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EXTRATOS DE CEREJEIRA-DO-RIO-GRANDE (*Eugenia involucrata*): AVALIAÇÃO DA BIOATIVIDADE EM MODELOS *IN VITRO*

Santa Maria, RS

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EXTRATOS DE CEREJEIRA-DO-RIO-GRANDE (*Eugenia involucrata*): AVALIAÇÃO DA BIOATIVIDADE EM MODELOS *IN VITRO*

Dissertação apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, Área de Concentração em Ciência e Tecnologia de Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Mestra em Ciência e Tecnologia dos Alimentos.**

Orientadora: Drª. Tatiana Emanuelli
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Santa Maria, RS
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Declaro, FRANCIELE ALINE SMANIOTTO, para os devidos fins e sob as penas da lei, que a pesquisa constante neste trabalho de conclusão de curso (Dissertação) foi por mim elaborada e que as informações necessárias objeto de consulta em literatura e outras fontes estão devidamente referenciadas. Declaro, ainda, que este trabalho ou parte dele não foi apresentado anteriormente para obtenção de qualquer outro grau acadêmico, estando ciente de que a inveracidade da presente declaração poderá resultar na anulação da titulação pela Universidade, entre outras consequências legais.

Franciele Aline Smaniotto

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Aprovado em 29 de novembro de 2022:

_____

Tatiana Emanuelli, Drª. (UFSM)
(Presidente/Orientador)

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Ana Teresa Serra, Drª. (iBET)

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Jesús Lozano-Sánchez, Dr. (UGR)

Santa Maria, RS
2022

DEDICATÓRIA

Dedico este trabalho a minha família e amigos, que sempre acreditaram em mim!

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RESUMO

EXTRATOS DE CEREJEIRA-DO-RIO-GRANDE (*Eugenia involucrata*): AVALIAÇÃO DA BIOATIVIDADE EM MODELOS *IN VITRO*

AUTORA: Franciele Aline Smaniotto
ORIENTADORA: Drª. Tatiana Emanuelli
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A Eugenia involucrata DC. é uma espécie vegetal nativa subexplorada, presente nas regiões sul e sudeste do Brasil. Estudos recentes relatam a presença de grandes quantidades de compostos fenólicos potencialmente benéficos para a saúde humana em diferentes partes (folhas, polpa e sementes) desta espécie. O diabetes é uma doença caracterizada pela hiperglicemia, a qual resulta na glicação de proteínas e geração excessiva de espécies reativas de oxigênio (EROs) que leva ao estresse oxidativo e a formação de produtos finais de glicação avançada (AGEs), ambos envolvidos na patogênese da doença. Dessa forma, uma das estratégias de tratamento e/ou prevenção do diabetes é promover a redução dos níveis de glicose sanguínea, através da inibição de enzimas envolvidas no metabolismo de carboidratos e lipídeos. Até o presente momento não foi encontrado na literatura cientifica nenhum estudo realizado com extratos da polpa e semente de *E. involucrata* na inibição de enzimas envolvidas na digestão de carboidratos e lipídeos. Sendo assim, o objetivo deste estudo foi investigar *in vitro* o potencial anti-hiperglicêmico e anti-obesogênico de extratos etanólicos de polpa e semente de *E. involucrata*, por meio de sua capacidade de inibir enzimas envolvidas na digestão de carboidratos e lipídios, e avaliar a inibição da formação de AGEs, e a capacidade de neutralizar espécies reativas de importância biológica. O extrato de semente de *E. involucrata* apresentou maior teor compostos fenólicos totais (11.550,5 ± 633,0 μg. mL^{-1}), compostos por ácidos fenólicos e flavonóides, que o extrato da polpa da fruta (440,7 ± 28,1 μg. mL^{-1}), composto por ácidos fenólicos, flavonóides e antocianinas. Os valores de IC_{50} (expressos como μg de compostos fenólicos. mL^{-1}) revelaram que os compostos fenólicos dos extratos de *E. involucrata* apresentaram potência superior a da acarbose para inibir as enzimas digestivas α-amilase (IC_{50} polpa: 0,2 ± 0,0 μg. mL^{-1}; IC_{50} semente: 1,3 ± 0,2 μg. mL^{-1}; acarbose: 39,9 ± 3,9 μg. mL^{-1}), α-glicosidase (IC_{50} polpa: 0,3± 0,0 μg. mL^{-1}; IC_{50} semente: 1,5 ± 0,0 μg. mL^{-1}; acarbose: 2.019,0 ± 81,3 μg. mL^{-1}), mas inferior a do orlistat para inibir a lipase pancreática (IC_{50} polpa: 0,7 ± 0,0 μg. mL^{-1}; IC_{50} semente: 0,6 ± 0,0 μg. mL^{-1}; orlistat: 0,1 ± 0,0 μg. mL^{-1}). Também inibiram a formação de AGEs (IC_{50} polpa: 6,8 ± 0,3 μg. mL^{-1}; IC_{50} semente: 30,9 ± μg. mL^{-1}), e foram capazes de remover espécies radicalares de importância biológica (radical peroxil: IC_{50} polpa: 0,02 ± 0,0 μg. mL^{-1}, IC_{50} semente: 0,12 ± 0,0 μg. mL^{-1}; e radical hidroxil: IC_{50} polpa: 3,9 ± 0,0 μg. mL^{-1}, IC_{50} semente: 4,5 ± 0,1 μg. mL^{-1}). Apesar dos compostos fenólicos extraídos da polpa da *E. involucrata* terem apresentado maior potência na inibição de enzimas digestivas e remoção de espécies reativas em comparação com fenólicos da semente da fruta, a extração da semente apresenta maior rendimento, resultando em concentração de fenólicos 26 vezes superior à do extrato da polpa, e maior potência do extrato de semente quando considerada a quantidade de polpa ou semente necessária para obtenção dos extratos. Os resultados obtidos neste estudo fornecem a base para estudos futuros em modelos *in vivo* e estudos clínicos visando a formulação de novos fármacos ou nutracêuticos, bem como de produtos alimentícios com propriedades funcionais.

Palavras-chave: cereja-do-rio-grande; compostos bioativos; diabetes; enzimas digestivas; produtos finais de glicação avançada.

ABSTRACT

EXTRACTS OF CHERRY-OF-RIO-GRANDE (*Eugenia involucrata*): PHYTOCHEMICAL CHARACTERIZATION AND EVALUATION OF BIOACTIVITY IN *IN VITRO* MODELS

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Eugenia involucrata DC. is an underexplored native plant species, found in the Southern and Southeastern regions of Brazil. Recent studies report the presence of large amounts of phenolic compounds that potentially benefit human health in different parts (leaves, pulp, and seeds) of this species. Diabetes is characterized by hyperglycemia, which results in the glycation of proteins and excessive generation of reactive oxygen species (ROS) that lead to oxidative stress and the formation of advanced glycation end products (AGEs). Thus, one of the strategies for the treatment and/or protection of diabetes is to promote the reduction of blood glucose levels, through the inhibition of enzymes involved in carbohydrate digestion. So far, no study has been found in the literature on the effects of extracts from the pulp and seed *E. involucrata* towards enzymes involved in the digestion of carbohydrates and lipids. Therefore, the aim of this study was to investigate in vitro the anti-hyperglycemic and anti-obesogenic potential of phenolic compounds of ethanolic extracts from *E. involucrata* pulp and seed, through their ability to inhibit enzymes involved in the digestion of carbohydrates and lipids, and to evaluate the inhibition of AGEs formation, and the potential ability to neutralize reactive species of biological importance. The seed extract had higher content of total phenolic compounds (11,550.5 ± 633.0 μg. mL^{-1}), being composed of phenolic acids and flavonoids, than the pulp extract (440.7 ± 28.1 μg. mL^{-1}) that was composed of phenolic acids, flavonoids and anthocyanins. The IC_{50} values (μg of phenolic compounds. mL^{-1}) revealed that the extracts had higher potency than acarbose towards the inhibition of α-amylase (0.2 ± 0.0 for pulp; 1.3 ± 0.2 for seed; 39.9 ± 3.9 for acarbose) and α-glucosidase (0.3 ± 0.0 for pulp; 1.5 ± 0.0 for seed; 2,019.0 ± 81.3 for acarbose), but lower potency than orlistat to inhibit pancreatic lipase (0.7 ± 0.0 for pulp; 0.6 ± 0.0 for seed; 0.1 ± 0.0 for orlistat). Extracts also inhibited (IC_{50} values as μg of phenolic compounds. mL^{-1}) the formation of AGEs (6.8 ± 0.3 for pulp; 30.9 ± 1.7 for seed; 256.6 ± 13.7 for aminoguanidine) and were able to remove free radical species of biological relevance, namely peroxyl radical (0.02 ± 0.0 for pulp; 0.12 ± 0.0 for seed), hydroxyl radical (3.9 ± 0.0 for pulp; 4.5 ± 0.1 for seed). Despite the higher potency of phenolic compounds extracted from *E. involucrata* pulp towards all biological effects investigated when compared to the phenolics from seed extract, seed extraction has greater yield. Therefore, the concentration of phenolic compounds was 26 times higher in seed than pulp extract and seed extract had higher potency than pulp extract when taking into account the amount of pulp or seed that is necessary to obtain each extract. The results obtained in this study provide a basis for further studies in animal models and clinical studies for the development of new drugs or nutraceuticals, as well as food products with functional properties.

Keywords: cherry-of-rio-grande; bioactive compounds; diabetes; digestive enzymes; advanced glycation end products.

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SUMÁRIO

1. INTRODUÇÃO

A biodiversidade do ecossistema brasileiro é considerada uma das mais ricas do mundo, sendo uma fonte muito valiosa e ainda pouco explorada de plantas e frutos nativos, os quais podem fornecer soluções sustentáveis com a finalidade de diversificar a produção de alimentos e melhorar a segurança alimentar da população (SCHULZ et al., 2020; SCHMIDT et al., 2020; INFANTE et al., 2016). Além disso, no Brasil, a Política Nacional de Alimentação e Nutrição (PNAN) apresenta entre seus propósitos, a melhoria das condições de alimentação e nutrição da população brasileira, através da promoção de estratégias com foco na prevenção e controle das doenças associadas à alimentação (MINISTÉRIO DA SAÚDE, 2013). Aliado aos objetivos do PNAN, compostos derivados de espécies vegetais tem despertado o interesse da comunidade científica quanto ao seu potencial para a prevenção e/ou tratamento das doenças crônicas, como o diabetes (ARAUJO et al., 2021). A identificação do potencial nutricional e farmacológico da flora nativa brasileira possui grande potencial para valorizar as cadeias produtivas nacionais, impulsionando sua exploração sustentável e estando alinhado aos Objetivos de Desenvolvimento Sustentável (ODS) propostos pela Organização das Nações Unidas (ONU) aos países membros.

A cerejeira-do-rio-grande (*Eugenia involucrata*) é uma espécie nativa da família Myrtaceae encontrada nas regiões sul e sudeste brasileira, e ainda pouco explorada, sendo que seus frutos são consumidos principalmente *in natura*. Recentemente, alguns pesquisadores vêm estudando os compostos bioativos, mais especificamente os compostos fenólicos presentes nesta espécie, bem como sua capacidade antioxidante e potencial para prevenção e/ou tratamento de algumas doenças crônicas (INFANTE et al., 2016; NICÁCIO et al., 2017; GIRARDELO et al., 2020; SCHMIDT et al., 2020; MANNINO et al., 2022).

Os compostos fenólicos são uma classe de compostos naturais com destacado potencial antioxidante, tendo distribuição ampla no reino vegetal e estando presentes em praticamente todas as partes das plantas (XU et al., 2017). Além da capacidade de remover radicais livres, também foi relatado para alguns compostos fenólicos a capacidade de inibir enzimas digestivas necessárias para a absorção de hidratos de carbono e lipídios da dieta e, portanto, relevantes para a redução do risco de diabetes e a obesidade, as quais vem acometendo grande parte da população (NOWICKA et al., 2018; SIMONATO et al., 2019).

O diabetes é considerado um problema de saúde pública mundial, que desafia os gestores e profissionais da saúde por reduzir a qualidade de vida e estar relacionado a diversas outras patologias na população, o que aumenta as despesas dos serviços de saúde (LALEGANI

et al., 2018). Trata-se de uma doença crônica caracterizada principalmente por hiperglicemia, onde o pâncreas não é mais capaz de produzir insulina ou a insulina produzida não é empregada de maneira adequada pelo organismo. A insulina é um hormônio produzido pelo pâncreas e é responsável pela entrada da glicose sanguínea nas células, onde será utilizada como fonte de energia. O diabetes tipo 2 é o mais prevalente e atinge principalmente adultos acima de 40 anos e idosos (RODACKI et al., 2022; IDF, 2017).

De acordo com dados divulgados na décima edição do Atlas da Federação Internacional do Diabetes (IDF), existem cerca de 537 milhões de pessoas no mundo todo com diabetes, e entre 2019 e 2021 houve um aumento de 74 milhões de casos (IDF, 2021). O Brasil encontra-se na 6° posição de países com maior incidência de diabetes no mundo, sendo estimado que atualmente existem cerca de 15,7 milhões de adultos na faixa etária de 20 a 79 anos com a doença, e que em 2045 a incidência chegue a 23,2 milhões (IDF, 2021). Estes dados alarmantes acerca do diabetes têm levado diversos pesquisadores a estudarem as questões envolvidas na maior incidência da doença e a buscarem novas alternativas em fontes naturais, com destaque aos compostos fenólicos, com foco na prevenção e/ou tratamento do diabetes (ARAUJO et al., 2021).

