cristina Stein [b], Luana Mota Ferreira [a], Tatiana Emanuelli [c] Luana Haselein Maurer [c], Leticia Cruz [a*]

[a]Departamento de Farmácia Industrial, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
[b]Departamento de Ciências da Saúde, Curso de Farmácia, Universidade Regional Integrada do Alto Uruguai e das Missões, Frederico Westphalen, RS 98400-000, Brazil
[c]Departamento de Tecnologia e Ciência dos Alimentos, Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria RS 97105-900, Brazil

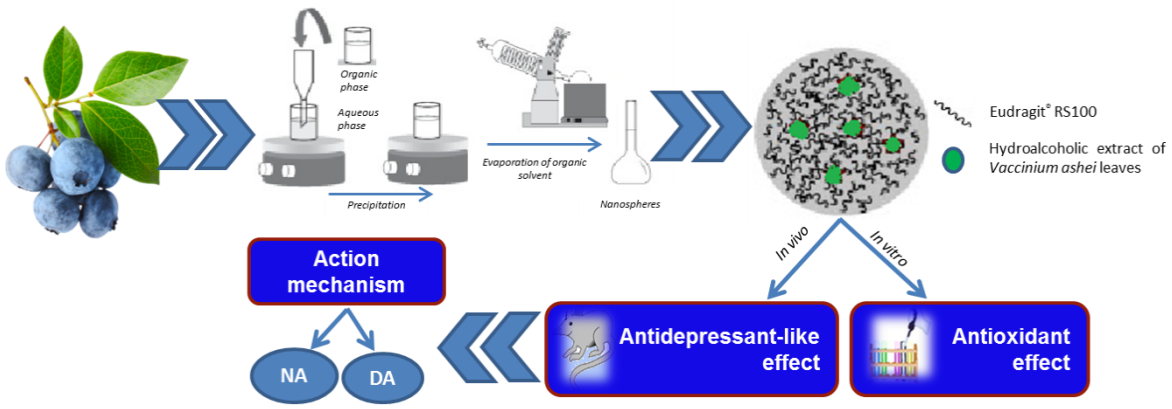
*Corresponding author:
Letícia Cruz, Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, Santa Maria, 97105-900, Brazil.
Phone: +55 55 32209373. Fax: +55 55 32208149.
E-mail: leticiacruz@smail.ufsm.br

E-mail addresses:
verciane@uri.edu.br (V. S. Cezarotto), duda-piovesan@hotmail.com (E. P.Franceschi), anacristina@uri.edu.br (A. C. Stein), luanamotaferreirra@gmail.com (L. M. Ferreira); leticiacruz@smail.ufsm.br (L. Cruz) luanahmaurer@gmail.com (L. H. Maurer); tatiemanuelli@gmail.com (T. Emanuelli)

Abstract

Hydroalcoholic extract of *Vaccinium ashei* leaves (HEV) was used for the preparation of polymeric nanoparticles (NPHEV) to evaluate and compare their *in vivo* antidepressant-like and *in vitro* antioxidant effects. NPHEV were developed using Eudragit® RS100 by the interfacial deposition of a preformed polymer method. Particle size, zeta potential, polydispersity index, pH and rutin and chlorogenic acid contents were evaluated. The antioxidant activity was analyzed by DPPH and ORAC methods. The *in vivo* antidepressant-like activity of NPHEV was assessed in mice by tail suspension and forced swimming tests. The involvement of the monoaminergic neurotransmission on the antidepressant-like activity of NPHEV was also evaluated *in vivo*. The results showed that NPHEV presented physicochemical characteristics compatible with nanosystems for drug delivery, and the antioxidant effect was confirmed by ORAC increase. Furthermore, NPHEV exhibited antidepressant effect demonstrated by the significant reduction of the immobility time in tail suspension and forced swimming tests, without altering the locomotor activity of mice in the open field test. The pretreatment with SCH 23390 (15 µg/kg, s.c., dopamine D1 receptor antagonist), sulpiride (50 mg/kg, i.p., dopamine D2 receptor antagonist), prazosin (1 mg/kg, i.p., α1-adrenoceptor antagonist) and yohimbine (α2-adrenoceptor antagonist) before NPHEV administration (5 mg/kg, p.o.) significantly prevented the anti-immobility effect in tail suspension test. These data provide the first evidence of the antidepressant-like activity of NPHEV, which is due to an interaction with dopaminergic and noradrenergic neurotransmission. Therefore, NPHEV is a potential new formulation intending depression treatment.

Graphical abstract:



Key Words:
Blueberry, polymeric nanoparticles, antidepressant-like, antioxidant activity, monoaminergic neurotransmission

1 Introduction

Plants are natural sources of various compounds with diverse biological activities offering treatment for several diseases (ARMENDÁRIZ-BARRAGÁN et al., 2016). Among plants with biological interest, *Vaccinium ashei* (*V. ashei*) fruit tree of the family Ericaceae, popularly known as blueberry (GOLDMEYER et al, 2014; SOUSA; PEIXOTO; TOLEDO, 1995), is worldwide recognized due to its high content of bioactive compounds and health benefits given by its consumption (FERLEMI; LAMARI, 2016; GIACALONE et al, 2011; JOSEPH; EDIRISINGHE; BURTON-FREEMAN, 2014; RAMIREZ et al., 2010; RODRIGUES et al., 2011).

Despite great interest in Blueberry fruits, the use of the leaves can also be considered an advantageous approach due to its high phenolic content, being reported three times higher than those observed in the fruits (EHLENFELDT; PRIOR, 2001; FERLEMI; LAMARI, 2016; PILJAC-ZEGARAC; BELSCAK; PILJAC, 2009; VYAS et al., 2013). For this reason, in recent years there is a growing scientific interest in them.

Blueberry leaves are considered a waste or byproduct of berry cultivation. In a previous study (CEZAROTTO et al., 2017), we have demonstrated high levels of chlorogenic acid and rutin in a blueberry leaves hydroalcoholic extract. These compounds have been associated with the following activities: antileukemic (SKUPIEN et al., 2006), antihypertensive (SAKAIDA et al., 2007), hypolipidemic (LI; LI; GENG, 2011; NAGAO et al., 2008; YUJI et al., 2013), atherosclerosis prevention (BASU et al., 2010) and anticancer (MECHIKOVA et al., 2010). In another recent study of our group, it was demonstrated for the first time that the acute administration of the blueberry leaves hydroalcoholic extract significantly reduced the immobility time in both forced swim and tail suspension tests, without altering the locomotor activity in the open field test (unpublished dada).

Regarding the literature, the clinical use of plant extracts represent a challenge because of their complex composition, low absorption of many compounds due to their water solubility and inadequate molecular size, and low active compounds amounts in the extract, which is related to a modest potency (BONIFÁCIO et al., 2014). In addition, some compounds including chlorogenic acid, anthocyanin, and gallic acid tend to be biologically unstable and prone to

degradation or oxidation (COIMBRA et al., 2011; HAN et al., 2015). This way, in order to propose a dosage form for the blueberry leaves hydroalcoholic extract, the development of nanoparticle suspensions was explored in this study.

Polymeric nanoparticles are solid colloidal particles ranging from 100 to 500 nm. Due to their size and unique physicochemical characteristics, nanoparticles offer some advantages to the encapsulated products, such as enhanced stability, controlled release, improved pharmacokinetics, decreased side effects, reduced dose with consequent improved activity (DONG et al., 2016; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Considering the nanoparticles potentialities and the antioxidant and antidepressant-like activities of *V. ashei* leaves, the aim of this study was to develop polymeric nanoparticles containing hydroalcoholic extract of *V. ashei* leaves (NPHEV) and to evaluate and compare with free extract their *in vivo* antidepressant-like and *in vitro* antioxidant effects. Besides, the involvement of the dopaminergic, noradrenergic and serotonergic neurotransmission in the NPHEV antidepressant-like effect was investigated. It is important to highlight that, as far as we know, this is the first report on the blueberry leaves extract nanoencapsulation.

2. Materials and methods

2.1. Chemicals

The following compounds and reagents were obtained from Sigma Aldrich (São Paulo, Brazil): methanol, ethanol, ascorbic acid, gallic acid, chlorogenic acid, DPPH radical (1,1-diphenyl-2-picrylhydrazyl), coumarin, 4-hidroxycoumarin, catechin, quercetin, rutin, chrysin, kaempferol, rosmarinic acid and caffeic acid, polysorbate 80 (Tween 80®), and sorbitan monooleate (Span 80®). Eudragit® RS 100 (Röhm Pharma, Germany) was supplied by Almapal (São Paulo, Brazil). Fluorescein (3‘,6‘-dihydroxyspiro[isobenzofuran-1[3H],9‘[9H]-xanthen]-3-one), Trolox (6-Hydroxy-2,5,7,8tetramethylchroman-2-carboxylic acid) and (AAPH) 2,2’-azobis-2-amidinopropanewere obtained from Aldrich (Milwaukee, WI). For the ORAC assessment, a fluorescein stock solution (407 μmol/L) was prepared in potassium phosphate buffer (75 mmol/L; pH 7.4) and kept at 4°C in the dark. The work solution of fluorescein (81 ηmol/L) was freshly prepared after dilution with phosphate buffer. For the behavioral experiments, the following drugs were used: fluoxetine and imipramine hydrochloride from Galena® (Porto Alegre, Brazil). *p*-chlorophenilalanine methyl ester (pCPA), (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepinehydrochloride

(SCH23390), prazosin and yohimbine were purchase from Sigma Chemical Co. (São Paulo, Brazil).

