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Efeitos inibitórios de bebidas preparadas com variedades de *Camellia sinensis* sobre a absorção de amido e triglicerídeos

TAMIRES BARLATI VIEIRA DA SILVA

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TAMIRES BARLATI VIEIRA DA SILVA

"EFEITOS INIBITÓRIOS DE BEBIDAS PREPARADAS COM VARIEDADES DE *CAMELLIA SINENSIS* SOBRE A ABSORÇÃO DE AMIDO E TRIGLICERÍDEOS".

Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

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BIOGRAFIA

Tamires Barlati Vieira da Silva nasceu na cidade de Apucarana, Paraná. Possui graduação em Engenharia de Alimentos pela Universidade Tecnológica Federal do Paraná (UTFPR), campus Campo Mourão e mestrado em Tecnologia de Alimentos pelo Programa de Pós-Graduação em Tecnologia de Alimentos (PPGTA) da UTFPR campus Campo Mourão.

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Esta tese de doutorado é composta de um artigo de revisão e dois artigos experimentais.

Silva, T.B.V., Vieira, T.F., Bracht, A., Peralta, R.M. (2021). Efeitos inibitórios das antocianinas sobre a absorção de amido e triglicerídeos: uma revisão. Flávia Michelon Dalla Nora (Org). Compostos Bioativos e suas Aplicações. Cap. 2, p. 19-48. Mérida Publishers https://doi.org/10.4322/mp.978-65-994457-7-4.c2

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[1].

GENERAL ABSTRACT

INTRODUCTION AND OBJECTIVES – *Camellia sinensis* is the plant that gives rise to green, black, white and oolong teas. The names are associated with the parts of the plant used and the post-harvest drying and fermentation processes. These beverages that are prepared with them have several beneficial health effects, which are attributed to their polyphenol content, mainly catechins and catechin derivatives, including (−)-epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC) and epicatechin-3-gallate (ECG). *C. sinensis* extracts have been proposed to be useful in the prevention and/or treatment of various disorders, including cancer, cardiovascular disease, diabetes mellitus, and hepatitis. A new variety of *C. sinensis* produced in Kenya has attracted the attention of many researchers. Thanks to the high content of anthocyanins of this variety, the leaves have a purple color and the drink obtained by infusion is called purple tea. It is known that anthocyanins are phenolic compounds belonging to the flavonoid family, commonly found in red, purple and blue flowers, fruits and tubers. It has been suggested that regular consumption of anthocyanins may reduce the risk of various diseases such as dyslipidemia and diabetes, resulting in potential health benefits. Delayed digestion of starch by inhibiting intestinal amylases and alpha-glucosidase can be considered an appropriate treatment to control postprandial hyperglycemia. Likewise, delayed absorption of fatty acids from dietary triglycerides, due to inhibition of pancreatic lipase, can be considered a therapeutic approach to the control of dyslipidemia. The objectives of this work were: Chapter 1: to provide an up-to-date, comprehensive, and critical review of the inhibitory action of anthocyanins on the absorption of starch and triglycerides, mainly as a result of their ability to inhibit the digestive enzymes amylases and lipases. Chapter 2. Compare the inhibitory effects of different *C. sinensis* beverages on starch digestion using the mouse starch tolerance test. Chapter 3. Compare the phenolic profiles and various bioactivities (antioxidant, anti-inflammatory, cytotoxic, antibacterial activities) of beverages prepared from green tea and purple tea with emphasis on their effects on fat absorption.

METHODS- For the review work, we searched the specialized literature for studies that used anthocyanins in the treatment of diabetes, obesity and/or metabolic syndrome. The selected works were evaluated according to the type of research and classified as: classical methods for evaluating the inhibitory activity of anthocyanins on amylases and lipases and methods for evaluating the molecular interactions of inhibitors with enzymes. For the experimental work, that is, chapters 2 and 3, the following materials were used: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (trolox), thiobarbituric acid, sulforhodamine B, salivary and pancreatic amylases, pancreatic lipase, which were purchased from Sigma-Aldrich. Acarbose and orlistat were purchased from local pharmacies. Green, oolong, white and black teas were purchased from in the local market (Maringá, Brazil), and Kenyan purple tea (purple tea) was purchased from Justea.com, Vancouver, Canada. To carry out the experiments, the extracts were prepared mimicking consumption where the leaves were placed in contact with water for 15 minutes promoting infusion. The extracts were filtered, frozen and lyophilized and stored at -20 ºC until use. The extracts were characterized and quantified for their phenolic compounds profiles by HPLC-MS and the total anthocyanins content was quantified by the pH differential method. The inhibitory potential of the extracts on the pancreatic α-amylase was evaluated by *in vitro* and in vivo methods. In the *in vitro* method, the inhibitory effect on pancreatic alpha-amylase was evaluated by quantifying the release of reducing sugars from starch by the 3,5 dinitrosalicylic acid method in the absence and presence of different concentrations of the extracts. In *in vivo* assays, extracts

derived from the various teas were administered to mice by gavage at doses of up to 500 mg/kg before starch administration. Acarbose, a classic inhibitor of starch hydrolysis and digestion, was administered in doses up to 50 mg/kg before starch. Plasma glucose was determined at 0, 30, 60, 90 and 120 min after starch administration. Blood samples were collected from the tail vein and analyzed using a glucometer (AccuChek®). For the study described in Chapter 3, experiments were carried out using only green and purple tea extracts. The extracts were analyzed for antioxidant activity, anti-inflammatory activity, cytotoxicity against four human tumor cell lines and antimicrobial activity. Pancreatic lipase inhibitory activity by purple and green tea extracts was evaluated by *in vitro* and *in vivo* methods. For the *in vitro* pancreatic lipase assay, p-nitrophenyl-palmitate was used as substrate. For lipase inhibition *in vivo* the extracts were administered by gavage to mice prior to the addition of an olive oil load. Plasma levels of triglycerides were determined at 0, 90, 180, 270 and 360 minutes after administration of olive oil. The determination of the concentration of triglycerides in blood was performed using an AccutrendPlus® Roche triglyceride meter. The inhibitory effects of the extracts were compared to those caused by orlistat. Statistical analyses of experimental data were performed using the GraphPad Prism (version 8.0) and Scientist software (MicroMath Scientific Software, Salt Lake City, UT).

MAIN RESULTS, DISCUSSION AND CONCLUSION – In chapter 1, it was concluded that *in vivo* studies and especially clinical studies are highly necessary to confirm and give relevance to the numerous data already obtained under *in vitro* conditions. Efforts are also needed to allow a more secure attribution of the observed effects to well-defined molecular species, since, in general, experiments have been conducted with extracts that contain a great diversity of compounds. In Chapter 2, it was observed that purple tea was the most potent inhibitor of pancreatic α-amylase, a property that caused a very significant reduction in starch digestion and consequent reduction in the postprandial glycemic peak. A stimulatory effect of α-amylase was observed *in vitro* at lower concentrations of green tea, which may explain its lower inhibitory effect on starch digestion *in vivo*. Our results allow us to conclude that purple tea exerts a beneficial postprandial antihyperglycemic action in healthy or diabetic individuals. In the third chapter, it was observed that in terms of the analyzed bioactivities (antioxidant, anti-inflammatory, antibacterial and antifungal) the extracts of green and purple teas were very similar. However, purple tea had a significantly superior inhibitory activity on the pancreatic lipase, causing a marked inhibition of triglyceride digestion. We can generally conclude that purple tea was an efficient inhibitor of starch digestion (Chapter 2) and triglycerides digestion (Chapter 3) when compared to other *C. sinensis* beverages. These results, when combined, suggest that regular consumption of purple tea may be helpful in managing obesity and diabetes.

Keywords: starch, amylases, anthocyanins, *Camellia sinensis*, digestive enzymes, lipases, triglycerides.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – A *Camellia sinensis* é a planta que dá origem aos chás verde, preto, branco e oolong. As denominações estão associadas às partes da planta utilizadas e aos processamentos de secagem e fermentação pós-colheita. Essas bebidas tem vários efeitos benéficos à saúde, que são atribuídos ao seu conteúdo em polifenóis, principalmente catequinas e derivados de catequinas, incluindo (−)-epigalocatequina-3-galato (EGCG), epigalocatequina (EGC) e epicatequina-3 -galato (ECG). Extratos de *C. sinensis* tem sido propostos como úteis na prevenção e/ou tratamento de vários distúrbios, incluindo câncer, doenças cardiovasculares, diabetes mellitus e hepatite. Uma nova variedade de *C. sinensis* produzida no Quênia vem atraindo atenção de muitos pesquisadores. Graças ao alto teor de antocianinas desta variedade, as folhas apresentam uma coloração púrpura e a bebida obtida por infusão recebe o nome de chá roxo. É sabido que as antocianinas são fenólicos pertencentes à família dos flavonoides, comumente encontrados em flores, frutos e tubérculos vermelhos, roxos e azuis. Tem sido sugerido que o consumo regular de antocianinas pode reduzir o risco de várias doenças, como dislipidemia e diabetes, resultando em potenciais benefícios à saúde. A digestão retardada do amido por inibição das amilases e da alfa-glicosidase intestinal pode ser considerada um tratamento apropriado para controlar a hiperglicemia pós-prandial. Da mesma forma, a absorção retardada de ácidos graxos dos triglicerídeos da dieta devido à inibição da lipase pancreática, pode ser considerada uma abordagem terapêutica para o controle da dislipidemia. Os objetivos deste trabalho foram: Capítulo 1: escrever uma revisão atualizada, ampla e crítica sobre a ação inibitória das antocianinas sobre a absorção do amido e dos triglicerídeos, como resultado, principalmente, de sua capacidade em inibir as enzimas digestivas amilases e lipases. Capítulo 2. comparar os efeitos inibitórios das diferentes bebidas de *C. sinensis* na digestão do amido usando o teste de tolerância ao amido em camundongos. Capítulo 3. comparar os perfis fenólicos e diversas bioatividades (atividades antioxidantes, anti-inflamatórias, citotóxicas, antibacterianas) de bebidas preparadas a partir de chá verde e chá roxo com ênfase no efeito causado na absorção de gorduras.

MÉTODOS- Para o trabalho de revisão, buscou-se na literatura especializada estudos que utilizassem antocianinas no tratamento de diabetes, obesidade e/ou síndrome metabólica. Os trabalhos selecionados foram avaliados de acordo com o tipo de pesquisa e classificados como: métodos clássicos para avaliação da atividade inibidora das antocianinas sobre amilases e lipases e métodos para avaliação das interações moleculares dos inibidores com as enzimas. Para os trabalhos experimentais, ou seja, capítulo 2 e 3 foram utilizados os seguintes materiais 2,2-difenil-1-picrilhidrazil (DPPH), ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico (trolox), ácido tiobarbitúrico, sulforrodamina B, amilases salivares e pancreáticas, lipase pancreática foram adquiridos da Sigma-Aldrich. Acarbose e o orlistat foram adquiridos em farmácias locais. Os chás verde, oolong, branco e preto foram adquiridos no mercado local (Maringá, Brasil), e o chá roxo queniano (chá roxo) foi adquirido da empresa Justea.com, Vancouver, Canadá. Para realizar os experimentos, os extratos foram preparados mimetizando o consumo e onde as folhas foram colocadas em contato com a água por 15 minutos promovendo a infusão, Os extratos foram filtrados, congelados e liofilizados sendo armazenados a -20 ºC até uso. Os extratos foram caracterizados quanto aos perfis de compostos fenólicos por HPLC-MS e teor de antocianinas totais pelo método de diferencial de pH. O potencial inibitório dos extratos sobre a α-amilase pancreática foi avaliado por métodos *in vitro* e

in vivo. No método in vitro, o efeito inibitório sobre a alfa-amilase pancreática foi avaliado quantificando-se a liberação de açúcares redutores a partir do amido pelo método do ácido 3,5 dinitrosalicílico na ausência e presença de diferentes concentrações dos extratos. Nos ensaios *in vivo* os extratos derivados dos vários chás foram administrados a camundongos por gavagem em doses de até 500 mg/kg antes da administração do amido. Acarbose, um inibidor clássico da hidrólise e digestão do amido, foi administrada em doses de até 50 mg/kg antes do amido. A glicose plasmática foi determinada nos tempos 0, 30, 60, 90 e 120 min após a administração do amido. Amostras de sangue foram coletadas da veia da cauda e analisadas por meio de glicosímetro (AccuChek®). Já para o capitulo 3, foram realizados experimentos utilizando apenas os extratos de chá verde e roxo. Os extratos foram analisados quanto à atividade antioxidante, atividade anti-inflamatória, citotoxicidade contra quatro linhagens de células tumorais humanas e atividade antimicrobiana. Atividade inibitória da lipase pancreática pelos extratos dos chás roxo e verde foi avaliada por métodos in vitro e in vivo. Para o ensaio de lipase pancreática *in vitro* foi utilizado p-nitrofenil-palmitato como substrato. Para a inibição da lipase *in vivo* os extratos foram administrados por gavagem em camundongos anteriormente à adição de óleo de oliva. Os níveis plasmáticos de triglicerídeos foram determinados aos 0, 90, 180, 270 e 360 minutos após a administração do óleo de oliva. A determinação da concentração dos triglicerídeos no sangue foi realizada por meio de um medidor de triglicerídeos AccutrendPlus® Roche. Os efeitos inibitórios dos extratos foram comparados aos obtidos pelo orlistat. As análises estatísticas dos dados experimentais foram realizadas usando o software GraphPad Prism (versão 8.0) e o software Scientist da MicroMath Scientific Software (Salt Lake City, UT).

PRINCIPAIS RESULTADOS, DISCUSSÃO E CONCLUSÃO – No capítulo 1, pode-se concluir que estudos *in vivo* e sobretudo estudos clínicos são altamente necessários para confirmar e dar relevância aos inúmeros dados já obtidos nas condições *in vitro.* Também são necessários esforços que permitam uma atribuição mais segura dos efeitos observados a espécies moleculares bem definidas, já que de um modo geral os experimentos têm sido conduzidos com extratos que contém grande diversidade de compostos. No capítulo 2, foi observado que o chá roxo foi um inibidor mais potente da α-amilase pancreática, o que causou uma redução bastante significativa da digestão do amido e consequente redução do pico glicêmico pós-prandial. Foi observado *in vitro* um efeito estimulador da α-amilase em concentrações mais baixas do chá verde, que pode explicar o menor efeito inibidor da digestão do amido in vivo. Nossos resultados permitem concluir que o chá roxo apresenta ação anti-hiperglicêmica pós-prandial benéfica em indivíduos saudáveis ou diabéticos. No terceiro capítulo, foi observado que em termos das bioatividades analisadas (antioxidante, anti-inflamatória, antibacteriana e antifúngica) os extratos dos chás verde e roxo foram muito semelhantes. Entretanto, o chá roxo foi significativamente superior na atividade inibitória da lipase pancreática, causando uma inibição acentuada na digestão dos triglicerídeos. Podemos concluir de modo geral que o chá roxo foi eficiente inibidor da digestão do amido (Capítulo 2) e triglicerídeos (Capítulo 3) quando comparado às outras bebidas de *C. sinensis.* Esses resultados, quando combinados, sugerem que o consumo regular de chá roxo pode ser útil no controle da obesidade e do diabetes.

Palavras-chave: amido, amilases, antocianinas, *Camellia sinensis*, enzimas digestivas, lipases, triglicerídeos.

CAPÍTULO 2

Efeitos inibitórios das antocianinas sobre a absorção de amido e triglicerídeos: uma revisão

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Resumo

As antocianinas são compostos fenólicos, pertencentes à família dos flavonoides, normalmente encontrados em flores, frutos e tubérculos de coloração vermelha, roxa e azul. Tem sido proposto que o consumo regular das antocianinas reduz o risco de desenvolvimento de várias doenças, como aterosclerose, dislipidemias e diabetes, com consequentes benefícios potenciais para a saúde. O retardo da digestão de materiais amiláceos e dissacarídeos pela inibição das amilases (salivar e pancreática) e alfa-glucosidases intestinais pode ser considerada como uma abordagem terapêutica adequada para controlar a hiperglicemia pós-prandial em pré-diabetes. Da mesma forma, pode-se considerar o retardo da absorção dos ácidos graxos provenientes dos triglicerídeos da dieta em virtude da inibição da lipase pancreática ou de outra etapa do processo de absorção, que é bastante complexo, como uma abordagem terapêutica para controlar dislipidemias. Esta revisão tem como objetivo compilar e discutir recentes evidências da inibição de amilases e lipases pelas diferentes antocianinas, bem como comparar métodos in vitro e in vivo utilizados na avaliação destes fenômenos.

Palavras-chave: amido, amilases, antocianinas, enzimas digestivas, lipases, triglicerídeos.

1. Introdução

A obesidade e o diabetes são problemas de saúde pública mundial, com custos sociais incalculáveis (GAMBOA-GÓMEZ *et al.*, 2015). Muitos medicamentos têm sido utilizados para controlar tanto a obesidade quanto o diabetes ao longo dos anos. Uma das abordagens farmacológicas para o

controle da obesidade e do diabetes é a utilização de inibidores das enzimas digestivas, amilases e α-glucosidase intestinal e lipase pancreática (BIALECKA-FLORJANCZYK *et al.*, 2018; RÍOS; FRANCINI; SCHINELLA, 2015).

As α-1,4-glucan-4-glucanoidrolases (EC 3.2.1.1.) são enzimas pertencentes à família 13 das glicosídeo-hidrolases (GH), encontradas na saliva e no suco pancreático e conhecidas pelo nome comum de α-amilases (DASTJERDI; NAMJOYAN; AZEMI, 2015; PROENÇA *et al.*, 2019). Nos seres humanos, as α-amilases são produzidas principalmente no pâncreas e nas glândulas salivares. As α-amilases salivar e pancreática humana, junto com as glucosidases intestinas, promovem a digestão dos materiais amiláceos, hidrolisando as ligações α-1,4-glicosídicas de polímeros como amido, amilose, amilopectina, glicogênio e vários maltooligossacarídeos (SANTOS; FREITAS; FERNANDES, 2018; SUN; WARREN; GIDLEY, 2019). O retardo da absorção de glicose pela inibição da α-amilase e da α-glucosidase no trato digestivo tem sido sugerido como uma abordagem terapêutica para o diabetes tipo 2 e como uma estratégia de combate ao sobrepeso e à obesidade.

A lipase humana (EC 3.1.1.3) pertence a um grupo de enzimas que inclui lipases pancreáticas, endoteliais, hepáticas e outras lipases lipoproteicas que possuem semelhança estrutural. As lipases pancreáticas são enzimas exócrinas do suco pancreático, essenciais para a digestão de gorduras alimentares no lúmen intestinal, desempenhando um papel fundamental na decomposição do triglicerídeos dietéticos em ácidos graxos livres circulantes e monoacilgliceróis (RAJAN; PALANISWAMY; MOHANKUMAR, 2020; SEYEDAN *et al.*, 2015). Por ser responsável pela hidrólise dos triglicerídeos, a lipase pancreática contribui para que a gordura proveniente da dieta seja absorvida pelas células intestinais. Sua inibição pode auxiliar no controle da absorção de triglicerídeos no intestino, sendo então eficaz no combate ao sobrepeso e à obesidade (FABRONI *et al.*, 2016; RAJAN; PALANISWAMY; MOHANKUMAR, 2020).

Inibidores das enzimas envolvidas na digestão do amido como a acarbose, miglitol e voglibose são amplamente utilizados como drogas orais para controlar a glicemia, pois diminuem a hiperglicemia pós-prandial o que também atenua a secreção de insulina. Embora apresentem um bom perfil de

segurança, efeitos adversos gastrointestinais podem limitar a adesão a longo prazo à terapia (NEUSER *et al.*, 2005).

No caso das lipases pancreáticas o inibidor clássico é o orlistat (tetraidrolipstatina). O composto, um derivado sintético da lipstatina, produzido naturalmente por *Streptomyces toxytricini*, é um inibidor potente, específico e irreversível tanto das lipases pancreáticas como gástricas (HADVÁRY; LENGSFELD; WOLFER, 1988). Sua atividade é exercida através da formação de uma ligação covalente com o sítio ativo das lipases gástricas e pancreáticas no lúmen do trato gastrointestinal. Essa ação evita que essas enzimas hidrolisem a gordura alimentar (na forma de triglicerídeos) em ácidos graxos livres absorvíveis e monogliceróis, sendo os triglicerídeos não digeridos eliminados pela via fecal (HADVÁRY *et al.*, 1991). Contudo, assim como para os inibidores das glucosidases, o uso contínuo e prolongado do orlistat causa efeitos colaterais acentuados, como desconforto gastrointestinal, flatulência, cólicas abdominais e diarreia (AWOSIKA; ALUKO, 2019; GUTIÉRREZ-GRIJALVA *et al.*, 2019).

Compostos naturais presentes na alimentação têm sido explorados como potenciais ferramentas seguras no tratamento da obesidade e do diabetes. Alimentos ricos em compostos fenólicos da dieta têm atraído muito interesse devido às suas propriedades biológicas benéficas, que podem desempenhar um papel importante na manutenção da saúde humana. Diversos estudos têm demonstrado que extratos vegetais ricos em ácidos fenólicos, flavonoides e antocianinas exercem efeitos inibitórios sobre as lipases e amilases e poderiam, em tese, ser utilizados para reduzir a absorção de lipídeos e carboidratos complexos (CHIOU *et al.*, 2018). As antocianinas são excelentes opções para serem utilizadas como adjuvantes no controle da obesidade e do diabetes tipo 2, uma vez que, além das potenciais atividades inibidoras de lipases e amilases, apresentam consideráveis capacidades antioxidantes e anti-inflamatórias (FALLAH; SARMAST; JAFARI, 2020; LUNA-VITAL *et al.*, 2020; ZHANG *et al.*, 2020).

O presente capítulo tem como objetivo fornecer uma contribuição atualizada, ampla e crítica sobre a ação inibitória das antocianinas sobre a absorção do amido e dos triglicerídeos, como resultado, principalmente, de sua capacidade em inibir as enzimas digestivas amilases e lipases. Estudos *in vitro*

e *in vivo* foram considerados. Para a seleção dos artigos científicos, foram utilizadas as bases de dados Science Direct, Pubmed e Web of Science considerando preferencialmente os artigos publicados nos últimos 5 anos (2016-2020). Os termos de busca foram "inibidores de amilase'" (*amylase inhibitors*) em associação com antocianinas (*anthocyanins*) e "inibidores de lipase" (*lipase inhibitors*) em associação com antocianinas (*anthocyanins*).

2. Antocianinas

As antocianinas são compostos fenólicos pertencentes à família dos flavonóides, normalmente encontradas em flores, frutos e tubérculos de coloração vermelha, roxa e azul. Estes compostos têm sido utilizados nas indústrias de alimentos, normalmente como corantes, e na medicina popular, devido aos seus efeitos benéficos. Sua estabilidade varia com sua estrutura e é dependente do pH, da luz e da temperatura (KHOO *et al.*, 2017; LÓPEZ *et al.*, 2019). Sua estrutura molecular geral está mostrada na Figura 1.

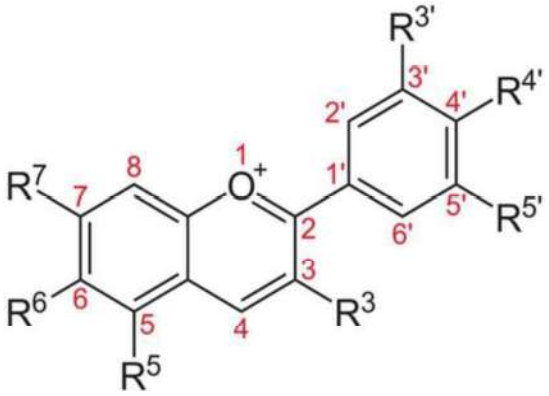

Figura 1. Estrutura básica das antocianinas. Fonte: KHOO *et al.* (2017).

Estes compostos aparecem frequentemente conjugados a açúcares (glicosídeos) que por sua vez podem estar acilados (antocianinas aciladas) (KHOO *et al.*, 2017). Quando encontrados em sua forma genuína (aglicona), são conhecidos como antocianidinas, sendo agrupadas em 3-hidroxiantocianidinas, 3-desoxianocianidinas e antocianidinas *O*-metiladas (CELLI, TAN, SELIG, 2019; KHOO *et al.*, 2017).

Existem mais de 500 tipos de antocianinas na natureza, mas somente 23 agliconas (antocianidinas) são conhecidas, das quais apenas 6 são onipresentes, sendo essas: cianidina, delfinidina, petunidina, peonidina, pelargonidina e malvidina. Elas diferem entre si principalmente pelo número de grupos hidroxila, conforme ilustra a Tabela 1, que relaciona os diferentes grupos R e R' de cada antocianidina. As antocianinas diferem pela natureza e número de açúcares em sua estrutura (ligados aos grupos OH), pelos carboxilatos alifáticos ou aromáticos ligados aos açúcares e pelas posições dessas ligações (ALI; ALMAGRIBI; AL-RASHIDI, 2016; SUI; ZHANG; ZHOU, 2016).

Tabela 1. As seis principais antocianidinas encontradas em plantas com os grupos passíveis de substituição para formar as antocianinas (ver detalhes no texto).

Antocianidinas	Padrão de substituição						
	R3	R5	R6	R7	R3’	R4’	R5’
Cianidina	OH	OH	H	OH	OH	OH	H
Delfinidina	OH	OH	H	OH	OH	OH	OH
Peonidina	OH	OH	H	OH	OCH_3	OH	H
Pelargonidina	OH	OH	H	OH	H	OH	H
Petunidina	OH	OH	H	OH	OH	OH	OCH_3
Malvidina	OH	OH	H	OH	OCH_3	OH	OCH_3

Fonte: KHOO *et al*. (2017).

