# Effects of Natural Products on Sugar Metabolism and Digestive Enzymes

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The candidate confirms that the work submitted is his/her own. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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## Abstract

The risk of diabetes is increasing and it is anticipated that people with diabetes will double by 2030 with about 90% of patients having type-2 diabetes. The use of herbal remedies in the treatment of diabetes has increased because of the side effects (flatulence, diarrhoea, tiredness and upset stomach) of some common drugs. To prevent or delay type-2 diabetes, the blood glucose level needs to be controlled. The objective of this research was to make a preliminary assessment of the capacity of PFS (Plant Food Supplement) extracts to reduce glucose, fructose and sucrose transport (acarbose-like activity) across the Caco-2 cell monolayers and inhibit digestive enzymes by PFS extracts. Sucrase activity is responsible for the hydrolysis of sucrose to fructose and glucose in the brush border membrane of the small intestine. Accordingly, inhibiting glucose uptake in the intestine may be beneficial for diabetic patients in controlling their blood glucose level. The initial steps of the *in vitro* tests development involved determining the activities of sucrase, maltase, isomaltase and human salivary α-amylase in an acetone-extract of rat intestinal tissues, improving on a previously published method by analysing glucose concentration via the hexokinase assay, and analysing the effect of PFS on sugar transporters using a previously published method using the Caco-2 cell monolayer. The literature evidence for the inhibition of cellular glucose uptake and transport by polyphenols across Caco-2 cells is limited. Also, to the best of our knowledge, this research is the first report regarding the analysis of cellular uptake and transport of $^{14}$C-sucrose and $^{14}$C-fructose using the Caco-2 cell monolayer with polyphenol-containing extracts. Additionally, $^{14}$C radioactivity was used due to its easy detection and allowed high sensitivity. Glucose, fructose and sucrose transport across the Caco-2 cell monolayer was significantly attenuated in the presence of PFS. Green tea, German chamomile and *Vitis Viniferae* extracts inhibited the transport of glucose, fructose and sucrose when tested independently. However, the *Vitis Viniferae* extracts were not able to achieve 50% inhibition for the sucrose and fructose transport. While the cellular uptake of glucose and fructose was inhibited by the PFS extracts, they were effective on the cellular uptake of sucrose. By contrast, Pelargonium and Echinacea were ineffective for both the transport and cellular uptake of sugars. Purified German chamomile and green

tea extracts were found to be moderate inhibitors of α-amylase digestion of amylopectin and α-glucosidase enzymes.

Due to the acarbose-like activity of the PFS extracts, they may have a potential role to reduce the risk of diabetes by inhibiting the hydrolysis of starches and reducing post-prandial blood glucose spikes. PFS may be seen as beneficial for use by diabetics as part of a nutritional intervention and in combination with exercise and drug treatment.

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## Abbreviations

| | |
|---|---|
| 6-PG | 6-phosphogluconate |
| ACN | Acetonitrile |
| ATP | Adenosine triphosphate |
| C | Catechins |
| C3R | Cyaniding-3- α-O-rhamnoside |
| C3S | Cyaniding 3-sambubioside |
| Caco-2 | Human colon adenocarcinoma cells |
| CAM | Complementary alternative medicine |
| COMT | Catechol-O-methyl transferase |
| COX-2 | Cyclooxygenase-2 |
| CQA | Caffeoylquinic acid |
| diCQA | Dicaffeoylquinic acid |
| DMSO | Dimethyl sulfoxide |
| DNS | Dinitrosalicylic acid |
| EC | Epicatechin |
| ECG | Epicatechin gallate |
| EFSA | European Food Safety Authority |
| EGC | Epigallocatechin |
| EGCG | Epigallocatechin gallate |
| EtAC | Ethyl acetate |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| G-6-P | Glucose-6-phosphate |
| G-6-PDH | glucose-6-phosphate dehydrogenase |

| | |
|---|---|
| GLUT2 | Sodium- independent transporter 2 |
| GLUT5 | Sodium- independent transporter 5 |
| GTDS | Green tea dietary supplement |
| HCl | Hydrochloric acid |
| LDL | Low density lipoprotein |
| NADH | Nicotinamide adenine dinucleotide |
| NaOH | Sodium hydroxide |
| NK | Natural killer |
| NOD | Non-obese diabetic |
| P3G | Pelargonidin-3- α-*O*-glucoside |
| P3R | Pelargonidin-3- α-*O*-rhamnoside |
| PFS | Plant food supplement |
| PG | Prostaglandin |
| Q3R | Quercetin-3-*O*-rhamnoside |
| SGLT1 | Sodium-dependent glucose transporter |
| STZ | Streptoztocin |
| SULTs | Sulfotransferases |
| UGTs | UDP-glcuronosyltransferases |
| WHO | World Health Organisation |
| β-CBG | Cytosolic β glucosidase |

# CHAPTER 1 LITERATURE REVIEW

High intake of sugars may have negative effects on health and this situation could feed metabolic abnormalities, such as type-2 diabetes (Johnson *et al.*, 2009). Type-2 diabetes, cardiovascular disease and metabolic syndrome risk is increased with repeated high post-prandial glucose spikes (Liu *et al.*, 1999; Liese *et al.*, 2005; Flynn, *et al.*, 2005; Manzano and Williamson, 2010; Hauner *et al.*, 2012). There are some *in vitro* animal and human intervention studies, which reported the effect of various dietary polyphenols on carbohydrate metabolism. The possible mechanisms are inhibition of digestive enzymes for glucose production, inhibition of glucose transporters in the intestine, stimulation of insulin secretion from the pancreatic β-cells, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, modulation of glucose release from the liver and modulation of intracellular signalling pathways and gene expression (Hanhineva *et al.*, 2010).

The highest concentration of post-prandial polyphenols is in the gut lumen compared to any other site in the body (Williamson, 2012). After consumption of high-polyphenol beverages, such as coffee, tea and orange juice, the amount of polyphenols reach millimolar concentrations in the gut lumen (Williamson, 2012). Therefore, glucose metabolism may be affected in the gut lumen. In this study, the Caco-2 cell line was used as it has been extensively employed as a model of the intestinal barrier in the literature. The Caco-2 cell line is the best choice to assess the inhibition of sugar transport because several morphological and functional characteristics of the mature enterocyte are able to be expressed by Caco-2 cells (Sambuy et al., 2005; Angelis and Turco, 2011). However there is also a limitation in the use of Caco-2 cells because it is not possible to mimic all biological activity of the human intestine.

## 1.1 Overview of Polyphenols

Polyphenols are plant metabolites and they are found in most plants or plant-derived foods and beverages (Bravo, 1998). Polyphenols protect plants from stress and excessive ultraviolet light exposure (Williamson, 2004). In addition,

they contribute organoleptic properties, taste and flavour of food products, such as red wine, tea, coffee and they have health benefits (Tomas Barberan & Espin, 2001; Williamson, 2004). There are numerous studies that have shed light on the effects of polyphenols, and reached the conclusions that these dietary antioxidants may protect against free radical damage (Tamara *et al.*, 2004), and promote health by preventing cardiovascular disease, cancer, osteoporosis, neurodegenerative diseases, diabetes and inflammatory disorders (Carughi, 2008; Kaliora *et al.*, 2009). In addition, polyphenols affect the sensory and nutritional qualities of plant foods. Anthocyanins are present in plants as major polyphenol pigments, and these pigments give the red, purple or blue colour (yellow is less frequent) to plants (Cheynier, 2005). The content of the phenolic compounds affects the astringency and bitterness of foods and beverages (Cheynier, 2005). Also, during processing or storage the degree of oxidation of polyphenols may cause either beneficial or undesirable characteristics in food products (Bravo, 1998). Flavonoids are the major plant phenolics and are classified into groups, such as the flavonols, flavanols, flavanones, flavones, anthocyanidins and isoflavones (Wacha *et al*, 2007) (Table 1).

**Table 1** Classes of phenolic compounds in plants (Balasundram, *et al.*, 2006).

| **Class** | **Structure** |
|---|---|
| Simple phenolics, benzoquinones | $C_6$ |
| Hydroxybenzoic acids | $C_6$–$C_1$ |
| Acethophenones, phenylacetic acid | $C_6$-$C_2$ |
| Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes) | $C_6$-$C_3$ |
| Napthoquinones | $C_6$-$C_4$ |
| Xanthone | $C_6$-$C_1$-$C_6$ |
| Stilbenes, anthraquinone | $C_6$-$C_2$-$C_6$ |
| Flavonoids, isoflavonoid | $C_6$-$C_3$-$C_6$ |
| Lignans, neolignan | $(C_6$-$C_3)_2$ |
| Biflavonoids | $(C_6$-$C_3$-$C_6)_2$ |
| Lignins | $(C_6$-$C_3)_n$ |
| Condensed tannins (proanthocyanidins or flavolans) | $(C_6$-$C_3$-$C_6)_n$ |

Table 1 indicates the natural polyphenols that consist of simple molecules (as phenolic acids) and highly polymerised compounds (tannis). Mainly, they are conjugated with one or more sugar units linked with hydroxyl groups. The linked sugars can be formed of monosaccharides, disaccharides or oligosacharrides (Bravo, 1998). Glucose is the most common sugar residue (Bravo, 1998).

Flavonoids are the largest class of polyphenols and are identified by the diphenylpropanes ($C_6$-$C_3$-$C_6$) structure. Diphenylpropanes consist of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (A, B and C ring) (Bravo, 1998). Figure 1 presents the structure and structural variations.

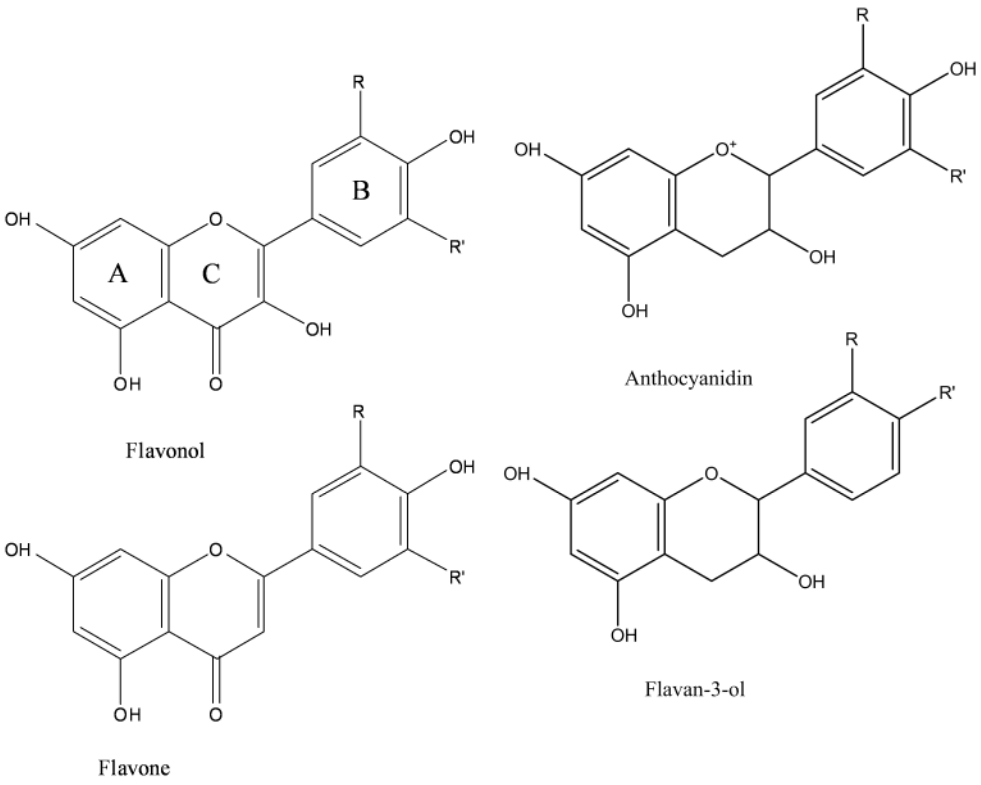



**Figure 1** Flavonoids ($C_6$-$C_3$-$C_6$); basic structure and structural variations.

Generally, flavonoids are most commonly found as glycoside derivatives (Bravo, 1998). Figure 1 indicates the two kinds of rings (A and B). The A ring is generated from a molecule of resorcinol (phloroglucinol) and the shikimate pathway derives the B ring (Henning *et al.*, 2004). Heterocyclic C-ring

flavonoids can be subdivided into six groups, including flavones (e.g. apigenin, luteolin, diosmetin), flavonols (e.g. quercetin, myricetin, kaempferol), flavonones, catechins, anthocyanidins and isoflavones (Henning *et al.,* 2004). According to their polarity and chemical structure, most of these compounds are soluble in aqueous and organic solvents (e.g. water, acetone, and methanol) and have relatively low molecular weights (Bravo, 1998). The B ring shows the characteristics of catechin flavanol structures by a dihydroxyl or trihydroxyl substitution, the A ring has the 5,7-dihyroxyl substitution and the C ring has a 3-OH substitution. (Yang *et al.,* 2001). The shikimate and acetate are the main two pathways that polyphenols biogenetically arise from (Bravo, 1998). Mostly, they are produced using intermediates of carbohydrate metabolism in the shikimate pathway (Tomas Barberan & Espin, 2001). The content of polyphenols in food is affected by many factors that contribute towards phenolic stability, biosynthesis and degradation (Tomas Barberan & Espin, 2001).

The main dietary sources of flavonols and flavones are tea and onions (Tamara *et al.*, 2004). The intake of flavonoids is difficult to estimate, since the average intake will vary greatly within the population. “Estimates range from 26 mg/day of flavonols in the Dutch diet to a median intake of total flavonoids of 239 mg/day, with a very wide range of 0.6 to 3,524 mg/day, among 34,708 U.S. women” (Williamson, 2004). One gram per day is the estimated amount of the daily polyphenol intake (Scalbert and Williamson, 2000; Scalbert *et al.,* 2005; Vauzour *et al.,* 2010). Current epidemiological studies report that consuming polyphenol-rich foods, fruit and vegetables lowers the risk of suffering from a stroke, Parkinson’s disease and dementia, such as Alzheimer‘s disease, in elderly people (Bastianetto *et al.,* 2008). Also, these findings concur with animal and *in vitro* studies suggesting that polyphenols derived from either beverages, fruits or plant extracts exhibit neuroprotective abilities or are able to reverse cognitive deficits (Bastianetto *et al.,* 2008). The consumption of plant foods lowered the risk of chronic diseases including diabetes, cardiovascular diseases and cancer (Scalbert *et al.,* 2005; Crozier *et al.,* 2009; Clifford *et al.,* 2004; Knekt *et al.,* 2002).

## 1.2 Plant Food Supplements

Due to the promising health benefits of polyphenols, their interest has increased recently. A complex mixture of polyphenols (flavonol, flavanol, flavone, anthocyanidin) or flavonoids alone has been used therapeutically as a dietary supplement in European countries and in the United States (Moini *et al.*, 2000). Weiss *et al.* (2006) reported that over 85% of Americans use dietary supplements (botanicals or herbals, vitamins, and minerals) regularly due to their promising health benefits (CVD, type-2 diabetes, cancer, inflammation and neurodegenerative disorders) and are a multi-billion dollar business (Weiss *et al.*, 2006; Seeram *et al.*, 2006). Dragan *et al*, (2015) reported that about 30% of patients with type-2 diabetes are using CAM. PFS contain concentrated sources of plants, their extracts or derivatives and are able to deliver physiological effects. Throughout history, plants and herbs have been part of the diet not only because they provide nutrients, but also for their health-promoting properties. Several reviews indicate the benefits of traditionally consumed plants for the management of diabetes (Bailey and Day 1989; Gougen and Leiter 2001; Shane 2001; Dey *et al.*, 2002; Liu *et al.*, 2002; Yeh *et al.*, 2003; Rahimi *et al.*, 2005; Chang *et al.*, 2007; Rizvi and Mishra, 2013; Ezuruike and Prieto, 2013; Lao *et al.*, 2014; Shikov *et al.*, 2014). It was observed that some herbal medicines and nutritional supplements were able to decrease blood glucose levels through various mechanisms that could be used in the management of diabetes (

Table 4). Chang *et al.* (2007) reviewed the extensive use of CAM (such as nutritional supplements and herbal medicines) for diabetic people with 18 studies from 9 countries and found that it varied between 17 and 73% between patients (Chang *et al.*, 2007). Also, WHO (2003) reported that 65 to 80% of the world population uses traditional medicines for health care.

Due to the side effects (flatulence, diarrhoea, tiredness and upset stomach) of some common drugs (such as acarbose, which prevents blood glucose levels from increasing too much after eating a meal, metformin, which reduces the glucose that is released from the liver to the bloodstream, sulphonylureas, which increase insulin secretion from the pancreas, and glitazones, which increase the sensitivity of body cells to increase) in the treatments of diabetes, the use of

herbal remedies has increased; 2 to 3.6 million diabetic patients in the USA use herbal remedies for the management of diabetes (Dabaghian *et al.*, 2012).

It was reported that people prefer to use plants in the belief that plants are more efficient, safer (as they have no side effects compared to synthetic drugs) and cheaper (Mall and Sahani, 2013; Durmuskahya and Ozturk, 2013). Plants used for the treatment of diabetes show differences between regions due to their traditional uses and where they grow (e.g. variations on account of the weather). Also, depending on the plant and region the leaf, bark, fruit, flower or the whole plant may be used. For instance, in India the most commonly used plants are *Allium sativum, Aegle marmelos, Azadirachta indica, Curcuma longe, Emblica officianalis, Gynema sylvestre, Magnifera indica, Trigonella foenum-graecum* and *Terminelia chebula* (Mall and Sahani, 2013). *Punica granatum, Rosa damascene, Plantago psyllium, Coriandrum sativum, Portulaca oleracea* and *Rumex patientia* are the most popular traditionally used plants used in Iran due to their anti-diabetic effects (Dabaghian *et al.*, 2013). In Turkey, the most commonly used plants for diabetic improvement are reported as *Zizyphus jujube Mill., Origanum onites L., Ceracus mahaleb L.* and *Trigonella foenum-graecum* (Durmuskahya and Ozturk, 2013). The aim of this investigation was to determine the effect of polyphenols from the following plant food supplements: *Camellia Sinesis* (Green tea), *Matricarcia Recutita* (German chamomile), *Vitis Viniferae* (grape leaf), Pelargonium and Echinacea on cellular glucose uptake and the transport of glucose from the apical to the basolateral compartment using a Caco-2 intestinal cell model. This study is a part of EU framework 7 project PlantLIBRA – Plant food supplement: Levels of intake, benefit and risk assessment. The project aimed to investigate science-based decision-making and the safe use of PFS (see 2.1). PFS extracts were chosen based on the project partner's suggestion (see ANNEX 1 page 234), missing data reported by EFSA experts and the results produced by the study group about the estimation of PFS intake. There was several PFS, whose effects were analysed, such as *Echinacea purpea, Andrographis paniculata, Pelargonium sidoides, Vitis vinifera* (grape leaf), *Matricaria recutita* (chamomile), *Camelllia sinensis* (green tea) and *Bowelliae res spir.* All the extracts were received from one of the project partners: PhytoLab Co. KG.h (Vestenbergsgreuth, Germany). The water

solubility of received extracts and the effect of 1 mg/ml of each PFS extract on glucose transport was analysed using Caco-2 cells, and based on those results the supplements were chosen.

### 1.2.1 Composition and protective health effects of Green tea

*Camellia sinensis* is the plant species of green tea and the leaves of the tea are roasted at a high temperature to inhibit polyphenol oxidation. Green tea has the highest concentration of flavanol content compared to other teas (Cai and Chow, 2004), which is given in

Table 2. Catechins, which are the major compounds of green tea, are phenolic compounds that are contained in dietary supplements (Weiss *et al.*, 2006). The common catechins in all green teas are EGC, C, EGCG, EC and ECG

Table 2. Narotzki *et al.* (2012) reported that 59% of the total catechins in green tea is EGCG with 19% of EGC, 13.6% of ECG and 6.4% of EC. One cup (240 ml) of brewed green tea contains up to 200 mg EGCG (Mukhtar and Ahmet, 2000).

**Table 2.** Polyphenol content of green tea (Cabrera *et al.,* 2003).

| Class | Polyphenol |
|---|---|
| **Flavan-3-ols** | (-)- epicatechin |
| | (-)- epicatechin-3-gallate |
| | (-)- epigallocatechin |
| | (-)- epigallocatechin gallate |
| | (+)- catechin |
| | (+)- gallocatechin |
| | theaflavin |
| | theaflavin-3-3'-digallate |
| | theaflavin-3'-gallate |
| | theaflavin-3-gallate |
| | thearubigins |
| **Flavones** | apigenin |
| | luteolin |
| **Flavonols** | kaempferol |
| | myricetin |
| | quercetin |
| **Others** | gallic acid, chlorogenic acid, caffeic acid |

Polyphenols are antioxidants. *In vitro* studies showed that green tea polyphenols could be direct antioxidants by scavenging reactive oxygen species or chelating transition metals and may act directly by up-regulating phase-II antioxidant enzymes. EGCG and ECG have the highest ROS activity (Henning *et al.,* 2004; Foster and Lambert, 2011). Armoskaite *et al.* (2011) analysed green tea from three different regions (Japan, China and Sri Lanka). They reported that green tea from China has the highest polyphenol content while Sri Lanka has lowest. Green tea from Japan was reported as having the highest antioxidant capacity while Sri Lankan tea had the lowest (Armoskaite *et al.,* 2011). Thus, standardization (EGCG and EGC levels) should be implemented on commercial GTDS or GTDS should be biologically standardized according to antioxidant capacity (Seeram *et al.,* 2006).

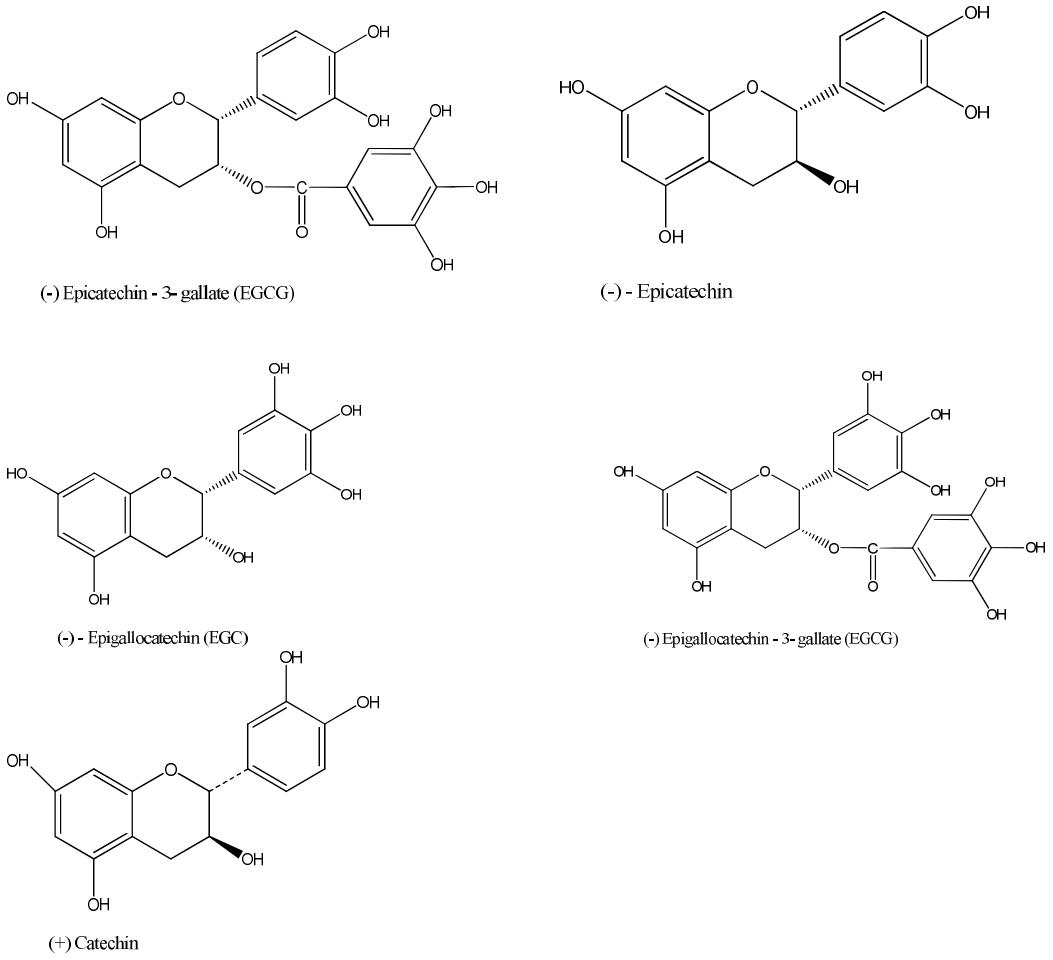



**Figure 2.** Chemical structures of green tea catechins.

### 1.2.1.1 Absorption of polyphenols and green tea catechins

Polyphenol absorption and metabolism are affected by their chemical structure, such as conjugation with other phenolics, molecular size, degree of polymerisation and solubility (Bravo, 2008). However, the absorption and metabolism of all flavonoids (from the poorly absorbed anthocyanins to the well-absorbed isoflavones) are following the same pathways (Williamson *et al.,* 2000). The concentration of flavonoid metabolites in the plasma is between 0 to 4 μM with an intake of 50 mg aglycone equivalents (Manach *et al.*, 2005), and in many human studies it did not exceed 1 μM.

The absorption of flavonoids occurs in the small intestine; however, depending on their ingested form it can also take place in the large intestine or stomach (Jagannath *et al.,* 2006). Deglycosylation is the initial step for the absorption

process of glycosylated flavonoids, which is essential if further metabolism exists (Williamson *et al.*, 2000). They are conjugated by intestinal enzymes and transported to the serosal or mucosal sides (Williamson, 2004). Deglycosylation can potentially occur at several sites in the duodenum and jejunum:

(1) within the intestinal lumen;

(2) brush border hydrolases; or

(3) intracellular hydrolases after transport of the flavonoid into the enterocyte (Day *et al.*, 2003; Williamson, 2004; Farrell *et al.*, 2013).

The absorption of flavonoids occurs in two pathways as shown in Figure 3 (Williamson, 2004).

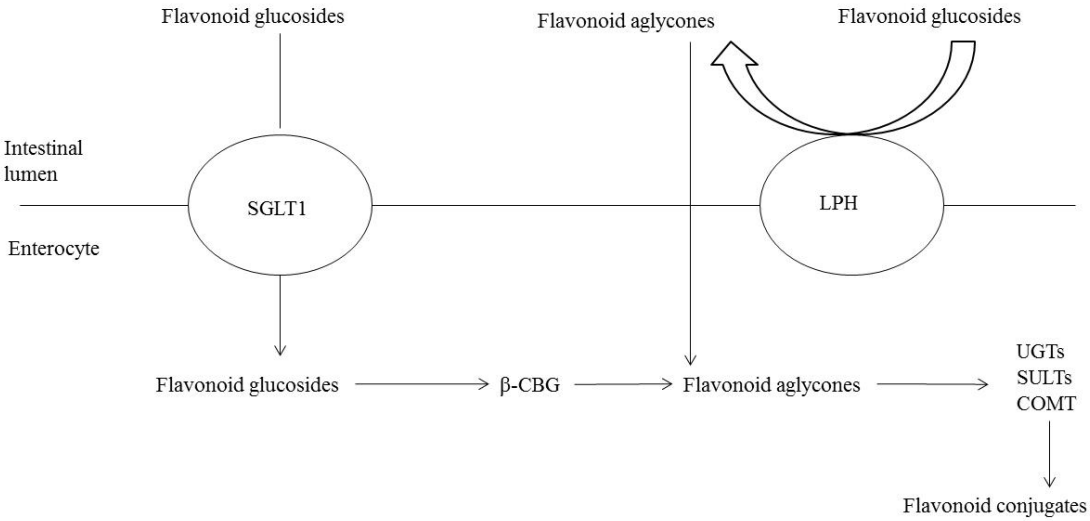



**Figure 3** Absorption and metabolism of flavonoid glucosides in the small intestine.

The first step utilises an enzyme, lactase phlorizin hydrolase (LPH), which is found in the brush border of the small intestine, to catalyse deglycosylation of glycosylated flavonoids. LPH is an enzyme that hydrolyses lactose and is located in the brush border of the small intestine (Williamson, 2004). Aglycones, which are the product of the deglycosylation reaction, can then diffuse into epithelial cells in two ways: passive or facilitated diffusion (Williamson, 2004).

An alternative step is flavonoid glycoside transport into the enterocyte via the function of a sugar transporter such as SGLT1. After deglycosylation, flavonoid glycosides are hydrolysed by β-CBG to flavonoid aglycones (Gee *et al.,* 2000; Williamson, 2004; Wolffram *et al.,* 2002). Some polyphenols, which are not absorbed in the small intestine, will pass to the colon and will be metabolised by gut microflora. Flavonoid aglycones are substrates for colonic bacteria forming phenolic acids. It was estimated that about 90 to 95% of total polyphenol intake will be accumulated in the large intestine up to the millimolar range and metabolised by gut microflora into the low molecular weight phenolic metabolites (Cardona *et al.*, 2013). Flavonoids linked with rhamnose are absorbed in the large intestine after degradation by the gut microbiota (*Bifidobacterium dentium*) (Marin *et al.*, 2015). After biliary excretion, green tea flavonoids are further metabolised to phenolic acids by colonic bacteria. Gonthier *et al.,* 2003 reported that among catechin metabolites are hydroxyphenylpropionic acid, hydroxybenzoic acid, hydroxyhippuric acid, hippuric acid, *p*-coumaric acid, vanillic acid and hydroxyphenylacetic acid in the colon. (-)- Epicatechin and procyanidin B2 are broken down into a mixture of phenolic acids with 5-valeric acid, 3-propionic acid and phenylacetic acid as the major catabolites (Stoupi *et al.,* 2009). These catabolites are absorbed into the circulation often at greater quantities compared to their parent aglycones. Williamson (2004) reported that both free and conjugated flavonoids in the colon can re-enter the enterohepatic cycle before being further metabolised. The conjugated form of green tea flavonoids has been found in plasma and urine. Henning *et al.* (2013) reported a human green tea intervention study. After consumption of 6 cups of green tea daily for 3 to 6 weeks, it was found that only phenolic acids (3,4-dihyroxyphenylacetic acid and 4-hydroxyphenylacetic acid) significantly changed compared to baseline plasma.

Following the absorption of the aglycones or final derivatives (in the small intestine or colon), this undergoes some degree of phase II metabolism (UGTs, SULTs and COMT). UGTs, SULTs and COMT are the important metabolic enzymes for the conjugation of flavonoids (Day *et al.,* 2000; Boersma *et al.,* 2002). The multiple hydroxyl groups in flavonoids are the major targets for

conjugation by human metabolic enzymes. The initial step of flavonoid conjugation takes place in the small intestine. UGT1A1 and UGT1A8, and SULT1A1 and SULT1A3 enzymes are responsible for the conjugation of green tea polyphenols in the small intestine. Following the conjugation of flavonoids in the small intestine, they are transferred to the liver by the bloodstream and may be subjected to more metabolic enzymes before being transported to the blood stream again until excretion takes place in the urine or bile. UGT1A9 and SULT1A1 enzymes are involved in modifications of green tea polyphenols in the liver (Pai *et al.,* 2001; Williamson, 2004; Lambert *et al.,* 2007).

The study by Williamson *et al.* (2005) showed that flavonoids are extensively metabolized during absorption, resulting in the glucuronide, sulfate and methyl conjugates being the predominant metabolites present in human plasma. The physiochemical properties of conjugated flavonoids found in the plasma are different to the aglycone forms. As methylation improves lipid solubility, most flavonoids found in the plasma are also conjugated with glucuronic acid or sulfate, which dramatically enhances hydrophilicity and molecular weight.

#### 1.2.1.2 Protective health effects of green tea

The studies on the health benefits of green tea facilitate investigation on the green tea extracts production (Seeram *et al.,* 2006). There are many *in vitro* and *in vivo* assessments reported in the literature for the consumption of green tea. Green tea shows benefits in more than one element of metabolic syndrome and some of these researches are summarized below.

According to studies in cell lines and animal models, tea flavanols have antiproliferative, antiangiogenic and anticarcinogenic activities. For example, Steffen *et al.* (2008) reported that the NADPH oxidase activity in HUVEC cells is inhibited by tea catechin and epicatechin, decreases the generation of endothelial superoxide, and potentially promotes nitric oxide longevity. The Hooper *et al.* (2008) study showed that green tea consumption significantly reduced LDL cholesterol. Also, in other researches, green tea has been shown to have positive effects on bone health (Dew *et al.,* 2007), arthritis (Ahmed, 2009) and cognitive function and neuroprotection (Bastianetto *et al,* 2008; Nathan *et*

*al.*, 2009). The popularity of consumption and the absence of toxicity in tea may make it a strong candidate for use in cancer prevention (Henning *et al.*, 2004; Sturgeon *et al.*, 2009).

The level of obesity is increasing worldwide and it is one of the leading preventable causes of death. Hence, to promote weight loss and maintain weight, the use of green tea is common. Some studies show that green tea catechins with caffeine are effective in weight loss and maintaining weight (Hursel *et al.*, 2009; Phung *et al.*, 2010).

According to the *in vivo* and *in vitro* studies above, there needs to be more research with regards to the beneficial effects of green tea consumption.

#### 1.2.1.3 Bioavailability of green tea catechins

The FDA defines bioavailability as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action." The biological effects of polyphenols depend on their bioavailability. The bioavailability of polyphenols is affected by their absorption, metabolism and excretion processes (Jagannath *et al.*, 2006). Also, polyphenol absorption and metabolism are affected by their chemical structure. Bravo (2008) states that the degree of glycosylation, acylation, their basic structure (e.g. benzene or flavones derivatives), conjugation with other phenolics, molecular size, degree of polymerization; pH and solubility also affect the bioavailability of polyphenols. Changes in pH affect the degradation of green tea catechins (these are stable at low pH and degradation is rapid at neutral or above pH). As the stomach has a low pH (1-3, gastric juice), catechin stability is unaffected; however, in the intestine the pH is ~7-8 (pancreatic juice), and therefore the stability of catechins is greatly reduced (Chen *et al.*, 1998; Desesso and Jacobson, 2001). Ferruzzi (2010) reported that when the catechins reach a neutral pH in the intestine, there is ~ 80% of loss.

There are other studies regarding the effect of adding other food constituents to catechins on the absorption of green tea catechins. Chen *et al.* (1998) reported that the stability of green tea catechins improved in the presence of ascorbic acid

at neutral pH. In a human intervention study it was reported that the consumption of cocoa with bread and sugar increased the bioavailability of catechins and epicatechins (Schramm *et al.,* 2003). Peters *et al.* (2010) observed the bioavailability of green tea in ascorbic acid- and sucrose-fed rats and found that the bioavailability of catechins was improved. Egert *et al.* (2013) studied the plasma catechins of 24 volunteers who consumed decaffeinated green tea with the addition of protein (skimmed milk, casein or soy milk). It was observed that the bioavailability of total catechins was reduced. Therefore, the addition of other food constituents may influence the bioavailability of catechins.

### 1.2.2 Chamomile composition and protective health effects

Chamomile belongs to the daisy family called *Asteraceae* or *Compositae* (Khan *et al.,* 2014) and it is represented by the two most known varieties, German chamomile (*Matricaria recutita*) and Roman chamomile (*Chamaelum nobile*). German chamomile is the most common variety used for medical purposes (Srivastava and Gupta, 2007). It consists of the fresh or dried flower heads of *Matricaria recutita* and their preparations. Chamomile contains several bioactive phytochemicals that could provide therapeutic effects; therefore, it is used as a herbal medication from the ancient times till today. Srivastava *et al.* (2010) reported that chamomile was traditionally used for centuries to treat ulcers, wounds, eczema, gout, haemorrhoids, ear and eye infections, cracked nipples, chicken pox, diaper rash and nasal inflammation.

It was reported that chamomile contains ~120 secondary metabolites including 28 terpenoids and 36 flavonoids, such as apigenin, quercetin, patuletin as glucosides, and various acetylated derivatives (Srivastava *et al.,* 2010). Both species contains flavonoids (apigenin, luteolin, patuletin, and quercetin), coumarins (herniarin, umbelliferone) and hydroxycoumarins and are considered to be major bioactives in chamomile (Srivastava *et al.,* 2010). Apigenin is present in very small quantities as free apigenin, but predominantly exists in the form of various glycosides (Srivastava *et al.,* 2010). Other common flavonoids are luteolin glucosides, quercetin glycosides and isohamnetin. McKay and Blumberg (2006) reported that chamomile has high levels of polyphenol content, such as flavonoids and coumarins. Approximately 0.1% of the total constituents

are coumarins, umbelliferone and esculetin (McKay and Blumberg, 2006). In another study, cinnamic acid derivatives, ferulic and caffeic acid were found in large amounts (39%) (Mulinacci *et al.*, 2000). Due to the equipment (GC-MS, ESI/-MS, LC-$MS^2$) or methods (in organic solvent or water solubility) that were used to analyse the composition of chamomile some differences in the results emerged (Ignat *et al.*, 2011; Lin and Harnly 2012). However, the major component of chamomile (flowers and plant) was reported as apigenin in many studies except Mulinacci *et al.* (2000). It was reported that the major polyphenols in chamomile are ferulic and caffecic acid (Table 3). See Table 7 for the composition of chamomile is presented in detail.

