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Isolation And Characterization Of Natural Alpha-Glucosidase Inhibitors From Antioxidant Rich Red Wine Grapes (vitis Vinifera)

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ISOLATION AND CHARACTERIZATION OF NATURAL α-GLUCOSIDASE INHIBITORS FROM ANTIOXIDANT RICH RED WINE GRAPES (*VITIS VINIFERA*)

by

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DISSERTATION

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DEDICATION

This work is dedicated in loving admiration to my parents, Chaouki and Khadije, whose endless love and support have nourished every step I took forward in my life. While my father is my idol in dedication and hard work, my mother symbolizes the art of sacrifice. Without these values along with persistent encouragement, I certainly would have never pursued my dream of earning a doctorate in science.

In this exciting but long journey, I had an unbeatable source of laughter and hope: my sister and best friend Mona, the funniest person I know. Her amazing sense of always seeing the full half of the cup managed to be contagious at times, and the unforgettable sister trips she put together made these years fly in no time. I dedicate this thesis to her.

Gratitude to an exceptional family to whom I am in dept for my making is difficult to express. It is thanks to you that I am who I am today.

I would also like to dedicate this work to my loving fiancé Houssam Halawi who only chooses to believe in me. Knowing he is there all these years made the hardest of achievements worth accomplishing. As we both struggle with perfectionism, he always manages to convince me that “it really looks great, it does not need to be better”.

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CHAPTER 1

Introduction

1.1. The Obesity Pandemic

Obesity rate in the US has doubled in the last two decades with more than one third of adults and almost 17% of children and adolescents being obese [1, 2]. There has been a synchronized increase in obesity rates in almost all countries, likely due to the increase in affordable processed food worldwide which in turn has created a global overconsumption of energy [3]. While public health initiatives to manage the prerequisites of obesity are crucial, treating its drawbacks is currently a major global concern. This pandemic has shown a detrimental impact on health-related quality of life of affected individuals due to obesity-related comorbidity, specifically cardiovascular disease, type-2 diabetes, obesity-related cancers, osteoarthritis, and psychological disturbance [4]. Needless to say, the resulting economic burden has expanded dramatically [5, 6].

1.2. Obesity, Oxidative Stress and Diabetes

Excessive body fat is directly correlated with an increased generation of systemic reactive oxygen species coupled with a significant reduction in the body's antioxidant capacity [7]. The state of chronic inflammation and oxidative stress that is thus created is believed to play a role in promoting obesity-related complications [8, 9]. In fact, these impairments are thought to directly lead to an inhibition of insulin responses, hence giving rise to insulin resistance and type-2 diabetes [7, 10]. Although the exact mechanism linking oxidative stress with altered insulin signaling is not fully understood,

there is a consensus that impaired insulin signaling is one of the outcomes of oxidative stress, likely through multiple pathways (Fig. 1.1) [11]. Another common metabolic attribute linked to obesity is hyperglycemia [8, 12], which in turn has been associated with the precipitation of oxidative stress and inflammation [13, 14], thus further promoting diabetes and its complications [15-17]. Therefore, oxidative stress appears to partake in both the initiation and the progression of diabetes and its related complications [11].

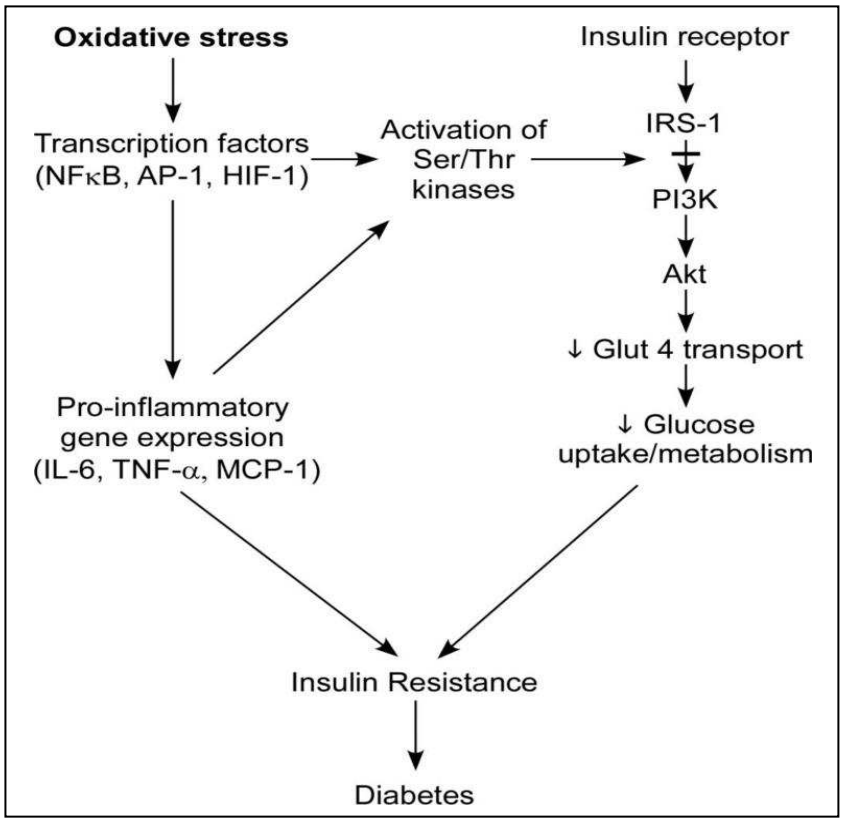

Fig. 1.1. The Effect of Chronic Oxidative Stress on the Insulin Signaling Pathway. Adapted from Rains et al, Free Radic Biol Med. 2011.

1.3. Diabetes in the 21st Century

Given the above stated facts, it is of no surprise that the prevalence of diabetes has risen at an alarming rate (Fig. 1.2). Diabetes currently affects 25.8 million people, that is 8.3% of the U.S. population [18], a number that is projected to double or triple by 2050 [19]. In addition, based on fasting blood glucose or glycated hemoglobin (HbA1c) levels, an estimated 79 million American adults have prediabetes and therefore an increased risk of developing type-2 diabetes and other chronic conditions [18]. Particularly disturbing is the significant increase in the prevalence of type-2 diabetes among children and adolescents, making it no longer an adult-only disease [20]. The diabetes epidemic has become a massive health burden significantly decreasing quality of life and increasing morbidity and mortality among Americans, all at a huge economic cost [18, 21]. The aforesaid statistics are mostly based on an expanding prevalence of type-2 diabetes, a condition depicted by insulin resistance and ß-cell failure with an underlying genetic propensity profoundly influenced by lifestyle and diet [22]. This form of diabetes accounts for about 90-95% of diabetics and begins with a state of slowly progressing hyperglycemia [23]. Patients are at an increased risk for microvascular and macrovascular complications which in turn lead to disability and eventually death. In terms of microvascular complications, they typically tend to develop retinopathy, neuropathy and nephropathy especially at advanced stages of the disease [24]. In fact, diabetes is the leading cause of blindness and non-traumatic lower limb amputations among US adults, and accounts for a considerable percentage of end-stage renal disease occurrences [18]. In terms of macrovascular disease, the incidence of cardiovascular disease and stroke increases significantly with diabetes, due to high

levels of circulating glucose in the blood [24]. In 2004, heart disease and stroke were noted on 68% and 16% of diabetes-related death certificates among US seniors, respectively. The total estimated cost of diabetes in the US was around $175 billion in 2007, and medical expenditures among diabetics were estimated to be around 2.3 times higher than those of non-diabetics [18].

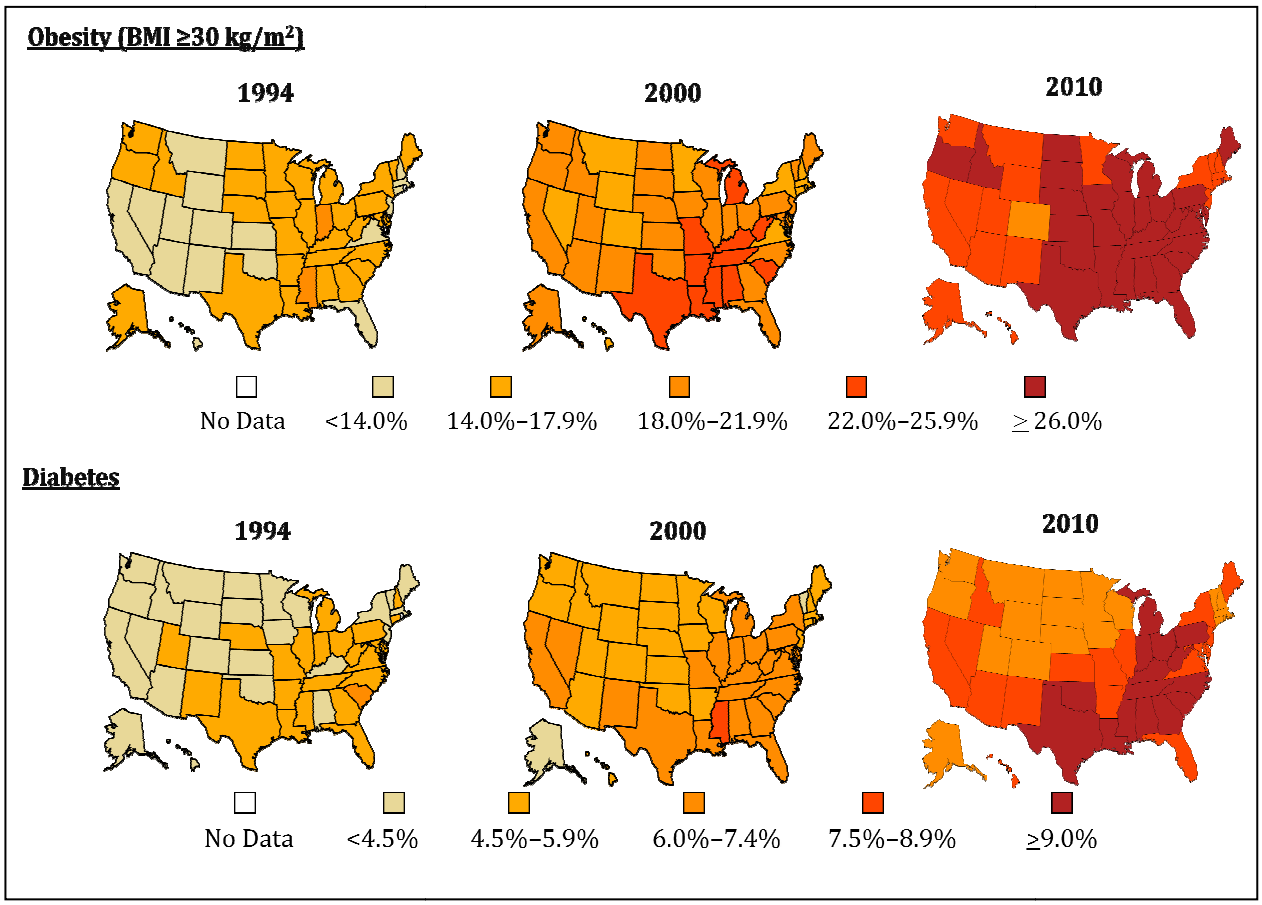

Fig. 1.2. Age-adjusted Prevalence of Obesity and Diagnosed Diabetes Among U.S. Adults Aged 18 Years or Older. Source: CDC's Division of Diabetes Translation. http://www.cdc.gov/diabetes/statistics.

1.4. Conventional Management of Diabetes

Anti-hyperglycemic therapy entails two aspects of treatment: lifestyle modifications and pharmaceutical interventions, with the latter being the major focus in glycemic control. Lifestyle interventions are comprised of dietary and physical activity regimens that promote weight loss through controlling the intake and expenditure of energy resulting in better glycemic control [25]. In terms of pharmaceutical agents, the biguanide metformin is the most commonly used first-line drug for type 2 diabetes. It is an insulin sensitizer that mainly reduces hepatic glucose production [25, 26]. A less common class of insulin sensitizers, thiazolidinediones, reduce blood glucose levels by improving insulin sensitivity in skeletal muscle and reducing hepatic glucose output [27]. Insulin secretion from pancreatic ß-cells may be stimulated by insulin secretagogues, namely sulfonylureas and meglitinides. A newer class of insulin secretagogues, the injectable glucagon-like peptide-1 (GLP-1) receptor agonists, were introduced recently and are focused on the incretin physiology in stimulating pancreatic insulin release [28]. When oral agents are contraindicated or become insufficient, insulin replacement therapy is typically required [29]. Treatment regimens vary among patients and are often modified based on the body's response and disease progression.

1.5. Limitations in Diabetes Management

Conventional anti-hyperglycemic agents seem to be insufficient to contain the widespread problem of diabetes and side effects often limit treatment choices [30, 31]. The most commonly prescribed oral therapies for type-2 diabetes, metformin and sulfonylureas, are successful in initial reduction in blood glucose and complication rates. However, they are often unable to provide durable glycemic control, resulting in the

need for complicated treatment regimens [25]. Additionally, hypoglycemia and weight gain have been reported with sulfonylureas use [32]. Thiazolidinediones have become limited by their association with serious side effects such as weight gain, fluid retention and bone loss [32, 33]. Despite being the most effective treatment, insulin is associated with weight gain, hypoglycemia [30] and considerable economic costs [34]. Even newer agents carry concerns. For instance, GLP-1 receptor agonists are accompanied by nausea and vomiting in addition to an increased risk of pancreatitis [35]. Therefore, there is an urgent need for alternative therapeutic strategies that will provide a broader range of choices while addressing safety and patient-tailored treatment.

1.6. Targeting Postprandial Hyperglycemia

While the traditional goal in managing diabetes is to control fasting blood glucose and HbA1c levels, treatment of postprandial hyperglycemia has become a compelling target to improve overall glycemic control [36-40]. Postprandial hyperglycemia develops early in the course of type-2 diabetes when insulin secretion becomes compromised [39]. It has been accused of the induction of glucose toxicity and ß-cell function deterioration which can ultimately give rise to an irreversible state of diabetes [39, 41]. It is also an independent risk factor for the development of cardiovascular disease, the leading cause of morbidity and mortality among diabetics [42, 43]. Postprandial hyperglycemia is linked to the amount of consumed starch and its rate of digestion, being the chief source of blood glucose [36]. Reducing the amount and rate of carbohydrate digestion and absorption can be an effective approach for postprandial hyperglycemia treatment [44-46]. This can be achieved by inhibiting starch hydrolyzing

enzymes in the digestive tract [46-50], perhaps through the use of food-derived phytochemicals [36, 50].

1.7. Control of Carbohydrate Digestion: α-Glucosidase Inhibition

Mammalian starch digestion takes place primarily in the small intestine through the action of α-amylase, resulting in both linear maltose and branched isomaltose oligosaccharides that are additionally hydrolyzed by α-glucosidases to yield glucose [50-52]. Natural as well as synthetic compounds are known to reduce postprandial hyperglycemia by inhibiting major carbohydrate digesting enzymes in the small intestine, such as α-glucosidase (Fig. 1.3) [53-55]. Inhibition of α-glucosidases has been shown to be effective in both preventing and treating type-2 diabetes through reducing postprandial hyperglycemia [38, 52, 55]. However, commercial inhibitors that have been used for diabetes treatment (i.e. Acarbose) were found to exhibit a non-specific inhibition of α-amylase, resulting in excessive accumulation of undigested carbohydrate in the colon, thus generating undesirable gastrointestinal side effects [36, 56, 57]. Research aiming at identifying novel inhibitors has increased in the last three decades. For instance, numerous plant extracts rich in polyphenols and phenolic compounds isolated from plants have been investigated and reported to be powerful inhibitors of carbohydrate hydrolyzing enzymes [58-60].

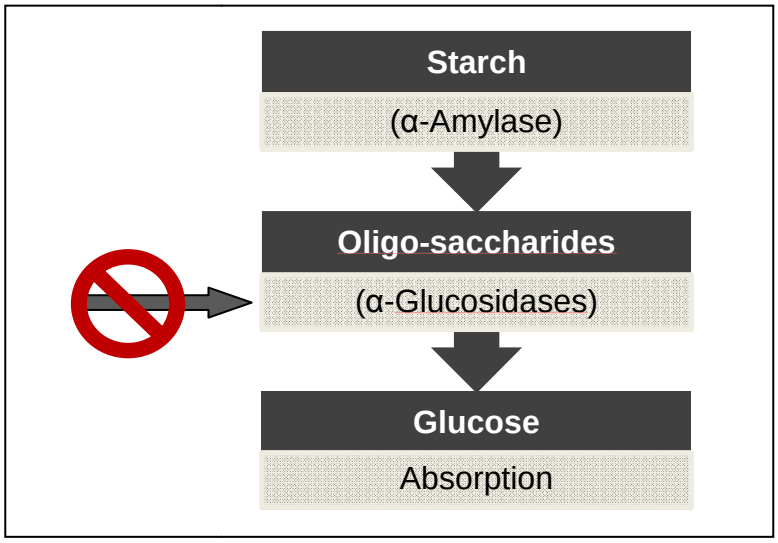

Fig. 1.3. Simplified Schematic of Carbohydrate Digestion and Absorption in the Small Intestine; Inhibition of α-Glucosidases to Reduce Glucose Absorption.

1.8. Natural Products in Drug Discovery

Natural products, either as extracts or as pure compounds, possess an immense potential as new drug leads due to their exceptional chemical diversity [61]. Plant sources continue to serve as an inexhaustible source of bioactive compounds [62]. Concurrently, consumer demand for alternative treatments is persistently increasing. In fact, the abundance of plant compounds is customary in natural food stores, and unconventional therapy is becoming a common choice among many consumers. Nevertheless, the purity of these substances is uncertain and available information regarding dosage is limited [62, 63]. The public perception of gentleness of natural medicines, the high cost of currently available synthetic medicines, and adverse side-effects of pharmaceuticals have together created a need to further develop natural products. The screening of natural preparations has become pivotal in the discovery of

various drugs [64]. For instance, in a screening for natural, food-derived α-glucosidase inhibitors, we identified a red grape pomace extract to be the most potent in inhibiting α-glucosidases, among over 300 extracts and compounds tested [31]. Further investigation yielded promising specificity and effectiveness data *in vitro* and *in vivo*, respectively [31, 65]. However, comparison of a wider range of grape pomaces and identification of the components responsible for the inhibitory activity have not been performed, to our knowledge.

1.9. Grape-Derived Bioactives and Grape Pomace

Grapes, namely red wine cultivars, are known to be among the highest antioxidant containing fruits [66, 67] and their pomaces have particularly been found to be rich in polyphenols and other antioxidants [68-70]. Grape pomace is the solid remains of grape following pressing for juice. It consists of the dry pulp with intact skin, seeds and stem, thus retaining a considerable amount of functional compounds that normally reside in these parts [71, 72]. Yet, grape pomace is considered a waste byproduct generated in the winemaking industry [73]. As a result, a waste-management issue arises from the accumulation of big loads of this byproduct annually [74]. The very limited uses of grape pomace include recycling as animal feed, organic fertilizers, and manure [31]. Meanwhile, grapes continue to be studied and recognized as a natural source of prominent bioactive compounds with potential health promoting and disease preventing properties [66, 68, 75]. For instance, remarkable amounts of polyphenolic compounds are found in grapes, grape seed extracts and wine [75-78]. Due to their ability to inhibit peroxidation chain reactions, dietary antioxidants have been associated

with a reduced risk of type 2 diabetes [79, 80]. However, literature on the potential of grape pomace as an alternative bioresource for diabetes management is very minimal.

CHAPTER 2

BACKGROUND, HYPOTHESIS AND SPECIFIC AIMS

Objective and Significance of the Study

Given the overwhelming rise in diabetes, it is imperative to explore novel approaches to prevent and control it. The current research evaluated the anti-diabetic potential of a selection of six red wine grape pomaces by determining their α-glucosidase inhibiting and antioxidant activities. After selecting the most potent variety, we isolated and identified the components responsible for the inhibiting activity, studied their specificity and dose response, and determined their stability, cytotoxicity and antioxidant capacity. This research may provide a foundation for the future development of a food-derived α-glucosidase inhibitor from grape pomace for preventing and treating diabetes, thus establishing a novel, safe dietary anti-diabetic strategy.

Hypothesis

We hypothesized that antioxidant rich red wine grape pomace contains components that possess an anti-diabetes functional food potential through specifically inhibiting intestinal α-glucosidases.

To test our hypothesis, we pursued 3 studies as highlighted next.

STUDY 1: To Identify a Specific Grape Variety with Anti-Diabetes Functional Food Potential through α-Glucosidase-Inhibiting and Antioxidant Capacities (Fig. 2.1).

Screening of plant-derived compounds for biological activity usually begins with an initial screening involving crude extracts of multiple plants or varieties of a plant [81]. α-glucosidases play a significant role in carbohydrate digestion and therefore postprandial blood glucose, a target for diabetes management [82]. The comparison of the α-glucosidase inhibitory potential of several crude grape pomace extracts allows the identification of the grape variety that is potentially rich in the inhibiting compounds.

Therefore, six red wine grape varieties were selected for screening: Chambourcin (hybrid), Merlot (Vitis vinifera), Norton (Vitis aestivalis), Petit Verdot (Vitis vinifera), Syrah (Vitis vinifera) and Tinta Cão (Vitis vinifera), and α-glucosidase inhibition assay was utilized to compare their inhibiting potential.

Additionally, numerous health protective functions have been attributed to antioxidants over the last few decades [75, 83, 84], suggesting that a bioactivity exhibited by a grape extract may be related to its antioxidant content. A review of literature on plant-derived α-glucosidase enzyme inhibitors indicates that known antioxidant compounds such as polyphenols, flavonoids and others have exhibited inhibitory activity *in vitro [82, 85]*. This brought about the need to investigate and compare the antioxidant makeup of our 6 grape varieties. Total Phenolic Content (TPC) assay is a popular method for quantifying antioxidants in a sample, as gallic acid equivalents [86]. Antioxidant capacity is usually further assessed by evaluating the sample's ability to scavenge free radicals. Two assays commonly serve this purpose.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay [87] and Oxygen Radical Absorbance Capacity (ORAC) Assay with 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) as the free radical generator [88]. Finally, inference on individual antioxidants in samples can be obtained via High Performance Liquid Chromatography (HPLC) profiling using known standards for comparison [89, 90].

Hence, the abovementioned universal antioxidant assays were employed to quantify the antioxidant content while HPLC profile comparison allowed the detection of major differences as well as specific antioxidant compounds.

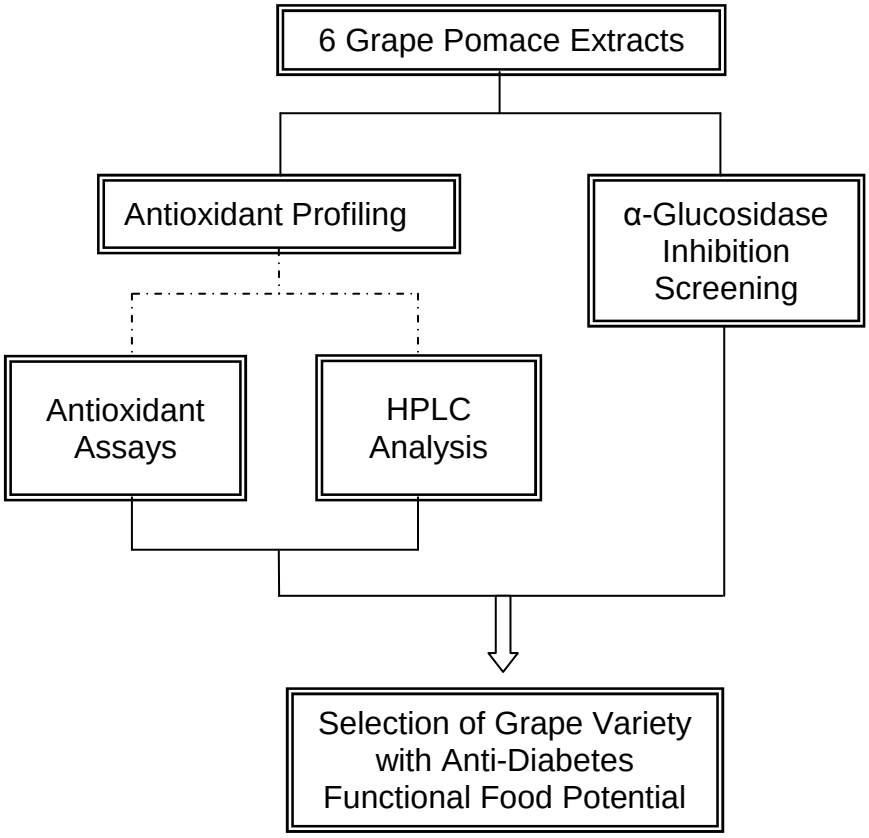

Fig. 2.1. Schematic of the Study Design for Study 1.