2.2 Plant material and extract preparation

Leaves of *Vaccinium ashei* (cv. climax) were collected in the city of Erechim, state of Rio Grande do Sul - Brazil (coordinates 27°38'3 "S and 52 º 16 '26" W) in March 2014. The *voucher* specimens were deposited at the herbarium of the Federal University of Rio Grande do Sul under the registered number ICN 186814. The leaves were dried in an oven (40 °C) and finely ground (80 µm). Hydroalcoholic extract (HEV) was obtained from dried and powdered plant material (60 g) extracted 3 times (3 aliquots of 400 mL) by maceration using water:ethanol (1:1, v/v) for 72 h at room temperature (CEZAROTTO et al., 2017). The extract was evaporated under reduced pressure at 50 °C to eliminate the solvent and dried by freeze-drying. The extracts were stored in amber glass at 10 °C to further analysis. The yield of the extract was 59.2% w/w.

2.3 Preparation of the nanoparticle suspension containing HEV (NPHEV)

Nanoparticles were prepared using Eudragit® RS100 by the method of nanoprecipition described by Fessi et al. (1986) with some modifications. Briefly, 50, 100 or 250 mg of HEV were dissolved in an organic phase (ethanol) containing 0.1% Eudragit® RS100 and 0.077% sorbitan monooleate. An aqueous phase containing 0.077% polysorbate 80 was also prepared. Both phases remained separately under moderate magnetic stirring at 40 °C. After 60 min, the organic phase was injected into the aqueous phase. After 10 min of stirring, ethanol was eliminated and water was concentrated by evaporation under reduced pressure to achieve 10 mL final volume. For comparison purposes, blank nanoparticles (NPHEV-b), without extract, were also prepared.

2.4. Nanoparticle suspensions characterization

2.4.1. Particle size analysis, polydispersity index, pH and zeta potential.

The mean particle size and polydispersity index (PDI) (n = 3) were measured at 25 °C by photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments, UK) after diluting the samples in ultrapure water (1:500). Zeta potential analyses (ZP) were performed

using the same instrument after samples dilution in 10 mM NaCl (1:500). The nanoparticles pH values were determined by directly immersing the electrode of a calibrate potentiometer in the formulations. Measures were performed at room temperature (25 ± 2 °C) in triplicate.

2.4.2. HPLC-DAD phenolic content analysis and encapsulation efficiency

The total phenolic content present in NPHEV (n = 3) was determined by diluting an aliquot of the samples in 10 mL methanol followed by sonication for 10 min. Samples were filtered through a 45 µm membrane and injected into the High performance liquid chromatography (HPLC-UV/DAD) system HPLC-UV/DAD was performed with Prominence Auto-Sampler (SIL-20A) equipped with Shimadzu LC-20AT (Shimadzu, Kyoto, Japan) pumps connected to a DGU-20A5 degasser and a CBM-20A integrator. UV-VIS detector DAD SPD-M20A and software LC Solution 1.22 SP1 were used. Analyses were carried out with a Phenomenex C_{18} column (4.6 mm x 250 mm; 5 µm). Injection volume was 40 µL and the gradient elution was conducted according to the slightly modified method (EVARISTO; LEITÃO, 2001). The UV absorption spectra were recorded in the 200-400 nm range. The compounds identification was performed by comparing their HPLC retention time and UV absorption spectrum with the respective commercial standards. Stock methanolic solutions of standards were prepared in the concentration range of 2.5-60.0 µg/mL. Quantification was carried out by integrating the peaks using external standard method at 327 nm wavelength for chlorogenic acid and 365 nm for rutin. Chromatographic operations were carried out at room temperature and in triplicate.

For the determination of the encapsulation efficiency, an aliquot of each sample was placed in a 10,000 MW centrifugal device (Amicon® Ultra, Millipore) and free phenolic compounds were separated from the nanostructures by ultrafiltration at 2,200 g for 10 min. The difference between the total and the free concentrations of the compounds, determined in the nanostructures and in the ultrafiltrate, respectively, was calculated as the encapsulation efficiency (EE%), according to the equation: EE = Total content − Free content/Total content × 100.

2.5. Antioxidant activities

2.5.1 DPPH radical scavenging assay

The DPPH (2,2-diphenyl-1-picryhydrazyl) assay was used to measure radical scavenging activity (MENSOR et al., 2001). HEV and NPHEV were diluted to final concentrations of 25, 20, 15, 10 and 5 µg/mL, in ethanol. An aliquot of 0.3 mM DPPH ethanol solution (1 mL) was added to 2.5 mL of sample solutions and allowed to react in room temperature. After 30 min the absorbance values were measured at 518 nm. Ethanol (1.0 mL) plus sample solutions (2.5 mL) was used as a blank. DPPH solution (1.0 mL; 0.3 mmol/L) plus ethanol (2.5 mL) was used as a negative control. The positive controls were the ascorbic acid standard solutions. The inhibition percentage the DPPH solution was calculated using: AA%= 100 – {[(Abssample - Absblank) x 100]/ Abscontrol}**.** The results were expressed as concentration of the HEV or NPHEV required to scavenge 50% DPPH free radicals (IC_{50}) in µg/mL.

2.5.2 Oxygen radical absorbance capacity assay (ORAC)

ORAC assay was conducted to measure the peroxyl radical scavenging activity according to Ou; Hampsch-Woodill; Prior (2001) with slight modifications. An aliquot of 150 µL of a fluorescein (81 nmol/L) solution was added to 25 µL of the diluted HEV (25 mg/mL), NPHEV (5 and 10 mg/mL) or Trolox standards (0-96 µmol/L) prepared in phosphate buffer 75 mmol/L pH 7.4 in a black 96-well plate and incubated at 37°C for 10 min. The reaction was initiated with addition of 25 µL of the peroxyl radical generator 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) 152 mmol/L prepared few minutes before use. The fluorescence was measured (λexc= 485 nm and λem= 528 nm) every minute for 90 min using a SpectraMax M5 plate reader (Molecular Devices, USA) maintained at 37°C. Standards and samples were made in triplicate. Results for ORAC were determined by regression relating Trolox concentrations and the net area under the kinetic fluorescein decay curve. The ORAC value of each extract was expressed as mmol/L of Trolox equivalents.

2.6 Antidepressant activity like

2.6.1 Animals

Male adult Balb-C mice (30 days old, 25–35 g) purchased from the vivarium at Universidade Regional do Médio e Alto Uruguai e das Missões, Campus Frederico Westphalen (Brazil). The animals were housed in groups of five mice in plastic cages (rats, 42 cm x 27 cm x 16 cm; mice 17 cm x 28 cm x 13 cm) and were kept under a 12 h light/dark cycle (lights on at 7 a.m.) at constant temperature of (22 ± 2 ºC) and humidity (60% RH), with free access to standard certified rodent diet and tap water. All experimental protocols were approved by The Animal Care Local Ethical Committee (CEUA URI-FW; protocol 004/2015) and performed according to the Brazilian law (BRAZIL, 2008), which are in compliance with the International Guiding Principles for Biomedical Research Involving Animals (CIOMS, 1985).

2.6.2 Tail suspension test (TST)

TST was conducted according to Steru et al. (1985) with minor modifications. Animals were suspended by tail 60 cm above the floor using adhesive tape (1 cm from the tip of the end) in a dim light room. Immobility time was recorded (in seconds) by a blind observer to treatment during 6 min and mice were considered immobile when they hung passively and completely motionless. Different groups of mice were acutely treated, per os (10 mL/Kg), with HEV (5, 10, 25 or 50 mg/kg); NPHEV (1, 2.5, 5, 10 or 25 mg/kg); fluoxetine (30 mg/kg) and vehicle control group with 2% polysorbate 80 solution in saline for HEV or NPHEV blank for NPHEV.

2.6.3 Forced swimming test (FST) in mice

The FST is widely used to detect antidepressant activity and was carried out according to Porsolt; Bertin; Jalfre (1977) with minor modifications. Briefly, mice were individually placed in an inescapable acrylic cylinder (25 cm tall; 10 cm diameter) filled with water (23 ± 1 °C). The duration of the immobility (in seconds) was scored for 6 min after 1 h administration. Immobility time was recorded when the mouse remained floating motionless or making only the movements necessary to keep its head above water. NPHEV (1, 2.5, 5, 10 or 25 mg/kg); imipramine (20 mg/kg) and vehicle control group NPHEV blank.

2.6.4 Monoaminergyc mechanism of action investigation

To evaluate the effect of NPHEV on the dopaminergic system, different groups were pre-treated with sulpiride (50 mg/kg, i.p., a dopamine D_2 receptor antagonist) or SCH 23390 (15 µg/kg, s.c., a dopamine D1 receptor antagonist) 30 min before the administration of NPHEV (5 mg/kg p.o). Sixty minutes later, the immobility was measured by TST. To assess the possible involvement of the noradrenergic neurotransmission on the antidepressant-like effect of NPHEV in the TST, independent groups of animals were pre-treated with prazosin (1 mg/kg, i.p., an α-1 adrenoceptor antagonist); yohimbine (1 mg/kg, i.p., an α-2 adrenoceptor antagonist) or vehicle; after 30 min they received NPHEV (5 mg/kg, p.o.) or vehicle and were submitted to TST 60 min later. The doses of the antagonists were based on previous studies from Müller et al. (2012). In order to investigate the serotonergic neurotransmission, animals were pretreated with pCPA (100 mg/kg, i.p., an inhibitor of serotonin synthesis) or vehicle, once a day, for four consecutive days, as described by Wang et al. (2008). Twenty four hours after the last pCPA or saline injection, animals were treated with NPHEV (5 mg/kg, p.o.) or vehicle and were submitted to the TST 60 min later.