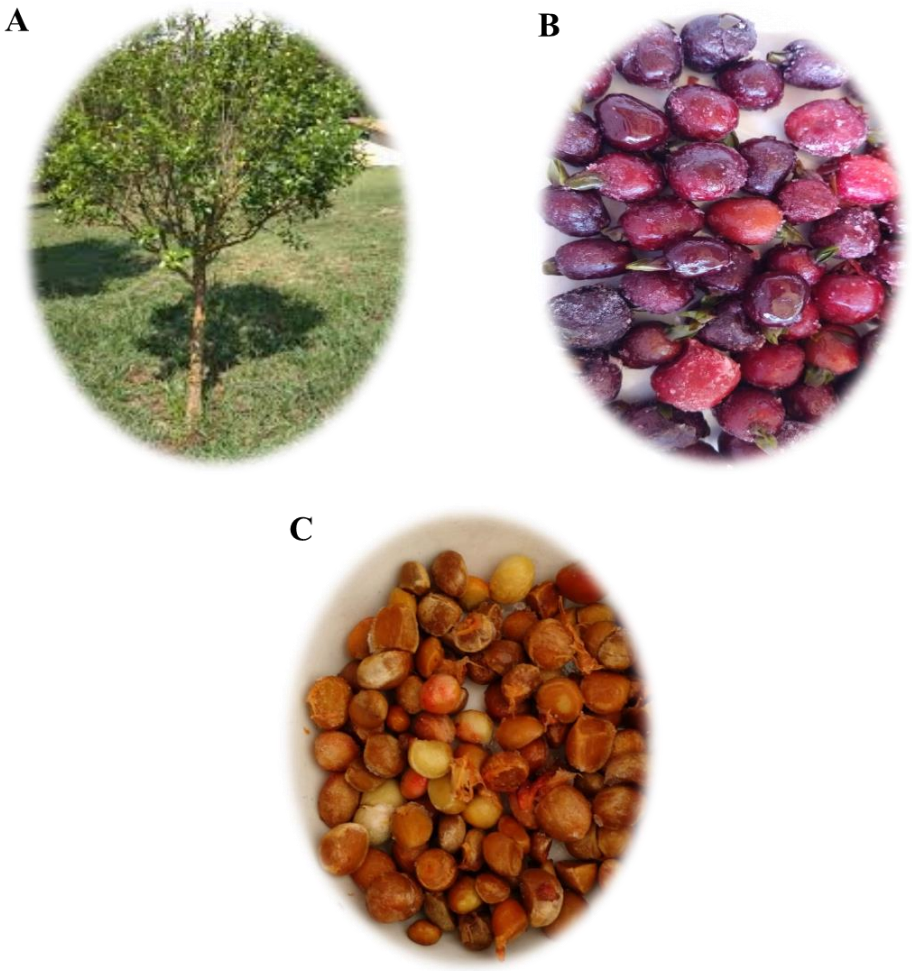
2. REVISÃO BIBLIOGRÁFICA

2.1 CEREJEIRA-DO-RIO-GRANDE (*Eugenia involucrata* DC.)

O gênero Eugenia é o maior pertencente à família Myrtaceae, possuindo aproximadamente 500 espécies de árvores e arbustos na América tropical e subtropical, sendo que 400 dessas espécies se encontram distribuídas no Brasil (FIGUEIRÔA, 2013).

A espécie *Eugenia involucrata* DC., conhecida popularmente como cerejeira-do-rio-grande ou também como cerejeira-do-mato, pertence à família Myrtaceae. É uma espécie vegetal nativa presente nas regiões sul e sudeste brasileiras, comumente encontrada em pomares domésticos (Figura 1A). A produção dos frutos ocorre entre os meses de setembro a dezembro, na região sul. Os frutos apresentam coloração verde quando imaturos e coloração que vai do vermelho ao roxo, quando maduros (Figura 1B). Apresentam-se como bagas piriformes e lisas, pesam em média 5 g e podem conter de 1 a 5 sementes, as quais possuem coloração amarelada e formato irregular (Figura 1C). A polpa da cereja-do-rio-grande possui coloração esverdeada e sabor agridoce, apresentando um aspecto suculento, podendo ser consumida *in natura* ou usada na elaboração de doces, geleias e licores (CARVALHO, 2009).

Figura 1 - Espécie *E. involucrata:* A) árvore, B) frutos e C) sementes.



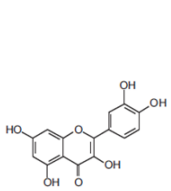
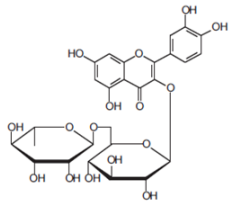
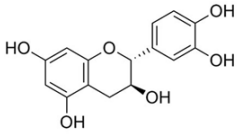
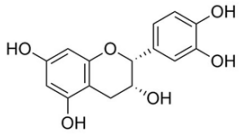
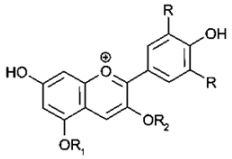
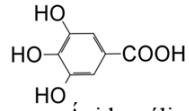
Fonte: Figura autoral.

Os frutos da cerejeira-do-rio-grande são ricos em compostos fenólicos, tanto os antociânicos como os não-antociânicos (MANNINO et al., 2022; GIRARDELO et al., 2020; SCHMIDT et al., 2020). Dentre as antocianinas encontradas destacam-se derivados de cianidina (cianidina-*3*-*O*-glicosídeo, cianidina-O-pentosídeo), derivado de delfinidina (delfinidina-O-hexosídeo, delfinidina-3-glicosídio) e derivado de pelargonidina (pelargonidina-3-O-glicosídeo, pelargonidina-glicosideo) (Figura 2). Já entre os compostos fenólicos não-antociânicos podemos destacar aqueles pertencentes às classes dos ácidos fenólicos, como o ácido gálico (derivado do ácido hidroxibenzóico), e à classe dos flavonoides, como a catequina e a epicatequina, a rutina e a quercetina (Figura 2) (MANNINO et al., 2022; GIRARDELO et al., 2020; SCHMIDT et al., 2020). No entanto, fatores ambientais como umidade, temperatura, intensidade de radiação solar, altitude e composição do solo, os quais

variam muito de uma região para outra, podem influenciar significativamente na composição química dos frutos e da planta como um todo (YANG et al., 2016).

Assim como os frutos, as sementes da cerejeira-do-rio-grande também são ricas em compostos fenólicos como o ácido gálico (INFANTE et al., 2016; NICÁCIO et al., 2017), a epicatequina (INFANTE et al., 2016; GIRARDELO et al., 2020) e a catequina (GIRARDELO et al., 2020) (Figura 2), e por isso essa parte dos frutos da cerejeira-do-rio-grande pode apresentar uma elevada ação biológica.

Figura 2 - Classes de compostos fenólicos, estrutura química e compostos fenólicos encontrados na cerejeira-do-rio-grande.

Classes e subclasses	Estrutura química	Compostos encontrados na cerejeira-do-rio-grande – Referencia
Flavonoides		
Flavonóis	 Quercetina  Rutina	Quercetina, Rutina (polpa) – Mannino et al., 2022; Girardelo et al., 2020; Schmidt et al., 2020.
Flavan-3-ois	 Catequina  Epicatequina	Catequina, Epicatequina (polpa e/ou semente) – Mannino et al.,2022; Girardelo et al., 2020; Schmidt et al., 2020; Nicácio et al., 2017; Infante et al., 2016.
Antocianinas	 Estrutura básica Antocianinas	Pelargonidina, Cianidina, Delfinidina (polpa) – Mannino et al., 2022; Girardelo et al., 2020; Schmidt et al., 2020.
Ácidos fenólicos		
Ácido hidroxibenzóico	 Ácido gálico	Ácido gálico (polpa e/ou semente) – Mannino et al., 2022; Girardelo et al., 2020; Nicácio et al., 2017; Infante et al., 2016.

Fonte: Figura autoral.

2.2 ATIVIDADE BIOLÓGICA DOS COMPOSTOS FENÓLICOS

Os compostos fenólicos possuem no mínimo um anel fenólico e um ou mais grupos hidroxila em sua estrutura e são considerados os antioxidantes naturais mais populares, devido as suas propriedades antioxidantes promissoras (AUGUSTI et al., 2021a; OLSZOWY, 2019). São metabólitos produzidos pelo metabolismo secundário das plantas com o intuito de protegê-las da ação de outros organismos (AUGUSTI et al., 2021a). Uma alimentação com ingestão regular de alimentos ricos em substâncias consideradas antioxidantes está associada a redução do risco de desenvolvimento e progressão de muitas doenças crônicas, como câncer, diabetes, doenças cardiovasculares e doenças neurodegenerativas (ABBAS et al., 2017; NOWICKA; WOJDYŁO; LASKOWSKI, 2018). Um fator comum dentre todas essas doenças é o chamado estresse oxidativo, que é o desequilíbrio entre a produção endógena de espécies reativas, as quais também incluem os radicais livres, e o sistema de defesa antioxidante celular (EL-SHIEKH et al., 2019).

As espécies reativas (ERs) podem ser agrupadas em dois grandes grupos: as espécies reativas de oxigênio (EROs) e as espécies reativas de nitrogênio (ERNs). As principais EROs são divididas em dois grupos: as radicalares [radical hidroxila ($HO^{\bullet}$), ânion radical superóxido ($O_2^{\bullet-}$), radical peroxila ($ROO^{\bullet}$) e radical alcoxila ($RO^{\bullet}$)] e as não-radicalares [peróxido de hidrogênio (H_2O_2) e ácido hipocloroso (HOCl)]. Entre as ERNs destacam-se o óxido nítrico ($NO^{\bullet}$), óxido nitroso (N_2O_3), ácido nitroso (HNO_2), nitritos (NO_2^-), nitratos (NO_3^-) e peroxinitritos ($ONOO^-$) (FRANÇA et al., 2013).

Normalmente durante os processos metabólicos ocorre a produção constante de ERs, pois estas possuem muitas vezes um papel fisiológico essencial para a manutenção da homeostase celular. As EROs e ERNs são naturalmente neutralizadas pelo sistema de defesa antioxidante, que tem como finalidade regular os níveis intracelulares dessas ERs e prevenir a ocorrência de danos celulares (FRANÇA et al., 2013). Em algumas condições patológicas ou de intenso estresse ao organismo, ocorre a redução da produção de antioxidantes celulares e o aumento na relação ERs /antioxidante a qual é identificada como estresse oxidativo (SIDDEEG et al., 2020).

Com relação aos antioxidantes, estes podem ser inicialmente classificados como endógenos e exógenos, sendo que o sistema antioxidante endógeno ainda pode ser dividido em enzimáticos e não enzimáticos. No sistema antioxidante endógeno enzimático destaca-se principalmente as enzimas superóxido dismutase (SOD), glutationa peroxidase (GSH-Px), glutationa redutase (GSH-Rd) e catalase (CAT), sendo que as enzimas SOD, GSH-Px e CAT

são responsáveis pela remoção do ânion radical superóxido, hidroperóxidos orgânicos e peróxido de hidrogênio, respectivamente (HALLIWELL & GUTTERIDGE, 2015; IGHODARO & AKINLOYE, 2017; FRANÇA et al., 2013). Dentre os antioxidantes endógenos não enzimáticos o destaque é a glutationa reduzida (GSH), que atua como a primeira linha de defesa celular frente ao excesso de ERs e à exposição a xenobióticos. Quanto aos antioxidantes exógenos, os de maior destaque podem ser facilmente obtidos através da ingestão alimentar como o α-tocoferol (vitamina-E), β-caroteno (carotenoide pró-vitamina-A), ácido ascórbico (vitamina-C) e os compostos fenólicos (HALLIWELL & GUTTERIDGE, 2015; IGHODARO & AKINLOYE, 2017; FRANÇA et al., 2013; VASCONCELOS et al., 2014).

Até certo tempo atrás, acreditava-se que o efeito antioxidante dos compostos fenólicos no organismo ocorria apenas em decorrência de sua ação direta na remoção de ERs, onde os compostos fenólicos ingeridos seriam absorvidos no intestino delgado, metabolizados e liberados na circulação sistêmica. No entanto, atualmente sabe-se que a biodisponibilidade dos compostos fenólicos é baixa (cerca de 5-10%) (AUGUSTI et al., 2021a; FRAGA et al., 2019) e assim efeitos antioxidantes diretos estariam relacionados mais a uma ação local no trato gastrointestinal (TGI), onde haveria uma elevada concentração desses compostos. Já os efeitos antioxidantes sistêmicos dos compostos fenólicos seriam mediados por mecanismos indiretos provenientes dessa pequena fração dos compostos fenólicos absorvidos e/ou de seus respectivos metabólitos (AUGUSTI et al., 2021a; FRAGA et al., 2019; GARCÍA-CONESA, 2017). Os compostos fenólicos não absorvidos restantes no intestino delgado seguem para o intestino grosso (cólon). No cólon, a microbiota colônica metaboliza mais uma fração (aproximadamente 90-95%) desses compostos, transformando-os em moléculas prontamente absorvíveis. Há evidências de que as bactérias do cólon desempenham atividade fundamental na absorção dos compostos fenólicos podendo determinar a sua biodisponibilidade na circulação sistêmica (FRAGA et al., 2019). Isso ocorre devido à baixa absorção intestinal da maioria dos compostos fenólicos, o que acaba resultando em baixas concentrações nos fluidos celulares, sendo insuficientes para exercer uma ação direta significativa na remoção das ERs (FRAGA et al., 2019; GARCÍA-CONESA, 2017). Além disso, há relatos na literatura de que a interação dos compostos fenólicos com a microbiota intestinal confere diversos benefícios a saúde humana, dentre os quais podemos destacar a modulação da microbiota (efeito prebiótico promovido pelos compostos fenólicos), aumento dos ácidos graxos de cadeia curta (AGCC), melhora da resposta imune e diminuição da proporção de bactérias intestinais patogênicas (*Firmicutes* e *Bacteroidetes*) (AUGUSTI et al., 2021a; MAURER et al., 2020a; SILVA et al., 2020; DANNESKIOLD-SAMSØE et al., 2019; LIN et al., 2019).