2.1. Fontes de antocianinas

As antocianinas perfazem grande parte dos pigmentos solúveis em água que conferem cores vermelha, azul e roxa às plantas, flores, frutas, vegetais e resíduos provenientes de frutas e vegetais, como casca e bagaço (GUO; XIA, 2018; LIU *et al.*, 2018; RODRIGUEZ-AMAYA, 2019). Elas estão presentes principalmente em chás; frutas como maçãs, uvas, ameixas, morangos, mirtilos e amoras; legumes como repolho roxo, cebola roxa e berinjela; e bebidas como vinho tinto e suco de uva (GUVEN; ARICI; SIMSEK, 2019; KENT *et al.*, 2017).

A variedade na composição em antocianinas dos alimentos está relacionada com cultivar/variedade, maturação, práticas de cultivo, localização das plantações, diferenças climáticas, processamento e armazenamento. Entre outros fatores, que também podem afetar a cor e a estabilidade das antocianinas, estas moléculas podem ser afetadas por temperatura, pH, luz, oxigênio, enzimas, íons metálicos, outros flavonóides e fenólicos, ácido ascórbico, açúcares e sulfitos (RODRIGUEZ-AMAYA, 2019). Na Figura 2 podemos observar alguns exemplos de alimentos que possuem alto teor de antocianinas.

Figura 2. Alimentos ricos em antocianinas. Fonte: Autoria própria.

Além das partes comestíveis das plantas, a recuperação de compostos bioativos, incluindo as antocianinas, a partir de resíduos e subprodutos industriais (partes não comestíveis) vem atraindo cada vez mais interesse de diversas indústrias, no âmbito de uma ótica de aproveitamento total e recuperação de compostos de alto valor agregado, com eficiência e sustentabilidade (BARBA *et al.*, 2016; YAMASHITA *et al.*, 2017). Após a extração desses compostos dos resíduos, eles podem ser aplicados como enriquecimento em produtos alimentícios, aprimorando suas propriedades

sensoriais, melhorando seu valor nutricional e os benefícios à saúde (MALIK; ERGINKAYA, ERTEN, 2019).

2.2. Antocianinas como inibidores de enzimas digestivas

2.2.1. Antocianinas como inibidoras de amilases

Diversos estudos experimentais descrevem o efeito antidiabético das antocianinas, apontando-as como compostos promissores, e dentre tais estudos, a maioria parte do princípio de que as antocianinas agem inibindo as enzimas digestivas de carboidratos, como a α-amilase e a α-glucosidase (GOWD; JIA; CHEN, 2017).

Esta atribuição não deixa de ter alguma incerteza, no entanto, já que normalmente extratos de frutas e flores são investigados. Estes extratos contêm vários compostos fenólicos, o que dificulta a atribuição da atividade inibidora a apenas um determinado composto (GOWD; JIA; CHEN, 2017).

Na Tabela 2 são descritos resumidamente estudos que evidenciam a associação das antocianinas com a inibição de enzimas, tais como α-amilases e α-glucosidases.

Tabela 2. Estudos que associam a inibição de amilases a extratos ricos em antocianinas.

Extratos e/ou substâncias puras	Principais resultados	Referência
Extratos de groselhas pretas (BC) e verdes (GC).	Foram identificados teores de antocianinas específicas em maiores quantidades nas BC quando comparados às GC. Embora os dois tipos de extrato tenham apresentado efeitos inibitórios significativos sobre a α-amilase salivar humana, somente o das BC inibiram a α-glicosidase de levedura e com maior intensidade do que a droga comercial acarbose.	(BARIK *et al.*, 2020)
Pelargonidina-3-O-rutinosídeo (Pg3R)	O Pg3R foi isolado de morangos, revelou-se um potente inibidor da α-glicosidase que demonstrou possuir potencial em melhorar a hiperglicemia pós-prandial. A análise de docking molecular permitiu obter uma visão aprofundada da relação estrutura-atividade no que se refere à inibição da α-glicosidase pelas antocianinas naturais em geral.	(XU *et al.*, 2019)
Cianidina-3-glicosídeo, cianidina-3-rutinosídeo e peonidina-3-glicosídeo	As antocianinas estudadas mostraram inibição competitiva da α-amilase pancreática suína *in vitro*, com diferentes potências, mas a maior delas foi observada para a cianidina-3-glicosídeo. Em todos os casos a inibição ocorreu em concentrações na faixa milimolar. Por meio do estudo *in silico* (docking molecular) verificou-se que as quatro antocianinas ocupavam o sítio ativo da α-amilase pancreática suína formando ligações de hidrogênio.	(SUI; ZHANG; ZHOU, 2016)
Extratos de mirtilo (*Vaccinium uliginosum* L.), mirtilo (*Vaccinium myrtillus* L.), amora (*Morus alba* L.) e oxicoco (*cranberry*; *Vaccinium*	Todos os extratos apresentaram inibição da α-glicosidase, sendo a maior atividade observada para o de amora, seguida pelo do mirtilo (*Vaccinium myrtillus* L.), mirtilo (*Vaccinium uliginosum* L.) e oxicoco, respectivamente. No ensaio enzimático da proteína tirosina fosfatase 1B (PTP1B), o maior efeito inibitório foi observado para o extrato de mirtilo (*Vaccinium uliginosum* L.), seguido pelo de mirtilo (*Vaccinium myrtillus* L.), amora e oxicoco,	(XIAO *et al.*, 2017)

oxycoccos L.)	respectivamente. O docking molecular revelou que as agliconas de cianidina apresentaram dois locais de ligação na PTP1B, apresentando assim a maior atividade de inibição desta enzima.	
Extrato de casca de feijão preto	O extrato de antocianinas da casca do feijão preto foi capaz de inibir mais efetivamente a α-glicosidase, seguido por α-amilase e dipeptidil peptidase-IV, respectivamente, além de diminuir as espécies reativas de oxigênio e, diminuir a captação de glicose, apresentando importante potencial biológico que pode contribuir para modular marcadores de diabetes.	(MOJICA *et al.*, 2017)
Extratos de mirtilo, groselha preta e madressilva azul	As antocianidinas extraídas do mirtilo, da groselha preta e da madressilva azul mostraram-se inibidoras do tipo misto da α-glucosidase. Por apresentarem mais grupos hidroxila no anel B, as antocianidinas obtidas a partir de groselha preta e madressilva azul apresentaram maior potencial inibidor. Por meio do ensaio de extinção de fluorescência, verificou-se que a inibição da α-glicosidase pela antocianidina consiste em um processo estático. O docking molecular revelou que todas as antocianidinas podem ligar ao sítio ativo da α-glicosidase e impedem o acesso ao p-nitrofenil-alfa -D-glucopiranosídeo (substrato).	(ZHANG *et al.*, 2019)
Cianidina 3-glicosideo, delfinidina 3-glicosideo, malvidina 3-glicosideo, peonidina 3-glicosideo e petunidina 3-glicosídeo e as correspondentes agliconas	Foi feito um estudo comparativo da atividade inibidora dos compostos relacionados ao lado mais a acarbose sobre a α-glicosidase de levedura. O IC_{50} da acarbose foi igual a $4{,}03 \times 10^{-4}$ M. Vários dos compostos relacionados, principalmente as agliconas, apresentaram IC_{50} inferiores a 10^{-4} M, mais potentes que a acarbose, portanto. Os glicosideos tenderam a apresentar valores de IC_{50} maiores, mais próximos ao da acarbose. O inibidor mais potente foi a aglicona delfinidina (IC_{50} de $1{,}25 \times 10^{-5}$ M); o menos potente foi o peonidina 3-glicosídeo (IC_{50} de $4{,}54 \times 10^{-4}$ M)	(ZHANG *et al.*, 2019)

Extratos de variedades de cereja azeda húngara e correspondentes cianidinas purificadas (cianidina-3-glicosídeo, cianidina-3-rutinosídeo, malvidina-3-glicosideo e malvidina-3,5-diglicosídeo)	Estudos cinéticos revelaram que os extratos de cereja azeda e as antocianinas selecionadas inibiram competitivamente a hidrólise catalisada pela alfa-amilase salivar humana. As variedades de cereja azeda húngara, especialmente “Cigánymeggy” e “VN1”, podem ser consideradas como fontes potenciais de antocianinas para aplicações em escala industrial.	(HOMOKI *et al.*, 2016)
Extratos de cultivares de batata roxa e vermelha	Os extratos de tubérculos de polpa roxa apresentaram maiores conteúdos totais de antocianinas quando comparado aos obtidos em batata vermelha e mostraram inibir mais eficazmente a α-amilase, a α-glicosidase e a aldose redutase. Os estudos cinéticos mostraram que as antocianinas são inibidores não competitivos da α-amilase e da α-glicosidase.	(KALITA *et al.*, 2018)
Extrato de cenoura preta (*Daucus carota* subespécie *sativus* var. *atrorubens* Alef.)	O estudo revelou que o extrato de cenoura preta purificado inibe as enzimas α-amilase, α-glicosidase e DPP-IV de modo mais efetivo do que a acarbose e a vildagliptina. O estudo *in silico*, usado para identificar a antocianina individual que contribuiu amplamente para a inibição, mostrou que o cianidina 3-xilosil galactosídeo interagiu fortemente com a enzima, sendo esta considerada a molécula mais promissora a ser investigada por suas propriedades antidiabéticas.	(KARKUTE *et al.*, 2018)

Tabela 3. Estudos que associam a inibição da lipase a extratos ricos em antocianinas ou antocianinas purificadas.

Extratos e/ou substâncias puras	Principais resultados	Referência
Extratos de laranja sanguínea, romã, amora preta, amora, sumagre, casca de laranja sanguínea, brotos jovens de limão, repolho roxo, couve-flor violeta, feijão preto, arroz preto e casca de arroz preto.	A atividade inibitória sobre a lipase pancreática *in vitro* dos extratos liofilizados foi avaliada e uma forte relação foi observada entre o conteúdo total de antocianinas e a inibição da lipase pancreática. As maiores inibições foram observadas para os extratos de laranja sanguínea e suco de romã, que apresentaram o maior conteúdo de antocianinas totais. Foi levantada a hipótese de que o alto potencial em inibir a lipase pancreática dos extratos contendo antocianinas está relacionado a uma ação sinérgica das antocianinas individuais.	(FABRONI *et al.*, 2016)
Cianidina e cianidina-3-glicosídeo derivados de *Vigna unguiculata*	O estudo identificou e avaliou os inibidores da lipase pancreática da leguminosa *Vigna unguiculata* revelando que a glicosilação das antocianidinas reduz significativamente a inibição da lipase. A inibição foi do tipo não-competitiva.	(VIJAYARAJ; NAKAGAWA; YAMAKI, 2019)
Extratos de arroz negro (*Oryza sativa* L.)	O estudo avaliou sistematicamente a inibição da absorção do colesterol. Foi verificado que os extratos aquosos de arroz negro (BRE), os extratos etanólicos e uma fração obtida do fracionamento com uma resina macroporosa causaram a redução da absorção do colesterol pela inibição da lipase pancreática, diminuindo a solubilidade do colesterol micelar e suprimindo a captação do colesterol nas células Caco-2. A inibição foi positivamente associada ao conteúdo de antocianinas (cianidina-3-glicosídeo (Cy-3-G) e peonidina-3-glicosídeo (Pn-3-G)) dos extratos, que apresentaram modo inibitório competitivo. Assim, considerou-se o arroz preto como fonte natural ideal para reduzir a absorção do colesterol, podendo suas antocianinas serem aplicadas na prevenção e no tratamento da hipercolesterolemia.	(YAO, S. *et al.*, 2013)
Cianidina-3-	Os mecanismos hipolipemiantes da C3R por meio da inibição da digestão e	(THILAVECH;

rutinosídeo (C3R)	absorção lipídica *in vitro* foram investigados por ensaios fluorométricos enzimáticos e colorimétricos enzimáticos, e demonstrou-se que o comportamento da C3R foi de inibidor competitivo de tipo misto da lipase pancreática, inibindo também a esterase do colesterol pancreático em cerca de 5-18%, a formação de micelas de colesterol e a ligação do ácido biliar. Houve também redução da micelização do colesterol. Sugeriu-se, portanto, o C3R como um composto com propriedades anti-hiperlipidêmicas promissoras.	ADISAKWATTANA, 2019)
Fitoquímicos da arônia	O objeto deste estudo foi uma possível ação preventiva dos fitoquímicos da arônia sobre o acúmulo de gordura visceral. O estudo avaliou a hiperlipidemia pós-prandial após a carga de óleo de milho em ratos, a atividade da lipase pancreática *in vitro* e a resposta glicêmica plasmática após a carga de sacarose. Os fitoquímicos inibiram significativamente os aumentos nos níveis de triglicerídeos no plasma nos testes de tolerância ao óleo de milho administrado oralmente. Também inibiram significativamente os aumentos nos níveis de glicose no sangue nos testes de tolerância à sacarose, sugerindo que tais fitoquímicos, ricos em antocianinas, suprimem o acúmulo de gordura visceral e a hiperglicemia, inibindo a atividade da lipase pancreática e/ou a absorção intestinal de lipídios.	(TAKAHASHI *et al.*, 2015)
Extratos de *Cornus mas* (Cm) *e Cornus alba* (Ca)	Foram investigadas e comparadas as atividades inibitórias *in vitro* sobre a lipase pancreática (PL) e sobre a α-amilase dos extratos aquoso e etanólico de frutas frescas liofilizadas e frutas secas adquiridas comercialmente de *C. mas*, bem como de frutas frescas de *C. alba*. Por meio da identificação de constituintes guiada por bioensaio, o estudo revelou que os flavonóides e derivados do ácido fenólico da *Ca* provavelmente inibem a atividade da PL em maior extensão do que os componentes da *Cm.* Considerando a atividade do Cm, foi estabelecido que as antocianinas, podem ser responsáveis pela inibição das enzimas pancreáticas. No entanto, a composição complexa dos extratos pareceu ser mais eficaz devido ao potencial de atividade sinérgica ou aditiva de seus constituintes. Os resultados mostraram também que os extratos de frutas secas disponíveis no mercado foram menos ativos do que os de frutas frescas colhidas e liofilizadas, provavelmente devido à menor quantidade de antocianinas, indicando que o consumo de frutas frescas traz	(SWIERCZEWSKA *et al.*, 2018)

	benefícios à saúde. Todos os extratos testados foram inibidores da atividade da PL mais fortes do que os inibidores da atividade da α-amilase.	
Chá *Sunrouge* (SR), SR segunda safra (SR2), SR terceira safra (SR3) e *Yabukita* (YK)	No estudo *in vitro*, SRs ricos em antocianinas produziram forte inibição da lipase, em comparação ao YK. No entanto, embora SR2 contivesse altos níveis de antocianinas, não mostrou o maior efeito inibitório contra a lipase. As catequinas do tipo galato, como galato de epigalocatequina, podem representar um dos componentes inibidores da lipase do SR. O estudo *in vivo* mostrou que o purê de SR2 pode inibir a atividade da lipase e suprimir a absorção de gordura do intestino delgado, já que o aumento na concentração plasmática de triacilglicerol após a administração oral de óleo a camundongos foi significativamente suprimido. Nos grupos YK e controle a concentração plasmática de TG não diferiu significativamente.	(SHIRAI, 2017)
Extrato da casca de jabuticaba (*Myrciaria jaboticaba*) e cianidina-3-O-glicosídeo	A cianidina-3-O-glicsídeo é um importante constituinte do extrato da casca da jabuticaba. No estudo os efeitos do extrato sobre a absorção de triglicerídeos e amido em camundongos foram comparados com os da cianidina-3-O-glicosídeo. O extrato inibiu a absorção de triglicerídeos e amido *in vivo*, mas foi aproximadamente 100 vezes mais potente no primeiro caso do que neste último, com 50% de inibição com uma dose de 3,5 mg/kg. Já a cianidina-3-O-glicosídeo não inibiu a absorção de amido com doses de até 20 mg/kg, mas produziu 72% de inibição da absorção de triglicerídeos na dose de 2 mg/kg. Tanto o extrato como a cianidina-3-O-glicosídeo inibiram a lipase pancreática, mas o efeito *in vivo* não pode ser explicado somente em termos desta ação. Muito provavelmente há também uma inibição direta de pelo menos uma das etapas que levam à absorção dos ácidos graxos livres, já que ambos, extrato e cianidina-3-O-glicosídeo, inibiram a co-absorção de ácido oleico livre e glicerol. A conclusão é que o extrato de casca de jabuticaba como também a cianidina-3-O-glicosídeo apresentam perspectivas amplamente favoráveis ao seu uso como adjuvantes medicamentosos.	(CASTILHO *et al.*, 2021)

2.2.2. Antocianinas como inibidoras de lipases

Diversos estudos já empregaram compostos purificados e extratos naturais para avaliar o efeito inibitório das antocianinas sobre a lipase (SEYEDAN et al., 2015; VIJAYARAJ; NAKAGAWA; YAMAKI, 2019). Na Tabela 3, estudos que evidenciam o poder inibitório das antocianinas sobre as lipases são descritos. Não há muitos estudos *in vivo,* o que seria altamente desejável. Neste aspecto cabe destacar o estudo de Yao *et al.* (2013) sobre os efeitos dos extratos de arroz negro sobre a absorção de colesterol, o de Takahashi *et al.* (2015) sobre a inibição do acúmulo de gordura visceral pelos fitoquímicos da arônia e o recente estudo de Castilho *et al.* (2021) acerca dos efeitos do extrato da casca de jabuticaba sobre a absorção de triglicerídeos. Este último estudo merece também destaque pelo fato de ter sido feita uma comparação dos efeitos *in vivo* do extrato da casca com os efeitos de um dos seus constituintes cianidínicos, mais precisamente, a cianidina-3-*O*-glicosídeo.

2.2.3. Métodos clássicos para avaliação da atividade inibidora das antocianinas sobre amilases e lipases

A caracterização de inibidores é fundamental para qualquer pesquisa que busque a descoberta de novos medicamentos ou adjuvantes em tratamentos que visem a melhoria da qualidade de vida de indivíduos afetados pela obesidade ou diabetes tipo 2. Devido ao grande número de parâmetros que precisam ser avaliados, é importante determinar quais são os mais importantes e manter estes sob foco constante (CESCO *et al.*, 2017). Uma abordagem multidisciplinar é necessária quando se utilizam plantas como fonte farmacológica, pois um bom resultado dependerá diretamente de uma série de ensaios *in vitro* e *in vivo* cuidadosamente selecionados. A escolha dos métodos deve ser ajustada prezando sempre uma boa reprodutibilidade (ATANASOV *et al.*, 2015).

2.2.3.1. Métodos *in vitro*

Os métodos de avaliação da inibição enzimática *in vitro* são relativamente simples. Na maioria das vezes, em ensaios enzimáticos, são utilizados substratos de preferência naturais ou análogos sintéticos em concentração fisiológica e o pH do experimento é alterado de acordo com o

adequado para cada tipo de enzima. Eventualmente podem ser utilizados substratos sintéticos, sobretudo quando isto facilita as medidas. A inibição é avaliada com base na determinação da velocidade da reação inicial catalisada pela enzima na ausência e presença de um possível inibidor. A inibição das enzimas pode ser quantificada *in vitro* por uma variedade de ensaios de atividade que frequentemente consistem em medidas espectrofotométricas. Métodos espectrofotométricos no caso das amilases são, por exemplo, a formação do complexo iodo-amido, os vários métodos de açúcares redutores e o método do p-nitrofenil-α-D-maltosídeo. Alternativamente, pode-se usar no ensaio das amilases derivados fluorogênicos como o p-nitrophenyl-maltopentoaosídio. A hidrólise deste composto resulta num aumento da fluorescência que é proporcional à atividade hidrolítica da enzima.

Para as lipases frequentemente são utilizados substratos lipídicos insolúveis em água como o butirato de p-nitrofenil (PNPB) e o 2,3-dimercapto-1-propanol tributirato (BALB) (RAHIM, TAKAHASHI, YAMAKI, 2015; BARRETT, FARHADI, SMITH, 2018; SUN, WARREN, GIDLEY, 2019; KANBARKAR, MISHRA, KHANAL, 2020). Outros substratos cromogênicos alternativos para as lipases são os ésteres de *p*-nitrofenol com ácidos orgânicos. Sua determinação também pode ser feita na presença de polissorbato e um sal de cálcio, fornecendo o precipitado de cálcio e um ácido graxo que dispersa a luz (POHANKA, 2019).

O grau de inibição é geralmente caracterizado pelo valor de "IC_{50}" do sistema experimental, que representa a concentração de inibidor necessária para reduzir a atividade de uma enzima específica em 50%. No entanto, este valor é função da concentração do substrato. No caso das inibições competitiva e mista, aumentos na concentração do substrato levam a aumentos no valor de IC_{50}. O contrário ocorre com a inibição incompetitiva, um tipo de inibição que é bastante raro, no entanto. Assim, uma boa prática é a de sempre especificar a concentração do substrato quando o valor de IC_{50} for determinado (BARRETT; FARHADI; SMITH, 2018; SUN; WARREN; GIDLEY, 2019).

Após a identificação da inibição da enzima por algum inibidor isolado ou extrato, torna-se necessário avaliar que tipo de inibição foi causada. Tanto as amilases quanto as lipases são enzimas que obedecem à cinética de Michaelis-Menten,

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad (1)$$

onde v é a velocidade de reação, [S] a concentração do substrato, K_M a constante de Michaelis-Menten e V_{max} a velocidade máxima (função da concentração da enzima). Deve-se mencionar, no entanto, que as lipases podem apresentar o fenômeno da inibição por substrato (CASTILHO et al., 2021).

Medir a curva de saturação do substrato na presença de diferentes concentrações do inibidor possibilitará avaliar se o inibidor causa uma inibição competitiva, não-competitiva, mista ou mesmo incompetitiva (ARSALAN; YOUNUS, 2018; SUN; WARREN; GIDLEY, 2019). Estas situações, inibição competitiva, mista e incompetitiva caracterizam-se, respectivamente, pelas equações a seguir, que resultam de uma modificação da equação de Michaelis-Menten:

$$v_i = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]}{K_{I1}}\right) + [S]} \quad (2)$$

$$v_i = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]}{K_{I1}}\right) + [S]\left(1 + \frac{[I]}{K_{I2}}\right)} \quad (3)$$

$$v_i = \frac{V_{max}[S]}{K_M + [S]\left(1 + \frac{[I]}{K_{I2}}\right)} \quad (4)$$

Os símbolos K_{I1} e K_{I2} designam as constantes de dissociação dos complexos EI e ESI, respectivamente. Para a inibição não-competitiva pura vale a equação (3) com $K_{I1} = K_{I2}$. A determinação da velocidade máxima (V_{max}), da constante de Michaelis-Menten (K_M) e das constantes de inibição usando o gráfico de Lineweaver-Burk, assim como outros métodos gráficos, tem várias limitações. Principalmente quando concentrações de substrato bem menores

do que o K_M são utilizadas, a determinação das inclinações das retas no plote duplamente recíproco (inverso da velocidade de reação contra o inverso da concentração do substrato, i.e., 1/v versus 1/[S]) torna-se incerta o que, por sua vez, torna difícil a distinção entre inibição competitiva, incompetitiva e mista (CORNISH-BOWDEN, 1974; SUN *et al.*, 2019).

Deste modo, outros métodos podem ser utilizados para auxiliar na análise do tipo de inibição, tais como o gráfico de Dixon, que consiste em plotar o inverso da velocidade de reação (1/v) contra a concentração do inibidor ([I]). A representação de 1/v versus [I] deverá resultar numa reta caso não haja a formação de complexos enzima-inibidor múltiplos. Se a curva for parabólica ou hiperbólica o mecanismo de inibição certamente será bastante complexo e uma análise numérica mais elaborada se fará necessária. A melhor solução para estes problemas será utilizar programas específicos para ajustes não-lineares de mínimos quadrados (CASTILHO et al., 2021). Programas deste tipo permitem distinguir entre vários mecanismos possíveis através de indicadores de precisão estatística.

2.2.3.2. Métodos *in vivo*

Os compostos isolados ou extratos que resultaram promissores nas análises *in vitro* necessitam ser testados *in vivo*, ou seja, é necessário verificar se os efeitos inibitórios são fisiologicamente relevantes. Vale ressaltar que os testes *in vivo* devem sempre ser conduzidos dentro de princípios éticos relacionados ao uso de animais (ATANASOV *et al.*, 2015).

Um método experimental que pode mostrar esses resultados é realizado através dá quantificação da glicemia após a alimentação do animal com amido, no caso das análises que visam a inibição das amilases. Para as análises que avaliam a inibição da lipase, realiza-se a quantificação de triglicerídeos presentes no sangue após a administração por gavagem de azeite de oliva na ausência ou presença de diferentes concentrações dos potenciais inibidores (KATO-SCHWARTZ *et al.*, 2020; OLIVEIRA *et al.*, 2015).

2.2.4. Métodos para avaliação das interações moleculares dos inibidores com as enzimas

Os ensaios *in vitro* e *in vivo* são muito importantes para avaliar a capacidade inibitória de antocianinas puras ou extratos ricos em antocianinas, mas apresentam algumas limitações, associadas às respostas e às perguntas de como ocorrem as ligações e interações do inibidor com a enzima e suas possíveis interações. Abaixo, são descritas algumas das técnicas utilizadas para avaliar tais interações moleculares.