STUDY 2: To Isolate and Identify Grape Pomace Components with α-glucosidase Inhibiting Activity via Bioactivity-Guided Fractionation (Fig. 2.2).

A crude plant extract is a complex combination of bioactive compounds and phytochemicals, out of which only one or a few are responsible for the functional property of interest. Separation hence poses a challenge, usually involving various steps and multiple fractionation techniques [91]. Liquid-liquid extraction is a helpful initial step to break down the crude extract into parts based on their relative solubility in two different immiscible liquids [92]. When an active fraction is identified through bioassay, a separation plan is made based on the predicted characteristics inferred from partition, such as polarity, solubility, etc. Column chromatography is a popular method used to purify individual chemical compounds from complex mixtures [93].

Therefore, the active grape pomace extract (GPE) was separated into fractions via liquid-liquid extraction and sub-fractions via column chromatography on a bioactivity guided fractionation basis to select the most active GPE sub-fraction.

Column chromatography often generates simple fractions of unknown concentrations. A sensitive purification and quantification technique must hence follow. High Performance Liquid Chromatography (HPLC) is a powerful analytical tool that is usually the method of choice [94]. The determination of the chemical structure and formula of the isolated compound is then achieved via combinatorial chemistry, utilizing compound libraries. Integration of mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectral data is the gold standard for structure verification in analytical chemistry [95].

Compounds within the active sub-fraction were then separated and purified using column chromatography and HPLC and active compounds were selected by bioassay. NMR and MS were employed to characterize the active compounds.

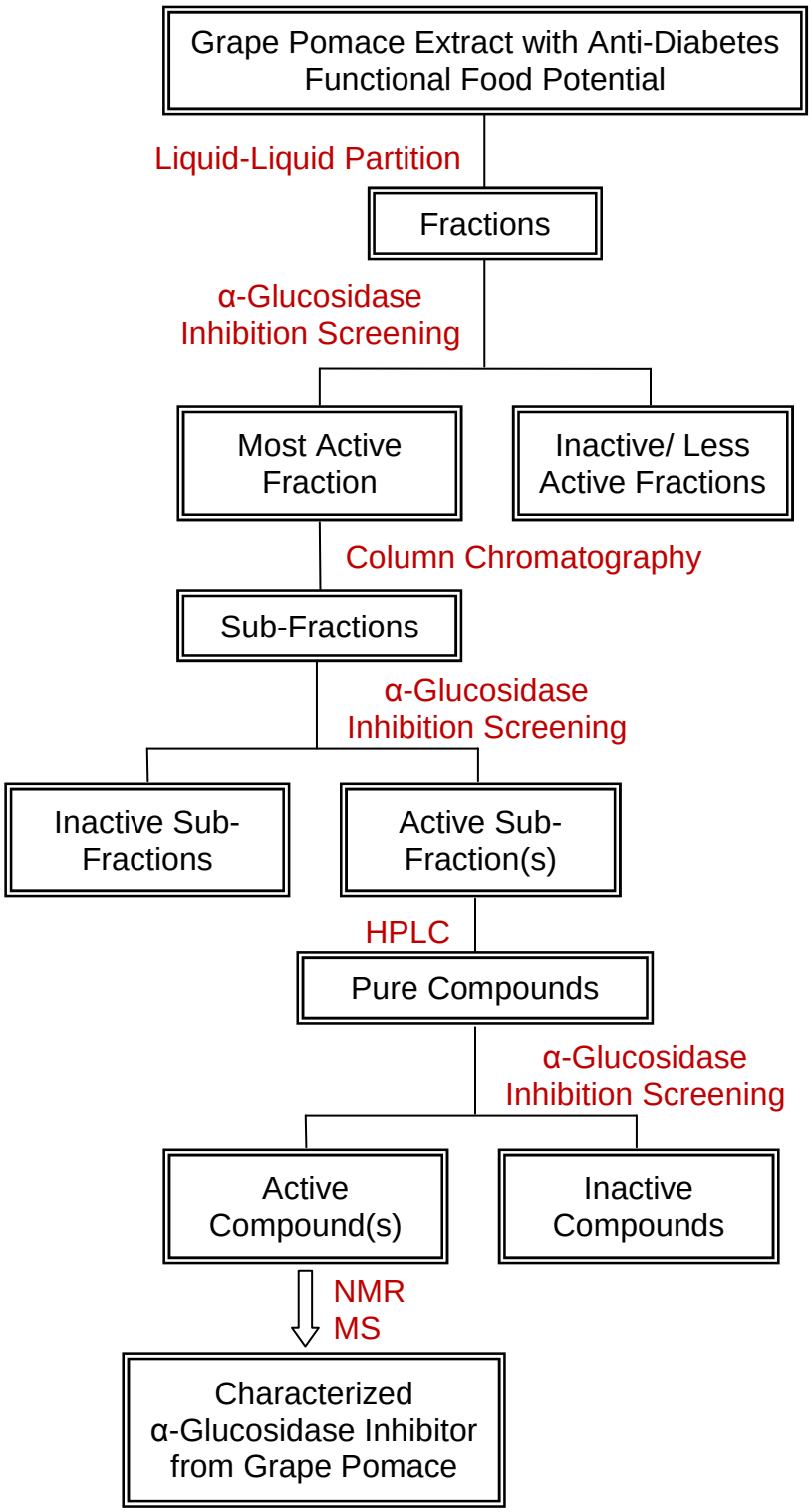

Fig. 2.2. Schematic of the Study Design for Study 2.

Study 3: To Determine the Applicability of the Isolated Grape Pomace Component as a Natural Inhibitor of α-Glucosidases (Fig. 2.3).

The process of drug development requires specific measures of mode of action and effectiveness prior to the procession to toxicological, pre-clinical and clinical testing. Mammalian intestinal α-glucosidase enzyme complex is comprised of three enzymes: sucrase, maltase and isomaltase, responsible for the digestion of sucrose, maltose and isomaltose, respectively [96]. It is necessary to identify which enzyme(s) in the complex is/are inhibited by the tested GPE components. Also, it has been reported that enzymes falling under the glycoside hydrolase family 13 such as α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) share a common reaction mechanism and several short conserved sequences [97], which is why a non-specific inhibitor will likely inhibit both enzymes. α-Amylase inhibition by our compound must be ruled out. It is also essential to understand whether the observed bioactivity is dose-dependent. Lastly, it is important to evaluate the antioxidant capacity of the isolated compound to draw possible relations between α-glucosidase inhibition and antioxidant activity, and to understand the compound's scope of bioactivity.

For the above reasons, the active component's enzyme inhibitory activity was tested against α-amylase and three individual α-glucosidases to identify and verify specificity, and antioxidant assays were employed to evaluate the antioxidant activity of the active compound.

The problem of instability is often encountered with natural medicines. In the course of development of an herbal drug, the determination of stability of the drug in the

proposed active form is essential. Stability can be affected by environmental factors such as temperature, light, air and humidity. Intrinsic factors such as particle size, pH, and solvent properties can also have a significant impact on stability [98]. Hence, heat treatment and pH treatment are required to determine if the product has potential use as a commercial bioactive applicable to food products. Another necessary safety measure is cytotoxicity studies. Given the fact that plants have been eaten and used in traditional medicine for centuries, it is not uncommon to believe that all compounds derived from natural sources are safe for human consumption. However, an isolated active compound requires testing to ensure it is safe in pure form.

The effect of heat treatment on the activity of the active component was assessed via bioassay. Similarly, bioassay was used to observe activity under various pH levels. The inhibitory activity of the isolated compound was also assessed via bioassay following storage under various conditions for a number of months. Lastly, the compound was tested for cytotoxicity using a normal animal cell line.

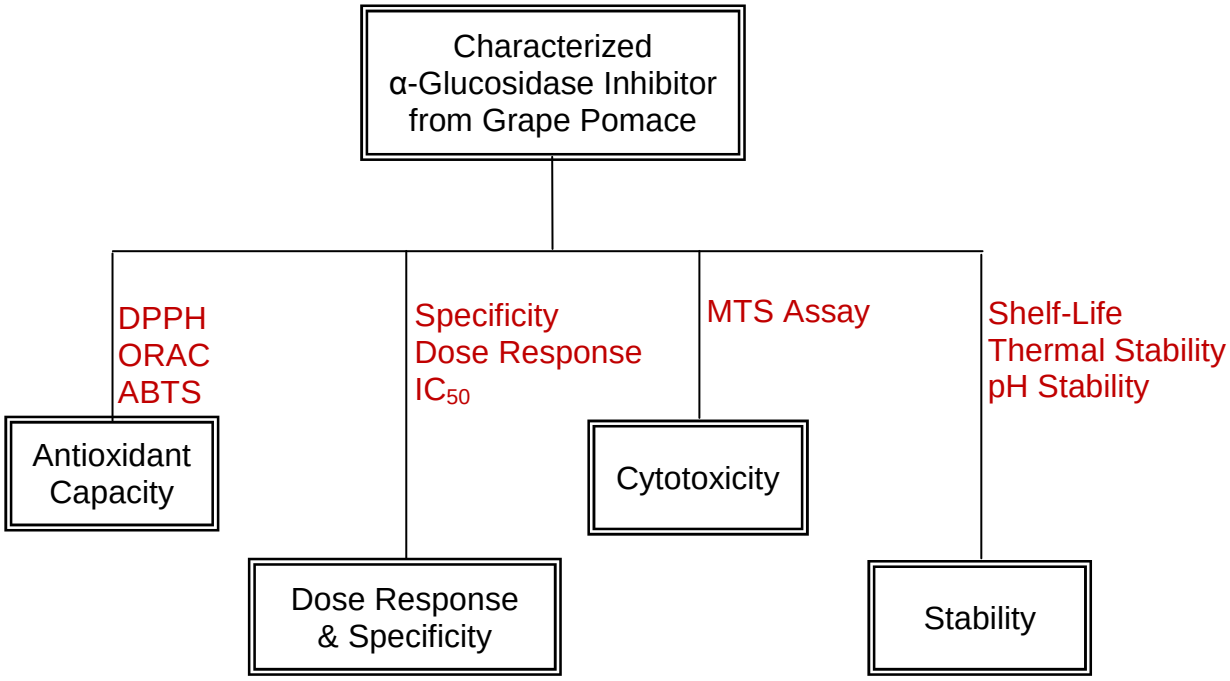

Fig. 2.3. Schematic of the Study Design for Study 3.

CHAPTER 3

α-Glucosidase Inhibiting Activity and Antioxidant Properties of Six Red Wine Grape Pomace Extracts

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Abstract

Dietary antioxidants have been associated with a reduced risk of type 2 diabetes. Grape pomace contains considerable amounts of polyphenols and it has been reported that grape pomace also exhibits specific inhibitory activity against alpha- glucosidases. This study aims to investigate the anti-diabetes potential of Chambourcin, Merlot, Norton, Petit Verdot, Syrah and Tinta Cão red wine grape pomaces by assessing their rat intestinal α-glucosidase inhibiting activity and antioxidant properties as well as their relationship. Among the selected pomaces, Tinta Cão, Syrah and Merlot extracts were the most potent inhibitors of α-glucosidase. These three varieties also appeared to have the highest respective total phenolic content. Chambourcin, Merlot and Tinta Cão exhibited the highest DPPH radical scavenging capacity, while Tinta Cão exceeded all other varieties in oxygen radical (AAPH) absorbing capacity. A strong positive correlation was observed between these results, suggesting that the α-glucosidase inhibiting potency of grape pomace extracts may be related to their richness in antioxidants. The phenolic compounds in the extracts were further purified and profiled using HPLC, and major differences in the concentrations of the profiled antioxidants were detected. However, none of these antioxidants individually was able to inhibit intestinal α-glucosidases in bioassay. Red grape pomace, namely Tinta Cão, appears to be a promising functional food for the future development of a food-derived α-glucosidase inhibitor for preventing and treating diabetes.

Key words: Grape Pomace; Antioxidant; α-Glucosidase; Diabetes

Introduction

The prevalence of obesity in the US has magnified in the last 20 years [2]. The state of chronic inflammation and oxidative stress that obesity has been associated with is believed to play a role in promoting obesity-related complications such as insulin resistance and type-2 diabetes [8, 9]. Another common metabolic attribute linked to obesity is hyperglycemia [8, 12], which in turn has been associated with the precipitation of oxidative stress and inflammation [13, 14], thus further promoting diabetes and its complications [15-17]. It is hence of no surprise that diabetes currently affects 25.8 million people in the U.S. and the number of Americans with prediabetes is on the rise. The costs associated with diabetes and its consequences have become a significant burden in the American society [18].

Type-2 diabetes is a chronic condition characterized by insulin resistance and ß-cell failure resulting from lifestyle habits that interact with an underlying genetic susceptibility [22]. Given the overwhelming rise in this disease, it is imperative to explore novel approaches to prevent and control it, particularly in the light of the side effects and limited long-term durability associate with conventional anti-hyperglycemic agents [30]. Inhibition of α-glucosidases has been shown to be effective in both preventing and treating type-2 diabetes through reducing postprandial hyperglycemia [38, 55]. However, commercial inhibitors often come with gastrointestinal side effects due to their non-specific inhibitory activity [56, 57]. This necessitates the search for alternatives. Meanwhile, plant sources continue to serve as an inexhaustible source of bioactive compounds [62]. In a screening for natural, food-derived α-glucosidase inhibitors, we identified a red grape pomace extract possessing specific α-glucosidase

inhibitory activity [31]. However, comparison of a wider range of grape pomaces and obtaining inference on the components responsible for the inhibitory activity have not been achieved, to our knowledge.

Grape pomace, the solid remains of grape after pressing, is commonly considered a waste byproduct generated in the winemaking industry [73]. On the other hand, grapes and wines are widely acknowledged as an important source of antioxidants, namely polyphenolic compounds such as flavanols, catechins, anthocyanins, and proanthocyanidins [75-78]. Since grape pomace is chiefly comprised of the skins and seeds, it is surmised that this biomass is a rich source of antioxidants [68, 69, 71]. While the literature associates dietary antioxidants with a reduced risk of type 2 diabetes [79, 80], it provides very limited information on the potential of grape pomace as an alternative bioresource for diabetes management.

The aim of this study is to evaluate the anti-diabetic potential of a selection of six red wine grape pomaces by determining their α-glucosidase inhibiting and antioxidant activities as well as their relationship. This research may lay the foundation for the future development of a safe, food-derived α-glucosidase inhibitor from grape pomace for preventing and treating type 2 diabetes.

MATERIALS AND METHODS

Materials

The organic solvents for grape pomace extraction and HPLC analysis were HPLC grade (Fisher Scientific, Atlanta, GA). Intestinal acetone powders from rat, 4-nitrophenyl-α-D-glucopyranoside (pNPG), Folin–Ciocalteu reagent, gallic acid, 2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) and phenolic standards including caffeic acid,

delphinidin chloride, gallic acid, malvin chloride, malvidin chloride, quercetin hydrate and quercetin 3-O-glucoside were purchased from Sigma-Aldrich (St. Louis, MO). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Acarbose and other phenolic standards including catechin, epicatechin gallate, kaempferol, myricetin and resveratrol were obtained from LKT Laboratories, Inc. (St. Paul, MN). Fluorescein and phenolic standards including cyanidin chloride and *p*-coumaric acid were purchased from Fluka Analytical (Buchs, Switzerland). Trolox and rutin were purchased from ACROS (Geel, Belgium).

Grape Pomace

Six red wine grape varieties: Chambourcin (hybrid), Merlot (*Vitis vinifera*), Norton (*Vitis aestivalis*), Petit Verdot (*Vitis vinifera*), Syrah (*Vitis vinifera*) and Tinta Cão (*Vitis vinifera* were kindly provided by Chrysalis Vineyards (Middleburg, VA). The pomaces were shipped immediately after pressing. Upon receipt of the samples, they were immediately dried in a food dehydrator at 95 ^{o}F for 28 h.

Sample Extraction

The pomaces were separated from stems and ground to a powder consistency followed by the manual removal of visible solid impurities. Grape pomace powder was soaked and stirred overnight at 450 rpm in aqueous acetone at a concentration of 0.1g/ml and supernatants were spun at 1,000 rpm for 5 minutes. Supernatants were retained and filtered using a 20 µm Whatman filter paper via suction filtration with pump-generated vacuum. The filtered extract was then transferred to a Buchi Rotavapor where the solvent was isolated via evaporation at 50 to 180 RPM and 40 to 60 ^{o}C, in gradual increments, and condensation at 4-8 ^{o}C to obtain a solvent-free grape pomace

extract in pure water. The extract was frozen at -80 ^{o}C, lyophilized and stored in powder form at 4^{o}C for use in screening. The prepared grape pomace extract (GPE) powders were reconstituted with aqueous acetone and diluted with ddH_2O to a concentration of 0.5 mg/ml.

α-Glucosidase Inhibition Screening

i. Preparation of rat α-Glucosidases

Intestinal acetone powders from rat were extracted with 0.05 M phosphate buffer (PB) pH 6.8 at a concentration of 25 mg/ml. The solution was soaked and stirred overnight at 450 RPM and supernatants were isolated and spun at 1,000 rpm for 5 minutes. Supernatants were retained and filtered via vacuum filtration using a 20 µm Whatman filter paper. The filtered solution was frozen at -80^{o}C, lyophilized and reconstituted with 0.05 M PB pH 6.8 to a concentration of 25 mg/ml. Ready-to-use aliquots of this concentration were stored at -20^{o}C.

ii. α-Glucosidase Inhibition Assay

α-Glucosidase enzyme at 25 mg/ml was used from prepared aliquots. 4-nitrophenyl-α-D-glucopyranoside (pNPG) was used as a substrate at a 4 mM concentration. Briefly, α-glucosidase enzyme complex hydrolyzes pNPG and releases *p*-nitrophenol (pNP). Reading the absorbance quantitates the release of pNP thus representing enzymatic activity [36]. Acarbose, known to inhibit α-glucosidase enzyme complex and used as an oral blood glucose lowering drug in diabetes [57], served as a positive control at 50 µg/ml. Enzyme, substrate and positive control solutions were prepared in the blank reagent (0.05 M PB pH 6.8) which is in turn used as a negative

control. GPE samples, prepared as described above, were screened using this assay. Ninety six-well bioassay microplates were prepared to contain 115 µl of sample or control, 90 µl of enzyme solution and 45 µl of substrate solution per well, mixed thoroughly. Absorbance at a wavelength of 405 nm was obtained at start of the reaction using a Perkin Elmer HTS 7000 Bio Assay Reader and software (Perkin Elmer, Norwalk, CT). The microplate was then incubated at 37 ^{o}C and absorbance reading was obtained again at 30 and 90 minutes with intense shaking between cycles. The absorbance reading, representing the concentration of pNP, was then used to compare the activity of the tested samples: the lower the reading, the less active the enzyme, and thus the more active the sample. Percent inhibition by all samples was calculated and compared to controls to determine potency, using the following formula:

% Inhibition = 100 – {(Abs_{sample}/ $Abs_{control}$) x 100}.

Antioxidant Assays

i. Total Phenolic Content (TPC) Assay

TPC was evaluated with Folin–Ciocalteu's phenolic reagents. Samples were diluted to 2 mg/mL with aqueous acetone. Gallic acid was used as a standard for preparing the standard curve. All the samples and standards were run in triplicates. Each test tube contained 25 µL of a sample or standard and 250 µL distilled water. 750 µl Folin-Ciocalteu's phenol reagent was then added to each tube and mixed using a vortex mixer. Then, 500 µL of 200 mg/ml sodium carbonate was added to each tube and mixed thoroughly. Samples and standards were incubated for 2 h at room temperature in the dark. Absorbance was detected at 765 nm and the TPC of each sample was expressed as milligrams of gallic acid equivalents (GAE) per mg GPE.

ii. DPPH Radical Scavenging Assay

This assay measures the ability of our samples to quench DPPH radicals. Samples were diluted to 5 mg/ml with aqueous acetone and then centrifuged at 6900 ×*g* for 20 min to eliminate residues [99]. 100 µl of each sample was mixed with 150 µl of DPPH radical solution in a 96-well microplate and absorbance was measured at room temperature every 5 min for 2 h at 500 nm. All samples were prepared in triplicates. After subtracting sample backgrounds at all time points, the percent scavenging capacity was calculated using the following equation:

Scavenging Effect (%) = $\{(Abs_{start} – Abs_{timepoint}) / Abs_{start}\} \times 100$.

iii. Oxygen Radical Absorbing Capacity ($ORAC_{FL}$) Assay

$ORAC_{FL}$ assay was performed as described by Zhou et al with slight modifications. Samples were diluted with aqueous acetone to a concentration of 0.1 mg/ml. Trolox at a concentration gradient served as standard [100]. All samples and standards were assayed in triplicates. In each well of a 96-well microplate, 200 µL of 8 µM fluorescein (in 75 mM PB pH 7.4) was mixed with 40 µL of sample or standard. The plate was then shaken and incubated for 15 min at 37 ℃ and 35 µL of 0.36 M AAPH was added to each sample, then fluorescence was measured every 5 min for 90 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Results are expressed as µmol trolox equivalent (TE)/g dried GPE weight.

High Performance Liquid Chromatography Analysis

Fifteen antioxidant compounds, typically reported in grape and wine, were used as antioxidant standards to identify and quantify antioxidants in our GPE samples. The

extracts were first cleaned using solid phase extraction (Oasis HLB 6 cc extraction cartridge, Waters Corporation, Milford, MA) to remove sugar and other contaminants. After drying with nitrogen gas, each sample/ standard was dissolved in methanol and filtered using a 0.45 micron, 3 mm syringe filter. Reversed-phase HPLC was employed to profile individual antioxidants in the cleaned extracts against known phenolic standards, using a Hitachi HPLC system (Model L-2455 Diode Array Detector, Model L-2200 Autosampler, Model L-2100/2130 Pump) from Hitachi High-Tech Technologies (Tokyo, Japan). A Phenomenex Aqua 5 µm C_{18} 250 x 4.6 mm analytical column (Phenomenex, Torrance, CA) represents the stationary phase while methanol, 0.5% acetic acid in 50% acetonitrile and 2% acetic acid were utilized as mobile phase solvents A and B, respectively. Twenty µL of each sample was injected via the autosampler at a 0-5689 psi pressure range, under room temperature. Gradient systems were used as follows: 10–26% A, 0–8 min; 26% A, 8-15 min; 26-30% A, 15–20 min; 30-55% A, 20–42 min; 55-87% A, 42–75 min; 87-100% A, 75-78 min; 100% A, 78-83 min; 100-10% A, 83-85 min; 10% A, 85-90 min. Flow rate was set at 1 ml/min. Samples and standards were monitored by UV detection and profiled at a wide range of wavelengths (200–700 nm), selecting the optimal wavelength for comparison. Profiles of standards and samples were compared and antioxidants were detected and quantified on the basis of their retention time and UV spectrum.

α-Glucosidase Inhibition Screening of Antioxidant Standards

The protocol described earlier was utilized. Antioxidant standards were reconstituted in aqueous acetone to a 0.5 mg/ml concentration and screened for α-glucosidase inhibiting activity.

