Submitted in partial fulfilment of the requirements for the Degree of Bachelor of Science

Investigating the inhibitory effects of dietary polyphenols on human salivary $\alpha$-amylase \textit{in vitro}.

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Abstract

Diabetes mellitus is a global health condition reaching epidemic proportions. One of the treatments for type 2 diabetes, acarbose, acts as an enzyme inhibitor, but it causes undesirable side effects. Thus, a new alternative is desirable. Polyphenols are functional foods that have the potential to reduce hyperglycaemia by $\alpha$-amylase inhibition. This hypothesis was tested in this project. The following report was one of the first pieces of research to be undertaken \textit{via} an \textit{in vitro} $\alpha$-amylase assay at optimum conditions, and with a solid phase extraction carried out on the polyphenols. Results found for the first time that quercetin ($IC_{50}=14$) and myricetin ($IC_{50}=24$) are strong inhibitors of $\alpha$-amylase, while galangin and myricetin derivatives were found to be poor inhibitors of $\alpha$-amylase. Additionally, a combination of polyphenols showed no significant additional increase in percentage inhibition. Myricetin inhibitors were found to be poor inhibitors, with none achieving $IC_{50}$ values. It is concluded that myricetin and quercetin have the potential to decrease $\alpha$-amylase activity and slow the digestion of starch into sugars and thus blunt the spikes in hyperglycaemia. A possible explanation to this could be regarding different polyphenols’ chemical structure. Further research should be performed with quercetin and myricetin \textit{in vivo} in order to explore the absorption and bioavailability of these polyphenols in humans.

1. Introduction

1.1 The burden of disease

Diabetes mellitus is a condition that occurs worldwide. It is estimated that in 2013 over 382 million people were diagnosed with diabetes globally. At least 85-95% of these patients were type 2 diabetes, and this figure is predicted to rise (Weng et al., 2015). Health surveys and epidemiological studies which included 199 countries and over 2.7 million participants, found that over 28 years, the incidence of diabetes increased by 194 million new cases (Danaei et al., 2011). If this incidence does not begin to show a decline, then it will not only affect individuals, but the expenditure on diabetes health care will also rise.

The cost of diabetes (both type 1 and 2) is already straining health services in the UK. Hex et al. examined the past costs and predicted the future costs of diabetes for 2010/2011 and 2035/2036 in the UK. Results found that in 2010/2011 diabetes cost £23.7 billion in the UK alone, and it was predicted that in 2035/2036 diabetes would account for 17% of the total amount spent on health costs in this country, at a staggering £39.8 billion (Hex et al., 2012). This demonstrates that as the occurrence of diabetes increases, so does the cost, so finding a solution to
this problem is crucial. Out of all the predicted diabetes costs, the large majority of expenses (£35.6 billion) were going into treating type 2 diabetic patients (Hex et al., 2012).

Type 2 diabetes is characterised by elevated blood sugar levels, due to the body’s inability to respond to insulin as normal (Martin et al., 1992). Therefore, studies have shown that postprandial hyperglycaemia is typical of type 2 diabetes (Hirsch, 2005). Moreover, fluctuations in blood sugar levels, both postprandial and interpandial, are observed in type 2 diabetes patients (Monnier et al., 2008). Studies have shown that fluctuations in blood glucose levels could also increase the risk of developing cardiovascular disease (CVD), as evidence shows a well established correlation between diabetes and an increased risk of heart disease (Haffner et al., 1998).

For these reasons, devising a control mechanism that regulates blood sugar and blunts the spiked fluctuations in these levels is key in helping those with type 2 diabetes mellitus, and subsequently may also be important in decreasing the risk of cardiovascular disease.

1.2. Inhibition of enzymes

Glucose homeostasis is key in preventing and controlling type 2 diabetes. Certain enzymes play a crucial role in the breakdown of starch, which produce sugars that cause hyperglycaemic peaks. Amylases (including α-amylase) are a family of enzymes which are responsible for hydrolysing starch (amylose) by causing the α-1,4, glycosidic links to break (Nyambe-Silavwe et al., 2015). This reaction produces the sugar maltose which is further broken down into glucose and can be absorbed into the blood. Thus, α-amylase (found in both the saliva and pancreas) plays a vital role in starch degradation into sugar, which can then be absorbed in the blood from the small intestine (Aiyer, 2005). Naturally, the rate of amylose starch breakdown by α-amylase affects blood glucose spikes. Promising evidence has suggested that inhibiting these enzymes could play an important role in controlling type 2 diabetes, due to the regulation of maltose production and thus the slowing of absorption of sugar into the blood stream due to less maltose being broken down into glucose (da Silva et al., 2014). Therefore, enzyme inhibitors are an important diabetic treatment (Weng et al., 2015).