**Table 3** Polyphenol content of German chamomile (McKay and Blumberg, 2006).

| Polyphenol | Percentage of total polyphenols |
|---|---|
| Apigenin | 16.8 |
| Luteolin | 1.9 |
| Quercetin | 9.9 |
| Patuletin | 6.5 |
| Hydroxycoumarins | 0.1 |
| Ferulic and caffeic acid | 39.1 |
| Unidentified phenolic derivatives | 25.8 |

The medical benefits and widespread use of chamomile enables chamomile tea to be perceived as a popular tea, with consumed over one million tea cups consumed per day (Srivastava and Gupta, 2007).

#### 1.2.2.1 Absorption of chamomile flavones; apigenin-7-glucoside

Apigenin-7-*O*-glucoside (A7G) is deglycosylated to apigenin in the intestine once it is absorbed (LPH or SGLT1) from the intestinal tract and then metabolized to UGT or SULT conjugates. Also, by the gut microflora, apigenin is further cleaved to 3-(4-hydroxyphenyl) propionic acid. Hanske *et al.* (2009) analysed the bioavailability of A7G in germ-free and human microbiota-associated (HMA) rats. They reported the main metabolites of A7G as apigenin,

naringenin and 3-(4-hydroxyphenyl) propionic acid, detected both in urine and faecal suspension. However, in whole blood samples, only apigenin conjugates and phloretin were detected. Chen *et al.* (2002) reported that apigenin is metabolised rapidly by UGT1A1 in the HepG2 cell line. After conjugation, the metabolites circulate in the blood and are then excreted into the bile and urine. Apigenin is rapidly glucuronidated in both human and mouse liver microsomes and the rate of glucuronidation is faster than sulfation (Nielsen *et al.,* 1999). Chen *et al.* (2007) reported the urinary excretion of apigenin in rats and found that the concentration of apigenin was low in urine. Therefore, they assumed that more apigenin may be metabolized to simple compounds (glucuronidated and sulphated apigenin) in the gut and absorbed, and may be further metabolised in the liver. They also detected apigenin in plasma. Lu and colleagues (2011) reported that apigenin in plasma, urine and bile was found mainly in conjugated form. Zhang and colleagues (2012) reported that the absorption of apigenin mainly occurs in the duodenum part of the intestine and the *in vivo* intestinal absorption may be through either passive transport in the ileum and colon segments or active carrier-mediated transport both in the duodenum and jejunum segments. Hu and colleagues (2003) analysed the intestinal disposition of apigenin metabolism in the Caco-2 cell line. They concluded that glucuronidated apigenin and apigenin sulphate conjugates were biotransformed in the cells and transported back to the apical compartment mainly by MRPs and possibly OATs. Teng and colleagues (2012) analysed the absorption and initial metabolism of apigenin using the intestinal perfusion model *in situ*. They found that 29% of the administered apigenin was transported back to the vascular side. They also reported the percentage concentration of apigenin glucoronides, sulphates and free apigenin, as 9, 4 and 16% at the vascular side respectively.

#### 1.2.2.2 Protective health effects of chamomile

The Srivastava *et al.,* (2009) findings demonstrate that apigenin glucosides enter into the cancer cells and are actively hydrolysed within the cells (Srivastava, *et al.,* 2009). The cancer cell growth rate is reduced by apigenin glucosides via the deconjugation of glycosides (Srivastana *et al.,* 2009). Using German chamomile as a mouthwash has some benefits in plaque and gingival reduction. Pourabbasa

and colleagues (2005) reported that compared to the control rinse ($p \leq 0.001$), German chamomile mouthwash significantly decreased both plaque and gingivitis without an adverse effect on teeth staining. One of the animal model studies indicated an antipruritic effect of German chamomile (Pourabbasa *et al.,* 2005). In mice, the compound 48/80-induced scratching behaviour significantly decreased after feeding them for 11 days with GC (Kobayashi *et al.,* 2003). German chamomile was offered as a herbal tea (vervain, liquorice, fennel, balm mint) to children to observe the treatment of colic (Gardiner, 2007). After 7 days of treatment (each day <150 ml/dose, no more than three times) the parents reported that tea eliminated the colic in 57% of cases, whereas the placebo helped only 26% without any adverse effect (Gardiner, 2007).

Rafraf and colleagues (2014) studied 64 individuals with type-2 diabetes in a single blind randomized controlled trial. They consumed 3g chamomile/150ml hot water three times for 8 weeks straight after meals. They reported that chamomile tea significantly reduced serum insulin levels. Kato and colleagues (2008) reported the inhibition of rat intestinal maltase and sucrase activities in the presence of chamomile and some of the active components of chamomile. Chamomile extract inhibited maltase and sucrase activity with an $IC_{50}$ value of 2.6 and 0.9 mg/ml respectively. Cemek *et al.* (2008) observed the effect of the chamomile ethanolic extract (70 mg/kg body weight) on hyperglycemia using streptoztocin (STZ)-induced diabetic rats. They found that treatment with chamomile extract significantly reduced post-prandial hyperglycemia. Emam (2012) analysed the antidiabetic activities of the water extracts of *Rosmarinus Officinalis* and *Matricaria recutita* (200 mg/kg for 21 days) using STZ-induced diabetic rats. They found a significant decrease in the fasting blood glucose level. The Ramadan *et al.* (2012) study also supported that the water extract of chamomile has a potential to reduce the blood glucose level of STZ-induced diabetic rats (100 mg/kg for 21 days). Another recent study also analysed the hyperglycaemic effect of the ethanolic extract of chamomile using STZ-induced diabetic rats (500 mg/kg for 4 weeks) (Al Musa and Al Hashem, 2014). They found a reduction in blood glucose level without improving insulin levels.

Liang and colleagues (1999) analysed the anti-inflammatory activity of apigenin and found that COX-2, PG and nitric oxide production was inhibited by apigenin in lipopolysaccharide-activated macrophages. McKay and Blumberg (2006) also reported that apigenin and quercetin showed the highest inhibitory activity of histamine release from human basophils, 89 and 96% respectively, at a concentration of 50 µM. This study showed that chamomile's active components have the potential of an anti-allergenic activity by inhibiting the antigen-induced histamine release from basophils.

Savino and colleagues (2005) investigated the effect of chamomile consumption for the treatment of intestinal colic in breastfed infants (randomized, double-blind, placebo-controlled trial). Infants were fed with the herbal mixture extract containing 71 mg/kg/d chamomile or the placebo for one week and it was found that the colic improved after consumption of the herbal extract. Another supportive double-blind human intervention study reported that infants fed with the herbal mixture containing chamomile had a significantly higher intestinal colic improvement (57%) compared with the placebo (26%) (Weizman *et al*., 1993).

Srivastava and Gupta (2009) analysed the anticancer properties of methanolic and aqueous extracts of chamomile. Chamomile showed anti-proliferative and apoptotic activity in human cancer cells. They also found that apigenin glucosides entered the cancer cells and were effectively hydrolysed within them. Using the BT-474 human breast cancer cell line it was reported that the ethanolic extract of the chamomile gel capsules showed weak estrogenic and progestational activity; however, they did not exhibit androgenic activity (Rosenberg Zand *et al.,* 2001).

In brief, those researches showed that German chamomile exhibits different health benefits.

#### 1.2.2.3 Bioavailability of chamomile flavones; apigenin-7-glucoside

The biological effects of polyphenols depend on their bioavailability. As explained previously in chapter 1 section 1.2.1.3, polyphenol's absorption and metabolism are affected by their chemical structure (Bravo, 2008) and food

matrix (Manach *et al.,* 2004). Hanske and colleagues (2009) investigated the influence of human intestinal microbiota on the bioavailability of A7G and they concluded that human microbiota have a big impact on A7G metabolism. Wen and Walle (2006) analysed the effect of structure on the bioactivity of dietary polyphenols in comparison with methylated and unmethylated polyphenols using the human liver S9 fraction and the Caco-2 cell line. They concluded that the combined use of the hepatic S9 fraction and Caco-2 cells has promising results on the oral bioavailability of dietary polyphenols. Methylated polyphenols were more attractive than unmethylated polyphenols for intestinal absorption and hepatic metabolic stability. Manach and colleagues (2004) reported that plant foods generally contain both dietary fiber and polyphenols. Dietary fiber intake improves the microbial metabolites in the intestine. Therefore, the bioavailability of polyphenols may have an effect without a food matrix, and food that is consumed with polyphenols affects their absorption and plasma concentration levels.

### 1.2.3 *Vitis Viniferae* composition and protective health effects

The biological effects of polyphenols depend on their bioavailability. As explained previously in chapter 1 section 1.2.1.3, polyphenol's absorption and metabolism are affected by their chemical structure (Bravo, 2008) and food matrix (Manach *et al.,* 2004). Hanske and colleagues (2009) investigated the influence of human intestinal microbiota on the bioavailability of A7G and they concluded that human microbiota have a big impact on A7G metabolism. Wen and Walle (2006) analysed the effect of structure on the bioactivity of dietary polyphenols in comparison with methylated and unmethylated polyphenols using the human liver S9 fraction and the Caco-2 cell line. They concluded that the combined use of the hepatic S9 fraction and Caco-2 cells has promising results on the oral bioavailability of dietary polyphenols. Methylated polyphenols were more attractive than unmethylated polyphenols for intestinal absorption and hepatic metabolic stability. Manach and colleagues (2004) reported that plant foods generally contain both dietary fiber and polyphenols. Dietary fiber intake improves the microbial metabolites in the intestine. Therefore, the bioavailability of polyphenols may have an effect without a food matrix, and food that is

consumed with polyphenols affects their absorption and plasma concentration levels.

### 1.2.4 *Vitis Viniferae* composition and protective health effects

One of the most widely grown fruit crops in the world is *Vitis Viniferae* and it belongs to the *Vitaceae* family (Giribabu *et al.*, 2014). The polyphenolic composition of *Vitis Viniferae* leaves has been analysed by several researchers and they are rich sources of anthocyanins, hydroxycinnamic acids, flavanols (catechin, epicatechin and epigallocatechin) (Shi *et al.,* 2003) and flavonol glycosides and stilbenes, such as resveratrol (Monagas *et al.*, 2006; Roopchand *et al.,* 2013; and Kammerer *et al.,* 2014). Aqueous extract of leaves contains flavonols, hyroxycinnamic acid and catechins. It was also reported that the skin, stems and seeds of the *Vitis Viniferae* are an important source of PROs. While seeds contain procyanidins polymers (catechin, epicatechin and ECG), the skin and stems contain prodelphinidins (EGC, EGCG, gallocatechin) (Monagas *et al.*, 2006).

Due to the polyphenol content of *Vitis Viniferae*, it has several pharmacological uses, such as an antidiabetic and antioxidant (Saada *et al.,* 2006; Orhan *et al.*, 2011), antimicrobial (Delgado-Adamez *et al.,* 2011; Oliveira *et al.,* 2013), anticarcinogenetic (Yi *et al.,* 2005; Mertens-Talcott *et al.,* 2006; Sharma *et al.*, 2012) and an inhibitor of platelet aggregation and low density lipoprotein (LDL) oxidation (Castilla *et al.,* 2006; Shanmuganayagam *et al.,* 2012).

Giribabu and colleagues (2014) investigated the effect of *Vitis Viniferae* seeds on carbohydrate-metabolizing enzymes and the other enzymes in the liver in diabetes using STZ-diabetic male rats (fed with 250 and 500 mg/kg/day *Vitis Viniferae* seed ethanolic extract for 28 days). They concluded that *Vitis Viniferae* may help to protect the liver in diabetics by decreasing liver oxidative stress. Pandey and Rizvi (2014) reviewed the effect of quercetin, myricetin and resveratrol obtained from red grapes on diabetes and observed that under diabetic conditions they protect against high glucose-induced oxidative stress.

Normal, glucose-hyperglycaemic and STZ-induced diabetic rats were fed with an aqueous extract of *Vitis Viniferae* (250 and 500 mg/kg for 15 days) to

investigate the extract's acute and subacute hypoglycaemic and antihyperglycaemic effects. They reported that the *Vitis Viniferae* extract has significant antihyperglycaemic and antioxidant activity compared to the positive control, tolbutamide, in diabetic rats (Orhan *et al.*, 2006). Banini *et al.* (2006) observed the effect of muscadine grape juice (MJ), muscadine grape wine (MW) and dealcoholized muscadine grape wine (dMW) on the blood glucose levels of healthy and type-2 diabetic subjects over 28 days. Daily intake of 150 ml of MW and dMW consumption with meals decreased blood glucose levels compared to diabetics administered with MJ.

### 1.2.5 Pelargonium composition and protective health effects

Pelargonium belongs to the Geraniaceae family and contains ~280 species that are mostly grown in South Africa (Mabbereley, 1997). Pelargonium contains essential oils, flavonoids, tannins and phenolic acids (ellagic acid, isoquercetrin, quercetrine, kaempferols, caftaric acid and rutoside) (Lalli *et al*., 2006; Kokalou and Souleles, 1988; Kolodziej *et al*., 1995; Contour and Louguet, 1985; Avila *et al*., 2013). Pantev and colleagues (2006) also reported on myricetin, apigenin and catechin in *Pelargonium sanguineum*. Due to its polyphenol content, Bown, (1995), Brendler and Wyk (2008) and Avila and colleagues (2013) indicated that it was traditionally used for ulcers, whooping cough, kidney pain, staunch bleeding, heal wounds, uterine haemorrhage, diarrhea and skin disorders. The essential oil of *Pelargonium* has also been used traditionally as an antiallergenic, antioxidant, antiviral, antibacterial, antidiarrhetic, antihepatotoxic, stomachic and antidiabetic (Brendler and Wyk, 2008; Boukhris *et al*., 2012). Boukhris and colleagues analysed the effect of pelargonium leaf oil on hyperglycaemia using alloxan-induced diabetic rats, and concluded that the essential oil of *Pelargonium* may be helpful in the management of diabetes. The essential oil of *Pelargonium* was traditionally used in the treatment of inflammation, haemorrhoids, diarrhea, gastric ulcers, liver problems, diabetes and urinary stones (Slima *et al*., 2013). Lizogub and colleagues (2007) conducted a randomized, placebo-controlled double blind trial of 103 adults to observe the effect of the *Pelargonium* sidoides liquid drug for the common cold and found that the symptoms and duration of the common cold was shorter compared to the

placebo. Koutelidakis and colleagues (2008) analysed the total antioxidant capacity (TAC) of plasma and organs using mice that were fed with *Pelargonium purpureum* (8 g/100 ml of water). It was found that this increased the TAC in the plasma and lungs. The antioxidant effects of *Pelargonium radula* were investigated by researchers (using DPPH free radical, reducing power and LDH leakage assays) and reported that due to its promising antioxidant capacity, the leaf extract may have a role in the management of diabetes (Latte and Kolodziej, 2004; Lalli *et al*., 2008; Karagozlera *et al*., 2008; Petlecski *et al*., 2012). The antidiabetic effect of the extract was only analysed due to its essential oil content, not because of its polyphenol content.

### 1.2.6 Echinacea composition and protective health effects

Echinacea is one of the most commonly consumed plants, with nine species in North America. For medicinal use, *Echinacea angustifolia*, *Echinacea pallida* and *Echinacea purpurea* species are preferred (Mishima *et al.,* 2004; Vimalanathan *et al.,* 2005; Shah *et al.,* 2007; Georgieva *et al.,* 2013). While the FDA approves it as a food, the Federal Bureau of Health (Germany) recognizes it as a medicine (Mishima *et al.,* 2004). In the current study, *Echinacea purpurea* was used to analyse the transport of carbohydrates across the Caco-2 cell monolayer. There are ~800 products (tablets, extracts, juice and tea) present in Europe using different parts of *Echinacea purpurea* or with different extraction and formulation used as a herbal medicine (Shah *et al.*, 2007; Georgieva *et al.,* 2014).

The polyphenolic composition of *Echinacea* has been reported as caffeic acid derivatives (cichoric acid, caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside) and ferulic acid derivatives (Perry *et al.,* 2001; Pellati *et al.,* 2005; Georgieva *et al.,* 2014). *Echinacea* is commonly used as a cold and flu treatment (Barrett, 2003; Lee at al., 2006). The phenolic acid composition of *Echinacea* supports immunostimulatory properties, promotes *in vitro* and *in vivo* phagocyte activity, shows anti-inflammation activity and inhibits free radical production and lipid peroxidation (Linde *et al.,* 2006; Georgieva *et al.,* 2014). Vimalanathan *et al.* (2005) reported that *Echinacea purpurea* has antiviral activity due to its caffeic acid content. Lee *et al*. (2006) reported that the *Echinacea* species

inhibited P450 enzymes *in vitro* and, therefore, it may increase antiretroviral drug concentrations. Aherne *et al.* (2007) analysed the effect of *Echinacea purpurea* extracts on the viability, membrane integrity, antioxidant activity and DNA integrity of Caco-2 cells and compared the results with rosemary, oregano and sage extracts. The authors indicated that *Echinacea* had the lowest toxic effect with an $IC_{50}$ value of 1421 µg/ml.

Ryan and colleagues (2001) indicated the *Echinacea*, garlic, herbal mixtures and glucosamine were the most commonly consumed alternative medicine for diabetes. Also, Dannemann and colleagues (2008) and Villa-Caballero and colleagues (2010) indicated that *Echinacea* was used as an alternative medicine for type-1 diabetes between type-1 diabetic patients. Delorme and Miller used non-obese diabetic (NOD) mice as a model for human type-1 diabetes and fed them with *Echinacea*. Due to its immune-stimulation effect of NK cells, it may be used in the management of type-1 diabetes (Delorme and Miller, 2005). To our knowledge, this is the first time the potential effects of *Echinacea* have been studied and reported in the literature on diabetic complications.

## 1.3 Carbohydrate metabolism and diabetes

### 1.3.1 Carbohydrates

Carbohydrates are the most important energy-containing components of the diet. They cover 56% of the usable energy in a diet of 12,500 kJ daily (Paulev & Zubieta, 2004). Carbohydrates are classified into 4 groups: mono-, oligo- and poly-saccharides, and their derivatives (Lindhorst, 2003). Oligo- and poly-saccharides are synthesised from mono-saccharides (two or more joined together), which are catabolised during digestion to the mono-saccharide form in order to provide energy. Nutrients occur from food degradation followed by absorption in the small intestine or bacterial fermentation in the large intestine. Sucrose, maltose and isomaltose are examples of disaccharides synthesised by the reaction between glucose and fructose and two glucose units respectively. The difference between maltose and isomaltose is the position of glycosidic links between glucose molecules, and is shown in Figure 4. Hydrolysis of sugars (sucrose, isomaltose and maltose) by digestive enzymes

Sucrose, maltose and isomaltose are hydrolysed by sucrase, maltase and isomaltase enzymes respectively located in the small intestine. One glucose and one fructose unit (α-1,2 glycosidic bond) make up one sucrose molecule, Mw 342.3. Two glucose units with an α-1,4 and α-1,6 linkage comprise maltose and isomaltose respectively.

The enzymatic hydrolysis of sucrose is catalysed by sucrase (EC 3.2.1.48) in humans. This enzyme is located at the luminal surface of enterocytes and is expressed by microvillus cells (Marieb, 2004; Beaulieu *et al.*, 1989). Gasteiger *et al.* (2003) reported that there are two enzymes present which catalyse sucrose hydrolysis directly: sucrose α-glucosidase (EC 3.2.1.48) and β-fructofuranosidase (EC 3.2.1.26). Maltose is catalysed by α-glucosidase (EC 3.2.1.20) and sucrose α-glucosidase (EC 3.2.1.48). Oligo-1,6 glucosidase (EC 3.2.1.10) catalyses the action of isomaltase, which is responsible for the hydrolysis of maltose and isomaltose. The active site of sucrase hydrolyses the α-1,2 and α-1,4 glycosidic bonds.

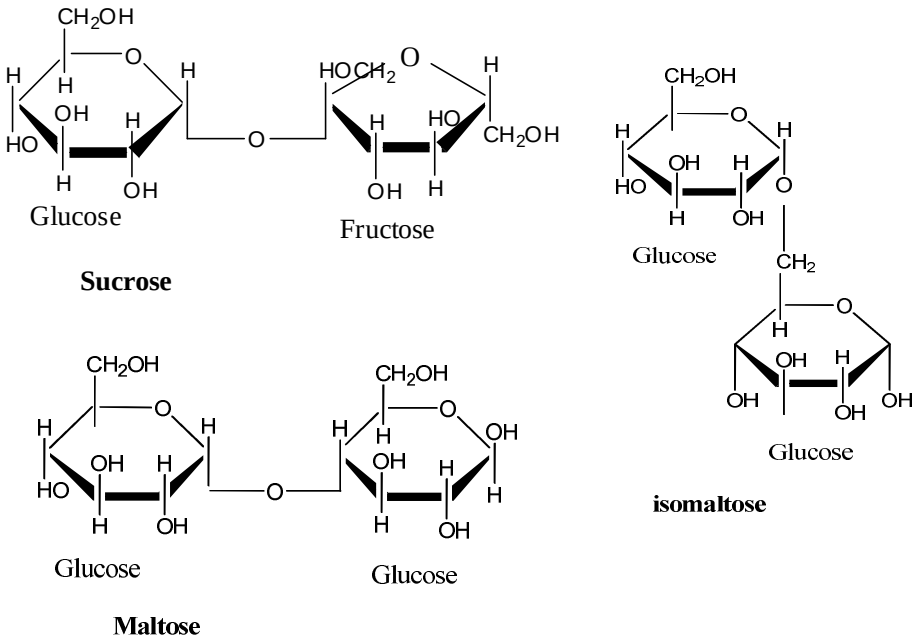



**Figure 4** Structure of sucrose, maltose and isomaltose.

### 1.3.1.1 Transport mechanism of glucose and fructose

Mono-saccharides are transported by active transport via the sodium-dependent glucose transporter SGLT1, and by facilitated sodium-independent transport via the glucose transporter GLUT2 (Goodman, 2010). The primary glucose carrier is

SGLT1, which is a protein composed of 664 amino acid residues (Wright *et al.*, 2011; Turk *et al.*, 1996). GLUT2, composed of 554 amino acids, can be recruited into the apical membrane to assist SGLT1 uptake of glucose when high concentrations of glucose are present in the intestinal lumen (Ait-Omar *et al.*, 2011). At low luminal concentrations, SGLT1 transports glucose against a concentration gradient with a $K_{0.5}$ (substrate concentration of half saturation-affinity) of 0.5 mM (Wright *et al.*, 2011). Two $Na^+$ molecules bind to SGLT1 to permit glucose binding to the transporter (Jiang *et al.*, 2012). After binding to SGLT1 on the luminal side of the intestinal brush border membrane, a conformational charge occurs that allows glucose binding. The second conformational charge allows glucose and $Na^+$ to enter the enterocytes (Figure 5) (Goodman, 2010). Glucose is released from the enterocyte via the GLUT2 passive transporter at the basal surface and enters the circulation, and the process of uptake and expulsion is simultaneous and continues until the concentration of glucose within the cell is <30,000 times that of the intestine (Figure 5) (Goodman, 2010; Nelson and Cox, 2000). Fructose enters and exits the cells via facilitated diffusion. Mono-saccharides generate adenosine triphosphate (ATP) to provide cellular energy (Marieb, 2004). The detailed transport mechanism for each sugar is given in the related chapters.

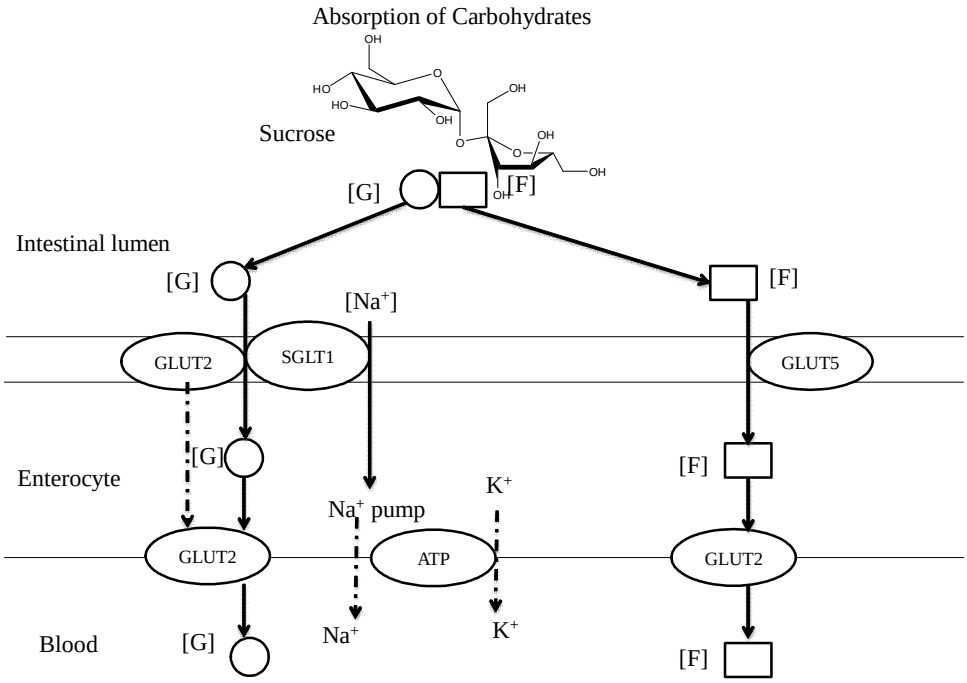



**Figure 5** Absorption of carbohydrate (adopted from Paulev & Zubieta, 2004). GLUT2 trafficking from basolateral to apical is depending on glucose [G] concentration.

### 1.3.2 Carbohydrate-related diseases

Carbohydrate-restricted diets are becoming popular for weight loss, while the prevention of obesity also encourages people to consume more animal products. However, the use of low carbohydrate diets may increase the risk of CVD due to the high amount of saturated fat and cholesterol associated with the consumption of animal products (Foster *et al.*, 2003; Holton *et al.*, 2006). Hauner and colleagues (2012) reported that high carbohydrate consumption increased total and LDL cholesterol but reduced HDL cholesterol.

Holton and colleagues used the data from 82,802 women in the Nurses' Health Study to analyse the relationship between a low carbohydrate diet and coronary heart disease. It was found that a low carbohydrate diet, and higher protein and fat intake were not related with increased risk of coronary heart diseases in women. Also, the risk of coronary heart diseases in women was reduced when the source of fat and protein was from vegetables (Holton *et al.*, 2006). Park and

colleagues (2008) analysed the relationship between carbohydrate intake and CVD with the data from the third Korea National Health and Nutrition Examination Survey (1536 men and 2235 women). It was reported that in women, high intake of carbohydrates increased the risk of higher BMI, blood pressure, fasting glucose, triglycerides and LDL-cholesterol levels, but high carbohydrate intake was inversely associated only with total cholesterol for men (Park *et al.,* 2008). Meyer *et al.* (2000) conducted a cohort study with 35,988 older Iowa women who did not have diabetes. After 6 years, the report of diabetes incident was related to 1141 women. Meyer and colleagues concluded that a diet that includes cereal fibre, grains and dietary magnesium prevents the development of diabetes in older women.

Barclay and colleagues (2008) observed the relationship between glycaemic index (GI), glycaemic load (GL) and the risk of chronic diseases. The comparison of inconsistent findings in the literature showed that low GI/GL diets were independently related with a reduced risk of chronic diseases but high post-prandial glycaemia was found to be related with disease progression (Barclay *et al.,* 2008). Liese and colleagues (2005) analysed the relation of fibre intake, GI, GL insulin sensitivity, fasting insulin, acute insulin response, disposition index, BMI and waist circumference from the data of 979 adults with normal and impaired glucose tolerance from the Insulin Resistance Atherosclerosis study between 1992 and 1994. It was found that GI and GL might not be associated with insulin sensitivity, insulin secretion and adiposity. Fibre intake has a beneficial effect on pancreatic functionality. Higher intake of carbohydrates with high GI increased the acute glucose and insulin response. Therefore, GI in the diet has an important role in glucose metabolism, insulin secretion and insulin resistance (Liese *et al.,* 2005).

### 1.3.3 Type-2 diabetes

There are 346 million people suffering from diabetes worldwide (WHO, 2008). This number is expected to double by the year 2030. Zhang and colleagues (2010) reported that the cost of diabetes treatment in Europe is 10% of the total healthcare costs ($196 billion) in Europe in 2010 and it is expected to rise to almost $235 billion in 2030. Glucose is an important carbohydrate that plays a

role in the development of diabetes. About 95% or more of all diabetics globally have type-2 diabetes. Type-2 diabetes develops due to insulin resistance or/and insulin deficiency. Type-2 diabetes is manifest when there is deficient or impaired insulin signalling for effective blood glucose absorption resulting in high blood glucose level although the sequence of events regarding insulin signalling dysfunction is not clear (e.g. whether insulin deficiency stimulated from β cell failure takes place first). Following the absorption of postprandial glucose in the blood (see Figure 5), it is transported to body cells via the bloodstream. The pancreas (β-cells) secretes insulin to maintenance glucose homeostasis in the body. Following insulin secretion into the blood, GLUT4 move from the intracellular membrane pool to the plasma membrane and enhanced glucose uptake into the muscle and adipose tissues. (Govers, 2014; Leney and Tavare, 2009). To prevent or delay type-2 diabetes, the blood glucose level needs to be controlled. Reducing the glucose in blood can occur in two ways: one is inhibiting the digestive enzymes for glucose production (α-amylase and/or α-glucosidase) and the second is inhibiting the glucose transporters that have a role in glucose absorption. Diabetes increases the risk of heart disease and complications which damage the kidney, retina and peripheral nerves (Williamson and Carughi, 2010). Therefore, controlling the blood glucose level will decrease the risk of those diseases and diabetes (Table 4, Table 5 and Table 6).

The normal blood glucose level in humans is ~4 mM (72 mg/dl) but after eating a meal this begins to increase the blood glucose level temporarily to <7.8 mM (140 mg/dl) (National Institute for Clinical Excellence, 2011). This increase stimulates the secretion of insulin and excess glucose is taken up by muscle cells and stored as glycogen (Nelson and Cox, 2000). By decreasing the blood glucose levels to normal, the insulin level begins to fall. Type-2 diabetes causes a high blood glucose level (hyperglycaemia) due to the lack of insulin production or the fact that the insulin produced is enough but ineffective (insulin resistance) (Nelson and Cox, 2000). The International Diabetes Federation reported that >382 million people around the world have diabetes and this may rise to >592 million by 2030, and 90% of all people will have type-2 diabetes. In the

UK, it is assumed that ~750,000 people have type-2 diabetes and have not been diagnosed while about 2.7 million are diagnosed (Diabetes, UK, 2012).

Therefore, lowering the blood glucose level via dietary intervention may assist in the prevention of progression to type-2 diabetes. There are several *in vitro* and *in vivo* studies which have explored such strategies and are discussed below.

Wilson and colleagues (2008) studied twelve healthy participants fed with sucrose (35 g) and berries (150 g of purée made of bilberries, blackcurrants, cranberries and strawberries providing ~800 mg polyphenols). Berries are a rich source of anthocyanins, flavonols, proanthocyanidins and phenolic acids. Participants who were fed without berries produced a different post-prandial glycemic response compared to the subjects who were fed with berries (Wilson *et al.,* 2008). Zhong and colleagues (2006) investigated whether tea extracts had the ability to affect carbohydrate absorption. The authors fed the human adult volunteers with 50 g white rice and a beverage containing either black, green or mulberry tea extract or a placebo. Then they measured the concentration of breath hydrogen and carbon dioxide at regular intervals. The ingestion of tea extracts significantly increased the breath hydrogen concentration compared to the placebo. Breath hydrogen concentration is indicative of the quantity and metabolic activity of anaerobic bacteria in the intestine.

Goodman (2010) analysed the glucose transporter in the presence of polyphenols using Caco-2 cells and intestinal brush border membrane vesicles or everted sacs. These studies claim that several flavonoids and phenolic acids can inhibit glucose transport. Phytochemicals are known to interact with SGLT-1 for the absorption of nutrients across the apical membrane of enterocytes (Buddington *et al.,* 2003). The phytochemical phloridzin is often used as a selective inhibitor. Buddington and colleagues (2003) reviewed some polyphenols that have the ability to reduce glucose uptake. By decreasing SGLT-1 activity, soy isoflavones partly affect post-prandial glucose levels; tea polyphenols were shown to lower the rate of glucose transport by non-specific binding to proteins; catechins and other phytochemicals were able to change brush border fluidity, which potentially influences the function of the transporters. Gao *et al.* (2007) studied

the inhibitory effect on the α-glucosidase enzymes of maltase, sucrose and isomaltase by the fruits of *Terminalia chebula* (Retz.). Phenolic compounds were extracted from the fruits and test samples with substrate (sucrose, maltose and isomaltose), and crude enzyme solutions were incubated. The authors found that the extracted polyphenols inhibited rat intestinal maltase activity; however, they did not inhibit sucrase or isomaltase activity. They concluded that phenolic compounds extracted from the fruits of *Terminalia chebula* (Retz.) may be useful as a natural source of α-glucosidase inhibitor that can be utilised in the suppression of post-prandial hyperglycaemia in type-2 diabetes management.

## 1.4 Hydrolysis of amylopectin by human salivary α-amylase

Carbohydrate-rich foods mainly contain starch in the human diet and they are responsible for the high level of blood glucose after intestinal digestion of a starch-containing meal. The hydrolysis of starch starts in the mouth with α-amylase and as the enzyme is degraded in the acidic content of the stomach, it has a minor role in the starch digestion and progress to the small intestine (Singh *et al.,* 2010). Salivary α-amylase catalyses starch to maltose, maltotriose and limits dextrins in the mouth (Butterworth *et al.,* 2011). The gene encoding salivary amylase, AMY1, is located on the short arm of the chromosome and the gene encoding the enzyme with Mws of ~56 000. The N-terminus of salivary amylase is blocked by a pyroglutamic acid residue and the total protein content of saliva is between 0.1 to 10 mg/ml (Butterworth *et al.,* 2011). Starch mainly consists of amylose (linear polymer of α-D glucose units linked together with α-1,4 glycosidic linkages and a degree of polymerisation (DP) ~500-6000 glucose residues) or amylopectin (branched polymer of α-D glucose units linked together with α-1,4 and α-1,6 glycosidic linkages and a DP ranging from $3x10^5$ to $3x10^6$ glucose units). Salivary amylase is an endo-amylase and it hydrolyses at the α-1,4 glycosidic bonds of the starch molecule, and different α-limit dextrins containing α-1,6 bonds are formed with amylopectin (Bijttebier *et al.,* 2009). Bread and cereal products, which are consumed daily, are rich in starch, which is rapidly digested and absorbed after a meal. This can cause an increase in high blood glucose level and insulin secretion. Nantanga and colleagues (2013)

reported that different foods with starch content have a similar amount of blood glucose level but the insulin level shows a difference.

The moisture content of starch (e.g. in cooking) has an effect on the salivary amylase activity. Low moisture processing starch allows amylase to show activity on fewer polymer chains compared to high moisture processing. Lower moisture content of starch is gelatinized primarily by helix-helix dissociation followed by partial helix-coil dissociation. Therefore, the enzyme is able to access the cluster chains more easily than raw starch. By contrast, a high moisture content is gelatinized at the dissociation of helix-helix and helix-coil and this allows salivary amylase to access and hydrolyse α-1,4 glycosidic bonds between unwound and cluster chains. By contrast, the enzyme more efficiently hydrolyses molecules without a helical structure (Nantanga *et al.*, 2013).