2.2.4.1. Espectroscopia de fluorescência

A espectroscopia de fluorescência é uma técnica altamente sensível para visualizar propriedades estruturais terciárias de proteínas que resultam do processo de dobramento/desdobramento de proteínas. Esta técnica tem sido utilizada para verificar se ocorre interação entre os compostos fenólicos e as proteínas em nível molecular e para explicar o modo como estas interações acontecem. Os resíduos aromáticos das proteínas, triptofano (Trp), tirosina (Tyr) e fenilalanina (Phe) são os responsáveis pela fluorescência das proteínas. A intensidade da fluorescência está diretamente relacionada à quantidade de proteína em solução (SUN; WARREN; GIDLEY, 2019; WU *et al.*, 2019). Mudanças nas condições do solvente em que uma proteína se encontra e a interação com ligantes, por outro lado, alteram as características espectrais de sua fluorescência. Isto se traduz tanto em mudanças no comprimento de onda das emissões bem como em alterações na intensidade destas mesmas emissões.

2.2.4.2. Calorimetria de varredura diferencial (DSC)

A DSC pode ser usada para caracterizar propriedades térmicas e monitorar transições de fases. Na prática, ela mede a mudança do calor associada à desnaturação térmica das moléculas quando aquecida a uma velocidade constante. Como resultado, a desnaturação térmica e a variação da entalpia podem ser obtidas após a proteína ser totalmente desnaturada na DSC (GUO *et al.*, 2018; SUN *et al.*, 2019; WANG *et al.*, 2018).

O processo de desnaturação da proteína é geralmente dividido em dois estágios. O primeiro é reversível, havendo perda parcial da atividade da

proteína, decorrente da ruptura das interações não-covalentes intermoleculares, pelo processo de desdobramento das proteínas. Já o segundo estágio, é irreversível, levando à desnaturação da molécula inicialmente desdobrada e a interação da enzima com o inibidor durante a análise de DSC (SUN; GIDLEY; WARREN, 2017).

O efeito da temperatura na inativação pode ser entendido por meio do termograma, que apresenta normalmente um pico endotérmico que representa a temperatura de fusão, e esta, por sua vez, indica que a enzima foi de fato inativada, sendo considerada um indicador de termoestabilidade. Via de regra, quanto maior o valor da temperatura de fusão, mais estável é a proteína (HAN *et al.*, 2020).

2.2.4.3. Dicroísmo circular (DC)

A espectroscopia de DC é uma técnica bem estabelecida para estudar a estrutura tridimensional das macromoléculas, uma vez que a formação de complexos compostos fenólicos-proteína afeta estruturalmente as proteínas, alterando a estrutura secundária de um ou dos dois ligantes após a interação, modificando seu espectro (GUO *et al.*, 2018; YAO *et al.*, 2018).

A técnica consiste em monitorar alterações moleculares através da absorção de luz em diferentes comprimentos de onda (ZENG *et al.*, 2016). Cada estrutura na proteína tem comprimentos de onda específicos para sua detecção, por exemplo, a α-hélice tem, tipicamente, uma banda positiva em 193 nm e bandas negativas em 222 e 208 nm, já as folhas pregueadas β tem bandas negativas em 218 nm e bandas positivas em 195 nm.

Portanto, o uso do dicroísmo circular (DC) em conjunto com outras técnicas como, espectroscopia de fluorescência e o DSC é de grande valia, pois podem corroborar para análise de resultados, uma vez que ambos os métodos possuem a mesma finalidade de avaliação das alterações estruturais da proteína (espiral aleatória ou desordenada), pós ligação com polifenóis. A composição da estrutura secundária das proteínas também pode ser determinada usando espectroscopia no infravermelho por transformada de Fourier (FT-IR) (CZUBINSKI; DWIECKI, 2017).

2.2.4.4. Calorimetria de titulação isotérmica (ITC)

Por meio da termodinâmica também é possível analisar as interações entre proteínas e outras moléculas. A ITC é a única técnica com capacidade de medir a afinidade de ligação e os componentes de entalpia, entropia da energia de Gibbs. Ambos são medidos com base na determinação do calor liberado ou absorvido durante a interação. Tais dados fornecem informações sobre todos os mecanismos de interação nos meios biológicos que afetam as propriedades da superfície do sistema (VELAZQUEZ-CAMPOY, LEAVITT, FREIRE, 2015; LIU *et al.*, 2018; PROZELLER, MORSBACH, LANDFESTER, 2019).

Normalmente, os dados são obtidos por meio de um gráfico de calor contra o número de injeção num dado tempo ($\mu cal \cdot s^{-1}$) e exibem uma série de picos para cada injeção. Esses dados devem ser normalizados pico a pico a fim de obter um gráfico da variação de entalpia por mol (ΔH, $KJ \cdot mol^{-1}$) em relação à razão molar polifenol/enzima (KARONEN *et al.*, 2015; SUN, GIDLEY, WARREN, 2017).

Entende-se que a técnica em questão é mais uma alternativa para avaliar as interações enzima-inibidor, neste caso, se tem uma alternativa de análise rápida das constantes de Michaelis e IC_{50} para os pares enzima-substrato ou enzima-inibidor, que pode ser utilizada em combinação com as demais técnicas citadas anteriormente com o intuito de corroborar os resultados.

2.2.4.5. Docking molecular

Os métodos computacionais são tecnologias que auxiliam na seleção de extratos naturais com atividade biológica. Através do docking molecular é possível realizar simulações em nível molecular, ou seja, encenar a ligação entre os ligantes de proteínas e as estruturas moleculares (ATANASOV *et al.*, 2015; KAZMI *et al.*, 2019; SUN, WANG, MIAO, 2020). Com os dados obtidos é possível selecionar os compostos naturais com potencial inibitório. Isto porque a modelagem computacional permite prever a estrutura conformacional do complexo inibidor-enzima, calcular a priori a energia de ligação do inibidor, e prever as possíveis interações envolvidas, inclusive com identificação dos resíduos de aminoácidos (ZHANG, ARYEE, SIMPSON, 2020). Esse tipo de abordagem *in sílico* tem sido amplamente utilizado na indústria farmacêutica

para selecionar fármacos que tenham afinidade para com o sítio ativo. Além disso, essa tecnologia também é utilizada para caracterizar o modo de interação ligante-proteína, podendo prever os resíduos de aminoácidos envolvidos nas ligações com os polifenóis (SUN, WANG, MIAO, 2020).

3. Limitações dos estudos

As antocianinas possivelmente desempenham um papel significativo como inibidores de enzimas. Muitas destas inibições, se adequadamente controladas, poderão eventualmente beneficiar a saúde humana. Apesar do potencial das antocianinas como auxiliares nos tratamentos da obesidade e do diabetes, o uso de extratos apresenta certas limitações devido às incertezas geradas pelo grande número de seus constituintes moleculares. Fazem-se, portanto, necessários estudos que utilizem antocianinas purificadas. Estas nem sempre estão disponíveis e quando estão são muitas vezes bastante caras, dificuldades que se espera que sejam superadas em breve como fruto do futuro desenvolvimento tecnológico.

4. Conclusão e perspectivas

O consumo de alimentos ricos em antocianinas, como mirtilo, amoras, framboesas, morangos, uvas vermelhas, entre outros, bem como o uso de extratos com altos teores de antocianinas, podem ser recomendados como coadjuvantes dos tratamentos convencionais.

A atividade inibitória das antocianinas sobre as enzimas amilase, glicosidase e lipase é determinada principalmente por sua estrutura molecular. Considera-se em geral que a determinação dos valores de IC_{50} (concentração que produz 50% de inibição) e a caracterização da cinética de inibição são abordagens preliminares indispensáveis. Estas abordagens preliminares devem ser seguidas de experimentos *in vivo* para certificar se o efeito de fato tem ou não relevância fisiológica. Como aprimoramento posterior do conhecimento acerca dos mecanismos da inibição a nível molecular podem ser feitas análises tais como, espectroscopia de fluorescência e calorimetria de titulação isotérmica. Simulações computacionais (docking molecular) podem ser úteis em qualquer fase das investigações para corroborar os dados cinéticos e sugerir possíveis abordagens experimentais para elucidar,

sobretudo, a participação de determinados grupos nas interações inibidor-proteína.

Em perspectiva pode-se dizer que estudos *in vivo* e sobretudo estudos clínicos são altamente necessários para confirmar e dar relevância aos inúmeros dados já obtidos nas condições *in vitro*. Também são necessários esforços que permitam uma atribuição mais segura dos efeitos observados a espécies moleculares bem definidas, já que de um modo geral os experimentos têm sido conduzidos com extratos que contém grande diversidade de compostos.

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The inhibitory action of purple tea on *in vivo* starch digestion compared to other *Camellia sinensis* teas

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ABSTRACT

In order to contribute to improve knowledge about the actions of *Camellia sinensis* extracts on starch digestion, several varieties were compared. The latter were green, oolong, white, black, and purple teas. The results are hoped to contribute to our understanding of the mode of action and potency of the various tea preparations as possible adjuvants in the control of post-prandial glycemia. The extracts were prepared in way similar to their form of consumption. All extracts decreased starch digestion, but the purple tea extract was the strongest inhibitor, their inhibitory tendency started at the dose of 50 mg/kg and was already maximal with 250 mg/kg. Maltose tolerance was not significantly affected by the extracts. Glucose tolerance was not affected by purple tea, but black tea clearly diminished it; green tea presented the same tendency. Purple tea was also the strongest inhibitor of pancreatic α-amylase, followed by black tea. The green tea, oolong tea, and white tea extracts tended to stimulate the pancreatic α-amylase at low concentrations, a phenomenon that could be counterbalancing its inhibitory effect on starch digestion. Based on chemical analyses and molecular docking simulations it was concluded that for both purple and black tea extracts the most abundant active component, epigallocatechin gallate, seems also to be the main responsible for the inhibition of the pancreatic α-amylase and starch digestion. In the case of purple tea, the inhibitory activity is likely to be complemented by its content in deoxyhexoside-hexoside-containing polyphenolics, especially the kaempferol and myricetin derivatives. Polysaccharides are also contributing to some extent. Cyanidins, the compounds giving to purple tea its characteristic color, seem not to be the main responsible for its effects on starch digestion. It can be concluded that in terms of postprandial anti-hyperglycemic action purple tea presents the best perspectives among all the tea varieties tested in the present study.

1. Introduction

Type 2 diabetes mellitus is the most common type of diabetes and its complications are a major global health problem (Wasana, Attanayake, Weerarathna, & Jayatilaka, 2021). According to estimates of the 2019 International Diabetes Federation (IDF), there are around 460 million adults aged between 20 and 79 in the world suffering from diabetes (Saeedi et al., 2019). A frequent treatment for type 2 diabetes mellitus consists in the prescription of inhibitors of carbohydrate digestive enzymes aiming at reducing the absorption of monosaccharides originating from starch and sucrose (Awosika & Aluko, 2019). Inhibition of starch digestion by ingesting α-amylase and α-glucosidase inhibitors is an especially effective way of diminishing post-prandial glycemia, but the drugs commonly used, such as acarbose, present several negative effects (Awosika & Aluko, 2019; Gutiérrez-Grijalva, Antunes-Ricardo, Acosta-Estrada, Gutiérrez-Uribe, & Basilio Heredia, 2019). For this reason, there has been continuous efforts at discovering natural anti-diabetic agents that could provide mild anti-hyperglycemic protection

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by virtue of a continuous ingestion in conjunction with the regular and daily food intake.

Infusions (teas) prepared with the various *Camellia sinensis* varieties have been suggested as potentially effective adjuvants in the protection against hyperglycemia episodes. The supporting evidence was derived mainly from *in vitro* measurements of the activities of α-amylases (salivary and pancreatic) and yeast α-glucosidase. Close analysis of the hitherto published results, however, reveals conflicting results. For example, it has been reported that a green tea extract inhibits the porcine pancreatic α-amylase with a IC_{50} of 4020 μg/mL, but also that it inhibits the yeast α-glucosidase with a IC_{50} of 4.4 μg/mL (Gao, Xu, Wang, Wang & Hochstetter, 2013). The authors claim that the inhibitory activity of the green tea extract on the yeast α-glucosidase enormously exceeds that of acarbose (the classical inhibitor of carbohydrate digestion), but the correct enzyme for comparisons should actually be the mammalian intestinal α-glucosidase, which is very strongly inhibited by acarbose (IC_{50} = 0.25 μg/mL; (Zhang et al., 2017). In another study, a IC_{50} of 2000 μg/mL for the inhibition of the human α-amylase by a green tea extract was found (Miao, Jiang, Jiang, Zhang, & Li, 2015). This would be similar to the value reported previously by porcine pancreatic α-amylase inhibition. (Gao et al., 2013). However, Nyambe-Silavwe et al. (2015) claim that their green tea preparation inhibited the human salivary α-amylase with a IC_{50} of 8.9 μg/mL when amylose was the substrate and of 25 to 69 μg/mL when amylopectin was the substrate, numbers that are not in agreement with those reported by Gao et al. (2013) and Miao et al.(2015). Yang and Kong (2016), on the other hand, reported biphasic effects for green, oolong and black tea extracts in addition to a polyphenolic preparation extracted from *Camellia sinensis*: stimulation at low concentrations, followed by inhibition at higher ones.

These and other contradicting reports (Yang et al., 2019) may have resulted from different assay conditions and preparations of the tea extracts. Contradictions, however, are of limited help when one tries to translate them to the *in vivo* situation. *In vivo* experiments are, thus, highly desirable at the present stage. There is one *in vivo* study in humans indicating that green tea may indeed affect carbohydrate digestion (Lochocka et al., 2015). In this study the influence of a green tea extract (4 g dose) was investigated on the $^{13}CO_2$ recovery in breath after the ingestion of ^{13}C labeled cornflakes. The study found a reduced $^{13}CO_2$ recovery in individuals who had ingested the green tea extract. This outcome was interpreted as representing inhibition of starch digestion caused mainly by the reported α-glucosidase inhibition (Gao et al., 2013). This is no doubt a reasonable interpretation in view of the available evidence, but one should not forget that there is a long metabolic way between starch digestion and CO_2 production, so that there are many other mechanistic alternatives. In this respect there is a more direct way of inferring about starch digestion, which consists in measuring the glycemic levels in animals just after a starch load. This technique has proven to be an adequate procedure for evaluating the action of starch digestion in the intestine (Kato-Schwartz et al., 2018, 2020). In order to contribute further for clarifying the effects of *Camellia sinensis* extracts on starch digestion, we are proposing in this work a comparative study of the effects of five varieties on starch digestion using the starch tolerance test in mice. The varieties to be investigated are green, oolong, white, black and purple tea. Emphasis will be given to purple tea, however, which represents a new and promising variety in terms of medicinal effects. The latter differs from the other tea varieties because of its unique purple leaves (Kerio, Wachira, Wanyoko, & Rotich, 2012; Lai et al., 2016). The leaves have this color because they contain anthocyanins in quantities that largely exceed the contents of other tea varieties. Anthocyanins are claimed to possess many functional and biological properties (Adisakwattana, Yibchok-Anun, Charoenlertkul, & Wongsasiripat, 2011; Kong, Chia, Goh, Chia & Brouillard, 2003), including beneficial effects in diabetic individuals (Rocha et al., 2019), but the pharmacologic potential of purple tea is much less known than that of green tea, for example. In line with the present attempts at finding antidiabetic agents that will allow mild anti-hyperglycemic protection by virtue of its continuous ingestion in parallel with the regular food intake, the extracts used in the present study will be prepared in a way that simulates their preparation for regular consumption. It is hoped that the results will contribute significantly to our understanding of the mode of action and potency of the various tea preparations as possible adjuvants in the control of post-prandial glycemia.

2. Material and methods

2.1. Material

Porcine pancreatic α-amylase (type IV-B), and potato starch were purchased from Sigma-Aldrich. Acarbose was obtained from local pharmacies. All reagent grade chemicals were from the highest possible degree of purity.

2.2. Preparation of the aqueous tea extracts

The commercial *Camellia sinensis* var. assamica products labeled as green tea, oolong tea, white tea, and black tea were purchased in the local market (Maringá, Brazil). Kenyan purple tea (purple tea) was purchased from Justea.com, Vancouver, Canada. Purple tea is a special type of tea produced mostly in Kenya. Differently from green, oolong, black, and white tea, purple tea is not a tea type characterized by its manufacturing, but a different cultivar called TRFK 306/1 (Kilel et al., 2013). It can be used for manufacturing all types of tea, but the sample used in the present study was manufactured followed the procedures used to prepare green tea.

The extracts were prepared according to the protocol described by Lochocka et al. (2015). The tea leaves (100 g) were ground, boiled in distilled water (1000 mL) and subsequently stirred for 15 min at 70 °C (repeated 3 times). The collected extracts were filtered, frozen and lyophilized under vacuum at −20 °C.

2.3. Analysis of phenolic compounds

The lyophilized extracts were dissolved in methanol/water (80:20, *v/v*) to a final concentration of 10 mg/mL and filtered through 0.22 μm disposable filter disks. Analysis was performed in a HPLC system (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) coupled to a diode-array detector (DAD, using 280 and 370 nm as preferred wavelengths) and a Linear Ion Trap (LTQ XL) mass spectrometer (MS, Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Separation was made in a Waters Spherisorb S3 ODS-2 C18 column (3 μm, 4.6 mm × 150 mm; Waters, Milford, MA, USA). The operating conditions were previously described by Bessada, Barreira, Barros, Ferreira & Oliveira (2016). The identification of phenolic compounds was achieved by comparing retention times, UV–Vis and mass spectra with available standard compounds. Otherwise, available data reported in the literature were applied to tentatively identify the compounds. Quantitative analysis was performed using a 7-level calibration curves of each available phenolic standard constructed upon the UV signal: apigenin-6-C-glucoside ($y = 107025x + 61531$, $R^2 = 0.9989$; LOD = 0.19 μg/mL; LOQ = 0.63 μg/mL); chlorogenic acid ($y = 168823x - 161172$, $R^2 = 0.9999$; LOD = 0.20 μg/mL; LOQ = 0.68 μg/mL); gallic acid ($y = 131538x + 292163$, $R^2 = 0.9969$; LOD = 0.28 μg/mL; LOQ = 0.87 μg/mL); B - catechin ($y = 84950x - 23200$, $R^2 = 1$; LOD = 0.17 μg/mL; LOQ = 0.68 μg/mL); myricetin-3-*O*-glucoside ($y = 23287x - 581708$, $R^2 = 0.9988$; LOD = 0.23 μg/mL; LOQ = 0.78 μg/mL); and quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL). For the Identified phenolic compounds with unavailable commercial standard these were quantified using the calibration curve of the most similar standard available. The analyses were carried out in triplicate and the results are expressed as mean values and standard deviations (SD), in mg/g of lyophilized extract.

2.4. Total anthocyanin assay

The total anthocyanin contents of the various tea extracts were evaluated by the pH differential method according to the pH differential method Lee et al. (2005). The pH 1.0 buffer was 0.025 M hydrochloric acid–potassium chloride buffer (HCl-KCl) and the pH 4.5 buffer was 0.4 M sodium acetate. The sample dilution was established by limiting the pH 1.0 absorbance at 520 nm between 0.2 and 1.4. The total anthocyanin content of each sample (C_{TA}; mg cyanidin-3-*O*'-glucoside equivalents per g extract) was calculated by the following formula:

$$C_{TA} = \frac{A \times mw \times DF \times 10^3}{\varepsilon \times \ell \times W} \quad (1)$$

A is the specific absorbance, *mw* the molecular weight of cyanidin-3′-*O*-glucoside (449.2 g/mol), DF the dilution factor, ε the molar extinction coefficient of cyanidin-3′-*O*-glucoside at 520 nm ($2.69 \times 10^4\ M^{-1}\ cm^{-1}$), ℓ the light path length (cm), *W* the amount that was extracted (in mg/100 g dry weight) and 10^3 a factor for the conversion of g into mg. The specific absorbance A is defined as:

$$A = (A_{520\ nm} - A_{700\ nm})_{pH\ 1.0} - (A_{520\ nm} - A_{700\ nm})_{pH\ 4.5} \quad (2)$$

2.5. Polysaccharide extraction

High-molecular weight components (mainly polysaccharides) were precipitated by addition of 3 volumes of cold absolute ethanol to each aqueous extract (Deng et al., 2015). The mixtures were maintained at 8 °C overnight and separated by centrifugation (8000 rpm at 4 °C, 30 min). The sediments were washed with ethanol twice and finally dissolved in water. These materials were called ethanol precipitated fractions (EP). Total sugars were assayed by the phenol–sulfuric method based on the absorbance at 490 nm of the phenol-carbohydrate complex (Dubois et al., 1956) and expressed as mg sugars/g extract. Glucose was used for constructing the standard curve.

2.6. Pancreatic α-amylase assay

Initial reaction rates catalyzed by the porcine pancreatic α-amylase were measured at 37 °C and pH 6.9 in absence or presence of various tea extract concentrations (Kato et al., 2017). The pH used is close to the optimum reported previously (da Silva et al., 2014). The substrate was potato starch up to 1.0 g per 100 mL prepared in 20 mmol/L phosphate buffer plus 6.7 mmol/L NaCl. The amount of enzyme added to each reaction mixture was 1 unit (specific activity 500 units/mg protein). Two different protocols were used. In the first one, the substrate and one of the tea extracts or redissolved ethanol precipitates (at various concentrations) were incubated for 30 min at 37 °C and the reaction was started by adding the enzyme. In the second protocol the enzyme and one of the tea extracts or redissolved ethanol precipitates (at various concentrations) were incubated for 30 min at 37 °C and the reaction was initiated by the addition of substrate. These two protocols are justified by the observation that the degree of inhibition of the α-amylase phenolic compounds is affected by the addition order of the reaction participants (D'Costa & Bordenave, 2021). For both protocols the reaction was allowed to proceed for 10 min. After this time the amount of reducing sugars in the reaction medium was quantified using the 3,5-dinitrosalicylic acid procedure (Miller, 1959). The standard curve was constructed with maltose. No changes in pH were observed during the incubation time.

2.7. Molecular docking

The structures of the phenolic compounds identified in Table 1 were obtained from the PubChem database (Kim et al., 2016) in the *.sdf format. All possible variations for a pentoside or hexoside that were available in PubChem were considered in order to cope for eventual

Table 1
Tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the extracts of the various tea varieties (*Camellia sinensis*) used in the present work. Full experimental details are given in the Section 2.

Tentative identification	Green tea mg/g extract	Purple tea	Oolong tea	White tea	Black tea
(Epi)gallocatechin isomer I[A]	0.72 ± 0.03	5.67 ± 0.07	0.127 ± 0.007	–	–
(Epi)gallocatechin isomer II[A]	2.52 ± 0.02	–	0.49 ± 0.01	–	–
(Epi)gallocatechin isomer III[A]	2.12 ± 0.08	–	0.31 ± 0.01	–	–
(+)-Catechin[A]	4.8 ± 0.2	1.76 ± 0.01	0.704 ± 0.006	0.082 ± 0.002	1.25 ± 0.03
Epigallocatechin gallate[A]	30.2 ± 0.4	11.6 ± 0.1	6.48 ± 0.05*	6.24 ± 0.08*	8.5 ± 0.1
3-*O*-Caffeoylquinic acid[B]	–	–	–	1.31 ± 0.02	–
4-*O*-Caffeoylquinic acid[B]	–	–	–	0.570 ± 0.001	–
5-*O*-Caffeoylquinic acid[B]	–	–	–	1.06 ± 0.02	–
Theasinensin B (or E)[C]	–	–	–	–	1.06 ± 0.05
Apigenin-*C*-hexoside-*O*-pentoside[D]	0.34 ± 0.02	0.046 ± 0.001*	0.042 ± 0.001*	0.061 ± 0.005*	0.031 ± 0.001*
Apigenin-*C*-hexoside-*O*-pentoside[D]	0.38 ± 0.01	0.028 ± 0.001	0.061 ± 0.001	0.074 ± 0.006	0.046 ± 0.001
Myricetin-3-*O*-rutinoside[E]	0.35 ± 0.01	0.674 ± 0.001	0.279 ± 0.001	0.257 ± 0.001	–
Myricetin-3-*O*-glucoside[E]	0.43 ± 0.01	0.742 ± 0.001	0.355 ± 0.006	0.280 ± 0.001	0.306 ± 0.001
Myricetin-*O*-hexoside[E]	0.59 ± 0.01	0.676 ± 0.002	0.302 ± 0.004	0.327 ± 0.001	–
Quercetin-*O*-deoxyhexosyl-hexoside-hexoside[F]	0.457 ± 0.008	0.138 ± 0.001	0.206 ± 0.001	0.149 ± 0.002	–
Quercetin-*O*-deoxyhexosyl-hexoside-hexoside[F]	0.594 ± 0.007	–	0.067 ± 0.001*	0.070 ± 0.001*	–
Quercetin-3-*O*-glucoside[F]	0.25 ± 0.02*	0.252 ± 0.001*	0.127 ± 0.002¶	0.129 ± 0.002¶	0.190 ± 0.001
Quercetin-3-*O*-rutinoside[F]	–	0.319 ± 0.001	–	–	0.104 ± 0.003
Quercetin-*O*-deoxyhexosyl-hexoside[F]		0.167 ± 0.001			0.085 ± 0.001
Quercetin-*O*-malonyl-hexoside[F]	–	0.196 ± 0.001	–	–	–
Kaempferol-*O*-deoxyhexosyl-dihexoside[F]	0.46 ± 0.01	–	0.156 ± 0.002	0.220 ± 0.003	–
Kaempferol-*O*-deoxyhexosyl-dihexoside[F]	0.359 ± 0.007	–	0.065 ± 0.001	0.102 ± 0.001	–
Kaempferol-3-*O*-rutinoside[F]	0.154 ± 0.001	0.274 ± 0.001	0.058 ± 0.001*	0.060 ± 0.001*	0.096 ± 0.001
Kaempferol-3-*O*-glucoside[F]	0.084 ± 0.001*	0.218 ± 0.002	–	–	0.086 ± 0.001*
Kaempferol-*O*-malonyl-hexoside[F]	–	0.213 ± 0.003	–	–	–
3,4-*O*-Dicaffeoylquinic acid[B]	–	–	–	0.155 ± 0.003	–
4,5-*O*-Dicaffeoylquinic acid[B]	–	–	–	0.127 ± 0.001	–
Total phenolic compounds	**44.76 ± 0.06**	**23.0 ± 0.2**	**9.83 ± 0.04**	**11.27 ± 0.07**	**11.8 ± 0.2**
Total phenolic acids	**–**	**–**	**–**	**3.22 ± 0.01**	**–**

(*continued on next page*)

Table 1 (*continued*)

Tentative identification	Green tea mg/g extract	Purple tea	Oolong tea	White tea	Black tea
Total flavan3-ols	**40.31 ± 0.02**	**19.1 ± 0.2**	**8.11 ± 0.04**	**6.32 ± 0.08**	**10.8 ± 0.2**
Total other flavonoids	**4.46 ± 0.08**	**3.943 ± 0.004**	**0.047 ± 0.001**	**1.73 ± 0.02**	**0.945 ± 0.002**

Standard calibration curves: A - catechin (y = 84950x – 23200, R^2 = 1; LOD = 0.17 μg/mL; LOQ = 0.68 μg/mL); B- chlorogenic acid (y = 168823x – 161172, R^2 = 0.9999; LOD = 0.20 μg/mL; LOQ = 0.68 μg/mL); C- gallic acid (y = 131538x + 292163, R^2 = 0.9969; LOD = 0.28 μg/mL; LOQ = 0.87 μg/mL); D - apigenin-6-C-glucoside (y = 107025x + 61531, R^2 = 0.9989; LOD = 0.19 μg/mL; LOQ = 0.63 μg/mL); E - myricetin-3-O-glucoside (y = 23287x – 581708, R^2 = 0.9988; LOD = 0.23 μg/mL; LOQ = 0.78 μg/mL); F- quercetin-3-O-glucoside (y = 34843x – 160173, R^2 = 0.9998; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL). Values labeled with the same symbols (* or ¶) in the same line do not differ statistically from each other, as indicated by variance analysis followed by Student-Newman-Keuls post hoc testing.

ambiguities resulting from the use of the LC-MS methodology.