Statistical Analysis

Results were analyzed via IBM SPSS 22.0 for Windows (IBM Corp., Armonk, NY) using one-way analysis of variance (ANOVA). Tukey's HSD post-hoc analyses were employed to compare outcomes using $P < 0.05$ as a cutoff point for statistical significance. Pearson's correlation was conducted to study the relationship between variables. Data for each dependent variable is reported as mean $\pm$ SEM.

RESULTS

Inhibition of Mammalian α-Glucosidases

Percent enzyme inhibition by GPEs is presented in Fig. 3.1. With the exception of Petit Verdot, the selected GPEs showed potent inhibition against rat intestinal α-glucosidases. At a concentration of 0.5 mg/ml, Tinta Cão exerted the strongest inhibition of intestinal α-glucosidases, measured as 95% ($P < 0.05$). Chambourcin, Norton, Merlot and Syrah also exhibited significant activity, ranging from 72% to 88% inhibition. The inhibitory effect of these samples surpassed that of Acarbose, a commercial α-glucosidase inhibitor which exerts ~50% inhibition at 50 µg/ml under the described assay conditions. Petit Verdot, on the other hand, demonstrated a poor inhibitory activity of 7%.

Total Phenolic Content (TPC)

As shown in Fig. 3.2, all the tested pomace samples contained noticeable amounts of phenolic compounds at the tested concentration of 2 mg/ml, with the exception of Petit Verdot. Merlot GPE contained the highest TPC (0.29 mg GAE/mg) followed by Syrah GPE (0.28 mg GAE/mg), Tinta Cão GPE (0.26 mg GAE/mg),

Chambourcin GPE (0.19 mg GAE/mg) and Norton GPE (0.14 mg GAE/mg), while Petit Verdot GPE contained the least TPC (0.06 mg GPE/mg, $P < 0.05$).

DPPH Radical Scavenging

Antioxidant capacity was evaluated by DPPH radical scavenging assay. Five milligrams of the Chambourcin, Merlot and Tinta Cão GPEs quenched 58%, 51% and 49% of DPPH radicals in the reaction at 120 min, respectively. A lesser yet remarkable quenching of the radical was observed with similar concentrations of Syrah (36%) and Petit Verdot (34%) GPEs. At the same conditions, Norton GPE scavenged 26% of the DPPH radical. All values were significantly higher than the control ($P < 0.05$), an identical reaction containing the sample solvent. Percent DPPH scavenging per tested GPE is portrayed in Fig. 3.3.

Oxygen Radical Absorbance Capacity ($ORAC_{FL}$)

The $ORAC_{FL}$ assay enabled the evaluation of the scavenging capability of the selected GPEs against peroxyl radicals (AAPH). As depicted in Fig. 3.4, the six varieties appeared to possess a notable oxygen radical scavenging activity at 0.1 mg/ml. Tinta Cão GPE exerted the highest $ORAC_{FL}$ value, presented as 3204. Closely, Syrah quenched the peroxyl radical effectively at 3169 µmol TE/g, followed by Norton, Chambourcin and Merlot GPEs which yielded the respective $ORAC_{FL}$ values of 2918, 2878 and 2832 µmol TE/g. Petit Verdot again exhibited lower radical quenching, estimated as 1960 µmol TE/g ($P < 0.05$).

Correlation

Table 3.1 summarizes the strong positive correlation that was detected when comparing the trends observed in α-glucosidase inhibition, phenolic content, DPPH

quenching and AAPH absorbing activity. Using Pearson's Correlation, the association was noted when comparing each two assays. The correlation was significant between α-glucosidase inhibition and both TPC and ORAC ($P < 0.01$), as well as TPC with both ORAC and DPPH ($P < 0.01$ and $P < 0.05$, respectively). Although DPPH assay results did not exhibit a significantly strong correlation with either α-glucosidase inhibition or ORAC results, there was a positive correlation.

Phenolic Acid Composition

HPLC chromatograms of standards and samples are displayed in Fig. 3.5 and Fig. 3.6, respectively. All profiled antioxidants were detected in the 6 GPE samples, in varying concentrations. The highest and lowest concentrations of most antioxidants were observed in the Chambourcin and Petit Verdot varieties, respectively. The sum of concentrations of detected antioxidant compounds was highest by far in Tinta Cão GPE (460.6 mg/g), most attributable to the anthocyanin malvidin chloride (439.08 mg/g), and lowest in Petit Verdot (11.13 mg/g), with consistently low concentrations of most antioxidant compounds, except for caffeic acid (2.10 mg/g), which was most concentrated in Petit Verdot GPE among the tested varieties. Sum of concentrations of the profiled antioxidants ranged from 23.74 to 145.35 mg/g in the remaining varieties. Table 3.2 summarizes the computed data.

α-Glucosidase Inhibiting Activity of Antioxidant Standards

α-Glucosidase inhibition screening of the known antioxidants detected in the six GPE samples revealed no inhibitory activity.

DISCUSSION

Alpha-glucosidases play a significant role in carbohydrate digestion and absorption and therefore postprandial blood glucose, a target for diabetes management [82]. The comparison of the α-glucosidase inhibitory potential of several crude grape pomace extracts allows the identification of the grape variety that is potentially rich in the inhibiting compounds. Although yeast α-glucosidase is readily available in pure form and widely used for nutraceutical investigations [101, 102], α-glucosidase from mammalian source is more biologically relevant. The mammalian enzyme complex was hence extracted and purified from rat intestinal powder. The presented α-glucosidase inhibition data is consistent with our previous findings indicating that red wine grapes are strong inhibitors of the enzyme [31], with exception to Petit Verdot variety. Having obtained the grape pomaces from the same vineyards and followed a consistent sample preparation protocol, our findings suggest that Tinta Cão exceeds other tested varieties in inhibitory activity due to varietal differences rather than differences in growth and preparation conditions.

The richness of grapes and their pomaces in antioxidants [66-70], and the fact that numerous health protective functions have been attributed to antioxidants over the last few decades [75, 83, 84], together suggest that a bioactivity exhibited by a grape extract may be related to its antioxidant content. A review of literature on plant-derived α-glucosidase enzyme inhibitors indicates that known antioxidant compounds such as polyphenols, flavonoids and others have exhibited inhibitory activity *in vitro [82, 85]*. This brought about the need to investigate and compare the antioxidant makeup of our 6 grape varieties. Hence, universal antioxidant assays were employed to quantify the

antioxidant content while HPLC profile comparison allowed the detection of major differences as well as specific antioxidant compounds.

According to our results, the tested grape pomace varieties are rich in phenolic compounds, with the exception of petit verdot which had the lowest TPC value. Merlot, Syrah and Tinta Cão pomace extracts appeared to contain the highest amounts of phenolic compounds, with these compounds accounting for 29%, 28% and 27% of the dried weight extract of these varieties, respectively. Although higher TPC has been previously reported in red grape pomace extracts, like for example Norton (48%, 80% ethanol extract) [65] and Bangalore (36%, methanol extract) [71], differences may be attributed to source and extraction method/solvent. Interestingly, our observed trend appears to go in parallel with our aforementioned α-glucosidase inhibition results. Our results hence not only indicate that these three varieties are particularly rich in antioxidants, but also hint that the antioxidant content may have contributed to the observed enzyme inhibition potency.

DPPH radical quenching rate of 34-58% suggests that our GPE samples are strong free radical scavengers, compared to previously tested grape extracts. For instance, ranges of 12.5% to 66.7% have been reported with grape skin extracts [103, 104]. Amongst our tested varieties, Chambourcin, Merlot and Tinta Cão seem to exhibit the strongest antioxidant activity in terms of quenching the DPPH radical, while Norton demonstrated the least ability in scavenging the radical.

The tested GPEs had $ORAC_{FL}$ values ranging between 2970 and 4878 μmol TE/g dried pomace extract, which is remarkably higher than reported $ORAC_{FL}$ values of 5-92 μmol TE/g fresh weight of common fruits and vegetables [65]. For instance, we

have previously estimated the $ORAC_{FL}$ value of Norton grape to be 22.9 μmol TE/g fresh fruit weight [105]. It hence appears that pomace extracts exert remarkably higher peroxyl radical scavenging activity than fresh grapes. When comparing the tested varieties in the current study, Tinta Cão appeared to exhibit the highest $ORAC_{FL}$ while Petit Verdot was at the lower end of the range, in line with their α-glucosidase inhibiting capacity and TPC levels.

When conducting correlation tests to compare the stated trends, we observed a positive correlation between all four assays, though not particularly significant between all pairs. α-Glucosidase inhibition data correlated strongly with both TPC and $ORAC_{FL}$ data suggesting that the varieties with a stronger enzyme inhibition capacity also exhibited a stronger antioxidant capacity (peroxyl radical scavenging), likely due to their richness in phenolic compounds. TPC appeared to be significantly correlated with both DPPH and ORAC values. Although several studies correlating TPC and radical scavenging results in fruits found that higher TPC does not always correspond to higher radical absorbing ability [105, 106], many others have observed a strong positive correlation between these parameters [20, 107, 108]. Our data is consistent with the studies rendering high antioxidant activity to richness in phenolics [104]. Despite being positively correlated with all assays, DPPH results were only significantly correlated with TPC. This perhaps may be attributed to the fact that total phenolic content corresponds to a total that contains a variety of antioxidants possessing different mechanisms of action [109]. Furthermore, the strong α-glucosidase inhibiting activity exhibited by our samples may be related to those polyphenols that quench the peroxyl radical, but not necessarily the DPPH radical.

Finally, inference on individual antioxidants in our samples was obtained via HPLC profiling, to detect major differences that may explain the observed trends. The anthocyanins cyanidin chloride, delphinidin chloride, malvidin chloride and malvin chloride, the flavanols catechin and epicatechin gallate, the flavonols kaempferol, myricetin, quercetin hydrate and quercetin 3-o-glucoside, the flavone rutin, the hydroxycinnamates caffeic acid and *p*-coumaric acid, the stilbenoid resveratrol and the non-flavonoid phenolic compound gallic acid, have been abundantly reported in grapes, particularly red grapes and their extracts and wines [83, 110-112]. They were hence selected as standards for antioxidant profiling in the GPE samples. As expected, the profiled antioxidant compounds were all detected in the tested samples. Also, the total concentration of detected antioxidants was highest with Tinta Cão and lowest with Petit Verdot, in line with the aforementioned assay results indicating that the former possesses strong antioxidant ability while the latter exhibits the weakest antioxidant capacity among the tested varieties. Of interest was the search for antioxidant compounds that are particularly deficient in the poor α-glucosidase inhibiting variety, Petit Verdot, and antioxidant compounds that are particularly highly concentrated in the most potent α-glucosidase inhibiting variety, Tinta Cão. Catechin, *p*-coumaric acid, epicatechin gallate, quercetin 3-o-glucoside, malvidin chloride and resveratrol were particularly very low in Petit Verdot GPE. All tested anthocyanins were especially concentrated in Chambourcin and Tinta Cao varieties. The concentration of malvidin chloride in Tinta Cão was 3.27 times higher than the next most concentrated variety. This prompted the evaluation of the α-glucosidase inhibiting capacity of these compounds, to identify the compound(s) that may be responsible for the observed

differences between the GPE varieties. For this reason, all 15 antioxidant standards were screened for α-glucosidase inhibiting activity. To our surprise, none of the compounds exhibited this bioactivity, implying that the tested GPEs likely contain an unidentified bioactive component that strongly inhibits α-glucosidases and likely exhibits antioxidant properties. Of particular concern is the Tinta Cão variety which ranks on the top of the list in terms of α-glucosidase inhibition along with antioxidant capacity.

Conclusion

Red wine grape extracts, namely Tinta Cão GPE, appear to be novel food-derived extracts that potently inhibit mammalian α-glucosidases. This reported activity is new and likely specific to the grape variety and maybe related to its antioxidant content. Although comparing antioxidant activity and content of a sample to those in the literature can be difficult at times due to the absence of one universal method and reporting fashion, the current results do reveal high antioxidant content/activity that strongly correlates with α-glucosidase inhibition. These promising findings may provide a foundation for the future development of natural α-glucosidase inhibitors from Tinta Cão GPE to potentially use for diabetes management and prevention. Further investigation is required to validate and optimize this property.

FIGURES

Fig. 3.1. Percent α-Glucosidase Inhibition per GPE Sample. Enzyme activity was determined by measuring p-nitrophenol release from pNPG at 405 nm. Acarbose (50 µg/ml) is the standard and denoted as Std. C, Chambourcin. M, Merlot. N, Norton. P, Petit Verdot. S, Syrah. T, Tinta Cão. Bars marked with different superscripts are significantly different ($p<0.05$).

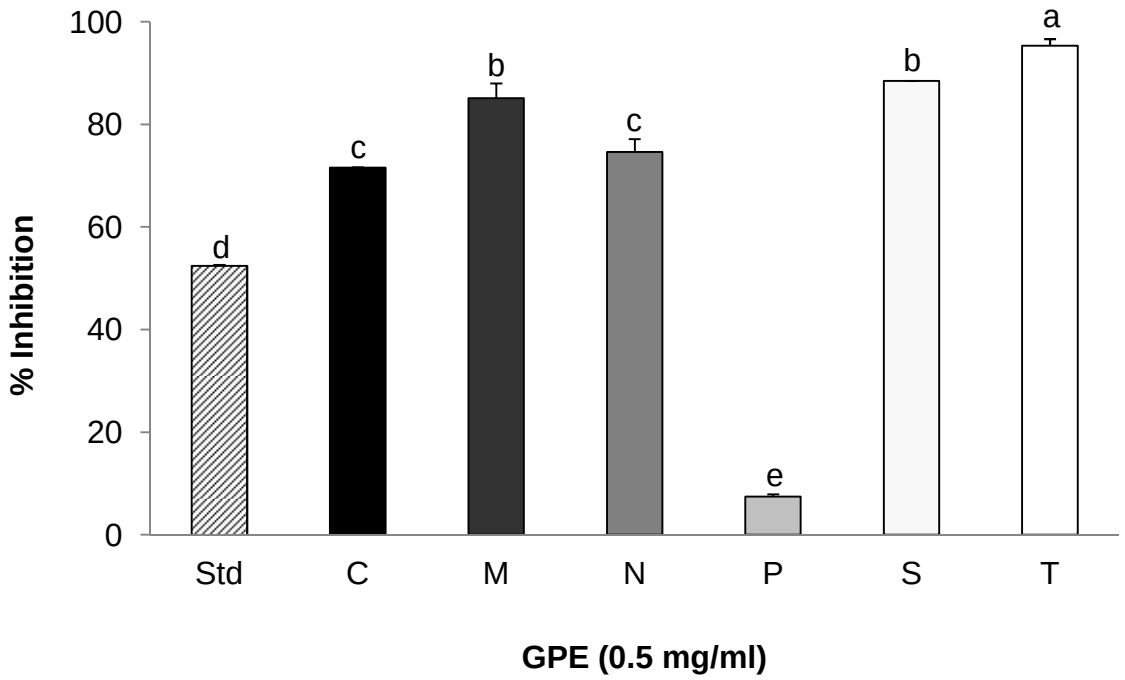


Fig. 3.2. Total Phenolic Content (TPC) per GPE Sample. TPC was determined using Folin-Ciocalteau's reagent. Data is presented as mg gallic acid equivalents (GAE) per mg dry GPE weight. C, Chambourcin. M, Merlot. N, Norton. P, Petit Verdot. S, Syrah. T, Tinta Cão. Bars marked with different superscripts are significantly different ($p<0.05$).

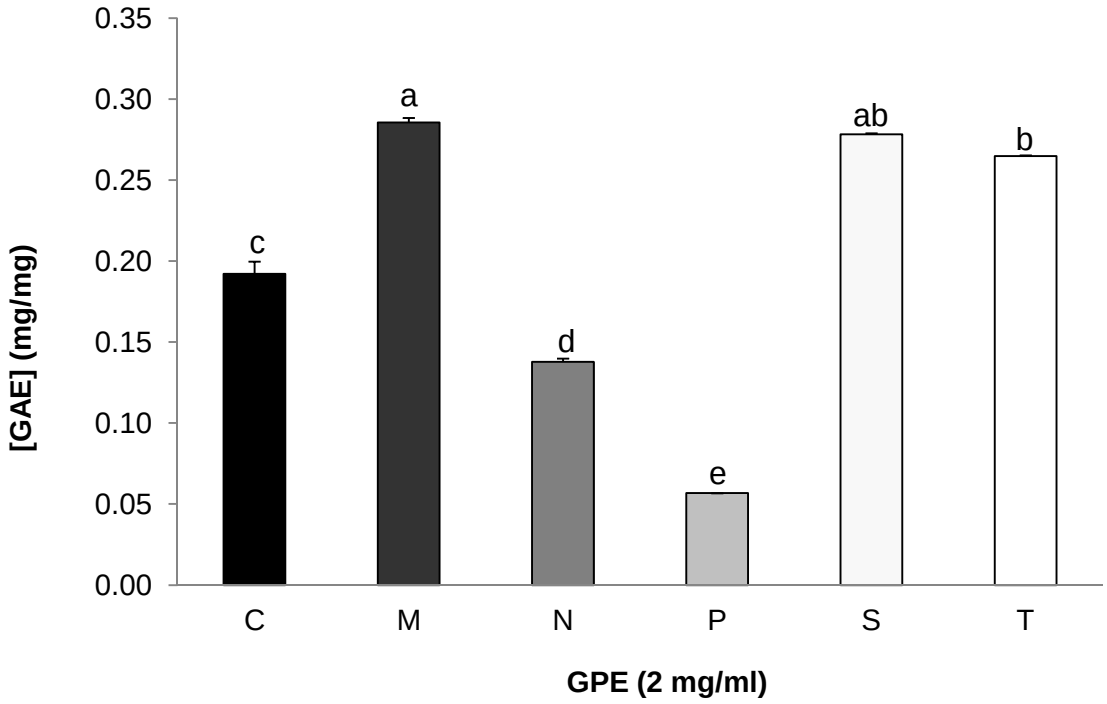


Fig. 3.3. Percent DPPH Scavenging per GPE Sample. Scavenging capacity was measured at 500 nm using 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) radical. The reaction was conducted for 120 min. C, Chambourcin. M, Merlot. N, Norton. P, Petit Verdot. S, Syrah. T, Tinta Cão. Control, identical reaction containing the sample solvent. Lines marked with different letters are significantly different ($p<0.05$).

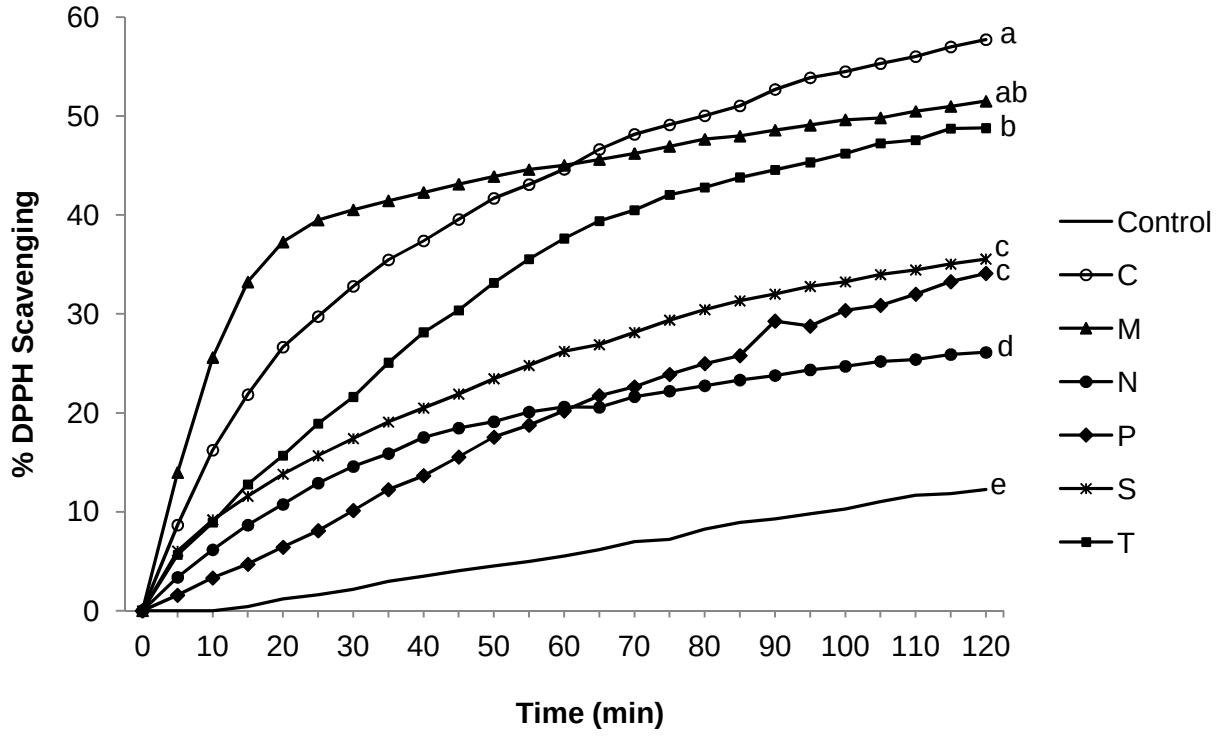


Fig. 3.4. Oxygen Radical Absorbance Capacity ($ORAC_{FL}$) per GPE Sample. $ORAC_{FL}$ was determined using 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator. Data is presented as μmol Trolox Equivalents (TE) per g dry GPE weight. C, Chambourcin. M, Merlot. N, Norton. P, Petit Verdot. S, Syrah. T, Tinta Cão. Bars marked with different superscripts are significantly different ($p<0.05$).

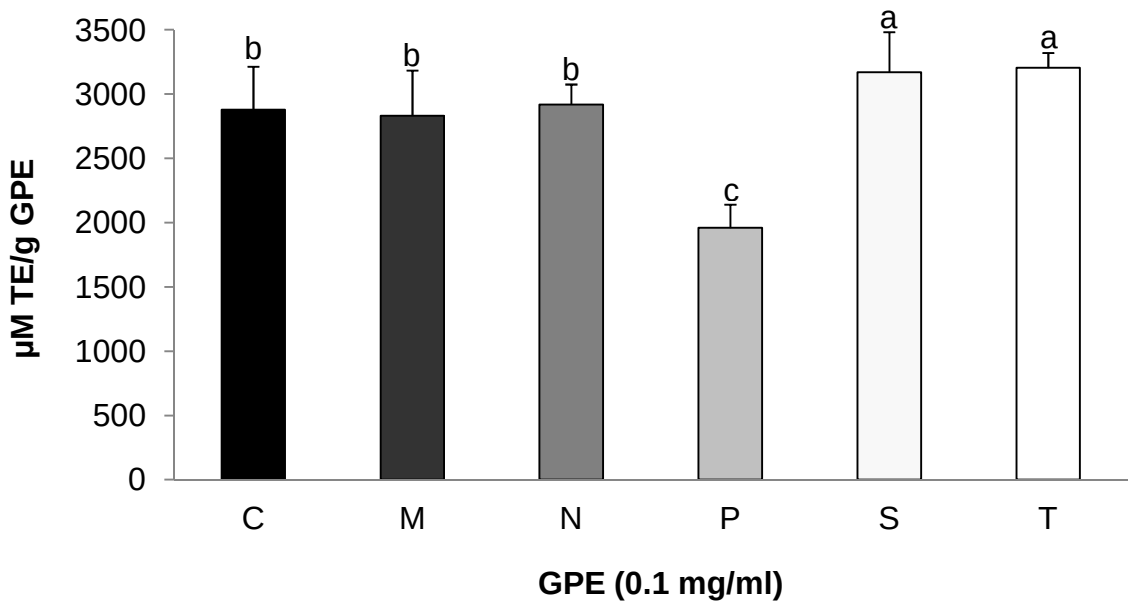


Table 3.1. Correlation between α-Glucosidase Inhibition and Antioxidant Capacity.

Correlation was measured using Pearson's *r*.

NA, not applicable.