2.6.5 Locomotor activity

The spontaneous locomotor activity was performed in the open-field test (OFT). Thus, 60 min after the administration of the NPHEV (5 mg/kg, p.o.), mice were individually placed in a transparent acrylic box (40 x 30 x 30 cm) with the floor divided into 24 equal squares, then, the number of crossings was recorded during a period of 6 min as described by (STEIN et al., 2012) with minor modifications.

2.7 Statistical analysis

Student's t-test was used for comparison between two means and a one or two-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test depending on the experimental design was used for comparison of more than two means. A difference was considered statistically significant when $p \leq 0.05$.

3. Results and discussion

3.1 Characterization of nanoparticle suspensions

Recently, nanocarriers have drawn increasing attention for their excellent and efficient delivery of active ingredients or fractions extracted from plants (LIU; FENG, 2015). Heretofore, the association of plant extracts has been described for liposomes, nanoemulsions and nanoparticles (either lipid or polymer-based nanoparticles). Factors such as polarity of active compound, solubility, presence of organic solvent and volatility must be taken into consideration when selecting the nanosystem and its preparation technique (ZORZI et al., 2015). Thus, due to the solubility characteristics of the HEV, which was soluble in ethanol, poorly soluble in water and insoluble in ethyl acetate, the preparation of polymer nanoparticles was chosen by the nanoprecipitation method using Eudragit® RS100 as polymer.

After preparation, nanoparticle suspensions at 5 and 10 mg/mL HEV (NPHEV-5 and NPHEV-10, respectively) presented homogeneous and milky appearance with no visible precipitation, compatible with other polymeric nanostructured suspensions. However, the formulation at 25 mg/mL of HEV (NPHEV-25) presented precipitates immediately after preparation, being discarded.

The results of the physicochemical characterization of the polymeric nanoparticle suspensions are in Table 1.

Table 1 – Physicochemical characteristics of the nanoparticles suspensions

	Average diameter (nm)	PDI	Zeta potential (mV)	pH
NPHEV-b	206 ± 7	0.163 ± 0.00	+8.8 ± 0.0	4.6 ± 0.03
NPHEV-5	143 ± 5	0.164 ± 0.03	+13 ± 10	3.9 ± 0.12
NPHEV-10	144 ± 1	0.142 ± 0.01	+15 ± 8	3.7 ± 0.10

Values are expressed as mean ± SEM (n = 3). PDI, polydispersity index; NPHEV-b, blank nanoparticles; NPHEV-5, nanoparticles suspension containing hydroalcoholic extract of *Vaccinium ashei* leaves at 5 mg/mL; NPHEV-10, nanoparticles suspension containing hydroalcoholic extract of *Vaccinium ashei* leaves at 10 mg/mL

It is possible to observe that the presence of HEV reduced the nanoparticles average diameter possibly due to the presence of compounds with surface activity in the extract. The nanoparticles loaded with HEV showed average diameters lower than 200 nm and PDI less than 0.2, which indicates a narrow distribution. It can be observed that the concentration of the extract did not influence these parameters. According Mohanraj; Chen; Chen (2006), particle size and size distribution are the most important characteristics of nanoparticle systems, since they determine the *in vivo* distribution, biological fate, toxicity and the targeting ability of

nanoparticle systems. In addition, they can also influence the drug loading, drug release and stability of nanoparticles.

The zeta potential was positive, a characteristic of nanoparticles prepared with Eudragit® RS100 due to its quaternary ammonium group (JANA et al., 2014; PIGNATELLO et al., 2002; 2006). The positive zeta potential can facilitate an effective adhesion of the nanoparticles with the negatively charged mucus of the gastro-intestinal tract, prolonging the effective residence time of the formulations (JANA et al., 2014). Furthermore, the formulations presented acid pH values, similar to other Eudragit® RS100 nanostructured systems (SANTOS et al., 2014; SCHAFFAZICK et al., 2006). In addition, these values can be attributed to the acid characteristics observed for HEV.

The results of the EE showed that almost 100% of rutin was entrapped within the particle (92% and 94% for NPHEV-5 and NPHEV-10, respectively). The same was not observed for chlorogenic acid where EE were next to 50% (54 % and 59 % for NPHEV-5 and NPHEV-10, respectively). The loading results ranged between 87 ± 6% to 100 ± 8%.

Depending on the lipophilicity and the nanostructure, the extract compounds can be basically associated to nanoparticles in three different ways: (i) solubilized in the external aqueous phase; (ii) adsorbed onto the surface of the carrier, and/or (iii) entrapped into the nanocarrier (ZORZI et al., 2015). In this way, the rutin was entrapped within the polymer in the moment of the nanoparticle precipitation and chlorogenic acid suffers partition into the aqueous phase being partially encapsulated.

The results showed in this study are in accordance to other reports. Nallamuthu; Devi; Khanum (2014) encapsulated chlorogenic acid into chitosan nanoparticles by ionic gelation method. The formulations exhibited size, polydispersity index and zeta potential of 146 ± 5 to 255 ± 10 nm; 0.23 to 0.43 and +27 ± 0.6 to 34 + 0.6 mV, respectively. The EE (%) of chlorogenic acid was 45 ± 0.3 to 59 ± 0.2% with loading efficiency of 5.2%. In a study performance by Almeida et al. (2010) rutin-loaded nanocapsules and rutin-loaded nanoemulsions showed average size of 124.3 ± 2.1 nm and 124.2 ± 1.8 nm, respectively, negative zeta potentials, polydispersity indexes below 0.20, and encapsulation efficiency close to 100% (93.3 ± 0.6 and 93.8 ± 0.4%, respectively).

3.2. Antioxidant activities

3.2.1. DPPH and ORAC assay

The antioxidant activity by DPPH and ORAC assay of the HEV and NEPHV was showed in Table 2. The results confirmed antioxidants properties of the *V. ashei* leaves previously reported (EHLENFELDT; PRIOR, 2001; PILJAC-ZEGARAC; BELSCAK; PILJAC, 2009; CEZAROTTO et al., 2017). In addition, it was found that the nanoencapsulation of HEV increased the antioxidant activity by ORAC method in comparison to the free extract ($P<0.05$), and this is dose-dependent (Table 2). Antioxidant activity determined by DPPH method was the same for both free and nanoencapsulated extract. In this case, the nanoencapsulation did not affect the HEV scavenging capacity.

Table 2- Antioxidant activity of HEV and NPHEV by DPPH and ORAC methods

	IC_{50} for DPPH (µg/mL)	ORAC values (mmol/L)
HEV	12.39 ± 0.02^{a}	70 ± 4
NPHEV-5	$16.3 \pm 0.5^{a,b}$	91 ± 8
NPHEV-10	14.4 ± 0.4^{b}	133 ± 11

One way ANOVA followed by Student Newmann-Keuls; Values are expressed as mean ± SEM (n = 3). Same letters no indicate significant differences ($p>0.05$). HEV hydroalcoholic extract of *Vaccinium ashei* leaves; NPHEV-5, nanoparticles suspension containing HEV at 5 mg/mL; NPHEV-10, nanoparticles suspension containing HEV at 10 mg/mL

The results of antioxidant evaluation were consistent with other previous studies in which extract encapsulation increased the stability or controlled the release of the phenolic compounds and, consequently, enhanced the antioxidant activity (BIDONE et al., 2014; HAN et al., 2015; KHALIL; ABDU, 2013; TSAI et al., 2012).

According to Zorzi et al. (2015), the control of antioxidants kinetic release generally is recognized as the main reason for the superior activity of nanoencapsulated antioxidants. However more studies are needed in this regard.

3.3. Antidepressant-like activity

In our previous study (unpublishec data), it was found that hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei;* cv. climax) leaves (HEV) collected in December/2013, a different period from this study, produced significant antidepressant-like effects without modifying the locomotor activity. It was observed that HEV (50 mg/Kg) treatment significantly reduced the immobility time in FST and TST acute models and chronic animal model of depression UCMS (Unpredictable Chronic Moderate Stress). These findings were possibly attributed to the presence of chlorogenic acid and rutin, important flavonoids, which have been

reported to possess antidepressant-like effect in studies involving animal models (GUAN; LIU, 2016).

In this study, after oral acute treatment of HEV, a one-way ANOVA revealed no significant effect on the immobility time in the TST in any of doses tested, when compared to the positive control fluoxetine. Post-hoc analysis revealed a significant decrease in the immobility time only with fluoxetine at 30 mg/kg in the TST [P<0.001], and no significant effects of HEV at 5, 10, 25 and 50 mg/kg in in the immobility time [P>0.05] (Fig. 1).

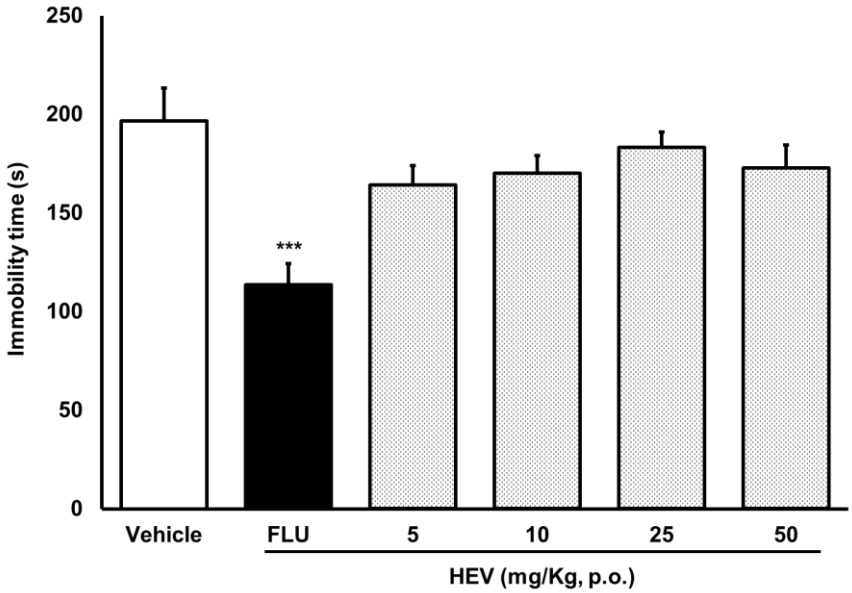


Fig.1. Effects of hydroalcoholic extract of rabbiteye blueberry (*Vaccinium ashei*) (HEV) after acute treatment on immobility TST in mice. Effect of 5, 10, 25 and 50 mg/Kg HEV and 30 mg/kg fluoxetine administration in mice TST. Values are expressed as mean ± SEM (n=7-10 mice per group). One-way ANOVA followed by Student-Newman-Keuls comparisons: ***P<0.001, compared to the respective control group (Vehicle).