1.3. Current diabetic treatment

As it stands, there are currently over ten drug types available for type 2 diabetes treatment. They work by an array of different mechanisms (Garber et al., 2013). Enzyme inhibition, however, remains the preferred treatment, with health professionals recommending it as the first, second, and third-line treatment for type 2 diabetes (Weng et al., 2015). One of the most popular drugs currently on the market is acarbose. Acarbose inhibits α-glucosidase, which is also found in the saliva and pancreas of animals including humans. When α-glucosidase is inhibited, less hyperglycaemia and blood sugar fluctuations take place as less polysaccharides are hydrolysed into glucose (Lordan et al., 2013; da Silva et al., 2014).

Although there is supporting evidence for acarbose as a means of controlling type 2 diabetes, there are also negative side effects associated with the drug. Due to acarbose preventing the majority of starch breakdown, the starch is not absorbed into the blood from the small intestine. It therefore travels to the large intestine where it ferments, causing undesirable
flatulence and diarrhoea in some individuals (Nyambe-Silavwe et al., 2015). For this reason, the use of acarbose is often discontinued (Nyambe-Silavwe et al., 2015). Furthermore, evidence from non-intervention studies shows that acarbose is more effective and well tolerated in certain populations, such as Southeast Asians rather than Caucasians (Weng et al., 2015). For this reason, a natural alternative to acarbose that is applicable worldwide would be beneficial.

1.4. Introduction to polyphenols

Polyphenols are compounds found naturally in plant matter. They contain many subgroups such as anthocyanins, flavonols and tannins, which are responsible for certain characteristics of some plants (such as colour and flavour) (Barrett et al., 2013). The research discussed in this project will look at the flavanol subgroup in particular, whose basic chemical structure is characterised by three benzene rings with a different number of hydroxyl groups (-OH) attached to each (Tsao, 2010). Common sources of dietary polyphenols include berries, grapes skins/wine, and tea (Tsao, 2010). Certain polyphenols have been shown to be protective against a number of conditions such as dyslipidaemia, atherosclerosis and possibly hypertension (Akkarachiyasit et al., 2010; Brown et al., 2009). Additionally, recent evidence has suggested that certain polyphenols may show strong inhibitory properties of enzymes, especially those enzymes involved in starch breakdown such as α-amylase (Chen and Kang, 2014). This was further consolidated by an animal study which found that giving certain polyphenols to rats lowered their blood glucose levels (Abesundara et al., 2004).

Although research has been carried out to investigate the inhibitory properties of different polyphenols on enzymes, little of this work has taken place under the correct assay conditions and the experiments have only been performed on a limited range of polyphenols. Nyambe et al., (2015) conducted multiple in vitro assays in order to deduce the optimum inhibition assay conditions for α-amylase, and to our knowledge, little research has followed which incorporates these recommended conditions (i.e. the correct pH, temperatures, and concentrations) (Nyambe-Silavwe et al., 2015). Furthermore, it is now well established that some polyphenols react with the DNS colour reagent that is used in inhibition assays which is a source of bias. A solid phase extraction (SPE) can be used to separate the polyphenols from the rest of the reagents to eliminate the DNS colour reagent and polyphenols from interacting and affecting results. Omission of a SPE makes previous research less reliable, as this gives skewed results (Nyambe-Silavwe et al., 2015).

1.5. Aim of this research

Human studies have revealed that multiple polyphenols are present in the blood plasma of humans after the ingestion of polyphenol rich foods (Kerimi et al., 2015). The polyphenols present were galangin, quercetin, and kaempferol and although myricetin was not seen to be present after it was consumed, it was proposed that it was transformed to quercetin or present as a myricetin derivative (Kerimi et al., 2015). These four polyphenols are found in abundance in the diet and, to our knowledge, no studies have looked at the effect of these polyphenols on α-amylase activity via an in vitro inhibition assay to deduce the mechanistic evidence for the effect on starch breakdown under the optimum conditions. This project seeks to address this issue by deducing a mechanism for the action of polyphenols on starch metabolism. If it can be shown that these polyphenols have
inhibitory properties of α-amylase activity *in vitro*, then this could potentially be applied in human studies in order to observe the effect it has on blunting postprandial hyperglycaemia peaks in diabetic patients. As a first step, however, this study will observe the inhibitory potential of galangin, myricetin (and its derivatives), quercetin, kaempferol and epigallocatechin gallate (EGCG) *in vitro* by an α-amylase inhibition assay, and will test the interaction of each polyphenol with the DNS colour reagent on beginning the experiment.

2. Experimental

2.1. Basis of the experiment

Inhibition assays are a commonly used method of assessing a compound's inhibiting potential *in vitro* (e.g. for new drug development), and are reliable, providing each assay is always carried out under the same conditions (Strelow et al., 2004). In the case of this experiment, an α-amylase inhibition assay was used to assess the polyphenol's inhibiting potential of α-amylase with amylose as the substrate and increasing concentrations of various polyphenols. The quantitative data was collected in the form of an absorbance reading from a plate reader (at 540 nm). The colour intensity being measured is detected by the DNS colour reagent which reacts with maltose. The darker the orange colour, the more maltose has been produced. Thus, a potent inhibitor would give a lower absorbance reading due to a lighter yellow colour being seen, as less maltose is being produced by the α-amylase – amylose complex. The statistical analysis of the absorbance readings given is described in section 2.8. This method was based on the methodology of that from Nyambie et al., (2015).