The modeled structure of porcine pancreatic alpha-amylase bonded to the inhibitor acarbose pentasaccharide (ARE) was used in the simulations (Kato-Schwartz et al., 2020). The details of the simulations followed the protocol described previously (Kato-Schwartz et al., 2020) with modifications. In summary, the structures of the compounds were evaluated by four different programs, in each case with four replications. The protocols for the Autodock (Morris et al., 2009), Molegro (Thomsen & Christensen, 2006) and Gold (Jones, Willett, Glen, Leach, & Taylor, 1997) programs were as described previously (Kato-Schwartz et al., 2020). The analyses with the DockThor program (Santos, Guedes, Karl & Dardenne, 2020) followed the default settings. The scores provided by each program were compared with the scores of the amylose pentasaccharide substrate or the modeled acarbose inhibitor. Since each program generates scores whose numerical values can not be directly compared, a mean relative score for each compound was calculated according to the following relation:

$$\text{Mean relative score} = \frac{1}{4}\left(\frac{Gold}{Gold_{max}} + \frac{Molegro}{Molegro_{max}} + \frac{DockThor}{DockThor_{max}} + \frac{Autodock}{Autodock_{max}}\right) \quad (3)$$

$Gold_{max}$, etc., corresponds to the score of the compound to which the maximal numerical value was attributed by each program. This normalization may be lumping together numbers that might have different meanings and should be regarded as an attempt at obtaining an unified comparative perspective over a great number of diverse substances.

2.8. Starch tolerance assay in mice

The starch tolerance test was done as described previously (Kato et al., 2017; da Silva et al., 2014) with modifications. Fasted (18 h) male Swiss mice (25–35 g) were used and the administration route was in all cases intragastric (by gavage). The number of animals for each experimental approach was between 3 and 4. Controls received solely commercial corn starch (1 g/kg body weight). Filtered tap water was administered for establishing a base line. The extracts derived from the various teas were administered at doses of up to 500 mg/kg before starch administration. Acarbose, a classical inhibitor of starch hydrolysis and digestion, was administered at doses of up to 50 mg/kg before starch administration. Plasma glucose was determined at times 0, 30, 60, 90, and 120 min after starch administration. The extract dose was based on literature data. Blood samples were collected from the tail vein and analysed by means of a glucometer (AccuChek®) for determining the plasma glucose concentration.

2.9. Glucose and maltose tolerance assays in mice

For the glucose and maltose tolerance assays 18 h fasted mice were used. Glucose or maltose were administered intragastrically to all animals (1.5 g/kg) except to those that received just filtered tap water to establish the basal line. The tea extracts were administered to different groups animals at the dose of 250 mg/kg before glucose or maltose administration. Blood samples were collected from the tail vein at times 0, 30, 60, 90 e 120 min and analysed by means of a glucometer (Accu-Chek®) for determining the plasma glucose concentration.

2.10. Statistical analysis and calculations

Statistical analysis was performed using the GraphPad Prism software (version 8.0). The results were expressed as the mean ± standard errors, and were analysed using one-way analysis of variance (one-way ANOVA), followed by post-hoc Student-Newman-Keuls testing. The 5% level was adopted as a criterion of statistical significance. Numerical interpolation (Stineman's formula) was used for evaluating the IC_{50} values of the α-amylase by the various tea extracts. The calculations were done using the *Scientist* software from MicroMath Scientific Software (Salt Lake City, UT).

3. Results

3.1. Chemical constituents of the extracts

Phenolic compounds are generally believed to constitute the main bioactive compounds and inhibitors of α-amylases in *Camellia sinensis* extracts and, for this reason, all extracts used in this study were characterized in terms of their contents in these compounds, using the analytical procedures described in the Section 2.

Table 1 gives the contents of each identified phenolic compound in the extracts of the various tea varieties. Details such as retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, and fragmentation pattern can be found in Supplementary Material (Tables 1S-5S). The identification of (+)-catechin, 5-*O*-caffeoylquinic acid, myricetin-3-*O*-rutinoside, myricetin-3-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside was made by comparison with authentic available standards. The remaining compounds were tentatively identified by comparing their chromatographic characteristics with results previously described in literature (Dou, Lee, Tzen, Lee, 2007; Kim, Goodner, Park, Choi, Talcott, 2011; Zielinski, Haminiuk, Beta, 2016). The contents are all expressed as quantities (mg) per gram extract and they represent the amounts that were effectively extracted using the procedure described in the methods section (mild hot water extraction). In terms of the contents of total phenolic compounds, the decreasing sequence is green tea > purple tea > black tea ≈ white tea > oolong tea. Individually, epigallocatechin gallate is the most abundant compound in all extracts, the decreasing sequence being green tea > purple tea > black tea > white tea ≈ oolong tea. The purple tea extract revealed to possess the by far highest content of the (epi)gallocatechin isomer 1, 7.9 times more than green tea, which ranked second in this respect. The green tea, however, exceeded the purple tea extract in having 2.7 times more (+) catechin and 2.59 times more epigallocatechin gallate. Finally, phenolic acids were found only in the white tea extract; on the other hand, a theasinensin, B or E, was found only in the black tea extract.

With respect to the total content of anthocyanins, the difference between the purple tea extract and all other extracts is remarkable. The purple tea extract revealed to possess 0.9 ± 0.1 mg cyanidin-3-*O*-glucoside equivalents/g extract, while all other extracts presented 0.100 ± 0.005 mg cyanidin-3-*O*-glucoside equivalents/g extract (black tea) or

less (oolong, white and green tea).

The content of polysaccharides, another possible source of inhibitory compounds (Deng et al., 2015), of white, oolong, green, purple and black tea were, respectively, 113 ± 10, 62 ± 1, 43 ± 2, 24 ± 1 and 23.7 ± 0.2 mg glucose equivalents per g extract.

3.2. Effects of the tea varieties on the porcine pancreatic α-amylase

Our first approach to the possible effects of the five tea extracts on the biological components involved in starch digestion was to measure the activity of the pancreatic α-amylase. The enzyme used was the commercially available porcine pancreatic α-amylase. Fig. 1A shows the results that were obtained when the reaction was initiated by the addition of the substrate (starch at a final concentration 1 g/100 mL) after pre-incubation of the enzyme with the various extracts. Data were expressed as relative rates, the control reaction rate being defined as unity. In this assay three extracts were clearly stimulatory at low concentrations, namely green tea, white tea and oolong tea. Inhibition, however, took place at higher concentrations, though it was not very pronounced for concentrations up to 10 mg/mL in the case of green, white and oolong teas. Black tea and purple tea, on the other hand, were inhibitory over the whole concentration range that was employed, purple tea being by far the most effective one with more than 90% inhibition at the concentration of 4 mg/mL. The IC_{50} value for purple tea was equal to 1.74 mg/mL and that of black tea 4.29 mg/mL. For all other teas the IC_{50} can be expected to be above 10 mg/mL.

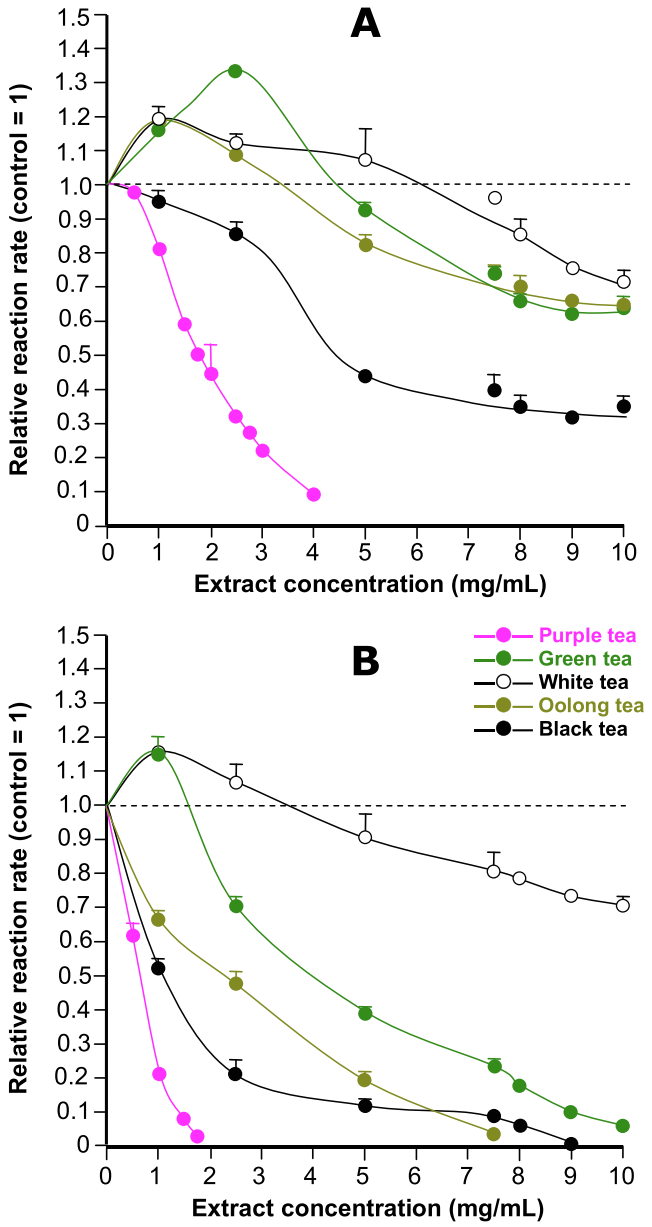

Fig. 1. Dependences of the activity of the porcine pancreatic α-amylase on the concentration of the various tea extracts. The reaction rates in the presence of the various extracts were represented as fractions of the rate measured in the absence of the extracts (control = 1). Each datum point is the mean of three determinations. Standard errors of the mean cannot be seen when smaller than the symbols. Panel A shows the reaction rates that were measured when the reaction was started by adding the enzyme after pre-incubation of the substrate with the extract; panel B shows the reaction rates that were measured when the reaction was started by adding the substrate after a pre-incubation of the enzyme with the extract (see details in the Material and Methods section).

The results that were obtained using the second protocol, i.e., when the reaction was started by addition of the substrate after pre-incubation of the extracts with the enzyme, are shown in Fig. 1B. Under this protocol, stimulation at low concentrations was absent in the case of oolong tea and considerably reduced and restricted to the lowest concentration ranges in the case of green and white teas. The inhibition degree, on the other hand, was substantially increased in all cases, as revealed by visual inspection and, with more precision, by the corresponding IC_{50} values which were: 0.63 mg/mL for purple tea, 1.09 mg/mL for black teat, 2.24 mg/mL for oolong tea and 3.97 mg/mL for green tea. Solely for white tea the IC_{50} value remained above 10 mg/mL.

The inhibition revealed by Fig. 1 can be caused by low molecular weight compounds (e.g., phenolic compounds), but macromolecules could equally be involved. Putative candidates are polysaccharides, which are present in *Camellia sinensis* preparations (Scoparo et al., 2016) and that have been demonstrated to be able to inhibit digestive enzymes (Deng et al., 2015). To get an insight into this possibility the solubilized extracts used in the experiments shown in Fig. 1 were treated with ethanol, which precipitates macromolecules, especially polysaccharides. The precipitates were solubilized in the same water volume as the original extract solution and used in α-amylase assays of the kind shown in Fig. 1. Fig. 2 allows to compare the activities of the pancreatic α-amylase in the presence of the total aqueous extracts (AE) of the various *C. sinensis* varieties with the activities in the presence of the corresponding re-solubilized ethanol precipitates (EP). For each pair of columns (AE and EP) the extract concentrations were those that produced the maximally observed inhibition in the experiments shown in Fig. 1. Panel A shows the reaction rates that were measured when the reaction was started by adding the enzyme after pre-incubation of the substrate with the aqueous extract or re-solubilized ethanol precipitate. Using this procedure, no inhibition was found for the re-solubilized precipitate. Instead, a tendency toward stimulation can be identified for purple tea. Panel B shows the reaction rates that were measured when the reaction was started by adding the substrate after a pre-incubation of the enzyme with the aqueous extract or re-solubilized ethanol precipitate. In this case, purple and oolong tea presented small inhibitions when compare to the great inhibitory activity of the total aqueous extract. The black tea re-solubilized ethanol precipitate, on the other hand, inhibited the α-amylase activity by 29%, comprising, thus, a significant fraction of the inhibition caused by the total aqueous extract.

3.3. In silico studies

The tea extracts contain many compounds (Table 1) and their isolation for testing the inhibitory activity on the α-amylases can be quite laborious. Computer simulations may give a hint about the compounds (or compound) that are more likely to act as inhibitors of the α-amylases.

The list in Table 1 presents some ambiguities that could not be resolved so that possible variations that were available in PubChem

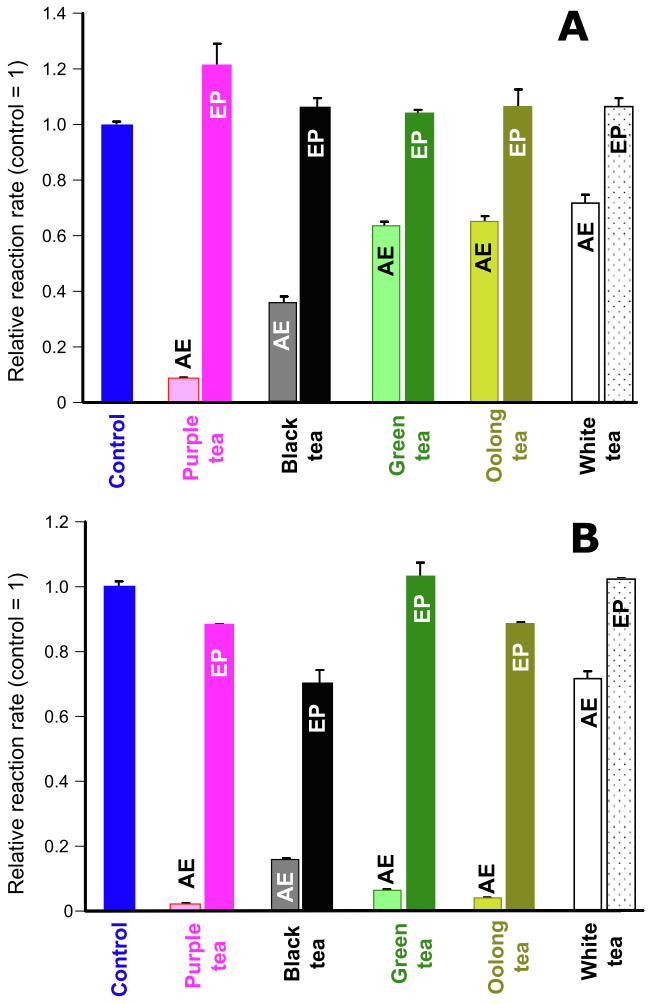

Fig. 2. Comparison of the activities of the pancreatic α-amylase in the presence of the total aqueous extracts (AE) of the various *C. sinensis* varieties with the activities in the presence of the corresponding re-solubilized ethanol precipitates (EP). For each pair of columns (AE and EP) the extract concentrations were those that produced the maximally observed inhibition in the experiments shown in Fig. 1. Panel A shows the reaction rates that were measured when the reaction was started by adding the enzyme after pre-incubation of the substrate with the aqueous extract or re-solubilized ethanol precipitate; panel B shows the reaction rates that were measured when the reaction was started by adding the substrate after a pre-incubation of the enzyme with aqueous extract or re-solubilized ethanol precipitate (see details in the Material and Methods section).

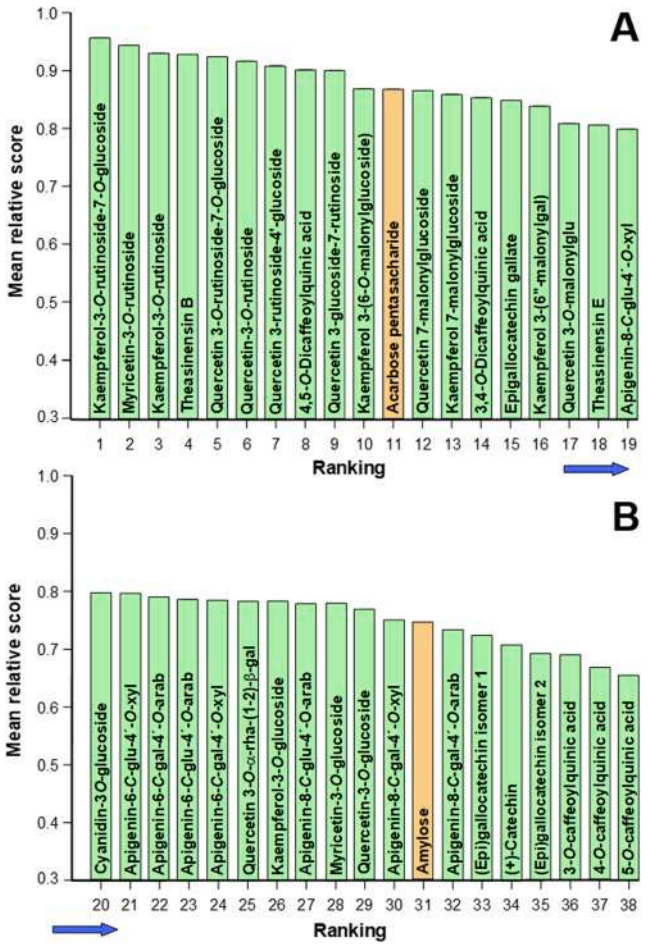

Fig. 3. The compounds quantified in the *Camellia sinensis* varieties displayed according to their decreasing mean relative scores, as defined by equation (3). Abbreviations: glu, glucoside; xyl, xyloside; gal, galactoside or galactopyranoside specifically for the compound ranked 25; arab, arabinoside; rham, rhamnopyranosyl.

were considered. On doing so, the library with the evaluated compounds ended up by containing 36 molecules plus the acarbose pentasaccharide inhibitor and the amylose pentasaccharide substrate. The list with the names and respective PubChem identification codes (CID) for the compounds used in the simulations is shown in Table 6S. The average scores obtained with four simulations for each docking program are shown in Table 7S. The graph of Fig. 3 shows in pictorial form the mean relative scores, calculated according to equation (3), in decreasing order.

The compound with the highest binding probability according to the mean relative scores shown in Fig. 3 is kaempferol-*O*-rutinoside-hexoside, (represented by the molecule kaempferol-3-*O*-rutinoside 7-*O*-glucoside, CID 21676298). The distance in terms of the mean relative score between this molecule and that one ranked in second place, namely myericetin-3-*O*-rutinosde, is more pronounced than the differences between the molecules ranked between 2 and 9. Notable, however, is the result that among the ten best ranked molecules, 7 of them are rutinosides. Fig. 3 also reveals that the catechins, often considered important bioactives of *Camellia sinensis*, especially green tea, do not appear in the first positions. The best ranked is epigallocatechin gallate (15), whereas (+)-catechin itself ranks only 33. Fig. 3 also reveals that cyanidin-3-*O*-glucoside, the main responsible for the characteristic colour of the purple tea leaves appears in position 20.

3.4. Effects of purple tea and other varieties on starch digestion

It is important to find out if the effects of tea extracts on the α-amylases have physiological relevance, i.e., if they also occur *in vivo*. Since starch must be hydrolyzed within the intestinal lumen before glucose absorption occurs, the data in Fig. 1 suggest that the purple tea should be the best inhibitor of the phenomenon. This question was approached in the present work by means of the starch tolerance test. Experiments were done with all the five tea extracts. The extracts were given intragastrically to mice at doses of 500 mg/mL. The extract administration was followed by intragastric starch administration and the plasma glucose concentration was followed at intervals from zero time to 120 min. All results of this experimental series are summarized in Fig. 4.

As observed from previous reports (Kato et al., 2017; da Silva et al., 2014) the administration of starch resulted in pronounced transiently elevated plasma glucose concentrations. Administration of water produced a small increment in glucose concentration, generally attributable to the mild stress to which the mice are undergoing in consequence of the experimental manipulations. Previous administration of the various

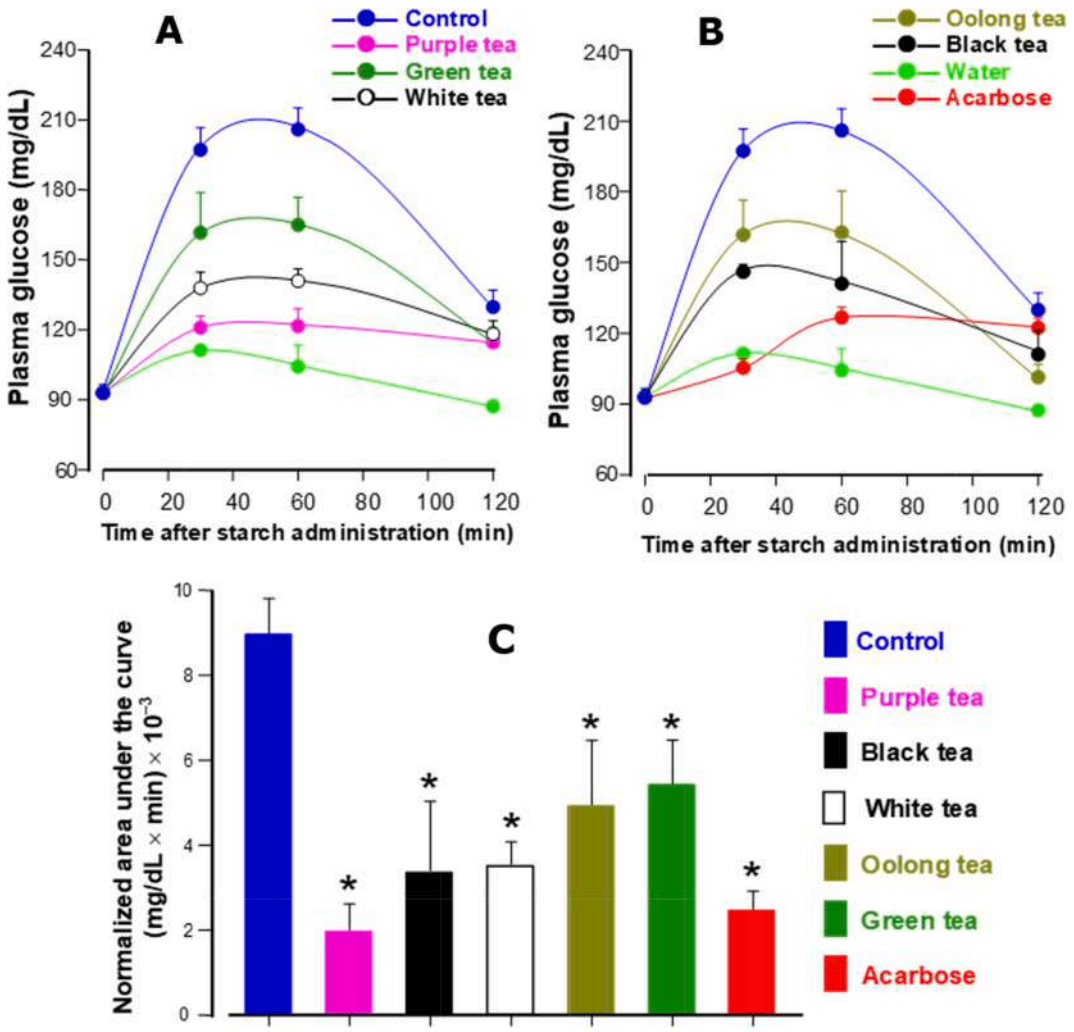

Fig. 4. Starch tolerance tests in mice and the influence of various tea extracts. Full details about the starch tolerance tests are given in the Section 2. Each series of data points represents the mean of 3 experiments (i.e., 3 different mice). Values are means ± standard errors. Panels A and B represent the time courses of the plasma glucose concentrations under the specified conditions. Panel C represents the areas between each of the various time response curves and the curve obtained when water was given to the mice. For each tea extract the administered dose was equal to 500 mg/kg. The acarbose dose was 50 mg/kg. Asterisks in panel C (*) represent statistical significance at the 5% level relative to the control condition according to the Student-Newman-Keuls test.

tea extracts modified the responses of the mice in all cases, with a kinetics that was somewhat different to that one observed when acarbose, the classical inhibitor of starch digestion, was given. Even visual inspection of the various curves in panels A and B of Fig. 4 allows to conclude that the actions of the various teas were different and that the purple tea extract was likely to be the most effective in diminishing glycemia in consequence of starch digestion. A more precise evaluation of the effects of the various teas is given by panel C of Fig. 4 in which the areas between the various test curves and the curve obtained when water was given alone to the mice were represented. These areas are generally regarded as a quantitative measure of the delay in starch digestion caused by specific agents including acarbose. Fig. 4C confirms that all tea extracts, at the doses that were administered in each case (500 mg/kg), caused inhibition of starch digestion, but that purple tea was the most effective one, with 80% inhibition. The least inhibitory was green tea, with a 40% diminution.