**Table 4** Summary of polyphenol rich food effect on glucose transport, uptake and inhibition of α-glucosidase and α-amylase enzymes (human intervention studies).

| **Analysed/ major polyphenols** | **Plant Food** | **Experimental model** | **Type of interaction** | **Effect** | **Reference** |
|---|---|---|---|---|---|
| Flavan-3-ols, theaflavins | Black tea | Healthy humans | Oral glucose load black tea | Late phase plasma glucose response reduced but the insulin level increased. | Brayns *et al.*, 2007 |
| Anthocyanins, proanthocyanins | Bilberry, blackcurrants, cranberries and strawberries | Healthy humans | Randomised controlled cross-over | Plasma glucose concentration significantly lower than control (15 & 30 min). | Torronen *et al.*, 2010 |
| Catechin, epicatechin, procyanidin flavonoids | Chocolate | Healthy subjects | Oral glucose tolerance | Protect against acute hyperglycaemia. | Grassi *et al.*, 2005 |
| Chlorogenic acid | Coffee | Overweight men | Randomized crossover trial | Glucose, insulin concentration reduced significantly. | Van Dijk *et al.*, 2009 |
| Catechins | Green tea | Type-2 diabetic patients | Double blind control study | Recovery of insulin secretory ability. | Nagao *et al.*, 2009 |
| Stilbene, quercetin, resveratrol | Muscadine grape | Type-2 diabetic patients | Fasting glucose concentration and insulin level | Fasting blood glucose decreased blood insulin glucose ratio increased. | Banini *et al.*, 2006 |

**Table 5** Summary of polyphenol rich food effect on glucose transport, uptake and inhibition of α-glucosidase and α-amylase enzymes (cell studies).

| **Analysed/ major polyphenols** | **Plant Food** | **Experimental model** | **Type of interaction** | **Effect** | **Reference** |
|---|---|---|---|---|---|
| Phloretin, quercetin, apigenin, myricetin | - | Caco-2 cells | $Na^+$-free / dependent | $Na^+$-dependent: no effect<br>$Na^+$-free: glucose uptake reduced | Johnston *et al.*, 2005 |
| Quercetin, myricetin, isoquercetin | - | *Xenopus laevis* oocytes | GLUT2 expressed *Xenopus laevis* oocytes | Complete inhibition of glucose transport with 50 µM quercetin | Kwon *et al.*, 2007 |
| Apple, Strawberry | Apple and strawberry | Caco-2 cell monolayer | $Na^+$-free / dependent | Q3R: 26%, phloridzin: 52%, diCQA: 12%, P3G: 26% | Manzano and Williamson 2010 |
| C3R, P3R, quercetrin | Acerola | Caco-2 cells | 3-O-[methyl-$^3$H]-D-glucose- uptake | $IC_{50}$ Acerola: 0.2 mg/ml<br>Inhibitory effect of P3R higher than Acerola | Hanamura *et al.*, 2006 |
| Chebulagic acid | *Terminalia chebula retz* | Caco-2 and acetone rat intestine extract | Glucose oxidase method | Inhibited sugar digestive enzymes and sucrose hydrolysis. | Huang *et al.*, 2012 |
| Catechin, EC, EGCG ,GCG, ECG | Pu-erh tea | HepG2 cells | Fluorescence- 485 nm | Glucose uptake increased. | Du *et al.*, 2012 |

**Table 6** Summary of polyphenol rich food effect on glucose transport, uptake and inhibition of α-glucosidase and α-amylase enzymes (animal and in vitro studies).

| **Analysed/ major polyphenols** | **Plant Food** | **Experimental model** | **Type of interaction** | **Effect** | **Reference** |
|---|---|---|---|---|---|
| Phlorotannins | Ascophyllum | Porcine pancreatic α-amylase<br>Acetone rat intestine extract | p-hydroxybenzoic acid<br>α-D-glycopranoside | $IC_{50}$ α-amylase 0.1, α-glucosidase 20 µg/ml GAE. | Nwosu *et al.*, 2010 |
| Neoericitrin, naringin, neo-hesperidin | Bergamot | Male Wistar rats | Blood and faecal | Decrease blood glucose level. | Mollace *et al.*, 2011 |
| Anthocyanins | Blueberry | C57/BI6 mice | Fasting and postprandial glucose | Reduced hyperglycaemia, body weight gain, serum cholesterol | Roopchand *et al.*, 2013 |
| Polyphenols, tannins | Castenea mollissima spiny burs | STZ induced diabetic rats | Glucose oxidase method | Hypoglycaemic and hypolipidemic | Yin *et al.*, 2011 |
| Rutin, protocatechuic acid | *Cynidoscolus chayamansa* | STZ induced Wistar rats | Fasting blood glucose levels | Acute hyperglycaemic effect | Loarca-Pina *et al.*, 2010 |

Table 6 continued.

| **Analysed/ major polyphenols** | **Plant Food** | **Experimental model** | **Type of interaction** | **Effect** | **Reference** |
|---|---|---|---|---|---|
| CQA, diCQA, feruloyl quinic acid | Coffee | Acetone rat intestinal sucrase and maltase | Glucose C2 test WAKO | $IC_{50}$ maltase 0.07 and sucrase 0.35 mg/ml. | Murase *et al.*, 2011 |
| Eugenin, trapain, 1,2,3,6 tetra-O-galloyl-β-D glucopyranose | Water chestnut (*Trapa Japonica*) | Human salivary α-amylase and α-glucosidase (acetone rat intestinal extract) | α-amylase: DNS<br>α-glucosidase: Glucose C2 test WAKO test | $IC_{50}$ α-amylase 53, maltase 159 and sucrase activity 654 µM. | Yosuda *et al.*, 2014 |
| Chlorogenic acid, cyanidin, quercetin malonl-glucoside, cyanidin malonl-glucoside, | Lettuce | High fat diet induced C57BL/6 mice | Alphatrak glucometer | No significant difference in plasma glucose level. | Cheng *et al.*, 2014 |
| Anthocyanins, proanthocyanidins | Grape-soybean flour complex | Hyperglycaemic C57BL/6J mice | Glucometer | Lower blood glucose level. | Roopchand *et al.*, 2013 |
| Ellagic acid, quercetin | Muscadine | α -glucosidase from *Saccharomyces cervisiae* | α-D-glycopranoside | $IC_{50}$ 1.92 mg/ml. | You *et al.*, 2012 |
| Curcumin | - | Male Sprague Dawley rats | 2-deoxy[$^{3}$H] glucose | Basal and insulin stimulated 2-deoxyglucose uptake inhibited. | Green *et al.*, 2013 |

## 1.5 Aim and objective

In order to prevent or delay type-2 diabetes, the blood glucose level needs to be controlled. Polyphenols have been show to affect sugar metabolism. The aim of this research is to investigate sucrase, maltase, isomaltase and human salivary α-amylase inhibition by PFS extracts and the effect of PFS on sugar transporters (SGLT1, GLUT2 and GLUT5). The evidence from the literature on the inhibition of cellular glucose uptake and transport by polyphenols across Caco-2 cells is limited. To the best of our knowledge, this research is the first report regarding the analysis of cellular uptake and transport of $^{14}$C-sucrose and $^{14}$C-fructose using the Caco-2 cell monolayer with polyphenol-containing extracts. The main objectives of the work are:

- To establish and characterise an *in vitro* model of the impact of PFS extracts on sugar transporters using Caco-2 cells, and on digestive enzymes using acetone-extract of rat intestinal tissues and human salivary α-amylase.
- To make a preliminary assessment of the capacity of PFS extracts to reduce glucose, fructose and sucrose transport (acarbose-like activity) across the Caco-2 cell monolayers and cellular uptake.
- To identify the mechanism by which polyphenol in German chamomile extract was effective on the transport across the monolayer and cellular uptake of glucose and fructose.
- To test whether modified German chamomile extract was affected on the transport across the monolayer and cellular uptake of glucose.
- To identify the effect of FBS starvation on cells 12 h before the experiment with/without German chamomile and sugars.
- PFS extracts were analysed for their possible interference with α-glucosidase and α–amylase assays and assessed by considering critical parameters affecting the final inhibition.

# CHAPTER 2 CHARACTERISATION OF PLANT FOOD SUPPLEMENTS AND INHIBITION OF HUMAN SALIVARY α-AMYLASE BY PLANT FOOD SUPPLEMENTS

## Abstract

This project is part of EU framework 7 project PlantLIBRA (Plant food supplement: Levels of intake, benefit and risk assessment). This project aims to investigate science-based decision making and safe use of PFS. This study concentrated on the benefits of PFS extracts within the wider project. This chapter is divided into two sections. The first section includes the effective compounds of polyphenols that inhibit the sugar transporters or digestive enzymes, and analyses the composition of PFS extracts. It was found that German chamomile extract contains high levels of ferulic acid hexosides and apigenin-7-*O*-glucoside, while EGCG was the most abundant catechin in green tea extract. The second section includes the inhibition of human salivary α-amylase by PFS extract. Human salivary α-amylase was chosen because it can help us understand the possible effects of PFS extracts in human α-amylase enzyme. The current methods found in the literature were optimised considering critical parameters that may affect the final inhibition of α-amylase (e.g. DNS reacts with polyphenols and affects the final inhibition). Acarbose is an anti-diabetic prescription drug used as a positive control for the treatment of type-2 diabetes. It inhibits the carbohydrate hydrolysis enzymes (α-glucosidase and α-amylase). PFS extracts might also be considered to have the same effect (acarbose-like effect). Both German chamomile and green tea extracts are found to be moderate inhibitors of human salivary α-amylase with $IC_{50}$ values of 2.5 and 1 mg/ml respectively. Also, acarbose at 0.1 mM concentration equals to 57% inhibition.

These findings indicate that glycaemic responses may be reduced when German chamomile and green tea extracts are taken during a meal due to their acarbose-like activity.

## 2.1 EU Project

The aim of this project is to encourage the safe use of food supplements. In this project, 23 universities from different European countries are working in different fields, sharing their work and expertise among themselves and with leading stakeholders towards an integrated science of botanicals. The development of PFS in Europe could bring substantial market growth. However, the present scientific and regulatory situation for PFS creates barriers to such growth. To protect consumers and exploit market opportunities, a new integrated approach is needed in research. Therefore, both risk and benefit assessments are undertaken in this project (http://plantlibra.eu).

This study is involved in the methodology for the benefit assessment of PFS, corresponding to the application and validation work package of this project. This work package aims to address the validation, achievement and application of a methodology in order to establish the benefits of PFS consumption using advanced *in vitro* approaches.

## 2.2 Characterization of PFS used for *in vitro* enzyme activity measurements

PFS were received from the EU project, PhytoLab Co. KG.h (Vestenbergsgreuth, Germany). While green tea extract is maltodextrin-free, German chamomile contains 50%, *Vitis Viniferae* contains 17%, Echinacea contains 33% and Pelargonium contains 75% of maltodextrin. Maltodextrin is added to increase the water solubility of the extracts by the supplier. Maltodextrin consists of a variable length of D-glucose units (between 3 to 17 glucose units long) linked with α-1,4 glyosidic bonds. Therefore, maltodextrin is a potent substrate for intestinal enzymes and can interfere with the measurement of inhibition.

The composition and purification of German chamomile was analysed by colleagues who were also involved in the EU project and were responsible for those parts of the project.

### 2.2.1 Removal of maltodextrin from PFS

To remove the maltodextrin from the German chamomile extract, a method was developed using the Akta Purifier 1.0 by a colleague Mr. J. Alberto. The purified fraction did not contain maltodextrin and the fraction was soluble in DMSO. This extract was dissolved in 100% DMSO. The concentration of DMSO in the final assay was 0.1%, which does not interfere with human salivary amylase. The final concentration of DMSO in the inhibition of digestive enzyme assay was 4%. The effect of DMSO to the enzyme activity was analysed and it was found that DMSO inhibited the enzyme activity by 8%. This was adjusted for in the relevant assays.

### 2.2.2 Identification of compounds in German chamomile using HPLC with triple-quad mass spectrometric detection

The composition of German chamomile extract was analysed using triple-quad mass spectrometric detection and single-quad mass spectrometric detection. German chamomile extract solubilised in water was analysed using an Agilent 1200 HPLC coupled with a 6410 LCMS triple quadrupole fitted with an electro-spray ionization source in the negative mode (Figure 6 and Figure 7). The standard compounds were supplied by PhytoLab Co. KG.h (Vestenbergsgreuth, Germany). Preliminary analysis identified many compounds, shown in Table 7.

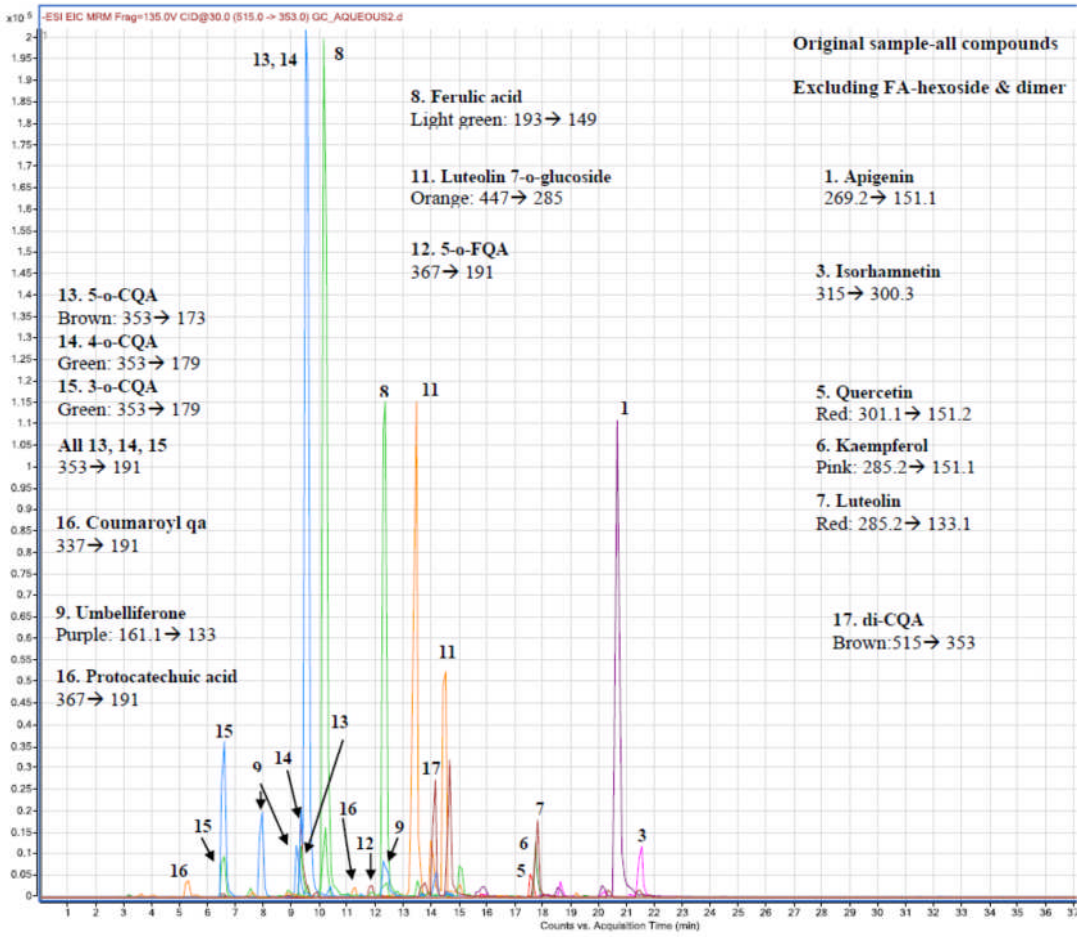



**Figure 6** Analysis of German chamomile in water using HPLC with triple-quad mass spectrometric detection excluding the ferulic acid hexosides, which otherwise would dominate the chromatogram.

Figure 6 represented the expanded format of Figure 7 and peaks labelled with same number in the figures represented the same compounds which were explained in the figures.

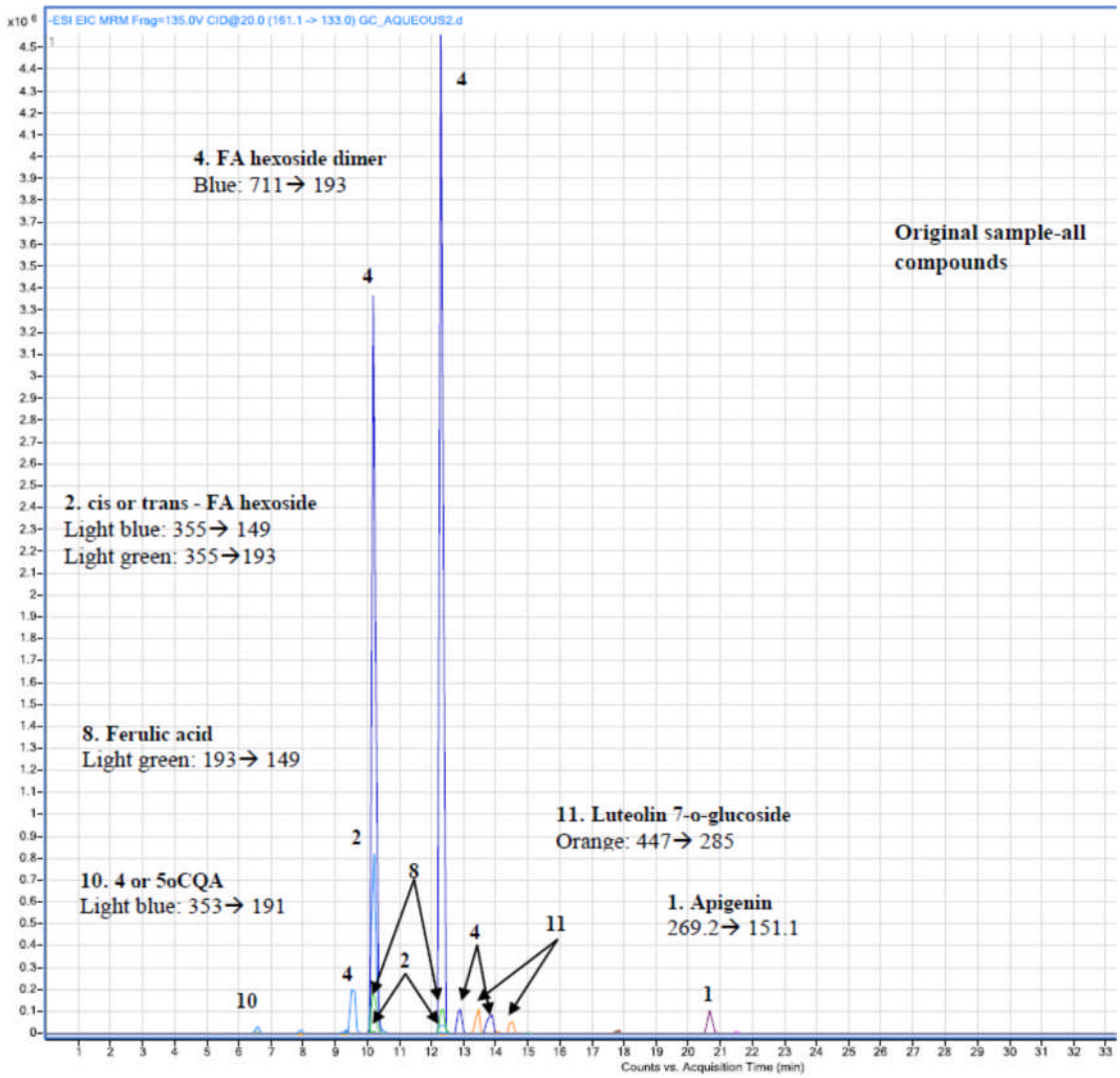



**Figure 7** Analysis of German chamomile in water using HPLC with triple-quad mass spectrometric detection.

**Table 7** Characteristics of compounds identified in German chamomile using HPLC with triple-quad mass spectrometric detection.

| **Compound name** | **Precursor ion** | **Product ion** | **Fragmentor** | **Collision energy** | **RT** | **RT** | **RT** | **RT** |
|---|---|---|---|---|---|---|---|---|
| Ferulic acid (FA) hexoside dimer | 711 | 193 | 135 | 35 | 10.1 | 12.2 | 12.8 | 13.8 |
| Quercetin 3-*O*-rutinoside | 609 | 301 | 135 | 35 | 12.3 | | | |
| Kaempferol 3-*O*-rutinoside | 593 | 285 | 135 | 30 | 13 | | | |
| di- caffeolyquinic acid (CQA) | 515 | 353 | 135 | 30 | 13.7 | 15.2 | 14.6 | |
| Luteolin *O*-acylhexoside | 489 | 285 | 135 | 30 | 14.6 | 15.9 | | |
| Myricetin *O*-hexoside | 479 | 317 | 135 | 25 | 12 | | | |
| Quercetin-3-*O*-glucuronide | 477 | 301 | 135 | 35 | 13.2 | 14.5 | | |
| Quercetin 3-*O*- glucoside | 463 | 301 | 135 | 25 | 12.5 | 13 | | |
| Luteolin 7-*O*-glucoside | 447 | 285 | 135 | 25 | 13.5 | 14.5 | | |
| Dimethoxy-cinnamic acid hexoside | 369 | 189 | 135 | 30 | 14.7 | 16.9 | 19.5 | |
| 5 *O*-feruloylquinic acid | 367 | 191 | 135 | 30 | 11.8 | 13.7 | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Cis/trans FA hexoside | 355 | 193 | 135 | 30 | 10.1 | | |
| Cis/trans FA hexoside | 355 | 149 | 135 | 30 | 10.1 | | |
| *O*-caffeolyquinic acid (CQA) | 353 | 191 | 135 | 30 | 9.5 | | |
| 3-*O*-CQA | 353 | 179 | 135 | 30 | 6.5 | | |
| 4-*O*-CQA | 353 | 173 | 135 | 30 | 9.3 | | |
| Coumaroyl quinic acid aglycone | 337 | 191 | 135 | 30 | 11.2 | | |
| Isohamnetin aglycone | 315 | 300.3 | 135 | 20 | 21.5 | | |
| Quercetin aglycone | 301.1 | 151.2 | 135 | 20 | 17.5 | | |
| Kaempferol aglycone | 285.2 | 151.1 | 135 | 20 | 17.7 | | |
| Luteolin aglycone | 285.2 | 133.1 | 135 | 25 | 17.8 | | |
| Apigenin aglycone | 269.2 | 151.2 | 135 | 20 | 20.6 | 12.3 | |
| FA aglycone | 193 | 149 | 135 | 10 | 10.2 | | |
| Umbelliferone aglycone | 161.1 | 133 | 135 | 20 | 7.9 | 9.2 | 12.3 |
| Protocatechuic acid aglycone | 153 | 109 | 135 | 10 | 5.3 | | |

### 2.2.3 Quantification of compounds in German chamomile using HPLC with single-quad mass spectrometric detection

German chamomile extract solubilised in water was analysed using Shimadzu LC-2010 HT coupled with a LCMS-2020 quadrupole mass spectrometer fitted with an electro-spray ionization source used in the negative mode. The standard compounds were supplied by PhytoLab Co. KG.h and they were paired with compounds in the German chamomile water extract solution, but due to lack of standards the amount of ferulic acid hexosides could not be quantified. Preliminary analysis identified many compounds, shown in Table 8. It was found that German chamomile contains many phytochemicals and apigenin-7-*O*-glucoside was predominant in the extract. Ferulic acid hexoside was also apparently at a high level.

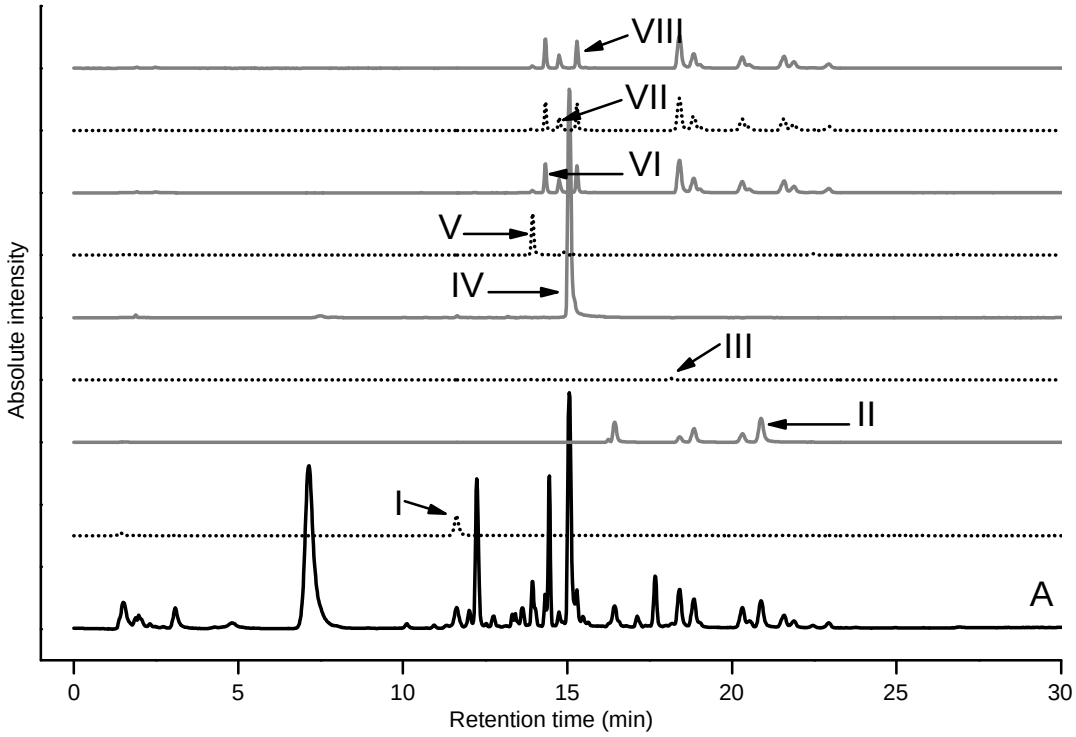



**Figure 8** Quantification of compounds in German chamomile dissolved in water using HPLC with single quadrupole mass spectrometry.

Total ion current (TIC) of German chamomile without hydrolysis (A) identifying the presence of umbelliferone (I) retention time (RT)= 11.7 min; apigenin (II) RT= 20.9 min; luteolin (III) RT= 18.1min; apigenin-7-glucoside (IV) RT= 15.1; luteolin-7-glucoside (V) RT= 13.9; 3,4-dicaffeoylquinic acid (VI) RT= 14.3 min; 3,5-dicaffeoylquinic acid (VII) RT= 14.7 min and 4,5-dicaffeoylquinic acid (VIII) RT= 15.3 min

**Table 8** The polyphenol profile of German chamomile for samples without hydrolysis ($H_2O$); fraction after hydrolysis and precipitation of proteins by ACN, followed by extraction into EtAC.

| | | | **$H_2O$** | **ACN** | **EtAC** |
|---|---|---|---|---|---|
| **Compound** | $m/z^{(-)}$ | Retention time (min) | Composition (%) excluding maltodextrin component | | |
| **Umbelliferone** | 161 | 11.7 | 0.07 | 0.15 | 0.14 |
| **Apigenin** | 269 | 20.9 | 0.35 | 0.46 | 0.30 |
| **Luteolin** | 285 | 18.1 | 0.01 | 0.01 | 0.01 |
| **Apigenin-7-glucoside** | 431 | 15.1 | 5.69 | 0.58 | 0.32 |
| **Luteolin-7-glucoside** | 447 | 13.9 | 0.28 | 0.01 | 0.0* |
| **3,4-dicaffeoylquinic acid** | 515 | 14.3 | 0.12 | 0.0* | 0.0* |
| **3,5-dicaffeoylquinic acid** | 515 | 14.7 | 0.08 | 0.01 | 0.01 |
| **4,5-dicaffeoylquinic acid** | 515 | 15.3 | 0.10 | 0.01 | 0.01 |

*0.0 equals to below detection limit.

Most of the phytochemicals present in PFS are linked with a sugar or organic acids by glycosidic or ester linkages. To absorb those phytochemicals, those linkages need to be removed as part of the digestive process with brush border β-glucosidases in the small intestine or esterases and glycosidases from bacteria in the colon. Then the product aglycone from the digestive process can be absorbed. To analyse the effect of digestion on the biological activity of German chamomile extract, the extract was subjected to enzymatic hydrolysis followed by extraction as a model of the first step of digestion in the gut with ACN and EtAC. Hydrolysis using hesperinidase and cellulose removed the sugars from the polyphenols in the German chamomile and cleaved the ester bonds between phenolic and quinic acid (the first step of digestion in the gut). This experiment indicates that the digestion affects the biological activity of German chamomile

due to glucose transport across the cell monolayer, which was decreased by ~50%.

#### 2.2.3.1 Polyphenol profile of the main compounds from German chamomile after hydrolysis

German chamomile hydrolysed samples were analysed using HPLC with single quadrupole MS (Figure 9 and Figure 10). Preliminary analysis identified many compounds, shown in Table 8. As expected after hydrolysis, the chromatogram of the extract was less complex and the aglycones were revealed.

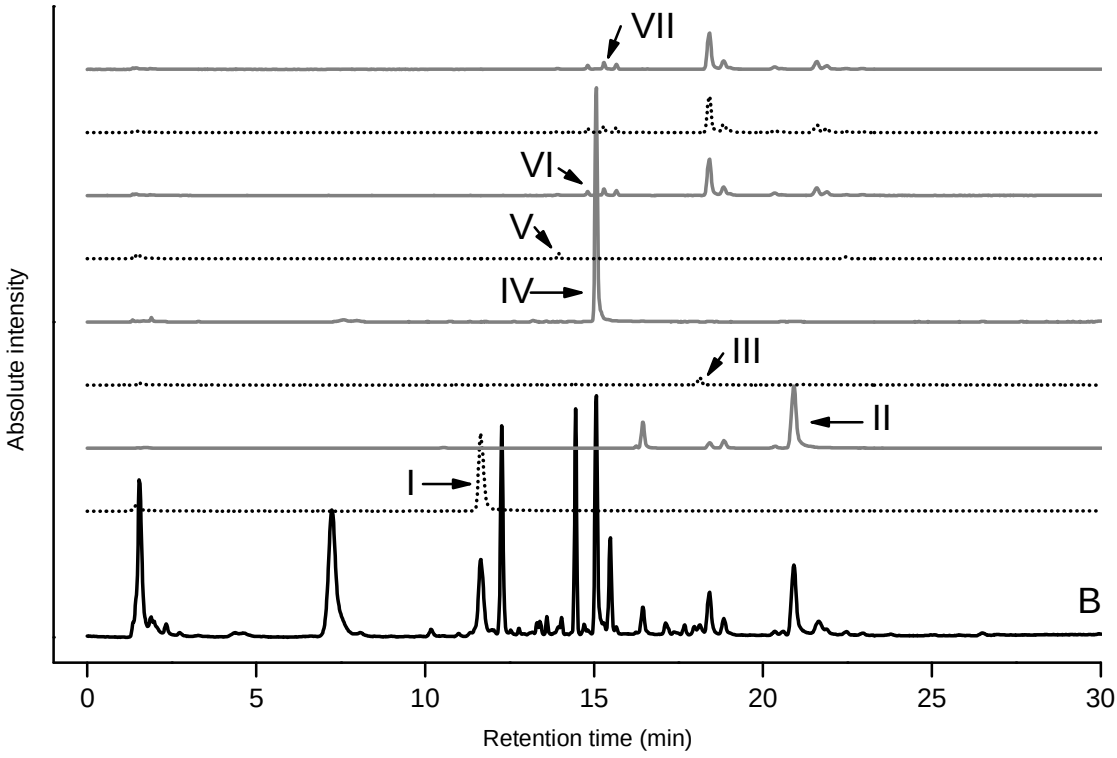



**Figure 9** Quantification of compounds in German chamomile after hydrolysis using HPLC with single quadrupole mass spectrometry.

Total ion current (TIC) of German chamomile with hydrolysis and extraction with acetonitrile (B) identifying the presence of umbelliferone (I) retention time (RT)= 11.7 min; apigenin (II) RT= 20.9 min; luteolin (III) RT= 18.1min; apigenin-7-glucoside (IV) RT= 15.1; luteolin-7-glucoside (V) RT= 13.9; 3,5-dicaffeoylquinic acid (VI) RT= 14.7 min and 4,5-dicaffeoylquinic acid (VII) RT= 15.3 min

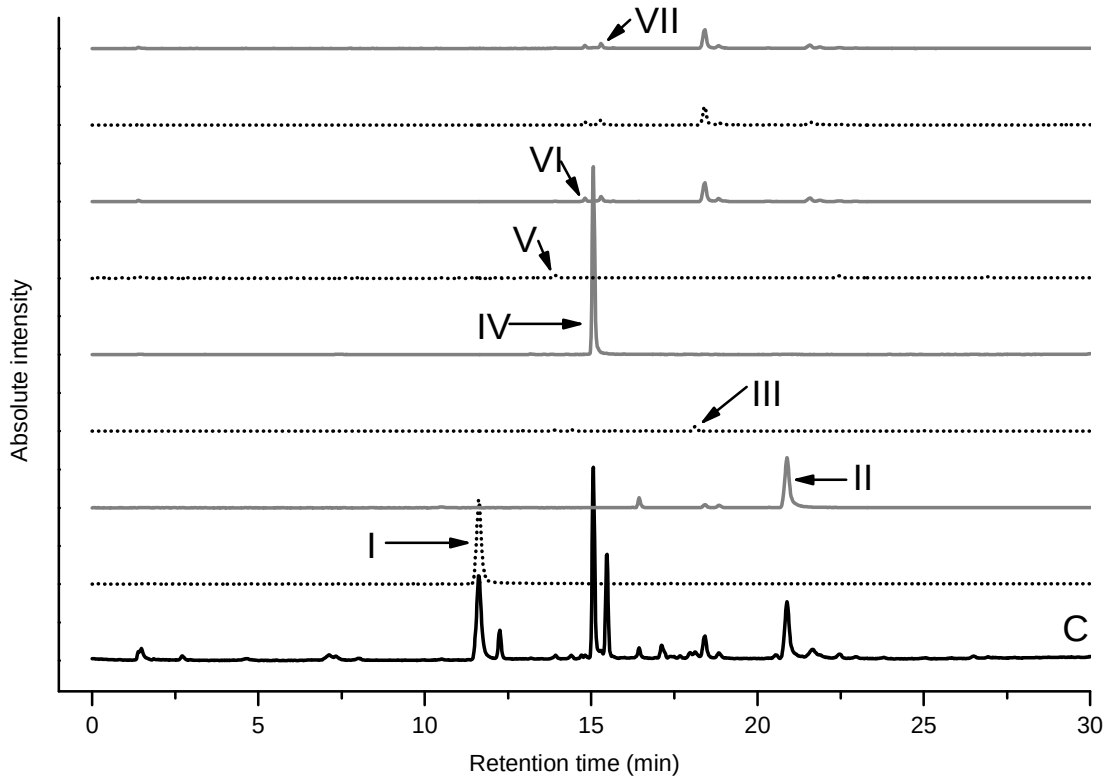



**Figure 10** Quantification of ethyl acetate-extracted compounds after hydrolysis of German chamomile using HPLC with single quadrupole mass spectrometry.

Total ion current (TIC) of German chamomile with hydrolysis and extraction with ethyl acetate (C) identifying the presence of umbelliferone (I) retention time (RT)= 11.7 min; apigenin (II) RT= 20.9 min; luteolin (III) RT= 18.1min; apigenin-7-glucoside (IV) RT= 15.1; luteolin-7-glucoside (V) RT= 13.9; 3,5-dicaffeoylquinic acid (VI) RT= 14.7 min and 4,5-dicaffeoylquinic acid (VII) RT= 15.3 min.

### 2.2.4 Quantification of compounds in the green tea extract using reverse phase HPLC

Samples of green tea extract were characterized using reverse phase HPLC. A 5 µl sample was separated using the Eclipse Plus C18, 18 µm, 2.1x100 mm column maintained at 30 $^{0}$C. (Phenomenex, Cheshire, UK). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. Elution started at 10% solvent B at a flow rate of 0.25

ml/min. Solvent B was increased to 15% after 9.5 min, and after 14.5 min it was increased to 95% until 27 min (cleaning column) and held at 10% until 30 min (re-equilibration of the column).

Standard curves of these compounds were run and used to quantify the compounds in the green tea extract and taxifolin was used as the internal standard (IS-10 µg/ml) (Figure 11, and Figure 12, Table 9). EGCG reported as the highest concentration of flavanol in a cup of green tea and catechin reported as the lowest (Cabrera *et al.,* 2003; Frejnage, (2007); Reto *et al.,* 2007). In the current study the most abundant catechin in green tea extract was EGCG and catechin was the lowest which is in the agreement of literature.

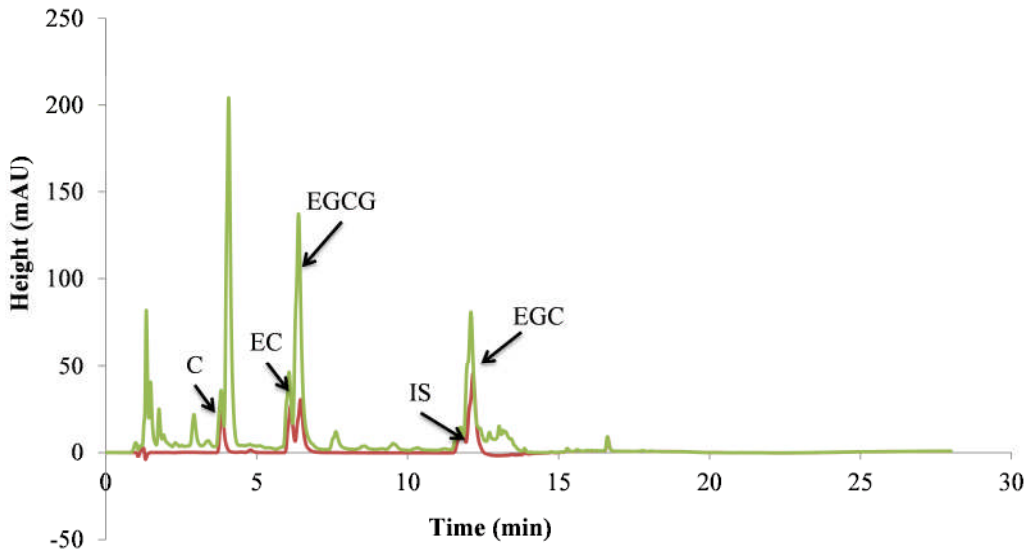



**Figure 11** Quantification of the green tea extract in water using HPLC.

The red line indicates the standard mixture (C, EC, EGCG, EGC and IS) at a concentration of 30 µg/ml and the green line indicates the 1 mg/ml green tea extract with 30 µg/ml standard mixture spiked. Detection wavelengths were 260, 280 and 320 nm.