2.2 Materials, reagents and standards

96 mM 3,5- dinitrosalicylic acid (catalogue number; D0550), amylose from potato (A0512), EGCG (E4143), dimethyl sulfoxide (DMSO), sodium potassium tartrate solution (5.3 M) (S2377), quercetin (Q4951), and salivary α-amylase (A1031), were all obtained from Sigma-Aldrich Co. Ltd, Dorset, UK. Galangin (1114 S), myricetin (1127 S), kaempferol (1124 S), myricetin-3-O-galactoside (1395 S), myricetin trimethylether (1310) and myricitrin (myricetin 3-O-rhamnoside) (1029 S) were purchased from Extrasynthase, Genay, France. Cartridges for the solid phase extraction were Oasis MAX 1 mL which were obtained from Waters Corporation Ltd., Milford, United States.

The following were prepared in bulk on beginning the experiments: 0.1 M phosphate buffer solution (PBS) (pH 6.9) was made up by dissolving 10.9 g disodium hydrogen phosphate, 3.2 g sodium hydrogen phosphate, and 90 g sodium chloride in 1 litre of ultrapure (miliQ) water. A pH of 6.9 was ensured by using sodium hydroxide (1 M) or hydrochloric acid (1 M) and adjusting on a pH metre.

400 μL of DNS colour reagent solution was prepared by adding sodium potassium tartrate solution 5.3 M to 95 mM 3,5 dinitrosalicylic acid solution with 120 ml water. Sodium potassium tartrate solution (5.3 M) was prepared by heating NaOH (80 mL) at 50-70 °C and dissolving 120 g sodium potassium tartrate solution in NaOH whilst on a heat and stirring plate. 96 mM 3,5-dinitrosalicylic acid solution was prepared by dissolving 4.38 g of 3,5- dinitrosalicylic acid in 20 mL of purified water and heated on a stirring plate.

2.2 Enzyme preparation and concentration
1 mL of phosphate buffer solution (0.1 M) was used to dissolve 1 mg human salivary \( \alpha \)-amylase enzyme to give the concentration of 117.5 units/ml. The desired concentration for the assay was obtained by using the equation \( M_1V_1=M_2V_2 \) (where \( M= \) concentration and \( V= \) volume). Initially, an enzyme concentration of 0.5 units/mL was used. This was later adjusted to increase five fold, resulting in a final assay concentration of 2.5 units/mL.

### 2.3 Preparation of amylose

Amylose starch from potato was prepared by weighing 25 mg amylose and dissolving it in 100 \( \mu \)L ethanol, 1 mL water, and 200 \( \mu \)L 10% NaOH. The mixture was then placed on a hot plate at 150 °C for 10 minutes. Once dissolved, the solution was made up to 10 mL with cold water, and the pH adjusted to 6.9 by adding either concentrated HCl, 0.1 M HCl, or 0.05 M HCl on a pH metre until the desired pH was met. The final amylose concentration was 1 mg/mL in 100\( \mu \)L of the final assay.

### 2.4 Preparation of the polyphenols

The polyphenols used were quercetin, EGCG, myricetin, galangin, kaempferol, and myricitrin derivatives (myricitrin, myricetin trimethyl ether, and myricetin galactoside). These were made up to the appropriate concentrations of 0, 20, 40, 60, 80 and 100 \( \mu \)M. The amount of DMSO required to dissolve the polyphenols (using the smallest amount of DMSO possible) was calculated by using the molecular weight of the polyphenol as well as the weight and final concentration required. All concentrations, including blanks, were balanced with DMSO to remove any error that the DMSO may cause by interacting with the DNS colour reagent. However, no interaction was observed.

### 2.5 Effect of polyphenol on DNS colour reagent

It was necessary to determine what affect different polyphenols may have on the DNS colour reagent to ensure reliable results were obtained. 1000 \( \mu \)L of DNS colour reagent was placed in a 2 mL Eppendorf tube with 450 \( \mu \)L PBS (pH 6.9, 0.01 M), and 50 \( \mu \)L polyphenol and DMSO (100 \( \mu \)M). A blank consisted of 1000 \( \mu \)L colour reagent, 450 \( \mu \)L PBS, balanced with the appropriate volume of DMSO (this being dependent on the polyphenol being used). Both samples were placed in 100 °C water bath for 10 minutes to allow colour to develop and a redox reaction to take place. Samples were left to cool on ice, and then 250 \( \mu \)L of each was placed on a 96 well plate and run through the plate reader at 540 nm. Absorbance readings showed whether each polyphenol interacted with the colour reagent. If the polyphenol interacted with the colour reagent, then a solid phase extraction was necessary as an additional step to remove the polyphenol content, as the polyphenol causes the interaction. This SPE was only done after the reaction has taken place and before the DNS colour reagent was added. This is because the polyphenol both the inhibitor and the cause of the DNS reaction, therefore removing it before the reaction would give no inhibition. The method for solid phase extraction can be seen in section 2.7.