Starch tolerance tests were also done in mice that received different doses of the purple tea extract in an attempt at establishing a dose–effect relationship. The results are shown in Fig. 5. The peak of the curve obtained with the 50 mg/kg dose of the purple tea was under the control peak (Fig. 5A), but the normalized area (Fig. 5B) was not significantly different from the control area under the statistical criteria adopted in this work. The effect reached statistical significance, however, with the 100 mg/kg dose and the 250 mg/kg dose already producing an effect that was no longer statistically different from that one observed with the 500 mg/kg dose.

Experiments were also done with different doses of the green tea extract in an attempt at detecting a possible stimulatory effect on starch digestion, which is suggested by the stimulatory effect of this tea variety on the α-amylase activity (see Fig. 1). The results of this experimental series are shown in Fig. 6. Low doses of up to 100 mg/kg were given because stimulation is expected to occur at low concentrations. Panel A in Fig. 6 reveals that the dose of 50 mg/kg was ineffective in modifying starch digestion, but the 100 mg/kg dose was clearly inhibitory. Stimulation, on the other hand, did not occur when the doses were reduced to 25 and 5 mg/kg, as revealed by panel B of Fig. 6. Actually, the doses from 5 to 50 mg/kg did not affect starch digestion at all, as clearly indicated by the areas under the curves that are shown in Fig. 6C. Singularly, the transition between absence of inhibition and maximal or nearly maximal inhibition took place within the interval between 50 and 100 mg/kg because the latter dose caused an inhibition that was very similar to that one previously found with the 500 mg/kg dose.

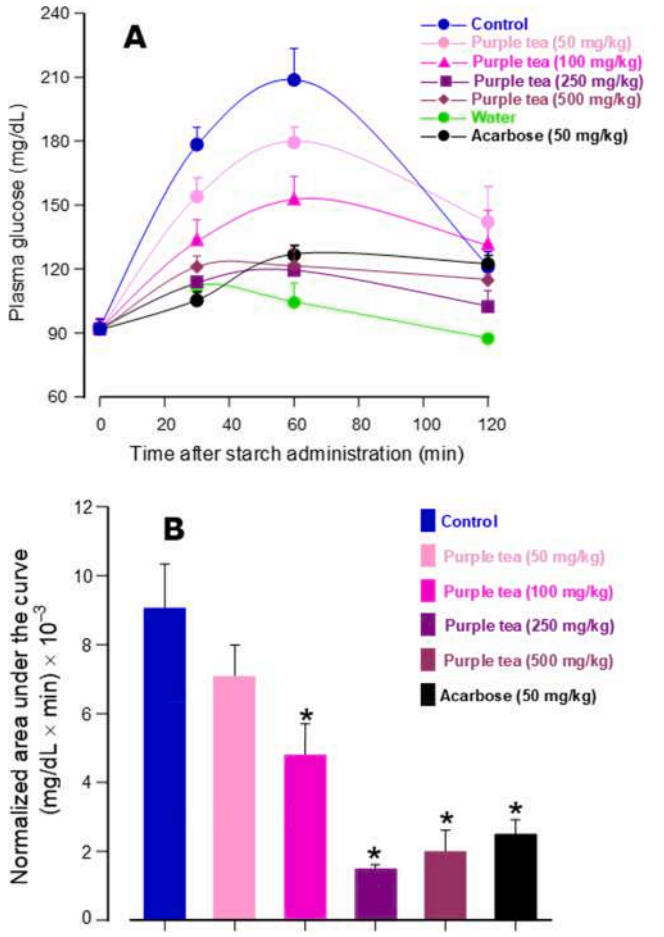

Fig. 5. Starch tolerance tests in mice and the influence of various purple tea extract doses. Full details about the starch tolerance tests are given in the Section 2. Each series of data points represents the mean of 3 experiments (i.e., 3 different mice). Values are means ± standard errors. Panel A represents the time courses of the plasma glucose concentrations under the specified conditions. Panel B represents the areas between each of the various time response curves and the curve obtained when water was given to the mice. Asterisks in panel B (*) represent statistical significance at the 5% level relative to the control condition according to the Student-Newman-Keuls test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. *Effects of purple tea and other varieties on maltose and glucose tolerance*

Diminution of the hyperglycemic burst in consequence of a maltose load can be expected if hydrolysis of this disaccharide in the intestine is strongly inhibited (Adisakwattana et al., 2011). In order to investigate this possibility maltose tolerance tests were performed using the same protocol employed in the starch tolerance tests. Since this work is focused mainly on the effects of purple tea, the dose of 250 mg/kg was chosen because it had already maximal effect on starch digestion. The results are shown in Fig. 7. From the time courses (Fig. 7A and B) it is apparent that the effects of the various tea extracts are minimal, excepting perhaps the white tea extract. The differential areas in Fig. 7C confirm this conclusion. There are very weak tendencies toward stimulation, especially in the case of oolong tea, but the statistical dispersion prohibits any definitive conclusion. Much stronger is the tendency of a delay in maltose digestion caused by the white tea extract. However, also in this case, statistical significance at the 5% level is lacking.

The final experiments that were done in this work were glucose tolerance tests. Glucose absorption depends of an active transport system (Thorsen, Drengstig & Ruoff, 2014), which could eventually be inhibited (or activated) by one or more of the extracts used in the present work. The results of the pertinent experiments are shown in Fig. 8. Here again the doses were 250 mg/kg. The curves obtained with the purple tea extract, shown in panel A, seem to run very close to the control curve. The same can be said about the curves obtained with the white tea extract (panel A) and oolong tea extract (panel B). The curves obtained after administrating both black and green extracts, however, showed a clear tendency of running well above the control curve, especially in their final portions. This was confirmed when the differential areas were calculated and represented in graphical form in panel C of Fig. 8. The black tea extract clearly diminished glucose tolerance (i.e., the hyperglycemic burst was prolonged) in comparison with the control. The green tea extract presented the same tendency, without statistical significance however.

4. Discussion

4.1. *General aspects of the action of the Camellia sinensis extracts on starch digestion*

The main question to be responded by this study was if aqueous extracts of different *Camellia sinensis* varieties, prepared under identical conditions, can eventually act as similar or different inhibitors of the pancreatic α-amylase and of starch digestion in mice. In this respect the data revealed that purple tea was undoubtedly the best inhibitor of both pancreatic α-amylase and starch digestion. Black tea was the next best inhibitor of both α-amylase and starch digestion. However, with respect to the latter phenomenon no strict correlation could be established between the inhibition of the α-amylase and starch digestion. White tea, for example, was the by far least effective inhibitor of the α-amylase, but its capacity in inhibiting starch digestion was similar to that of the black tea. With respect to green tea, which ranked among the least effective inhibitors of starch digestion, the results are particularly surprising for two main reasons: (1) firstly because green tea has been proposed by several investigators as an effective inhibitor of starch digestion based mainly on results obtained with isolated enzymes (Gao et al., 2013; Miao et al., 2015; Nyambe-Silavwe et al., 2015), but also on at least one *in vivo* study (Lochocka et al., 2015); (2) the green tea extract used in the present work was the preparation that presented the highest content in total phenolic compounds, actually nearly twice as much as the purple tea extract. Several phenolic compounds, including some that are abundant in green tea, have been reported to act as α-amylase or α-glucosidase inhibitors (D'Costa & Bordenave, 2021; Desseaux, Stocker, Brouant & Ajandouz, 2018), but in the present work green tea was also a relatively weak inhibitor of the α-amylase under our assay conditions. The role of α-amylase inhibition seems to be predominant for the *in vivo* effects observed in the present study. This is based on the observation that maltose tolerance was not affected by any of the various extracts, except perhaps for a non-significant tendency that was observed with white tea. A possible inhibition of α-glucosidase *in vivo* should have affected maltose tolerance. The results of the glucose tolerance tests, on the other hand, may be taken as an indication that the various tea extracts are not able to inhibit glucose absorption by the intestine, which is an energy-dependent process (Thorsen et al., 2014). Remarkably, however, black tea diminished glucose tolerance in a significant way and green tea presented the same tendency. This kind of effect is difficult to interpret because there are several possibilities, all of them contributing to the modifications in the starch tolerance test, which would no longer be reflecting solely the hydrolysis inhibition. Stimulation of glucose absorption is one of these possibilities. Another one is inhibition of glucose transformation. There are no data available, however, to distinguish between these or other possibilities.

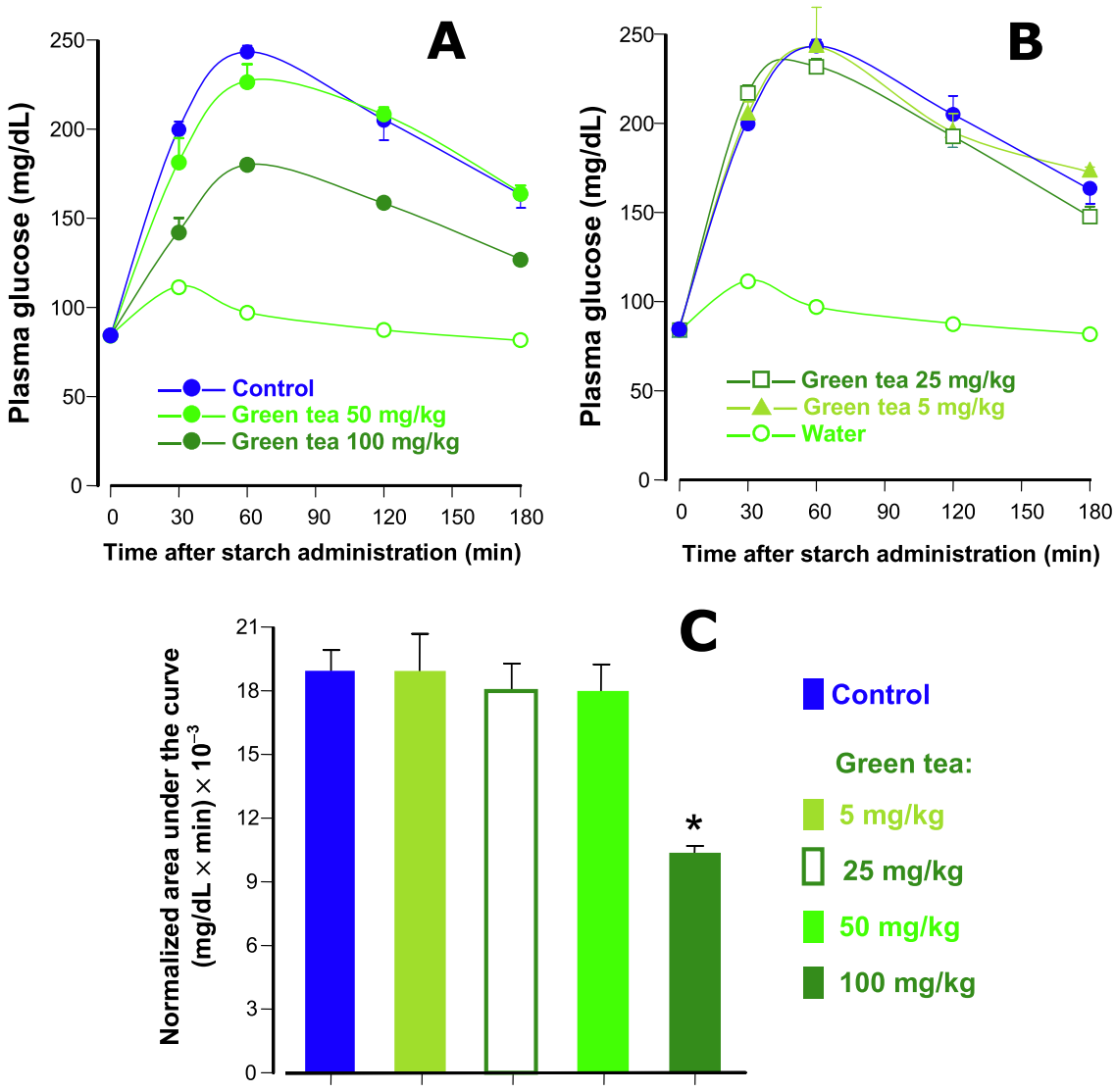

Fig. 6. Starch tolerance tests in mice and the influence of various green tea extract doses. Full details about the starch tolerance tests are given in the Section 2. Each series of data points represents the mean of 3 experiments (i.e., 3 different mice). Values are means ± standard errors. Panels A and B represent the time courses of the plasma glucose concentrations under the specified conditions. Panel C represents the areas between each of the various time response curves and the curve obtained when water was given to the mice. Asterisks in panel C (*) represent statistical significance at the 5% level relative to the control condition according to the Student-Newman-Keuls test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. *The role of α-amylase*

In favor of the widespread hypothesis that the pancreatic α-amylase plays an important role in starch digestion and that its inhibition may also lead to a diminution of the phenomenon is the observation that the purple tea extract was by far the most potent inhibitor of the enzyme. Black tea was the second most effective inhibitor, an observation that also agrees with the action of this tea variety on starch digestion. The other types of tea, however, were actually stimulators of the pancreatic α-amylase at low concentrations, the inhibitory activity being effective only at quite high concentrations. Stimulation at low concentrations is not so surprising as it seems, because the phenomenon has already been reported before (Yang & Kong, 2016). Actually, as mentioned in the Introduction, data on the *in vitro* α-amylase and α-glucosidase inhibition by *Camellia sinensis* extracts present extreme variations from report to report so that translation to the *in vivo* conditions becomes extremely uncertain. An at least partly positive correlation between the *in vitro* and *in vivo* inhibitory actions, as it was the case in the present study with the purple tea and black tea extracts, seems not to be very frequent. This may be caused by the fact that it is very difficult to reproduce in the test tube the conditions that predominate in the intestine once both starch and the various chemical constituents of a given extract get in contact. In this respect it looks important to list the various factors that may be different in the *in vitro* systems when compared to the *in vivo* situation. In most cases the porcine pancreatic α-amylase is used, but the animal used for experimentation is frequently a rodent and enzymes from different species may react differently to the inhibitors. This is a reasonable supposition, but there are yet no experimental data corroborating it. Furthermore, the initial reaction rates are generally measured *in vitro* under standardized conditions without pre-incubation. Pre-incubation with the enzyme, however, clearly enhances the inhibitory effects, as it has been demonstrated in the present study, corroborating previous observations with tea extracts and their pure constituents (D'Costa & Bordenave, 2021; Yang & Kong, 2016). In this respect it is worth to recall that the starch tolerance test takes at least two hours to be completed, so that there would be enough time for an enhancement of the inhibitory effects. Furthermore, we were not able to demonstrate stimulation of starch digestion, a fact that may be revealing that net

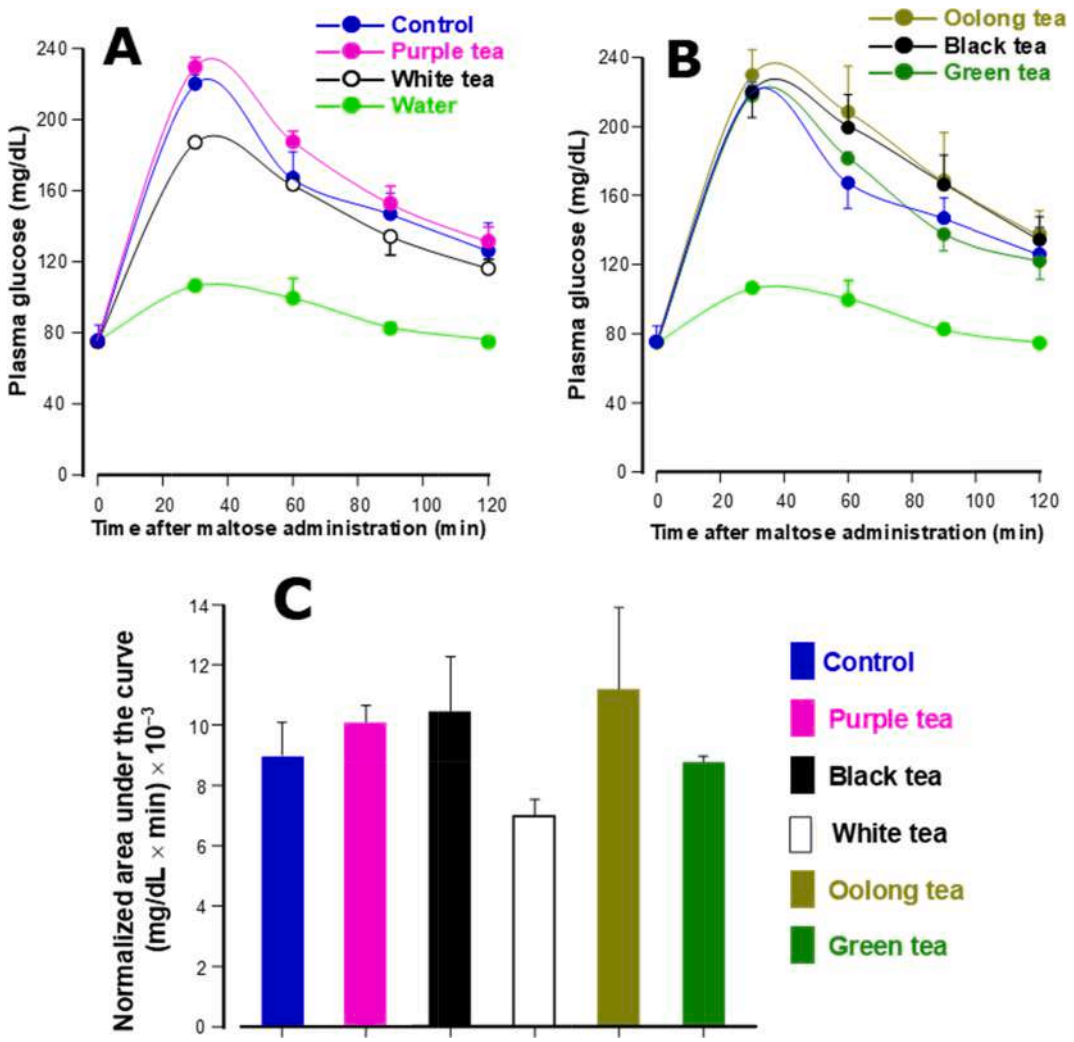

Fig. 7. Maltose tolerance tests in mice and the influence of various tea extracts. Full details about the maltose tolerance tests are given in the Section 2. Each series of data points represents the mean of 3 experiments (i.e., 3 different mice). Values are means ± standard errors. Panels A and B represent the time courses of the plasma glucose concentrations under the specified conditions. Panel C represents the areas between each of the time response curves and the curve obtained when water was given to the mice. For each tea extract the administered dose was equal to 250 mg/kg.

inhibition of α-amylase is predominating *in vivo*. Another point is that the local concentrations of the inhibitors and the local concentrations of enzyme and substrate cannot be predicted, as the intestinal lumen is not homogeneous. This may also lead to different degrees of inhibition, especially if competition for the active site is significant. Transformation of the active principles may also occur in the intestinal lumen by virtue of the action of microorganisms (Correa et al., 2017), a phenomenon that can, in theory at least, generate more potent inhibitors than those originally present in the extract.

4.3. On the significance of the computational simulations

Implicit in the docking simulations was the idea that phenolic compounds are certainly involved because they are known inhibitors of α-amylases at relatively low concentrations (10–50 μM; Zhang et al., 2017; D'Costa & Bordenave, 2021). Listed in Table 1 are those polyphenolic compounds that were effectively detected in the aqueous extracts used in the present study. Exhaustive hydroalcoholic extraction revealed an increased richness in compounds, at least for black and green tea, and probably also higher contents (Scoparo et al., 2012). However, the purpose of the present work was to investigate the action of extracts more closely resembling the daily brewed beverages. The computational investigation simulated a competitive inhibitory effect, where the phenolic compounds were docked to the active site of the enzyme. Although the results obtained with the docking simulations may suggest the compounds with the highest probability of binding to the α-amylase active site, such compounds presented different ways of fitting (poses) with very close scores. This is due to the large number of rotatable bonds present in these molecules, what increases the degree of freedom of movement and generates different spatial conformations for the same molecule. Thus, the number of conformational species for the same molecule increases exponentially, what translates into interactions of different conformations of the same molecule with the active site of the enzyme. It should be remarked that since the simulations refer to binding to the active site, they necessarily refer solely to the phenomenon of inhibition. Stimulation could eventually be brought about by binding to other sites of the enzyme, a possibility that was not considered in the simulations.

4.4. The molecular components involved in α-amylase inhibition

An important question that arises whenever the effects of complex extracts are investigated is one about the compounds that are responsible for the detected effects. This is a question that is actually connected to the topics that were discussed in the preceding sub-sections as it equally bears relation to the inhibitory action on the digestive enzymes.

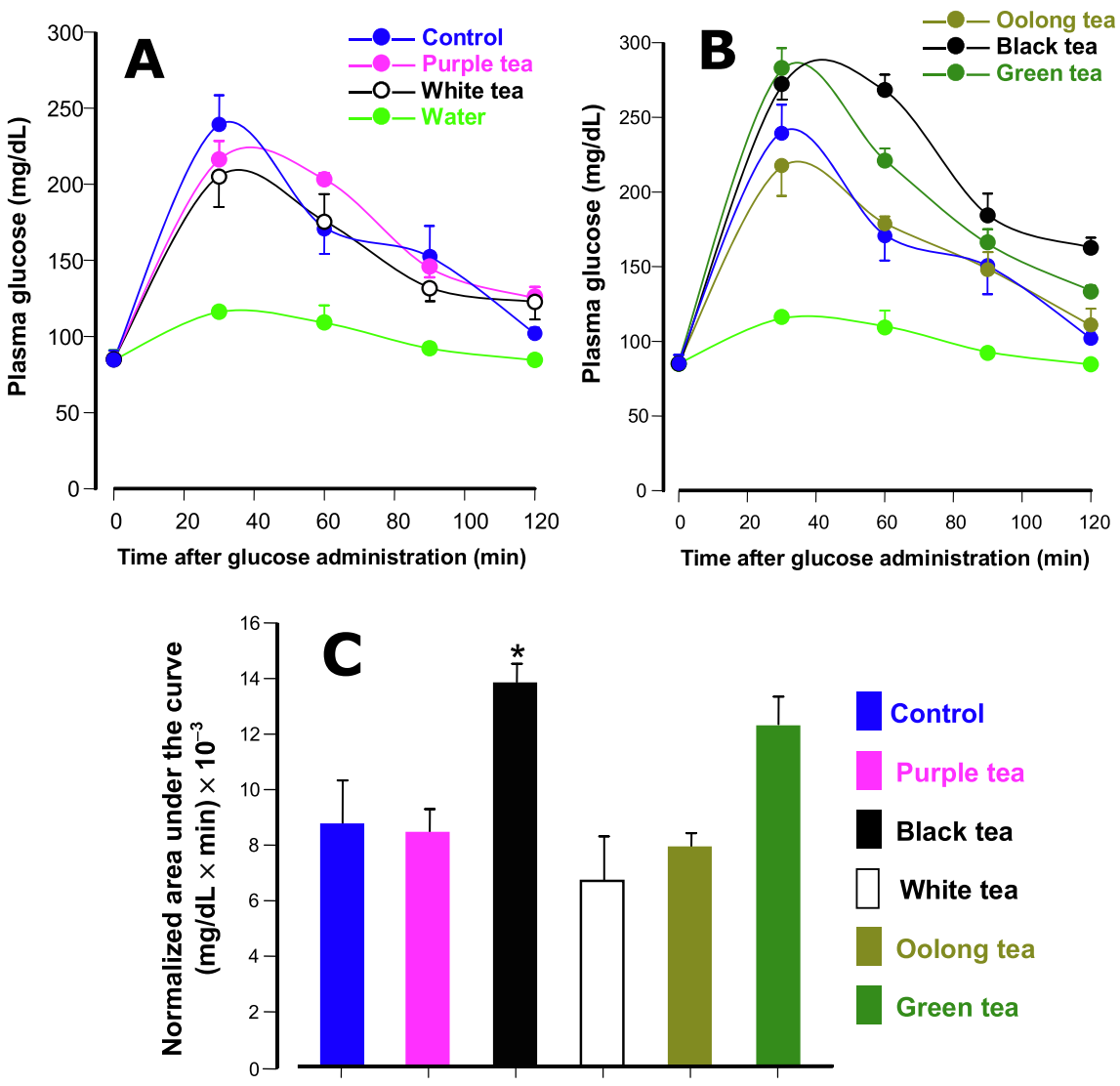

Fig. 8. Glucose tolerance tests in mice and the influence of various tea extracts. Full details about the glucose tolerance tests are given in the Section 2. Each series of data points represents the mean of 3 experiments (i.e., 3 different mice). Values are means ± standard errors. Panels A and B represent the time courses of the plasma glucose concentrations under the specified conditions. Panel C represents the areas between each of the various time response curves and the curve obtained when water was given to the mice. For each tea extract the administered dose was equal to 250 mg/kg. The asterisk in panel C (*) represents statistical significance at the 5% level relative to the control condition according to the Student-Newman-Keuls test.