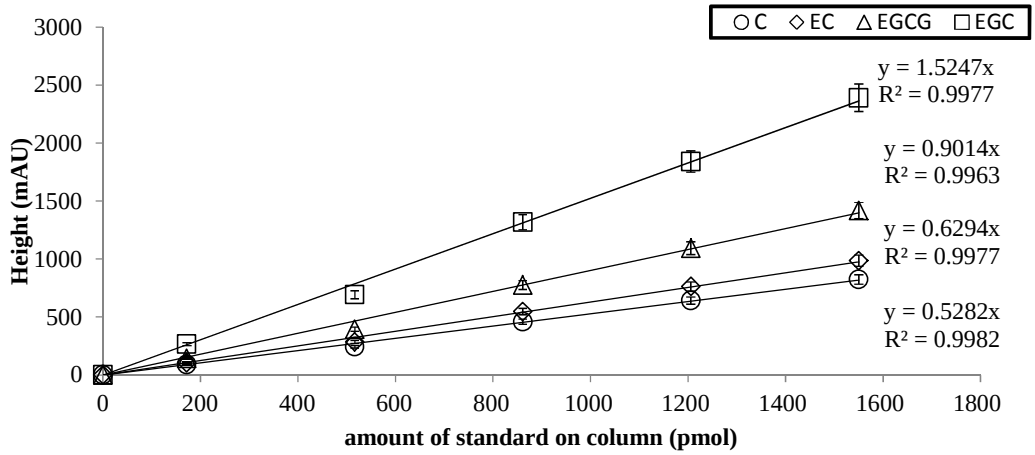



**Figure 12** Standard curve of catechin (C), epicatechin (EC), epigallocatechin gallate (EGCG) and epigallocatechin (EGC) at the concentrations of 0, 10, 30, 50, 70, and 90 µg/ml.

**Table 9** Polyphenol profile of the green tea extract.

| Compound | Retention time (min) | Composition (%) | µg/ml |
|---|---|---|---|
| Catechin | 3.8 | 1.7 | 7.1 |
| Epicatechin | 6.07 | 4 | 17.3 |
| Epigallocatechingallate | 6.4 | 24 | 138.8 |
| Epigallocatechin | 12.1 | 7 | 11.2 |

The maltodextrin content of *Vitis Viniferae* extract did not removed because this project mainly interested on the German chamomile and green tea extracts as their inhibitory effect on transporters and digestive enzymes were higher than *Vitis Viniferae* extract.

## 2.3 INHIBITION OF HUMAN SALIVARY α-AMYLASE BY PLANT FOOD SUPPLEMENTS

### Abstract

In this present work, it was investigated whether the presence of PFS extracts can inhibit human salivary α-amylase. The initial steps of method development involved determining the activities of human salivary α-amylase on amylopectin and improving a previously published method by analysing reducing sugar production from amylopectin. Purified German chamomile and green tea extracts were found to be moderate inhibitors of α-amylase digestion of amylopectin with $IC_{50}$ values of ~ 2.5 and ~1 mg/ml, respectively. Acarbose was also tested as a positive control: at 0.1 mM, 57% inhibition was observed. Thus, 1 mg/ml green tea was approximately equal to 0.0645 mg/ml acarbose concentration. Due to the acarbose-like activity of PFS extracts, they may have a potential role to reduce the risk of diabetes by inhibiting the hydrolysis of starches, and reducing post-prandial blood glucose spikes.

### 2.3.1 Introduction

The digestion of starch starts in the mouth by salivary α-amylase, which is hydrolysed to maltose, maltriose and small dextrins. Pancreatic α-amylase continues the digestion in the small intestine. The enzymes (maltase, sucrase, and lactase (α-glucosidase enzymes)) that are located in the brush border of the small intestine complete the digestion process to glucose (Grabitske and Slavin, 2009). α-Amylase is produced by the salivary glands and pancreas, and both enzyme isoforms have considerable sequence homology (Williamson, 2013). Amylose and amylopectin are hydrolysed by α-amylase by hydrolysis of α-1,4 glycosidic bonds, but the branch points (α-1,6 glycosidic bonds) are not hydrolysed (Figure 13). The hydrolysis occurs randomly on the starch chain (endo-acting enzyme). Acarbose is an anti-diabetic prescription drug used for the treatment of type-2 diabetes. It inhibits the carbohydrate hydrolysis enzymes (α-glucosidase and α-amylase). Miglitol and voglibose are also anti-diabetic prescription drugs used for the treatment of type-2 diabetes but both are an inhibitor of α-glucosidase. PFS extracts might also be considered to lead the same effect (acarbose-like effect). Since acarbose has side effects such as flatulence, diarrhoea and nausea, PFS extracts could be used in the prevention of type-2 diabetes.

Starch digestion starts in the mouth (with human salivary α-amylase) quickly enough to contribute to the energy requirements of individuals. Human salivary α-amylase was chosen because the aim was to understand the possible effect of PFS extracts in the human source of the α-amylase enzyme, while it was also a lot cheaper than human pancreatic α-amylase. The difference between human and pancreatic α-amylase originates in their active site region. Two substitutions (Thr 163 and Leu 196) in salivary α-amylase may have an effect on substrate binding (Brayer *et al.,* 1995). Salivary α-amylase is an endo-amylase and it hydrolyses at the α-1,4 glycosidic bonds of the starch molecule (Bijttebier *et al.,* 2009).

Several studies have shown that polyphenol-rich plants and herbal extracts were able to inhibit α-amylase activity (Kashket and Paolino, 1988; Zhang and

Kashket, 1998; Piparo *et al.*, 2008; Boath *et al.*, 2012; McDougall *et al.*, 2005; Shimizu *et al.*, 2000; Podsedek *et al.*, 2014).

A recent study tested the inhibitory activity of α-amylase from porcine pancreas, with 30 commonly consumed fruits purchased from a local supermarket. The $IC_{50}$ values of the tested fruits for pancreatic α-amylase were between 1 to 80 mg/ml. Only half of the fruits at a 200 mg/ml concentration were able to inhibit α-amylase activity by >50% (Posedek *et al.*, 2014). The most effective inhibitors were red and green gooseberries, chokeberry and redcurrant with $IC_{50}$ values <2 mg/ml. Posedek and colleagues concluded that, as those fruits slow down the release of glucose in the blood, they may be of use in the management of type-2 diabetes.

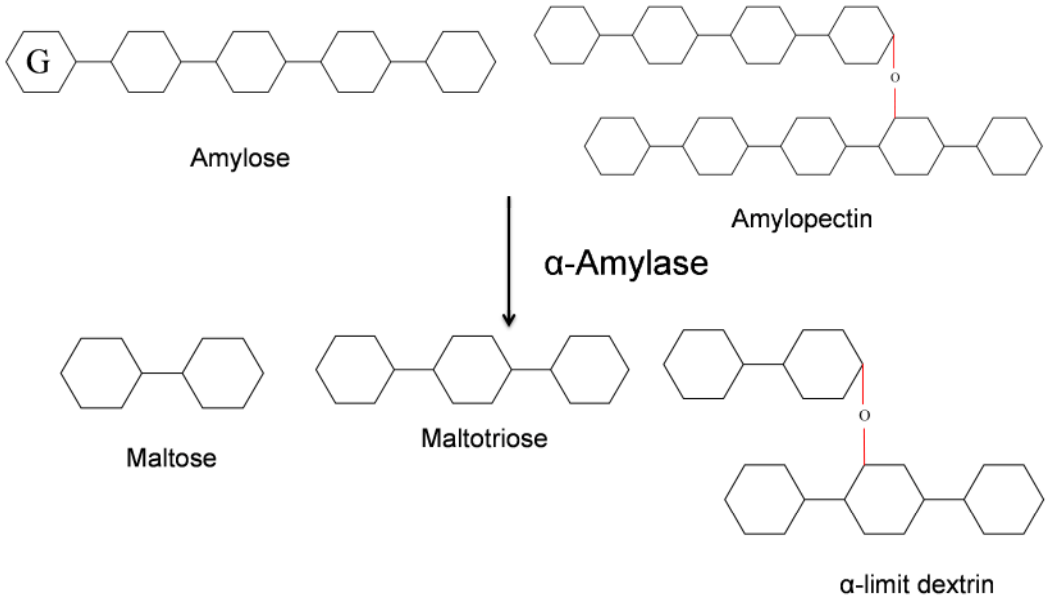



**Figure 13** The hydrolysis of amylose and amylopectin by α-amylase.
The red bond represents the α-1,6 glycosidic bonds (G: glucose).

Phenolic-rich extract (PRE) and tannin-rich fraction (TRF) of edible seaweed (Ascophyllum nodosum) were analysed for their porcine pancreatic α-amylase inhibitory activity. The PRE extract inhibited α-amylase with an $IC_{50}$ ~0.05 µgGAE/ml whereas the inhibitory activity of TRF was three-fold less potent.

The astringency of the tannin-like components were also reported to be related to their inhibitory activity of amylase (Pantidos *et al.,* 2014).

Phyllanthus amarus Schum and Thonn (Phyllanthaceae) herbs have been traditionally used in the treatment of diabetes. Fawzi and Devi (2014) analysed the aqueous and methanol extracts of leaf and stem of this herb against α-amylase activity, and compared it with acarbose. The aqueous extraction of stem ($IC_{50}$ value of 35 µg/ml) showed the highest inhibitory effect against α-amylase and it was significantly lower than acarbose ($IC_{50}$ value of 58 µg/ml). Methanol leaf extract ($IC_{50}$ value of 45 µg/ml) was also significantly lower than acarbose. Aqueous leaf and methanol stem extracts exhibited $IC_{50}$ values of 62 and 57 µg/ml, respectively.

Hara and Honda (1990) analysed the inhibitory effect of four different kinds of catechins and theaflavins for salivary α-amylase. The effect of theaflavins was stronger than catechins. The effect of α-amylase inhibition was observed in the descending order of potency of theaflavins digallate> theaflavins monogallate> theaflavins> catechins gallate> gallocatechin gallate> epicatechin gallate> epigallocatechin gallate.

As explained above, previous studies have shown that polyphenol consumption has an effect on type-2 diabetes. Polyphenols have the potential to inhibit α-amylase, which in turn would slow down the formation of products. We tested whether the PFS' green tea, German chamomile, and *Vitis Viniferae* could inhibit α-amylase. The assay was set up and validated, and the results of this are presented in the methods and results section.

## 2.4 Material and methods

The initial steps of method development involved determining the activities of human salivary α-amylase on amylopectin and improving a previously published method (Akkarachiyasit *et al.,* 2010) by analysing the reducing sugar production from amylopectin.

### 2.4.1 Chemical and reagents

Acarbose (A8980-IG), dinitrosalicylic acid solution (DNS; S2377), sodium potassium tartrate solution (D-0550), human salivary amylase type XIII-A (A-1031-5KU), maltose (M5885), monosodium phosphate (S8282) and disodium phosphate (S9763) were purchased from Sigma-Aldrich Inc (St Louis, MO, USA). Amylopectin was purchased from Fluka Biochemika. DNS and sodium potassium tartrate solutions were prepared based on the Sigma protocol for human salivary amylase (EC 3.2.1.1) (Colour reagent solution).

Stock solutions of amylopectin were dissolved in hot water, and prepared freshly for each experiment.

### 2.4.2 Extract preparation

PFS was received from EU project, PhytoLab Co. KG.h (Vestenbergsgreuth, Germany). Stock solutions of PFS were dissolved in millipore water at room temperature, prepared freshly for each experiment, and then centrifuged at 17000 g for 5 min. Following this, supernatants were collected and used for analysis. German chamomile contains 50% of maltodextrin which is also a potential substrate of digestive enzymes. Therefore, this extract was purified by Jose Alberto using the Akta Purifier 1.0. This extract was dissolved in 100% DMSO. The concentration of DMSO in the final assay was 0.1%, which does not interfere with human salivary amylase.

### 2.4.3 Enzyme preparation

Human salivary amylase was dissolved in 20 mM sodium phosphate buffer with 6.7 mM sodium chloride (S/3160/53 from Fisher Scientific, Loughborough, UK) at pH 6.9 at room temperature. The enzyme solution was prepared freshly for each experiment.

### 2.4.4 Preparation of sodium tartrate solution

Into the 8 ml of 2 M NaOH, 12 g of sodium tartrate was added and placed on a heating plate to dissolve. It was added to prevent oxidation of product (maltose) and stabilise the colour.

### 2.4.5 Preparation of DNS solution

DNS powder (0.438 g) was added into 20 ml of deionized water and directly placed on a heating plate to dissolve. DNS was used as the colour reagent for the α-amylase reaction. When α-amylase hydrolyses starch it releases the reducing sugars as a product. DNS reacts with the free carbonyl group of the reducing sugars under an alkaline condition and forms 3-amino-5-nitrosalicylic acid, which could be measured at 540 nm (Goncalves *et al.*, 2010). DNS changed the colour as reducing sugars are released.

Colour reagent solution: Both prepared sodium tartrate and DNS solution were added together with 40 ml of deionized water and stored in an amber bottle at room temperature.

## 2.5 Inhibition of human salivary α-amylase by PFS

The practical steps of the method are summarised in Figure 14. The assay was conducted by mixing 200 µl of the substrate solution (different concentration for each sugar), 50 µl of 10 mM pH 7.0 sodium phosphate buffer, 50 µl of PFS extract or buffer with 200 µl enzyme solution (different concentration for each sugar) and vortexed for 10 seconds. The assay was carried out in triplicate. Following this, the samples were incubated at 37 ºC for 10 min. After incubation, 1 ml of colour reagent solution was added to determine the production of reducing sugar and vortexed for 10 seconds. To stop the enzyme reaction, the samples were placed in a boiling water bath (GLS Aqua 12 plus) for 10 min and then transferred into ice to cool down to room temperature. The sample volume for analysing the production of reducing sugar was 250 µl and it was transferred to a 96-well plate (Nunc A/S., Roskilde, Denmark). The absorbance was read at 540 nm with PHERAstar FS microplate reader (BMG LABTECH). Results are presented as percentage inhibition relative to the blank control using Equation 1.

Enzyme activity was measured in the presence of a variety of green tea and German chamomile supplement extracts, and acarbose was used as a positive control. At the concentration of 0.1 mM, acarbose inhibited α-amylase activity by 57 %.

$$\%= \frac{\text{Abs control-Abs sample}}{\text{Abs for control}} \times 100$$

**Equation 1**

It was observed that the colour reagent solution activity was promoted by green tea and German chamomile extracts as it is known that the reducing potential of polyphenols could interfere with the development of colour, and hence affect the assay. Therefore, before adding the colour reagent solution, samples were transferred to a boiling water bath for 10 min to stop the enzyme reaction. Then they were transferred into ice to cool down to room temperature. Following this, an SPE column (Waters Oasis MAX Cartridge 003036349A) was used to remove the polyphenols from the green tea and German chamomile extracts. 1 ml of colour reagent solution was added to each sample and the samples were transferred to a boiling water bath for 10 min again. Then the absorbance was read at 540 nm.

## 2.6 Statistical analysis

IBM SPSS Statistics 22 was used for the analysis of the data. The Levene test was used to evaluate the homogeneity of the means groups. If the criterion was met the Tukey HSD post hoc test was applied: otherwise, the Dunnett C followed the one way Anova. The values shown represent the mean values and the error bars indicate the standard deviation (SD). Unless otherwise stated, differences were considered as statistically significant when $p \leq 0.05$.

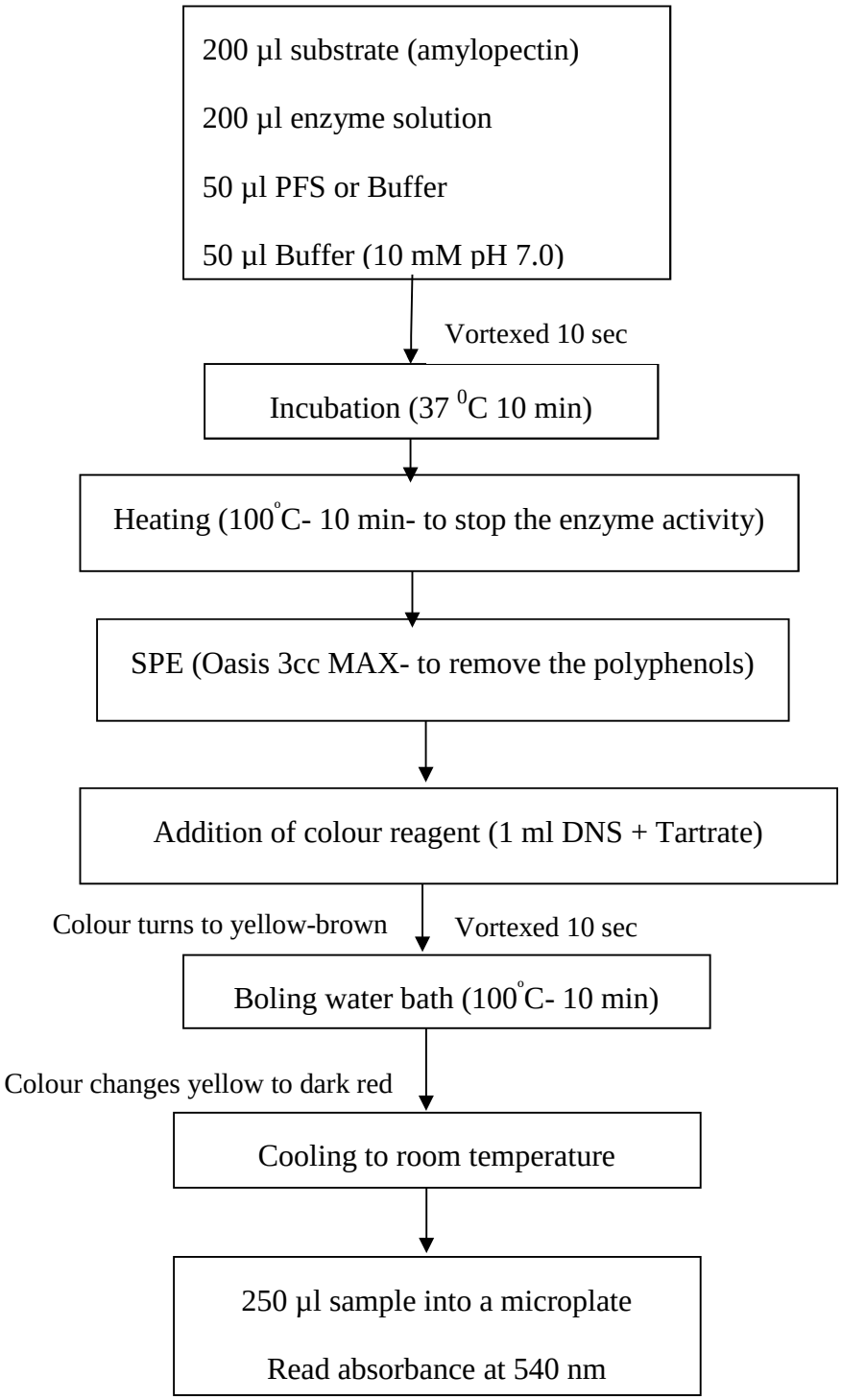



**Figure 14** Summary of the practical steps in the inhibition of human salivary α-amylase assay.

## 2.7 Results

The standard curves obtained with pure maltose standard solutions were linear and reproducible. The concentration range was between 0 to 10 mM and the average of all the standard curves from each experiment are shown in Figure 15. To establish and characterise, the $K_m$ for amylopectin with human salivary α - amylase was measured (Figure 17). Time dependence was also assessed for three different concentrations of enzyme (Figure 18). Figure 16 represented the variations of absorbance reading for different days.

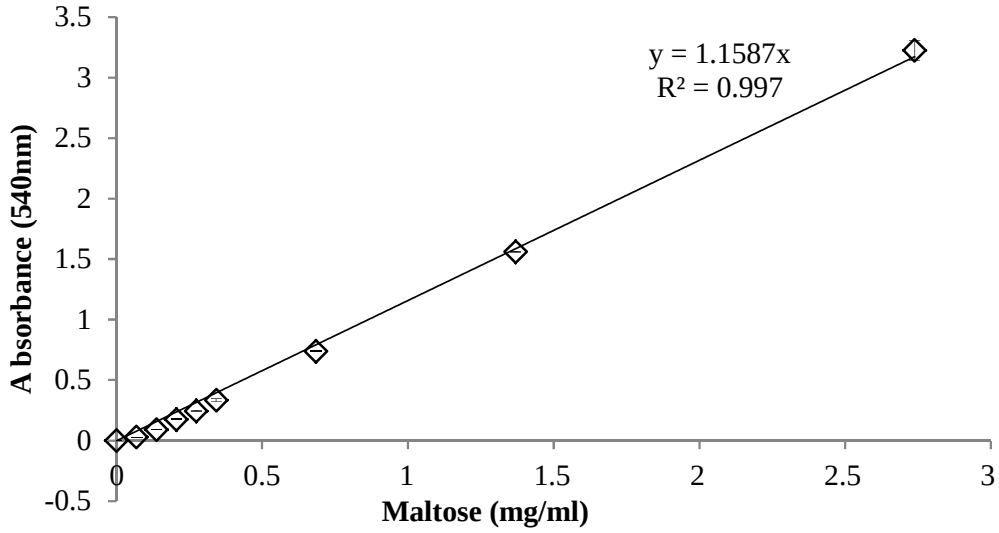
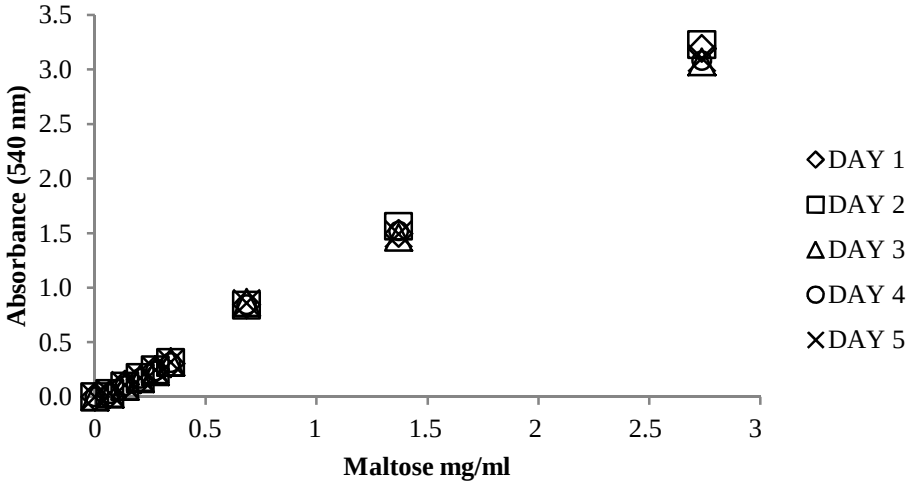



**Figure 15** Standard curve of maltose in the reducing sugar assay.

Mean± SD (n=3 per concentration of maltose).

**A**




**B**

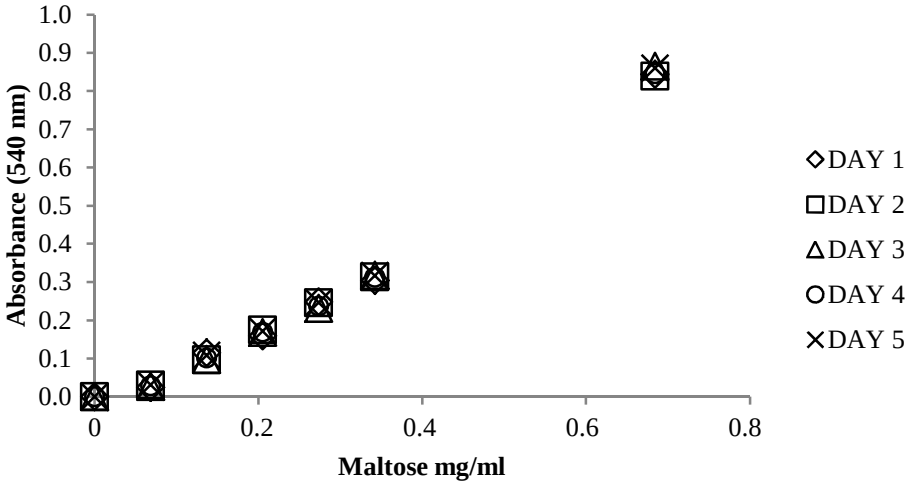



**Figure 16** A: Standard curve of maltose for different experiment days.

B: Expanded format of A. Three replicates for each concentration for each days. Mean± SD (n=3 per concentration of maltose for each day).

The Michaelis-Menten kinetic parameters of human salivary α-amylase are shown in Figure 17. Using a chosen enzyme concentration and different incubation times, the kinetic parameters of $K_m$ and $V_{max}$ were determined with the Lineweaver-Burk plot. The $K_m$ value for the measurement of maltose production from amylopectin was 1 mg/ml and $V_{max}$ was 0.12 mg substrate

hydrolysed/min for human salivary α-amylase. The substrate concentration was adopted as 1 mg/ml, which was equal to the $K_m$ value.

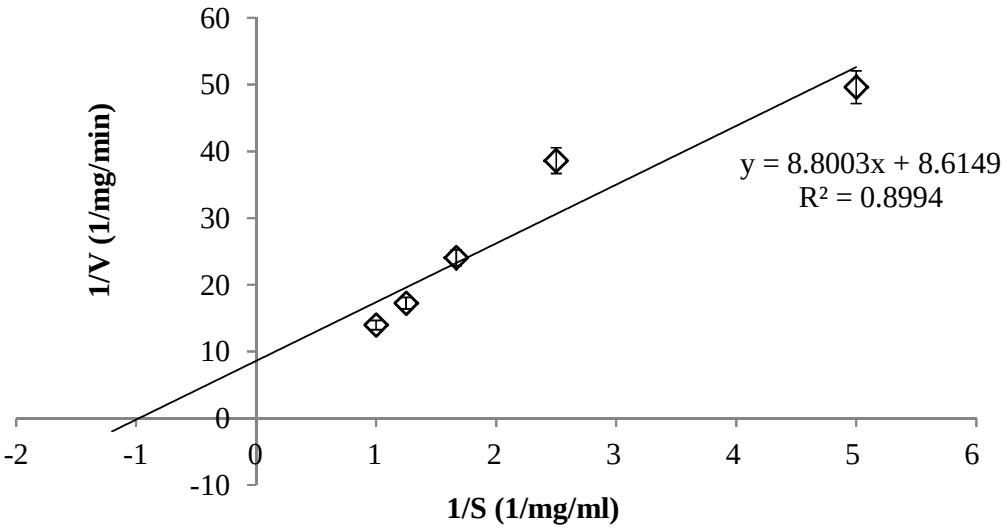



**Figure 17** Lineweaver- Burk plot for amylase digestion of amylopectin.

From this, $K_m$= 1 mg/ml amylopectin and $V_{max}$= 0.12 mg substrate hydrolysed/min.

Mean± SD (n=3 for each concentrtion).

Maltose production from amylopectin was observed at the different concentrations of human salivary α-amylase (Figure 18). The time dependence assessed for different concentrations of human salivary α-amylase was linear up to 10, 10 and 6 min for 1, 3 and 5 U enzyme concentrations, respectively. Therefore an incubation time of 10 min and 3 U enzyme was chosen as the optimum assay conditions using 1 mg/ml amylopectin. Different concentrations of enzyme showed the same pattern of maltose production as in all cases, the curve flattens at a certain time point. Hydrolysis of amylopectin became constant with the increasing time points. The enzyme substrate complex rate was constant during the steady state. The rate slows as substrate concentration continues to increase until the curve flattens. This situation shows that the reaction has

reached maximum velocity and all free enzymes are saturated with substrate (Nelson and Cox, 2000).

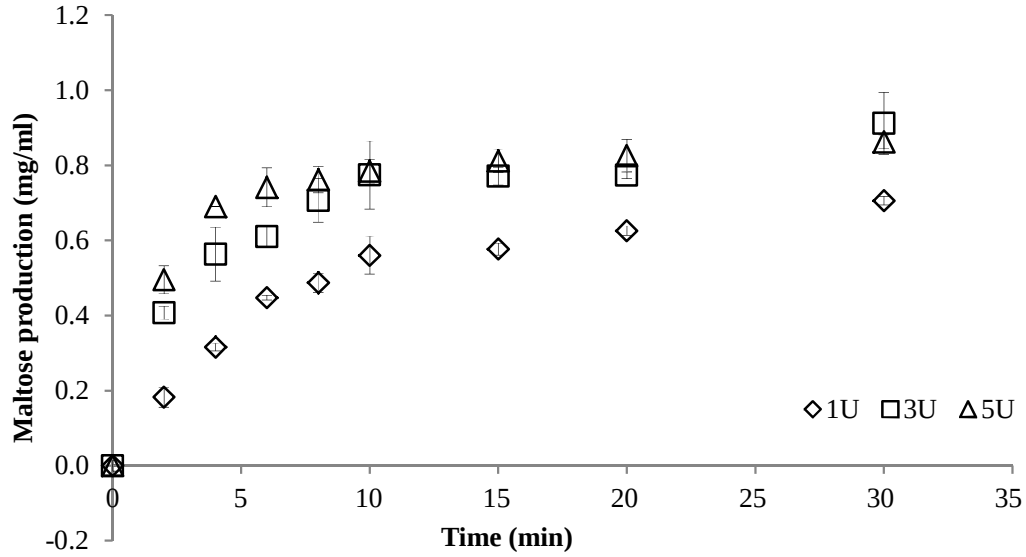



**Figure 18** Time dependence of amylase hydrolysis of amylopectin.
Three different amounts of enzyme were used, 1, 3 and 5 U/ml.
Mean± SD (n=3 for each time points per sample).

The rate of enzyme reaction depending on the enzyme concentration was shown in Figure 19. Increasing the concentration of enzyme also increases the rate of enzyme reaction linearly.

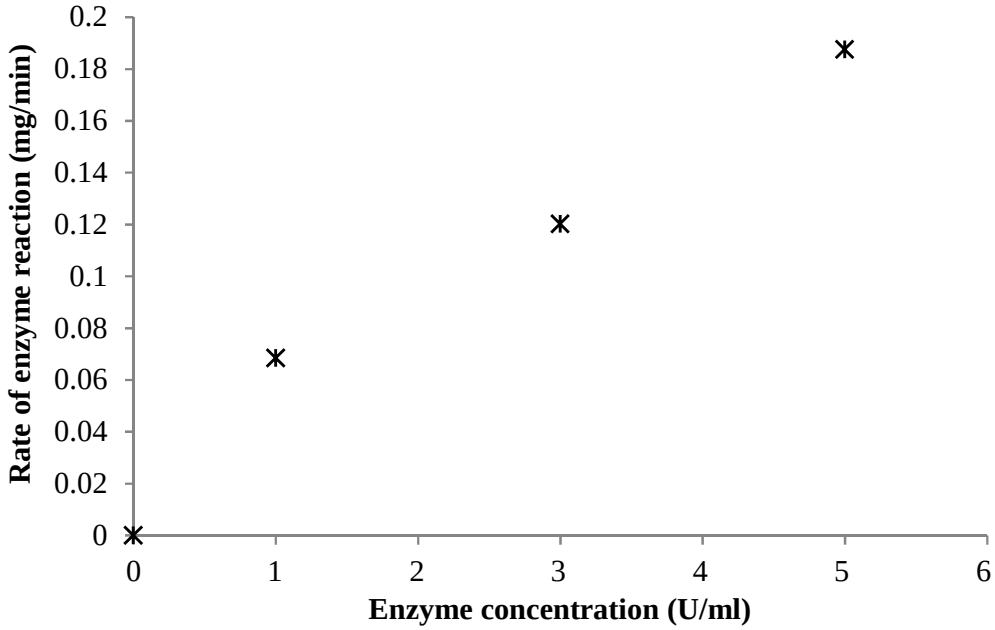



**Figure 19** Data from Figure 18 replotted to show the dependence of rate upon enzyme concentration.

### 2.7.1 Inhibition of α-amylase by green tea and German chamomile extracts

As German chamomile and *Vitis Viniferae* extracts contain some maltodextrin (3.4.4), 20 mg/ml of maltodextrin was used as a substrate in the assay. It was observed that maltodextrin was a potent substrate for α-amylase, and therefore *Vitis Viniferae* extract was not analysed. Purified German chamomile extract (maltodextrin content of extract was removed) were analysed. Initially 2 mg/ml of green tea and 0.4 mg/ml of purified German chamomile extracts were tested, but both appeared to be activating the enzymatic reaction. To confirm this, several experiments were performed; PFS extracts only were added to assay (without enzyme and substrate), and PFS with enzyme added to assay without substrate. It was found that the apparent activities were still higher than the control sample (without inhibitor but with enzyme and substrate), and so it was concluded that the polyphenol content of the extracts were interfering with the DNS reaction. To prevent this problem, SPE columns were used to remove the polyphenols after the assay but before the analysis. After the improvement of the

assay, 2 mg/ml of green tea and 0.4 mg/ml of purified German chamomile extract inhibited human salivary α-amylase activity by 40 and 30%, respectively.

Both purified German chamomile and green tea extracts were moderate inhibitors of amylase digestion of amylopectin with an $IC_{50}$ value of ~2.5 and ~1 mg/ml, respectively (Figure 20). Acarbose was also tested as a comparison and positive control. At the concentration of 0.1 mM (0.0645 mg/ml), this gave 57% inhibition of amylase hydrolysis. Thus, 1 mg/ml green tea was approximately equal to 0.0645 mg/ml acarbose.

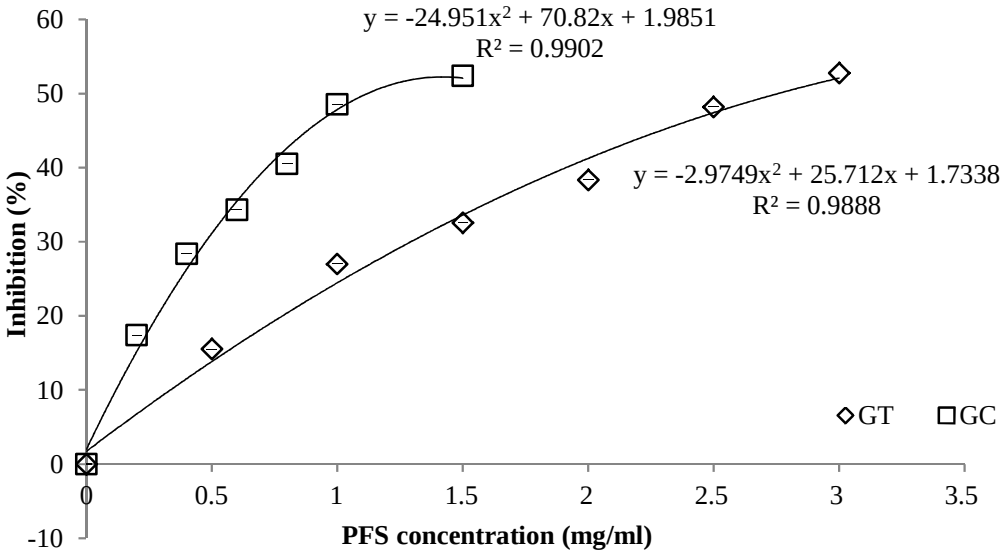



**Figure 20** Inhibition of human salivary amylase with different concentrations of green tea and German chamomile extracts.

Mean± SD (n=3 per PFS concentration). Tukey HSD test applied.

## 2.8 Discussion

Both purified German chamomile and green tea extracts were moderate inhibitors of amylase digestion of the starch component, amylopectin. However, due to the maltodextrin content of *Vitis Viniferae* extracts, the inhibitory effect on α-amylase could not be analysed. Even German chamomile extract contains maltodextrin, which could still be pre-purified to remove its maltodextrin content.

Zhang and Kashket (1997) observed the inhibitory effect of black and green tea on human salivary α-amylase activity. Volunteers were fed with salted crackers and then rinsed with black or green tea decoctions or water. They found that the production of maltose was ~70% less after rinsing with tea compared to water rinsing. The inhibitory activity of black teas was higher than green teas, and when the tannins were removed, the inhibitory activity of both teas decreased. Catechins were effective at a concentration of 2 mg/ml. In the current study, green tea extract inhibited human salivary α-amylase activity with an $IC_{50}$ value of 1 mg/ml. Thus, catechins may contribute to the inhibitory effect of the extract. Lee and colleagues (2010) analysed the inhibitory effect of black, green and oolong teas for α-amylase activity. They found that black tea had the strongest inhibitory activity with $IC_{50}$ value of 0.42- 0.67 mg/ml. In addition, theaflavins show better inhibition activity compared to catechins (1.5 to 20 mM). Zhang and Kashket (1997) also reported that the inhibition effect of black teas on α-amylase activity was higher than green tea. Gao and colleagues (2013) analysed the combined effect of green tea extract (GTE), green tea polyphenols (GTP) and EGCG with acarbose. Gao and colleagues found that GTE, GTP and EGCG (without acarbose combination) inhibited α-amylase activity with an $IC_{50}$ value of 4020, 1370 and 1849 µg/ml, respectively. The effect of their α-amylase inhibition was poor. When they combined GTE and GTP with acarbose, they had a synergistic effect on α-amylase when the inhibition percentage was <72 and 40%, respectively. By contrast, EGCG and acarbose combination had only an antagonistic effect at all concentrations.

In the literature there are some studies regarding the polyphenol content of green tea and German chamomile extracts. Those studies also supported that PFS

extracts may have a role in inhibiting α-amylase activity. The reported studies showed variations with regards to the type of the α-amylase (human, porcine or microorganisms) used in the assays.

He and colleagues (2006) study also supported that 0.05 mg/ml of catechins, epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin inhibited 61 % of α-amylase activity. Hara and Honda (1989) analysed the inhibitory effect of four different kinds of catechins and theaflavins for salivary α-amylase. The effect of theaflavins was stronger than catechins. The effect of amylase inhibition was observed in the descending order of potency of theaflavins digallate> theaflavins monogallate> theaflavins> catechins gallate> gallocatechin gallate> epicatechin gallate> epigallocatechin gallate. Green tea extract also contains catechins, epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin. Therefore, those studies also supported the inhibitory effect of green tea extract.