Da mesma forma, evidências recentes indicam que apesar da baixa concentração plasmática, alguns compostos fenólicos podem apresentar concentração mais elevada em determinadas biomoléculas. A quercetina e outros compostos fenólicos apresentaram grande afinidade por proteínas presentes na superfície das partículas de LDL, atingindo concentrações compatíveis com as que produzem efeito antioxidante direto (TUNG et al., 2020). Estes resultados ajudam a explicar a presença persistente de compostos fenólicos nas partículas de LDL e a proteção contra o risco de doenças cardiovasculares, observados após o consumo de vinho tinto, azeite de oliva e café (TUNG et al., 2020).

A capacidade antioxidante dos compostos fenólicos tem sido extensamente explorada nos últimos anos por pesquisadores do mundo todo, no entanto esses compostos também têm demostrado potenciais atividades anti-obesogênica, anti-inflamatória, antibacteriana, antitumoral e hepatoprotetora (WOJDYLO et al., 2019). Nosso grupo de pesquisa tem realizado diversos estudos *in vitro* e *in vivo*, ao longo dos últimos anos, com compostos fenólicos provenientes de frutas (jabuticaba, mirtilo, uva e seus subprodutos) a fim de investigar sua associação com efeitos benéficos a saúde, como: redução da resposta inflamatória oxidativa, recuperação da GSH e prevenção da apoptose do cólon em modelo de colite ulcerativa em ratos (MAURER et al., 2020a; MAURER et al., 2020b); aumento da produção de AGCC nas fezes fecais (MAURER et al., 2019; SILVA et al., 2020); atenuação de lesões gástricas provocada por etanol em ratos (SILVA et al., 2020); efeito antiproliferativo em modelo celular 3D de câncer colorretal (AUGUSTI et al., 2021b); e atenuação da hiperglicemia, resistência à insulina, hiperlipidemia e complicações hepáticas em modelo de diabetes tipo 2 em ratos (QUATRIN et al., 2018).

Evidências crescentes apontam que os compostos fenólicos, sobretudo os flavonoides, apresentam efeitos benéficos sobre o diabetes e a obesidade, podendo desencadear mecanismos que promovem a redução da resistência à insulina, da inflamação e do estresse oxidativo (FRAGA et al., 2019). Além disso, esses compostos também podem apresentar potencial para inibir algumas enzimas digestivas, como a α-amilase e a α-glicosidase, envolvidas na digestão de carboidratos, e a lipase pancreática, envolvida na digestão de lipídeos (SPÍNOLA; LLORENT-MARTÍNEZ; CASTILHO, 2020), o que reforçaria o papel dos compostos fenólicos na prevenção e auxílio ao tratamento do diabetes e da obesidade.

2.3 DIABETES E OBESIDADE

O diabetes e a obesidade são considerados como as comorbidades mais dominantes que afetam a população mundial, sendo também considerados fatores de risco para diversas outras patologias crônicas, como doenças cardiovasculares, diversos tipos de câncer, doenças respiratórias e demência (WONGSA; PHATIKULRUNGSUN; PRATHUMTHONG, 2022; EBRAHIMPOUR; ZAKERI; ESMAEILI, 2020).

O diabetes mellitus é o principal desfecho clínico associado ao desequilíbrio no metabolismo da glicose. De acordo com a Organização Mundial da Saúde (OMS), o diabetes é considerado uma doença metabólica crônica que possui como característica elevados níveis de glicose sanguínea (hiperglicemia) ou redução da sensibilidade tecidual à insulina, e a longo prazo, pode levar a complicações no coração, vasos sanguíneos, rins, visão e nervos. Dessa forma, pacientes diabéticos apresentam uma série de comorbidades associadas, tais como doenças cardiovasculares, nefropatia, retinopatia e neuropatia, que aumentam o risco de mortalidade (WHO, 2022). Em virtude da pandemia provocada pela COVID-19, pacientes diabéticos apresentam risco elevado de complicações graves provocadas pelo novo coronavírus e, consequentemente, maiores taxas de admissão em UTI, sendo que há maior risco de mortalidade em pacientes diabéticos do que em não diabéticos (HUSSAIN et al., 2020; CABALLERO et al., 2020; APICELLA et al., 2020).

No mundo todo existem cerca de 422 milhões de pessoas diagnosticadas com diabetes, e 1,6 milhão de mortes causadas diretamente pelo diabetes a cada ano, visto que a prevalência é maior em países de baixa e média renda (WHO, 2022). O aumento da prevalência do diabetes tem associação com a rápida urbanização, aumento do estilo de vida sedentário e do excesso de peso, mudança dos hábitos alimentares, maior oferta e acesso a alimentos industrializados, e o aumento do crescimento e envelhecimento populacional (SOCIEDADE BRASILEIRA DE DIABETES, 2019).

O diabetes é dividido em três tipos principais: diabetes tipo 1, diabetes tipo 2 e diabetes gestacional. Destaca-se o diabetes tipo 2, pois responde por 90 a 95% dos casos totais de diabetes e geralmente acomete indivíduos a partir dos 40 anos de idade. É caracterizado por graus variáveis de resistência tecidual à insulina e uma deficiência relativa na secreção desse hormônio pelas células β-pancreáticas, além de defeitos na regulação da produção hepática da glicose (RODACKI et al., 2022; SOCIEDADE BRASILEIRA DE DIABETES, 2019;).

Durante o tratamento do diabetes, uma das estratégias terapêuticas é promover a diminuição dos níveis de glicose pós-prandial por meio do retardo da absorção de glicose através da inibição das enzimas que hidrolisam os carboidratos no trato gastrointestinal, como a α-amilase e α-glicosidase (WONGSA; PHATIKULRUNGSUN; PRATHUMTHONG, 2022;

RASOULI et al., 2017). A acarbose, um dos fármacos mais usados no tratamento do diabetes tipo 2, age inibindo a ação da α-amilase e da α-glicosidase. No entanto, na literatura há relatos de que o uso prolongado da acarbose pode resultar em efeitos colaterais, provocar resistência a certos medicamentos, e até mesmo, provocar citotoxicidade (TONGKAEW et al., 2022; SPÍNOLA; LLORENT-MARTÍNEZ; CASTILHO, 2020; NYAMBE-SILAVWE et al., 2015). Dessa forma, tem se buscado alternativas a partir de compostos naturais para a inibição dessas enzimas, como os compostos fenólicos, que poderiam ter ação semelhante à acarbose, mas que apresentassem efeitos colaterais menores ou até mesmo ausentes quando comparados aos efeitos provocados pelo fármaco (TONGKAEW et al., 2022; NYAMBE-SILAVWE et al., 2015). Alguns compostos fenólicos, incluindo flavonoides e ácidos fenólicos, demonstraram capacidade de inibir as enzimas digestivas α-amilase e α-glicosidase (MAHNASHI et al., 2022; HANHINEVA et al., 2010). A catequina é um desses compostos que tem chamado a atenção, pois há indícios de apresentar fortes efeitos inibidores na atividade das enzimas α-amilase e α-glicosidase (LEE et al., 2020). Além disso, espécies do gênero Eugenia, dentre elas *E. uniflora*, já foram relatadas como inibidores de enzimas digestivas α-amilase e α-glicosidase (ARAUJO et al., 2021).

Um fator de destaque envolvido no desenvolvimento e progressão do diabetes tipo 2 e suas complicações é o estresse oxidativo (BAKSHI; SHARMA; NAGPAL, 2022). O estado de hiperglicemia persistente, que caracteriza o diabetes, provoca uma superprodução de ER, as quais contribuem para a destruição das células β das ilhotas pancreáticas a longo prazo. O excesso de ERs afeta a produção mitocondrial de trifosfato de adenosina (ATP), ocorrendo redução na síntese e secreção de insulina (LOIZZO et al., 2019).

Além disso, a formação excessiva de ERs induzida pela hiperglicemia pode causar danos às biomoléculas e depleção de sistemas antioxidantes, e aqui podemos destacar a interação da glicose com proteínas celulares que leva à formação dos produtos de Amadori e dos produtos de glicação avançada (AGEs, do inglês *Advanced Glycation End-products*). Os AGEs, por sua vez, ativam proteínas intra e extracelulares que através de receptores específicos para os AGEs (RAGE), ativam células inflamatórias e induzem a formação de ERs, que colaboram para a ativação da transcrição de fatores nucleares que resultam na expressão de genes envolvidos na inflamação vascular e disfunção endotelial (RUIZ; RAMASAMY; SCHMIDT, 2020; FREUND; CHEN; DECKER, 2018).

Os AGEs são compostos que podem ser formados de maneira endógena ou exógena. A formação endógena pode ocorrer por meio da hiperglicemia no diabetes ou por aumento do estresse oxidativo, já a formação exógena de AGEs ocorre principalmente em alimentos e no

tabaco (VAN DONGEN et al., 2022; URIBARRI et al., 2015). Os AGEs são formados por meio de reação não enzimática, através da ligação covalente de um açúcar redutor a um grupo amino de proteínas, podendo também ser formados por meio da oxidação de lipídios ou nucleotídeos. Quando essa reação ocorre em alimentos ela é denominada de reação de Maillard, enquanto a reação que ocorre em sistemas biológicos é denominada glicação. (HUANG et al., 2019; URIBARRI et al., 2015; SZAWARA-NOWAK et al., 2014; LUEVANO-CONTRERAS & CHAPMAN-NOVAKOFSKI, 2010).

A formação dos AGEs está associada ao estresse oxidativo e a processos inflamatórios em diversas doenças crônicas como o diabetes, sendo importante destacar que os AGEs podem causar o estresse oxidativo, e que ao mesmo tempo o estresse oxidativo também pode levar a formação dos AGEs (URIBARRI et al., 2015).

Em pessoas com idade mais avançada, é encontrado um acúmulo de AGEs, e este acúmulo acaba sendo maior em altas concentrações de glicose. O acúmulo de AGEs contribui para alterações no sistema cardiovascular, aumento da perda de massa e força muscular, surgimento de doenças renais, doença de Alzheimer e transtornos mentais (D'CUNHA et al., 2022; WU et al., 2022; LUEVANO-CONTRERAS & CHAPMAN-NOVAKOFSKI, 2010).

Os produtos da reação de glicação atuam como estressores oxidantes ou como agentes que contribuem para um elevado risco de complicações associadas ao diabetes. Dessa forma, reduzir ou retardar a formação de AGEs pode ser eficaz no retardo e prevenção principalmente das complicações do diabetes (WU et al., 2011). Existem compostos sintéticos que fazem o papel de inibidores dos AGEs, sendo que o mais conhecido é a aminoguanidina, a qual apresenta diversos efeitos colaterais, como citotoxicidade observada em ensaios clinicos (ZHAO et al., 2022; URIBARRI et al., 2015). Dessa forma, diversos estudos vêm sendo realizados com o objetivo de encontrar compostos naturais, presentes em plantas e alimentos, que possuam potencial de inibir a glicação de proteínas e a formação dos AGEs. Dentre os compostos estudados, destacam-se os compostos fenólicos, pois constituem o principal grupo de compostos que demonstraram a capacidade de inibir *in vitro* a formação de AGEs (URIBARRI et al., 2015; WU et al., 2011).

Um dos principais fatores de risco para o diabetes é a obesidade, outra doença que vem causando sérios impactos na saúde da população e tem gerado grande preocupação aos profissionais de saúde. A obesidade é uma doença crônica caracterizada pelo acúmulo excessivo de gordura corporal, sendo considerada um problema de saúde pública devido ao grande número de pessoas obesas e ao fato de estar intimamente associada ao aumento do risco de outras patologias, como as doenças cardiovasculares, hipertensão, hiperlipidemia, artrite,

acidente vascular cerebral, diabetes e câncer. Também existe forte associação da obesidade com maior morbimortalidade (EL-SHIEKH et al., 2019; YUN, 2010; HUANG et al., 2009).

A obesidade é causada pelo desequilíbrio entre a ingestão de alimentos e o gasto calórico, o que resulta em acúmulo de gordura no tecido adiposo e não adiposo (MELDRUM et al., 2017; EBRAHIMPOUR; ZAKERI; ESMAEILI, 2020). As mudanças na oferta de alimentos incluindo aumento da disponibilidade e comercialização de alimentos de baixo custo, prontos para consumo, com elevado valor energético, oferecidos em porções grandes, ricos em gordura e açúcares e pobres em proteína e fibra alimentar, juntamente com o estilo de vida sedentário, contribuem para o aumento da prevalência de obesidade observada nas últimas décadas (HALL et al., 2022).

A lipase pancreática é considerada a enzima mais importante envolvida no processo de digestão das gorduras provenientes da dieta, hidrolisando cerca de 50 a 70% das gorduras alimentares, e a sua inibição está associada com efeitos benéficos sobre o sobrepeso e a obesidade (WONGSA; PHATIKULRUNGSUN; PRATHUMTHONG, 2022; DUARTE et al., 2020). Existem fármacos que são usados com a finalidade de promover a inibição da enzima lipase pancreática e consequente redução de peso corpóreo, como é o caso do orlistat. Entretanto, esses fármacos comumente usados causam alguns efeitos colaterais como dor abdominal, esteatorréia, incontinência fecal, insônia, inquietação e problemas cardiovasculares (AHMAD et al., 2020). Devido aos efeitos adversos provocados pelos fármacos, tem-se investigado compostos naturais que promovam reduzidos efeitos colaterais, sendo que a inibição das enzimas que promovem a digestão e absorção de nutrientes, como a lipase pancreática, tem sido um dos mecanismos investigados na busca por compostos que poderiam apresentar um efeito anti-hiperglicêmico e anti-obesidade.

2.4 POTENCIAL BIOLÓGICO DE EXTRATOS DE CEREJEIRA-DO-RIO-GRANDE

2.4.1 CAPACIDADE ANTIOXIDANTE DE EXTRATOS DA CEREJEIRA-DO-RIO-GRANDE

Frutas e vegetais em geral são ricos em compostos bioativos como compostos fenólicos, que são considerados potentes antioxidantes pois possuem a capacidade de neutralizar EROs e ERNs (TSAO, 2010) e diversas evidências apontam que esses fitoquímicos poderiam auxiliar na proteção dos sistemas celulares contra danos oxidativos (AUGUSTI et al., 2021a; NICÁCIO et al., 2017; ZHANG et al., 2010).