Thus, no significant decrease in the immobility time triggered by the administration of HEV in any of doses tested observed in this study can be justified by the fact that chlorogenic acid and rutin were less concentrated in the extract of this collection (9.8 ± 0.01 mg/g dry weight and 4.38 ± 0.01 mg/g dry weight, respectively) when compared to the december/2013 collection (15.87 ± 0.03 and 12.13 ± 0.02 mg/g dry weight, respectively) (CEZAROTTO et al., 2017). It is well established that plant chemical composition is influenced by the seasons leading to different pharmacological properties (CEZAROTTO et al., 2017; COSTA et al., 2016).

In this sense, the development of a nanoparticle formulation containing HEV seemed to be a promising alternative. Thus, after acute treatment with NPHEV, a one-way ANOVA revealed a significant effect on the immobility time in the TST [F(6,59) = 9.768, P<0.01] (Fig.2) and FST [F(6,72) = 5.717, P<0.01] (Fig.3). Further post-hoc analysis revealed a significant

decrease in the immobility time in after administration of NPHEV at the doses of 5, 10 and 25 mg/kg and fluoxetine at 30 mg/kg in the TST [P<0.05, P<0.05, P<0.01 and P<0.001, respectively]. In another test of acute animal model of depression, the FST, the NPHEV administration at the doses of 5, 10 and 25 mg/kg and imipramine at 20 mg/kg also decreased the immobility time of these groups [P<0.05, P<0.05, P<0.05 and P<0.01, respectively], indicating that NPHEV increase antidepressant-like effects of the HEV in mice.

To verify that the changes in immobility time in the FST and TST were not attributed to non-specific side-effects, animals treated with NPHEV were tested in the spontaneous locomotor activity. Treatment with NPHEV at 5 mg/kg, the lowest effective dose, did not exhibit significant effect (P=0.936) in the general locomotor activity in mice (Fig. 4) when compared to the vehicle group.

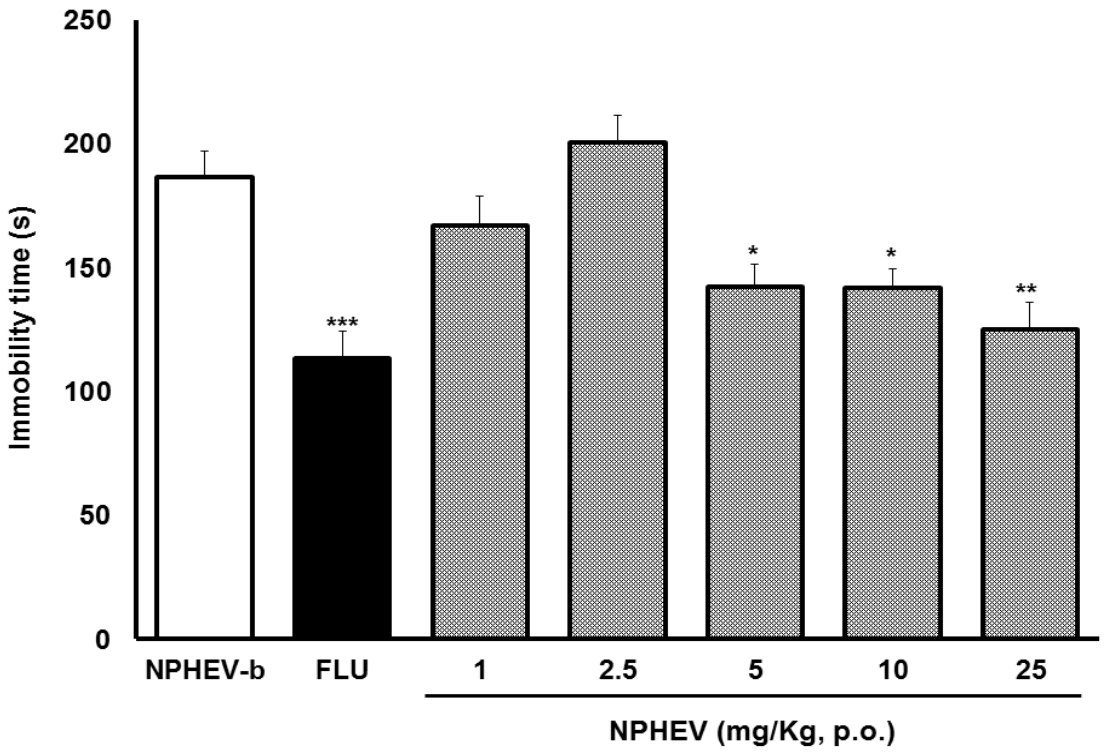


Fig.2. Effects of nanoparticle suspension containing HEV (NPHEV) after acute treatment on immobility TST in mice. Effect of 1, 2.5, 5, 10 and 25 mg/Kg NPHEV, blank nanoparticle suspension (NPHEV-b) and 30 mg/kg fluoxetine administration in mice TST. Value are expressed as mean ± SEM (n=7-10 mice per groups). One-way ANOVA followed by Student-Newman-Keuls comparisons: ***P<0.001, **P<0.01 and * P<0.05, compared to the respective control group (NPHEV-b).

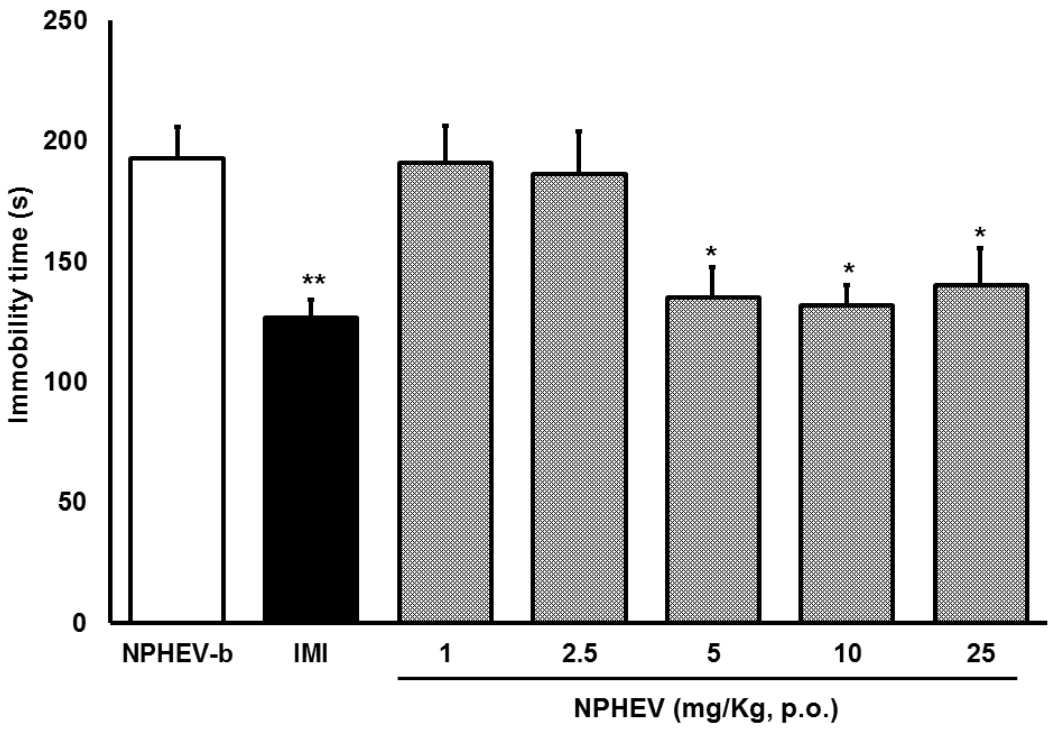


Fig.3. Effects of nanoparticle suspension containing HEV (NPHEV) after acute treatment on immobility FST in mice. Effect of 1, 2.5, 5, 10 and 25 mg/Kg NPHEV, blank nanoparticle suspension (NPHEV-b) and 20 mg/kg imipramine administration in mice FST. Value are expressed as mean ± SEM (n=7-10 mice per groups). One-way ANOVA followed by Student-Newman-Keuls comparisons: **P<0.01 and * P<0.05, compared to the respective control group (NPHEV-b).