The five extracts that were used at different concentrations certainly contain many different compounds and it is very difficult to define the exact compounds or group of compounds that are the main responsible for the effects. To this fact one has to add the observation that several tea extracts can also stimulate the α-amylase, so that the effect on starch absorption that was observed *in vivo* could be the resultant of two antagonistic effects. There are no data available about the compounds that cause stimulation. But purple tea, besides being the strongest inhibitor of both α-amylase and starch digestion, did not stimulate the enzyme and it should offer less difficulties to the attempt at identifying the main inhibitory molecular species based on the evidence produced in the present study. Purple tea is by far the richest one in anthocyanins (Kerio et al., 2012). Anthocyanins are in fact inhibitors of α-amylases or α-glucosidase, but their potency *in vitro* and *in vivo* is rather limited (Castilho et al., 2021; Wiese, Gärtner, Rawel, Winterhalter & Kulling, 2009) and the purple tea extract content in these compounds is relatively small in absolute terms. Corroborating this, in the docking simulations, cyanidin-3-*O*-glucoside was classified as a relatively weak ligand. For all these reasons it is not very likely that the anthocyanins in the purple tea extract are the main inhibitors of α-amylase unless phenomena like synergisms occur. On the other hand, epigallocatechin gallate is by far the most abundant polyphenolic in purple tea as well as in all other varieties investigated in the present work and it is well known that the compound inhibits α-amylase (Desseaux et al., 2018). It is not the strongest inhibitor according to our docking simulations as it ranks 15, five places after the classical inhibitor acarbose. However, due to its abundance it is likely that it contributes significantly to the inhibitory activity of purple tea. Furthermore, purple tea contains several of the most effective inhibitors of α-amylase according to our simulations which are the deoxyhexoside-hexoside-containing polyphenolics, especially the kaempferol and myricetin derivatives. Polysaccharides isolated from pu-erh tea, on the other hand, have also been demonstrated to inhibit the α-amylase (Deng et al., 2015). The total polysaccharide content of the extract used in the present study is not very pronounced (23 mg/g). However, it is apparent that polysaccharides and eventually other macromolecules that can be precipitated by ethanol, contribute to the total inhibitory activity on the α-amylase, even though the latter seems to be limited to 10% of the total inhibition. All these characteristics of the purple tea extract, combined with the absence of a significant stimulatory effect, are certainly

contributing to the relatively strong inhibitory activity of this tea variety on both the α-amylase and starch digestion.

A good contrast to purple tea is green tea, which was a weaker inhibitor of both α-amylase and starch digestion in spite of its high content in epigallocatechin gallate. The inhibitory action of green tea seems to be strongly influenced by the opposing stimulatory effect which was present even when the second assay protocol (30 min of pre-incubation with the enzyme and reaction initiation by the addition of starch) was employed. The fact that the green tea extract contains more epigallocatechin gallate than the purple tea extract by a factor of 2.59 and that it is at the same time a weaker net inhibitor does not disprove the participation of this compound as an important inhibitor. It is much more likely that it represents a case in which the inhibitory activity is effectively counterbalanced by the stimulatory effect so that the net resulting effect is a diminished inhibitory activity.

Black tea seems to occupy an intermediate position between purple and green tea in terms of its inhibitory activity. It should be recalled that the black tea extract has the lowest content in phenolic compounds and that its content in epigallocatechin gallate was only 73% that of purple tea. It is perhaps significant, however, that no net stimulation of the α-amylase by black tea was found. Furthermore, it is the only tea variety that contains theasinensin B (Tao et al., 2020), the compound that ranked in position 4 among the most likely to be bound by the α-amylase (Fig. 3). Black tea, finally, is the tea type for which polysaccharides and eventually other macromolecules that can be precipitated by ethanol gave the most significant contribution to the α-amylase inhibition (29%). Remarkably, this occurs in spite of the fact that the content in polysaccharides of the extract used in the present work is relatively low when compared to white tea, for example. This suggests the existence of a highly specific structure in black tea, whose identification, however, needs a specific experimental design as it cannot be done with the data already available in the literature (Deng et al., 2015; Scoparo et al., 2016).

The behavior of oolong and white tea is more difficult to explain in terms of their molecular constituents. This is especially true for white tea which was the weakest inhibitor of the α-amylase, but not necessarily the weakest inhibitor of starch digestion. Remarkably, the extracts of these two types of tea presented the highest contents in polysaccharides, but inhibition of the α-amylase by these constituents was absent. Especially the behavior of the white tea extract within the general context of this work is a clear indication that our present knowledge about the molecular and mechanistic details of the effects of plant extracts on starch digestion is still largely incomplete. There can be many factors involved that escape to our present understanding as already mentioned above in the first part of the Discussion.

5. Conclusion

To our knowledge this study is the first attempt at comparing the effects of several *Camellia sinensis* varieties, extracted in the same way, on starch digestion *in vivo* and under identical conditions. Furthermore, the preparation procedure of the extracts was very close to that one used when teas are brewed for human consumption. The actions of the various tea extracts on glucose, maltose, and especially starch tolerance certainly present many complexities. Although the extracts of all tea varieties were able to inhibit starch digestion to a certain extent it is clear that the purple tea extract was the most effective inhibitor. For this variety epigallocatechin gallate can be devised as the main compound involved. The contributions of kaempferol and myricetin derivatives to the inhibition are also likely to be significant. For black tea epigallocatechin gallate seems also to be most active component. For the other varieties the identification of the compounds that are involved is obscured by the superposition of inhibitory and stimulatory effects. The bulk of the results obtained in the present study allow to formulate the hypothesis that in terms of a beneficial postprandial anti-hyperglycemic action in both healthy or diabetic individuals, the action of purple tea presents the best perspectives among all the varieties tested in the present study. Additional experimental work is required in order to clarify the exact role of the various molecular species that are involved in the inhibition of starch digestion.

CRediT authorship contribution statement

Tamires Barlati Vieira da Silva: Conceptualization, Methodology. **Pâmela Alves Castilho:** Conceptualization, Methodology. **Anacharis Babeto de Sá-Nakanishi:** Data curation, Writing – original draft. **Flávio Augusto Vicente Seixas:** Data curation, Writing – original draft, Software. **Maria Inês Dias:** Conceptualization, Methodology. **Lillian Barros:** Visualization, Investigation, Writing – review & editing. **Isabel C.F. R. Ferreira:** Visualization, Investigation, Writing – review & editing. **Adelar Bracht:** Supervision, Validation, Writing – review & editing. **Rosane Marina Peralta:** Validation, Writing – review & editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110781.

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SUPPLEMENTARY MATERIAL

The inhibitory action of purple tea on *in vivo* starch digestion compared to other *Camellia sinensis* teas

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Table 1S. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the green tea extract.

Peak	Rt (min)	$\lambda_{max\ ((nm))}$	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg/g extract)
1	4.79	274	305	219(75),179(100),125(11)	(Epi)gallocatechin isomer I[A]	0.72±0.03
2	5.29	271	305	219(68),179(100),125(25)	(Epi)gallocatechin isomer II[A]	2.52±0.02
3	5.81	275	305	219(71),179(100),125(23)	(Epi)gallocatechin isomer III[A]	2.11±0.08
4	8.97	281/311	289	245(34), 203(16), 137(27)	(+)-Catechin[A]	4.8±0.2
5	10.01	274	457	331(79),305(73),169(100)	Epigallocatechin gallate[A]	30.2±0.4
6	13.11	335	563	545(30), 473(100), 443(79),383(32), 353(30), 311(2), 297(4)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.34±0.02
7	13.44	336	563	545(17), 473(71), 443(100),383(19), 353(22), 311(3), 297(5)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.38±0.01
8	14.19	344	625	317(100)	Myricetin-3-*O*-rutinoside[C]	0.35±0.01
9	14.61	352	479	317(100)	Myricetin-3-*O*-glucoside[C]	0.43±0.01
10	14.81	352	479	317(100)	Myricetin-*O*-hexoside[C]	0.59±0.01
11	15.31	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[D]	0.457±0.008
12	15.88	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[D]	0.594±0.007
13	17.16	349	755	285(100)	Kaempferol-*O*-deoxyhexosyl-dihexoside[D]	0.46±0.01
14	17.96	347	463	301(100)	Quercetin-3-*O*-glucoside[D]	0.25±0.02
15	18.51	348	755	285(100)	Kaempferol-*O*-deoxyhexosyl-dihexoside[D]	0.359±0.007
16	20.51	346	593	285(100)	Kaempferol-3-*O*-rutinoside[D]	0.154±0.001
17	21.74	344	447	285(100)	Kaempferol-3-*O*-glucoside[D]	0.084±0.001
					Total Phenolic Compounds	44.76±0.06
					Total Flavan3-ols	40.31±0.02
					Total Other Flavonoids	4.46±0.08

Standard calibration curves: A - catechin ($y = 84950x - 23200$, $R^2 = 1$; LOD = 0.17 µg/mL; LOQ = 0.68 µg/mL); B - apigenin-6-*C*-glucoside ($y = 107025x + 61531$, $R^2 = 0.9989$; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); C - myricetin-3-O-glucoside ($y = 23287x - 581708$, $R^2 = 0.9988$; LOD = 0.23 µg/mL; LOQ = 0.78 µg/mL); D- quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL). tr- traces (compounds below LOD amounts).

Table 2S. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the purple tea extract.

Peak	Rt (min)	$\lambda_{max\ ((nm))}$	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg/g extract)
1	4.71	274	305	219(75),179(100),125(11)	(Epi)gallocatechin isomer I[A]	5.67±0.07
2	8.7	281/311	289	245(34), 203(16), 137(27)	(+)-Catechin[A]	1.76±0.01
3	9.85	274	457	331(79),305(73),169(100)	Epigallocatechin gallate[A]	11.6±0.1
4	13.23	335	563	545(30), 473(100), 443(79),383(32), 353(30), 311(2), 297(4)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.046±0.001
5	13.96	336	563	545(17), 473(71), 443(100),383(19), 353(22), 311(3), 297(5)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.028±0.001
6	14.19	344	625	317(100)	Myricetin-3-*O*-rutinoside[C]	0.674±0.001
7	14.61	352	479	317(100)	Myricetin-3-*O*-glucoside[C]	0.742±0.001
8	14.81	352	479	317(100)	Myricetin-*O*-hexoside[C]	0.676±0.002
9	15.69	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[D]	0.138±0.001
10	17.00	353	609	301(100)	Quercetin-3-*O*-rutinoside[D]	0.319±0.001
11	17.71	353	609	301(100)	Quercetin-*O*-deoxyhexosyl-hexoside[D]	0.167±0.001
12	18.09	347	463	301(100)	Quercetin-3-*O*-glucoside[D]	0.252±0.001
13	19.62	342	549	505(25),301(100)	Quercetin-*O*-malonyl-hexoside[D]	0.196±0.001
14	20.20	346	593	285(100)	Kaempferol-3-*O*-rutinoside[D]	0.274±0.001
15	21.46	344	447	285(100)	Kaempferol-3-*O*-glucoside[D]	0.218±0.002
16	23.93	346	533	489(32),285(100)	Kaempferol-*O*-malonyl-hexoside[D]	0.213±0.003
					Total Phenolic Compounds	23.0±0.2
					Total Flavan-3-ols	19.1±0.2
					Total Other Flavonoids	3.943±0.004

Standard calibration curves: A - catechin ($y = 84950x - 23200$, $R^2 = 1$; LOD = 0.17 µg/mL; LOQ = 0.68 µg/mL); B - apigenin-6-*C*-glucoside ($y = 107025x + 61531$, $R^2 = 0.9989$; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); C - myricetin-3-*O*-glucoside ($y = 23287x - 581708$, $R^2 = 0.9988$; LOD = 0.23 µg/mL; LOQ = 0.78 µg/mL); D- quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL). tr- traces (compounds below LOD amounts).

Table 3S. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the oolong tea extract.

Peak	Rt (min)	λ_{max} ((nm))	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg/g extract)
1	4.52	274	305	219(75),179(100),125(11)	(Epi)gallocatechin isomer I[A]	0.127±0.007
2	5.48	271	305	219(68),179(100),125(25)	(Epi)gallocatechin isomer II[A]	0.49±0.01
3	5.96	275	305	219(71),179(100),125(23)	(Epi)gallocatechin isomer III[A]	0.31±0.01
4	9.11	281/311	289	245(34), 203(16), 137(27)	(+)-Catechin[A]	0.704±0.006
5	10.2	274	457	331(79),305(73),169(100)	Epigallocatechin gallate[A]	6.48±0.05
6	13.19	335	563	545(30), 473(100), 443(79),383(32), 353(30), 311(2), 297(4)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.042±0.001
7	13.51	336	563	545(17), 473(71), 443(100),383(19), 353(22), 311(3), 297(5)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.061±0.001
8	14.26	344	625	317(100)	Myricetin-3-*O*-rutinoside[C]	0.279±0.001
9	14.92	352	479	317(100)	Myricetin-3-*O*-glucoside[C]	0.355±0.006
10	15.52	352	479	317(100)	Myricetin-*O*-hexoside[C]	0.302±0.004
11	15.95	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[D]	0.206±0.001
12	17.00	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[D]	0.067±0.001
13	17.27	349	755	285(100)	Kaempferol-*O*-deoxyhexosyl-dihexoside[D]	0.156±0.002
14	18.56	347	463	301(100)	Quercetin-3-*O*-glucoside[D]	0.127±0.002
15	20.51	348	755	285(100)	Kaempferol-*O*-deoxyhexosyl-dihexoside[D]	0.065±0.001
16	21.78	346	593	285(100)	Kaempferol-3-*O*-rutinoside[D]	0.058±0.001
					Total Phenolic Compounds	9.83±0.04
					Total Flavan3-ols	8.11±0.04
					Total Other Flavonoids	0.047±0.001

Standard calibration curves: A - catechin ($y = 84950x - 23200$, $R^2 = 1$; LOD = 0.17 µg/mL; LOQ = 0.68 µg/mL); B - apigenin-6-*C*-glucoside ($y = 107025x + 61531$, $R^2 = 0.9989$; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); C - myricetin-3-*O*-glucoside ($y = 23287x - 581708$, $R^2 = 0.9988$; LOD = 0.23 µg/mL; LOQ = 0.78 µg/mL); D- quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL). tr- traces (compounds below LOD amounts).

Table 4S. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the white tea extract.

Peak	Rt (min)	λ_{max} ((nm))	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg/g extract)
1	4.89	324	353	191(100),179(49),173(5),161(5),135(8)	3-*O*-Caffeoylquinic acid[A]	1.31±0.02
2	6.18	322	353	191(22),179(52),173(100),161(5),135(9)	4-*O*-Caffeoylquinic acid[A]	0.570±0.001
3	6.72	325	353	191(100),179(23),173(32),161(5),135(5)	5-*O*-Caffeoylquinic acid[A]	1.06±0.02
4	9.34	281/311	289	245(34), 203(16), 137(27)	(+)-Catechin[B]	0.082±0.002
5	10.15	274	457	331(79),305(73),169(100)	Epigallocatechin gallate[B]	6.24±0.08
6	13.20	335	563	545(30), 473(100), 443(79),383(32), 353(30), 311(2), 297(4)	Apigenin-*C*-hexoside-*O*-pentoside[C]	0.061±0.005
7	13.51	336	563	545(17), 473(71), 443(100),383(19), 353(22), 311(3), 297(5)	Apigenin-*C*-hexoside-*O*-pentoside[C]	0.074±0.006
8	14.26	344	625	317(100)	Myricetin-3-*O*-rutinoside[D]	0.257±0.001
9	14.97	352	479	317(100)	Myricetin-3-*O*-glucoside[D]	0.280±0.001
10	15.41	352	479	317(100)	Myricetin-*O*-hexoside[D]	0.327±0.001
11	15.95	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[E]	0.149±0.002
12	16.71	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[E]	0.070±0.001
13	17.28	349	755	285(100)	Kaempferol-*O*-deoxyhexosyl-dihexoside[E]	0.220±0.003
14	18.59	347	463	301(100)	Quercetin-3-*O*-glucoside[E]	0.129±0.002
15	19.92	327	515	353(20),191(100),179(42),173(12),161(5),135(15)	3,4-*O*-Dicaffeoylquinic acid[A]	0.155±0.003
16	20.50	348	755	285(100)	Kaempferol-*O*-deoxyhexosyl-dihexoside[E]	0.102±0.001
17	21.48	346	593	285(100)	Kaempferol-3-*O*-rutinoside[E]	0.060±0.001
18	22.44	327	515	353(18),191(34),179(61),173(100),161(5),135(5)	4,5-*O*-Dicaffeoylquinic acid[A]	0.127±0.001
					Total Phenolic Compounds	11.27±0.07
					Total Phenolic Acids	3.22±0.01
					Total Flavan3-ols	6.32±0.08
					Total Other Flavonoids	1.73±0.02

Standard calibration curves: A- chlorogenic acid ($y = 168823x - 161172$, $R^2 = 0.9999$; LOD = 0.20 µg/mL; LOQ = 0.68 µg/mL); B - catechin ($y = 84950x - 23200$, $R^2 = 1$; LOD = 0.17 µg/mL; LOQ = 0.68 µg/mL); C - apigenin-6-*C*-glucoside ($y = 107025x + 61531$, $R^2 = 0.9989$; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); D - myricetin-3-*O*-glucoside ($y = 23287x - 581708$, $R^2 = 0.9988$; LOD = 0.23 µg/mL; LOQ = 0.78 µg/mL); E- quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL). tr- traces (compounds below LOD amounts).

Table 5S. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the black tea extract.

Peak	Rt (min)	$\lambda_{max\ ((nm))}$	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg/g extract)
1	4.74	280	761	609(45),591(100),423(5),305(5)	Theasinensin B (or E)[A]	1.06±0.05
2	8.96	281/311	289	245(34), 203(16), 137(27)	(+)-Catechin[B]	1.25±0.03
3	9.89	274	457	331(79),305(73),169(100)	Epigallocatechin gallate[B]	8.5±0.1
4	13.1	335	563	545(30), 473(100), 443(79),383(32), 353(30), 311(2), 297(4)	Apigenin-*C*-hexoside-*O*-pentoside[C]	0.031±0.001
5	13.44	336	563	545(17), 473(71), 443(100),383(19), 353(22), 311(3), 297(5)	Apigenin-*C*-hexoside-*O*-pentoside[C]	0.046±0.001
6	14.83	352	479	317(100)	Myricetin-3-*O*-glucoside[D]	0.306±0.001
7	17.22	353	609	301(100)	Quercetin-3-*O*-rutinoside[E]	0.104±0.003
8	17.96	353	609	301(100)	Quercetin-*O*-deoxyhexosyl-hexoside[E]	0.085±0.001
9	18.34	347	463	301(100)	Quercetin-3-*O*-glucoside[E]	0.190±0.001
10	20.48	346	593	285(100)	Kaempferol-3-*O*-rutinoside[E]	0.096±0.001
11	21.75	344	447	285(100)	Kaempferol-3-*O*-glucoside[E]	0.086±0.001
					Total Phenolic Compounds	11.8±0.2
					Total Flavan3-ols	10.8±0.2
					Total Flavonoids	0.945±0.002

Standard calibration curves: A- gallic acid ($y = 131538x + 292163$, R^2=0.9969; LOD = 0.28 µg/mL; LOQ = 0.87 µg/mL); B - catechin ($y = 84950x – 23200$, R^2= 1; LOD = 0.17 µg/mL; LOQ = 0.68 µg/mL); C - apigenin-6-*C*-glucoside ($y = 107025x + 61531$, R^2 = 0.9989; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); D - myricetin-3-*O*-glucoside ($y = 23287x – 581708$, R^2 = 0.9988; LOD = 0.23 µg/mL; LOQ = 0.78 µg/mL); E- quercetin-3-*O*-glucoside ($y = 34843x – 160173$, R^2 = 0.9998; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL). tr-traces (compounds below LOD amounts).

Table 6S. Names and identification codes (CID) of the compounds used in the docking simulations as obtained in the data base of PubChem.

Tentative identification	**CID**	**Tentative identification**	**CID**
(Epi)gallocatechin isomer 1	72277	**Quercetin-*O*-rutinoside-hexoside ***	
(Epi)gallocatechin isomer 2	10425234	Quercetin-3-*O*-rutinoside-7-*O*-glucoside	10190763
(+)-Catechin	9064	Quercetin-3-*O*-glucoside-7-*O*-rutinoside	44259182
Epigallocatechin gallate	65064	Quercetin-3-*O*-rutinoside-4'-*O*-glucoside	44259179
3-*O*-Caffeoylquinic acid	1794427	Quercetin-3-*O*-glucoside	5280804
4-*O*-Caffeoylquinic acid	9798666	Quercetin-3-*O*-rutinoside	5280805
5-*O*-Caffeoylquinic acid	5280633	**Quercetin-*O*-deoxyhexoside-hexoside ***	
Theasinensin B	467315	Quercetin-3-*O*-alpha-rhamnopyranosyl-(1-2)-beta-galactopyranoside	5748416
Theasinensin E	467317	**Quercetin-*O*-malonyl-hexoside ***	
Apigenin-*C*-hexoside-*O*-pentoside* #		Quercetin-3-*O*-malonylglucoside	5282159
Apigenin-6-*C*-glucoside-4´-*O*-arabinose	01a	Quercetin-7-*O*-malonylglucoside	135397936
Apigenin-8-*C*-glucoside-4´-*O*-arabinose	01b	**Kaempferol-*O*-rutinoside-hexoside ***	
Apigenin-6-*C*-galactoiside-4´-*O*-arabinose	01c	Kaempferol-3-*O*-rutinoside 7-*O*-glucoside	21676298
Apigenin-8-*C*-galactoiside-4´-*O*-arabinose	01d	Kaempferol-3-*O*-rutinoside	5318767
Apigenin-6-*C*-glucoside-4´-*O*-xyloside	01e	Kaempferol-3-*O*-glucoside	44258798
Apigenin-8-*C*-glucoside-4´-*O*-xyloside	01f	**Kaempferol-*O*-malonyl-hexoside ***	
Apigenin-6-*C*-galactoside-4´-*O*-xyloside	01g	Kaempferol-3-(6-*O*-malonylglucoside)	14162699
Apigenin-8-*C*-galactoiside-4´-*O*-xyloside	01h	Kaempferol-7-*O*-malonylglucoside	145864961
Myricetin-3-*O*-rutinoside	44259428	Kaempferol 3-*O*-(6"-malonylgalactoside)	44258761
Myricetin-3-*O*-glucoside	22841567	3,4-*O*-Dicaffeoylquinic acid	102162816
Cyanidin-3-*O*-glucoside	441667	4,5-*O*-Dicaffeoylquinic acid	102162817

* Generic name with representative structures available in PubChem.

[#] The program Marvin Sketch was used to design the chemical structures

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Table 7S. The average scores and standard deviation for each ligand assessed by docking in the porcine pancreaticα-amylase were obtained with four repetitions for each program. The red numbers represent the score used as the cut-off note (reference ligand) for each program. The numbers in bold represent the compounds with a better score than the reference ligand amylose or acarbose (ARE), therefore, the best ranked.