### 2.6 \( \alpha \) - amylase enzyme inhibition assay

To make up the samples, 2mL Eppendorf tubes were used which contained 200 \( \mu \)L substrate (amylose), 50 \( \mu \)L phosphate buffer solution (PBS) and 50 \( \mu \)L polyphenol inhibitor (inhibitor mixture plus PBS always equalled 100 \( \mu \)L; however volumes were
adjusted and balanced with DMSO depending on the desired concentration. Controls were made up to contain the same as the samples above, but with an additional 50 µL PBS instead of the polyphenol. Triplicates of each sample were used in order to produce repeats. The pre-made up α-amylase solution and samples were separately placed in the 37 °C water bath for 10 minutes in order to bring both mixtures up to their optimum temperature. After this time, 200 µL α-amylase enzyme was placed in each sample, mixed on a vortex machine, and immediately placed in the 37 °C water bath for 10 minutes to allow the reaction to take place.

After 10 minutes, the tubes containing the samples were placed in 100 °C water bath for 10 minutes to denature the enzyme and cease the reaction. Samples were placed on ice to cool, before being centrifuged (speed 13.3 xG for 5 minutes). A solid phase extraction then took place on the polyphenols which had demonstrated interaction with the colour reagent (see section 2.7).

Following the solid phase extraction (if required) 1000 µL DNS colour reagent was added to each sample. Blanks were also made up which consisted of 1000 µL colour reagent and 500 µL PBS. All samples were placed in 100 °C water bath for 10 minutes for the redox reaction (colour development from DNS colour reagent and maltose) to take place. If maltose was present, a colour change from yellow to orange was produced by the colour reagent.

After 10 minutes, samples were removed from the water bath and placed on ice to cool. A 96 well plate (non UV transparent) was used to hold 250 µL of each sample, and this was run through the plate reader at 540 nm to obtain absorbance readings and determine maltose levels produced by the α-amylase – amylose complex.

2.7 Solid phase extraction

The solid phase extraction removed the polyphenol from the sample before adding the colour reagent, due to the reaction between some polyphenols and the DNS colour reagent. The rest of the experiment was then be carried out as usual; and the maltose concentration measured. One Oasis MAX cartridge per sample was conditioned with 1 mL methanol, and dried out under vacuum (10 mm Hg for 10 minutes). The cartridges were then equilibrated with 1 mL miliQ water. 500 µL of each sample was loaded into the cartridges and allowed to run through (at 20 Hg). This removed all polyphenolic compounds. 500 µL of water was used to rinse the cartridges between samples to avoid cross-contamination. This process was repeated for all triplicates for each sample, which were collected in new Eppendorf tubes.

2.8 Calculating percentage inhibition and statistical analysis

The absorbance readings from the samples were detected by the plate reader. The blank reading was subtracted from each sample reading to give the absorbance value without the colour reagent. The amount of α-amylase inhibition from the polyphenol was calculated as a percentage of the control. The following equation was used:

\[
\% \text{ Inhibition} = \frac{\text{Control absorbance average} - \text{Sample absorbance reading}}{\text{Control absorbance average}} \times 100
\]

Averages and standard deviations were calculated for each sample and plotted in the form of a graph. The
graphs were used to determine the IC$_{50}$ value for each polyphenol. IC$_{50}$ values are commonly used in drug research, and represent the concentration of the inhibitor (polyphenol) required to achieve 50% inhibition of the $\alpha$-amylase (Brooks et al., 2004). Two tailed paired t-tests were performed. Results were classed as significant when the $p$ value was below or equal to 0.05. One-way ANOVA tests were carried out in order to assess significance between different samples.

3. Results

3.1. Interaction of polyphenols with DNS colour reagent

Each polyphenol was tested in order to determine its reactivity with the DNS colour reagent, to remove any bias which may have occurred due to this interaction. Results in fig. 1 show that the following compounds did not react with the DNS colour reagent: quercetin, galangin and kaempferol. No solid phase extraction was required for these compounds. However, myricetin showed a strong interaction with the DNS colour reagent. For myricetin, the difference between the absorbance readings for the blank and polyphenol was significantly higher (paired t-test (two-tailed) $p=0.00$ $n=0.18$) for the polyphenol-containing sample than for the blank. For all of the myricetin derivatives, a strong colour change from yellow to green occurred when testing the interaction with DNS colour reagent, demonstrating that there was a strong reaction between the two, and thus, a change in absorbance reading could be due to the colour change from this interaction rather than the inhibitory potential of the polyphenol. No absorbance reading was taken for the myricetin derivatives due to the obvious colour change noted from orange to green. For this reason, solid phase extractions were carried out for the myricetin derivatives. EGCG also required a solid phase extraction as it showed a strong interaction with DNS; however, due to the obvious interaction no reading was obtained.