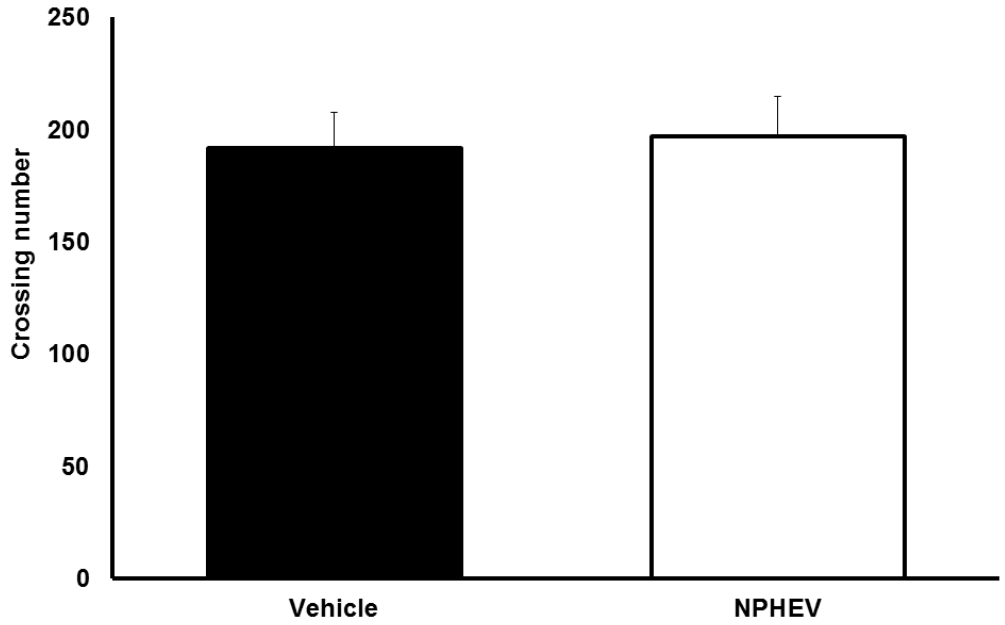


Fig.4. Spontaneous locomotor activity performed in the open-field of NPHEV. The crossing number was measured in mice receiving solution in saline (Vehicle) or 5 mg/Kg HEV. Student t test (P=0.936); values are expressed as mean ± SEM (n=10-13).

According to the literature, both chlorogenic acid and rutin (OLTHOF; HOLLMAN; KATAN, 2001; PACZKOWSKA et al., 2015) have low solubility and poor oral bioavailability. In this way, the nanostructures appear to have favored the bioavailability of these compounds, either by improving their dissolution or by increasing the permeability of these compounds across the blood-brain barrier. Similar results were observed by Paczkowska et al. (2015) where the development and characterization of nanostructures (Rutin/β-cyclodextrin inclusion complex) improved the solubility, stability and permeability of rutin, resulting in improved antioxidant and microbiological activities. In addition, a Tween 80® release system has been shown to be a promising technique to improve drug delivery through the blood-brain membrane (NAGPAL; SINGH; MISHRA, 2013).

3.3.1 Involvement of monoaminergic neurotransmission in the antidepressant-like effect of the NPHEV in the TST

In order to investigate the NPHEV mechanism of action, the involvement of monoaminergic neurotransmission in the antidepressant-like effect in the TST was performed. The results were showed in Fig. 6 (A, B, C, D and E). The Fig. 6A shows that the anti-immobility effect of the NPHEV (5 mg/kg, p.o.) was significantly prevented by pretreatment with SCH 23390 (15 µg/kg, s.c.) [two-way ANOVA, $F_{pre\text{-}treatment}$ (1,47) = 7.325, P = 0.01], $F_{treatment}$ (1,47) = 3.848, P= 0.056] and $F_{pre\text{-}treatment\times treatment}$ (1,47) = 7.325, P = 0.01]. The pretreatment of animals with sulpiride (50 mg/kg, i.p.) was also able to prevent the NPHEV anti-immobility effect (Fig. 6B) [Two-way ANOVA, $F_{pre\text{-}treatment}$ (1,44) = 3.81, P = 0.058], $F_{treatment}$ (1,44) = 17.05, P<0.001] and $F_{pre\text{-}treatment\times treatment}$ (1,44) = 5.425, P<0.05]. Still, the anti-immobility effect of the NPHEV (5 mg/kg, p.o.) was completely prevented by pre-treatment with prazosin, 1 mg/kg, i.p. (Fig. 6C) [two-way ANOVA, $F_{pre\text{-}treatment}$ (1,42) = 12.614, P=0.001], $F_{treatment}$ (1,42) = 19.905, P<0.001] and $F_{pre\text{-}treatment\times treatment}$ (1,42) = 4.281, P=0.045] and yohimbine, 1 mg/kg, i.p. (Fig. 6D) [two-way ANOVA, $F_{pre\text{-}treatment}$ (1,44) = 3.856, P=0.056], $F_{treatment}$ (1,44) = 17.292 P<0.001] and $F_{pre\text{-}treatment\times treatment}$ (1,44) = 5.532, P=0.024]. However, the serotonin synthesis inhibitor pCPA (100 mg/kg, i.p., once a day for four consecutive days), was not able to prevented the decrease in the immobility time elicited by NPHEV (5 mg/kg, p.o.) [two-way ANOVA, $F_{pre\text{-}treatment}$ (1,42) = 0.654, P=0.424], $F_{treatment}$ (1,42) = 18.932, P<0.001] and $F_{pre\text{-}treatment\times treatment}$ (1,42) = 0.49, P=0.488] (Fig. 6E).

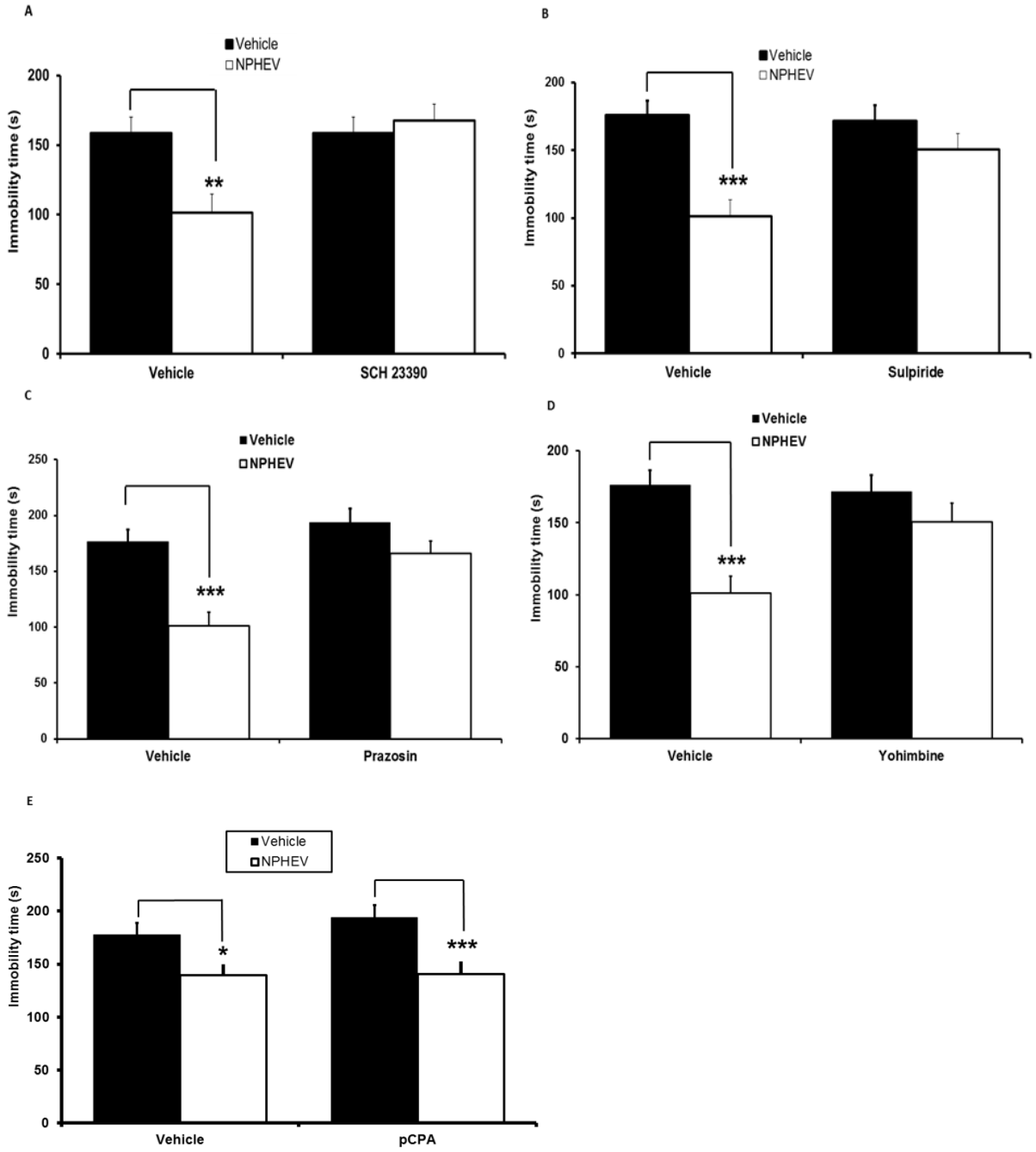


Fig.5. Pharmacological antagonism. (A) Effect of pre-treatment of mice with SCH 23390 (15 µg/kg, s.c.), (B) sulpiride (50 mg/kg, i.p.), (C) prazosin (1 mg/kg, p.o., panel C), (D) yohimbine (1 mg/kg, p.o.) and (E) pCPA (100 mg/kg/day, i.p.) on the NPHEV (5 mg/kg, p.o.) in mice TST. Each column represents the mean±S.E.M. (n = 8–12). Two-way ANOVA followed by Student–Newman–Keuls comparisons: *p<0.05; **p < 0.01; and ***p < 0.001 when compared to the vehicle treated group.