Compound (CID)		**Gold**	**Molegro**	**DockThor**	**Autodock**
21676298	Kaempferol-3-*O*-rutinoside-7-*O*-glucoside	**91.5**	**-191**	**-9.52**	**-7.6**
44259179	Quercetin-3-*O*-rutinoside-4'-*O*-glucoside	**88.1**	-184	**-9.14**	**-6.9**
102162817	4,5-*O*-Dicaffeoylquinic acid	**93.5**	**-193**	-7.14	**-7.6**
135397936	Quercetin-7-*O*-malonylglucoside	**90.6**	-161	-7.50	**-7.7**
44259428	Myricetin-3-*O*-rutinoside	**90.8**	-176	**-9.50**	**-7.9**
ARE	Acarbose	**94.2**	-187	-7.82	-6.0
5280805	Quercetin-3-*O*-rutinoside	83.0	-177	**-9.12**	**-8.0**
10190763	Quercetin-3-*O*-rutinoside-7-*O*-glucoside	**92.7**	**-195**	**-9.16**	**-6.5**
467315	Theasinensin B	**87.5**	-186	-8.13	**-8.5**
5318767	Kaempferol-3-*O*-rutinoside	**86.2**	-169	**-9.03**	**-8.6**
145864961	Kaempferol-7-*O*-malonylglucoside	**84.7**	-158	**-8.45**	**-7.3**
44259182	Quercetin-3-*O*-glucoside-7-*O*-rutinoside	**85.4**	-188	**-8.71**	**-7.1**
Amylose	Amylose	83.7	-191	-8.20	-2.2
01b	Apigenin-8-*C*-glucoside-4´-*O*-arabinose	74.8	-136	-6.95	**-7.8**
44258761	Kaempferol-3-*O*-(6"-malonylgalactoside)	83.4	-153	-7.93	**-7.4**
5282159	Quercetin-3-*O*-malonylglucoside	82.3	-168	-6.82	**-6.8**
65064	Epigallocatechin gallate	81.5	-150	-7.22	**-8.7**
102162816	3,4-*O*-Dicaffeoylquinic acid	81.3	**-195**	-7.19	**-6.9**
01c	Apigenin-6-*C*-galactoiside-4´-*O*-arabinose	79.3	-124	**-8.73**	**-6.7**
14162699	Kaempferol-3-*O*-(6-*O*-malonylglucoside)	82.9	-169	**-8.24**	**-7.5**
01d	Apigenin-8-*C*-galactoiside-4´-*O*-arabinose	73.0	-137	-6.83	**-6.4**
01a	Apigenin-6-*C*-glucoside-4´-*O*-arabinose	72.8	-116	**-8.69**	**-7.5**
467317	Theasinensin E	67.0	-139	**-8.46**	**-7.9**
5280804	Quercetin-3-*O*-glucoside	71.8	-134	-7.66	**-7.1**
44258798	Kaempferol-3-*O*-glucoside	69.7	-127	-8.02	**-7.8**
01f	Apigenin-8-*C*-glucoside-4´-*O*-xyloside	70.7	-134	**-8.42**	**-7.6**
72277	(Epi)gallocatechin isomer 1	70.1	-102	-7.26	**-7.5**
01g	Apigenin-6-*C*-galactoside-4´-*O*-xyloside	74.6	-118	-7.56	**-8.2**
5748416	Quercetin-3-*O*-alpha-rhamnopyranosyl-(1-2)-beta-galactopyranoside	68.9	-135	-8.04	**-7.5**
441667	Cyanidin-3-*O*-glucoside	72.7	-144	-8.09	**-7.2**
22841567	Myricetin-3-*O*-glucoside	68.9	-129	**-8.54**	**-7.2**
01e	Apigenin-6-*C*-glucoside-4´-*O*-xyloside	69.5	-120	**-8.94**	**-7.7**
9064	(+)-Catechin	66.4	-102	-7.08	**-7.4**
10425234	(Epi)gallocatechin isomer 2	65.4	-98	-7.42	**-6.9**
9798666	4-*O*-Caffeoylquinic acid	66.4	-115	-6.75	-5.8
01h	Apigenin-8-*C*-galactoiside-4´-*O*-xyloside	63.0	-127	**-8.65**	**-6.7**
1794427	3-*O*-Caffeoylquinic acid	63.7	-124	-6.53	**-6.6**
5280633	5-*O*-Caffeoylquinic acid	61.7	-107	-6.59	**-6.3**

Green tea and Kenyan purple tea have several similar biological activities but differ largely in their inhibitory potential against fat digestion

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Abstract

A new variety of classic green tea plant (*Camellia sinensis*) was recently developed and is exclusive to Kenya. Due to the high content in anthocyanins of the leaves, the beverage obtained from this new variety has a purplish color, what originated the name purple tea. The purpose of this study was to compare the phenolic profiles and some bioactivities of beverages prepared from green tea and Kenyan purple tea in a way similar to that for human consumption. The results obtained in the present study showed that the bioactive properties of green tea and Kenyan purple tea are similar. However, purple tea was a much stronger inhibitor of pancreatic lipase. This inhibition reflected very strongly on the triglyceride digestion *in vivo*, an activity that was inhibited solely by the purple tea extract. These results, when combined, suggest that the regular consumption of Kenyan purple tea can be useful in the control of both obesity and diabetes.

Key words: green tea, purple tea, catechins, anthocyanins, biological properties, enzyme

1. Introduction

Green tea (*Camellia sinensis*), a beverage originated in ancient China, has become increasingly popular all over the world in recent decades. Green tea has been extensively studied and its regular consumption is correlated with many benefits for human health (Guo et al., 2017, Sanlier et al., 2018). The bioactivities of green tea, such as, antioxidant, antibacterial, antitumoral, anti-inflammatory, anti-diabetic, anti-proliferative and anti-atherosclerotic, among others, have been revised by several authors (Teixeira & Sousa, 2021; Shirakami & Shimizu, 2018, Musial et al., 2020; Prasanth et al., 2019). These activities are usually attributed to polyphenolics such as epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallo-catechin-3-gallate, which is considered the most active catechin (Musial et al., 2020).

A new variety of the classic tea plant was recently developed and is exclusive to Kenya. This new variety is grown under cooler conditions, at elevations between 4,500 and 7,500 feet. This exposes the plants to more intense sun rays causing them to produce higher levels of protective anthocyanins, which confer a purple color to the leaves (Kerio et al., 2012; Kerio et al., 2013; He et al., 2018). Due to the high content in anthocyanins of the leaves, the beverage obtained from this new variety has a purplish color, what originated the name purple tea. The richness in anthocyanins in purple tea opens new prospects concerning its bioactivities. Recent research has shown that anthocyanins have numerous health beneficial properties which include antioxidant, anti-tumoral, anti-diabetic, anti-angiogenic, antiapoptotic and pro-apoptotic, and antimicrobial activities, among others (Alappat & Alappat, 2020, Khoo et al., 2017; Lin et al., 2012; Shipp & Abdel-Al, 2010, Sivamaruthi et al., 2018; Gonçalves et al., 2021; Shirakami & Shimizu, 2018).

A recent work of our laboratory reports a comparison of the abilities of 5 types of tea in inhibiting starch digestion in mice, namely purple, green, oolong, black and white teas (da Silva et al., 2021). The study revealed that purple is the most potent inhibitor of the pancreatic α-amylase *in vitro* and also the most potent inhibitor of starch digestion in mice. Inhibition of starch digestion by the various tea types seems to depend strictly on the effects on the α-amylase and not on the inhibition of the α-glucosidase because no diminution of maltose digestion was observed. It was concluded that in terms of postprandial anti-hyperglycemic action purple tea presents the best perspective among all the tea varieties that were tested, including green tea (da Silva et al., 2021). It is worth to emphasize that purple tea is not a tea type characterized by its manufacturing, but a different cultivar which is manufactured following the same procedures used to prepare green tea. The question that can be formulated based on the differences in their actions on starch absorption, is one about the possible occurrence of other differences in their bioactivities. As stated above, green tea presents many biological actions in mammals, and a comparison of these actions with those of its derivative cultivar might eventually produce novel and useful results. Taking this into account, the purpose of this study was to compare the phenolic profiles and several bioactivities (antioxidant, anti-inflammatory, cytotoxic, antibacterial activities) of beverages prepared from green tea and purple tea in a way similar to that for human consumption. Furthermore, taking into account that there are several reports that green tea and other varieties are able to inhibit the pancreatic lipase (Nakai et al., 2005; Gondoin et al., 2010; Gulua et al., 2018), experiments were also done in order to search for a possible inhibition of fat absorption by both purple and green tea preparations. In this specific case both *in vitro* and *in vivo* experiments were done under the assumption that the latter are indispensable for assuring a physiological significance to the observations.

2. Material and methods

2.1. Materials

2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), dexamethazone, orlistat (tetrahydrolipstatin), ellipticine, acarbose, thiobarbituric acid, sulforhodamine B, salivary and pancreatic amylases, pancreatic lipase were acquired from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals were commercially available materials at the highest grade.

2.2. Preparation of the aqueous tea extracts

The commercial *Camellia sinensis* var. *assamica* (green tea) was purchased in the local market (Maringá, Brazil). Kenyan purple tea (purple tea) was purchased from Justea.com, Vancouver, Canada. Purple tea is a different cultivar called TRFK 306/1, a special type of tea produced mostly in Kenya (Kilel et al., 2013).

The extracts were prepared according to the protocol described previously (Lochocka et al., 2015). The tea leaves (100 g) were ground, boiled in distilled water (1000 mL) and subsequently stirred for 15 minutes at 70 °C (repeated 3 times). The collected extracts were filtered, frozen and lyophilized under vacuum at –20 °C. After determining the extraction yields in %, the lyophilized materials were weighed in analytical balance.

2.3. Analysis of phenolic compounds

The lyophilized extracts were dissolved in methanol/water (80:20, *v/v*) to a final concentration of 10 mg/mL and filtered through 0.22 μm disposable filter disks. Analysis

was performed in a HPLC system (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) coupled to a diode-array detector (DAD, using 280 and 370 nm as preferred wavelengths) and a Linear Ion Trap (LTQ XL) mass spectrometer (MS, Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Separation was made in a Waters Spherisorb S3 ODS-2 C18 column (3 µm, 4.6 mm × 150 mm; Waters, Milford, MA, USA). The operating conditions were previously described (Bessada et al., 2016). The identification of phenolic compounds was achieved by comparing retention times, UV-Vis and mass spectra with available standard compounds. Otherwise, available data reported in the literature were applied to tentatively identify the compounds. For the identified phenolic compounds with unavailable commercial standard, the quantification was performed using the calibration curve of the most similar standard available. The analyses were carried out in triplicate and the results are expressed as mean values and standard deviations (SD), in mg/g of lyophilized extract.

2.4. Total anthocyanin determination

The total anthocyanin contents of the various tea extracts were evaluated according to the pH differential method (Lee et al., 2005). The pH 1.0 buffer was 0.025 M hydrochloric acid-potassium chloride buffer (HCl-KCl) and the pH 4.5 buffer was 0.4 M sodium acetate. The sample dilution was established by limiting the pH 1.0 absorbance at 520 nm between 0.2 and 1.4. The total anthocyanin content of each sample (C_{TA}; mg cyanidin-3-*O*'-glucoside equivalents per g extract) was calculated by the following formula:

$$C_{TA} = \frac{A \times mw \times DF \times 10^3}{\varepsilon \times \ell \times W} \quad (1)$$

A is the specific absorbance, *mw* the molecular weight of cyanidin-3'-*O*-glucoside (449.2 g/mol), DF the dilution factor, ε the molar extinction coefficient of cyanidin-3'-*O*-glucoside at 520 nm (2.69×10^4 M^{-1} cm^{-1}), ℓ the light path length (cm), *W* the amount that was extracted (in mg/100g dry weight) and 10^3 a factor for the conversion of g into mg. The specific absorbance A is defined as:

$$A = (A_{520\ nm} - A_{700\ nm})_{pH\ 10} - (A_{520\ nm} - A_{700\ nm})_{pH\ 4.5} \quad (2)$$

2.5. Antioxidant activity evaluation

To evaluate the antioxidant activity of the tea extracts, five methods were used. Three of them were traditional *in vitro* assays, namely the 2,2-diphenyl-1-picrylhydrazyl (DPPH), the reducing power of iron ions (FRAP) and the oxygen radical absorbance radical (ORAC) assays. The other two were cell-based methods, the oxidative hemolysis inhibition test (OxHLIA) and the inhibition of the production of thiobarbituric acid reactive substances (TBARS). Successive dilutions of the stock solution were made and used for assaying the antioxidant activity of the sample. The evaluation of FRAP and ORAC was carried out as previously described (Koehnlein et al., 2016). The DPPH assay was performed as previously described (Corrêa et al., 2015). The results of the FRAP and ORAC were expressed as μmol Trolox equivalents (TE)/mg of extract, and in the DPPH assay the results were expressed as IC_{50} values (sample concentration supplying 50% of antioxidant activity). The TBARS assay followed the methodology described by Lockowandt et al. (2019). The extracts capacity to inhibit the formation of thiobarbituric acid reactive substances (TBARS) was assessed using porcine brain cell tissues as oxidizable substrates, and the results were expressed as IC_{50} values (mg/mL), which correspond to the extract concentrations providing 50% of antioxidant activity. The

oxidative haemolysis inhibition assay (OxHLIA) was carried out as described previously (Lockowandt et al., 2019). IC_{50} values (μg/mL) were calculated for Δt of 60 min and translate the extract concentration required to keep 50% of the erythrocyte population intact for 60 min. Trolox was used as a positive control in both assays.

2.6. Anti-inflammatory activity

The anti-inflammatory activity of the extracts (at concentrations up to 400 μg/mL) was assessed based on the nitric oxide (NO) production by a lipopolysaccharide (LPS)-stimulated murine macrophage cell line (RAW 264.7, purchased from European Collection of Authenticated Cell Cultures, ECACC)). The NO production was quantified based on the nitrite concentration using the Griess Reagent System kit containing sulphanilamide, *N*-1-naphthylethylenediamine dihydrochloride and nitrite solutions, following a procedure previously described by Corrêa et al. (2015). Dexamethasone was used as a positive control, while no LPS was added in negative controls. The effect of the tested extracts on NO basal levels was also assessed by performing the assay in the absence of LPS. The results were expressed as IC_{50} values (μg/mL), which correspond to the extract concentrations providing 50% inhibition of NO production.

2.7. Cytotoxic activity

The cytotoxicity of the extracts was assessed by the sulforhodamine B assay against four human tumour cell lines (acquired from Leibniz-Institut DSMZ), namely MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma), following a protocol previously described by Spréa et al. (2020). Ellipticine was used as a positive control. The same assay was also used to evaluate the hepatotoxicity of the extracts against a non-tumour cell line (PLP2, porcine

liver primary cells) obtained as described by Spréa et al. (2020). The results were expressed in GI_{50} values (μg/mL), which translate the extract concentration providing 50% of cell growth inhibition.

2.8. Antimicrobial activity

The extracts were redissolved in 5% dimethyl sulfoxide (DMSO) to a concentration of 30 mg/mL and further diluted. The microdilution method (Soković et al., 2010) was performed to assess the antimicrobial activity against the Gram-negative bacteria *Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311) and *Enterobacter cloacae* (ATCC 35030), and the Gram-positive *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate) and *Listeria monocytogenes* (NCTC 7973). The antifungal activity was assessed against *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), and *Penicillium verrucosum* var. *cyclopium* (food isolate) (Corrêa et al., 2015). The minimum extract concentrations that completely inhibited bacterial growth (MICs) were determined by a colorimetric microbial viability assay, and minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were also calculated. Streptomycin, ampicillin, ketoconazole and bifonazole (Sigma-Aldrich, St. Louis, MO, USA) were used as positive controls, and 5% DMSO was used as a negative control.

2.9. In vitro pancreatic lipase assay

The porcine pancreatic lipase was assayed using p-nitrophenyl-palmitate as the substrate and spectrophotometrically recorded at 410 nm (Oliveira et al., 2015). The substrate solution was prepared by suspending 20 mg of *p*-nitrophenyl palmitate in 10 mL of

isopropanol. The suspension was sonicated until complete dissolution of *p*-nitrophenyl-palmitate. At the time of use, this stock solution was diluted with isopropanol to concentrations up to 0.5 mg/mL. The porcine pancreatic lipase was dissolved in Tris-HCL buffer (pH 8.0) at the concentration of 2 mg/mL. This suspension was centrifuged at 2000*g* for five minutes and the supernatant used as the source of enzyme. The final reaction mixture (2.4 mL) contained 100 mM Tris-HCl buffer (pH 8.2), 530 μM substrate, the tea extracts at varying concentrations and was 25% isopropanol. For running the reaction, two protocols were used, the first one without previous incubation of the tea extracts with the enzyme and the second one with pre-incubation of the enzyme with the extracts. In the first procedure the tea extracts (100 μL) and the enzyme (100 μL) were added to 1800 μL of the pre-warmed reaction buffer at 37 ^{o}C, the reaction was started by adding the substrate solution ((500 μL). In the second procedure the tea extracts (100 μL) and the enzyme (100 μL) were added to 1800 μL of the buffer solution and after incubating the mixture at 37 ^{o}C for 10 minutes, the substrate (100 μL) was added for starting the reaction. The reaction was stopped by transferring the reaction vessel to a bath of boiling water. After 10 min, the incubation was cooled to room temperature and centrifuged at 1500 g for 5 min. Absorbance of the supernatant at 410 nm was determined against a blank solution containing denatured enzyme.

2.10. Triglyceride tolerance tests

In vivo lipase inhibition was inferred from triglyceride tolerance tests, which were done as described previously (Oliveira et al., 2015; Castilho et al., 2021). Fasted (18 h) male Swiss mice (25-35 g) were used. Seven groups of mice (n = 3-4 per group) were utilized: (1) mice received solely olive oil (5 mL/kg) by gavage (controls); (2) only tap water

administration (negative controls for establishing the basal line); (3) olive oil plus orlistat administration (50 mg/kg; positive controls); (4) intragastric administration of olive oil plus purple tea extract at the dose of 100 mg/kg; (5) intragastric administration of olive oil plus 250 mg/kg purple tea extract; (6) intragastric administration of olive oil plus 500 mg/kg purple tea extract; and (7) intragastric administration of olive oil plus 500 mg/kg green tea extract. The extracts and orlistat administration preceded the olive oil administration. The plasma triglyceride levels were determined at 0, 90, 180, 270 and 360 minutes after olive oil administration in blood samples collected from the tail vein. Measurement of blood triglycerides was carried out by means of an AccutrendPlus® Roche triglyceride meter (Luley et al., 2000).

2.11. Statistical Analysis

Differences between two means were assessed by Student's t-test. Data sets composed of more than two means were subjected to univariate or multivariate variance analyses (ANOVA and MANOVA) according to the context, with post-hoc Newman-Keuls-testing. Significance was accepted when $p \leq 0.05$. The IC_{50} values for the lipase inhibition were computed by numerical interpolation (Stineman's formula).

3. Results

3.1. Phenolic profiles and anthocyanins contents of green tea and Kenyan purple tea

The yield of the green tea extract was significantly higher than that of the purple tea, 45.60 ±1.50% and 35.67±1.10%, respectively ($p \leq 0.05$). Table 1 gives the content of each identified phenolic compound in both extracts. Details such as retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, and

fragmentation pattern can be found in Supplementary Material (Tables 1S-2S). The identification of (+)-catechin, 5-*O*-caffeoylquinic acid, myricetin-3-*O*-rutinoside, myricetin-3-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside was made by comparison with authentic available standards. The remaining compounds were tentatively identified by comparing their chromatographic characteristics with results previously described in literature (Dou, Lee, Tzen, Lee, 2007; Kim, Goodner, Park, Choi, Talcott, 2011; Zielinski, Haminiuk, Beta, 2016). The contents are all expressed as quantities (mg) per gram extract, and they represent the amounts that were effectively extracted using the procedure described in the methods section (mild hot water extraction). A total of 17 phenolic compounds were identified in green tea and 16 in purple tea. In terms of the contents in total phenolic compounds, green tea was almost 2-fold superior to purple tea. Individually, epigallocatechin gallate (EGCG) was the most abundant compound in both extracts, 30.20 and 11.6 mg/g extract for green and purple tea, respectively. The purple tea extract revealed to possess the by far highest content of the (epi)gallocatechin isomer 1, 7.9 times more than green tea. However, the sum of the contents of all (epi)gallocatechin isomers of green tea, 5.36 mg/g, is very close to the content in (epi)gallocatechin isomer 1 of purple tea. The green tea extract exceeded the purple tea extract in having 2.7 times more (+) catechin.

With respect to the total content of anthocyanins, the difference between the purple tea extract and green tea extract is remarkable. The purple tea extract revealed to possess 0.9±0.1 mg cyanidin-3-*O*-glucoside equivalents per g extract, while the green tea extract presented 0.100±0.005 mg cyanidin-3-*O*-glucoside equivalents per g extract ($p \leq 0.05$).

3.2. Antioxidant activity

Table 2 shows the antioxidant activities of green and purple teas determined by 5 different methods, three chemical assays (DPPH, FRAP and ORAC), and two cell-based assays (TBARS and OxHLIA). It is of great value to use two or more tests to assess the antioxidant capacity of extracts, since the antioxidant compounds act by different mechanisms, and each mechanism has its own specific target in the reaction matrix (Corrêa et al., 2017; Garcia et al., 2019; Gonçalves et al., 2019). For the DPPH and FRAP assays, no significant difference was found for the extracts, while the ORAC assay showed a higher antioxidant activity for purple tea. Concerning the cell-based assays, despite the significantly different composistion of the extracts, both green tea and purple tea showed similar antioxidant activities.

3.3. Cytotoxic and anti-inflammatory activities

Table 3 presents the antitumoral effects of green and purple tea on the growth of four human tumor cell lines: (NCI-H460, HepG2, HeLa and MCF-7), expressed as concentrations that caused 50% of the inhibition of cell growth (GI_{50}). The tumor cell that showed the greatest sensitivity to the extracts was HepG2 (GI_{50} = 20.03 and 21.20 µg/mL for green and purple teas, respectively) followed by NCL-H460, HeLa, and MCF-7. The cytotoxicity on porcine liver primary cells (PLP2) of both extracts was low but far superior to the GI_{50} values obtained for tumor lines. The values of GI_{50} for green tea and purple tea did not generally differ statisticaly, excepting the cases of MCF7 and Hela cells. In relation to the anti-inflammatory activity, no significant difference was also observed between green tea and purple tea. Green and purple extracts presented cytotoxic and anti-

inflammatory activities significantly inferior to those of the positive controls ellipticine and dexamethasone, respectively.

3.4. Antimicrobial activity

Antibacterial and antifungal activities are presented in Table 4. The inhibitory concentration (MIC) values of the extracts were evaluated for three Gram-negative bacteria (*Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311) and *Enterobacter cloacae* (ATCC 35030)), and three Gram-positive bacteria (*Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate) and *Listeria monocytogenes* (NCTC 7973). The MIC values shown in Table 4 indicate that the extracts were active against Gram-negative and Gram-positive bacteria, with Gram-positive bacteria being slightly more sensitive to the extracts than Gram-negative bacteria. Concerning fungicidal activities, the inhibitory concentration values of the extracts were evaluated for six filamentous fungi. Both extracts present very similar inhibitory effects on all of them.

3.5. Action of on the pancreatic lipase

Figure 1 shows the concentration dependences of the effects of green and purple teas on the porcine pancreatic lipase *in vitro*. As described in the Materials and methods section the assays were performed with or without preincubation of the enzyme with the extract. Figure 1 reveals that the pancreatic lipase was much more sensitive to purple tea when compared to green tea and that the sensitivity increased after preincubation with the extracts. At the purple tea extract concentration of 400 μg/mL inhibition was almost complete when the activity was measured after pre-incubation. Also, the IC_{50} value, that

was equal to 218.7 μg/mL in the assay without preincubation, was reduced to 91.2 μg/mL upon preincubation. Inhibition of the lipase caused by green tea, on the other hand, was considerably less pronounced than that of purple tea, but it also increased when preincubation was done. The IC_{50} values, however, are certainly well above 400 μg/mL, the highest concentration that was used in the present assays.

3.6. Action on triglyceride absorption in mice

The results of the triglyceride tolerance experiments shown in Figure 2 should allow to infer if the lipase inhibitory activities of purple and green tea extracts also manifest themselves *in vivo*. The results in panel A reveal that the time courses of the plasma concentrations of triglycerides were clearly modified when purple tea was administered to the mice prior to the olive oil load. All curves obtained under the influence of purple tea were shifted in a dose-dependent manner in the direction of the baseline curve (when water was administered in place of olive oil). Panel B of Figure 2 shows that this was also the effect of orlistat, the classical inhibitor of triglyceride digestion. Panel 2 also reveals that the effect of green tea, even at the high dose of 500 mg/kg, did not produce modifications that could be be interpreted as an inhibition of triglyceride digestion. The latter assumption is corroborated by panel C, which shows the areas under the curves in panels A and C subtracted from the area under the base line, which is given by the curve obtained after water administration. It is generally accepted that these areas reflect with good approximation the net rate of the intestinal triglyceride absorption process. The area under the curve obtained after green tea administration did not differ from the control area. Different doses of purple tea, on the other hand, produced progressively smaller areas

under the curves. That one computed for the 400 mg/kg dose was almost the same as that one found when orlistat was administered.

4. Discussion

Catechins are the main polyphenolic compounds in the fresh leaf of the tea plant (*C. sinensis* (L.) O. Kuntze). In the preparations used in the present study, the total catechin content in green tea extract was significantly higher than that in the Kenyan purple tea extract. This observation disagrees with previous comparative studies of green tea and Kenyan purple tea (Kerio et al., 2012; Kerio et al., 2013) which showed similar catechin contents. During the last decades, leaves from Kenyan cultivars of *Camellia sinensis* (Kenyan purple tea) have been the object of studies seeking to characterize the main molecules responsible for their biological actions. In addition to the tea catechins usually identified in *C. sinensis*, namely epigallocatechin, catechin, epicatechin, epigallocatechin gallate, and epicatechin gallate, extracts from Kenyan purple tea also present anthocyanins and anthocyanidins. Malvidin is the predominant anthocyanidin, while cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside are the predominant anthocyanins (Kerio et al., 2012; Kerio et al., 2013; Kilel et al., 2013). Cyanidin-3-*O*-galactoside was also found to be the most abundant anthocyanin identified in the Zijuan purple tea extract obtained from the best known purple leaf and anthocyanin-rich tea cultivars in China (Lv et al., 2015). Epigallocatechin gallate is considered the most powerful bioactive of *C. sinensis*, and it is generally considered responsible for most of the health benefits derived from the habitual consumption of this herb and the derived beverage (Nagle et al., 2006; Suzuki et al., 2016; Muthu et al. (2016). The aqueous extraction used in this work, which mimetizes the conventional preparation of the beverage for human consumption, showed that the contents

of epigallocatechin gallate were significantly higher in the green tea extract when compared to the purple tea extract. In terms of their antioxidant, anti-inflammatory, cytotoxicity, antibacterial and antifungal activities, however, both tea extracts were fairly comparable. A definitive explanation for this observation cannot be inferred from the available data. It is perfectly possible, however, that the much higher content in anthocyanins of the purple tea compensates its lower content in epigallocatechin gallate. Anthocyanins have in fact been found to have important biological activities, which include antioxidant, anti-inflammatory, and anticarcinogenic properties (Joshi et al., 2017). Anthocyanins have also been shown to induce apoptosis in cancerous cells (Lee et al., 2009), besides having the capacity of protecting cells against oxidative stress-induced apoptosis (Elisia & Kitts, 2008).