![Fig 1](image_url) - Interference of polyphenols by interaction with DNS colour reagent in comparison with the blank. All polyphenols were used at a concentration of 100 $\mu$M. Standard deviation bars are shown in black (samples: n=3).
3.2. Effect of original polyphenols on $\alpha$-amylase activity.

Fig. 2 shows $\alpha$-amylase inhibition from the four original polyphenols investigated in this experiment. All polyphenols exhibited at least partial inhibition of $\alpha$-amylase activity at concentrations 0-100 µM. Galangin demonstrated the least inhibition of $\alpha$-amylase, reaching a maximum inhibition of just 8.1% (SD=±9.8). However, galangin at the highest concentration (100 µM) inhibited $\alpha$-amylase activity by 4.9% more than the lowest concentration of galangin (20 µM). This difference was found to be statistically significant (paired t-test (two tailed), $p=0.03$). Despite this, galangin, did not achieve 50%
inhibition and therefore no IC\textsubscript{50} value was obtained (table 1), even at the maximum concentration (100 \(\mu\)M).

Kaempferol proved to have slightly greater inhibitory potential to that of galangin, with fig. 2 and table 1 showing 50\% inhibition was achieved, giving a relatively low IC\textsubscript{50} value of 18 \(\mu\)M. Kaempferol reached a maximum inhibition of 70.1\% (SD=±0.8) at a low concentration of just 40 \(\mu\)M. Although the inhibition was seen to drop between 40 \(\mu\)M and 100 \(\mu\)M, a paired t-test (two tailed) revealed that this decrease was not statistically significant (\(p=0.5, n=13.2\%\)). This means that kaempferol maintained its maximum inhibition (70.1\%).

Table 1 shows that myricetin reached the highest percentage inhibition of \(\alpha\)-amylase of all polyphenols, at 99.1\% (SD=± 0.2). The standard deviation bars shown on fig. 2 are small and therefore it is likely that the myricetin reached significantly higher inhibition than the other polyphenols. At 20 \(\mu\)M, so the bars overlap with kaempferol and quercetin, however, a one-way ANOVA showed that the percentage inhibitions were all still significantly different to one another (\(F= 7.37, p=0.02\)). Although myricetin proved to reach the highest inhibition out of all of the polyphenols, myricetin in fact obtained the highest IC\textsubscript{50} value (24 \(\mu\)M) out of the polyphenols that achieved one. This means that although myricetin filled the highest amount of active sites on \(\alpha\)-amylase, it also required the highest concentration to do so. A one-way ANOVA found that the similarities shown in fig. 2 in final inhibition (% at 100 \(\mu\)M) between myricetin and quercetin were statistically significant, with a large F value meaning that the null hypothesis (that the percentage inhibitions are the same) can be rejected, and the small \(p\) value indicating statistical significance (\(F = 39.7, p=0.003\)).

Quercetin obtained the lowest IC\textsubscript{50} value (14 \(\mu\)M) and a maximum inhibition of \(\alpha\)-amylase of 93.9\% (SD=±1.5). Max inhibition was marginally lower than myricetin; however, quercetin had a much lower IC\textsubscript{50} value then myricetin. Statistical tests found that quercetin had technically reached its maximum inhibition at 40 \(\mu\)M, due to the decrease in percentage inhibition between this concentration and the final concentration (100 \(\mu\)M) not being statistically significant (paired t-test (two tailed); \(p=0.68 n=0.79\)). Interestingly, the findings from these four polyphenols show that the highest percentage max inhibition does not necessarily give the lowest IC\textsubscript{50} value.

The inhibitory properties of EGCG were also studied. Table 1 shows that EGCG reached a maximum inhibition of 38\%. However these results are not comparable with the other four inhibitors, as the concentrations used were only up to 10 \(\mu\)M, as opposed to 0 to 100 \(\mu\)M. EGCG did however increase in percentage inhibition of \(\alpha\)-amylase as the concentration increased, and it might be expected that this linear inhibition would continue to increase with increasing concentration.
3.3. Combined polyphenols on α-amylase activity

Following the findings that quercetin, myricetin and kaempferol were strong inhibitors, it was concluded that an inhibition assay with combinations of these polyphenols would be beneficial in order to determine whether polyphenols interact with each other in vitro. When studying kaempferol and quercetin combined (fig 3A) a maximum of concentration 10 µM was used. At 10 µM, percentage inhibition 31.6% (SD=±6.0). This is low considering the inhibitory effects of quercetin alone were strong (34% at 10 µM), and even higher for kaempferol (41% at 10 µM). It was expected that the sum of the individual inhibitors at 10 µM (i.e. 75%) would be seen, but in fact it appears that having these two polyphenols in combination may slightly impede each other and lower the percentage. Furthermore, no IC₅₀ value was obtained.