These results demonstrated that pre-treatment with dopaminergic (SCH 23390 and sulpiride) and alpha-adrenergic blocker prazosin (α1-adrenoceptor antagonist) and yohimbine (α2-adrenoceptor antagonist) significantly attenuated the NPHEV-induced antidepressant-like activity in the TST. Whereas, the pre-treatment with pCPA, an inhibitor of 5-HT biosynthesis, did not show the same activity, indicating that the effect of the NPHEV depends mainly on the

noradrenergic (via α-1 and α-2 receptors) and dopaminergic (via D1 and D2 receptors) neurotransmissions.

These results were not expected considering that in the previous research of our group *Vaccinium ashei* Reade leaves (cv. climax) (CEZAROTTO et al., 2017) showed antidepressant-like effect and this activity was attributed to high total contents of chlorogenic acid and rutin. In accordance with the literature the potential antidepressant-like activity of these compounds has been associated with the serotonergic pathway. According to Park et al. (2010), chlorogenic acid isolated from *Artemisia capillaris* Thunb. has shown antidepressant-like activity and the suggested mechanism of antidepressant action was through the opioidergic pathway. Wu et al. (2016) demonstrated that the extract of *Eucommia ulmoides*, rich in chlorogenic acid is able to cross the blood-cerebrospinal fluid barrier to exhibit its neuron protection and promotion of serotonin release through enhancement synapsin I expression. In addition, Machado et al. (2008) carried out a study exploring the effect of rutin isolated from the ethanolic extract of *Schinus molle* using FST and TST in mice and suggested the involvement of the serotonergic and noradrenergic system. Furthermore, extracts of *Hemerocallis Citrine*, rich in rutin, increased serotonin and dopamine levels in the central nervous system proving that the antidepressant-like activity of extract might be related to the serotonergic and dopaminergic systems (DU et al. , 2014).

Thus, the nanoencapsulation of the *Vaccinium ashei* Reade leaves extract showed other mechanism of action, which is dependant mainly on noradrenergic and dopaminergic neurotransmissions. These results may be attributed to synergistic or additive effects among the compounds present in the formulation or by the influence of the nanocarrier, which could have transported the compounds to specific locations for action.

These findings are in accordance to Muller et al. (2012) that reported the antidepressant-like effect of supercritical CO_2 *Valeriana glechomifolia* extract in mice FST, which was mediated by noradrenergic and dopaminergic neurotransmissions, with no significant dependence on the serotonergic neurotransmission. Sah et al. (2011) reported similar results for *Valeriana wallichii* dichloromethane extract.

According to the literature, the monoamine theory of major depression has been well-accepted by researchers. According to this theory, there is a decrease in the levels of various monoaminergic neurotransmitters such as norepinephrine, serotonin and dopamine in brains of depressed patients (PATHAK, 2013). Thus, the antidepressants currently available increase the synaptic action of one or more of the three monoamines (serotonin, noradrenalin and dopamine) by blocking the neuronal reuptake of these neurotransmitters, although most pharmacologic

treatment strategies for depression enhance only serotonin and norepinephrine neurotransmission (MARKS et al., 2008).

Nevertheless, there is a dire need for novel therapies that could improve conventional therapies (AKIL, 2017). In this context, natural products research has been considered as an option for the development of drugs with innovative mechanisms of action and/or conceivably minimized adverse side effects (MULLER et al., 2012). In this sense, the results from this study suggest that the NPHEV presents a dual-action different from most of the conventional antidepressants. In recent years, antidepressant combination therapies with multifunctional pharmacologic mechanisms have been used to enhance therapeutic outcomes (PEHRSON; SANCHEZ, 2014). Agents that interact with several complementary targets or with distributed cerebral networks (or with both) offer greater hope for the broad-depression based and efficacious treatment of both cardinal and comorbid symptoms of depression (MILLAN, 2009). Dual-action agents show promise for alleviating depressive symptoms that do not resolve with single-action agents. Adjunctive therapies and dosing options are given for common residual symptoms, including sleep difficulties, sexual dysfunction, and pain, for patients to truly regain their quality of life must be provided (BLIER, 2013). In particular, the noradrenergic action plays an important clinical effect in different antidepressant classes, as confirmed by the efficacy of dual action antidepressants such as the noradrenergic and dopaminergic reuptake inhibitor (NDRI), bupropion which enhance the noradrenergic transmission. The noradrenergic action seems to be related to improvement to the motor activity, attention, and arousal of the patients (DEL OSSO et al., 2011). Considering these literature data, the dual-action of the NPHEV observed point to as promising agents for developing new antidepressants to treat those patients for whom the serotonergic action is not enough.

In conclusion, nanoparticles loaded with Blueberry leaves hydroalcoholic extract were successfully prepared and presented adequate physicochemical characteristics. The *in vivo* results provided evidence that the nanoencapsulation is a promising approach to enhance the antidepressant effect of Blueberry leaves hydroalcoholic extract, which was mediated by increased dopamine and noradrenalin in the brain. Therefore, NPHEV is a potential new formulation intending depression treatment.

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6 DISCUSSÃO GERAL

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Dentre o grupo de pequenas frutas, os mirtilos ou *blueberry*, nomes populares dado ao gênero *Vaccinium* spp., vem destacando-se em todo o mundo devido as excelentes propriedades sensoriais e ao elevado teor de compostos bioativos. A presença destes compostos está relacionada a diversos benefícios à saúde decorrentes de seu consumo (CHU et al., 2017; FERLEMI; LAMARI, 2016; WU et al., 2017). Contudo, apesar do interesse nos frutos, para as folhas, consideradas material de desperdício (DENG et al., 2014; FERLEMI; LAMARI, 2016; ZHU et al., 2013), diversos estudos tem demonstrado elevados teores de compostos fenólicos (LI et al., 2013).

Os compostos fenólicos presentes no gênero *Vaccinium* spp. compreendem uma ampla variedade de metabólitos secundários divididos em ácidos fenólicos, flavonoides e taninos condensados e hidrolisáveis (FERLEMI; LAMARI, 2016), os quais têm demonstrado inúmeras atividades farmacológicas (AGATI et al., 2012). Além disso, as folhas são uma fonte rica de ácido clorogênico, o qual tem demonstrado propriedades antioxidantes, anti-inflamatórias, cardioprotetoras e neuroprotetoras (FERLEMI; LAMARI, 2016; MATSUO et al., 2010; NACZK et al., 2006).

Sendo assim, observa-se um interesse científico crescente em relação à composição química e às propriedades biológicas das folhas, tornando-as uma possível fonte alternativa de compostos bioativos (FERLEMI; LAMARI, 2016).

Contudo, estudos têm demonstrado que a síntese e regulação dos metabólitos secundários presentes em plantas é frequentemente influenciada por diversos fatores, os quais provocam variações na composição e, consequentemente nas atividades biológicas (GOBBO-NETO; LOPES, 2007; SONI; BRAR; GAUTTAM, 2015).

Dentre estes fatores pode-se destacar os fatores genéticos, tais como a cultivar, além de fatores ambientais, fatores agronômicos, coleta e fatores de estocagem, bem como o processamento do material vegetal (COSTA et al., 2016; ROUTRAY; ORSAT, 2014; TIWARI; CUMMINS, 2013; VENSKUTONIS et al., 2016). Desta forma, é de suma importância conhecer os fatores que induzem a estas variações durante o desenvolvimento de produtos à base de plantas (MICHEL et al., 2017; PAVARINI et al., 2012; SCOGNAMIGLIO et al., 2014).

Desse modo, o primeiro capítulo deste trabalho foi delineado no sentido de verificar a influência do período de coleta e da cultivar sobre a composição fenólica e propriedades

antioxidantes de folhas de *V. ashei*. Esta espécie foi selecionada, pois é a mais promissora para as regiões do Sul do Brasil, devido a menor exigência de frio (FACHINELLO, 2008).

Neste contexto, foi avaliado o teor de compostos fenólicos e flavonoides totais de diferentes cultivares de folhas de *V. ashei* (Clímax, Bluegem, Aliceblue, Powderblue e FloridaM) coletados em um Pomar de produção orgânica localizado no município de Erechim (RS). O material vegetal foi coletado em dois períodos vegetativos da planta, em dezembro/2013 que corresponde ao período de colheita dos frutos e em março/2014, período que antece a poda em que a colheita já encerrou. Além disso, foi avaliado o perfil de compostos metabólicos por CLAE-UV/DAD e o potencial antioxidante das amostras. Ao final os dados foram correlacionados através do coeficiente de Pearson.

Os resultados demonstraram que as folhas coletadas em março/2014 apresentaram maiores teores de fenólicos e flavonoides totais. Estes resultados estão de acordo com (GRACE; LOGAN; ADAMS, 1998) que relata que as mudanças sazonais influenciam notavelmente sobre o crescimento da planta, alterando o conteúdo de metabólitos secundários. Os compostos fenólicos são frequentemente produzidos pelas plantas como resposta a agentes estressores externos, como exposição à UV, herbivoria, seca e/ou danos mecânicos (PERCIVAL; MACKENZIE, 2007). Segundo (VENSKUTONIS et al., 2016), durante o período de maturação, a planta concentra a produção de metabólitos secundários para os frutos e, mais tarde, após o período de colheita, esta mesma produção é redirecionada para as folhas, iniciando um novo ciclo.