The inhibitory activity of luteolin for amylase was tested by Kim and colleagues (2000). They reported that the inhibitory activity of luteolin was effective, but however, not as potent as acarbose. They also tested 22 flavonoids for their inhibitory effect on amylase activity. Luteolin, luteolin-7-*O*-glucoside and kaempferol-3-*O*-glucoside inhibited α-amylase activity with $IC_{50}$ values of 50-500 µg/ml, 5 mg/ml and 5 mg/ml, respectively. In the current study, purified German chamomile extract also contained luteolin and luteolin-7-*O*-glucoside, and the Kim and colleagues study supports that they may contribute to the inhibitory effect of extract. Tadera and colleagues (2006) observed that luteolin, quercetin and myricetin inhibited α-amylase with $IC_{50}$ values of 0.36, 0.50 and 0.38 mM, respectively. By contrast, they found that apigenin, kaempferol, fisetin and cyanidin insignificantly inhibited the α-amylase activity. They concluded the inhibitory effect of the flavonols in the descending order of potency of isoflavone >flavone >flavonol >anthocyanidins >flavanone=flavan-3-ol. Purified German chamomile extract also contains a high amount of apigenin-7-*O*-glucoside. One study reported that apigenin-7-*O*-glucoside inhibited porcine pancreatic α-amylase activity (Funke and Melzig, 2006). Funke and Melzig observed the effect of traditionally used plants from Africa and Europe for

diabetes, the leaves of Tamarindus indica inhibited 90% of the α-amylase activity. They also tested some of the phenolic compounds and the effect of porcine pancreatic α-amylase inhibition were observed in the descending order of potency of acarbose> tannic acid> apigenin-7-*O*-glucoside> luteolin> luteolin-7-*O*-glucoside> fisetin> chlorogenic acid. Therefore, those studies are also in agreement of the current study regarding the inhibitory effect of German chamomile extract.

There are also different polyphenols or fruits reported for their α-amylase inhibitory activity. The variety of studies is regarding the inhibition of α-amylase with berry species. Grussu and colleagues (2011) reported the inhibitory effect of raspberry and rowanberry on α-amylase activity with an $IC_{50}$ value of 21 and 4.5 µg/ml, respectively. They concluded that proanthocyanidins inhibited α-amylase activity more than the anthocyanidins content of berries. Among all the berries, McDougall *et al.*, 2005 reported that strawberry and raspberry were the most effective inhibitors of human salivary α-amylase with $K_i$ values of 120 and 150 µg of phenols/assay, respectively. They further analysed red grape juice and red wine, which inhibited salivary α-amylase with $K_i$ values of ~20 µg/assay. By contrast, gallic acid and ellogallaic acid were ineffective.

Purified German chamomile and green tea extracts were found to be moderate inhibitors of human salivary α-amylase digestion of the starch component, amylopectin. Therefore, we may conclude that the glycemic response may be reduced when it is taken during a meal. These results indicate that PFS reduces the digestion of starch-containing foods. These results illuminate the role of PFS in reducing the diabetes risk.

# CHAPTER 3 INHIBITION OF DIGESTIVE ENZYMES BY PLANT FOOD SUPPLEMENTS


## Abstract

The purpose of the study was to determine the inhibitory effect of PFS on carbohydrate digesting enzymes (rat intestinal sucrase/isomaltase and maltase). The initial steps of method development involved determining the activities of sucrase, maltase and isomaltase in an acetone-extract of rat intestinal tissues and improving a previously published method by analysing glucose production from sucrose, maltose and isomaltose using hexokinase.

Green tea extract inhibited maltase, sucrase and isomaltase activities *in vitro* with $IC_{50}$ values of 0.95± 0.05, 0.44± 0.04 and 0.69± 0.02 mg/ml, respectively. Due to the maltodextrin content of German chamomile and *Vitis Viniferae* extracts, their inhibitory effect on carbohydrate digestive enzymes could not be analysed. Since maltodextrin is a substrate of digestive enzymes after maltodextrin was removed from the German chamomile extract, this activated isomaltase and sucrase but was ineffective on maltase ($p<0.01$). Acarbose (0.1 mM) as a positive control inhibited sucrase, isomaltase and maltase by 90± 3.5, 64± 4.8 and 100± 2 %, respectively. The findings demonstrate that green tea might influence carbohydrate digestion via inhibition of gut α-glucosidases.

## 3.1 Introduction

Carbohydrates are major energy-containing components of the diet and are classified into 4 groups: mono-, oligo- and polysaccharides and their derivatives (Lindhorst, 2003). Oligo- and polysaccharides are synthesised from monosaccharides (two or more joined together), and also, during digestion they are catabolised to the monosaccharide form to provide energy. Sucrose, maltose and isomaltose are examples of disaccharides synthesised by the reaction between glucose and fructose and two glucose units, respectively. The difference between maltose and isomaltose is the position of glycosidic links between glucose molecules, as shown in Figure 21. Sucrose, maltose and isomaltose are hydrolysed by sucrase/isomaltase and maltase/glucoamylase enzymes, which are located in the small intestine. Both enzymes have α-glucosidase activity (Williamson, 2013). One glucose and one fructose unit comprise one unit of sucrose, which is shown in Figure 21. Two glucose units with α-1,4 and α-1,6 linkage comprise maltose and isomaltose, respectively.

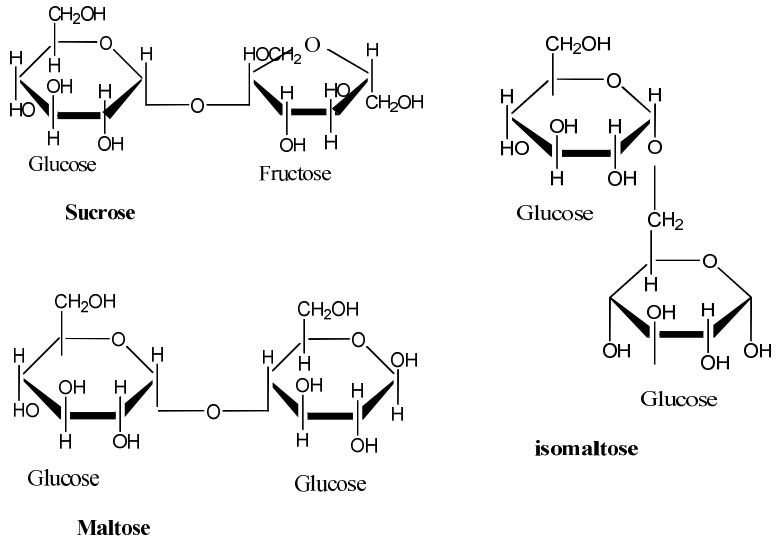



**Figure 21** Structure of sucrose, maltose and isomaltose.

In the vast literature, *α*-glucosidase inhibitors are found as therapeutic drugs for diabetes, and they inhibit carbohydrate metabolism (Yuk *et al.*, 2011). In the majority of the studies, phenolic compounds were investigated as α-glucosidase inhibitors (Bhandari *et al.*, 2008; He *et al.*, 2006; Kim *et al.*, 2000; Lee *et al.*, 2008; Song *et al.*, 2005; Zhang *et al.*, 2007). The positive effects of polyphenols on glucose homeostasis were observed in a large number of *in vitro* and animal model studies. These models are supported by epidemiological evidence on polyphenol-rich diets (fruit and vegetables, tea, coffee and some herbal supplements) (Hanhineva *et al.*, 2010; Boath *et al.*, 2012; Hamer and Chida, 2007; Sakulnarmrat *et al.*, 2014; Kamiyama *et al.*, 2010; Huxley *et al.*, 2009; van Dieren *et al.*, 2009).

Gao and colleagues (2007) studied the inhibitory effect on maltase, sucrose and isomaltase by the fruits of *Terminalia chebula* Retz. The authors found that the extracted polyphenols inhibited rat intestinal maltase activity; however, it did not inhibit sucrase or isomaltase activity. Gao and colleagues concluded that the phenolic compounds extracted from the fruits of *Terminalia chebula* Retz may be useful as a natural source of α-glucosidase inhibitor. It can potentially be utilised in the suppression of post-prandial hyperglycaemia in type-2 diabetes management.

One of the recent studies reported that blackcurrant (rich in anthocyanins, ~70% of the total) and rowanberry (rich in chlorogenic acids, ~65% of the total) inhibited the α-glucosides with $IC_{50}$ values of 20 and 30 µgGAE/ml. They found that both berry extracts were as effective as the pharmaceutical inhibitor, acarbose ($IC_{50}$ ~40 µg/ml) (Boath *et al.*, 2012).

Kamiyama and colleagues (2010) investigated the inhibition of digestive enzymes by green tea extract. The $IC_{50}$ value for the inhibition of rat intestinal maltase was reported to be 45 µg/ml. The polyphenol content of green tea extract; catechin-3-gallate, gallocatechin-3-gallate, epicatechin-3-gallate and epigallocatechin-3-gallate, had $IC_{50}$ values of 62, 67, 40 and 16 µM, respectively.

Sakulnarmrat and colleagues analysed the inhibition on α-glucosidase with Davidson's Plum (DP) and quandong (QD), which are widely using in various

food products (jam, sauces, wine or herbal tea) in Australia (Sakulnarmrat *et al.*, 2014). The polyphenol composition of DP contains ellagic acid and ellagitannins together with the flavonoids myricetin, quercetin, rutin and anthocyanins, and QD is rich in hydroxycinnamic acids, quercetin and cyaniding-3-glucoside. The inhibitory activity of 1 mg/ml DP and QD was 93 and 65%, respectively. It was concluded that the development of food supplements/nutraceuticals with multifunctional polyphenols may be helpful to prevent metabolic syndrome.

Another study recently conducted tested the inhibitory activity of rat intestinal α-glucosidase with 30 commonly consumed fruits purchased from their local supermarket. The $IC_{50}$ values of tested fruits were between 39.9 to 400 mg/ml. The strongest $IC_{50}$ value observed was for blue honeysuckle. Other potent inhibitors were green gooseberry> blueberry> bilberry> blackcurrent> sweet cherry> pink *Vitis Viniferae* and red gooseberry (Posedek *et al.*, 2014). They concluded that as those fruits slow down the release of glucose in the blood, they may be of use in the treatment of type-2 diabetes.

The *Phyllanthus* amarus Schum and Thonn (*Phyllanthaceae*) herbs have been traditionally used in the treatment of diabetes. Fawzi and Devi (2014) analysed the aqueous and methanol extracts of the leaf and stem of this herb against α-glucosidase activity. They compared this with acarbose inhibitory activity. Methanol extraction of the leaf ($IC_{50}$= 0.674 µg/ml) showed the highest inhibitory effect against α-glucosidase and it was significantly lower than acarbose ($IC_{50}$= 6.77 µg/ml). Methanol stem extract ($IC_{50}$= 6.73 µg/ml) inhibitory activity was comparable to acarbose. Aqueous leaf and stem extracts had $IC_{50}$ values 8.97 and 8.60 µg/ml, respectively. The total phenolic content of the extracts was highest in the aqueous stem (~0.08 gGAE/ml) and followed by the aqueous leaf (~0.045 gGAE/ml)> methanol extract of stem (~0.045 gGAE/ml) > methanol extract of leaf (~0.025 gGAE/ml).

The phenolic-rich extract (PRE) and tannin-rich fraction (TRF) of edible seaweed, *Ascophyllum nodosum*, were analysed for their α-glucosidase inhibitory activity. Both extracts inhibited α-glucosidase equally effectively ($IC_{50}$~10 gGAE/ml). The $IC_{50}$ value for α-glucosidase inhibition was reported as 40 µg/ml (similar to the Boath *et al.*, 2012 study). When TRF and acarbose were co-

incubated at half their $IC_{50}$ values, the inhibition was significantly increased. This result indicates that the TRF components and acarbose are binding to different sites on the enzyme. Therefore, they concluded that reducing the dose of acarbose with the addition of TRF may provide an advantage for the diabetics who react poorly to acarbose.

Polyphenols have the potential to inhibit digestive enzymes, which in turn would slow down the products of the substrate (e.g. glucose and fructose) reaching the blood. Previous studies have shown that polyphenol consumption has an effect on type-2 diabetes. We tested whether plant food supplements (PFS; green tea and purified German chamomile) could also lower blood glucose levels through inhibition of digestive enzymes (α-glucosidase (sucrase/isomaltase and maltase)). The assay has been set up and validated, and the results of this are presented in the methods and results sections.

## 3.2 Material and methods

The initial steps of method development involved determining the activities of sucrase, maltase and isomaltase in an acetone-extract of rat intestinal tissues and improving a previously published method (Gao *et al.,* 2007) by analysing glucose production from sucrose, maltose and isomaltose with the hexokinase assay.

### 3.2.1 Chemicals and reagents

Sucrose (S9378), maltose (M5885), isomaltose (I7253), acarbose, glucose hexokinase reagent (G3293), monosodium phosphate (S8282) and disodium phosphate (S9763) were purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA). Glucose anhydrous (G/0450/60) and fructose (F/1950/50) purchased from Fisher Scientific (Leicestershire, UK). Acetone (022928.K2) was purchased from Alfa Aesar (Lancashire, UK). The PFS were provided by as a part of the EU framework 7 project PLANTLibra. Stock solutions of carbohydrates were dissolved in 10 mM (pH 7.0) sodium phosphate buffer, prepared freshly for each experiment.

### 3.2.2 Extract preparation

Stock solutions of PFS were dissolved in millipore water at room temperature, prepared freshly for each experiment. The solutions were centrifuged at 17000 g for 5 min. Following this the supernatant was collected and used for analysis. German chamomile contains 50 % of maltodextrin, which is also a substrate of digestive enzymes. Therefore, this extract was purified by Mr. J. Alberto using the Akta Purifier 1.0 to remove the maltodextrin from the extract. Purified German chamomile extract was dissolved in 100 % DMSO. The final concentration of DMSO in the assay was 4 %. The effect of DMSO to the enzyme activity was analysed and it was found that DMSO (4 %) inhibited the enzyme activity by 8 % and the results corrected based on this inhibition.

### 3.2.3 Enzyme preparation

Intestinal acetone rat powder (I1630) was purchased from Sigma-Aldrich, Inc., and was prepared in 1 ml of 10 mM (pH 7.0) sodium phosphate buffer at the desired concentration of powder. Subsequently, it was vortexed for 30 seconds followed by centrifugation at 17000 g for 10 min. The supernatant was removed and used for analysis. It was prepared freshly for each experiment.

### 3.2.4 Hexokinase assay

This method of detection and measurement of D-glucose in samples was based on the detection at 340 nm of NADH produced using the assay. It is based on a series of catalytic reactions between glucose and the glucose assay reagent. The first reaction is catalysed by the hexokinase, where glucose is phosphorylated by adenosine triphosphate (ATP). The glucose-6-phosphate (G-6-P) formed is then oxidised to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenine dinucleotide (NAD). This reaction is catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH) (Figure 22). The consequent increase in NADH concentration is directly proportional to the glucose concentration and can be measured at 340 nm.

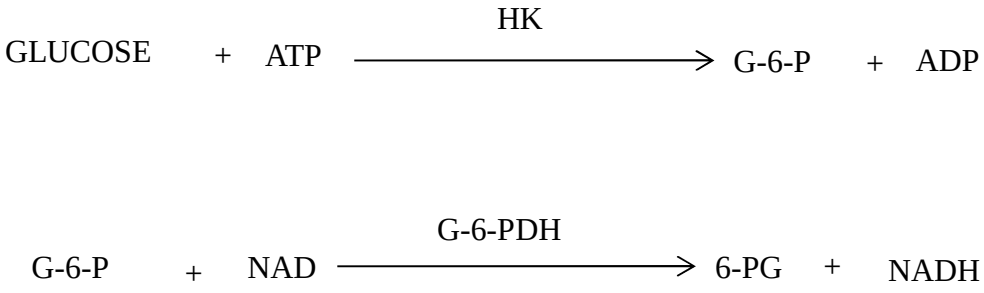



**Figure 22** Catalytic reactions between glucose and the glucose assay reagent (hexokinase- HK).

### 3.2.5 Inhibition of digestive enzymes by PFS

The assay was conducted by mixing 200 µl of the substrate solution (different concentration for each sugar), 50 µl of 10 mM (pH 7.0) sodium phosphate buffer, 50 µl of PFS extract or buffer with 200 µl enzyme solution (different concentration for each sugar) and vortexed for 10 seconds. The assay was carried out in triplicate. Following this, the samples were incubated at 37 °C for 20 min. Following the incubation process, 750 µl of acetone was added to stop the enzyme reaction and vortexed for 10 seconds. The samples were centrifuged at 17000 g for 5 min. Nitrogen was used to remove the acetone and centrifugation was repeated (17000 g for 5 min). Instead of using the genevac to remove the acetone, a nitrogen cylinder was used as the genevac evaporated the acetone in variable time (the time range was between 15 to 75 min). By contrast, it takes ~ 20 min using nitrogen gas (Figure 23).

The hexokinase assay kit and method was carried out to detect the amount of glucose produced. The sample volume for this determination was 10 µl, which were transferred into a UV transparent 96-well plate (Greiner UV-star M3812, Sigma). For this method, the hexokinase reagent was ordered individually and the D-glucose standard was prepared freshly for each set of experiment. Subsequently, 250 µl hexokinase reagent was added to those samples and incubated at 37°C for 15 min. Absorbance was read at 340 nm with the PHERAstar FS microplate reader (BMG LABTECH). Results are presented as percent inhibition relative to the blank control.

It was observed that the hexokinase assay reagent was inhibited by green tea and German chamomile extracts. In this case, polyphenols had a role in the hexokinase activity. Therefore, the polyphenol content of the samples needed to be removed. Before adding the hexokinase reagent into the samples, SPE columns (Waters Oasis MAX Cartridge 003036349A) were used to remove the polyphenols (after solid phase extraction there were not any hexokinase inhibition occurred) from the green tea and German chamomile extracts and the hexokinase assay kit was then employed to determine glucose production.

## 3.3 Statistical analysis

IBM SPSS Statistics 22 was used for the analysis of the data. The Levene test was used to evaluate the homogeneity of the means groups. If the criterion was met the Tukey HSD post hoc test was applied: otherwise, the Dunnett C followed the one way Anova. The values shown represent the mean values and the error bars indicate the standard deviation (SD). Unless otherwise stated, differences were considered as statistically significant when $p \leq 0.05$.

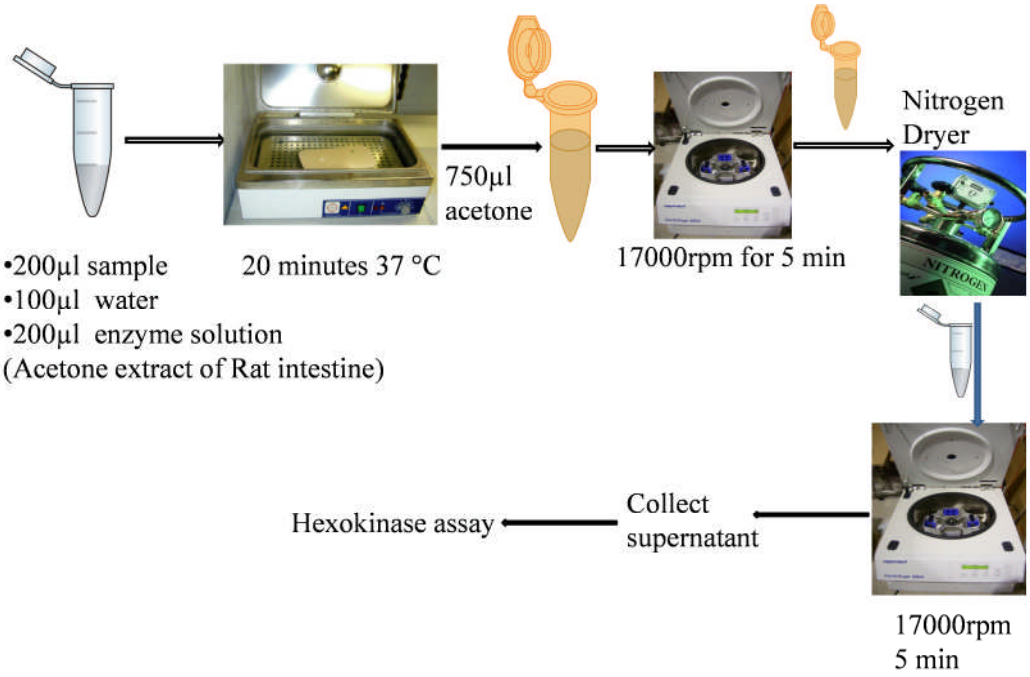



**Figure 23** Practical steps involved in determining inhibition of the digestive enzymes.

## 3.4 Results

Enzyme activity was measured in the presence of various concentrations of green tea and purified German chamomile (maltodextrin-free) extracts. Acarbose was used as a positive control.

The standard curves obtained with pure glucose standard solutions were all linear and reproducible. The concentration range of glucose was between 0.3 to 20 mM (Figure 24). The equation from the linear regression was used for the glucose concentration calculation in the samples.

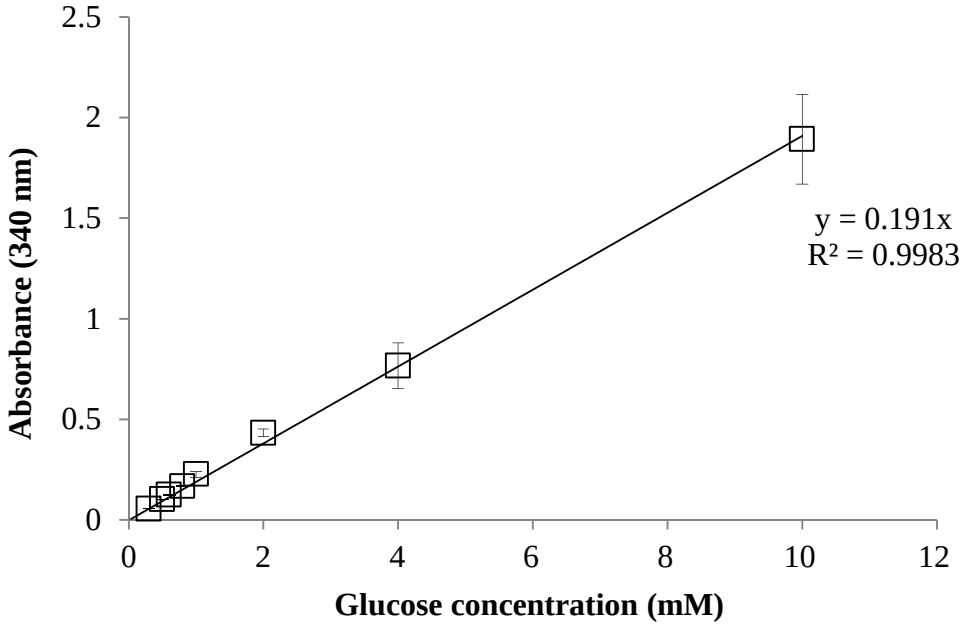



**Figure 24** Glucose standard curve with hexokinase assay (average of 22 experiment).

Mean± SD (n=3 per concentration for each 22 experiments).

The inhibition by the PFS extracts against rat intestinal sucrase/isomaltase and maltase activities was measured by increasing substrate concentration with or without PFS extracts at different concentrations. The enzyme inhibitory reaction results were calculated according to Michaelis-Menten kinetics. Using a chosen enzyme concentration and different incubation times, the kinetic parameters of $K_m$ and $V_{max}$ were determined using a Lineweaver-Burk plot. Those calculated kinetic parameters were compared with Hanes Woolf and Eadie Hofstee plots and the $K_m$ and $V_{max}$ accepted as the average of these plots for each of the digestive enzymes.

### 3.4.1 Inhibition of sucrase activity by PFS

To set up and validate the assay, the $K_m$ for sucrose with the rat intestinal acetone extract was measured. The $K_m$ value for the measurement of glucose production from sucrose was 18.3 mM and $V_{max}$ was 0.13 µmol substrate hydrolysed/min for rat intestinal acetone extract (Figure 25). This value was

close to the $K_m$ value of 18 mM determined for sucrase from mouse intestine (Lee *et al.,* 1998) and for purified sucrase it was 20 mM from Sprague-Dawley rats (Conklin *et al.,* 1975). The substrate concentration was adopted 16 mM due to the average of the Lineweaver-Burk, Hanes Woolf and Eadie Hofstee plots.

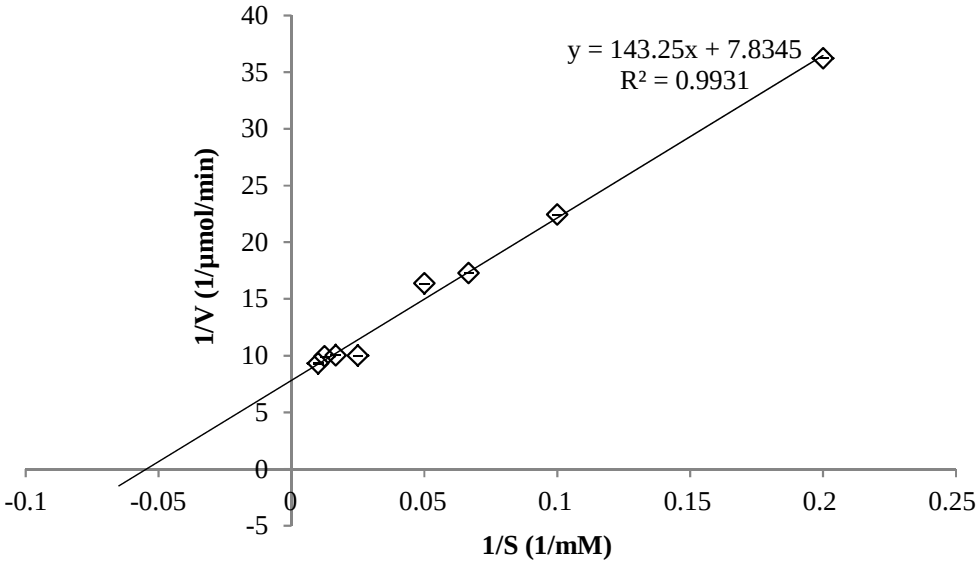



**Figure 25** Lineweaver-Burk plot for sucrase digestion of sucrose.
From this, $K_m$= 18 mM sucrose and $V_{max}$ 0.13 µmol substrate hydrolysed/minute. Mean± SD n=3.

Glucose production from sucrose was observed at different concentrations of rat intestinal extract sucrase (Figure 26).

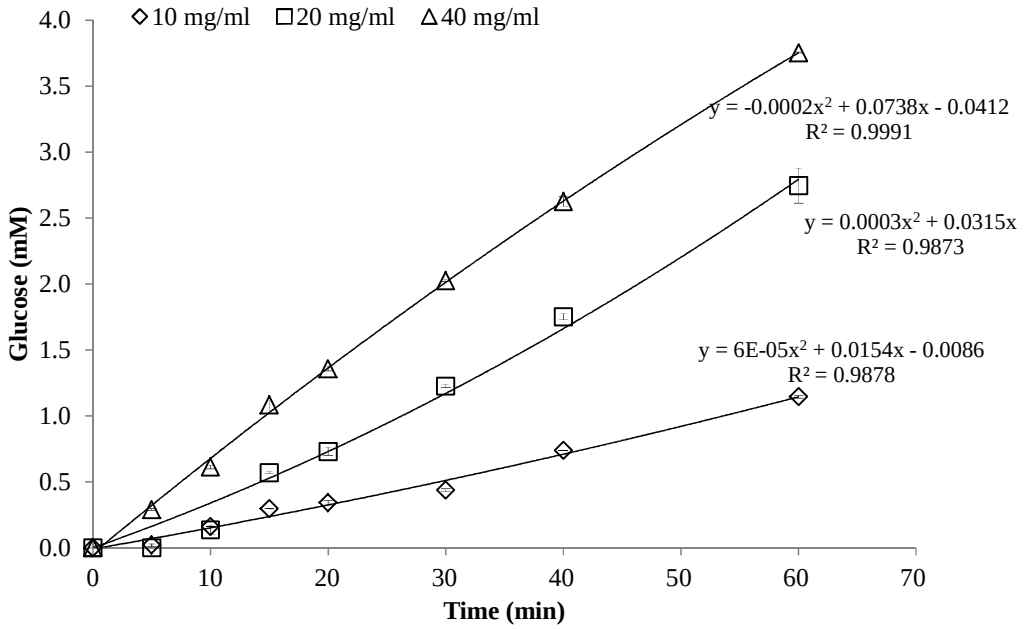



**Figure 26** Time dependence of glucose production from sucrose (16 mM) in the presence of different concentrations of acetone extract of rat intestinal sucrase (10, 20 and 40 mg/ml).

Mean ± SD (n=3 per time point). Tukey HSD test applied.

**Table 10** Absorbance (340 nm) and concentration of glucose production from sucrose with different concentrations of rat intestinal extract after 20 min incubation.

| **Enzyme concentration (mg/ml)** | **$A_{340}$** | **Glucose (mM)** | **Specific activity (µmol/min/g)** |
|---|---|---|---|
| **10** | 0.082 | 0.341 | 8.5 |
| **20** | 0.188 | 0.731 | 9.1 |
| **40** | 0.266 | 1.358 | 8.5 |

From this data, the incubation time and enzyme concentration were chosen to give the optimum assay conditions, 20 min and 20 mg/ml acetone rat intestinal sucrase extract (Table 10). It was suggested in the hexokinase assay kit product

information that the absorbance reading for glucose needed to be between 0.03 and 1.6.

The rate of reaction was dependent on the enzyme concentration (Figure 27).

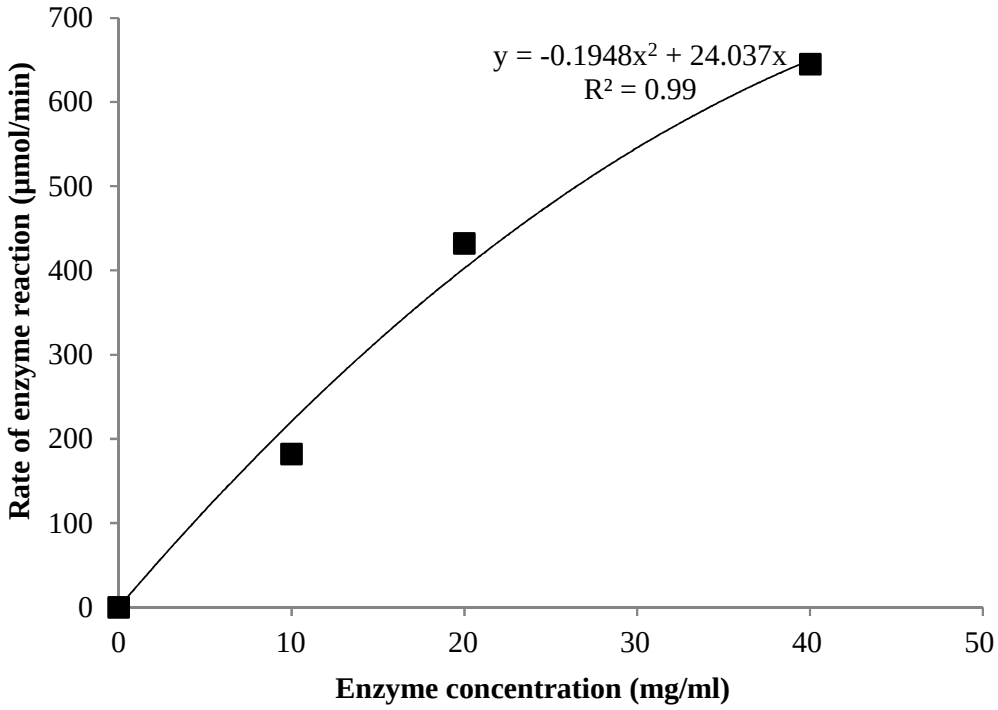



**Figure 27** Data from Figure 26 replotted to show the dependence of the rate upon enzyme concentration.

### 3.4.2 Inhibition of maltase activity by PFS

Using a chosen enzyme concentration and different incubation times, the kinetic parameters of $K_m$ and $V_{max}$ were determined with the Lineweaver-Burk plot. The $K_m$ value for the measurement of glucose production from maltose was 3.4 mM and $V_{max}$ was 0.26 mM substrate hydrolysed/min for rat intestinal maltase (Figure 28). This value was close to the $K_m$ value of other studies; 2.7 mM (Yoshikawa *et al.*, 1997) and 3.3-3.7 mM determined for maltase from rat small intestine. The substrate concentration adopted was 3 mM due to the average of the Lineweaver-Burk, Hanes Woolf and Eadie Hofstee plots.

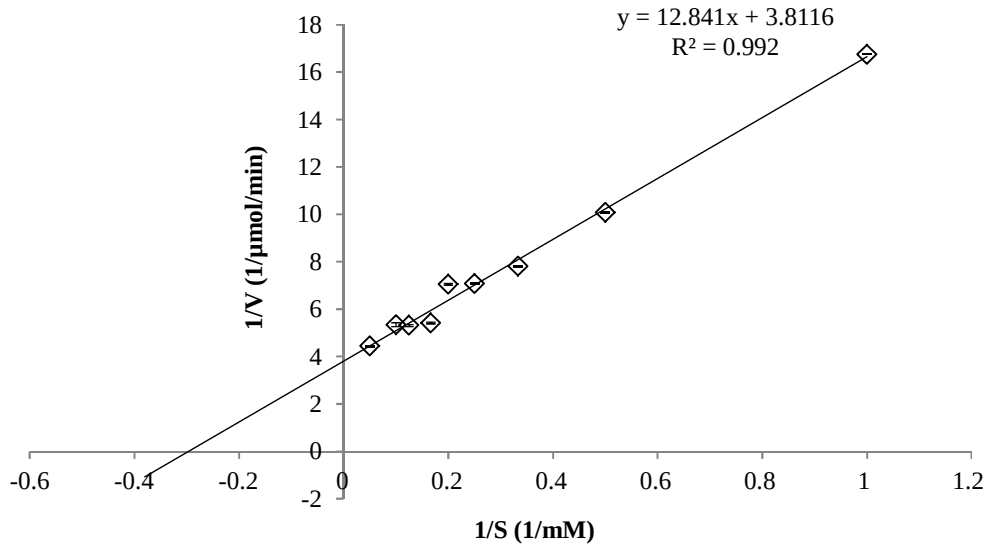



**Figure 28** Lineweaver-Burk plot for maltase digestion of maltose.

From this, $K_m$ 3.4 mM maltose and $V_{max}$ 0.26 µmol substrate hydrolysed/minute.

Mean± SD (n=3).

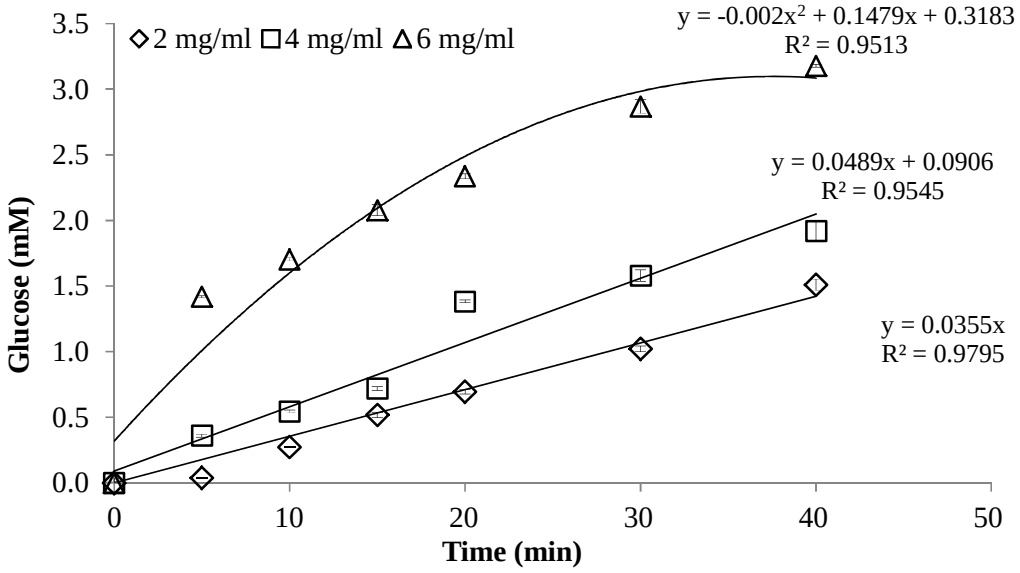



**Figure 29** Time dependence of glucose production from maltose (3 mM) at different concentrations of rat intestinal acetone extracts (2, 4, and 6 mg/ml).

Mean± SD (n=3 per time point for each enzyme concentration).

Glucose production from maltose was observed at different concentrations of acetone rat intestinal extract (Figure 29 and Figure 26). The concentration of glucose as product increased with enzyme activity.

The incubation time and enzyme concentration were chosen for optimum assay conditions as 20 min and 4 mg/ml acetone rat intestinal extract (Table 10).