For this reason, it was deemed appropriate to repeat the experiment, but to consider the inhibitory effects of the top two polyphenols shown in fig. 2- quercetin and myricetin. Fig. 3B displays these results. This time, a higher concentration of up to 20 µM was used, and quercetin and myricetin were studied individually but within the same inhibition assay, to create comparable results. Myricetin alone reached a maximum inhibition of 18% (SD=±2.7) (fig. 3B). Quercetin alone reached a maximum inhibition of 59% (SD=±3.3). When combined, the maximum inhibition reached was 65.6% (SD=±3.2, IC₅₀= 14.9), which means that the polyphenols’ individual inhibitory effects were not combined. However, a higher amount of inhibition was still seen when the polyphenols were in combination than when either
polyphenol was alone, as well as achieving a lower IC\textsubscript{50} value than each polyphenol alone. This proves that when two polyphenols are seen together \textit{in vitro}, they do not appear to impede the inhibitory effects of each other. Results do however show that when the polyphenols are combined, the sum of the maximum inhibition of each inhibitor alone is not seen, because if this was the case a maximum inhibition of 77% would be expected, whereas in this experiment only a maximum of 65.6% (SD=±3.2) was observed (table 1.). Furthermore, a one-way ANOVA compared the highest concentration of the combined inhibitors with quercetin alone. While the combined myricetin and quercetin did show increased percentage inhibition, the results are not statistically significant and therefore this result may have occurred by chance (p= 0.07, F= 5.77). So therefore, these polyphenols in combination has no significant additional benefit over quercetin alone.

3.4. Inhibition from Myricetin derivatives

As seen in fig. 2 and table 1, myricetin demonstrated the top inhibition of \( \alpha \)-amylase, reaching almost 100%. Therefore, most of \( \alpha \)-amylase’s active sites formed a complex with myricetin, and the enzyme activity was almost completely reduced, thus producing significantly less maltose. Due to this observation, it seemed appropriate to study the effects of its derivatives on enzyme activity.

Fig. 4 summarises the inhibition of \( \alpha \)-amylase from the assay for three myricetin derivatives: myricitrin, myricetin galactoside, and myricetin trimethylether. These three compounds all reacted with the DNS colour reagent and thus required a solid phase extraction.

None of the myricetin derivatives displayed effective inhibition of salivary \( \alpha \)-amylase activity (fig.4). The maximum inhibition reached was 27.8% (SD=±8) at 100\( \mu \)M, by myricetin galactoside. Additionally, none of the myricetin derivatives achieved an IC\textsubscript{50} value, meaning 50% inhibition of \( \alpha \)-amylase was not reached by any of these polyphenols at the concentrations used. Myricetin trimethylether was the poorest inhibitor, reaching a maximum of only 12% (SD=±5.79). Furthermore, the standard deviation is large, meaning there was a large spread of data around the mean, suggesting some of the percentage inhibitions are likely to have been well below 12%. It must be noted that although there were differences in the inhibitory properties of the three polyphenols, the differences were small. Furthermore, for the majority of the concentrations studied the standard deviations often overlapped, especially between myricitrin and myricetin trimethylether. Unexpectedly, an ANOVA revealed that the differences between the derivatives were statistically significant (F= 5.14, \( p = 0.02 \)).
To summarise the results in fig. 4: myricetin derivatives were poor inhibitors, indicating that α-amylase remained active and formed enzyme-substrate complexes, thus producing near-to-normal amounts of maltose. All three polyphenols showed a gradual increase in percentage inhibition with increasing concentration, and although they are statistically significant, it is unlikely that these small increases would alter blood glucose levels in vivo.

4. Discussion

Results presented in this research show new findings that add to those in the current literature. While Madeswaran et al., (2014) studied galangin in silico, and found it to have good binding site interactions with α-amylase, this does not necessarily mean it has good inhibitory properties, as the current study shows that galangin gave the lowest percentage of α-amylase inhibition (fig. 1) (Madeswaran et al., 2014).

Secondly, to our knowledge, this report is the first which finds kaempferol to be a strong inhibitor of α-amylase by an in vitro assay technique. In the diet, the largest known concentration of kaempferol is found in capers (0.1g/100 g), and kaempferol has also been shown to be present in the blood plasma of humans after ingestion (Inocencio et al., 2000; Kerimi et al., 2015). Therefore, depending on the levels of kaempferol in the diet and suggested bioavailability in the body, the current findings in the present work are significant and should be carried further.

The study by Kerimi et al. also found that no myricetin was present in the blood plasma of participants after it had been ingested as part of a polyphenol rich meal, and proposed that this could be either be converted to quercetin in vivo, or be present in other forms (Kerimi et al., 2015). This means that the strong inhibitory effects that quercetin showed are of considerable importance, as they could be present in larger amounts than other polyphenols after ingestion.

The findings from quercetin and myricetin shown in the current study are in partial agreement with previous research. Tadera et al. also found quercetin and myricetin to be strong inhibitors of α-amylase activity (Tadera et al., 2006). However, their research was performed on enzymes from the pancreas of pigs, unlike this experiment, which used human salivary α-amylase. Thus, the results presented here are more likely to be reliable and relatable to humans than previous work. The findings from the porcine pancreas showed that myricetin and quercetin were still inhibitors of pancreatic α-amylase, giving IC₅₀ values of 380 μM and 500 μM respectively (Tadera et al., 2006). However, no solid phase extraction was performed in this previous research. Our results, on the other hand, show that DNS greatly interacts with myricetin, and that a solid phase extraction was necessary here. This could explain the discrepancies between our findings with low IC₅₀ values and Tadera et al’s. (2006) findings of high IC₅₀ values.