Atualmente existem diversos ensaios disponíveis para avaliar a capacidade antioxidante, os quais podem ser classificados com base no tipo de reações químicas que sofrem, podendo ser divididos em ensaios baseados em transferência de elétrons e em ensaios baseados na transferência de átomos de hidrogênio. Em ensaios onde ocorre a transferência de elétrons é realizada a avaliação da capacidade que o antioxidante em estudo possui, de transferir elétrons e reduzir determinados compostos oxidantes, como carbonilas, metais e radicais. Nesse caso podemos citar os métodos de ensaio de ácido 2,2′-azinobis (3-etilbenzotiazolina-6-sulfônico) (ABTS), 2,2-difenil-1-picrilhidrazil (DPPH) e o poder redutor férrico (FRAP, do inglês *Ferric Reducing Antioxidant Power*) como exemplos (LEWOYEHU & AMARE, 2019; SIDDEEG et al., 2020).

Entretanto, em ensaios de transferência de átomos de hidrogênio, é avaliada a capacidade que um possível antioxidante apresenta para eliminar radicais livres através da doação de íons de hidrogênio, a partir de uma molécula estável. Como ensaios baseados na transferência de átomos de hidrogênio podemos citar o método de capacidade de absorção do radical oxigênio (ORAC, do inglês *Oxygen Radical Absorbance Capacity*) (LEWOYEHU & AMARE, 2019; SIDDEEG et al., 2020).

Além disso, é importante destacar que resultados da capacidade antioxidante de um composto podem variar quando diferentes métodos são usados, pois nos sistemas vivos existem diversos radicais e ERs, e também diversos mecanismos envolvidos no combate ao estresse oxidativo. Também, deve-se levar em conta a natureza química das moléculas usadas no teste, procurando-se empregar o ensaio mais adequado a fim de se obter resultados mais próximos da realidade. Devido a isso, existem diversos ensaios disponíveis para avaliar a capacidade antioxidante e deve-se destacar que há a necessidade de realização de diversos testes para contemplar todos os aspectos da capacidade antioxidante de um composto (LEWOYEHU & AMARE, 2019).

Alguns autores têm levantado críticas com relação a estes métodos de avaliação da capacidade antioxidante *in vitro*, alegando que alguns desses métodos se baseiam na remoção de radicais que são ausentes em organismos vivos, como DPPH e ABTS. Também alegam que a alta atividade antioxidante encontrada através de métodos *in vitro* não deve ser declarada como prevenção, tratamento ou até mesmo cura de determinadas doenças crônicas não transmissíveis, e que para este tipo de alegação é necessária a realização de ensaios *in vivo* (modelos animais e humanos) (GRANATO et al., 2018).

Por outro lado, os ensaios *in vitro* para avaliar a capacidade antioxidante, em sua maioria, são de baixo custo, de fácil execução, de curta duração, e não requerem equipamentos

ultrassensíveis, podendo ser usados para analisar compostos isolados e mistos. Esses ensaios são uma importante ferramenta de triagem usada na descoberta de potenciais fontes antioxidantes, apesar das suas limitações para prever efeitos *in vivo* (GRANATO et al., 2018).

Com relação à cerejeira-do-rio-grande, existem poucos estudos disponíveis na literatura cientifica que avaliaram a capacidade antioxidante de diferentes partes dessa planta. Infante e colaboradores (2016) compararam a capacidade antioxidante de extratos etanólicos de folhas, frutos e sementes de quatro espécies do gênero *Eugenia*, incluindo a *E. involucrata.* Os autores observaram que o extrato produzido a partir das folhas da cerejeira-do-rio-grande foi o que apresentou maior capacidade antioxidante avaliada através do método de remoção do radical peroxil (ORAC, do inglês Oxygen Radical Absorbance Capacity; 1.393,3 ± 69,6 μmol de equivalente de trolox (TE).g^{-1}) quando comparado ao extrato das sementes (168,7 ± 4,9 μmol TE.g^{-1}) e ao extrato dos frutos (321,7 ± 9,8 μmol TE.g^{-1}) da mesma espécie. No entanto, Girardelo e colaboradores (2020), observaram maior capacidade antioxidante do extrato etanólico de sementes da cerejeira-do-rio-grande (894,8 ± 84,4 μmol TE. mL^{-1}) do que no extrato da polpa (248,5 ± 33,5 μmol TE. mL^{-1}) através do teste de ORAC. Já Schmidt et al. (2020), que também avaliou a capacidade antioxidante de extratos hidroetanólicos de polpa de cerejeira-do-rio-grande através do método de ORAC, encontrou 126.7 ± 10.4 126 μmol TE. g^{-1} de extrato seco, para amostra 2 de extrato de cereja. Essas divergências de resultados, encontradas nesses estudos, podem ser devido aos diferentes métodos de extração usados, diferentes perfis químicos e conteúdo fenólico total encontrados.

Em ensaio de remoção do radical DPPH, Infante e colaboradores (2016) observaram que o extrato das sementes teve maior capacidade antioxidante que o extrato dos frutos, uma vez que a concentração de eficiência que reduz 50% da quantidade do radical (EC_{50}), para sementes foi 346,7 ± 14,1 μg.mL^{-1} e para os frutos a EC_{50} encontrado foi 988,5 ± 22,4 μg.mL^{-1}. No mesmo sentido, Nicácio e colaboradores (2017) também avaliaram a capacidade antioxidante de extratos etanólicos de folhas, frutos e sementes da cerejeira-do-rio-grande, e observaram maior capacidade antioxidante para o extrato das sementes e das folhas através de ensaios de remoção dos radicais $ABTS^{+}$ e DPPH, em comparação com o extrato dos frutos.

Os estudos conduzidos até o presente momento demostram que extratos obtidos a partir de diferentes partes da cerejeira-do-rio-grande apresentaram considerável capacidade antioxidante, com destaque para o extrato produzido a partir das sementes.

2.4.2. OUTRAS ATIVIDADES BIOLÓGICAS DOS EXTRATOS DA CEREJEIRA-DO-RIO-GRANDE

Extratos produzidos com diferentes partes de plantas pertencentes a espécies do gênero *Eugenia* tem sido usadas na medicina tradicional como agentes antimicrobianos e anti-inflamatórios (HUSSEIN, 2003). Entretanto, estudos a respeito da atividade biológica, além da atividade antioxidante, de extratos da cerejeira-do-rio-grande ainda são muito escassos na literatura cientifica, os quais avaliaram somente aspectos relacionados a atividade antitumoral e anti-inflamatória (INFANTE et al., 2016; GIRARDELO et al., 2020).

Recentemente, o potencial biológico de extrato de sementes da cerejeira-do-rio-grande foi avaliado por Girardelo e colaboradores (2020), através da avaliação da atividade antitumoral utilizando cultura celular de adenocarcinoma de pâncreas humano (PANC-1). Os pesquisadores concluíram que o extrato das sementes apresentou maior atividade antitumoral quando comparado ao fármaco Gemcitabina, considerado padrão ouro no tratamento de câncer de pâncreas. Neste estudo, o extrato apresentou citotoxicidade maior que a Gemcitabina em células PANC-1, sendo que o fármaco apresentou apenas 10% de citotoxicidade na concentração máxima avaliada (50 µM). Os pesquisadores acreditam que o resultado encontrado pode estar relacionado ao elevado conteúdo de compostos fenólicos presente nesta parte da planta.

Os extratos etanólicos de folhas, frutos e sementes da cerejeira-do-rio-grande também apresentam atividade anti-inflamatória (INFANTE et al., 2016). Através de um ensaio *in vivo* de migração de neutrófilos induzido por carragenina, os pesquisadores verificaram que o extrato de folhas reduziu em 40% a migração de neutrófilos, enquanto o controle positivo (dexametasona) reduziu em 64%, no entanto, os extratos de frutos e de sementes não afetaram a migração dos neutrófilos.

Apesar dos diferentes estudos *in vitro* e *in vivo* existentes na literatura, que avaliaram o potencial biológico de extratos de diversas partes da cereja-do-rio-grande, estudos sobre o potencial desses extratos frente a desordens no metabolismo da glicose são muito escassos, havendo apenas um estudo na literatura avaliando extratos das folhas frente a inibição da enzima α-glicosidase (CIPRIANI et al., 2022). Diversos estudos têm revelado que dietas ricas em compostos fenólicos possuem a capacidade de reduzir os níveis de glicose sanguínea, atuando através da inibição de certas enzimas digestivas. Dessa forma, pesquisas têm sido realizadas com o intuito de elucidar potenciais substâncias naturais que possuam efeito inibidor de enzimas digestivas e assim reduzir a hiperglicemia causada através da ingestão de alimentos que contenham amido em sua constituição (ARAÚJO et al., 2021; YAZDANKHAH; HOJJATI; AZIZI, 2019; SIMONATO et al., 2019).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Estudar *in vitro* a atividade biológica de extratos de sementes e frutos da cerejeira-do-rio-grande (*E. involucrata*) com foco no seu potencial anti-hiperglicêmico, antiobesogênico e antioxidante.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar a capacidade dos compostos fenólicos dos extratos para inibir a atividade de enzimas envolvidas na digestão de carboidratos e lipídios comparando sua potência e eficácia com inibidores de referência.
- Investigar *in vitro* a capacidade antioxidante dos compostos fenólicos dos extratos e seu potencial para inibir a formação de AGEs.

4 DESENVOLVIMENTO

4.1 MANUSCRITO:

Assessment of antidiabetic and anti-obesogenic potential of pulp and seed extracts from *Eugenia involucrata* fruits *in vitro*

Artigo a ser submetido ao periódico Food Research International (Qualis A1)

Assessment of antidiabetic and anti-obesogenic potential of pulp and seed extracts from *Eugenia involucrata* fruits *in vitro*

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Abstract

Eugenia involucrata DC. is an unexplored native plant species, present in the southern and southeastern regions of Brazil. Recent studies report the presence of large amounts of bioactive phenolic compounds (PC) in different parts (leaves, pulp, and seeds) of this species. The objective of this study was to investigate *in vitro*, the anti-hyperglycemic and anti-obesogenic potential of PC of extracts from *E. involucrata* DC. fruit pulp and seeds, through its ability to inhibit enzymes involved in the digestion of carbohydrates and lipids and the formation advanced glycation end products (AGEs), besides their potential to neutralize reactive species of biological relevance. The seed extract had higher content of total PC than the pulp extract. The extracts inhibited (IC_{50} values as μg of PC. mL^{-1}) the activities of α-amylase (0.2 for pulp vs. 1.3 for seed), α-glucosidase (0.3 for pulp vs. 1.5 for seed), and pancreatic lipase (0.7 for pulp vs. 0.6 for seed), the formation of AGEs (6.8 for pulp vs. 30.9 for seed), and had antioxidant capacity by scavenging peroxyl radical (0.02 for pulp vs. 0.12 for seed) hydroxyl

radical (3.9 for pulp vs. 4.5 for seed). Results obtained revealed the in vitro antidiabetic and antiobesogenic activity of *E. involucrata* with higher potency for phenolic compounds of fruit pulp than seed. The bioactive potential of phenolic compounds of pulp and seed must be further investigated *in vivo* to support its beneficial role in human health.
Keywords: cherry-of-rio-grande; bioactive compounds; diabetes; digestive enzymes; advanced glycation end products.

1. Introduction

The biodiversity of the Brazilian ecosystem is considered one of the richest in the world, being a source of valuable native plants and fruits, which can provide sustainable solutions to diversify food production and improve food security (SCHULZ et al., 2020; SCHMIDT et al., 2020; INFANTE et al., 2016). Manu underexplored native species are sources of bioactive compounds that exhibit biological activities potentially relevant for the prevention and treatment of chronic diseases, such as diabetes and cancer (ARAUJO et al., 2021; GIRARDELO et al., 2020).

The cherry-of-rio-grande (*Eugenia involucrata* DC.) belongs to the Myrtaceae family and is an underexplored native species found in the South and Southeast regions of Brazil. Its fruits are mainly consumed fresh. Some recent studies focused on the phenolic compounds of this species and their antioxidant, anti-inflammatory and antitumoral activity *in vitro* (INFANTE et al., 2016; NICÁCIO et al., 2017; GIRARDELO et al., 2020; SCHMIDT et al., 2020; MANNINO et al., 2022).

Phenolic compounds are formed by at least one aromatic ring with one or more hydroxyl functional groups attached, ranging from simple to highly polymerized molecules (SCHMIDT et al., 2020). They are a class of natural compounds with outstanding antioxidant potential, being able to scavenge free radicals, reducing oxidative stress, and protecting against the oxidation of biomolecules. Some phenolic compounds and phenolic-rich foods have also been demonstrated to inhibit digestive enzymes linked to diabetes and obesity (NOWICKA, WOJDYLO, & LASKOWSKI, 2018; SIMONATO et al., 2019; ARAUJO et al., 2021).

Diabetes affects 537 million people worldwide, and the number of cases increased by 74 million between 2019 and 2021 (IDF, 2021). It is a chronic disease characterized by a deficiency in the secretion of insulin and/or tissue insulin resistance that results in

hyperglycemia (IDF, 2021; RODACKI et al., 2022). Hyperglycemia promotes the glycation of proteins, lipids and DNA generating advanced glycation end products (AGEs) that promote oxidative stress and are implicated in the micro and macrovascular complications of diabetes (ARAUJO et al., 2021). Type 2 diabetes, which accounts for more than 90% of diabetes cases, and borderline diabetes patients can be benefited by the inhibition of digestive enzymes linked to carbohydrate digestion, such as α-amylase and α-glucosidase, that significantly reduces postprandial glycemia. Acarbose is a α-glucosidase inhibitor that is currently used in diabetes management (LEISTNER & HOLZGRABE, 2022). Another factor that contributes to the development and/or complications of diabetes is obesity, which leads to increased insulin resistance, promoting hyperglycemia. Obesity is characterized by the accumulation of body fat, mainly caused by excessive intake of caloric foods and a sedentary lifestyle (SPÍNOLA, LLORENT-MARTÍNEZ, & CASTILHO, 2020). The absorption of dietary fat also depends on the activity of digestive enzymes such as lipase (KUMAR & CHAUHAN, 2021). In fact, lipase inhibitors, such as orlistat, are approved by the Food and Drug Administration for the treatment of obesity (ZHANG et al., 2022).