**Table 11** Absorbance (340 nm) and concentration of glucose production from maltose at different concentrations of acetone rat intestinal extract after 20 min.

| **Enzyme concentration (mg/ml)** | **$A_{340}$** | **Glucose (mM)** | **Specific activity (µmol/min/g)** |
|---|---|---|---|
| **2** | 0.113 | 0.695 | 86 |
| **4** | 0.225 | 1.383 | 87 |
| **6** | 0.380 | 2.336 | 97 |

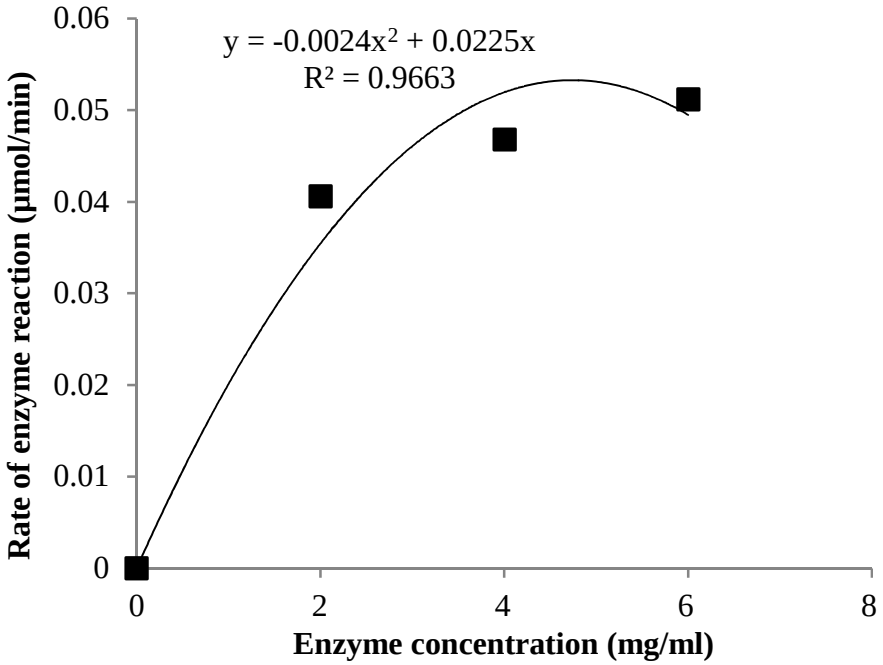



**Figure 30** Data from Figure 26 replotted to show the dependence of rate upon enzyme concentration (2, 4 and 6 mg/ml solid).

The rate of enzyme reaction depends on the enzyme concentration (Figure 30). Compared to sucrose hydrolysis, the hydrolysis of maltose was faster.

### 3.4.3 Inhibition of isomaltase activity by PFS

The $K_m$ value for glucose production from isomaltose was 5.7 mM and $V_{max}$ was 0.12 µmol substrate hydrolysed/min for rat intestinal acetone powder (Figure 31Figure 25). This value was close to the $K_m$ value of 4.5 mM (Yoshilawa *et al.*, 1997 and Oku *et al.*, 2006). The substrate concentration was adopted 6 mM due to the average of the Lineweaver-Burk, Hanes Woolf and Eadie Hofstee plots.

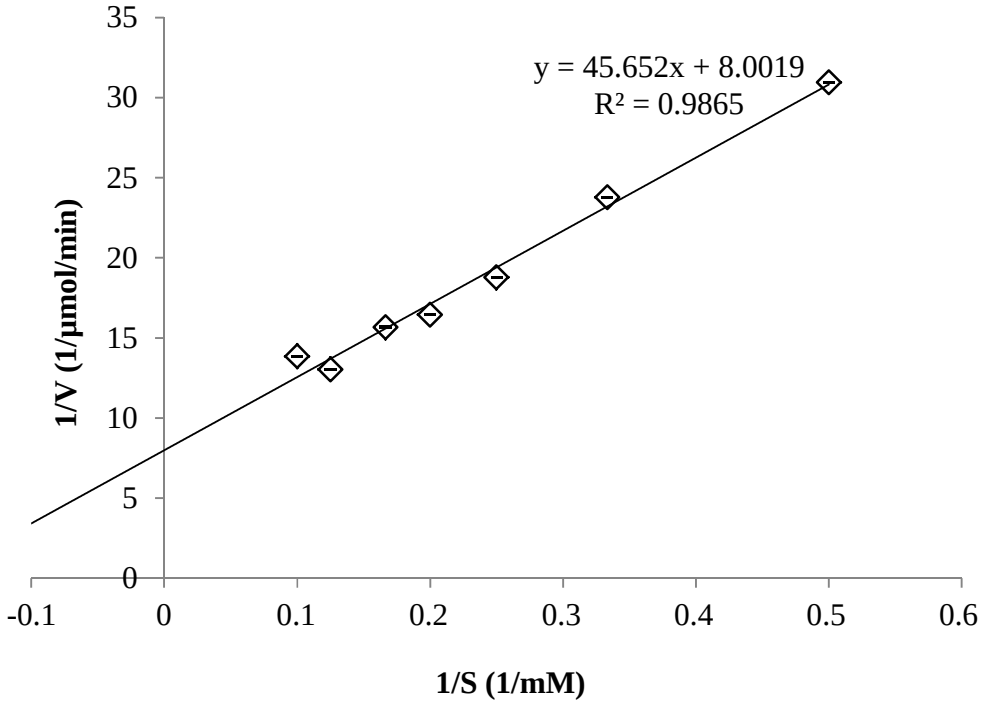



**Figure 31** Lineweaver-Burk plot for isomaltase activity.

From this, $K_m$= 5.7 mM isomaltose and $V_{max}$ 0.12 µmol substrate hydrolysed/minute. Mean± SD (n=3).

Glucose production from isomaltose was observed at different concentrations of acetone rat intestinal extract (Figure 32).

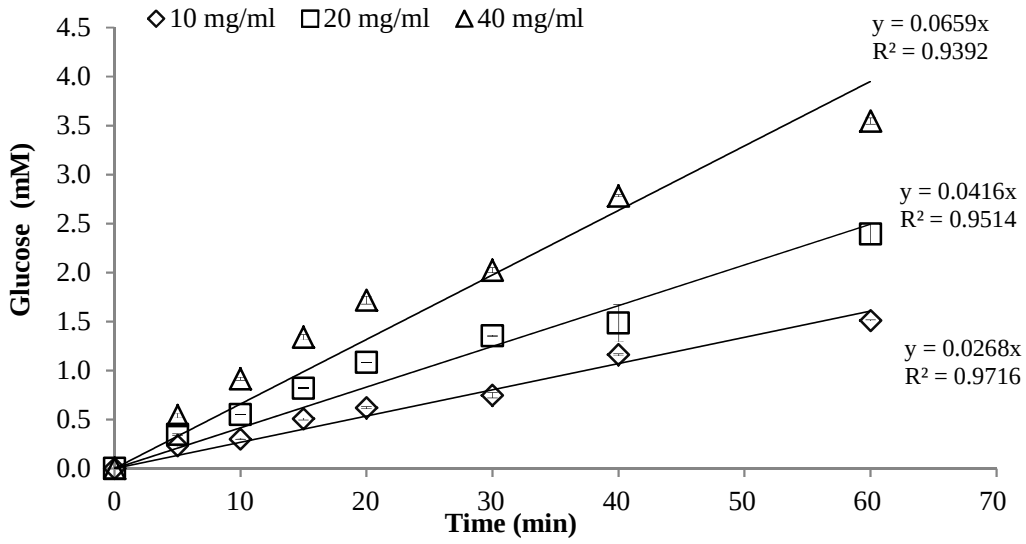



**Figure 32** Time dependence of glucose production from isomaltose (6 mM) at different concentration of rat intestine acetone extracts (10, 20, and 40 mg/ml).

The incubation time and enzyme concentration were chosen for optimum assay conditions as 20 min and 20 mg/ml acetone rat intestinal extract (Table 12).

**Table 12** Absorbance (340 nm) and concentration of glucose produced from isomaltose at different concentrations of acetone rat intestinal extract after 20 min.

| **Enzyme concentration (mg/ml)** | **$A_{340}$** | **Glucose (mM)** | **Specific activity (µmol/min/g)** |
|---|---|---|---|
| **10** | 0.102 | 0.621 | 15.5 |
| **20** | 0.195 | 1.084 | 14.6 |
| **40** | 0.329 | 1.719 | 10.7 |

The rate of enzyme reaction depends on the enzyme concentration (Figure 33).

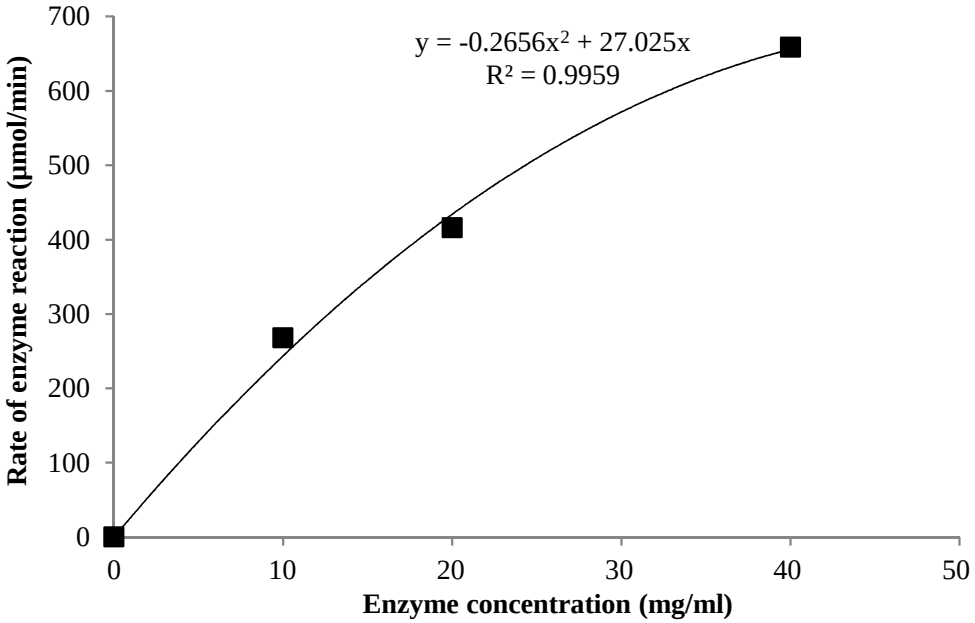



**Figure 33** Data from Figure 26 replotted to show the dependence of the rate upon enzyme concentration (10, 20 and 40 mg/ml).

### 3.4.4 Effect of PFS extracts and maltodextrin on glucose hexokinase reagent

PFS extracts contain maltodextrin (Table 14). To analyse any interference of the hexokinase assay by PFS extracts and the maltodextrin content of the PFS extracts, the test assay was modified. It was found that green tea extract was able to inhibit hexokinase activity. The inhibitory activity of green tea extract was from its polyphenol content (due to the reducing potential of polyphenols). To remove the polyphenol content of green tea extract in the assay, an SPE column was used, and two different columns were tested to choose the most effective column for polyphenol content removal of extract. Waters Oasis Max Cartridge (186000367) was more effective than Discovery DSC-18 SPE (52603-U) for removing the polyphenols from the green tea extract. After centrifugation of each sample to collect the supernatant (last process of inhibition assay steps), each sample was extracted through the Waters Oasis Max Cartridge to remove any polyphenols.

**Table 13** Added maltodextrin content of PFS extracts (PhytoLab information sheet).

| PFS extracts | Maltodextrin (%) |
|---|---|
| **Green tea** | 0 |
| **German chamomile** | 50 |
| ***Vitis Viniferae*** | 17 |

By contrast, maltodextrin did not have any inhibitory activity on hexokinase. Also, German chamomile extract did not show any inhibitory activity on hexokinase after removal of maltodextrin.

### 3.4.5 Effect of maltodextrin on the acetone rat intestinal extract

Maltodextrin is an oligosaccharide and consists of D-glucose units connected in variable lengths. The glucose units are linked with α-1,4 glycosidic bonds and it is easily digested and absorbed as quickly as glucose. Therefore, it may be a potential substrate for acetone rat intestinal extract. To analyse this, 2 mg/ml of each PFS extract was tested to observe their inhibitory effect on rat intestinal sucrase, maltase and isomaltase enzymes. Green tea extract inhibited maltase activity more strongly than isomaltase and sucrase. Conversely, German chamomile and *Vitis Viniferae* extracts were ineffective. Hence the effect of maltodextrin on acetone rat intestinal extract was analysed.

Various concentrations of maltodextrin were added to the assay as a substrate without any other sugar content and then the same process was performed with inhibition of the digestive enzyme assay. It was found that maltodextrin was a potent substrate for acetone rat intestinal extract. Therefore, analysis of PFS extracts, which contain maltodextrin, cannot occur unless the maltodextrin content is removed. Mr. J. Alberto purified the German chamomile extract using the Akta purifier. The inhibition effect of this purified extract on digestive enzymes was analysed.

### 3.4.6 Inhibition of digestive enzymes with green tea extract

Enzyme activity was measured in the presence of different concentrations of green tea extract. The maximum concentration of green tea extract was chosen as 2 mg/ml.

Maltose digestion was faster than isomaltose, and sucrose digestion was the slowest (Figure 34). Also, increasing the green tea concentration increases the percentage inhibition of sucrase, maltase and isomaltase (Figure 34).

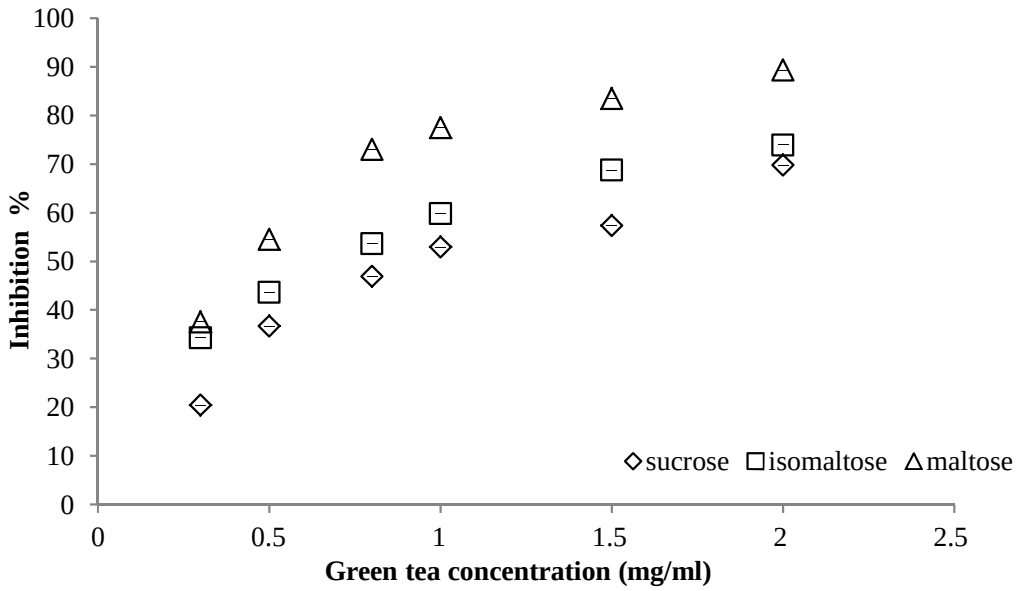



**Figure 34** Inhibition of digestive enzymes with different concentrations of green tea extract.

Mean± SD (n=3 for each concentration from each substrate). Tukey HSD test applied.

Figure 34 demonstrated that the inhibition of α-glucosidase enzymes was dose dependent. Both Figure 34 and Table 14 indicate that green tea extract inhibits maltase more strongly than isomaltase and sucrase. This also supports the study of Matsui and colleagues (Matsui *et al.*, 2007). They analysed the inhibitory effect of catechins and theaflavins, which are mainly present in green tea and maltase activity was inhibited more strongly than sucrase.

**Table 14** $IC_{50}$ values for rat intestinal α-glucosidases (sucrase, maltase and isomaltase) using green tea extract.

| Enzyme | $IC_{50}$ (mg/ml) |
|---|---|
| Sucrase | 0.95 ± 0.12 |
| Maltase | 0.44 ± 0.09 |
| Isomaltase | 0.69 ± 0.15 |

Mean± SD (n=3 for each concentration from each substrate). Tukey HSD test applied.

### 3.4.7 Inhibition of digestive enzymes with purified German chamomile extract

Purified German chamomile extract did not contain any maltodextrin and so it was suitable to assess any inhibitory effect on digestive enzymes. As the extract contained only polyphenols, the concentration to reach 50 % inhibition was expected to be lower than green tea extract. There was a limitation with this extract: it was not possible to dissolve this purified extract with deionized water but it was soluble in 100 % DMSO.

#### 3.4.7.1 Effect of DMSO on rat intestinal powder

The final concentration of DMSO in the assay was 4 %, therefore the effect of 4 % DMSO on acetone rat intestinal extract has been analysed. It was observed that 4 % DMSO inhibited 8 % of acetone rat intestinal extract activity. Therefore, the production of glucose needed to be corrected based on this result.

#### 3.4.7.2 The inhibitory effect of purified German chamomile extract on digestive enzymes

As German chamomile extract was purified, it was expected that with a lower concentration there will be more inhibitory activity compared to green tea extract. Intriguingly, the results showed that German chamomile extract activated the sucrase and isomaltase activity; however, it was ineffective on maltase activity.

Maltase activity was not affected by the purified extract (Table 15). To confirm that purified extract was not affecting the glucose hexokinase reagent, the Waters Oasis Max Cartridge was used to remove the polyphenol content of the purified German chamomile extract (no inhibitory activity on hexokinase observed after solid phase extraction) and it did not show any effect on the hexokinase reagent.

**Table 15** Effect of 0.4 mg/ml purified German chamomile extract on sucrase, maltase and isomaltase activity.

| Enzyme | GC (mg/ml) | $A_{340}$ | Glucose (mM) | Difference (%) |
|---|---|---|---|---|
| **Sucrase** | 0 | 0.131± 0.009 | 0.76 | |
| | 0.4 | 0.205± 0.007* | 1.19 | 156 |
| **Isomaltase** | 0 | 0.135± 0.006 | 0.78 | |
| | 0.4 | 0.193± 0.009* | 1.12 | 143 |
| **Maltase** | 0 | 0.236± 0.008 | 1.37 | |
| | 0.4 | 0.249± 0.011 | 1.44 | 105 |

Asterisk denote significant difference compare to control sample (*$p<0.01$).
Mean± SD (n=3 per inhibitor concentration). Tukey HSD test applied.

Isomaltase and sucrase enzymes are attached together with two active sites, so the inhibition of both enzymes should be close or the same as each other. It is quite close for green tea extract inhibition and also purified German chamomile extract has a similar activation level for sucrase and isomaltase, whereas the activation of maltase was low (Table 15).

Further analyses should be performed to understand which compound was activating the sucrase/isomaltase in the German chamomile extract.

The concentration of all the substrates was increased four fold (12 mM maltose, 64 mM sucrose and 24 mM isomaltose) and incubation time and enzyme

concentration of each substrate assay remained the same and the effect of purified German chamomile extract assessed (Table 16).

**Table 16** Inhibitory effect of 0.4 mg/ml purified German chamomile extract on sucrase, maltase and isomaltase with a four times higher concentration of substrates (64 mM sucrose, 24 mM isomaltose and 12 mM maltose).

| Enzyme | GC (mg/ml) | $A_{340}$ | Glucose (mM) | Difference (%) |
|---|---|---|---|---|
| **Sucrase** | 0 | 0.270±0.010 | 2.0 | |
| | 0.4 | 0.357±0.009* | 2.7 | 132 |
| **Isomaltase** | 0 | 0.263±0.007 | 2.0 | |
| | 0.4 | 0.356±0.014* | 2.7 | 135 |
| **Maltase** | 0 | 0.388±0.005 | 2.9 | |
| | 0.4 | 0.370±0.007 | 2.8 | 95 |

Mean± SD (n=3 per concentration). Asterisk denote significant difference compare to control sample (*$p<0.05$). Tukey HSD test applied.

**Table 17** Effect of DMSO at a 4-times higher concentration of substrates (64 mM sucrose, 24 mM isomaltose and 12 mM maltose).

| Substrate | $A_{340}$ | Glucose (mM) | Inhibition (%) |
|---|---|---|---|
| **Sucrose** | 0.389± 0.009 | 2.7 | |
| **Sucrose+ DMSO** | 0.263± 0.011* | 1.8 | 24 |
| **Isomaltose** | 0.276± 0.01 | 1.9 | |
| **Isomaltose+ DMSO** | 0.266± 0.008 | 1.9 | 2 |
| **Maltose** | 0.420± 0.009 | 2.9 | |
| **Maltose+ DMSO** | 0.399± 0.005 | 2.8 | 4 |

Mean± SD (n=3 per concentration). Asterisk denote significant difference compare to DMSO free sample (*$p<0.05$). Tukey HSD test applied.

These results demonstrated that the effect of DMSO is different at higher concentrations of substrate. According to Table 17, with a higher concentration of sucrose, the inhibitory activity of DMSO was higher than the usual sucrose concentration (16 mM- 8% inhibition). However, it was lower with isomaltose and maltose concentrations. Therefore, Table 17 showed the inhibitor effect of DMSO on sucrase/isomaltase and maltase activity, with a higher concentration of substrate having a higher inhibition.

### 3.4.8 Effect of acarbose on digestive enzymes

There are certain drugs that are used for the treatment of type 2 diabetes, such as acarbose; miglitol and voglibose are some of the examples of α-glucosidase inhibitors. However, these drugs may have side effects including diarrhea, abdominal discomfort and flatulence.

In this experiment, due to the proven acarbose inhibitory activity (commonly used in the literature), it was used as a positive control. With 0.1 mM acarbose concentration the percentage inhibition of sucrase, isomaltase and maltase were 90± 3.5, 64± 4.8 and 100± 2 %, respectively.

## 3.5 Discussion

There are prescription drugs available for the treatment of diabetes but these may have unpleasant side effects (Kwon *et al.*, 2007). Therefore, investigating alternatives for management of diabetes has become popular in research.

Green tea extract inhibited maltase, sucrase and isomaltase activities *in vitro* with $IC_{50}$ values of 0.95, 0.44, and 0.69 mg/ml, respectively. This supports the findings of Matsui (Matsui *et al.*, 2007) where they analysed the inhibitory effect of catechins and the flavins, which are mainly present in black tea and green tea, and maltase was more strongly inhibited than sucrase. By contrast, Gupta and colleagues (2007) reported that gallic acid, which is also present in tea, inhibited sucrase more potently than maltase in rats.

Yilmazer- Musa and colleagues (2012) reported that grape seed extract strongly inhibited α-glucosidases with an $IC_{50}$ value of 8.7 µg/ml compared to green tea extract with an $IC_{50}$ value of 34.9 µg/ml. The authors also tested some of the green tea polyphenols and found that EGCG, GCG and ECG inhibited α-glucosidase with $IC_{50}$ values of 24, 17 and 27 µg/ml, respectively. However C, EC and EGC were weak in α-glucosidase inhibition. Gao and colleagues (2013) analysed the combined effect of green tea extract (GTE), green tea polyphenols (GTP) and EGCG with acarbose. They found that GTE, GTP and EGCG without acarbose combination inhibited α-glucosidase activity with $IC_{50}$ values of 4.4, 10 and 5.2 µg/ml, respectively. Acarbose also inhibited with an $IC_{50}$ value of 4.8 µg/ml. When they combined GTE, GTP and EGCG with acarbose, they showed a synergistic effect at low concentration. By contrast, the higher concentrations showed an antagonistic effect (combination index). The combination of GTE, GTP and EGCG with acarbose had a synergistic effect on α-glucosidase when the inhibition percentage was below 47, 62.5 and 68%, respectively. The results of this investigation regarding green tea extract appear to be in agreement with others that describe the effect of green tea and its polyphenols on sucrase and maltase activity. Kamiyama and colleagues (2010) investigated the inhibition of maltase by green tea extract and catechins. Green tea extract inhibited rat intestinal maltase with $IC_{50}$ value of 45 µg/ml. The authors analysed the $IC_{50}$

values for catechin 3-gallate (CG), gallocatechin 3-gallate (GCG), epicatechin 3-gallate (ECG) and epigallocatechin 3-gallate (EGCG). They were good inhibitors of maltase, however, EGCG showed good inhibition towards maltase expressed in Caco-2 cells, with an $IC_{50}$ value of 27 µM. The gallated catechins showed significant inhibition while the ungallated catechins did not show significant effects. They concluded that gallated catechins or the green tea extract containing gallated catechins in dietary supplementation would contribute to the protection or improvement of type-2 diabetes and it may also explain the medicinal use of green tea as an anti-diabetic treatment in some cultures.

The inhibition of α-glucosidase and α-amylase was analysed with luteolin. The inhibitory activity of luteolin for α-glucosidase was stronger than acarbose. Luteolin, at 0.5 mg/ml, inhibited α-glucosidase by 36% (Kim *et al.*, 2000). They also tested 22 flavonoids for their inhibitory activity of α-glucosidase and α-amylase. The inhibitory activity of luteolin on yeast α-glucosidase was stronger than acarbose with $IC_{50}$ 0.5 and 5 mg/ml, respectively. Luteolin-7-*O*-glucosidase also inhibited the activity of α-glucosidase significantly. Those analysed compounds are present in the German chamomile extracts (GCE) and in contrast to the current study, luteolin-7-*O*-glucoside and luteolin represented the inhibition effect on enzymes. Kato and colleagues (2008) reported the inhibition of pancreatic α-amylase and rat intestinal maltase and sucrase activities in the presence of chamomile and some of the active component of chamomile. Chamomile extract inhibited maltase and sucrase activity with $IC_{50}$ values of 2.6 and 0.9 mg/ml, respectively. The effect of major components of chamomile (esculetin, apigenin, luteolin and quercetin at 400 µM) has also been tested in a hot water extract on sucrase and maltase activity. It was found that inhibition of maltase with quercetin was the highest (63 %), apigenin was about 35 % and luteolin was 15 %. Also, the inhibitory effect of esculetin was higher on sucrase activity (72.5 %) and quercetin showed the lowest effect (8.5 %). However, GCE and all compounds were weaker inhibitors than acarbose and, therefore it was concluded that the hot water extract of GCE had a weak inhibitory activity against rat intestinal maltase and sucrase. Esculetin and quercetin inhibited with $IC_{50}$ values of 72 and 71 µM, respectively. They also analysed the effect of hot

water extract, esculetin and quercetin on the blood glucose levels of fasted mice. After sucrose loading (2.5 g/kg body weight) to fasted mice, it was observed that GCE and quercetin decreased the blood glucose concentration at 30 and 60 min. Esculetin (50 mg/kg) showed a suppressive effect at 15 and 30 min on blood glucose level. By contrast, findings of this project showed that purified German chamomile extract activated the sucrase and isomaltase activity and it was ineffective on maltase activity.

Oku *et al.*, 2006 investigated the inhibitory effect of the leaves of Morus alba (ELM) on human intestinal disaccharides and the inhibitory activities of ELM were investigated. Sucrase, maltase and isomaltase of the human small intestine were conspicuously inhibited by ELM, whereas trehalase and lactase were not so strongly inhibited. The inhibitory effects became significant as the concentration of ELM increased. All of the inhibitory activities by ELM for sucrase, maltase and isomaltase of the rat small intestine were competitive inhibitory activities.

In the current study, green tea, German chamomile and *Vitis viniferae* extracts was analysed for their possible interference with α-glucosidase assays and assessed by considering critical parameters affecting the final inhibition (maltodextrin content of the extract, polyphenol reduction potential (the number of OH groups in the chemical structure) causing an interference with hexokinase reagent (Rice-Evans *et al.*, 1996) and the effect of DMSO to enzyme activity). After the enzymatic hydrolysis of substrates the potential reactive inhibitors were removed by SPE and then hexokinase reagent was used to assess the product. Therefore, in many published studies the reducing capacity of polyphenols was ignored and the results of the inhibition may be inconsistent.

The results of this investigation appear to be in agreement with others that describe the effect of polyphenols on sucrase, maltase and isomaltase activity for the effect of green tea extract.

Whilst it can be considered that inhibition of α-glucosidase enzymes could potentially reduce postprandial blood glucose levels, specific activity of the enzymes in human intestine and reactivity of polyphenols with other compounds

must be taken into account. The findings of this study revealed that green tea extract could inhibit sucrase, maltase and isomaltase activity. As a result it might be useful as a supplement for use in suppressing postprandial hyperglycaemia in the management of type-2 diabetes.

# CHAPTER 4 INHIBITION OF GLUCOSE TRANSPORT AND METABOLISM BY PLANT FOOD SUPPLEMENTS


## Abstract

Plant food supplements may have a major role in mediating intestinal glucose transport across the brush border membrane of enterocytes and so they may improve the management of diabetes (acarbose-like activity). Glucose transport across the Caco-2 cell monolayer was significantly attenuated in the presence of PFS. Green tea, German chamomile and *Vitis Viniferae* extracts inhibited both the uptake and transport of glucose when tested independently. By contrast, Pelargonium and Echinacea were ineffective. Using $Na^+$-containing and $Na^+$-free conditions in the presence of German chamomile, we concluded that the inhibition of GLUT2 was greater than SGLT1. Accordingly, the results indicate that apical GLUT2 is the primary target of the compounds from the German chamomile extract.

Some of the compounds that are present in German chamomile extract were also tested to determine their contribution to the inhibition of transport and uptake of glucose. Luteolin was the most effective compound to inhibit glucose transport; on the other hand, luteolin-7-*O*-glucoside produced the most effective inhibition of cellular glucose uptake. To analyse the effect of digestion on the biological activity of German chamomile extract, the extract was subjected to enzymatic hydrolysis followed by extraction as a model of the first step of digestion in the gut. Hydrolysis using hesperinidase and cellulose removed the sugars from the polyphenols in the German chamomile and cleaved the ester bonds between phenolic and quinic acid (the first step of digestion in the gut). This digestion affects the biological activity of German chamomile due to glucose transport across the cell monolayer, which was decreased by approximately 50%. The response of the Caco-2 cell line to stress (FBS starvation or glucose load overnight) was assessed after treatment with German chamomile or additional glucose. Both transport and uptake of glucose were tested. FBS starvation or addition of German chamomile overnight (12 h) did not have any effect on the

cellular uptake and transport of glucose. However, overnight incubation with a lower glucose concentration (1 mM) increased the transport of glucose significantly, but did not affect the cellular uptake. Transport of deoxyglucose was significantly inhibited by the same PFS treatments that affected glucose. In contrast to glucose, deoxyglucose uptake was not changed by any PFS treatments. In summary these results show, PFS may be effective on the management of diabetes due to their effect on glucose transport and metabolism.

## 4.1 Introduction

Carbohydrates and sugars make up the most important energy containing components of the diet. After consumption of carbohydrates or sugars, digestive processes lead to the appearance of glucose in the blood. Glucose is the main product of sugar and starch digestion, and is transported to the blood from the intestinal lumen by active transport via SGLT1 and by facilitated sodium-independent transport via GLUT2 (Goodman, 2010). After two $Na^+$ ions bind to SGLT1 on the luminal side of the intestinal brush border membrane, a conformational change takes place that allows glucose binding. A second conformational change allows glucose and $Na^+$ to enter the enterocyte (Goodman, 2010). Glucose is released from the enterocyte via the GLUT2 passive transporter at the basal surface and enters circulation. The process of uptake and expulsion is simultaneous (Figure 35) (Goodman, 2010). It should also be noted that the transporters have different $K_m$ values and specificity for the carbohydrate substrates. Hediger and Rhoads (1994) reported a $K_m$ for SGLT1 of 0.8 mM, whereas GLUT2 has a high capacity for glucose transport with a $K_m$ value of 17 mM (Thorens, 1996).

Consumption of plant foods is thought to lower the risk of chronic diseases including diabetes, cardiovascular diseases and cancer (Scalbert *et al.*, 2005; Crozier *et al.*, 2009; Clifford *et al.*, 2004; Knekt *et al.*, 2002). The highest concentration of polyphenols is in the gut lumen compared to any other tissue in the body (Williamson, 2012). After consumption of polyphenol-rich beverages, such as coffee, tea and orange juice, polyphenols can reach millimolar concentrations in the gut lumen (Williamson, 2012). Therefore, glucose transport and metabolism in the gut lumen may potentially be affected by polyphenols. However, as their concentrations are <0.005 mM in the blood and tissues, polyphenol effects in peripheral tissues are expected to be less potent (Williamson, 2012).

A plethora of *in vitro*, *ex vitro*, animal and human intervention studies has shown that various dietary polyphenols may influence carbohydrate metabolism at many levels. The possible mechanisms proposed include inhibition of digestive

enzymes for glucose production, inhibition of glucose transporters in the intestine, stimulation of insulin secretion from the pancreatic β-cells, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, modulation of glucose release from the liver, and modulation of intracellular signalling pathways and gene expression (Hanhineva *et al.*, 2010).

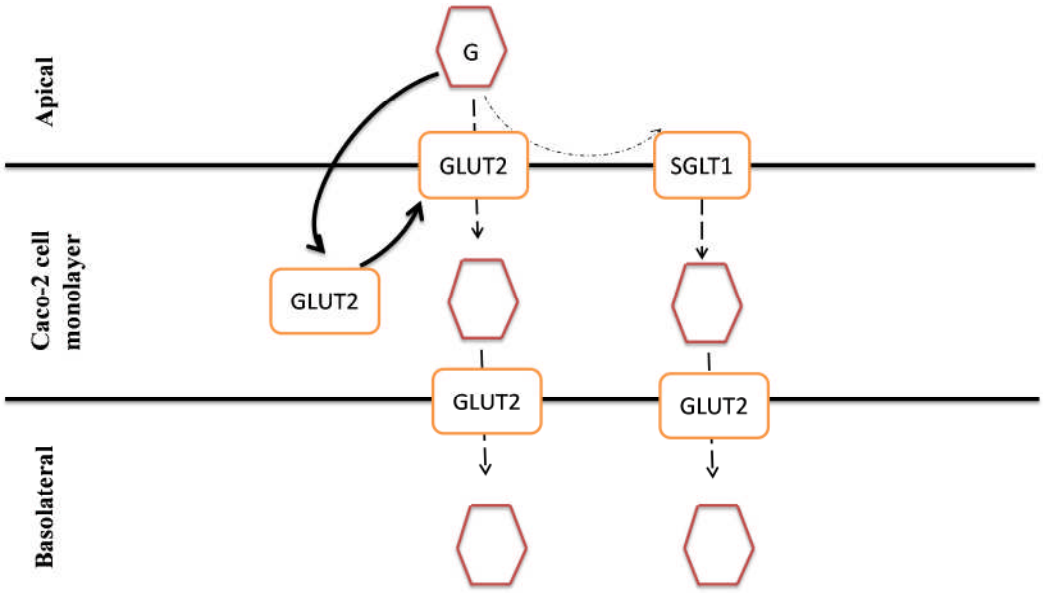



**Figure 35** Mechanism of glucose absorption to basolateral compartment in a Caco-2 cell system (G: glucose).

Phytochemicals are known to affect the apical membrane SGLT-1. Phloridzin is a commonly used inhibitor of nutrient absorption across the apical membrane of enterocytes (Buddington *et al.*, 2003). By decreasing SGLT-1 activity (some polyphenols inhibit the transport of glucose), soy isoflavones partly affect post-prandial glucose levels; tea polyphenols have been shown to lower the rate of glucose transport by non-specific binding to proteins; and catechins and other phytochemicals are able to change the brush border fluidity potentially influencing the function of transporters. A concentration of 100 µM (+)-catechin, (-)- epicatechin, epicatechin gallate, epigallocatechin and

epigallocatechin gallate also reduced sodium-dependent glucose uptake (Johnston *et al.,* 2005).

Manzano and Williamson (2010) reported that strawberry and apple extracts inhibited glucose transport and uptake across a Caco-2 cell monolayer dose-dependently. GLUT2 inhibition was more potent than SGLT1 based on the $IC_{50}$ values. Johnston *et al*. (2005) and Kwon *et al.* (2007) also investigated glucose uptake inhibition by quercetin at physiologically relevant concentrations. Treatment with 100 µM epicatechin gallate and quercetin-3-*O*-glucoside were also reported to competitively inhibit GLUT2 (Chen *et al*. 2007).

A more recent investigation of glucose uptake in Caco-2 cell monolayers was reported in the presence of an anthocyanin-rich berry extract in two conditions; after acute exposure glucose uptake was significantly decreased for both SGLT1 and GLUT2 and during a longer term exposure the extract significantly reduced both GLUT2 and SGLT1 mRNA and protein levels (Alzaid, 2013).

Although many studies exist on the health effects of polyphenol-rich foods, such as fruits & vegetables (including oranges, apples, strawberries and onions) or beverages (including coffee, tea, wine and orange juice), limited studies have been performed to specifically determine the effect of PFS on glucose transport. Yeh *et al*. (2003) reviewed 58 control clinical trials involving individuals with diabetes (mostly type 2) or impaired glucose tolerance (36 herbs used). A total of 44 out of 58 (76%) subjects showed improved glucose control with the tested supplements (e.g. *Coccinia indica, American ginseng, Chromium, Gymnema sylvestre, Aloe vera,* Vanadium, *Momordica charantia,* and Nopal). However, no definitive conclusions were made regarding the activity of these herbs and supplements for diabetes. In this regard, further analysis is needed.

Another recent review regarding herbal supplements was published by Suksamboon *et al*. (2011). The review was related to glycaemic control. The review showed that *Ipomoea batatas, Silybum marianum* and *Trigonella foenum-graecum,* but not *Cinnamomum cassia*, may improve glycemic control in Type 2 diabetes.

PFS contain concentrated sources of plants, their extracts or derivatives, and they are able to deliver physiological effects. Throughout history, plants and herbs have been part of the diet not only because they provide nutrients, but also for their health-promoting properties. This remains the case today, and the consumption of plant based foods has been increasing (Moini *et al.,* 2000; Weiss *et al.,* 2006; Seeram *et al.,* 2006; Dabaghian *et al.,* 2012). The aim of this investigation was to determine the effect of polyphenols from different plant food supplements (green tea, German chamomile, *Vitis viniferae*, Pelargonium, and Echinacea) on cellular glucose uptake and transport of glucose from the apical to basolateral compartment using a Caco-2 intestinal cell model.