With regards to amounts of polyphenols in the diet, quercetin is found in large quantities in dark chocolate (25 mg/100 g), black elderberry (42 mg/100 g), and oregano (42 mg/100 g) (Counet et al., 2006; Kaack and Austed, 1998; Lin et al., 2007). As for myricetin, the main source includes red wine (up to 1.79 mg/100 ml) (Burns et al., 2000). Due to the time restraints in the present
study, quercetin derivatives were not explored, although research shows that quercetin derivatives are in high levels in certain foods such as red onion (Price and Rhodes, 1997). Therefore, future work should further investigate quercetin derivatives via an in vitro assay that uses the same conditions as the current study.

In terms of our findings for EGCG, a study on mice found that EGCG reduced postprandial blood sugar levels, and also that in vitro, EGCG inhibited pancreatic α-amylase by 34% at a concentration of 20 µM (Forester et al., 2012). The findings presented in the current project show that at half this concentration (10 µM) greater inhibition was shown (38%). This can be attributed to the fact that Forester’s study did not use an enzyme inhibition assay. There also was no mention in their work of whether the ‘red starch’ used in their method interacted with the EGCG, whereas the DNS colour reagent used in our experiment is known to react with the EGCG; for this reason a solid phase extraction was carried out. This could explain the lower IC₅₀ obtained in previous research.

Combinations of polyphenols were also studied in this project due to previous evidence showing that polyphenol rich foods contain a wide range of different polyphenols in combination (Wada and Ou, 2002). This means that studying their interaction with one another is important. Additionally, human studies have demonstrated that multiple polyphenols are found in blood plasma after ingestion of certain foods (Kerimi et al., 2015). Therefore, observing how they interact with each other is crucial in determining whether the inhibition results from the polyphenols alone are still valid when in combination.

To our knowledge, this project is the first study to look at in vitro polyphenol combinations via an α-amylase inhibition assay. Although some studies have looked at dietary polyphenols in combination with acarbose, this is not relevant to this particular study which aims to find a weaker, natural alternative to this drug. This is because combinations of acarbose and polyphenols are still likely to induce the negative gastro-intestinal side effects of acarbose alone (Akkarachiyasit et al., 2010).

As we have seen, quercetin and myricetin are strong α-amylase inhibitors. However, the results from myricetin derivatives are particularly important, as research shows that it is likely that the body transforms myricetin into its derivatives after ingestion (Kerimi et al., 2015).

Myricitrin has been briefly studied by an α-amylase assay (Figueiredo-González et al., 2016). The results from this study state that myrcia is a type of plant species which contains high levels of quercetin and myricetin derivatives. It suggests that the myricetin derivatives present are partly responsible for the strong enzyme inhibitory effects seen by the myrcia plant (Figueiredo-González et al., 2016). However, as with much of the other literature published, this previous study did not carry out a solid phase extraction, and this omission could explain their disputed results of finding low IC₅₀ values for myricitrin in particular (IC₅₀= 5.8–29 µg mL⁻¹). However, this could partly be accounted for by the use of quercetin derivatives in their study. Our study proved that a solid phase extraction was necessary for myricetin derivatives, as their strong interaction with DNS colour reagent turned the DNS from orange to green upon contact, demonstrating that an undesirable reaction occurs.
Fig. 4 shows that at the maximum concentration (100 µM), the myricetin derivatives demonstrated no significant α-amylase inhibition, and extremely high concentrations would need to be used to achieve an IC_{50} value. In fact, most foods are unlikely to contain such high levels of polyphenols. Evidence shows that myricitrin and myricetin galactoside are found in some of the greatest amounts in high-bush blueberries (1.03mg/100g and 8.99mg/100g respectively). This may partly counteract for the poor inhibition seen if consumed in large amounts in the diet (Cho et al., 2004; Zheng and Wang, 2003). Overall, the myricetin derivatives were poor inhibitors and did not inhibit α-amylase activity and thus affect maltose production, so they are unlikely to blunt the peaks in high blood glucose levels.

The main findings from this research show that with a solid phase extraction, the inhibitory effects of dietary polyphenols range from strong to virtually no inhibition at all. A mechanism to explain these findings can be proposed. Research by Xiao et al. (2011) studied the molecular structure of different polyphenols in relation to their affinity to α-amylase. It was found that the affinity for the α-amylase depends on the different chemical structures of the polyphenols (Xiao et al., 2011). It would be expected that the higher the affinity for α-amylase, the greater the percentage inhibition seen. However, the findings presented here do not agree with this theory. Xiao et al. (2011) found that galangin and kaempferol had a higher affinity for α-amylase than myricetin and quercetin did (Xiao et al., 2011). Their study found that the addition of a hydroxyl group reduced the binding affinity of the polyphenol to α-amylase. Fig. 5 shows the structures of the four polyphenols looked at in the current study. Xiao et al. (2011) found, when studying A to B and B to C, that adding a hydroxyl group decreases the affinity for α-amylase by 1.44 fold and 4.36 fold, respectively (Xiao et al., 2011). Contrary to this, results from the current study show the opposite: that adding the hydroxyl group actually increases the percentage inhibition.