Superscripts denote statistical significance: [a] $p<0.05$; [b] $p<0.01$

Pearson's Coefficient	α-Glucosidase Inhibition	TPC	DPPH	ORAC
α-Glucosidase Inhibition	NA	0.882[b]	0.345	0.946[b]
TPC	0.882[b]	NA	0.503[a]	0.802[b]
DPPH	0.345	0.503[a]	NA	0.246
ORAC	0.946[b]	0.802[b]	0.246	NA

Fig. 3.5. HPLC Chromatogram of the 15 Selected Antioxidant Standards. Standards were profiled in triplicate (one shown) to determine the anticipated retention time range for each compound. UV spectrum is shown at 280 nm. 1, gallic acid. 2, malvin chloride. 3, catechin. 4, delphinidin chloride. 5, caffeic acid. 6, cyanidin chloride. 7, *p*-coumaric acid. 8, epicatechin gallate. 9, rutin. 10, quercetin 3-o-glucoside. 11, malvidin chloride. 12, myricetin. 13, resveratrol. 14, quercetin hydrate. 15, kaempferol.

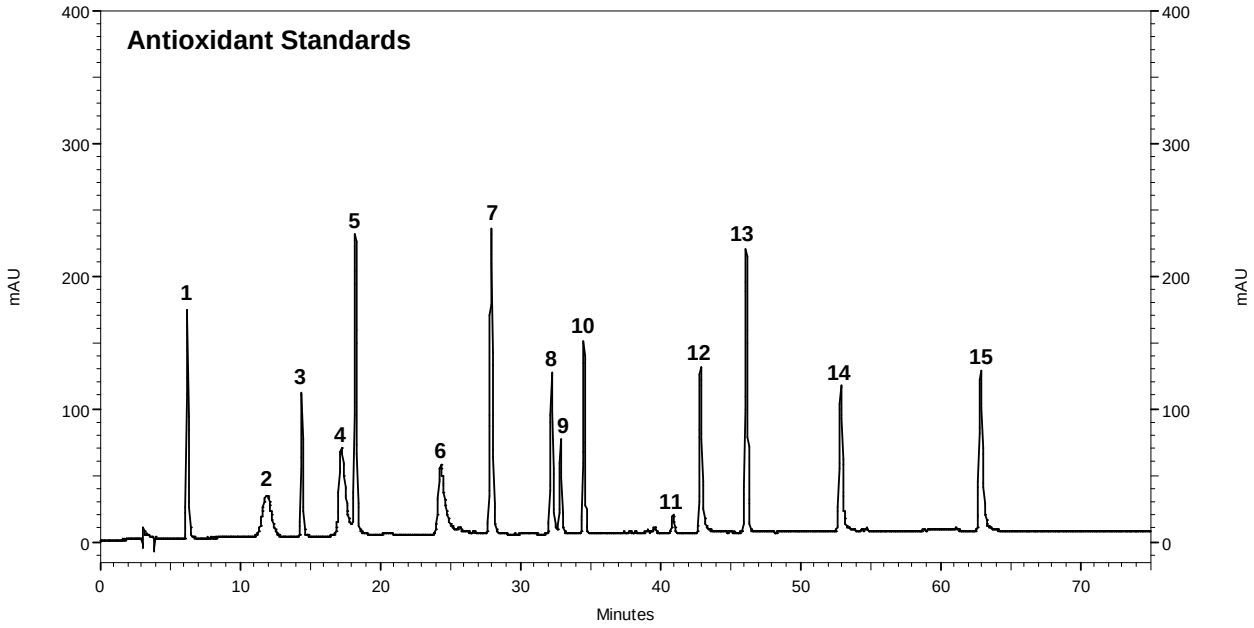
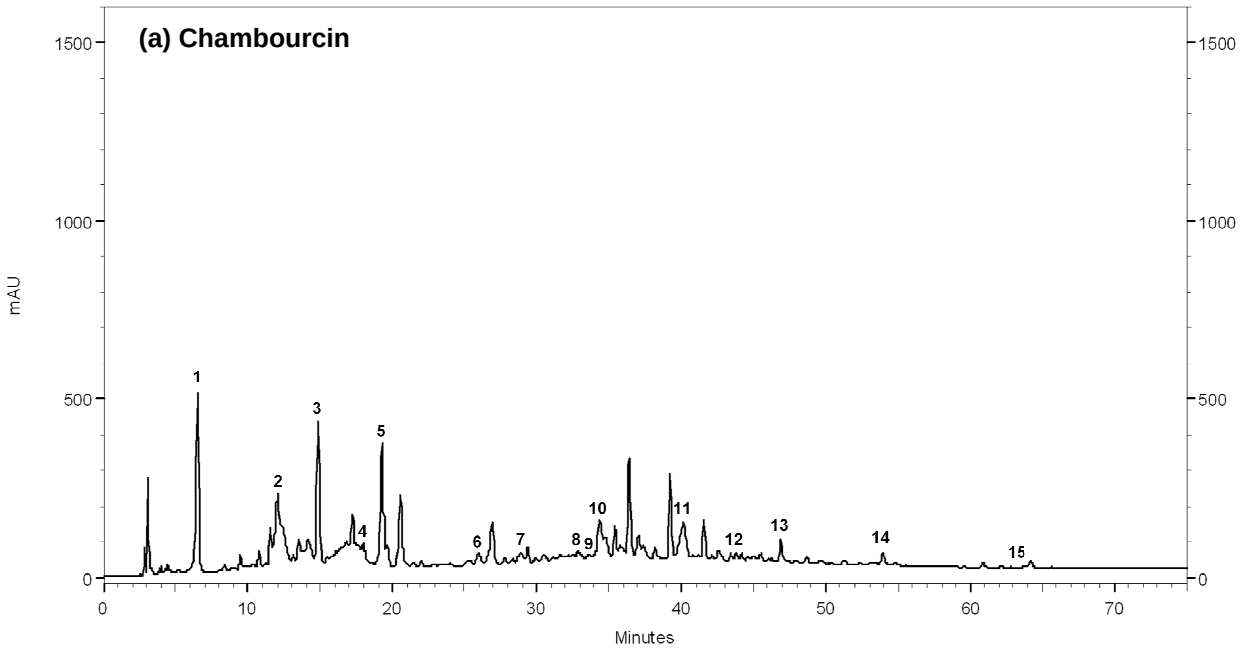
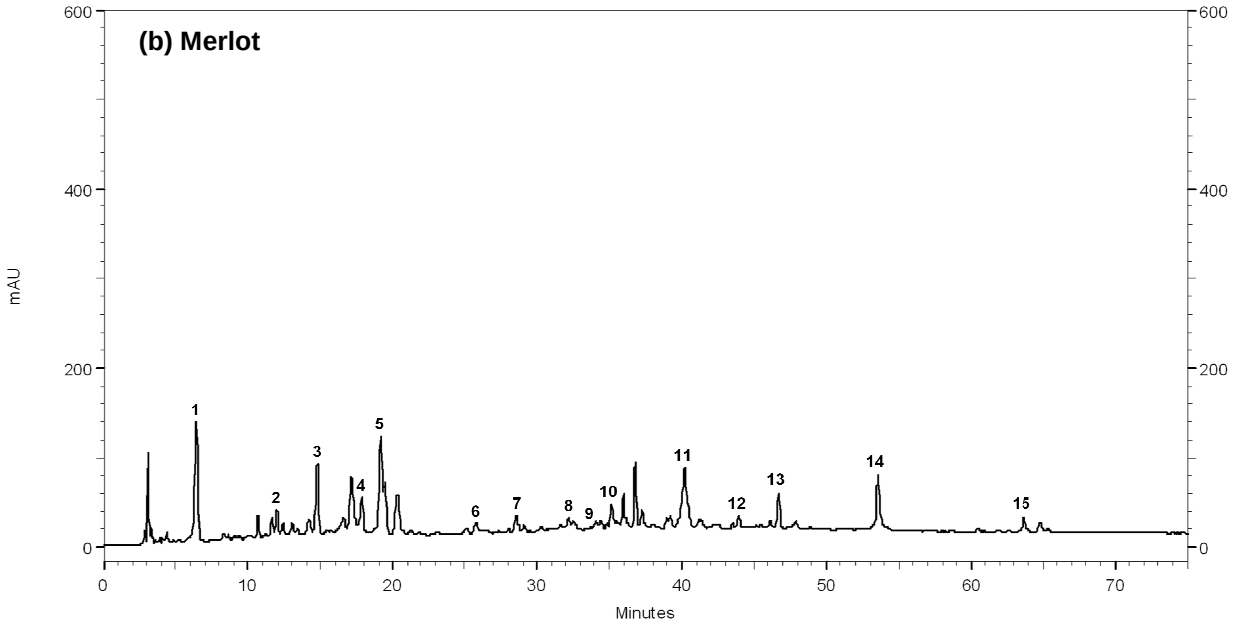
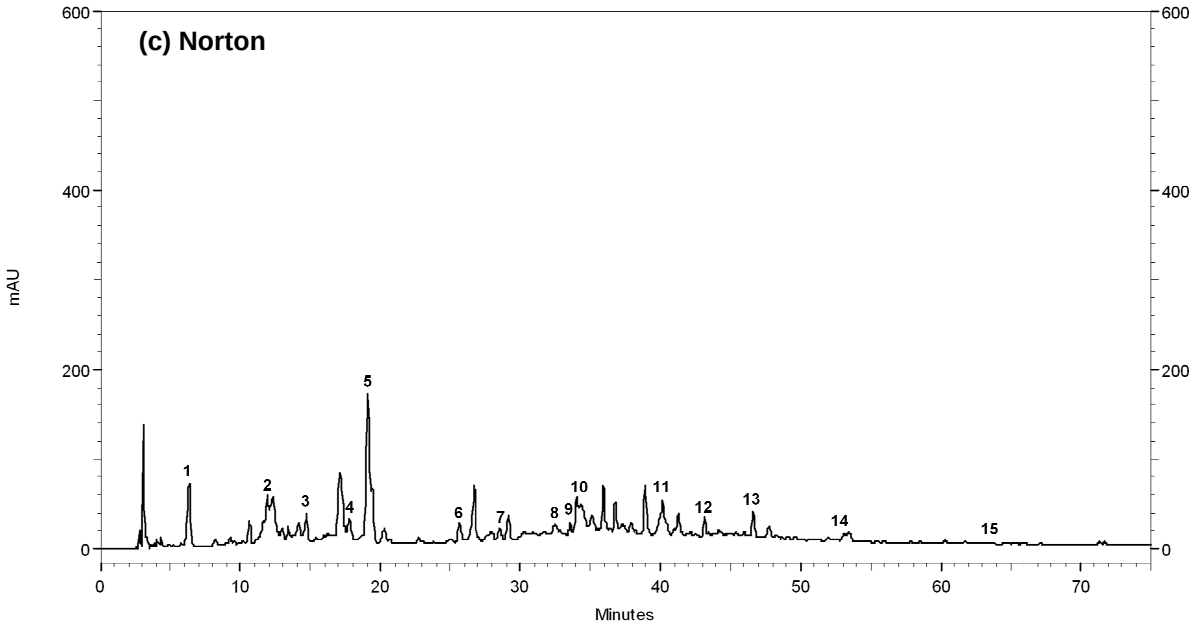


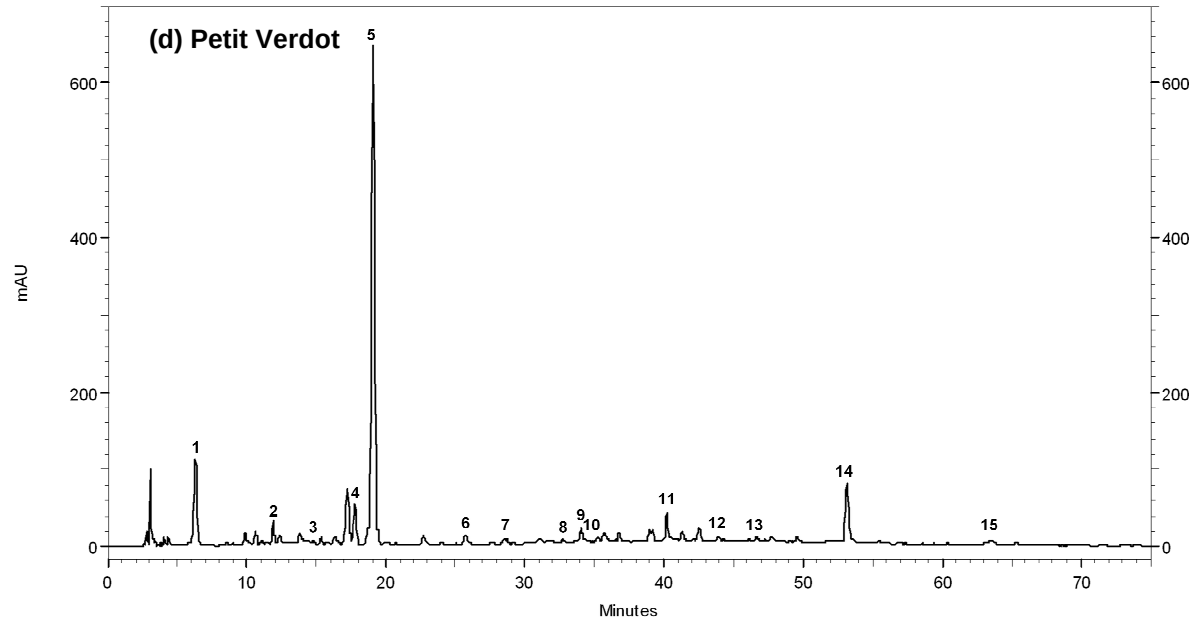
Fig. 3.6. HPLC Chromatograms of the Six Selected GPE Samples. Antioxidant rich concentrates isolated by solid phase extraction were profiled. Each sample is a complex mixture of compounds, including the profiled antioxidants. Peak numbers represent detected antioxidant standards. Spectra are displayed at 280 nm.

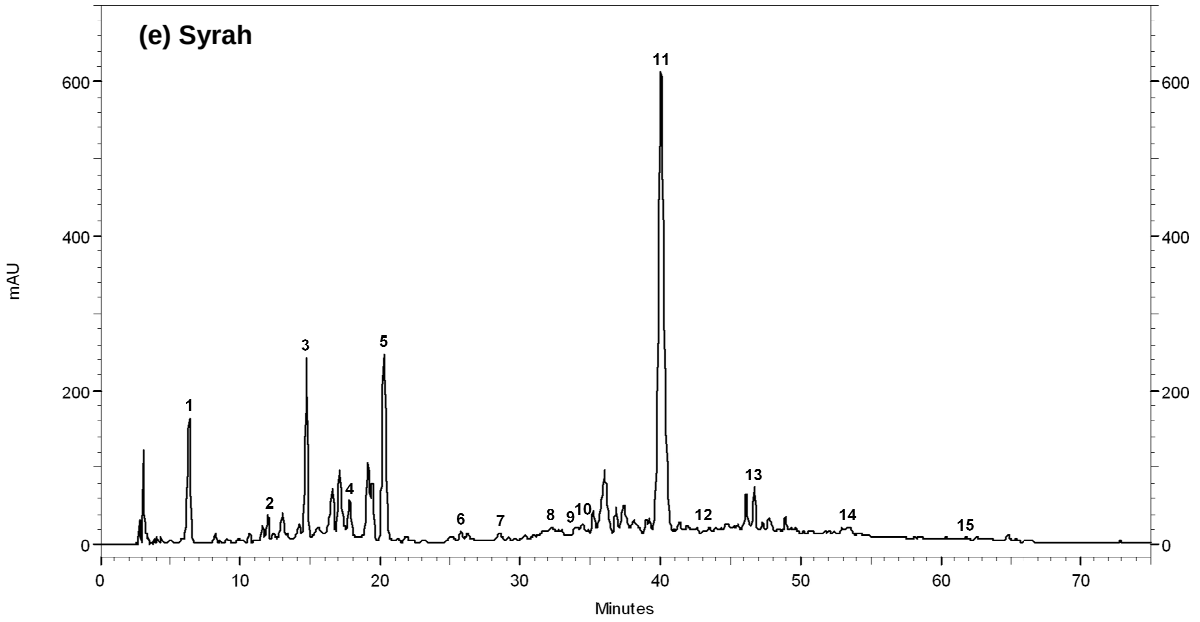
(a) Chambourcin. (b) Merlot. (c) Norton. (d) Petit Verdot. (e) Syrah. (f) Tinta Cão.











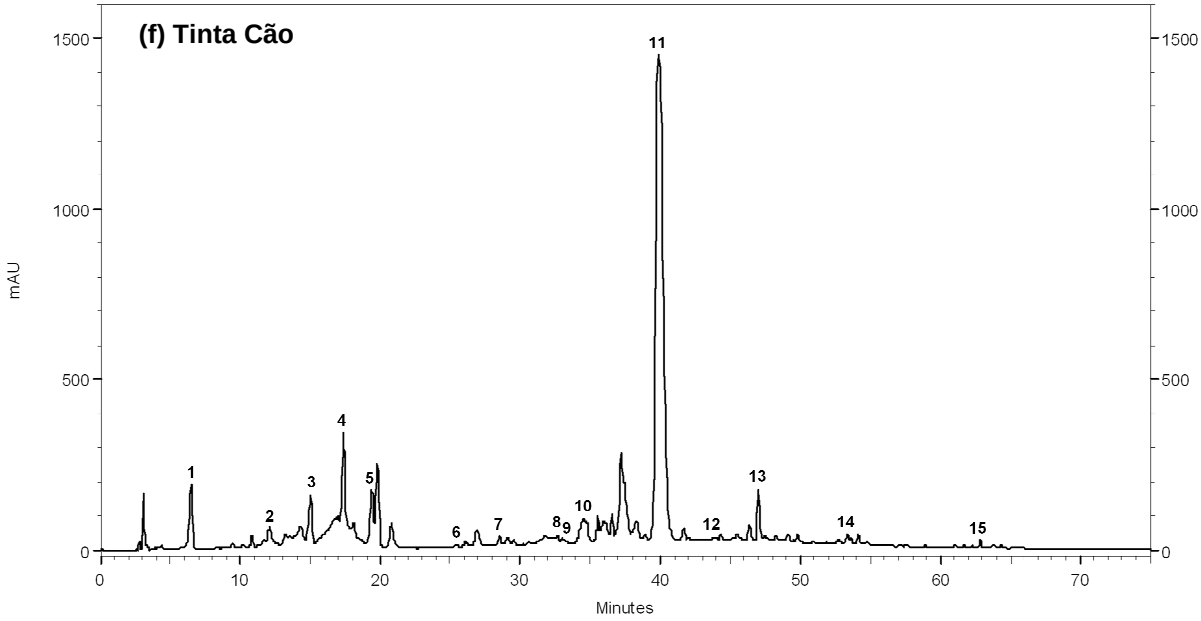


Table 3.2. Concentrations of the Detected Antioxidants in the GPE Samples. Following antioxidant detection based on retention time (RT), concentration was determined by measuring and comparing peak area of each detected compound in the sample and the standard chromatogram. Data is presented as milligrams of detected antioxidant per gram of crude GPE, and numbers in green and red represent highest and lowest concentration per row, respectively. C, Chambourcin. M, Merlot. N, Norton. P, Petit Verdot. S, Syrah. T, Tinta Cão.

Peak	Antioxidant Assignment	RT Range (minutes)	GPE Samples (mg/g crude extract)					
			C	M	N	P	S	T
1	Gallic acid	6.2-6.5	**2.83**	0.82	**0.44**	0.55	0.93	1.27
2	Malvin chloride	11.9-12.3	**3.44**	0.34	0.67	**0.23**	0.36	1.18
3	Catechin	14.4-15.1	**7.38**	1.70	1.05	**0.12**	4.31	3.78
4	Delphinidin chloride	17.2-17.8	0.85	0.70	0.52	**0.41**	0.76	**4.95**
5	Caffeic acid	18.2-19.2	1.51	**0.48**	0.74	**2.10**	1.14	0.86
6	Cyanidin chloride	24.3-26.2	**1.24**	0.30	0.45	**0.17**	0.25	0.45
7	*p*-Coumaric acid	27.9-29.2	**0.42**	0.13	0.13	**0.03**	0.12	0.21
8	Epicatechin gallate	32.2-33.5	**1.16**	0.26	0.38	**0.02**	0.43	0.44
9	Rutin	32.9-33.8	2.55	**0.39**	1.11	1.27	0.91	**2.57**
10	Quercetin 3-o-glucoside	34.5-35.5	**4.05**	0.37	1.59	**0.05**	0.72	3.29
11	Malvidin chloride	40.9-41.6	37.65	17.93	15.38	**4.57**	134.14	**439.08**
12	Myricetin	42.9-44.1	**0.86**	0.31	0.48	**0.18**	0.30	0.47
13	Resveratrol	46.1-47.3	0.65	0.21	0.27	**0.08**	0.38	**0.88**
14	Quercetin hydrate	52.9-54.0	**1.26**	1.10	**0.29**	1.18	0.33	0.84
15	Kaempferol	62.9-64.1	0.21	0.24	0.23	**0.17**	0.27	**0.33**
	Total		66.06	25.27	23.74	**11.13**	145.35	**460.60**

CHAPTER 4

Isolation and Purification of a Natural α-Glucosidase Inhibitor from Tinta Cão Grape Pomace

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Abstract

Alpha-glucosidases play a major role in controlling starch digestion and therefore postprandial blood glucose, a target for diabetes management. This study aims to prepare and purify active components in Tinta Cão grape pomace extract (GPE) that inhibit intestinal α-glucosidases. Tinta Cão GPE, previously shown to potently inhibit the enzyme, was partitioned into water, butanol and ethyl acetate extracts which were evaluated for rat intestinal α-glucosidase (25 mg/ml) inhibiting activity. The active extract was fractionated via several open column chromatography techniques and the retained fractions were tested. The most active fraction was further separated via HPLC and the collected fractions were evaluated. The active compound was then identified using NMR and MS analysis. At 0.5 mg/ml, the ethyl acetate fraction was the most effective inhibitor of α-glucosidase (68.14% inhibition, compared to 16.28-53.4%). Aqueous ethanol-eluted fractions of the HP-20 column outweighed the standard (Acarbose 50 µg/ml, 50% inhibition) at 69.82% inhibition. HPLC purification yielded an active compound that was later determined to be D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}. Results were significant, suggesting that a potent inhibitor of intestinal α-glucosidases can be isolated from Tinta Cão grapes for the potential development of a novel anti-hyperglycemic dietary supplement.

Key words: Grape Pomace; Tinta Cão; α-Glucosidase; Diabetes

Introduction

The prevalence of diabetes has become overwhelming. Diabetes currently affects 8.3% of the U.S. population [18], a number that is projected to double or triple by 2050 [19]. This epidemic has become a massive health burden significantly decreasing quality of life and increasing morbidity and mortality among Americans, all at a huge economic cost [21].

This alarming global rise in diabetes rates has made it necessary to explore novel approaches to prevent and control the disease. Traditional anti-hyperglycemic agents have shown limited long-term efficacy and often come with considerable side effects [31]. The huge economic costs, inability to provide durable glycemic control as well as the development of side effects ranging from hypoglycemia to impaired gastrointestinal function have raised concerns regarding the use of common anti-hyperglycemic agents, namely metformin, sulfonylureas, thiazolidinediones, GLP-1 receptor agonists, and even insulin [25, 29, 30, 32-35]. Therefore, it is crucial to develop alternative therapeutic strategies that will broaden treatment options and provide a safe and affordable substitute to currently available therapies.

In the shift from the traditional management of blood glucose, treatment of postprandial hyperglycemia has become an intriguing target to improve overall glycemic control [36-40]. Postprandial hyperglycemia, one of the earliest signs of type-2 diabetes, is thought to aggravate the disease by inducing glucose toxicity and ß-cell function deterioration which can ultimately give rise to an irreversible state of diabetes [39, 41]. Since it is linked to the amount of consumed starch and its rate of digestion [36], postprandial hyperglycemia can be managed by controlling carbohydrate digestion and absorption [44-46], specifically by inhibiting digestive enzymes responsible for the

break-down of starch [46-50]. For instance, the inhibition of α-glucosidases is effective in both preventing and treating type-2 diabetes through reducing postprandial hyperglycemia [52]. However, available inhibitors that have been used for diabetes treatment (i.e. Acarbose) were found to exhibit a non-specific inhibition of α-amylase, resulting in excessive accumulation of undigested carbohydrate in the colon, thus generating undesirable gastrointestinal side effects [36]. Research aiming at identifying novel inhibitors has increased in the last three decades. We have recently found several antioxidant rich red grape pomace extracts (GPEs), namely the Tinta Cão variety, to possess an impressive α-glucosidase inhibiting property (Chapter 3). The components responsible for this activity are unknown, to our knowledge.