## 4.2 Material and methods

### 4.2.1 Standards and reagents

D-[U-$^{14}$C] glucose 250 µCi (9.25 MBq) was purchased from Perkin Elmer (Boston, USA). D-glucose was purchased from Fisher Scientific Ltd, (Leicestershire, UK G/0450/60). The PFS were supplied by PhytoLab & Co (Vestenbergsgreuth, Germany) within a European FP7 project framework (PlantLibra). Dulbecco's Modified Eagle's Medium DMEM; including 1000 mg glucose/L, L-glutamine, $NaHCO_3$ and pyridoxine HCl) were purchased from Sigma (Dorset, UK; D6046). Caco-2 cells (HTB-37) were obtained from the American Type Culture Collection (LGC Promochem, Middlesex, UK).

### 4.2.2 Cell Culture

Cells were routinely grown in DMEM media containing low D-glucose (5 mM) supplemented with 15% (v/v) fetal bovine serum (FBS; F7524), 100 U/ml penicillin, 0.1 mg/ml streptomycin out of a solution containing (10,000 U/ml-10 mg/ml; P0781), and 0.25 µg/ml amphotericin B (250 µg/ml, A2942). Cells were propagated in 25 $cm^2$ (Corning® 430639) and 75 $cm^2$ (Corning® 430641) cell culture flasks in a 95% air/5% $CO_2$ incubator at 37°C. Every second or third day the growth medium was replaced with fresh medium. When cells reached 80-90% confluence they were detached from the flask using 0.25% trypsin-EDTA

solution (T4049). Caco-2 cells were plated on Transwell® plates (Corning® 3412) at a density of 6.43 x $10^4$ cells/$cm^2$. Growth media contained 10% FBS (v/v), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25µg/ml amphotericin B in DMEM media and replaced every other day in both compartments until full differentiation of the monolayer (between 21 and 23 days).

## 4.3 Glucose transport in Caco-2 cells

### 4.3.1 Preparation of Reagents

All the reagents were prepared freshly on the day of the experiment. A solution (TBS) prepared to use in the transport experiment with no glucose content. TBS was prepared according to the HBSS formulation without the addition of glucose. TBS contained 5.4 mM potassium chloride (P5405), 0.441 mM potassium phosphate (P0662), 0.352 mM sodium phosphate dibasic (S9763), 4.2 mM sodium bicarbonate (S5761), 1.8 mM calcium chloride dihydrate (C7902) and 0.1 mM ascorbic acid (A4544) (all from Sigma, Dorset, UK) and 137 mM sodium chloride (Fisher Scientific Ltd, Leicestershire, UK S5886), dissolved in water and the pH was adjusted to 7.4 with 1M HCl before being sterile filtered (Corning® 430049). The buffer was pre-warmed at 37°C.

### 4.3.2 Glucose

A stock solution of 10 mM glucose was prepared. Briefly, 180 mg of glucose (Fisher Scientific; G/0450/60) was dissolved in 100 ml of transport buffer solution. 1 ml of stock solution with/without PFS was added to TBS to a final volume of 10 ml containing 9 µl of 0.1 µCi/µl $^{14}$C-glucose (Perkin-Elmer; product number: NEC042X250UC). The pH was adjusted to 7.4 at 37 $^0$C for each sample with 1 M HCl or NaOH.

### 4.3.3 Plant Food Supplement extracts preparation

10 mg of each PFS was weighed and dissolved in 1 ml of TBS (v/v) and centrifuged at 17000 g for 5 min. The supernatant was collected and the relevant volume added to 10 ml TBS solution containing 1 mM glucose and 0.045 µCi/ml glucose.

To increase the water solubility of the supplements (except for the green tea extract), different concentrations of maltodextrin were added by the supplier (PhytoLab). Maltodextrin consists of D-glucose units linked by α-1, 4 glycosidic bonds with variable length. However, the use of radiolabelled glucose mixed with non-radiolabelled substrate allows for high sensitivity of the obtained counts. The relatively small amount of radiolabelled glucose (1 mM glucose including 9 µl of 0.1 µCi/µl D-[$^{14}$C]-glucose stock solution) mixed with sufficient unlabelled glucose (1 mM) means that the maltodextrin contained in the PFS did not interfere with the assay. The amount of maltodextrin in every PFS was: 50 % in German chamomile and Echinacea, 13 % in grape extract and 70 % in Pelargonium. We also determined that maltodextrin (0.5 mg/ml) to does not interfere with the assay (Figure 49). The final PFS concentration was corrected for the maltodextrin content.

### 4.3.4 Preparation of German chamomile active components

The individual compounds (apigenin, A7G, luteolin, L7G, diCQA, umbelliferone) tested were diluted in TBS from a 30 or 50 mM stock solution prepared in 100 % DMSO. All of the compounds were supplied by PhytoLab & Co. (Vestenbergsgreuth, Germany). The final concentration of DMSO in the assay did not exceed 0.1 %.

## 4.4 Inhibition assay protocol

Cells between passage 36 and 50 were utilised in the investigation of glucose transport. A minimum of four or six replicates were allocated per experimental condition.

On or after 21 days, transport studies were initiated by replacement of growth medium with TBS in both compartments, and cells were washed three times with addition of TBS to both compartment. After washing cells, 2 ml TBS was added to both apical and basolateral sides and pre-incubated for 30 min at 37 °C under a humidified atmosphere. Differentiation of the monolayer was assessed by measurement of trans-epithelial electrical resistance (TEER) of the cell monolayer. Only Transwell® inserts with a resistance exceeding a blank membrane by 300 Ω were utilized in the experiment. A low TEER value may

indicate less well-formed tight junctions and a potential increase in paracellular transport of glucose across the membrane. After equilibration, TEER values were taken from three positions per insert and TBS was carefully aspirated to waste. For experiments, 2 ml TBS containing 1 mM glucose solution and 0.045 µCi/ml glucose with or without PFS extracts (pH 7.4) was added to the apical compartment and 2 ml TBS in the basal compartment. Cells were incubated for 25 min at 37°C. Following incubation, TEER values were measured again as above and solutions from apical and basolateral compartments were removed in to 2 ml centrifuge tubes and immediately placed on ice. To wash cells of any residual radioactivity, 2 ml TBS was added to each compartment and then removed to 2 ml centrifuge tubes and placed on ice. Following this, 1 ml of 1 M NaOH solution was added to detach the cells from the membrane and plates were shaken for 30 min on a horizontal shaker. Suspended cells were transferred to eppendorf tubes. To neutralize the NaOH, 1 ml of 1 M HCl was added per tube. To analyse for counts in the different compartments the samples were vortexed to ensure homogeneity, and 0.25 ml from apical and sample blanks, and 0.5 ml from the cell suspension, basolateral and wash buffer were transferred to scintillation vials containing 5 ml of scintillation liquid. Radioactivity was determined using a Packard 1600 TR Liquid Scintillation Analyser (Figure 36). Light emission was counted for 10 min per vial and the number of counts per min (CPM) was calculated by the equipment software.

### 4.4.1 Liquid scintillation counting (LSC)

A Packard 1600 TR Liquid Scintillation Analyser was used for liquid scintillation counting (LSC). In this experiment, the D-[$^{14}$C]-glucose isotope (Perkin-Elmer, NEC042V250UC) was used for the assessment of glucose transport and cellular uptake.

The carbon-14 isotope emits high-energy beta radiation which is absorbed by the scintillation cocktail into detectable light pulses. This scintillation cocktail serves as an energy-collecting solvent to transfer the emitted energy efficiently to light. Beta particles emitted from the [$^{14}$-C]-glucose isotope transfer their energy to solvent molecules. This energy is absorbed by the scintillator (phosphor facilities). The absorption of energy excites the phosphor causing it to emit light

energy that is detected by the photomultiplier tube (PMT) of the liquid scintillation counter (Figure 37).

## 4.5 Statistical analysis

IBM SPSS Statistics 22 was used for the analysis of the data. The Levene test was used to evaluate the homogeneity of the means groups. If the criterion was met the Tukey HSD post hoc test was applied: otherwise, the Dunnett C followed the one way Anova. The values shown represent the mean values and the error bars indicate the standard deviation (SD). Unless otherwise stated, differences were considered as statistically significant when $p \leq 0.05$.

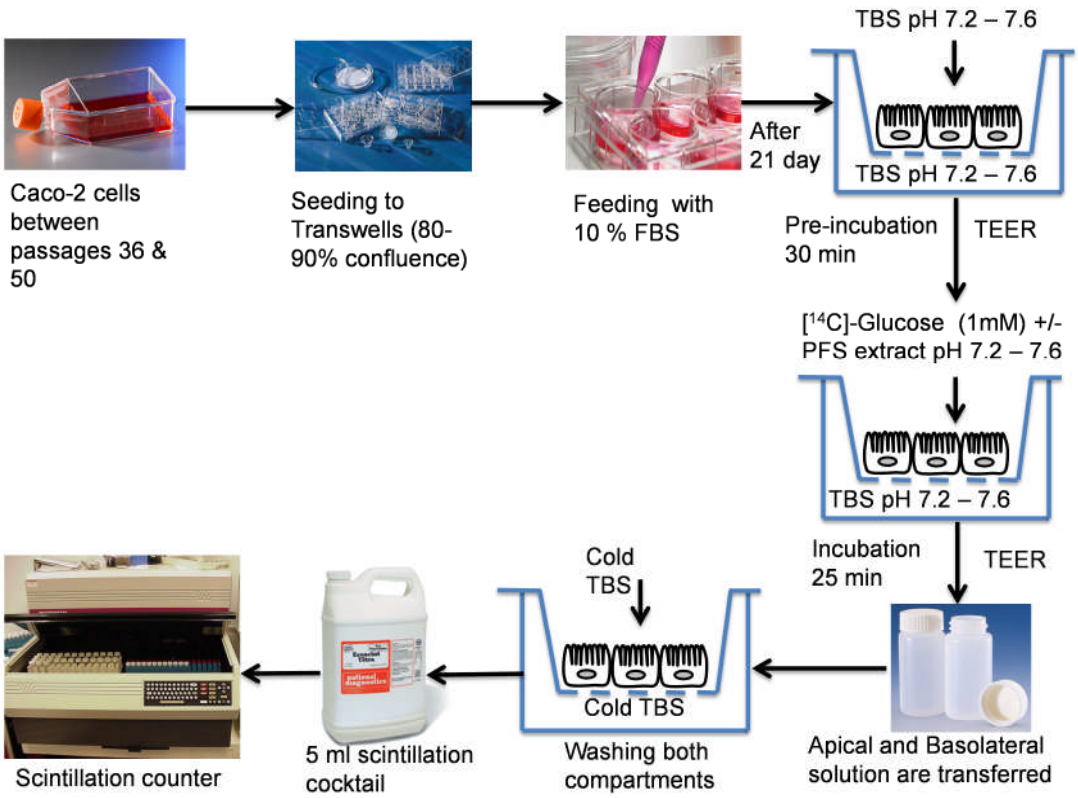



**Figure 36** Summary of the practical steps involved in the glucose transport assay.

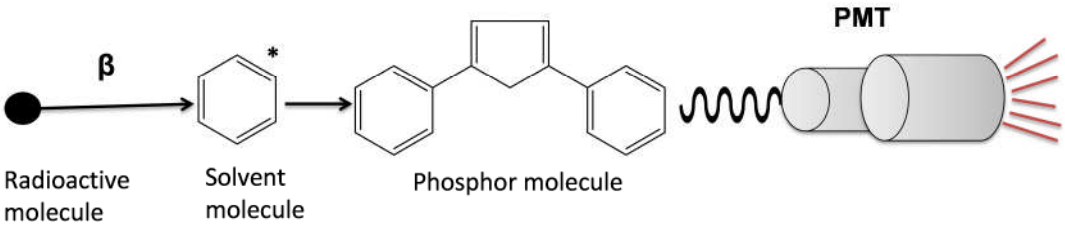



**Figure 37** Summary of the scintillation process.

After $^{14}C$ molecules emit beta (β) radiation the solvent molecules become excited and their energy is transferred to the phosphor molecule to emit light. The light is detected by PMT.

## 4.6 Results

### 4.6.1 Set up and validation of the D-[$^{14}$-C]-glucose transport across Caco-2 cells

A calibration curve of radioactivity as a function of [$^{14}$-C]-glucose quantity was prepared. With the y-intercept fixed at zero the gradient was calculated as 135756 CPM/µmol and the adjusted $R^2$ was 0.9999. This data indicates a good proportionality of radioactivity and molar quantity (Figure 38). Counting precision of the equipment was assessed by comparing the reference calibration solution of 123000 CPM/KBq (the typical count was average 118549 CPM). The results were corrected for the counter efficiency based on background absorbance standard signals (the typical count was on average 28.8 CPM, the solutions involved in the experiment (HCl+ NaOH mixture) had a typical count of 38.6 CPM, and TBS was 36.1 CPM).

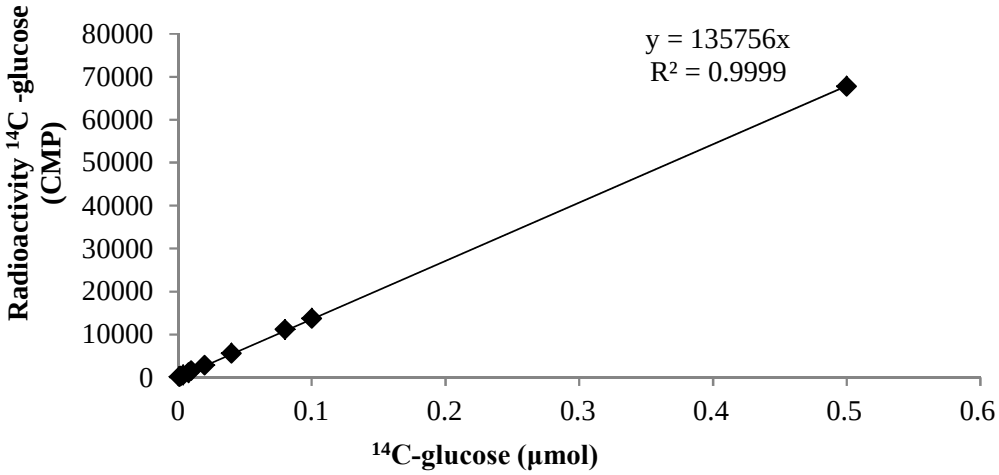



**Figure 38** Glucose standard curve for radioactivity measurements (CPM = D-[$^{14}$C]-glucose counts per min).

Where not visible, error bars are smaller than the size of the data point. Mean± SD (n=6 measurements from per experiment for each 21 experiment).

Caco-2 cells were plated on 6-well plates at a density of 6.43 x $10^4$ cells/cm$^2$. The experiments were performed after the cell monolayer reached the full differentiation. The differentiation of the monolayer was assessed by measurement of the TEER values of the cell monolayer. Only Transwell® inserts with a resistance exceeding a blank membrane by 300 Ω were utilized in the experiment. TEER values <300 Ω may be demonstrating the less well-formed tight junctions between the cells. The TEER value of the cells was measured after the pre- and post-incubation process. There was no significance in the pre-incubation TEER values of each well. However, after exposing cells to glucose and PFS extracts (post-incubation), the TEER values were significantly different for some PFS extracts (Table 18). It was hypothesized that higher concentrations of green tea and *Vitis Viniferae* extracts may improve the electrical resistance of the monolayer.

**Table 18** Blank corrected TEER of Caco-2 monolayers measured after pre-incubation with TBS (30 min) and post-incubation with test substrate (25 min) in the absence (control) and presence of PFS extracts.

| PFS extracts | Concentration of PFS (mg/ml) | TEER (pre-incubation) | TEER (post-incubation) | % Difference (pre-post incubation) |
|---|---|---|---|---|
| **Control** | 0 | 519.7± 12 | 412.1 ± 29 | 20 |
| **German chamomile** | 1 | 479.3±8 | 386.2 ± 40 | 19 |
| **Green tea** | 1 | 486.2±22 | 486.7 ± 46 * | 0 |
| ***Vitis Viniferae*** | 3 | 535.4±36 | 537.1 ± 16 * | 0 |

% difference demonstrates the difference between pre- and post-incubation TEER values. The asterisk denotes a significantly different TEER measurement relative to control TEER measurement ($p<0.05$). Mean ± SD (n=6 measurement per sample and 18 times readings from each sample).

The rate of transport from apical to basolateral cell culture medium was 10.4 nmol/min cell culture medium and cellular uptake of D-[$^{14}$C]-glucose (1 mM) was 0.5 nmol/min in the control sample (Figure 41 and Figure 42). The effect of PFS extracts on the transport of glucose from apical to basolateral and cellular uptake of glucose was investigated. Some of the active components of German chamomile were also individually assayed to explore the possibility that they were able to moderate glucose uptake and transport at concentrations relevant to the German chamomile extract. The inhibition of glucose transport by German chamomile extract was also investigated with/without $Na^+$ in the transport cell culture medium. It is expected that SGLT1 and GLUT2 will be active at the apical surface (with $Na^+$ but not under $Na^+$ free conditions) and GLUT2 will be

active at the basolateral side in the media with and without $Na^+$. Under $Na^+$-free conditions, only GLUT2 will operate at the apical surface, and SGLT1 will be inactive.

To analyse the effect of digestion on the biological activity of German chamomile extract, it was subjected to enzymatic hydrolysis followed by extraction with ACN and EtAC as a model of the first step of digestion in the gut. Hydrolysis using hesperinidase and cellulase removed the sugars from the polyphenols in the German chamomile and cleaved the ester bonds between phenolic and quinic acid. This experiment indicates that digestion affects the biological activity of German chamomile since glucose transport across the cell monolayer decreased approximately 50 %. German chamomile enzymatic hydrolysis was performed by Ms R Gutierrez-Riviera.

Basolateral transport of D-[$^{14}$C]-glucose (1 mM) was analysed at different time points with and without overnight FBS starvation to analyse the effect of different time starvation stress on Caco-2 cells. There was a linear increase of glucose transport with time (Figure 39). Overnight FBS starvation had no effect on D-[$^{14}$C]-glucose transport from the apical to basolateral side at any time point. Cellular uptake of glucose increased linearly with time (Figure 40). By contrast, cellular uptake of glucose decreased with overnight FBS starvation at both the 10 and 30 min time points ($p<0.01$) (Figure 40).

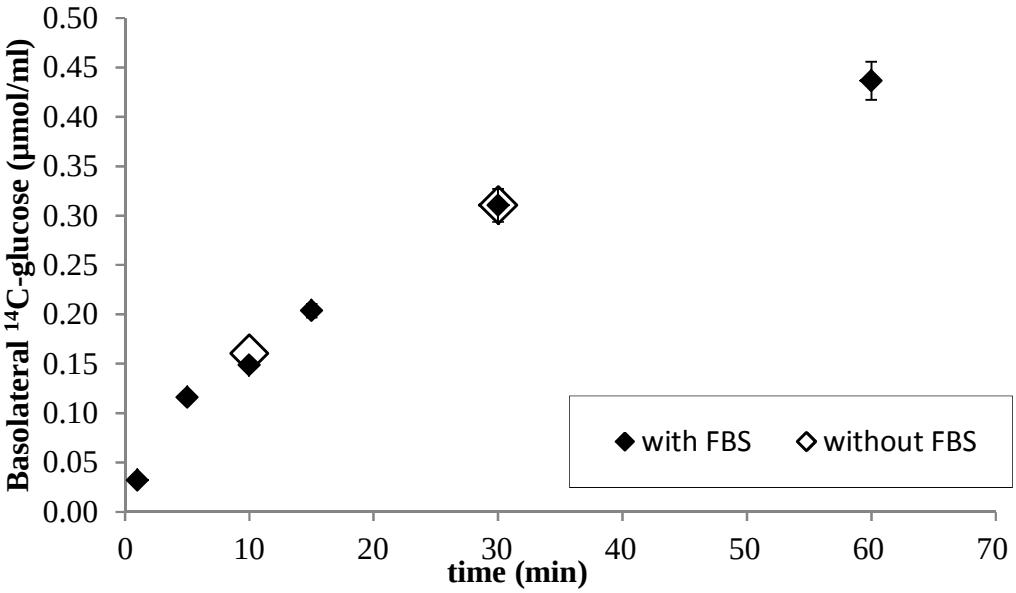



**Figure 39** Transport of D-[$^{14}$C]-glucose from the apical to basolateral side with and without FBS pre-treatment.

Transport of $^{14}$C-glucose was significantly different to zero at all time points ($p<0.01$). Transport of $^{14}$C-glucose was not significantly different and without FBS pre-treatment points ($p<0.05$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per time point). Tukey HSD test applied.

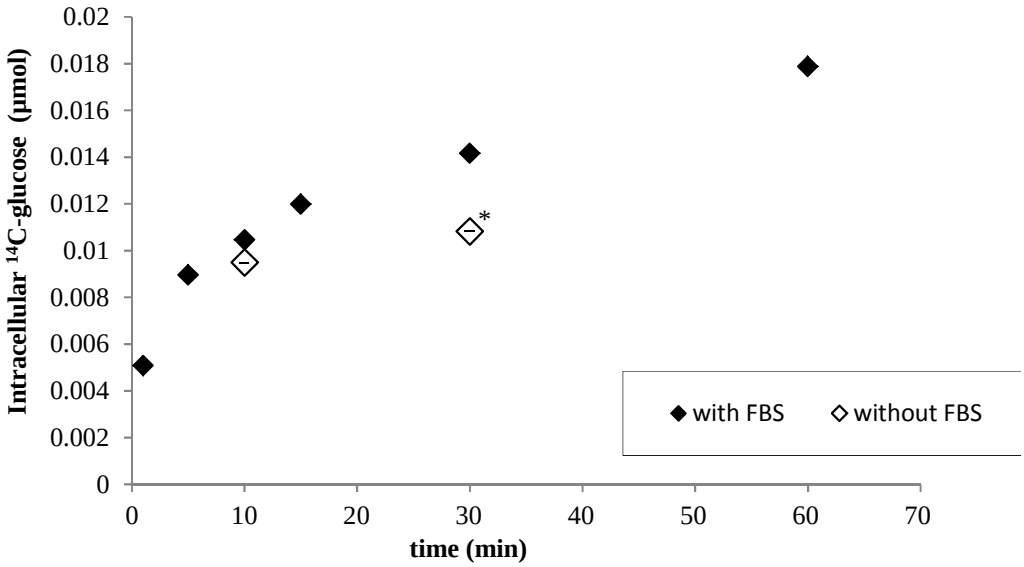



**Figure 40** Intracellular glucose after incubation with apical 1 mM glucose with and without FBS pre-treatment.

Cellular uptake of $^{14}$C-glucose was significantly different to zero at all time points ($p<0.01$). The asterisk denotes significant difference from the treatment with FBS and pre-treatment without FBS ($p<0.05$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per time point). Tukey HSD test applied.

$V_{max}$ and $K_m$ of apical to basolateral transport was calculated using nonlinear regression analysis using the Michaelis-Menten equation for cells incubated only with glucose and when co-incubated with 1 mg/ml German chamomile extract. The rate of apical to basolateral glucose transport was significantly decreased dose-dependently in the presence of German chamomile extract ($p<0.01$). The transport of glucose from the apical to basolateral compartment increases with substrate concentration, consistent with transporter-mediated processes following Michaelis Menten kinetics ($K_m$ was equal to 8.1 mM and $V_{max}$ was 0.09 µmol/min) (Equation 2).

Cellular uptake of glucose increased from the lowest to highest glucose concentration and uptake of cellular glucose was inhibited significantly in the

presence of 1 mg/ml German chamomile at all glucose concentrations ($p<0.01$) (Figure 42).

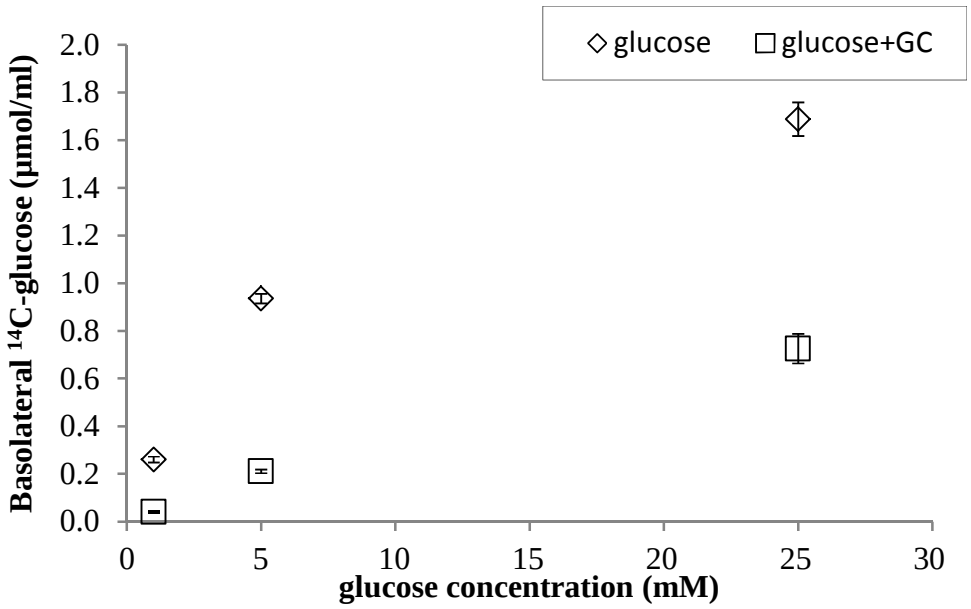
$$V_0 = \frac{V_{max}\,[S]}{K_m + [S]}$$

$$V_{max} = \frac{V_0\,(K_m + [S])}{[S]}$$

$$K_m = \frac{V_{max}\,[S]}{V_0} - [S]$$

**Equation 2**

**A**




**B**

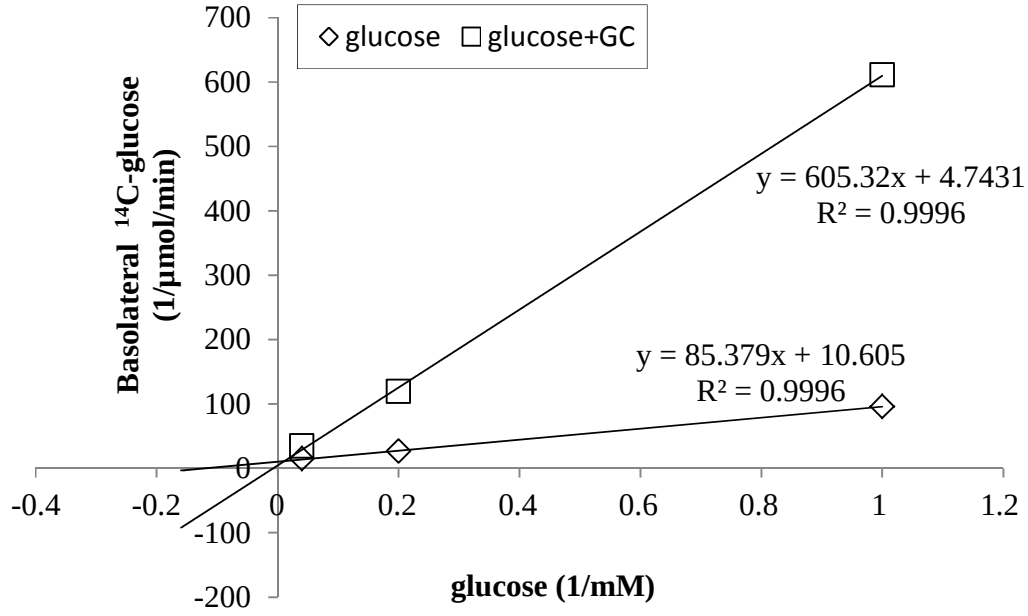
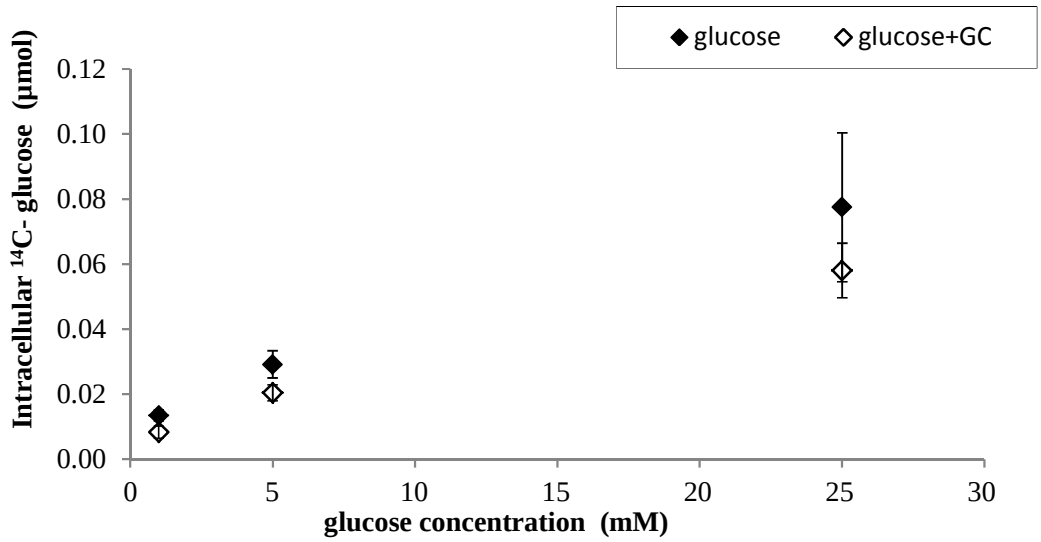



**Figure 41** A: Inhibition of glucose transport by 1 mg/ml German chamomile and transport of glucose at different concentrations of apical glucose.

B: Lineweaver-Burk plot for transport of glucose with/without 1 mg/ml German chamomile. From this, $K_m$ = 8.1 mM glucose and $V_{max}$ = 0.09 µmol substrate hydrolyzed/min. Plot indicative of competitive inhibition. Mean ± SD (n=6 per concentration point).

**A**




**B**

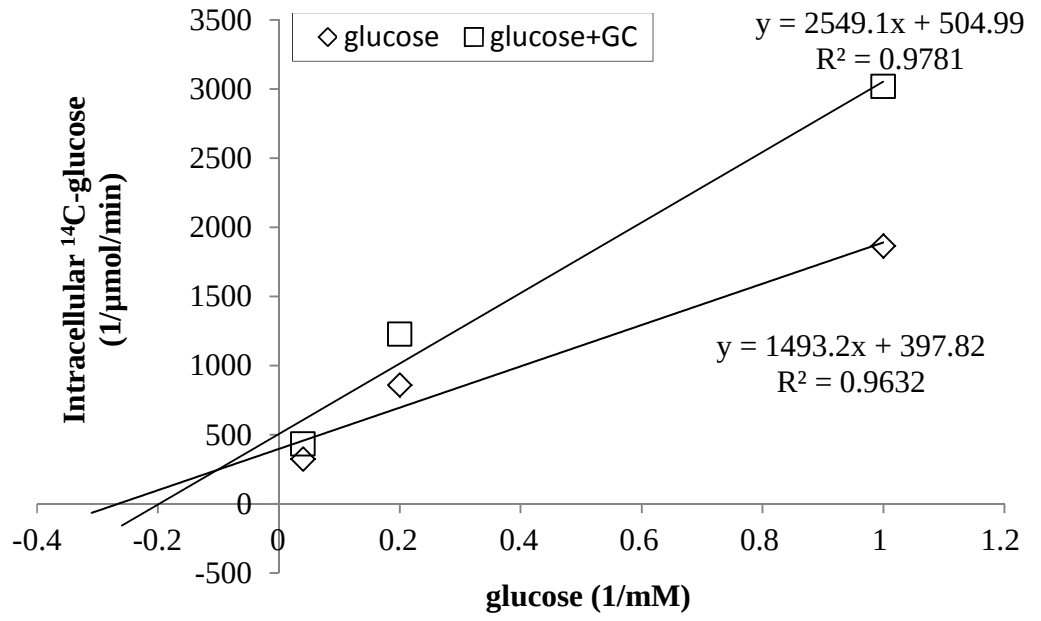



**Figure 42** Cellular glucose uptake by 1 mg/ml German chamomile at different concentrations of apical glucose was significantly decreased ($p<0.01$).

B: Lineweaver-Burk plot for cellular uptake of glucose with/without 1 mg/ml German chamomile. From this, $K_m$ = 8.1 mM glucose and $V_{max}$ = 0.09 µmol substrate hydrolyzed/min. Plot indicative of mix competitive inhibition. Mean ± SD (n=6 per concentration point).

### 4.6.2 Inhibition of glucose transport and uptake by PFS

German chamomile (Figure 43) and green tea (Figure 45) are very effective at attenuating the rate of glucose cellular uptake and basolateral transport. According to the experimental results, *Vitis Viniferae* extract was less effective and Pelagonium and Echinacea were ineffective (Figure 46, Figure 47 and Figure 48). In the intestine, glucose transport is mediated by SGLT1 and GLUT2 at the apical side and exported to the basolateral side via GLUT2. Under $Na^+$-free conditions, only GLUT2 will actively transport the glucose and export to the basolateral side via GLUT2 (Kellett and Brot-Laroche, 2005). Under $Na^+$-dependent conditions and in the absence of German chamomile extract, 9.9 nmol/min of $^{14}C$-glucose was transported into the basolateral compartment. Co-incubation of 1 mM glucose with 1 mg/ml German chamomile extract inhibited the apical to basolateral transport and cellular uptake of glucose up to 82 and 53 %, respectively. Under the same conditions but without $Na^+$ in the transport buffer and in the absence of German chamomile extract, 6.47 nmol/min of $^{14}C$-glucose was transported into the basolateral compartment. The basolateral transport of glucose and cellular uptake of glucose was also inhibited up to 51 and 58 %, respectively (Figure 44). Each concentration of German chamomile extract with and without $Na^+$ significantly reduced the concentration of basolateral glucose ($p<0.01$ and $p<0.05$, respectively). These results demonstrated that the transport of glucose from the apical to basolateral side was lower with $Na^+$ treatment than without $Na^+$ treatment ($p<0.01$). However, cellular uptake of glucose remained the same in both conditions (no significant difference). There are numerous studies regarding green tea or the effect of its polyphenols on health in the literature but there are only a few studies regarding chamomile. Therefore, this study mainly focuses on the effect of German chamomile extract on glucose transport.

**Table 19** Effect of PFS on glucose transport across the intestinal monolayer model and on cellular uptake.

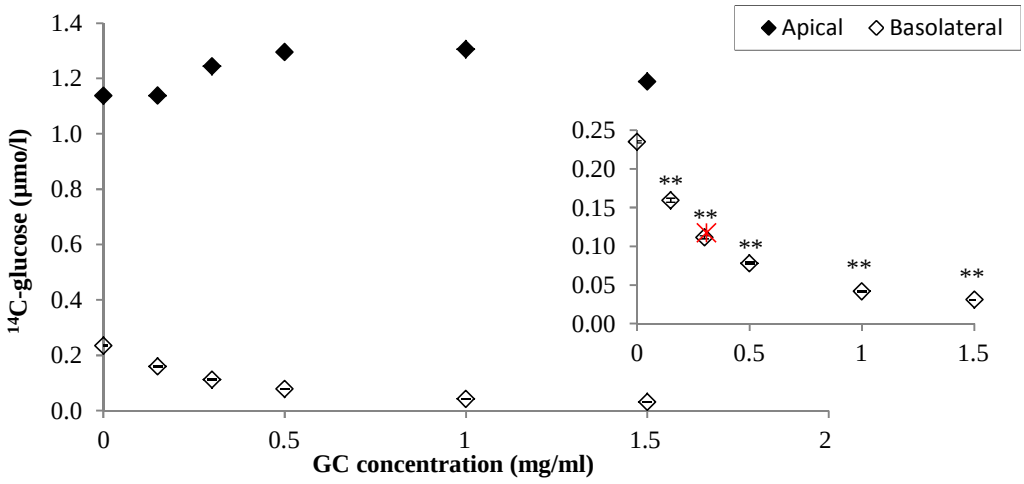
| **Supplement extract** | **$IC_{50}$ (mg/ml) transport** | **$IC_{50}$ (mg/ml) uptake** |
|---|---|---|
| German chamomile | $0.31 \pm 0.05^{\varphi\gamma}$ | $0.56 \pm 0.12^{\gamma}$ |
| Green tea | $0.26 \pm 0.04^{\varphi\gamma}$ | $0.55 \pm 0.17^{\gamma}$ |
| *Vitis Viniferae* | $0.91 \pm 0.07^{\alpha\beta}$ | $1.53 \pm 0.14^{\alpha\beta}$ |
| *Pelargonium* | None | None |
| *Echinacea purp rad* | None | None |
| German chamomile $Na^+$- free | $0.89 \pm 0.19^{\alpha\beta}$ | $0.60 \pm 0.10^{\alpha\gamma}$ |

For transport means of same row followed by Greek letters α represents significant difference compare to German chamomile and β denotes significant difference to green tea, φ represents significantly difference compare to German chamomile $Na^+$-free extract and γ represents significantly difference compare to *Vitis Viniferae* extract ($p<0.05$). Mean ± SD (n=4 per concentration of inhibitior). Tukey HSD test applied.

The concentrations required for the $IC_{50}$ value for basolateral transport and cellular uptake are shown in Table 19. According to Table 19, the green tea extract has the highest inhibitory activity for both uptake and transport of glucose.

The results of these assays indicate that apical GLUT2 is the primary target of the compounds from the German chamomile extract, and the absence of $Na^+$ (inactivation of SGLT1) did not greatly affect the uptake.