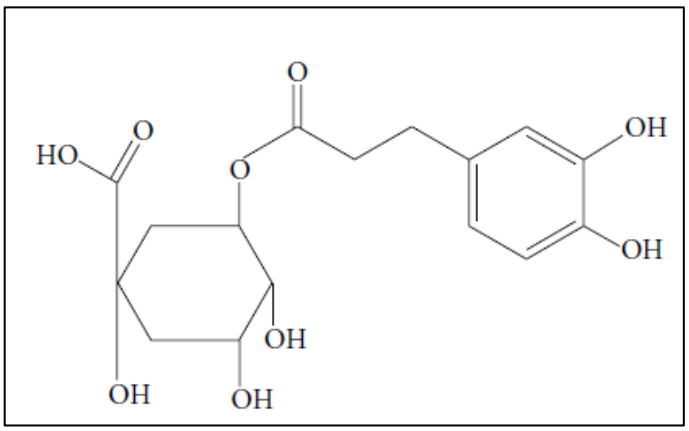
Com relação ao perfil cromatográfico por CLAE-UV/DAD, pode-se observar que o mesmo foi qualitativamente semelhante em todas as amostras analisadas, contudo a proporção relativa de cada composto teve influencia pela cultivar e pelo período de coleta. Como previamente descrito na literatura (FERLEMI et al., 2016) o ácido clorogênico foi o composto majoritário identificado em praticamente todas as cultivares, seguido da rutina. A quercetina não foi detectada em todas as amostras.

O ácido clorogênico (Figura 1) é um éster formado a partir de ácidos cinâmicos e ácido quínico, também chamado de ácido cafeoilquínico. O termo ácidos clorogênicos, no entanto, representa todo o conjunto de ésteres hidroxicinâmicos com ácido quínico, incluindo ácidos de cafeína, feruloyl, dicafetoílo e cumarilquinico. Além disso, existem várias formas isoméricas de ácido clorogênico para cada um dos subgrupos (MENG et al., 2013; TAJIK et al., 2017).

As propriedades biológicas do ácido clorogênico, além de seus efeitos antioxidantes e anti-inflamatórios, incluem regulação do metabolismo de glicose e lipídios e sobre os distúrbios

relacionados, por exemplo, diabetes, doenças cardiovasculares, obesidade, câncer e esteatose hepática (TAJIK et al., 2017).

Figura 1 – Estrutura química do ácido clorogênico

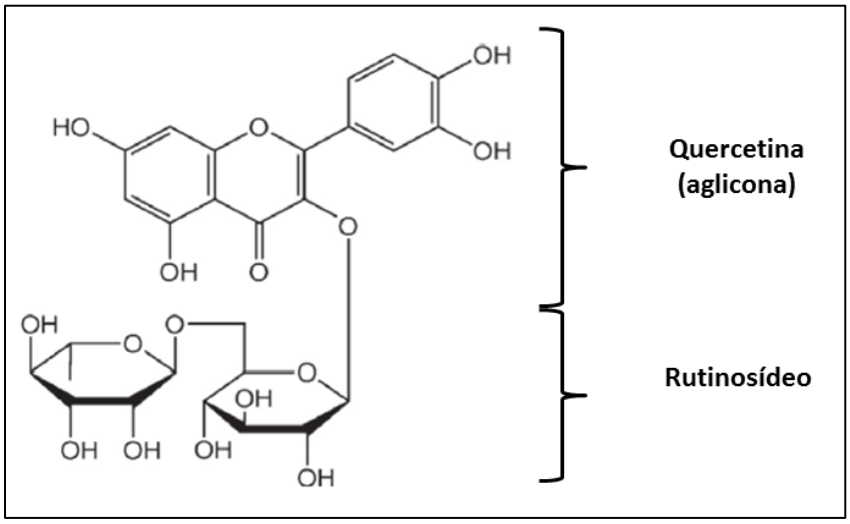


Fonte: Meng et al. (2013).

A rutina (3,3',4',5,7-pentahidroxiflavona-3-rhamnoglucosido) (Figura 2), por sua vez, é um importante flavonoide encontrado em muitas plantas e também é conhecida como vitamina P ou quercetina-3-*O*-rutinosideo. Estudos demonstram uma ampla gama de atividades biológicas incluindo atividade anti-inflamatória, antioxidante, neuroprotetora, nefroprotetora, hepatoprotetora e antidiabética (CHUA, 2013; GHORBANI, 2017). A rutina e a quercetina normalmente coexistem na natureza, uma vez que representa a forma glicona da quercetina e a hidrólise da rutina produz quercetina (CHUA, 2013).

Com relação ao potencial antioxidante, a cultivar bluegem de março/2014 foi a que apresentou maior atividade, em ambos os ensaios. Além disso, esta atividade demonstrou uma alta correlação com o conteúdo de rutina. De acordo com (YANG; GUO; YUAN, 2008), a rutina desempenha um importante papel em termos de atividade antioxidante e esta atividade é dose-dependente.

Figura 2 – Estrutura químicada rutina



Fonte: Adaptado de Chua (2013).

Até o momento, não havia relatos na literatura sobre a influência da cultivar e do período de coleta sobre a composição fenólica e atividade antioxidante de folhas de *V. ashei* produzidos nos Brasil. Desta forma, com este estudo foi possível fornecer informações quanto ao período de coleta e a cultivar ideal para as folhas de *V. ashei,* os quais resultam em elevados teores de metabólitos secundários e efeitos farmacológicos máximos.

Com o aumento constante do cultivo do mirtilo em torno do mundo, uma grande quantidade de folhas vem sendo descartadas após a poda. Sendo assim, explorar a aplicação dessas folhas como fonte alternativa de compostos bioativos seria muito benéfico para o desenvolvimento da agricultura (WANG et al., 2015), especialmente para o Brasil que tem demonstrado grande potencialidade para a cultura do mirtilo.

Dando continuidade aos trabalhos e considerando dois pontos importantes: os elevadores teores de ácido clorogênico e rutina presente nos extratos das folhas de *V. ashei* observados no perfil cromatográfico; e a relação destes compostos fenólicos com o potencial antidepressivo apontado pela literatura (DONATO et al., 2015; DU et al., 2014; GUAN; LIU, 2016; LI et al., 2016; MA et al., 2015; SHEWALE; PATIL; HIRAY, 2012) foi considerada a hipótese da avaliação antidepressiva para o extrato de *V. ashei*.

Desse modo, a primeira parte do segundo capítulo deste trabalho foi delineada no sentido de avaliar o efeito do extrato da cultivar Climax (coleta de dezembro/2014) nas doses de 10, 25 e 50 mg/kg (v.o.) sobre o tempo de imobilidade dos animais através de dois modelos agudos de depressão, Teste de Natação Forçada (TNF) e Teste de Suspensão pela Cauda (TST).

Estes testes são amplamente empregados para a avaliação da atividade antidepressiva (GONG et al., 2014; KRISHNAN; NESTLER, 2008). Durante o ensaio, a imobilidade exibida pelos animais submetidos ao estresse inescapável consegue refletir o estado de desespero comportamental, semelhante aos observados em pacientes depressivos (SHEWALE; PATIL; HIRAY, 2012).

Cabe salientar que, para esta etapa do trabalho foi selecionada somente a cultivar clímax que, embora não tenha apresentado os maiores teores de ácido clorogênico e rutina, foi a cultivar que apresentou uma distribuição mais homogênia entre estes dois compostos. Além disso, representa uma das cultivares com maior produção no Brasil, ocupando o segundo lugar em produtividade (ANTUNES et al., 2008; RADÜNZ et al., 2014).

Os resultados demonstraram que a administração aguda do extrato testado na dose de (50 mg/kg, p.o) foi capaz de reduzir o tempo de imobilidade dos animais em ambos testes (TNF e TSC). Além disso, a atividade locomotora espontânea dos animais em campo aberto, demostrou que os efeitos do extrato de *V. ashei* não altera a atividade espontânea dos animais, demonstrando que a diminuição do tempo de imobilidade não se deve a um efeito estimulante da droga.

Dando sequência ao trabalho, e a fim de reforçar a hipótese do potencial antidepressivo dos extratos, testou-se um modelo crônico de depressão, o Estresse Crônico Moderado e Imprevisível (ECMI). De acordo com a literatura, animais submetidos a este teste demonstram fenótipo do tipo depressivo em testes comportamentais como o TNF, após o regime de estresse (CASTAGNÉ et al., 2011; FILHO et al., 2015; PORSOLT et al., 1978; RUAN et al., 2014), que pode ser restaurado a níveis normais com o tratamento com agentes antidepressivos (CZÉH et al., 2016; WILLNER, 2017; WILLNER et al., 1987).

Deste modo, os animais foram submetidos a diferentes paradigmas de estresse diários de maneira alternada, a fim de tornar o teste imprevisível, por 40 dias. Durante este período foi realizado protocolo de pesagem a cada 5 dias e a partir do 20° dia os animais começaram a receber os tratamentos. Ao final do ensaio, avaliou-se o peso relativo dos órgãos, parâmetros hematológicos e o glicogênio cerebral e hepático dos animais.

Após avaliação dos resultados, contatou-se que o tratamento crônico com o extrato (50 mg/kg, p.o) foi capaz de reverter o comportamento do tipo depressivo, observado pela redução no tempo de imobilidade dos animais no TNF, o qual foi empregado na avaliação comportamental dos animais como desfecho do estudo. O ganho de peso corporal em ratos sob o regime de estresse foi significativamente alterado, o que pode ser atribuido ao efeito direto do estresse sobre o comportamento de ingestão alimentar dos animais. Uma vez que o

paradigma do ECMI produz uma série de mudanças crônicas relacionadas ao estresse, incluindo redução do ganho de peso corporal (ULRICH-LAI et al., 2006), esses resultados puderam confirmar o regime de estresse deste estudo.