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found that an addition of a sugar group to the polyphenols enhanced the affinity for α-amylase. This could explain why the myricetin derivatives were seen to be poor inhibitors; the addition of sugar groups to the general structure could further obstruct the α-amylase active site, in a similar mechanism to that proposed for galangin (fig. 6).

Overall, the current study has a number of strengths that set it apart from previous research. This is one of the first pieces of research to use an α-amylase inhibition assay with a solid phase extraction for the polyphenols that were shown to require it, the absence of which has been known to skew previous results. Many studies have found strong enzyme inhibitory effects in certain polyphenol-containing foods groups such as oolong tea and green tea (Yang and Kong, 2016). However, the current study is one of the few to have carried out a solid phase extraction, on the polyphenol extracts and under the optimum assay conditions. Furthermore, this experiment is relatable to humans as the α-amylase used is from human saliva, as opposed to that of previous experiments which have used pancreatic α-amylase, or worse, α-amylase from animals which are known to have different amino acids to the human enzyme (Brayer et al., 1995).

On the other hand, the present study does have some limitations. Firstly, due to the use of a lower concentration of EGCG, the results for this polyphenol are not directly comparable to the other four inhibitors. However, because of the strong inhibition seen at this low concentration (10 µM), it can be predicted that if this experiment were to be repeated then it is likely to agree with other researchers’ findings that EGCG is a strong

A suggested mechanism for this is shown in fig. 6. It is proposed that α-amylase is likely to have many active sites for the polyphenol inhibitor as well as other non-active sites. Thus, the high affinity of galangin could mean that a high proportion of galangin is binding to α-amylase but in the non-active ‘wrong’ sites, and therefore blocking the active binding site, and giving little inhibition. The more hydroxyl groups added, the less affinity is seen, and the less the active sites are blocked, which are therefore free to be inhibited by the polyphenols such as myricetin and quercetin and form complexes.

Further to this observation, Xaio et al. (2011) also

Fig.6- Possible mechanism for inhibition of α-amylase when amylose was used as a substrate. (A) Galangin’s high affinity shows it binding in the non-active site and thus blocking the rest of galangin to reach the active site. (B) Myricetin’s low affinity means that the active site is free to form a complex. A free active site as shown in (A) means amylose would be able to make a complex with α-amylase and thus more maltose would be produced.
inhibitor of α-amylase (Hara and Honda, 1990).

A further limitation is that the assay on kaempferol was repeated at concentrations 0, 10, 20, 30 and 40 µM. This was done in order to obtain a clearer trend, due to a very sharp rise in percentage inhibition seen between 0 and 40 µM in the initial assay. This could have introduced a potential source of bias, as kaempferol was the only polyphenol which was studied in further detail, so results at concentrations 10 and 30 µM may be more accurate for kaempferol than for the other polyphenols. However, because kaempferol reached its maximum inhibition at 40 µM in both the first and second assay carried out, the second assay is not likely to have significantly biased the findings.

A final consideration is with regard to fig. 3A. Here, the polyphenols were not looked at separately within the same experiment, therefore it is hard to compare individual and combined inhibition rates. On the other hand, fig. 2 does show that apart from galangin, all of the polyphenol percentage inhibitions rose rapidly between 10 µM to 30 µM. The fact that only a very low concentration of 10 µM was used could explain the low inhibition seen with the combined polyphenols, and may mean that the results are not truly representative. However, this was the reason for the repetition of this experiment using myricetin and quercetin at higher concentrations.

Despite these limitations, the inhibition assays carried out were of a high standard, with triplicates produced for each sample to increase the reliability of results. This gives a report that stands strong against other similar literature.

5. Conclusions

These findings have shown, for the first time, that quercetin, myricetin, and kaempferol are strong inhibitors of α-amylase activity and demonstrate slowing of the breakdown of amylose to maltose, via an in vitro inhibition assay. It was also found that galangin, myricitrin, myricetin galactoside and myricetin trimethylether are not potent inhibitors, and do not strongly reduce the amount of maltose produced. These results also reveal that myricetin and its derivatives greatly react with DNS colour reagent, and this could explain the discrepancies in data between this and previous research. Furthermore, our experiment was one of only a few that used human salivary amylase, and thus is likely to be more relevant to humans than those using pancreatic or porcine amylases. These findings call for further human studies to be undertaken into myricetin and quercetin in particular, with special consideration given to the levels of these found in foods, in order to assess the potential use of these polyphenols for a natural control for diabetes, by lowering maltose production and thus less maltose is available to be broken down into glucose and absorbed into the blood.
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