The current research aims to isolate and identify the component(s) in Tinta Cão GPE responsible for its observed α-glucosidase inhibiting activity. The results may pave the way for the future development of a natural α-glucosidase inhibitor from red wine grapes, thus establishing a novel anti-diabetic strategy.

MATERIALS AND METHODS

Materials

HPLC grade organic solvents were utilized for grape pomace extraction, column chromatography and HPLC analysis (Fisher Scientific, Atlanta, GA). Intestinal acetone powders from rat and 4-nitrophenyl-α-D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich (St. Louis, MO). Acarbose was obtained from LKT Laboratories, Inc. (St. Paul, MN). HP-20 Diaion Resin Styrenic Adsorbent was purchased from Sorbent Technologies (Atlanta, GA).

Grape Pomace Extract Preparation

Grape pomace extraction procedure described in Chapter 3 was followed. Tinta Cão (*Vitis vinifera*) grape pomace was kindly provided by Chrysalis Vineyards (Middleburg, VA) via the Agricultural Research Station at Virginia State University (Petersburg, VA). Briefly, Fresh pomace was dried in a food dehydrator at 95 ^{o}F for 28 h then ground to a powder consistency. Grape pomace powder was soaked and stirred overnight in aqueous acetone and supernatants were spun then filtered via suction filtration. The solvent was then isolated from the extract via evaporation and condensation. The water extract was lyophilized to then be reconstituted with aqueous acetone.

Bioactivity-Driven Fractionation of GPE

i. Liquid-Liquid Partition

A batch-wise single stage extraction method was followed. The water-GPE solution of aqueous acetone grape extraction was mixed with an equal volume of ethyl acetate in a separating funnel. The solution was exposed to repetitive intervals of vigorous shaking and rest until two distinct layers were observed. The isolated aqueous phase was exposed to another round of batch-wise single stage extraction with butanol. The ethyl acetate and butanol fractions were air-dried overnight and the water fraction was dried via rota-evaporation and sublimation. Powder fractions obtained were stored at 4 ^{o}C for future use.

ii. Column Chromatography Separation

Open glass columns were packed with silica gel (normal phase), diaion HP-20 (reversed-phase), C_{18} (reversed-phase), Sephadex LH-20 (molecular sizing), Cyano sorbent (universal phase) and Dowex resin (ion exchange). These stationary phases were examined for their capacity for separation with acetone, methanol, ethyl acetate and methylene chloride being used as eluents. The method yielding sub-fraction(s) with highest enzyme inhibition potency and potential for reproducibility was selected as the optimal fractionation method. After extensive evaluation and comparison, HP-20 open column was selected, using HP-20 Diaion Resin Styrene-Divinylbenzene Adsorbent, a polyaromatic adsorbent. Resin properties were as follows: 260 Å porosity, 250-850 µm particle size, 600 m^2/g surface area, 680 g/L bulk density, and 55-65% water content. The column used was a Synthware 45/50 ST joint, 2.7 x 22 in column (VWR International, Radnor, PA). Eluents were 100% H_2O, 30% ethanol, 50% ethanol, 70% ethanol, and 100% ethanol. Thin layer chromatography (TLC) and HPLC were then used to determine the point in the separation process when a single pure active compound has been isolated.

iii. High Performance Liquid Chromatography (HPLC) Purification

HPLC method previously utilized (Chapter 3) was employed with slight modifications. The rotaevaporation-dried GPE sub-fractions were dissolved in methanol and filtered using a 0.45 micron, 3 mm syringe filter. Reversed-phase HPLC was employed to study purity and separate compounds, using a Hitachi HPLC system from Hitachi High-Tech Technologies (Tokyo, Japan). A Macherey-Nagel Phenomenex 5 µm C_{18} 250 x 10 mm analytical column (Macherey-Nagel Inc., Bethlehem, PA) represents

the stationary phase while methanol and H_2O were utilized as mobile phase solvents A and B, respectively. Twenty µL of a sub-fraction was injected via the autosampler at a 0-5689 psi pressure range, under room temperature. Gradient systems were used as follows: 0–35% A, 0–3 min; 35% A, 3-10 min; 35-53% A, 10–13 min; 53% A, 13–16 min; 53-100% A, 16–20 min; 100% A, 20-22 min; 100-35% A, 22-25 min; 35% A, 25-28 min. Flow rate was set at 2.5 ml/min. Samples were monitored by UV detection (220-310 nm) thus determining whether each sample is a pure compound or a complex mixture. Complex mixtures were separated, and retained compounds/ simple fractions of the injected sample were collected in a Foxy Jr. Fraction Collector (Teledyne Isco, Lincoln, NE).

Chemical Structure Elucidation

NMR in combination with MS was performed in the Chemistry Department at Wayne State University to elucidate the structure of the isolated active compound(s). The compounds were analyzed on a Waters LCT Premier high resolution exact mass spectrometer (Waters Corp., Milford, Massachusetts). NMR spectra (^{1}H, ^{13}C, DEPT) was generated using a Varian Mercury 400 MHz instrument (Varian, Inc., Palo Alto, California). Through analysis of chromatograms and spectra, and comparison with data previously reported in the literature, the chemical structure of the compound(s) was determined.

α-Glucosidase Inhibition Screening

As previously described (Chapter 3), Intestinal acetone powders from rat were extracted with 0.05 M phosphate buffer (PB) pH 6.8, and retained α-glucosidase enzyme extract was reconstituted with the same buffer to a concentration of 25 mg/ml.

Four mM 4-nitrophenyl-α-D-glucopyranoside (pNPG) was used as a substrate while 50 µg/ml Acarbose served as a positive control. Ninety six-well bioassay microplates were prepared to contain 115 µl of GPE fraction/ sub-fraction or control, 90 µl of enzyme solution and 45 µl of substrate solution per well. Absorbance was obtained at a 405 nm wavelength at the start of the reaction and following a 30 min incubation at 37 ^{o}C, using a Perkin Elmer HTS 7000 Bio Assay Reader and software (Perkin Elmer, Norwalk, CT). Percent inhibition by tested samples was calculated using the following formula:

% Inhibition = 100 – {(Abs_{sample}/ $Abs_{control}$) x 100}.

Statistical Analysis

Results were analyzed using one-way analysis of variance (ANOVA) and Tukey's HSD post-hoc analyses, comparing outcomes with $P < 0.05$ indicating statistical significance. SPSS 22.0 for Windows (IBM Corp., Armonk, NY) was utilized to perform these tests. Data for each dependent variable is reported as mean ± SEM.

RESULTS

Inhibition of Mammalian α-Glucosidases

i. Activity of GPE Fractions

Two of the Tinta Cão GPE fractions significantly suppressed rat intestinal α-glucosidase enzyme activity. Percent enzyme inhibition by GPE fraction is presented in Fig. 4.1. At a concentration of 0.5 mg/ml, the ethyl acetate-soluble fraction of Tinta Cão GPE exerted the strongest inhibition of intestinal α-glucosidases, measured as 68.14% ($P < 0.05$). The water-soluble fraction exhibited a lesser yet remarkable inhibitory effect (53.4%),

comparable to the effect of the standard Acarbose, a commercial α-glucosidase inhibitor which exerts ~50% inhibition at 50 µg/ml under the described assay conditions.

ii. Activity of GPE Sub-Fractions

Following TLC-assisted elimination of redundant EA-GPE-derived sub-fractions, it was determined that 5 sub-fractions (at 0.5 mg/ml) outweighed the standard in enzyme inhibition. Fraction 2 of the HP-20 open column, eluted with 30% (v/v) ethanol, exhibited 69.82% inhibition. It was selected for further fractionation since it was significantly more active than all tested sub-fractions and the standard ($P < 0.05$), and it appeared more reproducible than the others. Furthermore, HPLC analysis revealed that this fraction is a mixture of a small number of compounds. Activity of sub-fractions is summarized in Fig. 4.2.

iii. Activity of GPE-Derived Pure Compounds

HP-20 fraction 2 underwent HPLC purification yielding four HPLC fractions, as shown in Fig. 4.3. Fractions 1, 2 and 3 consisted of single compounds, whereas fraction 4 was likely not a single compound. As portrayed in Fig. 4.4, upon α-glucosidase inhibition screening, it appeared that compounds 1 and 2 possessed the inhibitory activity under question, with 67.73% and 75.34% inhibition, respectively. On the other hand, no remarkable activity was observed with fractions 3 and 4. Compounds 1 and 2 were hence selected for chemical characterization.

Identification of Active Compound

Upon NMR and MS analysis, it appeared that compounds 1 and 2 are identical. They are conformational isomers of the same compound. The NMR and MS spectra of

the isolated active compound, shown to exhibit a strong α-glucosidase inhibition, are presented in Fig. 4.5. The structure, which was later elucidated, is presented in Fig. 4.6. The compound was determined to be: D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}, a phenyl glycoside. A review of the literature determines that it was reported once by Huang et al., isolated from *Picrorhiza scrophulariiflora* [113]. The compound is a yellowish powder, soluble in water, phosphate buffers and universal organic solvents. One Kg of dry Tinta Cão grape pomace yielded 7.65 mg of the active compound, following the above-mentioned fractionation and purification methods.

DISCUSSION

Research investigating the biological activity of plant-derived components commonly requires the isolation and characterization of bioactive compounds prior to proceeding to further evaluation [91, 114, 115]. A crude plant extract is a complex combination of bioactive compounds and phytochemicals, out of which only one or a few are responsible for the functional property of interest. Separation hence poses a challenge, usually involving various steps and multiple fractionation techniques [91]. Liquid-liquid extraction is a helpful initial step to break down the crude extract into parts with different properties, based on components' relative solubility in two different immiscible liquids [92]. The active components of interest in our search for α-glucosidase inhibiting components in Tinta Cão GPE were mostly concentrated in the ethyl acetate-soluble fraction. Column chromatography, a popular fractionation method [93], was then employed to further fractionate the ethyl acetate-soluble fraction of Tinta Cão GPE, followed by HPLC for further purification and analysis. The latter yielded four

simplified fractions of which three were determined to be pure compounds according to UV spectra. However, they were not identified as known compounds. Bioassay was again required to determine the activity of the generated samples and select the bioactive entity, and it suggested that two of the isolated compounds are strong inhibitors of α-glucosidase.

The determination of the chemical structure and formula of the isolated compound was then achieved via combinatorial chemistry. Confirmation usually relies on verification of information by comparison of liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) data. Utilizing these techniques along with reviewing the literature indicated that the active compounds were actually conformational isomers of the same compound and determined this compound to be D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}, a phenyl glycoside previously characterized by Huang et al [113]. The review of the literature also indicates that this compound has not been previously investigated for bioactivity, particularly α-glucosidase inhibition and antioxidant capacity. A natural, food-derived compound possessing the potential for the development of an anti-hyperglycemic supplement is a very promising future anti-diabetic strategy.

Conclusion

Tinta Cão grape is a biomass that possesses a remarkable ability to inhibit mammalian α-glucosidases. This property appears to be derived from at least one compound, D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}, isolated from the pomace of this grape variety (Fig. 4.7). These findings are new and carry promising potential for the future development of a novel food-derived natural supplement for

diabetes management and prevention. Further assessing the safety and applicability of this compound will aid in determining the future directions.

FIGURES

Fig. 4.1. Percent α-Glucosidase Inhibition per GPE Fraction. Enzyme activity was determined by measuring p-nitrophenol release from pNPG at 405 nm. Acarbose (50 µg/ml) is the standard and denoted as Std. H_2O, water fraction. EA, ethyl acetate fraction. BuOH, butanol fraction. Bars marked with different superscripts are significantly different ($p<0.05$).

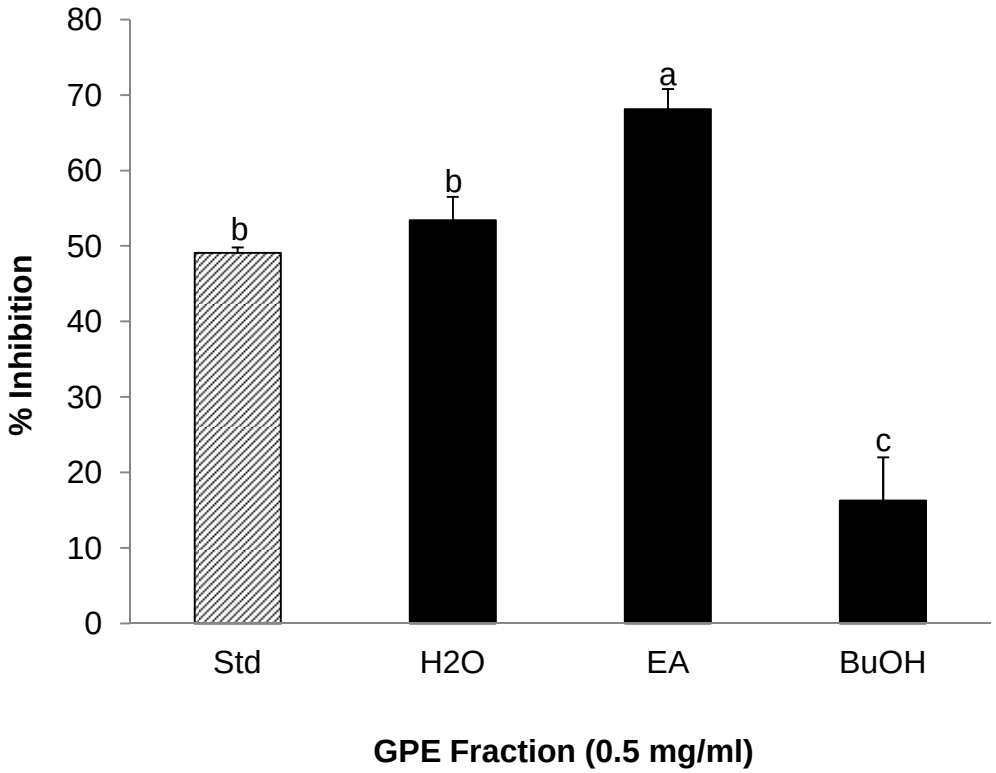


Fig. 4.2. Percent α-Glucosidase Inhibition per GPE Sub-Fraction. Enzyme activity was determined by measuring p-nitrophenol release from pNPG at 405 nm. Acarbose (50 µg/ml) is the standard and denoted as Std. C18, reverse phase C18 column. HP20, diaion resin HP-20 column. SPE, solid phase extraction. S1, silica gel column 1. S2, silica gel column 2. Bars marked with different superscripts are significantly different ($p<0.05$).

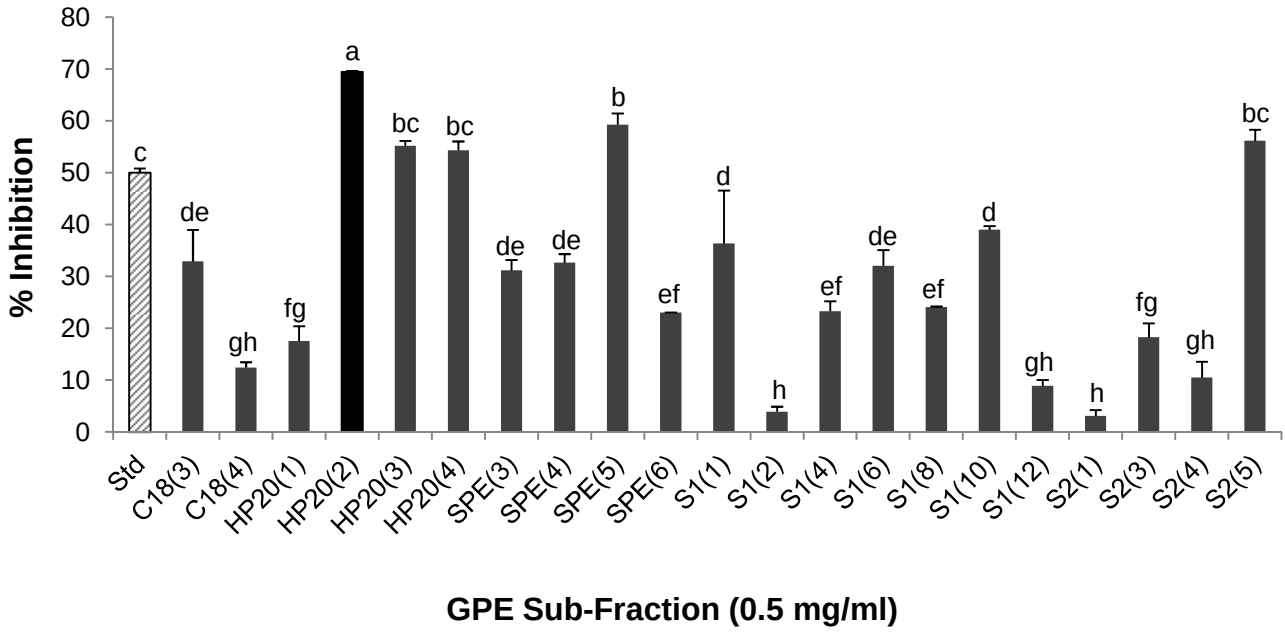


Fig. 4.3. HPLC Chromatogram of GPE-Derived Active Sub-Fraction. S, solvent peak. 1, fraction 1, single compound. 2, fraction 2, single compound. 3, fraction 3, single compound. 4, fraction 4, likely not a single compound.

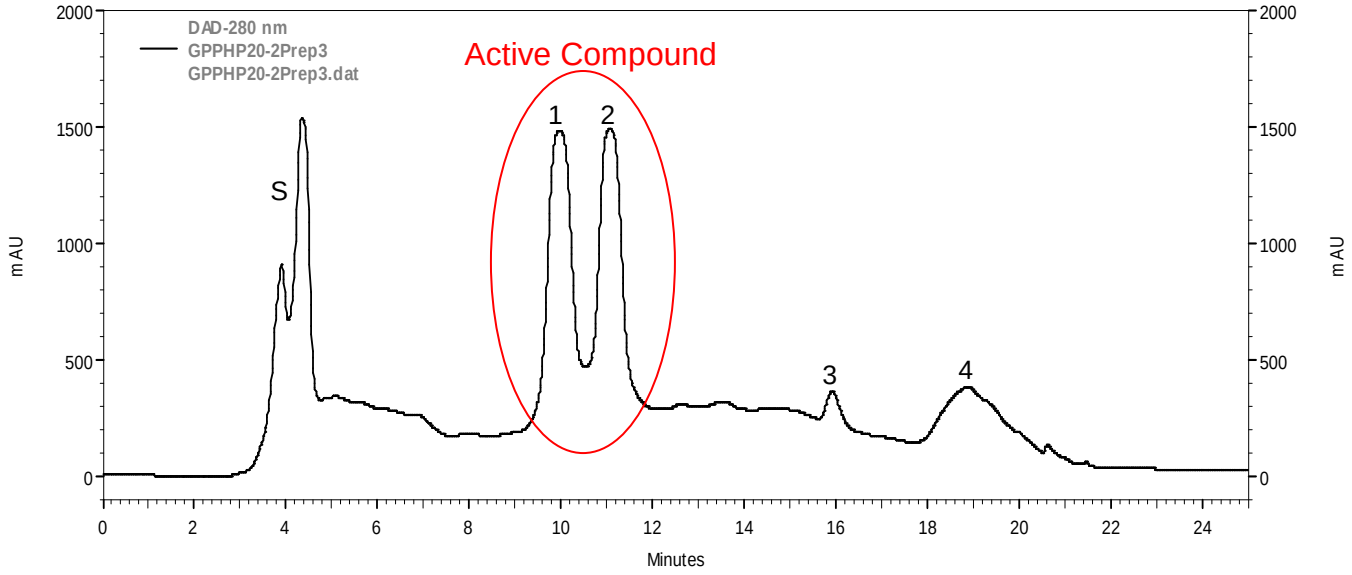


Fig. 4.4. α-Glucosidase Inhibitory Activity of GPE-Derived HPLC Fractions. Enzyme activity was determined by measuring p-nitrophenol release from pNPG at 405 nm. Acarbose (50 µg/ml) is the standard and denoted as Std. 1, compound 1. 2, compound 2. 3, compound 3. 4, HPLC fraction 4. Bars marked with different superscripts are significantly different ($p<0.05$).