Some *in vitro* and *in vivo* studies have evaluated biological properties of extracts from different parts of *E. involucrata* (INFANTE et al., 2016; NICÁCIO et al., 2017; GIRARDELO et al., 2020; SCHMIDT et al., 2020; MANNINO et al., 2022) but no study investigated the potential of pulp or seed extracts against disorders associated to glucose metabolism, and only one study that was focused on leaf extracts investigated its potential to inhibit α-glucosidase activity (CIPRIANI et al., 2022).

This study aimed to investigate *in vitro* the anti-hyperglycemic and anti-obesogenic potential of ethanolic extracts of *E. involucrata* fruit pulp and seeds, through their ability to inhibit enzymes involved in the digestion of carbohydrates and lipids, and their antioxidant activity as well as their potential to inhibit the formation of AGEs.

2. Materials and methods

2. 1 Plant materials

Specimens with fruits of *E. involucrata* were collected in November 2020 in the region of Curitibanos (Santa Catarina, Brazil) (27°8′29.30″S and 50°45′21.74″O) (SISGEN access number A967541) and taxonomically identified by a botanist. A voucher was deposited in the Federal University of Santa Catarina herbarium (CTBS: 3943). Immediately after collection,

the fruits were washed, refrigerated and protected from light, and immediately sent to the laboratory (UFSM, Santa Maria, Brazil) where they were kept at -20°C until analysis.

2.2 Chemical reagents

2,2′-Azobis(2-amidino-propane dihydrochloride (AAPH), thiobarbituric acid (TBA), bovine serum albumin, dimethylsulfoxide (DMSO), Ellman's reagent/5,5′- dithio-bis-2-nitrobenzoic acid (DTNB), α-glucosidase from Saccharomyces cerevisiae Type I, 4-nitrophenyl α-D-glucopyranoside (PNP-G) , α-amylase from porcine pancreas Type VI-B, lipase from porcine pancreas Type II, 4-methylumbelliferyl oleate (4-MUO), aminoguanidine hydrochloride, trolox, 2-deoxy-D-ribose, reduced glutathione (GSH), ethylenediaminetetraacetic acid (EDTA), cyanidin-3-glucoside (95%) protocatechuic acid (97%), 4-hydroxybenzoic acid (99%), vanillic acid (97%), catechin (98%), epicatechin (98%), myricetin (96%), p-coumaric acid (98%), syringic acid (95%), ellagic acid (95%), trans-ferulic acid (99%), kaempferol-3βD-glucopyranoside (97%), sinapic acid (99%), trans-cinnamic acid (99%), rutin (94%), quercetin (95%), gallic acid (98%), caffeic acid (98%), chlorogenic acid (95%), and resveratrol (99%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). D-Ribose, soluble starch were purchased from Êxodo Científica (São Paulo, Brazil). Acarbose were purchased from EMS Sigma Pharma Ltda (São Paulo, Brazil) and orlistat were purchased from Galena Química e Farmaceutica Ltda (São Paulo, Brazil). Absolute ethanol (99.4 °INPM), sodium phosphate monobasic, sodium phosphate dibasic, potassium phosphate monobasic and potassium phosphate dibasic were obtained from Comercial Neon© (Sao Paulo, Brazil). hydrogen peroxide (H_2O_2) was obtained by Dinâmica Química Contemporânea® (Sao Paulo, Brazil). Methanol, acetonitrile and formic acid (88%) were from J.T. Baker® (Center Valley, PA, USA). Liquid chromatography (LC)-grade water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Polytetrafluoroethylene syringe filter membrane (PTFE) were obtained by Waters (Milford, MA, USA).

2.3 Qualitative determination of cyanogenic glycosides

The presence of cyanogenic glycosides in the seeds of *E. involucrata* was assessed using the official method AOAC 936.11. We placed the chopped seed sample in tubes, added a few drops of chloroform and inserted a piece of moistened sodium picrate paper, which was attached to the lid of the tube. Tubes were tightly closed and incubated at 30 °C for 60 min.

Apple seeds were assessed in parallel tubes as positive control for the test. The presence of cyanogenic glycosides can be detected by a was indicated by color change from of picrate paper from yellow to brick red.

2.4 Pulp and seed extracts

The extracts were prepared according to Denardin et al. (2015) with brief modifications according to Girardelo et al. (2020). Fruit pulp (peel + flesh) was manually separated from seeds. Pulp (15 g) was minced an ultra-turrax, while the seeds (15 g) were previously manually cut with a knife and then minced in an ultra-turrax during 5 min with 45 mL of absolute ethanol (99.4 °INPM) (1:3, w/v). Thereafter, the sample was transferred to an Erlenmeyer flask and shaken for 30 min at 26°C. After stirring, the liquid fraction of the extract was separated, centrifuged (1500 × g for 5 min) and the supernatant obtained was stored under light protection. A second extraction was carried out by adding 45 mL of absolute ethanol (99.4 °INPM) to the solid residue of the minced sample, and all steps above were repeated once more. The supernatants obtained were mixed and evaporated in a rotary evaporator (40 °C/12 min). The extracts obtained from fruits and seeds were stored at −20 °C until analysis.

2.5 Phenolic compounds analysis

Phenolic compounds were analyzed by high performance liquid chromatography (HPLC) using a CBM-20A prominence chromatograph (Shimadzu, Japan) that was equipped with a degasser, manual injector, UV-vis and photodiode array (PDA) detectors.

Anthocyanins from pulp extract were quantified at 520 nm in a reversed phase C18 Core-Shell Kinetex (particle size 2.6 µm, 100 mm, 4.6 mm) at 38°C. The mobile phases were composed of solvent A (water: formic acid: acetonitrile (88.5:8.5:3, v/v/v)) and solvent B (water: formic acid: acetonitrile (41.5:8.5:50, v/v/v)). Samples (20 µL) were separated using a linear gradient for solvent B consisting of 0 min, 6%; 10 min, 30%; 30 min, 50%; 34 min, 100%; 36 min, 100%; 42 min, 6% run at 0.19 mL. min^{-1} (QUATRIN et al., 2019). Anthocyanins were identified based on the order of elution from previous studies (GIRARDELO et al., 2020; SCHMIDT et al., 2020) and quantified as equivalents of cyanidin-3-glucoside, concentration range 8 – 20 ppm (regression equation y = 0.000016875x – 3.8836; r = 0.9820). The limit of

detection (LoD) and limit of quantification (LoQ) for cyanidin-3-glucoside were, respectively, 0.020 and 0.068 ppm.

Non-anthocyanin phenolic compounds from pulp and seed extracts were quantified using a PDA detector and aC18 CLC-ODS column (150 mm, 4.6 mm, 5 μm particle size; Core-Shell technology, Phenomenex) at 35ºC. The method was described by Quatrin et al. (2019), the mobile phases being composed of Solvent A (5% (v/v) of methanol in acidified water (0.1% (v/v) of formic acid) and Solvent B (0.1% (v/v) v) of formic acid in acetonitrile).

The non-anthocyanin phenolic compounds from extracts were identified by comparison with the retention time of authentic standards and the spectral data obtained from UV–visible absorption spectra. To quantify phenolic acids and flavonoids, the chromatograms were obtained at 280 nm, 320, and 360 nm. Stock solutions of standards references were prepared in the initial mobile phase and were diluted in equidistant points within the concentration range of LOQ–60 ppm. Calibration curve for gallic acid: y = 79089x + 81326 (r = 0.9984); protocatechuic acid: y = 41570x + 28970 (r = 0.9977); syringic acid: y = 82930x + 40566 (r = 0.9995); trans-cinnamic acid: y = 235358x + 63507 (r = 0.998); catechin: y = 19861x + 21544 (r = 0.997); rutin: y = 53524x – 41979 (r = 0.978); quercetin: y = 64434x – 373423 (r = 0.8945); kaempferol 3-*O*-*β*-D-glucopyranoside: y = 57482x – 95671 (r = 0.9969). The limit of detection (LoD) and limit of quantification (LoQ) for gallic acid, protocatechuic acid, syringic acid, trans-cinnamic acid, catechin, rutin, quercetin, and kaempferol 3-*O*-*β*-D-glucopyranoside were, respectively, 0.012 and 0.037 ppm; 0.027 and 0.083 ppm; 0.008 and 0.024 ppm; 0.010 and 0.032 ppm; 0.026 and 0.078 ppm; 0.099 and 0.330 ppm; 0.146 and 0.444 ppm; 0.028 and 0.084 ppm. Unidentified compounds that are derivatives of one of the standard monomers were quantified by equivalence.

2.6 Evaluation of anti-hyperglycemic and anti-obesogenic potential *in vitro*

2.6.1 α-Amylase inhibition

The method for enzymatic inhibition assay was adapted from Apostolidie et al. (2007). Test-tubes containing different concentrations of cherry pulp (0.03 – 1.80 μg of phenolic compounds equivalent. mL^{-1}) or seed extracts (0.09 – 46.24 μg of phenolic compounds equivalent. mL^{-1}) or positive control acarbose (44.0 – 352.0 μg. mL^{-1}) were added 13 U/mL α-amylase solution (type VI-B from porcine pancreas in 0.02 M sodium phosphate buffer pH 6.9) and incubated at 25°C for 10 min before 1% soluble starch solution was added to each tube and incubated for further 10 min. Finally, dinitrosalicylic acid color reagent was added and the tubes

were placed in 100°C water for 5 min. The mixture was diluted with water and absorbance was read at 520 nm using a Hidex Sense plate reader (Hidex, Finland). The results were expressed as inhibition (%) of α-amylase activity according to the following formula:

$$\text{Inhibition (\%)} = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right] \text{X } 100$$

The inhibitory activity was expressed as IC_{50} (µg of phenolic compounds equivalent of pulp or seed. mL^{-1}), the concentration of extract required to cause a decrease of 50 % of the enzyme activity.

2.6.2 α-Glucosidase inhibition

The method for enzymatic inhibition assay was adapted from Apostolidie et al. (2007). Briefly, extracts of cherry pulp (0.11 – 1.10 µg of phenolic compounds equivalent. mL^{-1}) or seeds (0.72 – 2.89 µg of phenolic compounds equivalent. mL^{-1}) or positive control acarbose (1,075.0 – 2,1500.0 µg. mL^{-1}) were incubated at 25°C for 10 min in a 96-well plate containing 1 U/mL α-glucosidase solution (in 0.1 M sodium phosphate buffer pH 6.9). Then, p-nitrophenyl-α-d-glucopyranoside solution (5 mM) was added the absorbance was read at 405 nm using a Hidex Sense plate reader (Hidex, Finland), and incubated at 25°C for 5 min before the absorbance was read again at 405 nm. The results were expressed as the inhibition (%) of α-glucosidase activity according to the following formula:

$$\text{Inhibition (\%)} = \left[\frac{\Delta\text{Abs}_{\text{control}} - \Delta\text{Abs}_{\text{sample}}}{\Delta\text{Abs}_{\text{control}}}\right] \text{X } 100$$

The inhibitory activity was expressed as IC_{50} (µg of phenolic compounds equivalent of pulp or seed. mL^{-1}), the concentration of extract required to cause a decrease of 50 % of the enzyme activity.

2.6.3 Pancreatic lipase inhibition

The inhibition of lipase activity was determined by measuring the release of 4-methylumbelliferone from 4-methylumbelliferyl oleate using a fluorimetric method adapted from Podsedek et al. (2014) and Nakai et al. (2005). Extracts of cherry pulp (0.28 – 2.20 µg of phenolic compounds equivalent. mL^{-1}) or seed (0.29 – 14.44 µg of phenolic compounds

equivalent. mL^{-1}) or positive control orlistat (0.0 – 49.6 µg. mL^{-1}), lipase from porcine pancreas type II at 1 mg/mL (dissolved in buffer consisting of 20 mM Tris-base, 150 mM NaCl, and 1.3 mM $CaCl_2$, pH 7.4) were incubated in a 96-well plateat 37°C for 5 min. Then, 0.1 mM 4-MUO (dissolving f 4-MUO in DMSO and diluting 50-fold with the above Tris buffer) solution was added and incubated at 37°C for 20 min. Then, 0.1 M sodium citrate (pH 4.2) was added to stop the reaction, and the amount of 4-methylumbelliferone released by lipase was measured in a microplate reader Hidex Sense (Hidex, Finland) at an excitation wavelength of 360 nm and at an emission wavelength of 460 nm. The results were expressed as inhibition (%) of lipase activity according to the following formula:

$$\text{Inhibition (\%)} = \left[\frac{(F_{\text{control}} - F_{\text{control blank}}) - (F_{\text{sample}} - F_{\text{sample blank}})}{(F_{\text{control}} - F_{\text{control blank}})}\right] \text{ X } 100$$

The inhibitory activity was expressed as IC_{50} (µg of phenolic compounds equivalent of pulp or seed. mL^{-1}), the extract required to cause a decrease of 50 % of the enzyme activity.