Além disso, não foram observadas alterações significativas no peso relativo dos órgãos, nos parâmetros hematológicos e na concentração de glicogênio hepático e cerebral após a administração repetida, sustentando um baixo potencial de toxicidade para o extrato.

A depressão é uma das mais prevalentes desordens psiquiátricas com elevado risco de vida e elevado custo sócio-econômico (GARCÍA-GONZÁLEZ et al., 2017; MILLAN, 2006). Embora exista um grande arsenal de medicamentos antidepressivos disponível no mercado (BELMAKER; AGAM, 2008), estudos que visam à busca de novos agentes antidepressivos a partir de fontes naturais mais seguros e efetivos são de extrema importância (GUAN; LIU, 2016), demonstrando a relevância deste estudo. Somado a isso, até o presente momento, não há relatos da avaliação da atividade antidepressiva em extratos de folhas de *V. ashei* reforçando o caráter inédito deste estudo. Destaca-se apenas um estudo para o gênero em que foi investigado o papel do óxido nítrico na ação antidepressiva de frutos de *Vaccinium myrtillus* (billberry) em depressão induzida por ECMI em camundongos (KUMAR et al., 2012).

Com base nos resultados obtidos até então e, a partir da observação de que os extratos de folhas da cultivar clímax, coleta de março/2014 apresentaram teores de ácido clorogênico e rutina inferiores (9,8 ± 0,01 mg/g de peso seco e 4,38 ± 0,01 mg/g de peso seco, respectivamente) aos observados na coleta de dezembro/2013 (15,87 ± 0,03 e 12,13 ± 0,02 mg/g de peso seco, respectivamente), considerou-se a hipótese de variação no potencial antidepressivo dos extratos conforme discutido no Capítulo 1.

Sendo assim, e confirmando a hipótese levantada, pode-se observar que a administração aguda do extrato clímax, coleta de março/2014 não foi capaz de diminuir o tempo de imobilidade dos animais em TSC, em nenhuma das doses testadas, quando comparado com a fluoxetina.

Desta maneira, a segunda parte do capítulo 2 foi delineada no sentido de desenvolver nanopartículas poliméricas que pudessem potencializar as atividades farmacológicas propostas para este estudo.

Devido ao seu tamanho e características físico-químicas únicas, as nanopartículas oferecem uma ampla gama de vantagens aos produtos encapsulados, tais como, liberação controlada, estabilidade e farmacocinética melhorada, efeitos adversos diminuídos, bem como a possibilidade de redução na dose com consequente aumento da atividade (DONG et al., 2016; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Neste sentido, o extrato hidroalcoólico de folhas de *Vaccinium ashei* (cv. climax, coleta de março/2014) foi utilizado na preparação de nanopartículas poliméricas empregando o método de nanoprecipitação (NPEHV).

As nanopartículas preparadas foram caracterizadas quanto ao tamanho, potencial zeta, índice de polidispersidade, pH e os teores de rutina e ácido clorogênico foram quantificados por CLAE-UV/DAD a fim de avaliar o teor e a eficiência de encapsulamento (EE). A atividade antioxidante foi analisada pelos métodos DPPH e ORAC e a atividade antidepressiva por TSC e TNF.

Após análise dos resultados, pode-se verificar que as nanopartículas apresentaram características físico-químicas compatíveis com os nanosistemas destinados à administração de fármaco, exceto a formulação NPEHV na concentração de 25 mg/mL, que apresentou-se precipitada logo após o preparo, inviabilizando a utilização. Os resultados do EE mostraram que quase 100% da rutina foi aprisionada dentro da partícula, possivelmente devido as suas características mais lipofílicas (solubilidade em água e etanol: 0,13 g/L e 5,5 g/L, respectivamente; Log P 0,21; GULLON et al., 2017), diferentemente do ácido clorogênico que é hidrofílico (KITAGAWA et al., 2011) e que apresentou EE próximos a 50%.

Via de regra, o método empregado neste estudo no preparo das nanopartículas é usado para preparar nanosferas contendo princípios ativos lipofílicos. Os princípios ativos hidrofílicos têm afinidade reduzida para os polímeros utilizados, originando nanosferas com uma eficiência de encapsulação baixa, uma vez que este tipo de princípios ativos tende a difundir-se da fase interna para a fase externa, durante o processo de emulsificação espontânea. Os princípios ativos lipofílicos, por sua vez, não sofrem difusão para a fase externa, originado nanosferas com eficiência de encapsulação mais elevadas (SOUTO et al., 2012).

Com relação às atividades biológicas avaliadas, o efeito antioxidante foi confirmado pelo aumento de ORAC. Além disso, as nanopartículas apresentaram efeito antidepressivo demonstrado pela redução significativa do tempo de imobilidade em TSC e TNF, sem alterar a atividade locomotora no teste de campo aberto.

De acordo com a literatura, tanto o ácido clorogenico, quanto a rutina apresentam baixa solubilidade e biodisponibilidade (GULLON et al., 2017; OLTHOF; HOLLMAN; KATAN, 2001; PACZKOWSKA et al., 2015). Assim, as nanoestruturas parecem ter favorecido a biodisponibilidade destes compostos, melhorando a dissolução e aumentando a permeabilidade através das membranas biológicas.

De acordo com Gullon et al. (2017), na última década, a abordagem alternativa de entrega de fármacos a partir de nanoestruturas tem superado a baixa solubilidade de produtos

farmacêuticos em água. A redução no tamanho destes sistemas leva a um aumento da solubilidade, devido ao aumento da área superficial e a velocidade dissolução. Além disso, as nanopatículas poliméricas têm melhorado as propriedades físico-químicas de fármacos lipofílicos, além de evitar a degradação química e biológica (GULLON et al., 2017).

E por fim, com a finalidade de elucidar o mecanismo de ação envolvido na atividade antidepressiva, o envolvimento da neurotransmissão monoaminérgica na atividade do tipo antidepressiva das nanoestruturas foi avaliado *in vivo*. O pré-tratamento com SCH 23390 (15 μg / kg, sc, antagonista do receptor D1 da dopamina), sulpirida (50 mg/kg, ip, antagonista do receptor D2 da dopamina), prazosina (1 mg/kg, antagonista de adrenoceptor α1) e yohimbina (antagonista de adrenoceptores α2) antes da administração de NPV (5 mg/kg, po) impediu significativamente o efeito anti-imobilidade no TSC. Esses dados indicam que o efeito do tipo antidepressivo do NPHEV depende da ativação da neurotransmissão dopaminérgica e noradrenérgica.

Estes resultados não eram esperados uma vez que a atividade antidepressiva foi inicialmente associada à presença de ácido clorogênico e rutina e, segundo a literatura, estes compostos de maneira isolada apresentam, de modo geral, mecanismos de ação envolvendo receptores serotoninérgicos (DU et al., 2014; MACHADO et al., 2008; PARK et al., 2010; WU et al., 2016). Além disso, quando o extrato de *V. ashei* (cv. clímax, coleta de dezembro/203) foi avaliado quanto ao potencial antioxidante por TSC e TNF, o mesmo mostrou maior sensibilidade ao TSC, o que de acordo com Cryan; Mombereau; Vassout (2005) e Cryan; Valentino; Lucki (2005) é mais sensível para inibidores da receptação da serotonina, tais como a fluoxetina.

Esses resultados podem ser atribuidos a efeitos sinérgicos ou aditivos entre os compostos presentes na formulação, porém mais estudos neste sentido devem ser realizados.

Assim, os resultados sugerem que o extrato nanoencapsulado apresenta mecanismo de ação duplo diferente dos antidepressivos convencionais, tornando-se uma alternativa promissora para o tratamento de pacientes em que a ação setoninérgica não é suficiente.

Além disso, observa-se uma tendência crescente em terapias com mecanismo de ação multifuncional, as quais têm apresentado vantagens terapêuticas, com reduzidos efeitos adversos (PEHRSON; SANCHEZ, 2014).

7 CONCLUSÃO

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Este estudo pode contribuir para uma melhor compreensão da influência de diferentes cultivares e época de coleta sobre a composição fenólica e atividade antioxidante de folhas de *V. ashei*. Os resultados demonstraram que a variedade Bluegem colhida em março parece ser a mais promissora dentre as empregadas neste estudo.

Além disso, a avaliação da atividade do tipo antidepressiva sugeriu potencial terapêutico no manejo da depressão e outras desordens relacionadas reforçando as propriedades biológicas de *Vaccinium ashei* Reade. Esta atividade foi relacionada ao conteúdo de ácido clorogenico e rutina presente nos extratos, demonstrando perda da atividade dependendo da concentração destes compostos.

Sendo assim, o desenvolvimento de nanopartículas poliméricas carregadas com o extrato foi capaz de contornar este problema. Os nanosistemas foram preparados com sucesso e apresentaram características físico-químicas adequadas. Os resultados *in vivo* forneceram evidências de que a nanoencapsulação é uma abordagem promissora para melhorar o efeito antidepressivo do extrato, que foi mediado pelo aumento da dopamina e da noradrenalina no cérebro.

Dentro deste contexto, considerando o alto custo associado ao cultivo dos frutos, o uso das folhas pode ser considerado vantajoso, no momento em que amplia as aplicações de uso, gerando uma fonte alternativa de renda para os produtores. Os subprodutos derivados das folhas podem ser usados como coadjuvante em muitas condições em que o estresse oxidativo está envolvido, bem como no manejo da depressão e outras desordens relacionadas. Além disso, podem servir como produtos intermediários para a produção de formas farmacêuticas e nutracêuticas finais. Adicionalmente, o desenvolvimento de nanoestruturas contendo o extrato associado fornece a possibilidade de uma nova formulação com alto valor agregado, destinada ao tratamento de depressão.

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