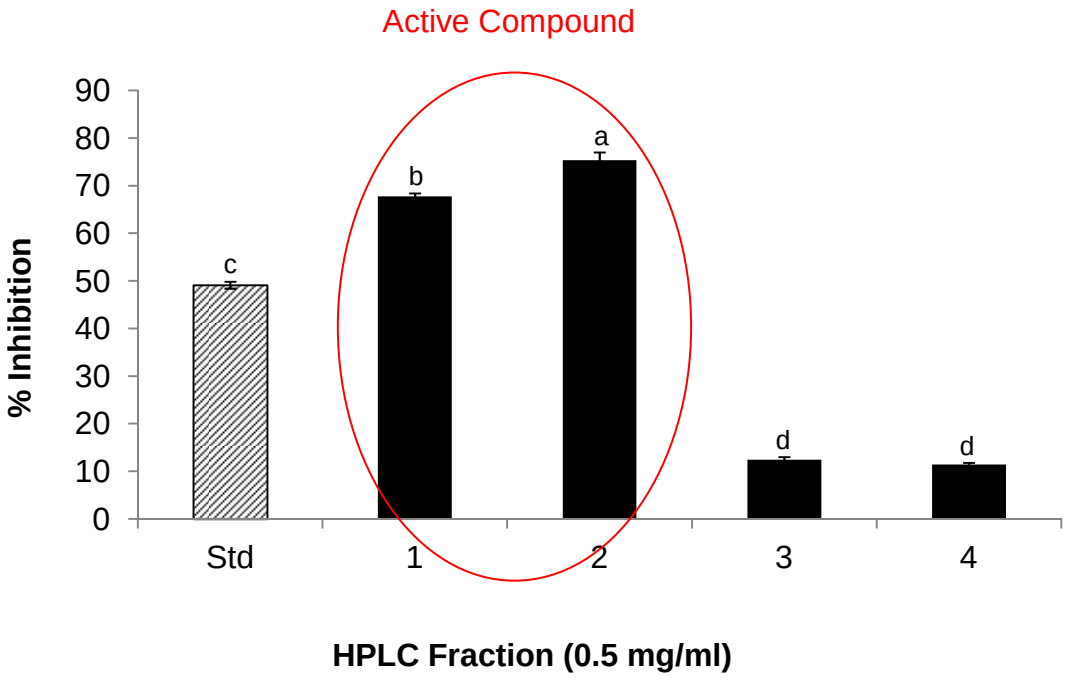
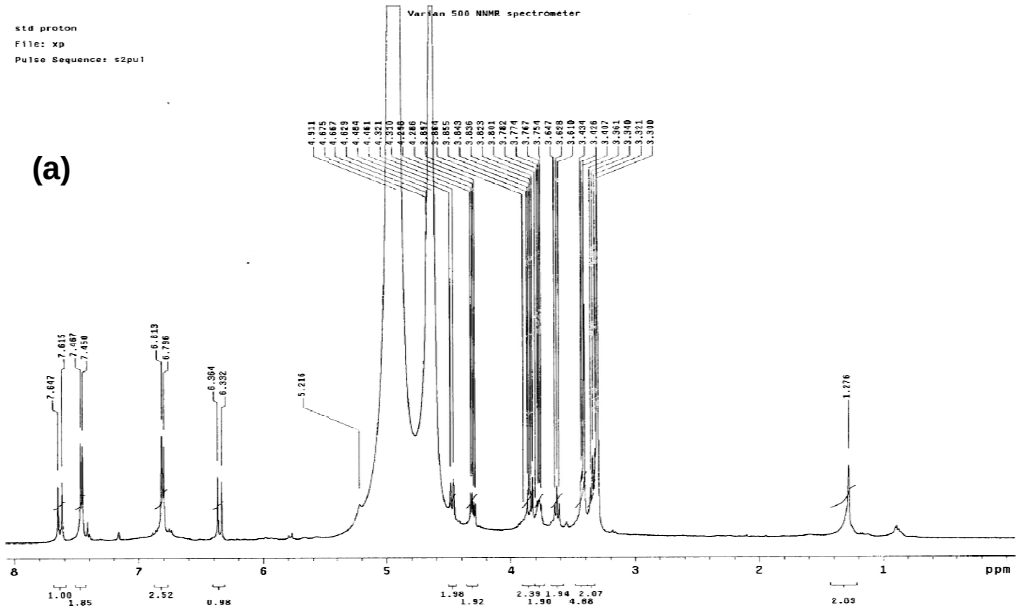
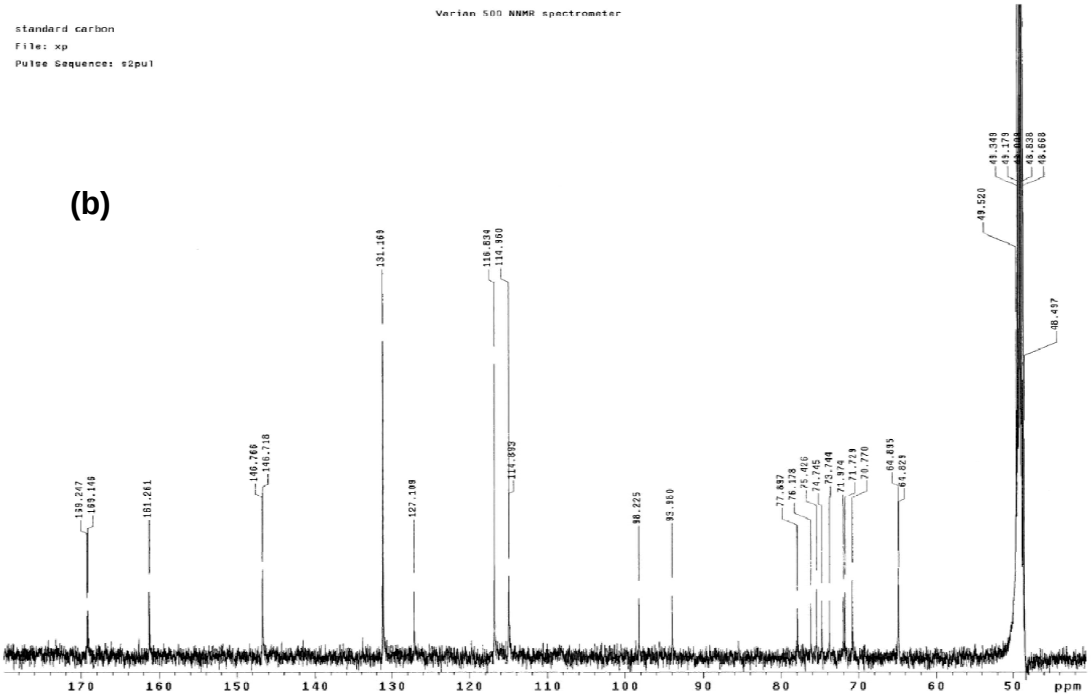


Fig. 4.5. NMR and MS Spectra of the Isolated Active Compound.

(a) H-NMR spectrum. (b) C-NMR spectrum. (c) MS spectrum.





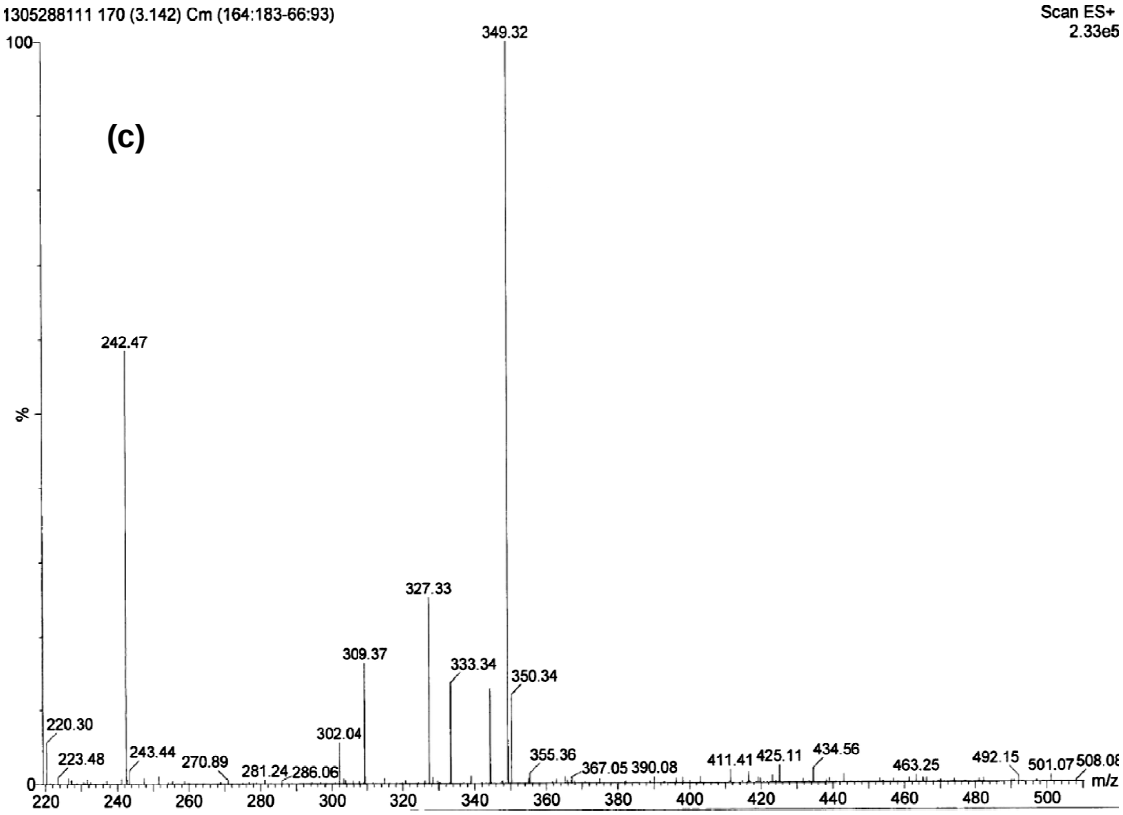
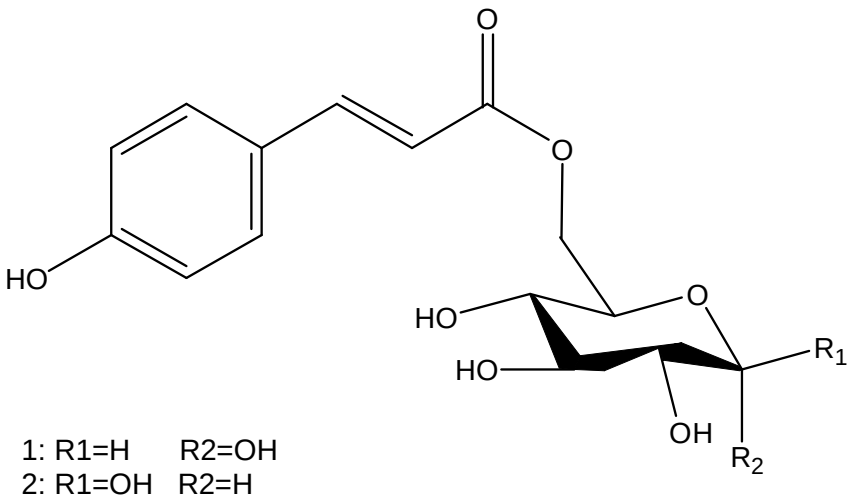


Fig. 4.6. Chemical Structure of the Isolated Active Compound. The compound was determined to be: D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}. Conformational isomer 1: R1=H, R2=OH. Conformational isomer 2: R1=OH, R2=H.



1: R1=H R2=OH
2: R1=OH R2=H

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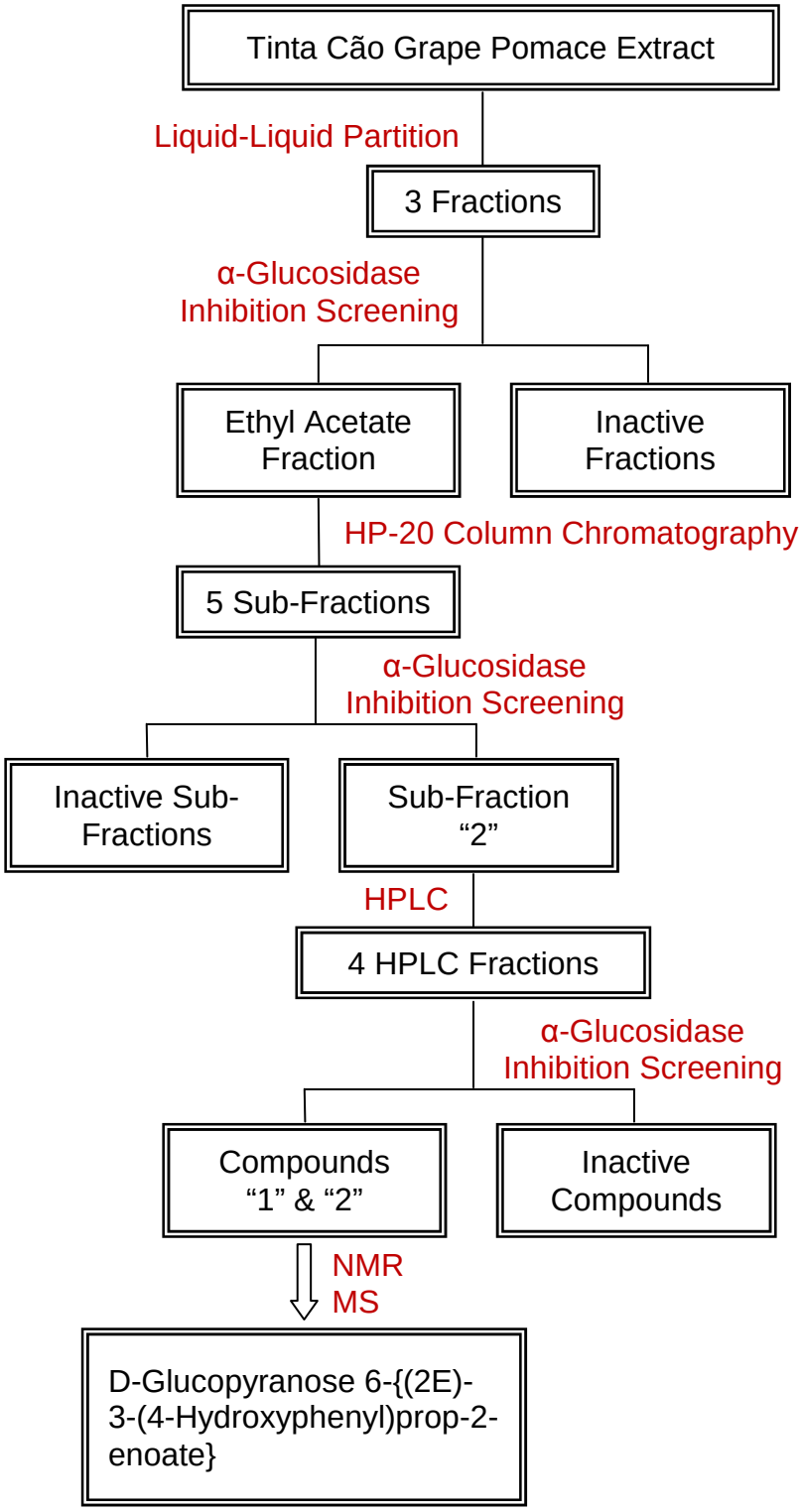
Fig. 4.7. Summary of the Active Compound Isolation Steps. Enzyme inhibition assay followed each step. Tinta Cão grape pomace extract was subjected to liquid-liquid partition yielding 3 fractions, of which the ethyl-acetate soluble fraction was determined to be the strongest α-glucosidase inhibitor. This fraction was further fractionated via multiple column chromatography techniques yielding numerous sub-fractions, of which fraction 2 of the HP-20 column was the most potent sub-fraction. This sub-fraction was purified via HPLC resulting in an active pure compound that was elucidated with NMR and MS and found to be consistent with D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}



CHAPTER 5

The Applicability of Grape Pomace-Derived D-Glucopyranose6-{(2E)R-3-(4-Hydroxyphenyl)prop-2-enoate} as a Natural Inhibitor of α-Glucosidases

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Abstract

This study was designed to evaluate the applicability of grape pomace-derived D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate} as a natural inhibitor of α-glucosidases. This compound was isolated from a Tinta Cão grape pomace extract previously shown to be a potent inhibitor of the enzyme complex. In the current study, the compound's dose response in inhibiting α-glucosidases was evaluated and specificity examination followed. Then, antioxidant activity of the compound was evaluated by free radical assays. Thermal, pH and shelf-life stability of the active sub-fraction/compound was then tested after exposing it to a range of temperature, pH and storage conditions. Lastly, cytotoxicity of the compound was determined through MTS assay utilizing NIH/3T3 cells. The isolated compound inhibits α-glucosidase and not α-amylase. Furthermore, it is a dose-dependent inhibition, exerted by predominantly inhibiting the maltase and isomaltase moieties of α-glucosidase. The compound also possesses an impressive antioxidant capacity. In terms of stability, it was shown to withstand temperature and pH extremes but lose activity upon prolonged storage and prolonged exposure to light. Lastly, at 25 and 50 µg/ml, the active compound was not cytotoxic to cells (90% cell viability). It was concluded that the compound, besides being a potent α-glucosidase inhibitor, is a strong antioxidant that is fairly stable under different environmental conditions and likely safe for human consumption. These results suggest that D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}, isolated from Tinta Cão grape pomace is a promising agent for the potential development of an anti-hyperglycemic dietary supplement, following pre-clinical and clinical testing.

Key words: Grape Pomace; α-Glucosidase; Diabetes; Specificity; Stability; Safety

Introduction

Diabetes rates are on a continuous dramatic increase worldwide, a trend that is anticipated to continue over the next two decades [116]. While this epidemic is uncontrollably spreading, currently available treatment options are often limited by suboptimal efficacy and side effects [30, 31]. This brings about the need to develop new approaches to prevent and control diabetes. Treatment of postprandial hyperglycemia, a promising therapeutic target for improving overall glycemic control [36, 37, 39], can be achieved by controlling carbohydrate digestion and absorption [44-46]. For instance, reduction of postprandial hyperglycemia through α-glucosidase inhibition has been effective in both preventing and treating type-2 diabetes [38, 52, 55]. Nonetheless, commercial inhibitors (i.e. Acarbose) were found to exhibit a non-specific inhibition of α-amylase due to similarities in the target enzymes, resulting in excessive accumulation of undigested carbohydrate in the colon, which in turn gives rise to gastrointestinal side effects [36, 56, 57].

We have observed a potent α-glucosidase inhibition exhibited by a Tinta Cão grape pomace- derived compound (Chapter 4); however, the specificity of this compound has not been determined. Another measure of specificity to be considered with mammalian intestinal α-glucosidase enzyme complex is the enzyme moiety responsible for the observed effect, as the complex consists of three enzymes: sucrase, maltase and isomaltase, responsible for the digestion of sucrose, maltose and isomaltose, respectively [96]. Once specificity is determined, the mechanism of action of the agent of interest can be identified. Dose response information is essential in drug development as it provides the necessary effectiveness and safety guidelines

associated with dosing [117]. Testing the likelihood of dose dependence *in vitro* can provide valuable information to be applied in preclinical and clinical studies.

Instability is a common problem in natural medicines. Stability tests to ensure product quality, safety, and efficacy are required prior to the approval of any pharmaceutical product [118]. Intermediate length testing should cover a minimum of six months duration, however, it is considered unnecessary to continue testing if a significant change in efficacy is seen in the first three months [119]. A loss of activity up to 85% can lead to failure in therapy, and is considered a significant loss of activity [118]. Another necessary safety measure is cytotoxicity studies. An isolated active compound requires testing to ensure it is safe in pure form. The edibility of a plant is no guarantee that its individual constituents are safe to consume, and vice versa. Plant research is currently separated into ethnopharmacology (ex. medicinal herbs) and toxicology (ex. poisonous plants), both leading to the production of drugs and lead compounds [120].

Lastly, the phenolic nature of the isolated compound, as shown in Chapter 4, in addition to the antioxidant and enzyme inhibition correlation observed in Chapter 3, prompt the investigation of the antioxidant capacity of this compound. Plant-derived phenolics are well known for their bioactive properties [121]. Particularly, these compounds have exemplary antioxidant functions [122].

The current research aims to assess the safety and applicability of the isolated compound by characterizing its inhibition mode and determining its stability and cytotoxicity. It also aims to understand the observed correlation between α-glucosidase inhibition and antioxidant capacity, noted in Chapter 3. This research may provide the

groundwork for the future development of a specific, food-derived α-glucosidase inhibitor from grape pomace for preventing and treating diabetes, thus establishing a novel, safe dietary anti-diabetic strategy.

MATERIALS AND METHODS

Materials

Intestinal acetone powders from rat, 4-nitrophenyl-α-D-glucopyranoside (pNPG), α-amylase from porcine pancreas type VI-B, 3,5-dinitrosalicylic acid (DNS), 2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO). Autokit Glucose CII and 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) were acquired from Wako Chemicals USA, Inc. (Richmond, VA). Maltose, sucrose and potato starch were purchased from Fisher Scientific (Pittsburgh, PA). Isomaltose was purchased from TCI America (Portland, OR). Acarbose was obtained from LKT Laboratories, Inc. (St. Paul, MN). Mouse embryonic fibroblast (MEF) cell line, NIH/3T3 (CRL-1658) was obtained from ATCC (Manassas, VA). CellTiter 96 AQ_{ueous} One Solution Reagent was obtained from Promega (Madison, WI). Fluorescein was purchased from Fluka Analytical (Buchs, Switzerland), and Trolox was purchased from ACROS (Geel, Belgium).

α-Glucosidase Inhibition Screening

Enzyme extraction and inhibition screening methods previously described (Chapters 3 and 4) were followed. Briefly, intestinal acetone powders from rat were extracted with 0.05 M phosphate buffer (PB) pH 6.8, and retained, lyophilized α-glucosidase enzyme extract was reconstituted with the same buffer to a concentration of 25 mg/ml. Four mM 4-nitrophenyl-α-D-glucopyranoside (pNPG) was used as a

substrate while 50 µg/ml Acarbose served as a positive control. Ninety six-well bioassay microplates were prepared to contain 115 µl of sample, 90 µl of enzyme solution and 45 µl of substrate solution per well. Absorbance was obtained at a 405 nm wavelength at the start of the reaction and following a 30 min incubation at 37 ^{o}C, using a Perkin Elmer HTS 7000 Bio Assay Reader and software (Perkin Elmer, Norwalk, CT). Percent inhibition by tested samples was calculated using the following formula:

% Inhibition = 100 – {(Abs_{sample}/ $Abs_{control}$) x 100}.

Mechanism of Action Tests

i. Dose Response Test

To understand whether the observed rat α-glucosidase inhibitory effect is dose-dependent, the previously described enzymatic assay was performed with multiple concentrations of the sample (0.1, 0.25 and 0.5 mg/ml) and percent inhibition trend in response to concentration change was calculated.

ii. Enzyme Specificity

ii.a. Pancreatic α-Amylase Inhibition Assay

Pancreatic α-amylase inhibition assay followed, using the protocol described by Zhang et al [36] with modifications. Briefly, 50 µl of the active compound (0.5 mg/ml, aqueous acetone) was incubated with 50 µl enzyme solution (0.17 mg/ml, ddH_2O) for 5 min at room temperature. Following preincubation, 100 µl substrate solution (0.5% potato starch, 20 mM PB pH 6.9) was added to the solution and the cocktail was incubated for 3 min at 37^{o}C. The reaction was stopped by adding 100 µl color reagent (DNS) and incubating for 10-15 min at 85-90 ^{o}C. After heating, the assay cocktail was allowed to cool for 10 min at room temperature. Fifty µl of the cocktail was then loaded

into a well containing 175 µl ddH_2O, in a 96-well assay plate. The assay was conducted in triplicate and absorbance, representing maltose release, was measured at 540 nm using a Perkin Elmer HTS 7000 Bio Assay Reader. The result was compared to that of control, prepared with sample solvent instead of sample. Percent enzyme inhibition was calculated using the following formula:

% Inhibition = $100 - \{(Abs_{sample} / Abs_{control}) \times 100\}$.

ii.b. Single α-Glucosidase Enzyme Inhibition Assay

The activity of the active compound against α-glucosidase was estimated utilizing the active sub-fraction from which it was extracted, with different substrates to identify specificity. The sub-fraction was reconstituted in aqueous acetone and diluted to 5 mg/ml. The sample was tested using the α-glucosidase enzyme inhibition assay described earlier, coupled with a Mutarotase-GOD Glucose assay. The previously described assay cocktail was prepared excluding pNPG. Instead, maltose (0.125 M), sucrose (0.5 M) and isomaltose (0.125 M) served as enzyme substrates in three separate assays. Glucose production in each assay represented enzymatic activity. Glucose was detected via an Autokit Glucose CII following the manufacturer's protocol. Results of the three assays were compared thus identifying the target enzyme in the multi-enzyme complex.

Antioxidant Evaluation of Active Compound

i. DPPH Radical Scavenging Assay

The protocol followed by Brand-Williams et al was modified [99]. The isolated active compound was tested at 0.5, 1 and 5 mg/ml (in aqueous acetone) for its ability to quench DPPH. 100 µl of sample was mixed with 150 µl of DPPH radical solution in a

96-well microplate and absorbance was measured at room temperature every 5 min for 2 h at 500 nm. Variants were prepared in triplicates. After subtracting sample backgrounds at all time points, the percent scavenging capacity was calculated using the following equation:

Scavenging Effect (%) = $\{(Abs_{start} - Abs_{timepoint}) / Abs_{start}\} \times 100$.

ii. Oxygen Radical Absorbing Capacity ($ORAC_{FL}$) Assay

$ORAC_{FL}$ assay described by Zhou et al [100] was performed with slight modifications. Active compound was diluted with aqueous acetone to 40 and 200 µM. Trolox which served as standard was prepared in concentrations of 0, 20, 40, 80, 100 and 200 µM in aqueous acetone. Sample variants and standards were assayed in triplicates. In each well of a 96-well microplate, 200 µL of 8 µM fluorescein (in 75 mM PB pH 7.4) was mixed with 40 µL of sample or standard. The plate was then incubated for 15 min at 37 ℃ followed by the addition of 35 µL of 0.36 M AAPH to each well, then fluorescence was measured every 5 min for 90 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Results are expressed as antioxidant power in relative fluorescence units (RFU).

iii. ABTS Radical Cation Decolorization Assay

The protocol described by Re et al [123] was modified. 2,2′-azinobis(3-ethylbenzothiazoline -6-sulfonic acid) (ABTS) was oxidized into a reactive cation by the addition of potassium persulfate to a concentration of 7 mM ABTS/ 2.5 mM potassium persulfate, and the cation solution was incubated in the dark at room temperature for 12 hours. After preincubation, the solution was diluted 70-fold. Then, 200 µl of the cation solution was mixed in a well of a 96-well plate with 10 µl of control (sample solvent),

standard (200 µM trolox) or sample (200 and 400 µM active compound). Absorbance, representing radical cation neutralization by standard/sample, was measured at 405 nm after 5 minutes of incubation at room temperature. Readings were recorded every minute for 31 minutes. Percent neutralization at each time point was calculated using the following formula:

% Neutralization = 100 – {(Abs_{sample}/ $Abs_{control}$) x 100}.