2.7 Inhibition of AGEs formation

Inhibition of AGEs formation was determined using BSA/D-ribose assay, a fluorimetric method adapted from Séro et al. (2013). Briefly, 10 mg.mL of BSA solution, 0.5 M of D-ribose solution (prepared in 50 mM potassium phosphate buffer pH 7.4 with NaN_3 0.02%), and different concentrations of extracts of cherry pulp (2.20 – 22.03 µg of phenolic compounds equivalent. mL^{-1}) or seeds (30.9 – 1.7 µg of phenolic compounds equivalent. mL^{-1}) or positive control aminoguanidine hydrochloride (55.0 – 5,525.0 µg. mL^{-1}) were added to a 96-well plate that was incubated at 37°C for 24 h. Thereafter, AGEs fluorescence was evaluated with a Hidex Sense microplate reader (Hidex, Finland) using 370 nm and 440 nm as the excitation and emission wavelengths. The results were expressed as inhibition (%) of the AGEs formation according to the following formula:

$$\text{Inhibition (\%)} = \left[\frac{F_{\text{sample}} - F_{\text{sample blank}}}{F_{\text{control}} - F_{\text{control blank}}}\right] \text{ X } 100$$

The inhibitory activity was expressed as IC_{50} (µg of phenolic compounds equivalent of pulp or seed. mL^{-1}), the concentration of extract required to cause a decrease of 50% of the AGEs formation.

2.8 Evaluation of antioxidant capacity *in vitro*

2.8.1 Oxygen radical absorbance capacity (ORAC) method

The antioxidant potential of all extracts was determined as their potential to protect against fluorescein oxidation by the peroxyl radical (ROO•) generated through the thermal degradation of AAPH, using the ORAC method (OU, HAMPSCH-WOODILL, & PRIOR, 2001). Briefly, the reaction was carried out at 37°C in 75 mM phosphate buffer (pH 7.4), and the final assay volume was 200 µl. Firstly, different concentrations of cherry pulp (0.02 – 0.05 µg of phenolic compounds equivalent. mL^{-1}) or seed extracts (0.07 – 0.41 µg of phenolic compounds equivalent. mL^{-1}) were prepared. Totally, 25 µL of sample (Trolox or extract) and 150 µL of fluorescein (81 nM, final concentration) were placed in the well of the black 96-well microplates. The mixture was pre-incubated for 10 min at 37°C. AAPH solution (25 µL; 152 mM) was rapidly added using a multichannel pipet. The plate was automatically shaken before the first reading, and the fluorescence was recorded every minute for 90 min. Fluorescence (λexc = 485 nm and λem = 528 nm) was measured using a Hidex Sense plate reader (Hidex, Finland). The ORAC values were calculated by a regression equation obtained with area under curve (AUC) of the fluorescein decay. This analysis was performed in triplicate and was expressed as IC_{50} (µg of pulp or seed equivalent. mL^{-1}). The IC_{50} is defined as the concentration of phenolic compounds of extract that inhibits 50% of the fluorescein oxidation.

2.8.2 Deoxyribose assay

The capacity of *E. involucrata* extracts to neutralize the hydroxyl radical (•OH) was evaluated by the deoxyribose method (HALLIWELL, GUTTERIDGE, & ARUOMA, 1987). After oxidation of deoxyribose by •OH generated during Fenton reaction, the produced malondialdehyde (MDA) reacts with 2-thiobarbituric acid (TBA) to form a pink/orange colored complex. Test-tubes containing 250 µL of potassium phosphate buffer (50 mM, pH 7.4), 1 mM $FeCl_3$, 1 mM EDTA, 60 mM 2-deoxyribose, 10 mM H_2O_2, 1 mM ascorbic acid and different concentrations of cherry pulp (2.94 – 14.69 µg of phenolic compounds equivalent. mL^{-1}) or seed extracts (2.57 – 15.40 µg of phenolic compounds equivalent. mL^{-1}) were prepared. The

samples were Incubated at 37°C for 1 h. Then, 250 µL of TBA (1%) and HCl (25%) was added and tubes were heated in a water bath at 100 °C for 15 min. After cooling at room temperature, 200 µL of each sample were transferred to a 96-well microplate and the absorbance was recorded at 532 nm using a Hidex Sense plate reader (Hidex, Finland). The results were expressed as IC_{50} (µg of phenolic compounds equivalent of pulp or seed. mL^{-1}).

2.8.3 Reduced glutathione (GSH) assay

The capacity of neutralization of H_2O_2 was evaluated by the GSH oxidation method (ELLMAN, 1959). One mM of potassium phosphate buffer (pH 6.8), 6 mM GSH, 5 mM H_2O_2, and cherry pulp or seed extracts (17.6 µg of phenolic compounds of pulp equivalent. mL^{-1} and 462.0 µg of phenolic compounds of seed equivalent. mL^{-1}) were added to test-tubes. The reaction mixtures were incubated in the dark at room temperature for 30 min. Then, 12 µL of the incubated solution was transferred to a 96-well microplate and 188 µL of 0.125 mM DTNB were added. After 5 min of incubation, the intensity of the yellow color was evaluated by a Hidex Sense plate reader (Hidex, Finland) at 412 nm. This analysis was performed in triplicate and the results were expressed as IC_{50} (µg of phenolic compounds equivalent of pulp or seed. mL^{-1}).

2.9 Statistical analysis

Data were statistically analyzed by analysis of variance (ANOVA), followed by Tukey's test for comparison of the means at a significance level of 5% ($p < 0.05$), using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The data were expressed as mean ± standard error of the mean (S.E.M.).

3. Results and discussion

3.1 Quantification and characterization of phenolic compounds

The concentration of phenolic compounds in the seed extract was 26 times higher than in the pulp (Table 1) as previously reported by Girardelo et al. (2020). However, the total amount of phenolic compounds found in our study differs from other authors (MANNINO et al., 2022; SCHMIDT et al., 2020; GIRARDELO et al., 2020; NICÁCIO et al., 2017; INFANTE et al., 2016), which may occur due to climate differences in the regions where the fruits were

collected (humidity, solar radiation, temperature, soil composition) or differences in the methods of extraction (GIRARDELO et al., 2020). For both pulp and seed extract, phenolic acids were the class of compounds found at the greatest amount (Table 1).

The phenolic compound found at greater amounts in the seed extract was protocatechuic acid, followed by epicatechin, and catechin, respectively (Table 1 and Figure 1A). In the pulp extract, rutin was found at greater amount, followed by epicatechin, and catechin (Table 1 and Figure 1B). Similar to Girardelo et al. (2020) and Nicácio et al. (2017), the pulp extract showed a greater diversity of phenolic compounds than the seed (Table 1 and Figura 1).

Anthocyanins were found only in the pulp extract, cyanidin-3-*O*-glucoside being the major one followed by cyanidin-*O*-pentoside, whereas the amounts of delphinidin-*O*-hexoside and pelargonidin-3-*O*-glucoside were lower than the limit of quantification (Table 1 and Figure 1C). These results are in line with the profile of anthocyanin phenolic compounds found by Girardelo et al. (2020) and Schmidt et al. (2020) in extracts from the pulp of *E. involucrata*.

Table 1 – Phenolic composition of pulp and seed extracts of *E. involucrata* fruits.

			Quantification (μg.mL^{-1} of extract)	
	Peak number*	**λ** (nm)	Pulp	Seed
Phenolic acids				
Gallic acid	1	280	ND	19.9 ± 0.7
Protocatechuic acid	2	280	4.8 ± 0.6	1,391.9 ± 117.5
Syringic acid	4	280	5.2 ± 0.2	254.9 ± 1.2
trans-Cinnamic acid	9	280	ND	43.3 ± 5.0
Sum of non-identified phenolic acids[1]			284.4 ± 3.7	7,627.4 ± 471.9
Total phenolic acids			294.3 ± 4.0	9,337.4 ± 593.9
Flavonoids				
Catechin	3	280	17.5 ± 0.9	921.6 ± 27.8
Epicatechin[2]	5	280	23.9 ± 6.8	1,105.7 ± 11.5
Rutin	6	360	43.8 ± 0.8	40.6 ± 0.2
Quercetin 3-glucoside[3]	7	360	3.6 ± 0.0	ND
Kaempferol 3-β-D-glucopyranoside	8	360	5.9 ± 0.2	ND
Quercetin	10	360	4.3 ± 0.1	38.9 ± 0.2
Sum of non-identified flavonoids[3]			41.6 ± 0.3	106.3 ± 0.1
Total flavonoids			144.7 ± 26.9	2,213.1 ± 39.1
Anthocyanins[4]				
Delphinidin-*O*-hexoside	11	520	< LoQ	ND
Cyanidin-3-*O*-glucoside	12	520	2.2 ± 0.9	ND
Pelargonidin-3-*O*-glucoside	13	520	< LoQ	ND
Cyanidin-*O*-pentoside	14	520	0.2 ± 0.1	ND
Total anthocyanins			2.4 ± 1.1	ND
Total phenolic compounds			440.7 ± 28.1	11,550.5 ± 633.0

Results are presented as mean ± standard error of the mean. ND = not detected. LoQ = limit of quantification. *Represent the peak numbers corresponding to each compound identified in the chromatograms. [1]Quantified as equivalents of gallic acid. [2]Quantified as equivalents of catechin. [3]Quantified as equivalents of quercetin. [4]Quantified as equivalents of cyanidin-3-*O*-glucoside.

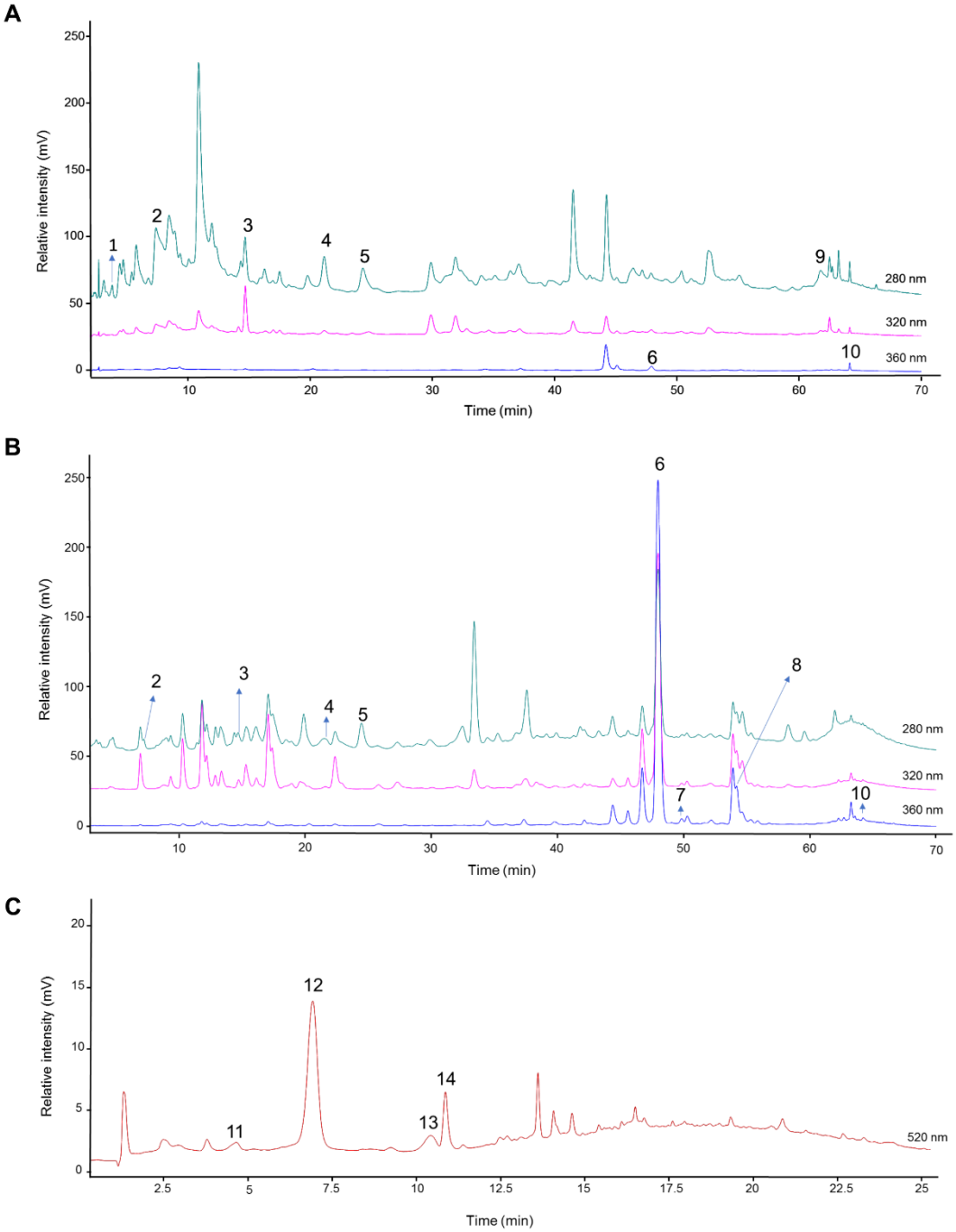


Figure 1 – Representative chromatogram of non-anthocyanin phenolic compounds in seeds (A) and pulp (B) extracts of *E. involucrata* fruits, and anthocyanin phenolic compounds in pulp (C). Chromatograms were acquired at 280 nm, 320 nm, and 360 nm for non-anthocyanin and 520 nm for anthocyanin phenolic compounds. Peak number identification: 1- Gallic acid; 2- Protocatechuic acid; 3- Catechin; 4- Syringic acid; 5- Epicatechin; 6- Rutin; 7- Quercetin 3-glucoside; 8- Kaempferol 3-*β*-D-glucopyranoside; 9- *trans*-Cinnamic acid; 10- Quercetin; 11- Delphinidin-*O*-hexoside; 12- Cyanidin-3-*O*-glucoside; 13- Pelargonidin-3-*O*-glucoside; 14- Cyanidin-*O*-pentoside.