Alongside the similar actions of both tea preparations on the various biological activities, just highlighted in the preceding paragraph, there is another activity in which the purple tea revealed to display clear superiority: this refers to the inhibitory activity on the pancreatic lipase which presumably also caused an enhanced inhibitory action on triglyceride digestion. In this respect it should be stressed beforehand that the superior action of purple tea on fat digestion plainly parallels the superior action of this tea variety on starch digestion, as reported reported recently by work of our group (da Silva et al., 2021).

The present report is not the first one in which effects of tea varieties on the pancreactic lipase are reported. Similar effects have been reported for green, oolong and white teas, for example (Nakai et al., 2005; Gondoin et al., 2010; Gulua et al., 2018). However, to our knowledge, these studies merely demonstrated *in vitro* effects on the pancreatic lipase and our study is, thus, the first demonstration that the inhibitory action also occurs *in vivo.* In this respect the purple tea extracts were clearly superior to the green tea extracts. This is valid for both the lipase inhibition *in vitro* and the triglyceride digestion *in vivo*. Actually,

no significant effect was found with a green tea even at the dose 500 mg/kg. Such a those of the purple tea extract almost entirely abolished triglyceride absorption in mice. The compounds that are involved in the inhibition of the pancreatic lipase might be several of the polyphenolics listed in Table 1. In fact, it since long known that the catechins, epicatechins and several other compounds found in the various tea varieties can inhibit the pancreatic lipase with various potencies (Nakai et al., 2005; Gondoin et al., 2010; Li et al., 2016). It is very difficult, however, to deduce the relative participation of each of the compounds listed in Table 1. The most abundant polyphenolic in both tea extracts, however, namely epigallocatechin gallate (EGCG), is unlikely to be the most important participant. If it were so, the action of green tea should be more pronounced, as it contains three times more EGCG than purple tea; and, as shown by a rare *in vivo* experiment in the literature, the action of EGCG on triglyceride absorption in rats is relatively poor even at the high dose of 100 mg/kg (Li et al., 2016). The latter is a high dose in terms of a pure substance. For comparison, doses of 500 mg/kg of green and purple tea exracts correspond to 15.1 and 5.8 mg/kg EGCG. So, the effects of the purple and green tea extracts result more likely from a combination of the actions of several inhibitors, that one in the purple tea extract being more favourable for producing the inhibitory effect. Considering the great number of compounds, one cannot exclude synergism and eventually even antagonisms, which are difficult to predict.

Although inhibition of the pancreatic lipase by polyphenolics is likely to play an important role in the inhibition of fat absorption, one should not disconsider the participation of anthocyanidins, which are much more abundant in purple tea in relative terms. Anthocyanidins are also inhibitors of the pancreatic lipase, though not very potent ones (Buchholz and Melzig, 2015; Castilho et al., 2021). This fact, combined with the low absolute quantitives that are present in purple tea, makes a significant contribution of

anthocyanidins to the inhibition of the pancreatic lipase an unlikely event. However, it has been recently shown that cyanidin-3-*O*-glucoside affects very strongly triglyceride and free oleate absorption in mice by a mechanism that does not involve the pancreatic lipase (Castilho et al., 2021). Even doses as low as 0.2 mg/kg can exert a significant inhibitory effect. If one considers that a 500 mg/kg dose of the purple tea extracts corresponds to the administration of at least 0.45 mg/kg of anthocyanidins, it would not be a complete surprise if it turns out that this class of compounds could be contributing in some way to the overall effect of purple tea on fat digestion that was detected in the present work. Of course, this is a point that deserves further investigations before a definitive conclusion can be reached.

5. Conclusion

The results obtained in the present study showed that the antioxidant, anti-inflammatory, antitumoral and antimicrobial properties of green tea and purple tea extracts are similar, but that the former purple tea is a much stronger inhibitor of the pancreatic lipase. This inhibition reflects very strongly on the triglyceride digestion *in vivo*, an activity that was inhibited solely by the purple tea extract. This finding is the first report of an *in vivo* inhibition of triglyceride absorption by a tea extract and it is comparable to the same effect reported for purple tea with respect to starch digestion. These results, when combined, suggest that the regular consumption of purple tea can be useful in the control of both obesity and diabetes.

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Conflict of interest

The authors declare no conflict of interest

Credit authorship contribution statement

Tamires Barlati Vieira da Silva: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original draft. **Carla Pereira**: Methodology, Formal analysis, Investigation, Writing review & editing. **Maria Inês Dias**: Methodology, Formal analysis, Investigation. **Filipa Mandim:** Methodology, Formal analysis, Investigation. **Marija Ivanov**: Methodology, Formal analysis. **Marina Soković**: Methodology, Formal analysis, Investigation. **Lillian Barros**: Writing review & editing. **Isabel C.F.R. Ferreira**: Writing review & editing. **Flávio Augusto Vicente Seixas:** Methodology, Formal analysis, Investigation. **Adelar Bracht**: Conceptualization, Formal analysis, Investigation, Writing review & editing. **Rosane Marina Peralta**: Conceptualization, Formal analysis, Investigation, Writing review & editing

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Table 1. Tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the extracts of the green tea and purple tea. Full experimental details are given in the Materials and Methods section.

Tentative identification	Green tea (mg/g extract)	Purple tea (mg/g extract)
(Epi)gallocatechin isomer I[A] (EGC)	0.72±0.03	5.67±0.07
(Epi)gallocatechin isomer II[A] (EGC)	2.52±0.02	-
(Epi)gallocatechin isomer III[A] (EGC)	2.12±0.08	-
(+)-Catechin[A]	4.8±0.2	1.76±0.01
Epigallocatechin gallate[A] (EGCG)	30.2±0.4	11.6±0.1
Apigenin-*C*-hexoside-*O*-pentoside[D]	0.34±0.02	0.046±0.001
Apigenin-*C*-hexoside-*O*-pentoside[D]	0.38±0.01	0.028±0.001
Myricetin-3-*O*-rutinoside[E]	0.35±0.01	0.674±0.001
Myricetin-3-*O*-glucoside[E]	0.43±0.01	0.742±0.001
Myricetin-*O*-hexoside[E]	0.59±0.01	0.676±0.002
Quercetin-*O*-deoxyhexosyl-hexoside-hexoside[F]	0.457±0.008	0.138±0.001
Quercetin-*O*- deoxyhexosyl-hexoside-hexoside[F]	0.594±0.007	-
Quercetin-3-*O*-glucoside[F]	0.25±0.02	0.252±0.001
Quercetin-3-*O*-rutinoside[F]	-	0.319±0.001
Quercetin-*O*-deoxyhexosyl-hexoside[F]		0.167±0.001
Quercetin-*O*-malonyl-hexoside[F]	-	0.196±0.001
Kaempferol-*O*- deoxyhexosyl-dihexoside[F]	0.46±0.01	-
Kaempferol-*O*- deoxyhexosyl-dihexoside[F]	0.359±0.007	-
Kaempferol-3-*O*-rutinoside[F]	0.154±0.001	0.274±0.001
Kaempferol-3-*O*-glucoside[F]	0.084±0.001	0.218±0.002
Kaempferol-*O*-malonyl-hexoside[F]	-	0.213±0.003
Total phenolic compounds	44.76±0.06$^{(a)}$	23.0±0.2$^{(b)}$
Total flavan3-ols	40.31±0.02$^{(a)}$	19.1±0.2$^{(b)}$
Total other flavonoids	4.46±0.08$^{(a)}$	3.943±0.004$^{(b)}$

Different letters overwritten to the averages of the same line indicate a significant difference at the level of 5%.

Quantitative analysis was performed using a 7-level calibration curves of each available phenolic standard constructed upon the UV signal: apigenin-6-C-glucoside (y = 107025x + 61531, R^2 = 0.9989; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); chlorogenic acid (y = 168823x – 161172, R^2= 0.9999; LOD = 0.20 µg/mL; LOQ = 0.68 µg/mL); gallic acid (y = 131538x + 292163, R^2=0.9969; LOD = 0.28 µg/mL; LOQ = 0.87 µg/mL); B - catechin (y = 84950x – 23200, R^2= 1; LOD = 0.17 µg/mL; LOQ = 0.68 µg/mL); myricetin-3-*O*-glucoside (y = 23287x – 581708, R^2 = 0.9988; LOD = 0.23 µg/mL; LOQ = 0.78 µg/mL); and quercetin-3-*O*-glucoside (y = 34843x – 160173, R^2 = 0.9998; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL).

Table 2. Antioxidant activities of green tea and purple tea

Antioxidant evaluation	**Green tea**	**Purple tea**
Chemical-based antioxidant activities		
DPPH (IC_{50} µg/mL)	10.5± 0.5[(a)]	9.1± 0.4[(a)]
FRAP (µmol of Trolox equivalent/mg extract)	3822± 167[(a)]	4178± 525[(a)]
ORAC (µmol of Trolox equivalent/mg extract)	3661 ± 1559[(a)]	5547 ± 428[(b)]
Cell-based antioxidant activities		
TBARS (IC_{50}, ng/mL)	16.00±0.001[(a)]	18.00±0.001[(b)]
OxHLIA (IC_{50}, µg/mL, Δt=60 min)	13±1[(a)]	12.7±0.4[(b)]

Different letters overwritten to the averages of the same line indicate a significant difference at the level of 5%. DPPH IC_{50} Trolox= 110±12 µg/mL; OxHLIA, Δt=60 min IC_{50} Trolox =21.80±0.20 µg/mL; TBARS IC_{50}, Trolox= 5.8 ± 0.6 µg/mL.

Table 3. Cytotoxic and anti-inflammatory activities of green tea and purple tea

Bioactivities	**Tea extracts**		**Positive control**
Cytotoxic activity (GI_{50}, µg/mL)	**Green**	**Purple**	**ellipticine**
NCI H460 (non-small cell lung carcinoma)	59±4[(a)]	58±3[(a)]	1.03±0.09
HepG2 (cervical carcinoma)	20±2[(a)]	21±2[(a)]	1.1±0.2
MCF7 (hepatocellular carcinoma)	68 ±5[(a)]	74±3[(b)]	1.1±0.2
HeLa (breast carcinoma)	64±3[(a)]	71±4[(b)]	1.91±0.06
PLP2 (porcine liver primary cells)	120±8[(a)]	121±3[(a)]	3.2±0.7
Anti-inflammatory activity (IC_{50}, µg/mL)	**Green**	**Purple**	**Dexamethasone**
RAW264.7	82±4[(a)]	76±5[(a)]	6±1

Different letters overwritten to the averages of the same line indicate a significant difference at the level of 5%.

Table 4. Antibacterial and antifungal activity of tested extracts (*Camellia sinensis*) (mg/mL).

	Green tea		Purple tea	
Bacteria	**MIC**	**MBC**	**MIC**	**MBC**
Staphylococcus aureus	3.00	6.00	1.50	3.00
Bacillus cereus	3.00	6.00	3.00	6.00
Listeria monocytogenes	3.00	6.00	3.00	6.00
Escherichia coli	1.50	3.00	3.00	6.00
Salmonella typhimurium	3.00	6.00	3.00	6.00
Enterobacter cloacae	1.50	3.00	3.00	6.00
Fungi	**MIC**	**MFC**	**MIC**	**MFC**
Aspergillus fumigatus	0.75	1.50	0.75	1.50
Aspergillus niger	0.75	1.50	0.38	0.75
Aspergillus versicolor	0.75	1.50	0.75	1.50
Penicillium funiculosum	0.75	1.50	0.75	1.50
Penicillium verrucosum	0.75	1.50	0.75	1.50
Trichoderma viride	1.50	3.00	0.75	1.50

MIC=minimum inhibitory concentration; MBC=minimum bactericidal concentration; MFC= minimum fungicidal concentration.

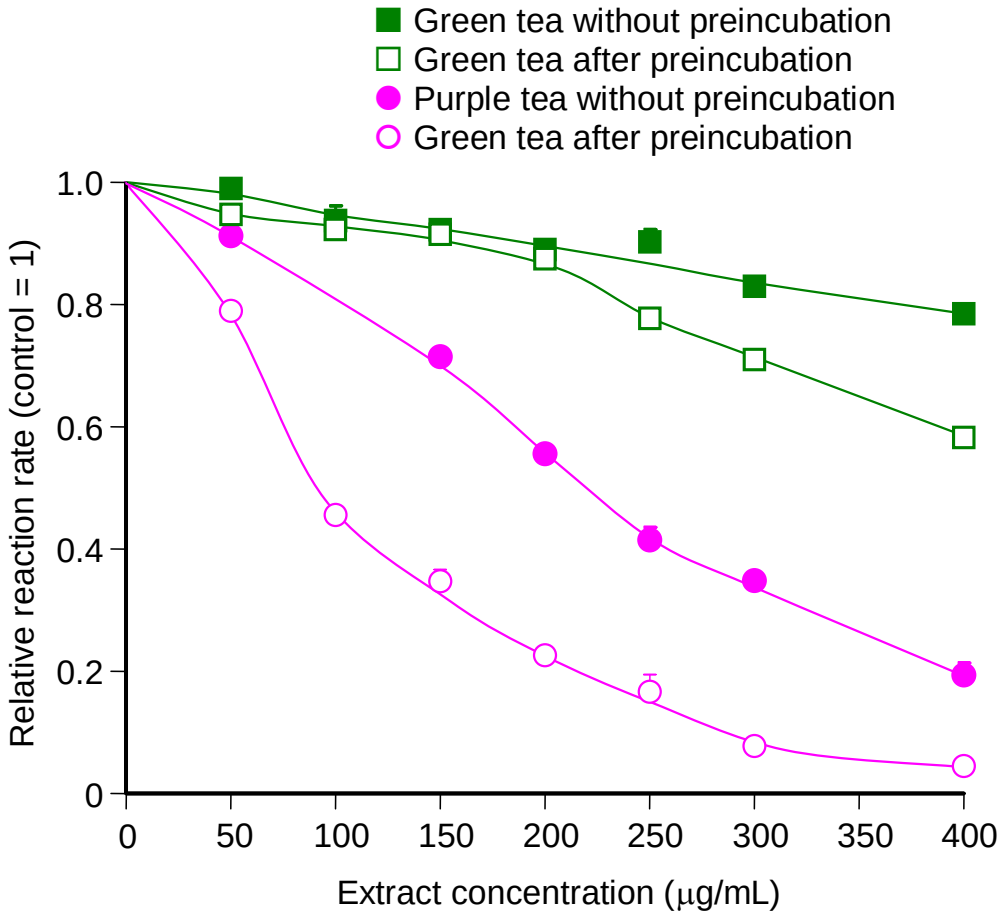

Figure 1. Concentration dependences of the inhibition caused by purple and green extracts on the porcine pancreatic lipase. The reaction rates were measured with or without prior incubation of the enzyme with the extract, as indicated at the top of the graph. Details are described in the Materials and Methods section. Each datum point is the mean of three determinations. Standard errors of the mean cannot be seen when smaller than the symbols.

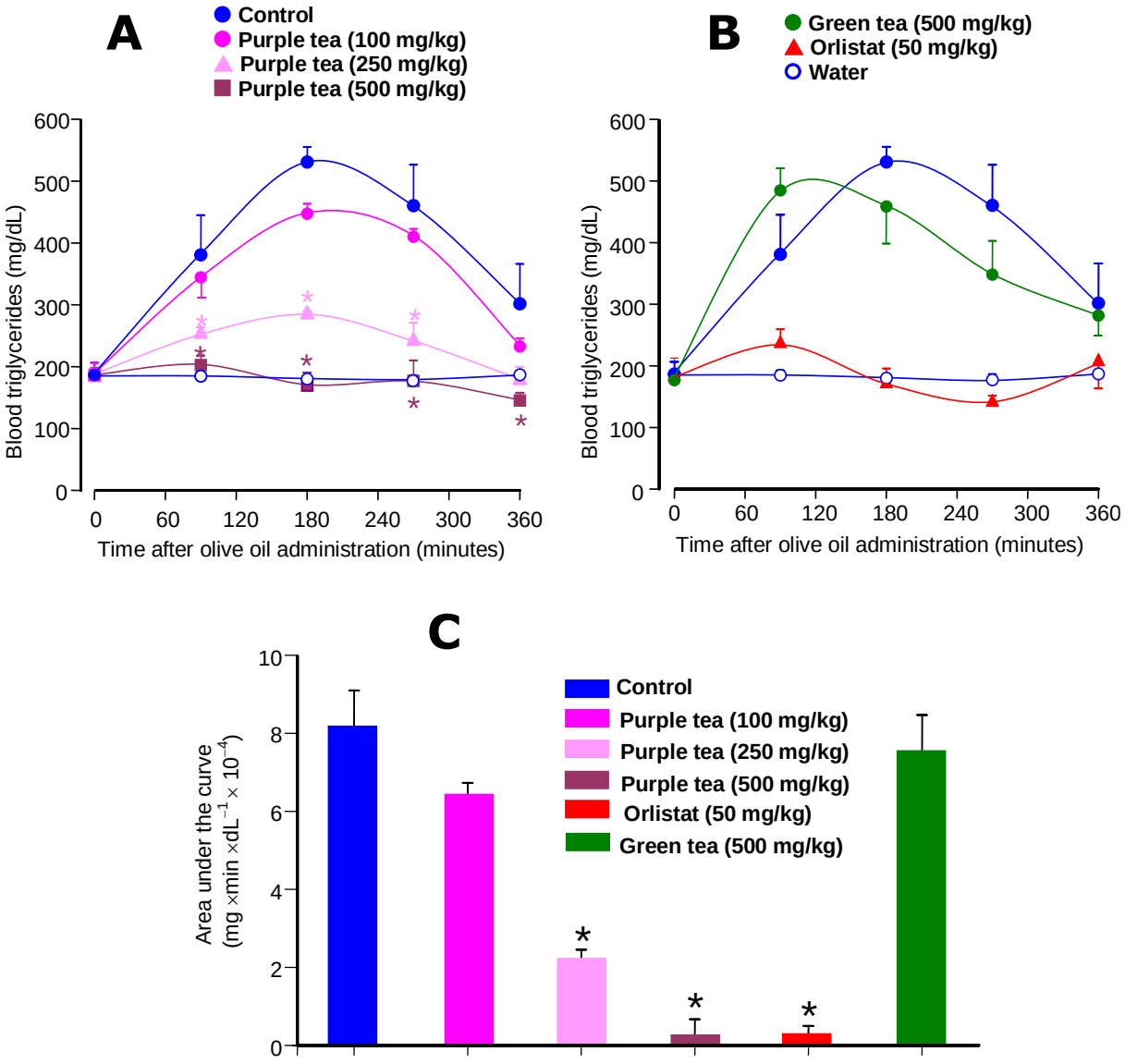

Figure 2. Actions of purple and green tea extracts on triglyceride digestion *in vivo*. A, blood triglyceride concentration profiles after intragastric olive oil loads in mice and the effects of the previous administration of various purple tea extract doses. B, blood triglyceride concentration profiles after intragastric olive oil loads in mice and the effects of previous administration of orlistat and a single dose of green tea extract. C, areas under the curves in panels A and B after subtraction of the area under the basal curve obtained after tap water administration. Asterisks indicate statistical significance relative to the control curve in A (Newman-Keuls post-hoc testing after MANOVA) and relative to the control bar in C (Newman-Keuls post-hoc testing after ANOVA).

SUPPLEMENTARY MATERIAL

Green tea and Kenyan purple tea have several similar biological activities but differ largely in their inhibitory potential against fat digestion

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Table 1S. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the green tea extract.

Peak	Rt (min)	$\lambda_{max\ ((nm))}$	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg/g extract)
1	4.79	274	305	219(75),179(100),125(11)	(Epi)gallocatechin isomer I[A]	0.72±0.03
2	5.29	271	305	219(68),179(100),125(25)	(Epi)gallocatechin isomer II[A]	2.52±0.02
3	5.81	275	305	219(71),179(100),125(23)	(Epi)gallocatechin isomer III[A]	2.11±0.08
4	8.97	281/311	289	245(34), 203(16), 137(27)	(+)-Catechin[A]	4.8±0.2
5	10.01	274	457	331(79),305(73),169(100)	Epigallocatechin gallate[A]	30.2±0.4
6	13.11	335	563	545(30), 473(100), 443(79),383(32), 353(30), 311(2), 297(4)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.34±0.02
7	13.44	336	563	545(17), 473(71), 443(100),383(19), 353(22), 311(3), 297(5)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.38±0.01
8	14.19	344	625	317(100)	Myricetin-3-*O*-rutinoside[C]	0.35±0.01
9	14.61	352	479	317(100)	Myricetin-3-*O*-glucoside[C]	0.43±0.01
10	14.81	352	479	317(100)	Myricetin-*O*-hexoside[C]	0.59±0.01
11	15.31	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[D]	0.457±0.008
12	15.88	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[D]	0.594±0.007
13	17.16	349	755	285(100)	Kaempferol-*O*-deoxyhexosyl-dihexoside[D]	0.46±0.01
14	17.96	347	463	301(100)	Quercetin-3-*O*-glucoside[D]	0.25±0.02
15	18.51	348	755	285(100)	Kaempferol-*O*-deoxyhexosyl-dihexoside[D]	0.359±0.007
16	20.51	346	593	285(100)	Kaempferol-3-*O*-rutinoside[D]	0.154±0.001
17	21.74	344	447	285(100)	Kaempferol-3-*O*-glucoside[D]	0.084±0.001
					Total Phenolic Compounds	44.76±0.06
					Total Flavan3-ols	40.31±0.02
					Total Other Flavonoids	4.46±0.08

Standard calibration curves: A - catechin ($y = 84950x - 23200$, $R^2 = 1$; LOD = 0.17 μg/mL; LOQ = 0.68 μg/mL); B - apigenin-6-*C*-glucoside ($y = 107025x + 61531$, $R^2 = 0.9989$; LOD = 0.19 μg/mL; LOQ = 0.63 μg/mL); C - myricetin-3-O-glucoside ($y = 23287x - 581708$, $R^2 = 0.9988$; LOD = 0.23 μg/mL; LOQ = 0.78 μg/mL); D- quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL). tr- traces (compounds below LOD amounts).

Table 2S. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the phenolic compounds present in the purple tea extract.

Peak	Rt (min)	$\lambda_{max\ ((nm))}$	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg/g extract)
1	4.71	274	305	219(75),179(100),125(11)	(Epi)gallocatechin isomer I[A]	5.67±0.07
2	8.7	281/311	289	245(34), 203(16), 137(27)	(+)-Catechin[A]	1.76±0.01
3	9.85	274	457	331(79),305(73),169(100)	Epigallocatechin gallate[A]	11.6±0.1
4	13.23	335	563	545(30), 473(100), 443(79),383(32), 353(30), 311(2), 297(4)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.046±0.001
5	13.96	336	563	545(17), 473(71), 443(100),383(19), 353(22), 311(3), 297(5)	Apigenin-*C*-hexoside-*O*-pentoside[B]	0.028±0.001
6	14.19	344	625	317(100)	Myricetin-3-*O*-rutinoside[C]	0.674±0.001
7	14.61	352	479	317(100)	Myricetin-3-*O*-glucoside[C]	0.742±0.001
8	14.81	352	479	317(100)	Myricetin-*O*-hexoside[C]	0.676±0.002
9	15.69	353	771	301(100)	Quercetin-*O*-deoxyhexosyl-dihexoside[D]	0.138±0.001
10	17.00	353	609	301(100)	Quercetin-3-*O*-rutinoside[D]	0.319±0.001
11	17.71	353	609	301(100)	Quercetin-*O*-deoxyhexosyl-hexoside[D]	0.167±0.001
12	18.09	347	463	301(100)	Quercetin-3-*O*-glucoside[D]	0.252±0.001
13	19.62	342	549	505(25),301(100)	Quercetin-*O*-malonyl-hexoside[D]	0.196±0.001
14	20.20	346	593	285(100)	Kaempferol-3-*O*-rutinoside[D]	0.274±0.001
15	21.46	344	447	285(100)	Kaempferol-3-*O*-glucoside[D]	0.218±0.002
16	23.93	346	533	489(32),285(100)	Kaempferol-*O*-malonyl-hexoside[D]	0.213±0.003
					Total Phenolic Compounds	23.0±0.2
					Total Flavan-3-ols	19.1±0.2
					Total Other Flavonoids	3.943±0.004

Standard calibration curves: A - catechin ($y = 84950x - 23200$, $R^2 = 1$; LOD = 0.17 µg/mL; LOQ = 0.68 µg/mL); B - apigenin-6-*C*-glucoside ($y = 107025x + 61531$, $R^2 = 0.9989$; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); C - myricetin-3-*O*-glucoside ($y = 23287x - 581708$, $R^2 = 0.9988$; LOD = 0.23 µg/mL; LOQ = 0.78 µg/mL); D- quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL). tr- traces (compounds below LOD amounts)