Stability Tests

i. Thermal Stability

The active sub-fraction (reconstituted in water) was heated to 50ºC, 80ºC, 100ºC, and 121ºC for 15 minutes using a Fisher Scientific Isotemp hot plate. Samples were removed to ice for 10 minutes for immediate cooling. Following heat treatment, the level of bioactivity of the samples at 1 mg/ml was assessed via α-glucosidase enzyme inhibition assay as previously described. Untreated sample was included as control.

ii. pH Stability

Buffers were prepared at pH 3, 4, 5, 6, 7, 8, 9, and 10. Solutions of the active sub-fraction were incubated in buffer for 4 hours. The level of bioactivity of the sub-fraction at different pH levels was assessed at 0.5 mg/ml via α-glucosidase enzyme inhibition assay as previously described. Buffers at each pH value were included in this assay as controls.

iii. Shelf-Life and Storage

The activity of the isolated active compound following storage in various conditions was evaluated. The conditions chosen for this test were: -20ºC freezer, 3-4ºC

fridge, room temperature (dark), and room temperature (light). They were tested for a period of 8 months, or until a significant loss of activity was exhibited.

MTS Assay for Cytotoxicity

The compound's cytotoxicity was determined by MTS assay using mouse embryonic fibroblast (MEF) cell line, NIH/3T3. MTS assay was conducted as described by Ji et al with slight modifications [124]. The active compound was dissolved in methanol to prepare a stock solution with a concentration of 1 mg/ml. Then, serial dilutions with DMEM were performed to make working solutions of 25 µg/ml and 50 µg/ml, in 2.5% and 5% methanol, respectively. The NIH/3T3 cells (5×10^3) were seeded in a 96-well culture plate and after overnight incubation, the medium was removed and replaced with a fresh medium containing methanol (solvent control, 2.5% or 5%) or active compound (25 or 50 µg/ml). After 72 h of incubation, 15 µl of CellTiter 96 AQ_{ueous} One Solution Reagent was added to each well. After 2 h incubation at 37 ℃ in a humidified, 5 % CO_2 atmosphere, absorbance was recorded at 485 nm on a Perkin Elmer HTS 7000. Each variant of the experiment was performed in octuplicate.

Statistical Analysis

Results were analyzed via IBM SPSS 22.0 for Windows (IBM Corp., Armonk, NY) using Student's *t*-test and one-way analysis of variance (ANOVA). Tukey's HSD post-hoc analyses were employed to compare outcomes using $P < 0.05$ as a cutoff point for statistical significance. Data for each dependent variable is reported as mean $\pm$ SEM.

RESULTS

Mechanism of Action of the Isolated Compound

i. Dose Response

Fig. 5.1 denotes the dose-response relationship between the isolated active compound and α-glucosidase enzyme inhibition. At 0.1 mg/ml, 0.25 mg/ml and 0.5 mg/ml of the active compound, 23.42%, 45.92% and 67.73% inhibition of the enzyme complex were observed ($P < 0.05$, $r^2 = 0.95$), respectively, indicating a dose-dependent relationship. The linear curve had a slope of 130.82 and intercept of 6.4689, from which IC_{50} was derived and determined to be 0.33 mg/ml.

ii. Enzyme Specificity

ii.a. Pancreatic α-Amylase Inhibition

Fig. 5.2 reveals that α-amylase, assayed utilizing an α-amylase inhibition assay, was active in the presence of the isolated compound, in comparison to the standard ($P < 0.05$). The isolated compound hence inhibits α-glucosidase but not α-amylase enzyme, at 0.5 mg/ml.

ii.b. Single α-Glucosidase Enzyme Inhibition

Sucrase, maltase and isomaltase activity was observed separately utilizing α-glucosidase enzyme extract with the substrates being sucrose, maltose, and isomaltose, respectively. As shown in Fig. 5.3, the active sub-fraction appears to exert its α-glucosidase-inhibiting activity by predominantly inhibiting maltase and isomaltase, among the 3 enzymes that make up the enzyme complex. It exerted 48%, 49% and 16% inhibition of isomaltase, maltase and sucrase, respectively. The standard

(acarbose), under the same conditions, predominantly inhibited maltase and sucrase, and only showed little isomaltase inhibition.

Antioxidant Activity of Active Compound

i. DPPH Radical Scavenging

Antioxidant capacity of the active compound was evaluated by DPPH radical scavenging assay. While 0.5 mg/ml of the compound exhibited nearly no quenching activity (1.59%), 1 and 5 mg/ml of the compound scavenged 11.09% and 34.87% of the DPPH radicals in the reaction at 120 min, respectively. Percent DPPH scavenging per tested compound concentration is presented in Fig. 5.4.

ii. Oxygen Radical Absorbance Capacity ($ORAC_{FL}$)

The active compound's antioxidant capacity was also evaluated via the $ORAC_{FL}$ assay which enabled the estimation of the scavenging capability of the active compound against peroxyl radicals (AAPH). As depicted in Fig. 5.5, the compound appears to possess a notable oxygen radical scavenging activity, namely by surpassing the standard at equal concentrations. Trolox, a known scavenger of the peroxyl radical, was used as the standard. At 200 µM, the $ORAC_{FL}$ of the active compound was 1.9 fold that of the standard. At 40 µM, the $ORAC_{FL}$ of the active compound was 2.9 fold that of the standard.

iii. ABTS Radical Cation Neutralization

The radical scavenging capacity of the active compound was further assessed utilizing an ABTS radical cation. The compound surpassed the standard in cation neutralization. At 31 minutes, 17.67%, 22.24% and 32.73% neutralization were

observed with the standard, sample at 200 µM and sample at 400 µM, respectively. ABTS cation neutralization by the active compound is presented in Fig. 5.6.

Stability of the Active Compound

i. Thermal Stability

Stability of the act*ive* sub-fraction at 37 ºC is previously established given that the reaction is carried out at this temperature. In this test, the sub-fraction's activity at room temperature was used as a reference to assess its activity at 50-121 ºC. At 50 ºC, no loss of activity was observed. At 80 and 100 ºC, 6.35% and 11.63 % loss of activity was observed, respectively. The greatest loss of activity was observed at 121 ºC, where the sub-fraction exhibited a 20.3% activity loss. Results are displayed in Fig. 5.7, indicating that the active sub-fraction is thermally stable.

ii. pH Stability

Table 5.1 represents activity of the active sub-fraction following treatment with buffers at a wide pH range. The greatest loss of activity (36.6%) was seen at a pH of 2, and activity loss ranging from 6% to 21% was observed at the pH range of 3 to 6. At basic pH levels, the activity of the sub-fraction appeared to increase by 4.7% and 10.5% at pH levels of 8 and 9, respectively, and decrease by 3% at pH level of 10.

iii. Shelf-Life and Best Storage Conditions

At all storage conditions, no loss of activity was observed with the isolated compound, when tested at 3 and 6 months, except when stored in the light (61% loss of activity at 3 months). However, at 8 months, a complete loss of activity was observed.

Cytotoxicity of the Isolated Compound

Fig. 5.8 represents the results of MTS assay characterizing cell viability in presence of two different concentrations of the active compound. Sixty percent confluent NIH/3T3 cells were incubated with the compound at 25 and 50 µg/ml. At 25 µg/ml, 87.06% of cell viability was maintained, whereas 90.6% viability was observed at 50 µg/ml. Cytotoxicity was assessed by comparing cell viability in solvent control and treatment wells to that of wells containing cells and growth media only. When assessing the effect of the compound with respect to the solvent control, 90.55% and 90.03% cell viability are observed with 25 µg/ml and 50 µg/ml of the compound, respectively.

DISCUSSION

Mammalian starch digestion takes place primarily in the small intestine through the action of α-amylase, resulting in both linear maltose and branched isomaltose oligosaccharides that are additionally hydrolyzed by α-glucosidases to yield glucose [50-52]. It was hence necessary to find out whether the active compound also exhibits a non-specific α-amylase inhibition, a problem previously reported with α-glucosidase inhibitors [36]. For instance Acarbose, the most widely acknowledged α-glucosidase inhibitor, produces undesirable gastrointestinal side effects such as flatulence and diarrhea due to this property [49, 56, 57]. The current finding indicating that the compound is not active against pancreatic α-amylase will have strong implications on the safety of the compound if it were to be recommended for human consumption in the future, as it likely eliminates concerns of gastrointestinal side effects. Also, mammalian intestinal α-glucosidase is actually a complex comprised of three individual enzymes: sucrase, maltase and isomaltase [36]. One of our goals was also to determine which

moiety in the complex is inhibited by the isolated compound, if not all 3 enzymes. While Acarbose inhibits maltase and sucrase, [125], our results suggested that the active sub-fraction (hence the active compound) predominantly inhibits the maltase and isomaltase moieties of intestinal α-glucosidase. Furthermore, the compound appears to exhibit a dose-dependent inhibition of the enzyme complex, a known characteristic of acarbose [126]. The half maximal inhibitory concentration (IC_{50}) of the compound was determined to be 0.33 mg/ml, suggesting that this compound may serve as a lead compound in the future development of a therapeutic agent. IC_{50} is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. It represents the concentration of a drug that is required for 50% inhibition *in vitro*. It is commonly used as a measure of antagonist drug potency in pharmacological research [127]. Although the IC_{50} of our pure compound is 6.6 times higher than that of Acarbose (50 µg/ml) under our assay conditions, this compound is natural, food-derived and possesses the advantage of specificity to α-glucosidases. These specificity and method of action properties lead to the proposition that the GPE-derived active compound will effectively and specifically reduce the amount and rate of carbohydrate digestion, without the risk of gastrointestinal discomfort. These characteristics will surely require verification *in vivo*.

It is important to note that further mechanism of action testing was limited by the inability to re-isolate the active compound. Detailed mode of action tests are often required before a substance is recommended for human consumption. Measurements of the rates of catalysis at different concentrations of substrate and inhibitor often answer this question [128]. These tests were not completed due to the unavailability of sufficient amounts of the isolated active compound. Bioactivity-guided fractionation

commonly yields minute amounts of bioactive components [129], often sufficient for a limited number of tests. Furthermore, when a bioactive component is isolated, it is not uncommon for its re-isolation to fail even when an identical protocol is employed. Also, the process of extract fractionation can lead to a reduction or loss of biological activity by compound break-down, which may result in the re-isolation of an inactive or less active component [61]. Due to the fact that plant material-derived bioactive compounds often reside in multi-component blends, their separation and isolation remains a challenge [91].

Our recent finding associating mammalian intestinal α-glucosidase inhibition with antioxidant capacity of 6 red wine grape pomace extracts (Chapter 3) sparked an interest in investigating the presence of this trend in the isolated active compound. In fact, the compound appeared to possess a remarkable antioxidant activity. Although its DPPH radical quenching capacity was not as prominent as that of the crude Tinta Cão GPE at similar concentration and conditions (Chapter 3), the active compound still scavenged 35% of the radical in the medium. On the other hand, $ORAC_{FL}$ values of the active compound were impressive. At the tested concentrations, it appeared to be at least 1.9-fold more active than trolox, a known potent scavenger of the peroxyl radical, at identical concentrations. When incubated with the ABTS radical cation, the compound also showed a potent inhibition, outweighing the standard trolox at a similar concentration. These striking findings suggest that the isolated compound is a novel antioxidant compound that has not been previously investigated for this property, to our knowledge. These results may partly explain the previously observed correlation between α-glucosidase inhibition and antioxidant capacity. Furthermore, antioxidant

activity exhibited by a compound found to potently inhibit α-glucosidase enzyme function may have strong implications on diabetes management. Oxidative stress has long been regarded as a leading factor in the progression of diabetes and development of its chronic, mostly irreversible complications [130-132].

Glycosides such as anthocyanins are known to be sensitive to high temperatures upon which they readily degrade or polymerize with other compounds in the medium [133]. It was hence anticipated that our GPE-derived active compound may lose activity upon boiling, given that it is a glycoside. Surprisingly, there was no significant loss of activity upon exposure of the active sub-fraction to high temperatures, indicating that the compound will likely withstand food processing temperatures if applied as a functional food in the future.

Although plant-derived phenolic compounds have been reported to be more stable in acidic than alkali media [134, 135], our findings suggest that the isolated compound exhibits up to 37% activity loss at very low pH levels but remains active at basic pH levels. The compound's stability at high pH levels may have important implications in its applications in food. Alkali treatment has become a common procedure in food processing, utilized for multiple purposes, ranging from protein recovery from cereals to the destruction of microorganisms [136].

Despite the current compound's stability under room temperature, fridge and freezer storage conditions for up to 6 months, it appears to lose activity after 8 months of storage. Moreover, it displays a clear instability upon light exposure. These features are not uncommon with plant-derived bioactives in general, and phenolic compounds in specific. These compounds are sensitive to light, which facilitates degradation reactions

[137]. Instability is often encountered with natural medicine, and while there may not be a wide range of options to overcome this limitation, scientists continue to invest in studying and developing a bioactive compound within the scope of its stability.

Cytotoxicity testing is fundamental in the process of drug discovery. It is essential to conduct cytotoxicity studies to ensure that the product under investigation is not toxic to animal cells [61, 138]. The current cytotoxicity results imply that the isolated compound is not cytotoxic to cells at the tested concentrations. To note, due to shortage of sample, only two- low concentrations- were selected for this test. However, given that cell viability was almost identical when comparing the wells containing 25 µg/ml of sample to those containing 50 µg/ml, it appears that cytotoxicity levels did not increase upon doubling sample concentration. Hence, it may be safe to assume that the compound is likely not cytotoxic at higher concentrations. This implies the likelihood that the compound is safe for human consumption, but animal testing is recommended prior to clinical testing.

Conclusion

Red wine grapes, specifically Tinta Cão, are a promising bioresource for the development of an effective and likely safe to consume α-glucosidase inhibitor for the management of diabetes, providing a dual benefit considering antioxidant capacity. At least one component, D-Glucopyranose6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}, has proven to possess these properties. Pre-clinical and clinical investigations are necessary future steps to validate these findings in vivo. Natural products continue to serve as drug leads and given the unlimited availability of plants, it is imperative to invest in research investigating the bioactive properties of plant components.

FIGURES

Fig. 5.1. Percent Inhibition of α-Glucosidase by the Isolated Compound at Different Concentrations. Enzyme activity was determined by measuring p-nitrophenol release from pNPG at 405 nm. The isolated compound was assayed at 0.1, 0.25 and 0.5 mg/ml. Points marked with different letters are significantly different ($p<0.05$).

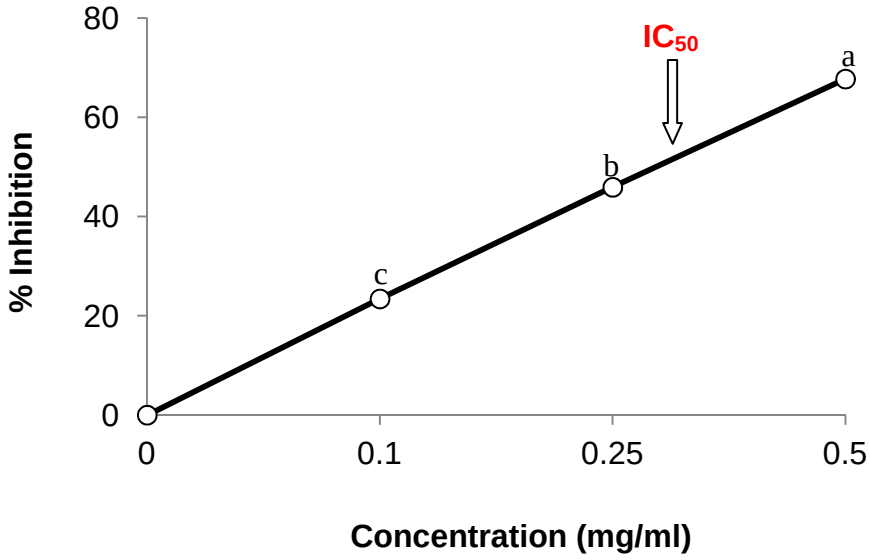

Fig. 5.2. Percent Inhibition of Pancreatic α-Amylase by the Isolated Compound. Enzyme activity was determined by measuring maltose release from starch at 540 nm. Acarbose (50 µg/ml) is the standard. Active compound was assayed at 0.5 mg/ml. Result is compared with α-glucosidase inhibition (p-nitrophenol release from pNPG at 405 nm) at similar sample and standard concentrations. Asterisk indicates significant difference compared to standard ($p<0.05$).

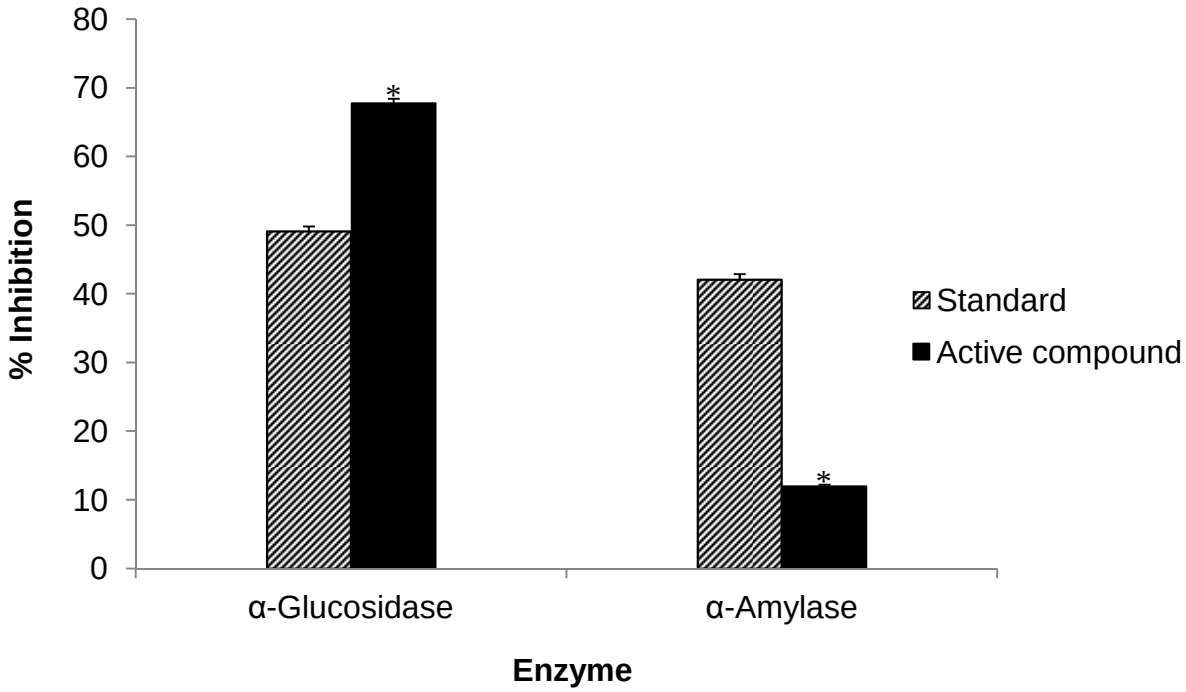

Fig. 5.3. Inhibition of Single α-Glucosidase Complex Enzymes by the Active Sub-Fraction. Enzyme activity was determined by measuring glucose release from oligosaccharides at 505 nm. Active sub-fraction (5 mg/ml) was incubated with maltose, isomaltose and sucrose, to estimate the activity of maltase, isomaltase and sucrase, respectively. Standard, acarbose, was subjected to similar testing.

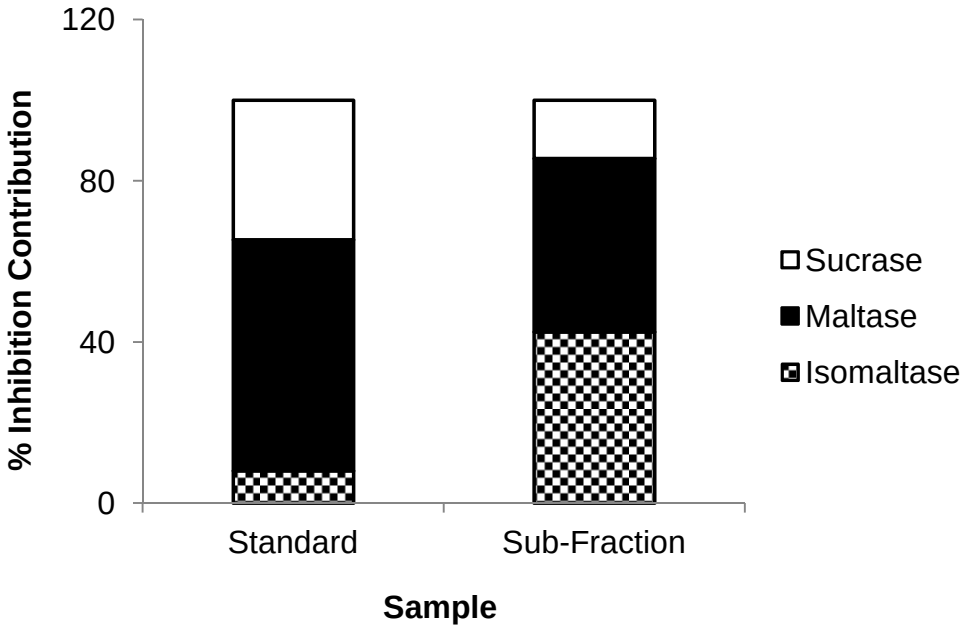

Fig. 5.4. Percent DPPH Scavenging per Active Compound Concentration. Scavenging capacity was measured at 500 nm using 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) radical. The reaction was conducted for 120 min. Control, identical reaction containing the sample solvent. Different letters represent significant difference ($p<0.05$).

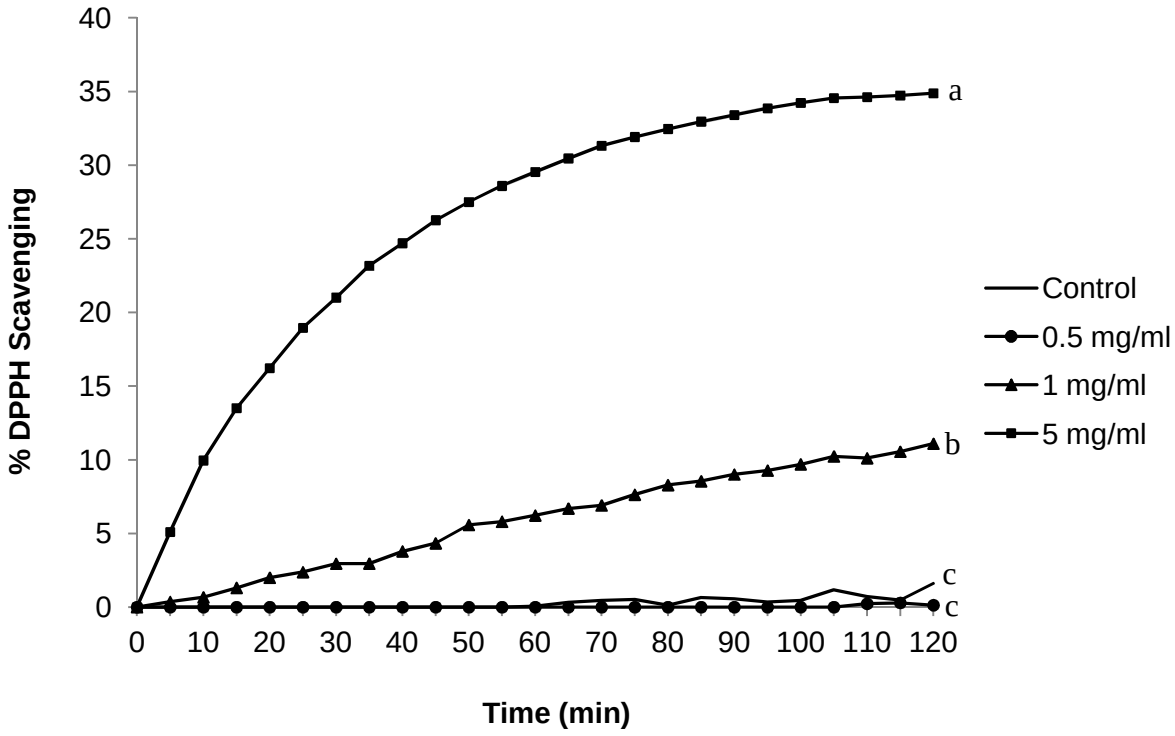

Fig. 5.5. Oxygen Radical Absorbance Capacity ($ORAC_{FL}$) of the Isolated Active Compound. $ORAC_{FL}$ was determined using 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator. Data is presented as antioxidant power in relative fluorescence units (RFU). Active compound was compared to standard (trolox) at equal concentrations. Asterisk indicates significant difference compared to standard ($p<0.05$).