3.2 Evaluation of anti-hyperglycemic potential *in vitro*

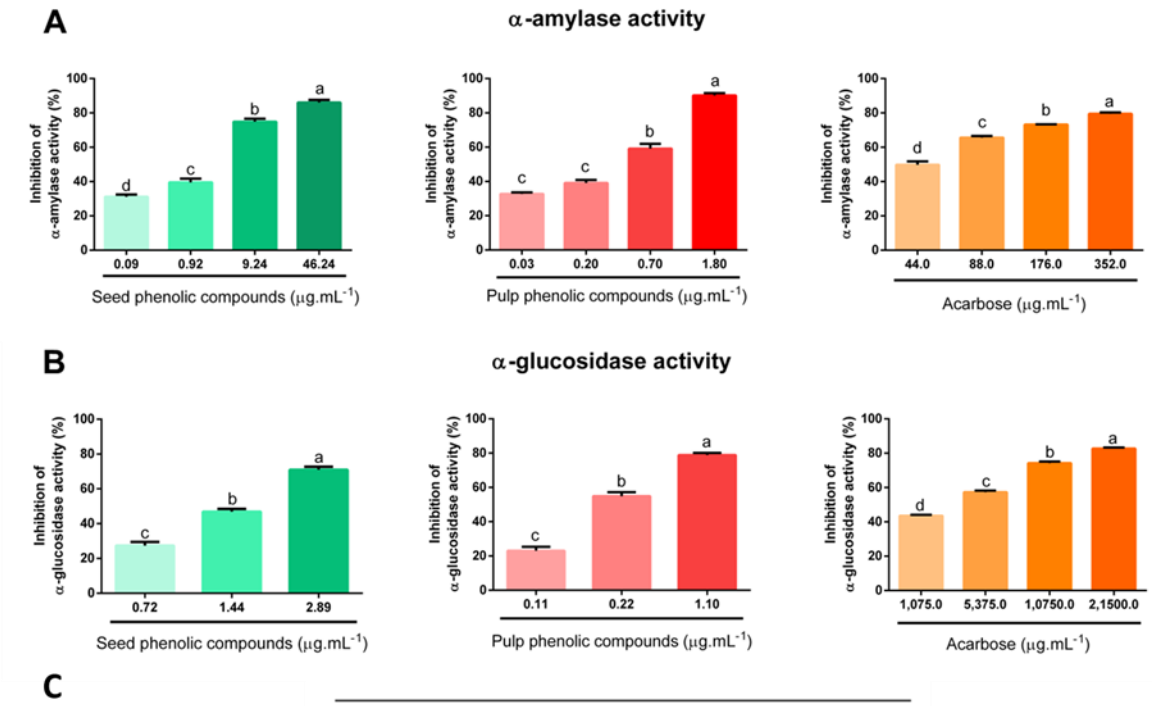
This study also aimed to evaluate the potential of *E. involucrata* pulp and seed extracts to inhibit the activity of digestive enzymes involved in the hydrolysis of carbohydrates (α-amylase and α-glucosidase) and lipids (pancreatic lipase), being the first study to assess the ability of these extracts to inhibit digestive enzymes. α-Amylase and α-glucosidase are primarily responsible for digesting dietary carbohydrates into smaller absorbable molecules (SPÍNOLA, LLORENT-MARTÍNEZ, & CASTILHO, 2020). α-Amylase carries out the first step of starch hydrolysis in the digestive tract (SUN, WARREN, & GIDLEY, 2019), whereas α-glucosidase is responsible for the final step of carbohydrate hydrolysis (VINHOLES et al., 2011). Currently, synthetic α-amylase and α-glucosidase inhibitors, such as acarbose, are used in the management type 2 diabetes to reduce postprandial glucose levels by delaying glucose absorption through the inhibition of carbohydrate digestion (RASOULI et al., 2017; SPÍNOLA, LLORENT-MARTÍNEZ, & CASTILHO, 2020). Because of the side effects such as drug resistance, and even toxicity reported for these synthetic drugs (NYAMBE-SILAVWE et al., 2015; SPINOLA, LLORENT-MARTÍNEZ, & CASTILHO, 2020), phenolic compounds have been prospected as natural alternative compounds that could have similar action with lower side effects (NYAMBE -SILAVWE et al., 2015).

To assess the possible inhibitory action of phenolic compounds of the extracts on digestive enzymes, we compared the results of these extracts with those of the reference drug acarbose. Similar to acarbose, pulp and seed extracts inhibited α-amylase activity in a dose-dependent manner (Figure 2. A). The phenolic compounds of the fruit pulp extract had greater inhibitory potency than the phenolic compounds of seed extract and the phenolic compounds of the two extracts were more potent than acarbose to inhibit α-amylase (IC_{50} values, Figure 2C). The phenolic compounds of extracts of pulp and seed of *E. involucrata* also had a dose-dependent inhibitory effect against α-glucosidase activity (Figure 2B). Phenolic compounds of pulp extracts had the highest potency to inhibit α-glucosidase (lowest IC_{50} values, $p < 0.05$) followed by phenolic compounds of seed extracts, whereas acarbose had the lowest inhibitory potency (about 6,000-fold higher IC_{50} values compared to phenolic compounds of the pulp extract ($p< 0.05$; Figure 2C). These data indicate that the phenolic compounds of the extracts evaluated in the present study had a promising potential to inhibit key enzymes in carbohydrate digestion, with an outstanding performance for the phenolic compounds of pulp and seed extract that had higher potency than acarbose. Different from acarbose that was 50-fold more potent to inhibit α-amylase than α-glucosidase, the phenolic compounds of seed and pulp extracts from

E. involucrata had similar inhibitory potency against both enzymes. Some phenolic compounds, including flavonoids and phenolic acids, have been demonstrated to inhibit α-amylase and α-glucosidase activity as reviewed (HANHINEVA et al., 2010). Catechin, one of major flavonoids of *E. involucrata* seeds (Table 1), has recently demonstrated strong inhibitory effects against α-amylase and α-glucosidase activity (LEE et al., 2020).

Other plant species of the genus *Eugenia* have already been reported to inhibit α-amylase and α-glucosidase enzymes, in both *in vitro* and *in vivo* assays (ARAUJO et al., 2021). Only Cipriani et al. (2022) has already studied such inhibitory effect for *E. involucrata* extracts but they studied the leaves. We demonstrated for the first time that the pulp and seed extracts of *E. involucrata* have inhibitory potency towards α-amylase and α-glucosidase activity. Although the higher content of phenolic compounds has been found in the seed extract compared to the pulp (11,550.5 vs. 440.7 µg of total phenolic compounds. mL^{-1}), higher inhibitory potency was observed for the pulp phenolic compounds (lower IC_{50} values). The greater inhibitory potency of phenolic compounds from pulp extract was likely associated to the presence of anthocyanins that are not found in the seed extracts. According to Borges et al. (2022), anthocyanins are considered one of the main active compounds that inhibit digestive enzymes, especially cyanidin-3-*O*-glucoside, which was found as the major compound in our pulp extract. Despite the higher inhibitory potency of phenolic compounds extracted from *E. involucrata* pulp when compared to the phenolics from seed extract, seed extraction has greater yield. Therefore, the concentration of phenolic compounds was 26 times in seed than pulp extract and seed extract had higher inhibitory potency than pulp extract when taking into account the amount of pulp or seed that is necessary to obtain each extract (Table S1, supplementary material).

Our study has some limitations regarding the α-glucosidase digestive enzyme assay, as the enzyme used in this study comes from yeast, which works very well for antidiabetic screening assays. However, the enzyme from mice is considered the most suitable model to validate the results, as it mimics the human digestive system (SPÍNOLA, LLORENT-MARTÍNEZ, & CASTILHO, 2019).



C

Sample	IC $_{50}$ (μg. mL^{-1})	
	α-Amylase	α-Glucosidase
Seed	1.3 ± 0.2^{b}	1.5 ± 0.0^{b}
Pulp	0.2 ± 0.0^{c}	0.3 ± 0.0^{c}
Acarbose	39.9 ± 3.9^{a}	2,019.0 ± 81.3^{a}

Figure 2 – Anti-hyperglycemic potential of ethanolic extracts from the seeds and pulp of *E. involucrata* evaluated by inhibition of α-amylase (A) and α-glucosidase activities (B); and their respective IC_{50} values (C). Data are presented as the mean ± standard error of 3 independent replicates. Different letters indicate results with a statistically significant difference ($p < 0.05$). IC_{50}: the concentration of extract that inhibits 50% of the enzyme activity was presented as μg of phenolic compounds equivalent. mL^{-1}. Acarbose was used as a positive control.

Supplementary Material

Table S1 - Inhibitory or scavenging potency of extracts from the seeds and pulp of *E. involucrata* expressed on a pulp or seed basis.

Inhibitory or scavenging effect evaluated	**IC_{50} values (g of pulp or seed equivalent.mL^{-1})**	
	Pulp	**Seed**
α-Amylase	0.0026 $g.mL^{-1}$	0.0005 $g.mL^{-1}$
α-Glucosidase	0.0028 $g.mL^{-1}$	0.0006 $g.mL^{-1}$
Pancreatic lipase	0.0079 $g.mL^{-1}$	0.0002 $g.mL^{-1}$
AGEs	0.0776 $g.mL^{-1}$	0.0133 $g.mL^{-1}$
ORAC	0.00005 $g.mL^{-1}$	0.0002 $g.mL^{-1}$
Deoxyribose	0.0439 $g.mL^{-1}$	0.0019 $g.mL^{-1}$
GSH	> 0.2 $g.mL^{-1}$	> 0.2 $g.mL^{-1}$

IC_{50}: the concentration of extract that inhibits 50% of the enzyme activity.

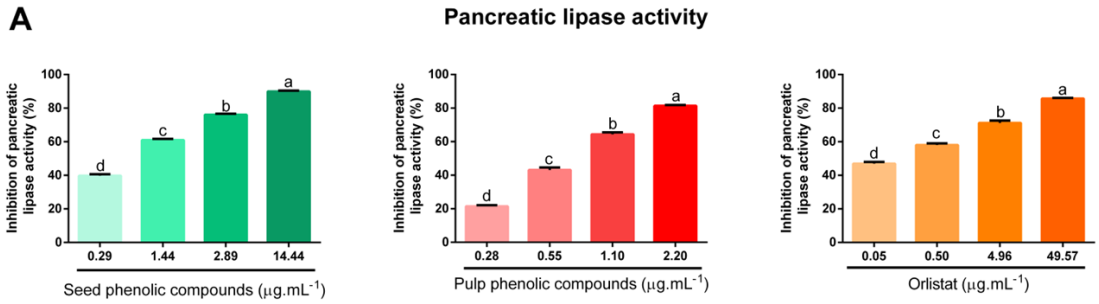
3.3 Evaluation of anti-obesogenic potential *in vitro*

In addition to hyperglycemia, another factor that contributes to the development of diabetes is obesity, characterized mainly by the excessive accumulation of body fat. Pancreatic lipase is responsible for the hydrolysis of dietary lipids during digestion, and its inhibition promotes the reduction of fat absorption and consequently contributes to the reduction of body weight (EL-SHIEKH et al., 2019; SPÍNOLA, LLORENT-MARTÍNEZ, & CASTILHO, 2020). There are several commercial drugs that inhibit the pancreatic lipase enzyme, such as orlistat, which, on the other hand causes some side effects such as steatorrhea, abdominal pain, and urgency to evacuate (SPÍNOLA, LLORENT-MARTÍNEZ, & CASTILHO, 2020).

The inhibitory potential of *E. involucrata* extracts against pancreatic lipase was also evaluated in the present study and compared to orlistat, which is approved as a lipase inhibitor for the management of obesity. Phenolic compounds of pulp and seed extracts, as well as orlistat had a dose-dependent inhibitory effect towards pancreatic lipase (Figure 3A). A greater inhibitory potency was observed for orlistat (lowest IC_{50} values), whereas the phenolic compounds of pulp and seed extract had lower inhibitory potency (Figure 3B). Borges et al. (2022), in a study carried out with phenolic compounds from jabuticaba peel and inhibition of pancreatic lipase, also observed greater inhibitory potency for orlistat than for compounds from jabuticaba peel. Although the concentration ranges that inhibited lipase were higher for phenolic compounds of pulp and seed extracts than orlistat, results indicate that both pulp and seed extracts of *E. involucrata* may be further investigated as potential alternatives to the use of commonly commercialized inhibitors. Similar to α-amylase inhibition, seed extract also had higher

inhibitory potency against pancreatic lipase activity than pulp extract when taking into account the amount of pulp or seed that is necessary to obtain each extract (Table S1, supplementary material). When investigating functional properties of extracts from inedible plant parts it is important to make sure that they do not contain toxic components. Seeds from other cherry species have been reported to contain cyanogenic glycosides (GIANCATERINO et al., 2023; SÉNICA et al., 2016), that are able to release cyanide, an inhibitor of oxygen transport and of the electron transport chain (MOSAYYEBI et al., 2020). However, qualitative assessment of cyanogenic glycosides revealed that they were not detected in the seeds of *E. involucrata* (data not shown).

A



B

Sample	IC $_{50}$ (μg. mL^{-1})
	Pancreatic lipase
Seed	0.6 ± 0.0^{b}
Pulp	0.7 ± 0.0^{a}
Orlistat	0.1 ± 0.0^{c}

Figure 3 – Anti-obesogenic potential of ethanolic extracts from seeds and pulp of *E. involucrata* fruits evaluated by the inhibition of pancreatic lipase enzyme activity (A) and its IC_{50} value (B). Data are presented as the mean ± standard error of 3 independent replicates. Different letters indicate results with a statistically significant difference ($p < 0.05$). IC_{50}: the concentration of extract that inhibits 50% of the enzyme activity was presented as μg of phenolic compounds equivalent. mL^{-1}. Orlistat was used as a positive control.

3.4 Inhibition of AGEs formation

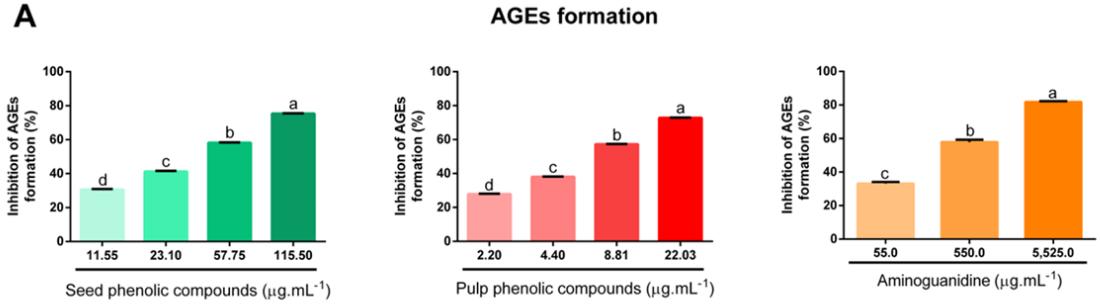
In addition to reducing hyperglycemia, another strategy for the management of diabetes would be to reduce the damage caused by hyperglycemia, such as that triggered by the formation of AGEs. AGEs are considered important biomarkers of diabetes complications (ARAÚJO et al., 2021). The endogenous formation of AGEs occurs through hyperglycemia and oxidative stress, even though AGEs also lead to oxidative stress and, consequently, to diabetes-related complications, such as cardiovascular changes, kidney disease, and even Alzheimer's (ARAÚJO et al., 2021; SPÍNOLA, LLORENT- MARTÍNEZ, & CASTILHO, 2020; VAN DONGEN et al., 2022; ZHAO et al., 2022). In this sense, reducing or preventing the formation of AGEs has been proposed to be effective for mitigating and/or preventing diabetes complications, as well as those caused by COVID-19 infection in diabetic individuals (SELLEGOUNDER et al., 2021). Aminoguanidine is a synthetic inhibitor of AGEs formation, which has several side effects, including toxicity observed in clinical trials (URIBARRI et al., 2015; ZHAO et al., 2022). Currently, studies have been carried out to find natural inhibitors of protein glycation and AGEs formation. Phenolic compounds constitute the main group of natural compounds that has been demonstrated to inhibit the formation of AGEs *in vitro* (URIBARRI et al., 2015; ZHAO et al., 2022).

This is the first study to evaluate the ability of phenolic compounds of ethanolic extracts from pulp and seed of *E. involucrata* to inhibit protein glycation and the formation of AGEs. We used aminoguanidine as a positive control. A dose-dependent inhibitory effect was observed for phenolic compounds of pulp and seed extracts as well as for aminoguanidine (Figure 4A). The inhibitory potency was higher for the phenolic compounds of pulp than for the seed extract, both extracts showed higher inhibitory potency compared to aminoguanidine (lowest IC_{50} values, $p < 0.05$), for the phenolic compounds of pulp extract the difference in the potency was more than 37 times higher (Figure 4B). Similar to enzyme inhibition, seed extract also had higher inhibitory effect against AGEs formation than pulp extract when taking into account the amount of pulp or seed that is necessary to obtain each extract (Table S1, supplementary material).

The phenolic compounds of extracts from pulp and seed of *E. involucrata* demonstrated a higher potency compared to aminoguanidine. Phenolic compounds of *E. involucrata* extracts can be an alternative to aminoguanidine, which is a synthetic compound that has not been approved in clinical trials because of its toxicity. In addition, there is evidence pointing to the ability of phenolic compounds to inhibit the glycation of proteins and the formation of AGEs, with emphasis on the class of anthocyanins, especially cyanidin-3-*O*-glucoside (BORGES et

al., 2022), and the class of phenolic acids and flavonoids from different plant species (KHAN et al., 2020). These last two classes of phenolic compounds were found in the pulp and seed extracts, with emphasis on the greater amount found in the seed extract.

A



B

Sample	IC $_{50}$ (µg. mL^{-1})
	AGEs
Seed	30.9 ± 1.7^{b}
Pulp	6.8 ± 0.3^{c}
Aminoguanidine	256.6 ± 13.7^{a}

Figure 4 – Inhibition potential of ethanolic extracts from seeds and pulp of *E. involucrata* fruits on AGEs formation (A) and its IC_{50} value (B). Data are presented as the mean ± standard error of 3 independent replicates. Different letters indicate results with a statistically significant difference ($p < 0.05$). IC_{50}: the concentration of extract that inhibits 50% of AGEs formation was presented as µg of phenolic compounds equivalent. mL^{-1}. Aminoguanidine was used as a positive control.

3.5 Evaluation of antioxidant capacity *in vitro*

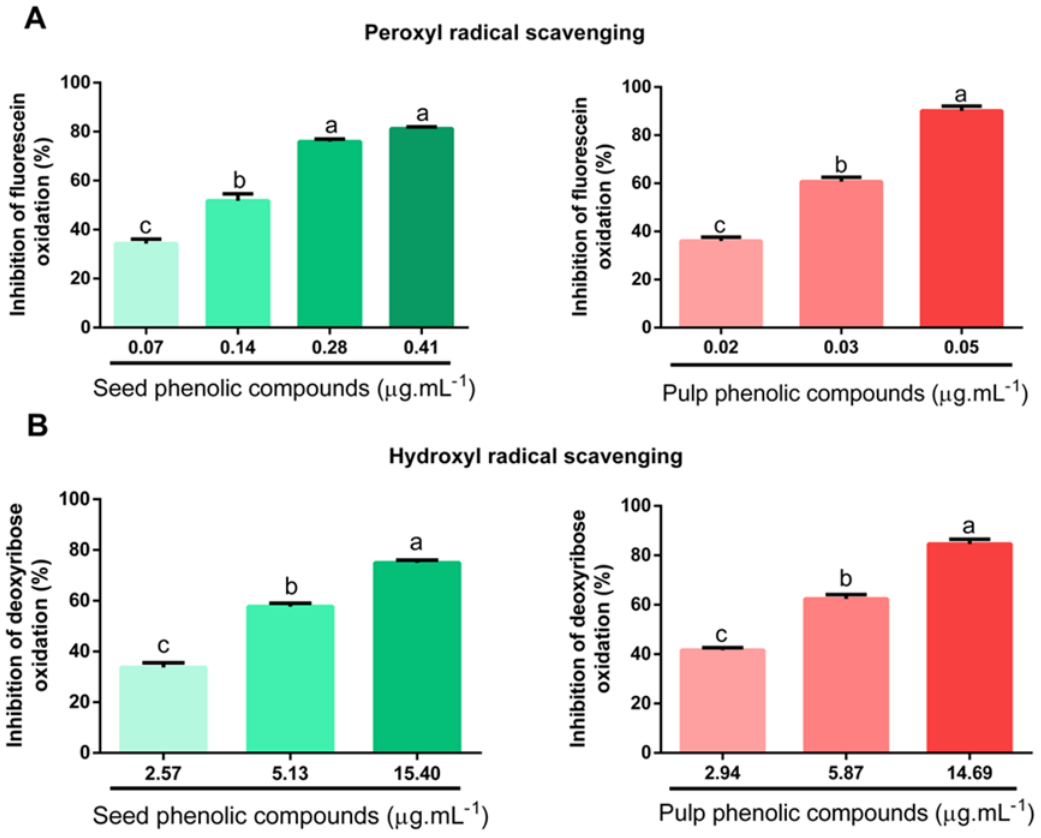
The state of persistent hyperglycemia, which characterizes diabetes, causes an overproduction of reactive species, which contribute to the long-term destruction of pancreatic islet β cells. Several free radical production pathways are related to hyperglycemia and are implicated in the pathology of diabetes: the polyol pathway; Amadori products and AGEs; protein kinase C (PKC); hexosamine; and the overproduction of superoxide anion ($O_2^{\cdot-}$) in the mitochondrial electron transport chain (IGHODARO, 2018). We investigated the ability of *E. involucrata* extracts to scavenge three different reactive species, namely, peroxyl ($ROO^{\bullet}$), hydroxyl ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2).

In the ORAC assay, phenolic compounds of pulp and seed extracts protected against fluorescein oxidation by the $ROO^{\bullet}$ radical generated through the thermal degradation of AAPH in a dose-dependent manner (Figure 5A). Our results showed higher $ROO^{\bullet}$ radical scavenging potency for the phenolic compounds of pulp extract when compared to the phenolic compounds of seed extract ($p<0.05$; Figure 5C; lower IC_{50} value). Girardelo et al. (2020) found greater antioxidant capacity in the ORAC method, for the seed extract when compared to the pulp of *E. involucrata*. However, other studies carried out previously revealed a higher antioxidant capacity by this same method for extracts from the pulp or fruits in comparison to that of the seeds (INFANTE et al., 2016; NICÁCIO et al., 2017). These differences found in our study when compared with others may be due to the difference in the way of expressing the results, difference in the content of phenolic compounds found and the different extraction methods and solvents used in the preparation of the extracts.

We assessed the ability of phenolic compounds of pulp and seed extracts to protect deoxyribose against the action of the $HO^{\bullet}$ radical generated by the Fenton reaction *in vitro* (HALLIWELL et al., 1987). All concentrations tested for phenolic compounds of the pulp and seed extract protected against deoxyribose oxidation in a dose-dependent manner (Figure 5B) the highest potency being observed for the phenolic compounds of pulp extract (lower IC50 value; Figure 5C). These data are in agreement with a previous study that described such activity for pulp extracts of *E. involucrata* (Schmidt et al., 2020).

We also investigated the ability of phenolic compounds of *E. involucrata* extracts to protect against the oxidation of GSH caused by H_2O_2 but none of the extracts tested were able to scavenge H_2O_2 (pulp $IC_{50} > 17.7$ $\mu g.mL^{-1}$ and seed $IC_{50} > 462.0$ $\mu g.mL^{-1}$) (Figure 5C), a result that is in line with that observed by Schmidt et al. (2020) for extracts from the pulp of *E. involucrata*, and also to those observed by Torma et al. (2017) for hydroethanolic extracts of açaí, which had no protective effect against the oxidation of GSH.

Our data show that the phenolic compounds of pulp extract have higher antioxidant capacity than those of *E. involucrata* seeds to scavenge ROO• and HO• radicals. These results may be related to the phenolic profile of pulp extracts, which contain anthocyanins that are absent in the seed extracts. However, when taking into account the amount of pulp or seed that is necessary to obtain each extract the seed extract had higher scavenging potency against ROO• and HO• radical than pulp extract (Table S1, supplementary material). Similar results were found by Girardelo et al. (2020) that observed higher antioxidant capacity for *E. involucrata* seed extracts than for the pulp.



C

Sample	IC_{50} (µg. mL^{-1})		
	ROO•	•OH	H_2O_2
Seed	0.12 ± 0.0[a]	4.5 ± 0.1[a]	> 462.0 µg/mL[a]
Pulp	0.02 ± 0.0[b]	3.9 ± 0.0[b]	> 17.6 µg/mL[b]

Figure 5 – Peroxyl (A and, hydroxyl (B) radical scavenging capacity and IC_{50} values (C) of ethanolic extracts from seeds and pulp of *E. involucrata* fruits. Data are presented as the mean ± standard error of 3 independent replicates.

IC_{50}: the concentration of extract that inhibits 50% of the substrate oxidation was presented as μg of phenolic compounds. mL^{-1}. Peroxyl radical ($ROO^{\bullet}$) scavenging was evaluated by ORAC (Oxygen Radical Absorbance Capacity) method. Hydroxyl radical ($HO^{\bullet}$) scavenging was evaluated by the deoxyribose method. Hydrogen peroxide (H_2O_2) scavenging was evaluated by the GSH (reduced glutathione) method. Different letters indicate results with a statistically significant difference ($p < 0.05$).

4. Conclusion

Phenolic compounds of seed and pulp extracts from *E. involucrata* fruits were demonstrated to inhibit enzymes involved in the digestion of carbohydrates (α-amylase and α-glucosidase) and lipids (pancreatic lipase) and therefore can be further investigated for the development of nutraceuticals and functional foods aimed to help in the management of hyperglycemia. Additionally, phenolic compounds of pulp and seed extracts also appear to be useful to reduce hyperglycemia damage, as they inhibited the formation of AGEs *in vitro* and were able to scavenge reactive species of biological importance. The greatest bioactive potency was found for the phenolics from the pulp extract compared to the seed extract, probably because of the presence of anthocyanins in addition to other class of phenolic compounds in the pulp extract, because the potency is related to the phenolic compounds. Despite the higher potency of phenolic compounds extracted from *E. involucrata* pulp towards all biological effects investigated when compared to the phenolics from seed extract, seed extraction had greater yield. Therefore, the concentration of phenolic compounds was higher in seed than pulp extract and seed extract had higher potency than pulp extract when taking into account the amount of pulp or seed that is necessary to obtain each extract.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5 CONCLUSÃO

A principal conclusão desse estudo é de que os compostos fenólicos dos extratos da semente e da polpa de *E. involucrata* foram capazes de inibir enzimas envolvidas na digestão de carboidratos (α-amilase e α-glicosidase) e lipídios (lipase pancreática) da dieta, assim como, inibir a formação de AGEs, e remover espécies reativas de importância biológica, *in vitro*. Os compostos fenólicos dos extratos de semente e de polpa de *E. involucrata* apresentaram eficácia superior ou comparável a inibidores de referência como a acarbose (inibidor clássico da α-amilase) e o orlistat (inibidor clássico da lipase pancreática), sendo a potência inibitória dos compostos fenólicos do extrato da polpa e da semente próxima ou superior aos inibidores de referência. Além disso, os compostos fenólicos dos extratos de semente e polpa de *E. involucrata* demonstraram potencial para substituir ao mesmo tempo inibidores de enzimas envolvidas na digestão de carboidratos e lipídios. Verificamos que os compostos fenólicos do extrato da polpa de cerejeira-do-rio-grande apresentaram maior potência antioxidante e inibitória da atividade de enzimas digestivas e da formação de AGEs, o que pode se explicar na maioria dos casos pelo maior volume desse extrato usado para inibição quando comparado ao volume do extrato da semente. Os resultados obtidos confirmam a hipótese do estudo revelando potencial bioativo promissor para as sementes e polpa das frutas de *E. involucrata* e fornecem a base para estudos subsequentes relacionados ao seu aproveitamento para o desenvolvimento de fármacos ou nutracêuticos, assim como na formulação de produtos alimentícios com propriedades funcionais.

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