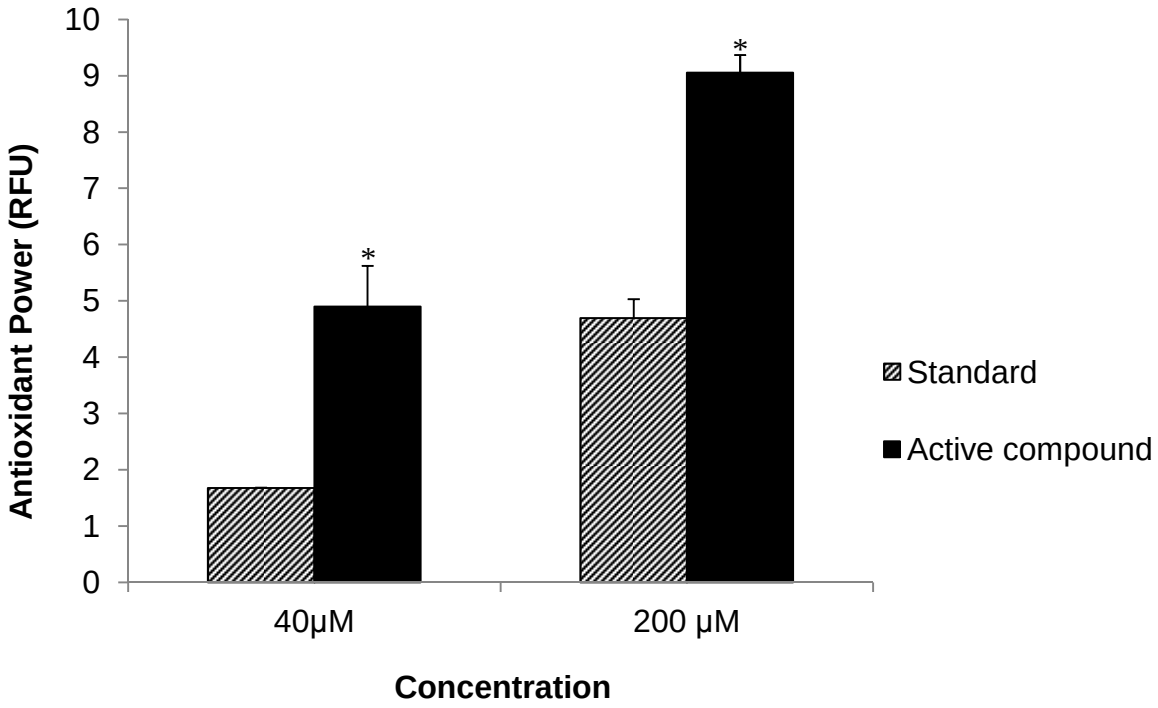

Fig. 5.6. Percent ABTS Cation Neutralization by the Active Compound. Neutralization of the 2,2′-azinobis(3-ethylbenzothiazoline -6-sulfonic acid) (ABTS) reactive cation was measured at 405 nm. The reaction was conducted for 31 min. Control, identical reaction containing the sample solvent. Different letters represent significant difference ($p<0.05$).

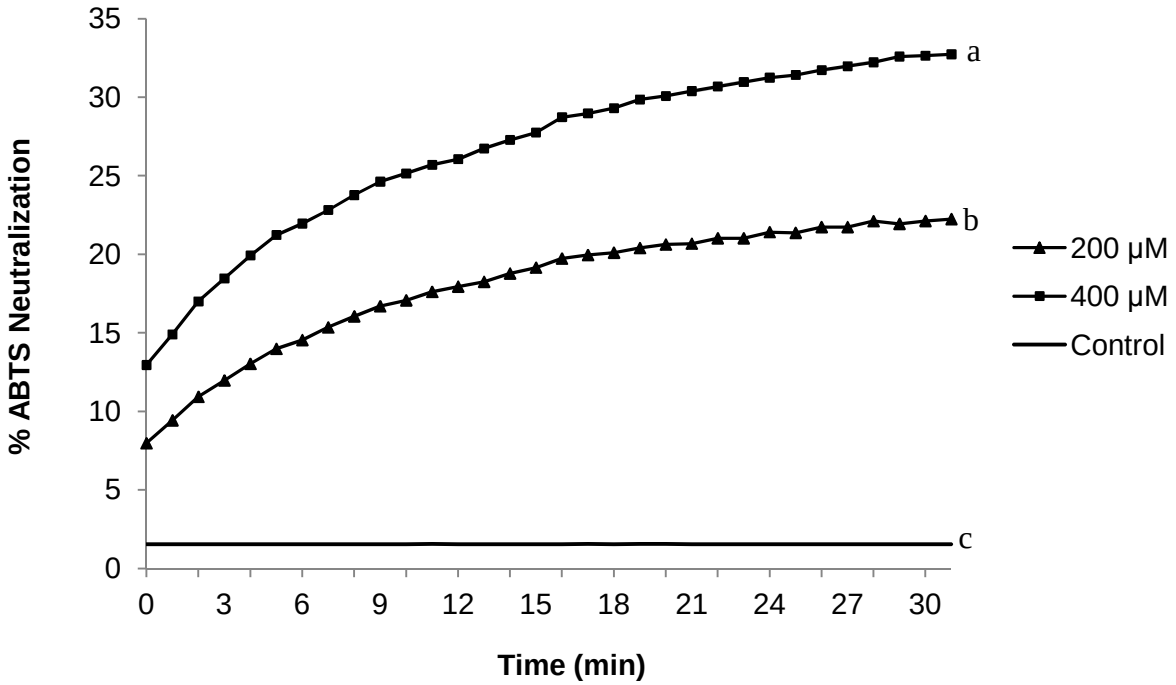

Fig. 5.7. Thermal Stability of the Active Sub-Fraction. Enzyme activity was determined by measuring p-nitrophenol release from pNPG at 405 nm. The active sub-fraction was assayed at 1 mg/ml after heat treatment at 50, 80, 100 and 121 *ºC*. Percent enzyme inhibition was assessed in comparison to untreated sample (room temperature), denoted as RT.

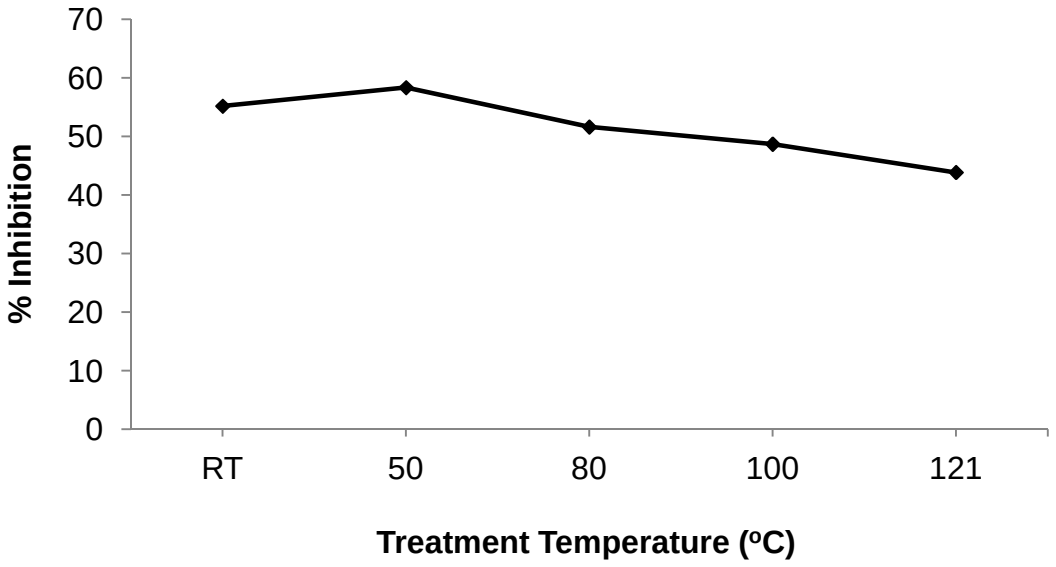

Table 5.1. pH Stability of the Active Sub-Fraction. Enzyme activity was determined by measuring p-nitrophenol release from pNPG at 405 nm. The sub-fraction was assayed at 0.5 mg/ml after treatment with buffers at pH 3, 4, 5, 6, 8, 9 and 10. Increase/reduction in enzyme inhibiting activity was assessed in comparison to untreated sample (pH 7).

pH Level	Maintained Activity (%)
2	63
3	79
4	94
5	83
6	92
7 (reference)	**100**
8	105
9	110
10	97

Fig. 5.8. Cytotoxicity of the Isolated Compound. NIH/3T3 cells were incubated with the compound at 25 and 50 µg/ml for 72 h. Respective solvent controls are 2.5% and 5% methanol, denoted as Control. CellTiter 96 AQ_{ueous} One Solution Reagent was utilized to quantify cell viability at 485 nm.

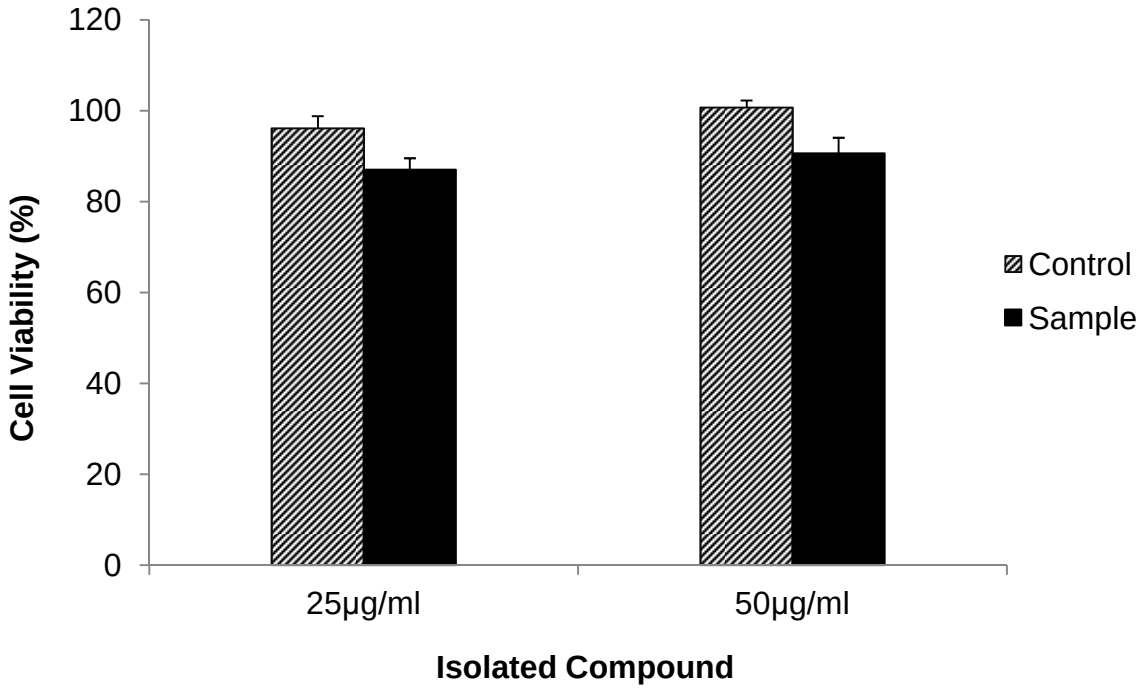

Table 5.2. Summary of the Active Compound Applicability Assessment. The isolated compound appears to inhibit α-glucosidase and not α-amylase. Furthermore, it is a dose-dependent inhibition, exerted by specifically inhibiting the maltase and isomaltase moieties of α-glucosidase. The compound also possesses an impressive antioxidant capacity. In terms of stability, it was shown to withstand temperature extremes but lose activity upon prolonged storage and prolonged exposure to light, and exhibit a partial loss of activity upon exposure to very acidic media. Lastly, at 25 and 50 µg/ml, the active compound was not cytotoxic to cells, proven by observing up to 90% cell viability.

Parameter	Result
Dose-Dependent	Yes
Specificity	Maltase, Isomaltase
Inhibits α-Amylase	No
Antioxidant Property	Yes
Heat Stable	Yes
pH Stable	Yes
Shelf Life	< 8 months
Storage	Dark
Cytotoxic	No

Summary and Future Directions

Study 1: The dramatic rise in obesity has led to a surge in type 2 diabetes rates, increasing illness, disability and mortality worldwide. Research aiming for the development of antidiabetic agents is hence on the rise. A now commonly studied target for diabetes and prediabetes management is the control of post-prandial hyperglycemia, as opposed to the traditional goal of targeting fasting hyperglycemia. This can be achieved by controlling carbohydrate digestion and absorption, and one method is inhibiting carbohydrate-digesting enzymes like α-glucosidase. We have previously reported that a red wine grape pomace extract (GPE) had the strongest inhibitory activity among hundreds of screened plant extracts. In the current study, a screening of six red wine GPEs (Chambourcin, Merlot, Norton, Petit Verdot, Syrah and Tinta Cão) indicated that the Tinta Cão variety possesses the strongest α-glucosidase inhibiting capacity. Antioxidant quantification in these grape varieties revealed an interesting trend: varieties with stronger α-glucosidase inhibitory capacity had a higher antioxidant content and ability. This prompted HPLC antioxidant profiling of the pomace samples. The 15 known antioxidants that appeared to vary in concentration among the 6 varieties were not able to suppress α-glucosidase activity, suggesting that the profiled antioxidant compounds are not in charge of the observed enzyme inhibition trend. Although the components responsible for the observed functions were not identified, the current results do reveal high antioxidant content/activity that strongly correlates with α-glucosidase inhibition. These promising findings may provide a foundation for the future use of grape pomace for the potential isolation and development of α-glucosidase

inhibitor for diabetes management. Further investigation is required to validate and optimize this property.

Future Directions: Having observed considerable differences in bioactivity among six varieties of grape pomace, it would be beneficial in the future to screen a wider range of grape varieties. The preparation of the enzyme in pure form as opposed to using an enzyme-rich extract would also generate more reliable and reproducible results. Lastly, antioxidant profiling would be more informative if more antioxidant standards are included, thus covering a wider variety of antioxidants. These strategies will provide a more confident foundation for the potential of utilizing GPEs for the development of an α-glucosidase inhibitor. However, to progress with the current results, *the components in Tinta Cão GPE responsible for the observed activity must be identified*.

Study 2: While diabetes rates continue to rise overwhelmingly, the main concern in treating diabetes is glycemic control. This is typically achieved by widely available oral medications as well as insulin and other injectables. While reliable in achieving short-term glycemic control, they often come with side effects and fail at certain points in time. Even agents targeting post-prandial hyperglycemia (as opposed to fasting hyperglycemia), such as Acarbose, appear to exert a non-specific α-amylase inhibition besides inhibiting α-glucosidases, generating undesirable gastrointestinal side effects. It therefore appears that currently available antihyperglycemic medications are insufficient to contain the problem. This brings about the need to explore novel agents. Among many scientific investigations searching for natural α-glucosidase inhibitors in the last decade, our investigation involving GPEs (study 1) yielded promising results prompting further testing to identify the GPE components responsible for the observed activity. An

extract is a complex combination of compounds and the search for a specific compound or group of compounds typically requires a series of bioactivity guided fractionation steps. In the current study, these steps were followed, yielding an active α-glucosidase inhibiting GPE fraction, a sub-fraction, and an active pure compound that was determined to be D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}. The isolation and identification of this compound may be a giant step forward for the future development of a natural α-glucosidase inhibitor from Tinta Cão GPE to potentially use for diabetes management and prevention.

Future Directions: Bioassay guided fractionation is a tedious process that must be carefully planned. In our study, we ran into the problem of insufficient amount of isolated compound. In the future, it is important to plan the separation with this point in mind. Separating large amounts of extracts surely involves magnified waste and cost, however, insures the isolation of a sufficient amount of the compound of interest. Also, while only the most active fraction was fractionated into sub-fractions, in the future it may be beneficial to attempt to fractionate the fraction that ranks next in activity (H_2O fraction in this study) which may have reduced the limitations related to chemical characteristics. However, to further develop the current results, *D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate} must be tested for applicability as a potential functional food*.

Study 3: When an agent is determined to possess a health-promoting bioactivity, it has to meet safety and stability measures before it is recommended for human consumption. The problem of instability is not uncommon with natural products. This makes it necessary to determine whether the product is stable under environmental

conditions that will likely be faced before and throughout consumption, such as storage, pH and temperature changes. It is also imperative to evaluate the safety of the product for human consumption. Cytotoxicity tests usually provide reliable information on whether the product will be expected to harm animal cells or not. In regards to α-glucosidase inhibition, another safety measure to take into consideration relates to possible side effects, given the nature of present inhibitors. It is important to rule out a non-specific α-amylase inhibition which in turn indicates the likelihood that no gastrointestinal side effects will be encountered. Alongside testing for enzyme specificity, inhibition mode is necessary information for the development of the product into a commercial bioactive agent. This study provided valuable inference on these aspects. D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate} appears to be fairly stable in terms of storage and environmental conditions, with minor limitations that can be taken into consideration in future applications. Also, it is likely safe for human consumption based on the negative cytotoxicity results and specificity to α-glucosidase, to which it is a dose-dependent inhibitor. Interestingly, this compound also exhibits a notable antioxidant capacity, which may partly explain the trend observed in study 1, and may represent a novel antioxidant compound. These results are fundamental for the future development of a natural, food-derived supplement for diabetes prevention and treatment, with the dual benefit of α-glucosidase inhibition and antioxidant activity.

Future Directions: Although the current results provide valuable information on the applicability of Tinta Cão GPE-derived D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate} as a functional food component, further measures of stability could be tested, such as photostability, compatibility with different storage

containers, storage form, ability to withstand simulated digestion, etc. Stability can further be understood by analyzing active compounds via NMR and MS to detect structural changes. Also, enzyme kinetics must be employed to reveal the mechanism of action. Moreover, cytotoxicity testing at higher concentrations may provide more relevant data. It is also important to conduct further measures of antioxidant capacity, namely more antioxidant assays (scavenging of additional radicals, lipid peroxidation inhibition, etc) and preferably analyze a wide range of known antioxidants for comparison, given the impressive antioxidant activity the isolated active compound appears to posses. However, to apply the current results, further testing is required prior to human consumption recommendation. First, pre-clinical testing should take place. This is achieved through the administration of the product to relevant animal models, for example diabetic mice/rats. Of interest, in addition to the effect on postprandial hyperglycemia, is the product's antioxidant effects *in vivo*, which should also be evaluated via relevant assays (ferric reducing ability of plasma, lipid peroxidation, inflammatory markers, etc). If the pre-clinical study validates the *in vitro* results with minimal to no side effects, then clinical testing is recommended. In a clinical test, human subjects with pre-diabetes will be observed for the effects of ingesting different doses of the product. Safety and efficacy of the product will render it appropriate for consideration for commercial development. These investigations will have very important implications given the current prevalence of diabetes and the urgent need to find alternative methods to control and prevent it.

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF NATURAL α-GLUCOSIDASE INHIBITORS FROM ANTIOXIDANT RICH RED WINE GRAPES (*VITIS VINIFERA*)

by

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Background: Diabetes is currently a global public health problem affecting people at all ages. Dietary antioxidants have been associated with a reduced risk of type 2 diabetes. Grape pomace contains considerable amounts of polyphenols and it has been reported to exhibit an inhibitory activity against alpha- glucosidases. Alpha-glucosidases, in turn, play a major role in controlling starch digestion and therefore postprandial blood glucose, a target for diabetes management.

Objective: This study aims to investigate the anti-diabetes potential of a selection of six grape pomaces and prepare and purify active components in the active variety that specifically inhibit intestinal α-glucosidases. The study was also designed to evaluate the applicability of the isolated active components as natural inhibitors of α-glucosidases.

Methods: Chambourcin, Merlot, Norton, Petit Verdot, Syrah and Tinta Cão red wine grape pomace extracts were assessed for their rat intestinal α-glucosidase inhibiting activity and antioxidant properties via biochemical assays and UV detection. Then, the grape pomace variety shown to potently inhibit the enzyme was subjected to

bioactivity-guided fractionation and the isolated active component was identified via analytical chemistry techniques. The characterized compound was then tested for functional food applicability via stability, enzyme specificity and cytotoxicity testing.

Results: Tinta Cão grape pomace extract was the most potent α-glucosidase inhibiting variety and possessed a remarkable antioxidant activity, both properties of which appeared to be correlated. HPLC analysis did not yield an antioxidant responsible for the observed trend. Hence, bioactivity-guided fractionation of the extract was pursued, yielding a pure active compound that was determined to be D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}, which also exhibited a strong antioxidant activity. Further testing indicated that the compound inhibits α-glucosidase and not α-amylase, and specifically inhibits the maltase and isomaltase moieties of α-glucosidase, in a dose-dependent fashion. The compound was fairly stable under different environmental and storage conditions, and it was also not cytotoxic to animal cells.

Conclusion: Red grape pomace, namely Tinta Cão, is a promising bioresource for the future development of a food-derived antidiabetic agent. At least one component, D-Glucopyranose 6-{(2E)-3-(4-Hydroxyphenyl)prop-2-enoate}, isolated from Tinta Cão grape pomace appears to potently and specifically inhibit mammalian intestinal α-glucosidases while exhibiting a notable ability to quench free radicals. It may thus represent an alternative future strategy for diabetes management and a novel antioxidant compound. Pre-clinical and clinical testing will validate the obtained results *in vivo*.

AUTOBIOGRAPHICAL STATEMENT

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Education

May 2010	Master of Science in Nutrition and Food Science, Wayne State University, Detroit, MI
May 2008	Coordinated Program in Dietetics, Wayne State University, Detroit, MI
Sept 2006	Dietetic Internship, Rafik Hariri University Hospital, Beirut, Lebanon
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Professional Experience

2014-Present	Graduate Research Assistant, Department of Nutrition & Food Science
2012-2013	Graduate Teaching Assistant, Department of Nutrition & Food Science
2011-2012	Part-Time Faculty, Department of Nutrition & Food Science
2008-2009	Instructional Assistant, Coordinated Program in Dietetics Wayne State University, Detroit, MI
2008-Present	Clinical Dietitian, Oakwood Hospital and Medical Center, Dearborn, MI
2008-Present	Member of Board of Directors, Southeastern Michigan Dietetic Association
2005-2006	Volunteer Research Assistant, Human Morphology, American University of Beirut

Publications

Kadouh, H. and Zhou, K. (2012) Nutraceutical and Health Properties of Common Beans (*Phaseolus vulgaris*), in Cereals and Pulses: Nutraceutical Properties and Health Benefits (eds L. Yu, R. Tsao and F. Shahidi), Wiley-Blackwell, Oxford, UK. doi: 10.1002/9781118229415.ch13

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Awards and Presentations

Recognized Young Dietitian of the Year, Michigan, Academy of Nutrition and Dietetics, 2010
Graduate Professional Scholarship, Wayne State University, 2007-2013
Mark & Arlene Christensen Endowed Scholarship for Math and Science Students, Wayne State University, 2011
Graduate Scholarship Award, Southeastern Michigan Dietetic Association, 2010
Dean's Honor list, American University of Beirut, 2004 and 2005

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