**A**




**B**

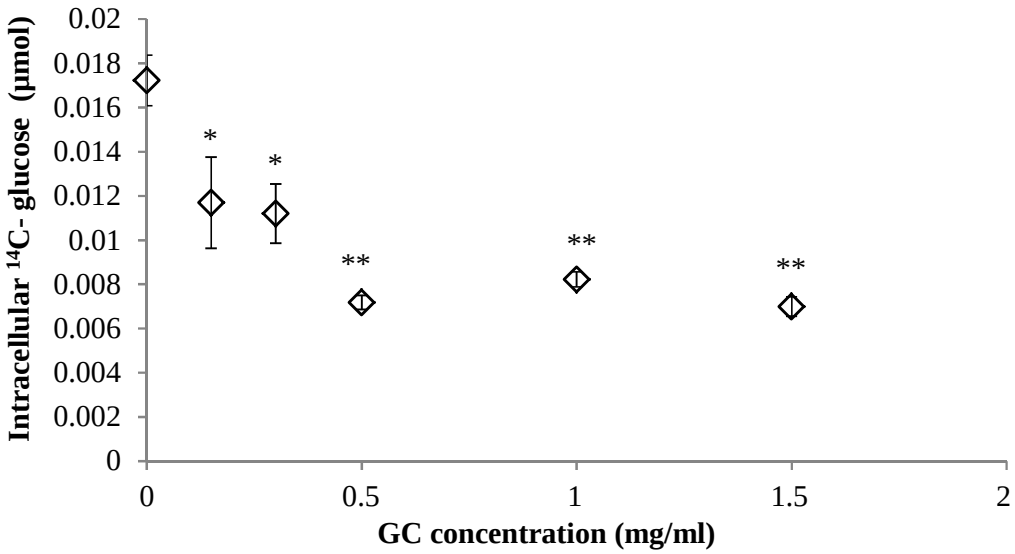
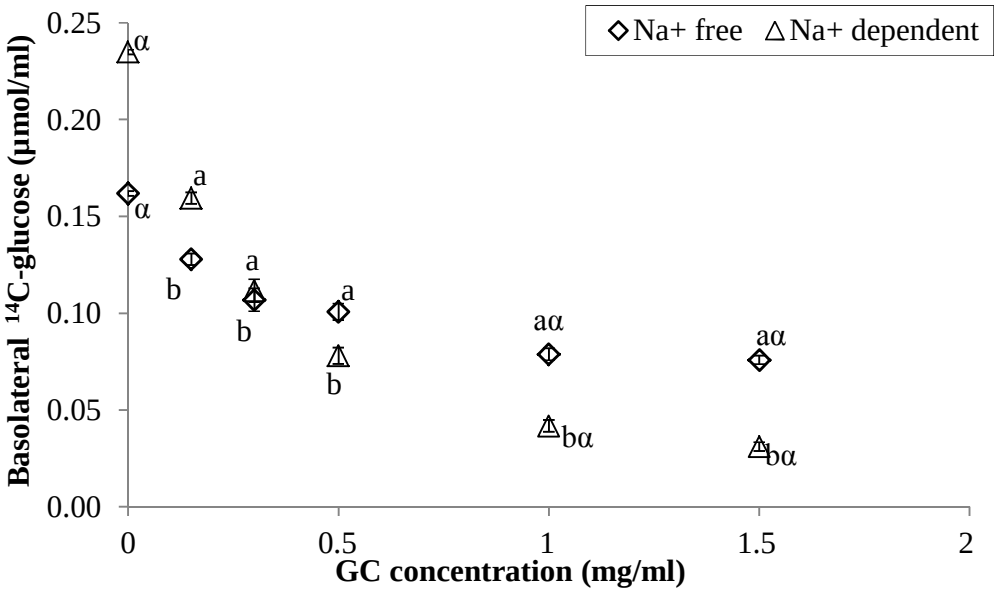
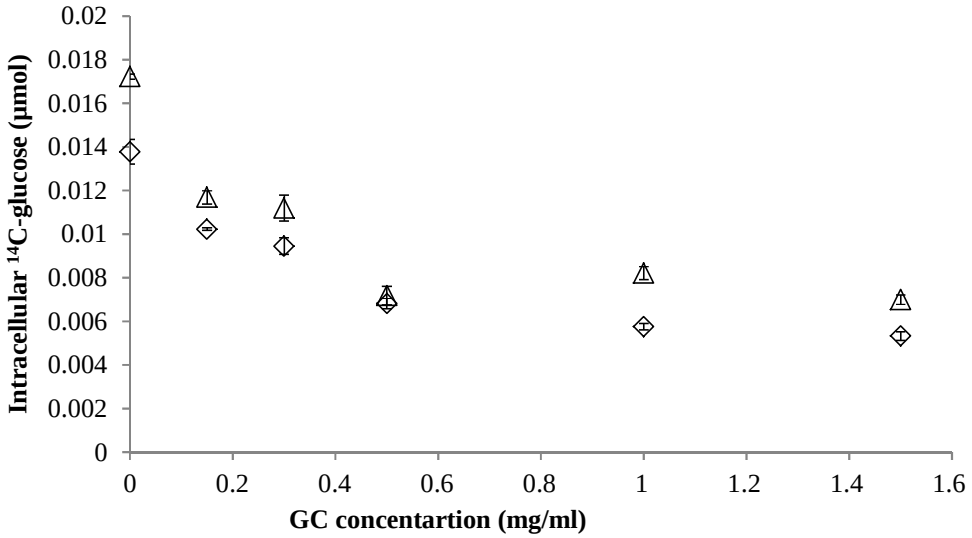



**Figure 43 A**: Inhibition of glucose transport across Caco-2 differentiated monolayers by different concentrations of German chamomile (GC); inset shows the magnified section with estimation of $IC_{50}$.

**B:** Cellular uptake of glucose at different concentrations of GC. The asterisks denotes the significantly different cellular concentration relative to control (*p<0.05, **p<0.01). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=4 per concentration point). Tukey HSD test applied.

**A**



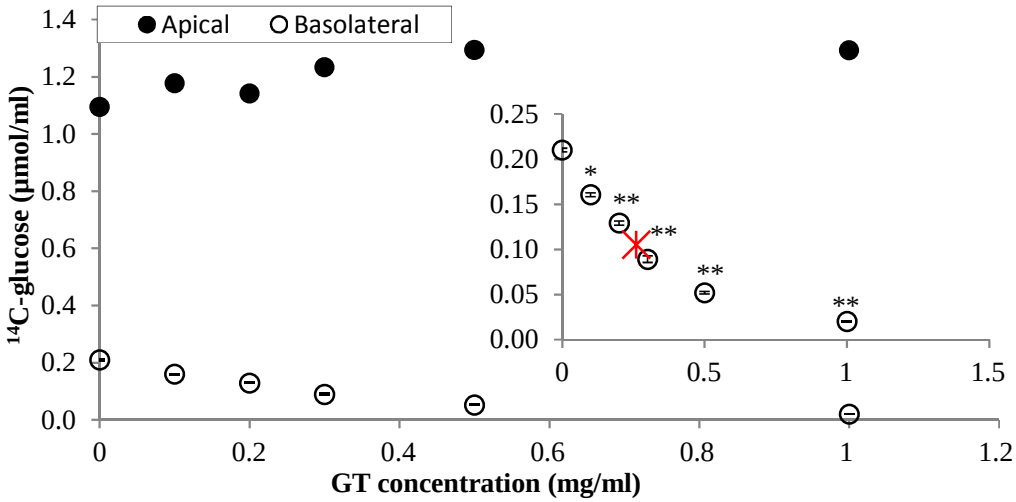




**B**

**Figure 44** A: Comparison of $Na^+$-dependent and $Na^+$-free glucose transport across the Caco-2 differentiated monolayers by different concentrations of GC.

B: Intracellular uptake of glucose with and without $Na^+$ in the transport buffer. The Latin letters denote the significantly different transport relative to the control of each treatment and the Greek letters denote the significantly different transport relative to the same concentration of extract for both treatments ($p<0.01$). Mean ± SD (n=6 per concentration point for each condition). Tukey HSD test applied.

**A**




**B**

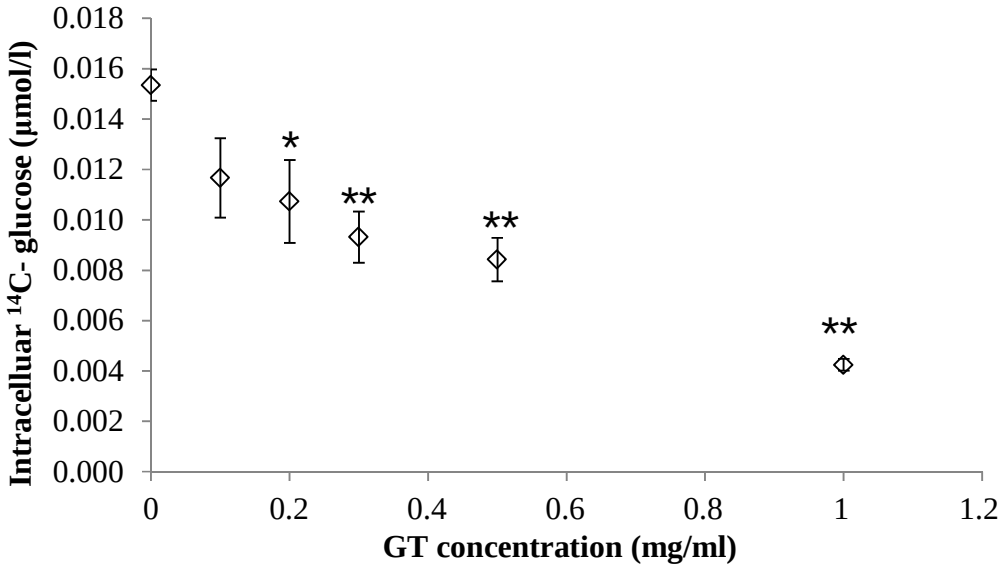
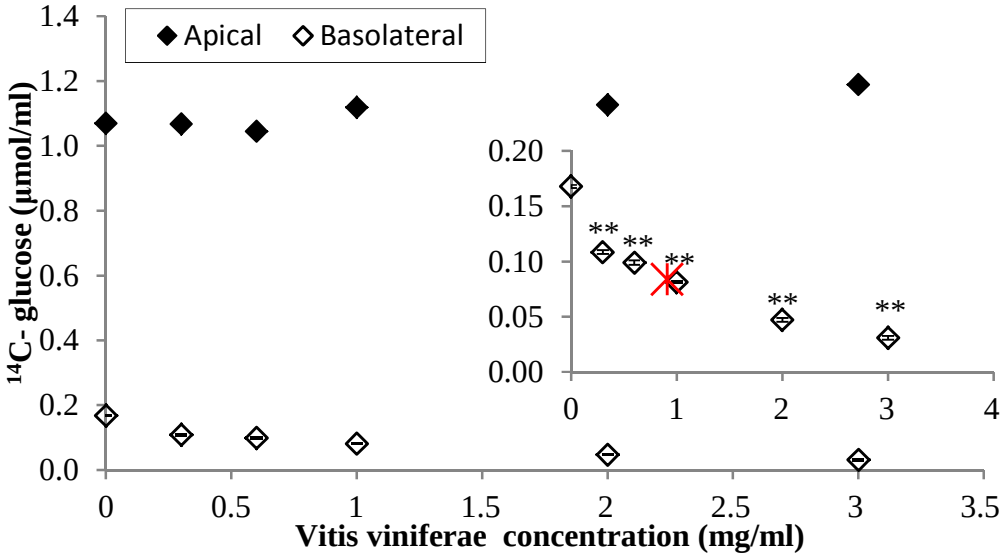



**Figure 45** A: Inhibition of glucose transport across the Caco-2 differentiated monolayers by different concentrations of green tea (GT); inset shows the magnified section with estimation of $IC_{50}$.

B: Cellular uptake of glucose at different concentrations of GT. The asterisks denote the significantly different cellular concentration relative to the control (*p<0.05, **p<0.01). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=4 per concentration point). Tukey HSD test applied.

**A**




**B**

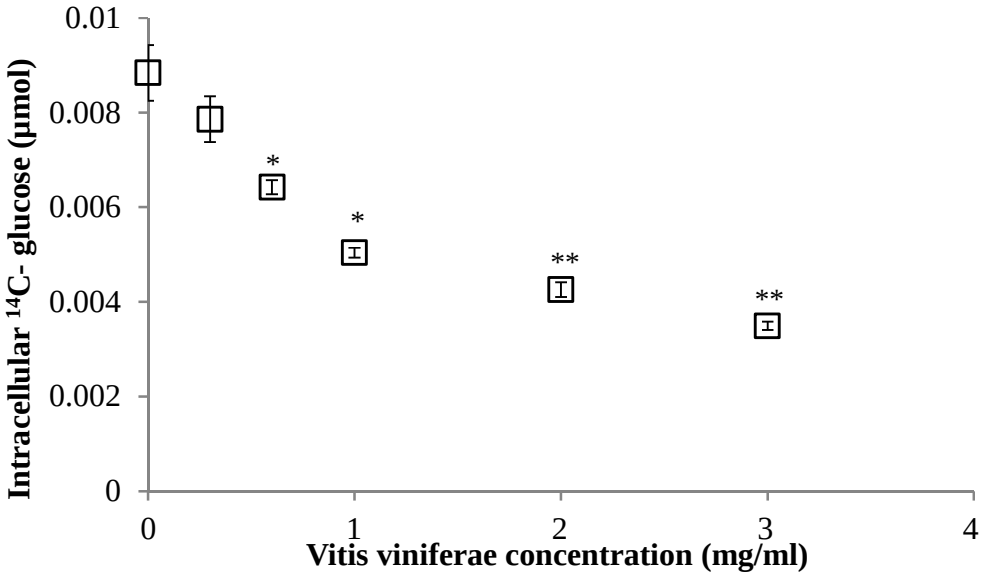
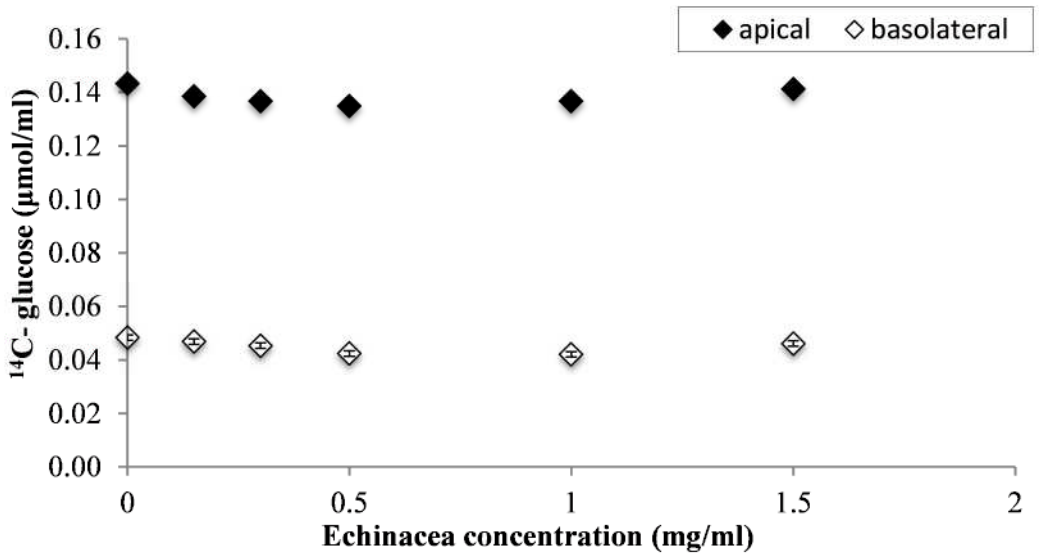



**Figure 46** A: Inhibition of glucose transport across the Caco-2 differentiated monolayers by different concentrations of *Vitis Viniferae.*

The inset shows the magnified section with estimation of $IC_{50}$. B: Cellular uptake of glucose at different concentrations of *Vitis Viniferae*. The asterisks denotes the significantly different cellular concentration relative to the control (*p<0.05, **p<0.01). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=4 per concentration point). Tukey HSD test applied.

**A**




**B**

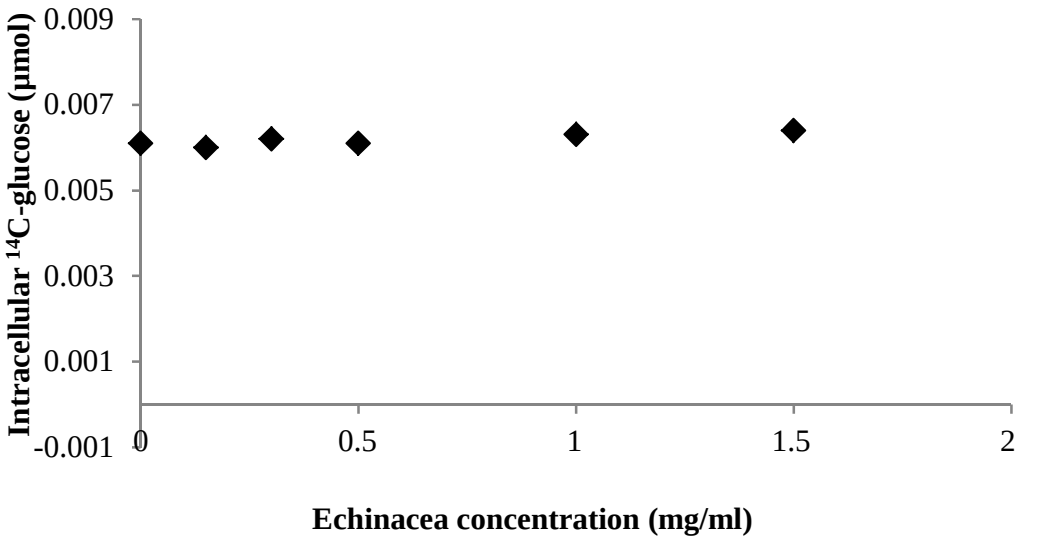
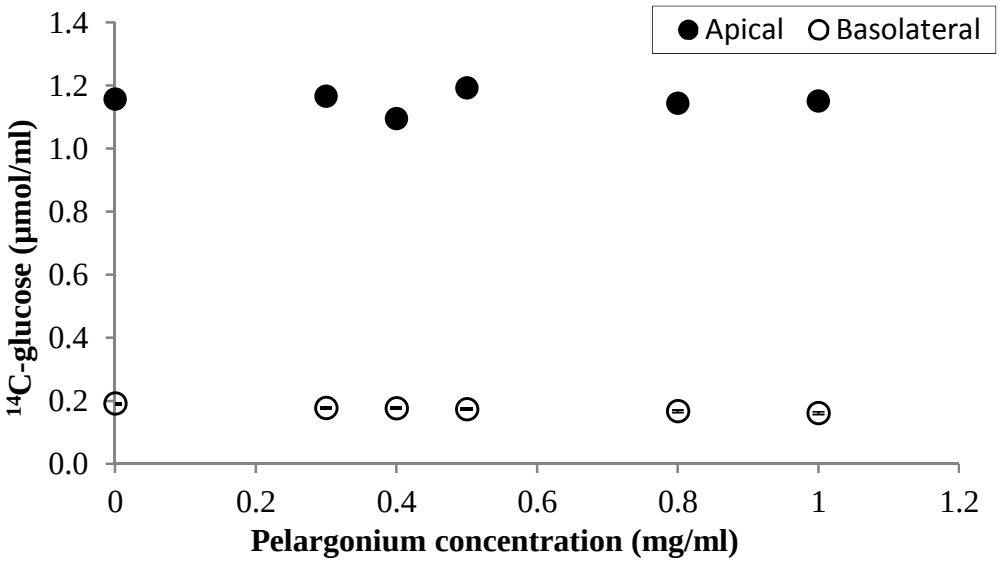



**Figure 47** A: Inhibition of glucose transport across Caco-2 differentiated monolayers by echinacea.

B: Cellular uptake of glucose at the different concentrations of echinacea. Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=4 n=4 per concentration point). Tukey HSD test applied.

**A**




**B**

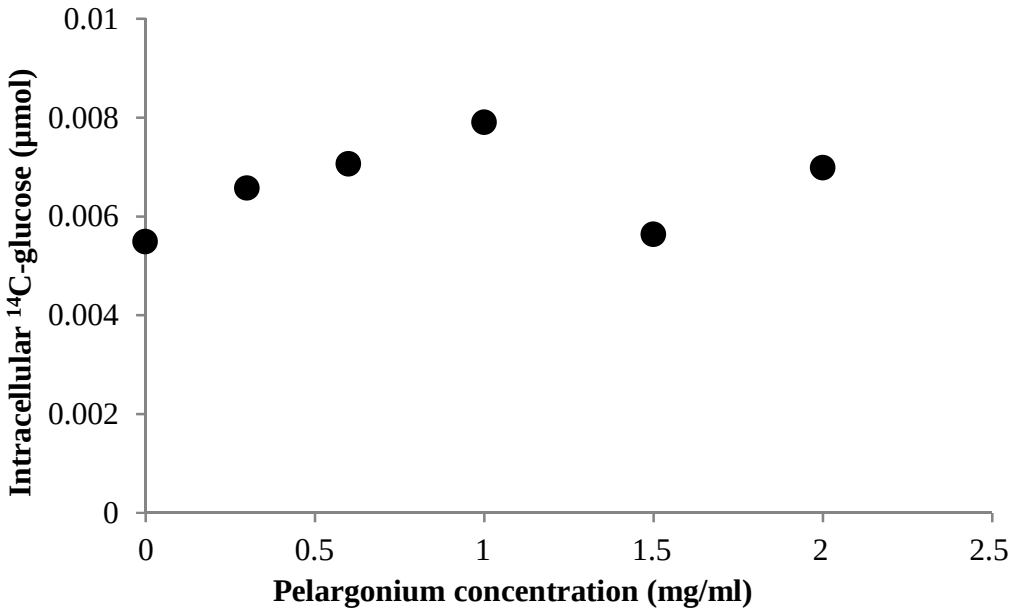



**Figure 48** A: Inhibition of glucose transport across the Caco-2 differentiated monolayers by different concentrations of pelargonium.

B: Cellular uptake of glucose at different concentrations of pelargonium. Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=4 per concentration point). Tukey HSD test applied.

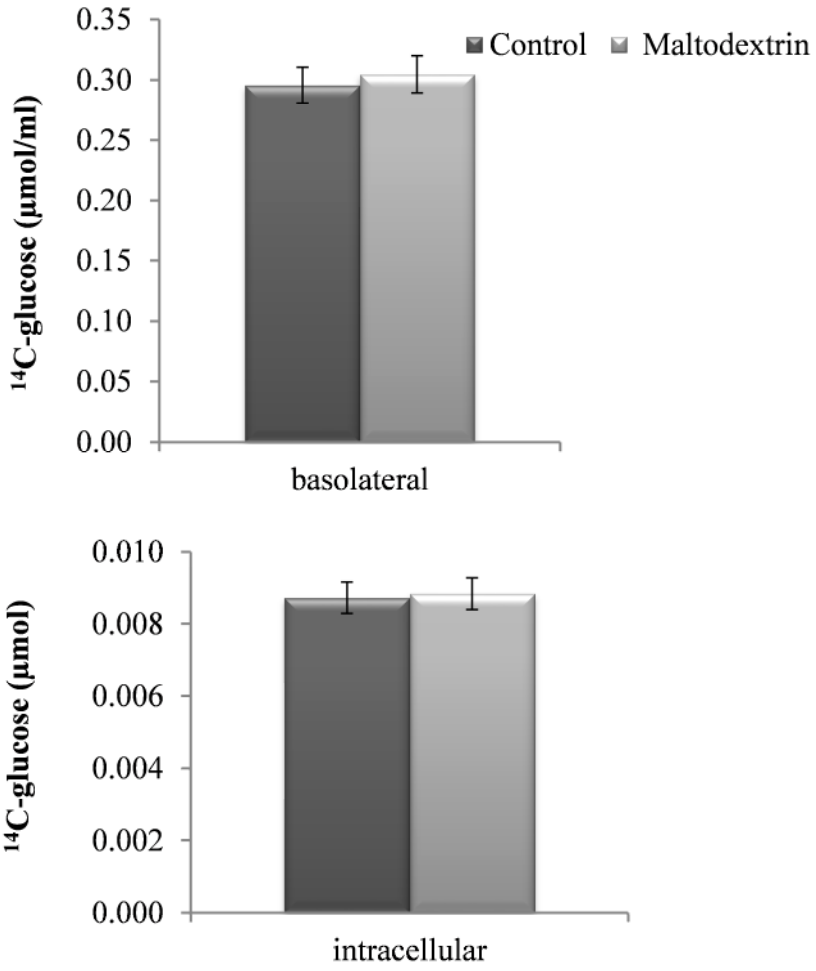



**Figure 49** Intracellular uptake and transport of D-[$^{14}$C]-glucose with/without 0.5 mg/ml maltodextrin.

Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

### 4.6.3 Inhibition of glucose transport with the hydrolysed and extracted form of German chamomile from ethyl acetate and acetonitrile

The impact of metabolism on the efficacy of inhibition was analysed after enzymatic hydrolysis of German chamomile. The extract of German chamomile was hydrolysed using the hesperidinase/cellulase method to resemble the digestion in the gut (Pimpao *et al.,* 2013). Hydrolysis using hesperinidase and cellulose removed the sugars from the polyphenols in the German chamomile

and cleaved the ester bonds between phenolic and quinic acid. As a part of the digestion process, sugars are removed from flavonoid glycosides by the brush border beta-glucosidases in the small intestine (the first step of digestion). This experiment indicates that the digestion affects the biological activity of German chamomile due to glucose transport across the cell monolayer has been decreased approximately 50 % (Figure 50).

One of the main components of German chamomile was apigenin-7-*O*-glucoside. Ferulic acid hexosides were also present at high levels but could not analysed due to absence of standard. German chamomile contains higher amount of apigenin-7-*O*-glucoside after hydrolysis and after exposing with solvent ACN (but its concentration decreased 10 times compared to the water extract due to deglycosylation), and also contains apigenin, umbelliferone and a low concentration of luteolin-7-*O*-glucoside and luteolin. The composition of German chamomile after hydrolysis and extraction using EtAC contains predominantly apigenin-7-*O*-glucoside, apigenin, umbelliferone and a low concentration of luteolin (see chapter 2 section 2.2.2 and 2.2.3).

The inhibition of glucose transport with untreated German chamomile and ACN dissolved sample at the same concentration (0.5 mg/ml) resulted in 67 and 32% inhibition, respectively. In comparison, the EtAC-extract sample (0.25 mg/ml) showed 34% inhibition of the glucose transport. Concentration of both extracted samples was different due to the volume of solvent used during hydrolysis of German chamomile process.

TEER measurements during pre- and post-incubation were both >300 Ω for all conditions. According to Table 20, the constituents of the hydrolysis assay have an effect on glucose transport. The matrix of the hydrolysis assay content had an effect on the glucose transport. Therefore the constituents of the hydrolysis reaction assay, not containing German chamomile, were tested to determine their affect on glucose transport (EtAC control- ACN control samples). Ultimately, these results were subtracted from the experiment containing hydrolysed German chamomile (Table 20). The transport and uptake of glucose with ACN-control sample decreased significantly compare to control sample (without extract in the glucose transport assay), but EtAC-control was not significant than control

sample. The transport of glucose with the German chamomile hydrolysed sample decreased significantly for both EtAC extraction and ACN treatment ($p<0.05$, $p<0.01$ respectively) (Table 20). The cellular uptake glucose was significantly decreased with German chamomile hydrolysed ACN treated sample but the cellular uptake of glucose with EtAC-extracted sample was significantly increased compare to control sample. However the cellular uptake of glucose with ACN-control sample and ACN treated sample were same therefore it was concluded that the background of the hydrolysis process is affecting the cellular uptake. The EtAC-extracted sample soluble at a final concentration of 0.01 % DMSO in TBS; however, it participated in the apical compartment after the incubation process. This might explain the reason for the increase in the cellar uptake of D-[$^{14}$C]-glucose. It may also come from the quenching effect of the EtAC extracted sample to the radiolabelled sucrose.

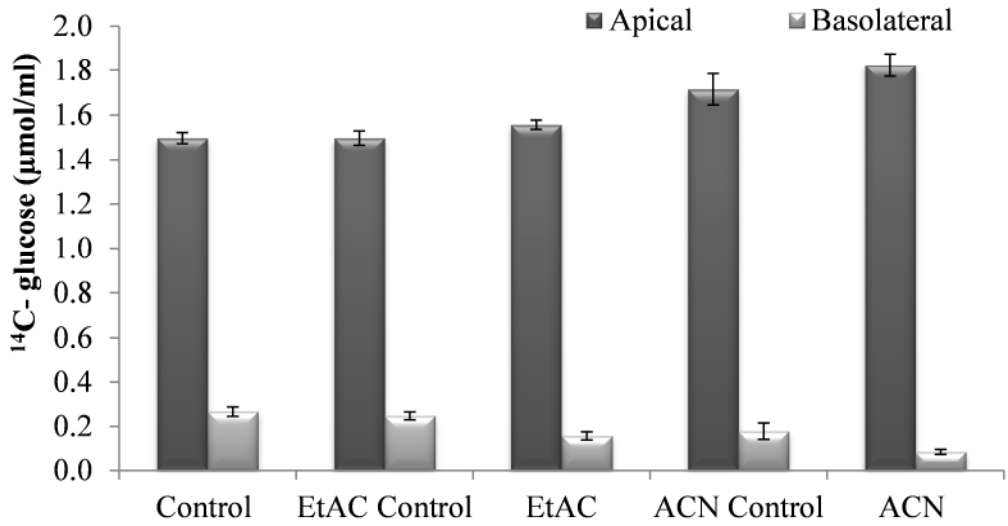
These results suggested that after ingestion of the PFS, the intact and hydrolysed polyphenols (in the gut lumen) have an important role in the attenuation of glucose absorption.

**Table 20** Cellular uptake and transport of D-[$^{14}$C]-glucose (nmol/min) with German chamomile, with hydrolysis followed by extraction with EtAC and ACN.

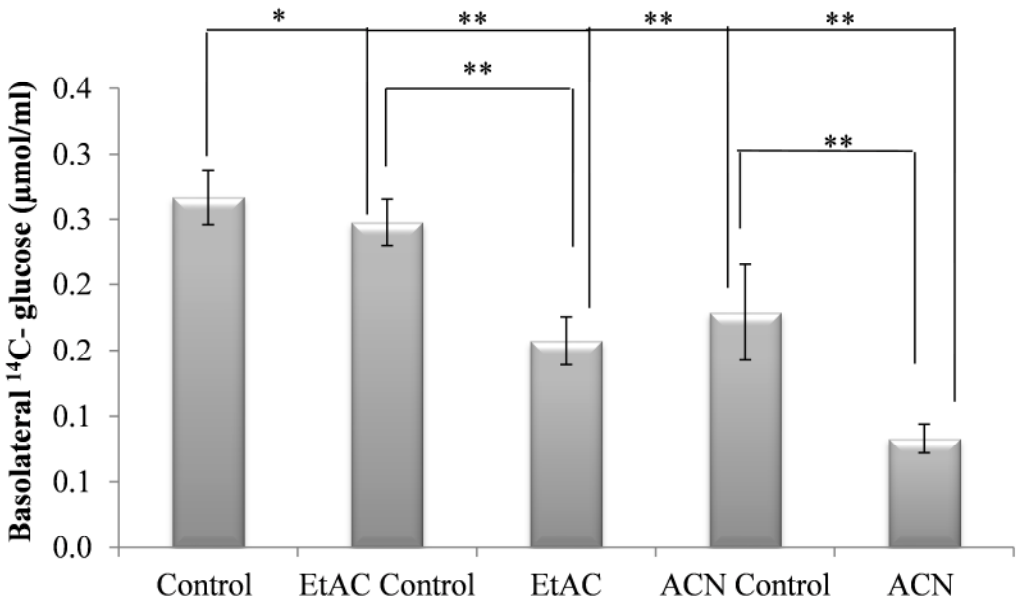
| Sample | Condition (nmol/min) | GC- EtAC (0.255 mg/ml) | GC- ACN (0.512 mg/ml) |
|---|---|---|---|
| Control | Transport | 10.7±0.010 | 11.8±0.012 |
| | Uptake | 1.6±0.004 | 1.6±0.002 |
| Non-GC hydrolysed (EtAC & ACN-control) | Transport | 9.9±0.013 | 7.2±0.015 * |
| | Uptake | 1.6 ±0.005 | 0.71±0.003 ** |
| Hydrolysed GC | Transport | 6.3±0.08 * | 3.3±0.005 * |
| | Uptake | 2.1±0.004 ** | 0.69±0.003 ** |

The hydrolysed control is the extract which is only the matrix of the hydrolysis process without any extract and hydrolysed is with GC. The asterisk denotes a significantly different glucose transport relative to the control (*p<0.01). The asterisk denotes a significantly different cellular concentration relative to the control (**p<0.05). Mean ± SD (n=6 per concentration). Tukey HSD test applied.

**A**



**B**




**C**

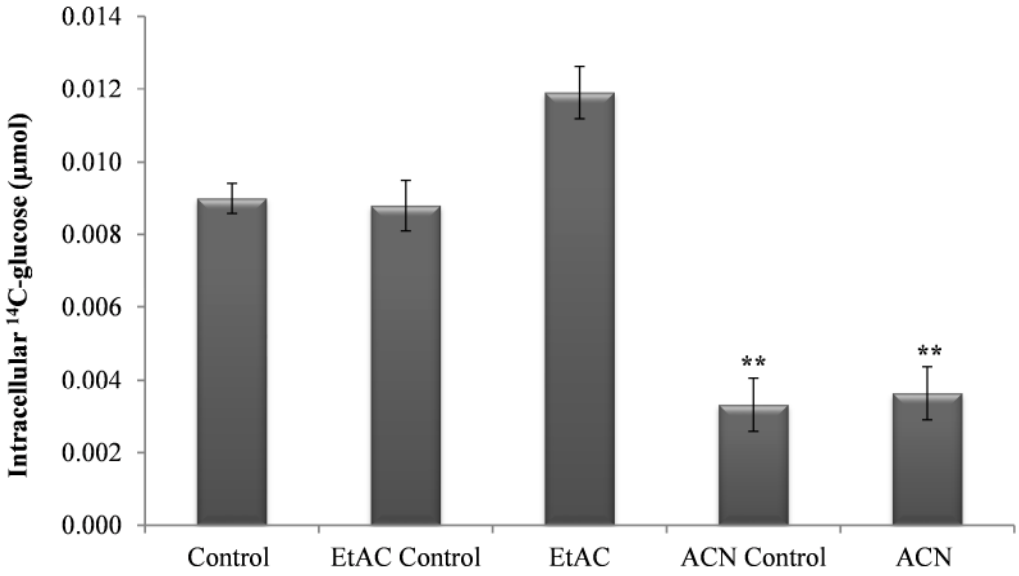



**Figure 50** A: Inhibition of glucose transport by the hydrolyzed and non-hydrolyzed German chamomile (ACN and EtAC) extracts.

B: Basolateral concentration of glucose. C: Intracellular glucose concentration. The asterisks denote the significant different concentrations relative to the control samples (*$p<0.05$, **$p<0.01$). Mean ± SD (n=6 per measurement). Tukey HSD test applied.

### 4.6.4 Inhibition of $^{14}C$- deoxyglucose by PFS extracts

The same method as described for the D-[$^{14}C$]-glucose transport was used to analyze [$^{14}C$]-deoxyglucose transport using the Caco-2 cell line. Deoxyglucose cannot be further metabolised in the intestine since the 2-hydroxyl group is replaced by hydrogen; therefore, it is phosphorylated rapidly and completely and is not effluxed following phosphorylation (Olefsky, (1978), Burant and Bell 1992). Deoxyglucose is not transported by SGLT1 (Wright *et al.*, 2001; Kwon *et al.*, 2006; Kellett, 2001; Kimmich and Randles, 1976; Shirazi-Beechey and Soraya, 1995) and is only transported by GLUTs (Waki *et al.*, 1998; Nelson *et al.*, 1996; Burant and Bell 1992). In the presence and absence of $Na^+$, the transport of glucose was 9.9 and 6.47 nmol/min, respectively, for the control samples. In the absence of PFS (control sample), the rate of deoxyglucose transport was 10.2 nmol/min. SGLT1 is inactive for the glucose transport under $Na^+$-free conditions and deoxyglucose transport. Therefore, the results of these experiments may show that there are other GLUTs that are responsible for the transport of deoxyglucose in the intestine which is in accordance with Burant and Bell (1992). According to Figure 51, there is a substantial inhibition of the [$^{14}C$]-deoxyglucose transport to the basolateral side ($p<0.01$). The transport of deoxyglucose to the basolateral side is comparable to the transport of glucose to the basolateral side as they are transported by the same transporters. Green tea and German chamomile extracts (1 mg/ml) inhibited D-[$^{14}C$]-glucose transport by 90 and 83 %, respectively. [$^{14}C$]-deoxyglucose transport is inhibited by green tea and German chamomile extracts (1 mg/ml) by 80 and 71%, respectively.

In contrast with the cellular uptake of glucose, deoxyglucose uptake into the cell was not inhibited by PFS. Inside the cell, glucose is converted into pyruvate by glycolysis. The first step is phosphorylation of glucose by hexokinase which adds a charged phosphate group to glucose (glucose-6-phosphate). Subsequently, glucose phosphate isomerases glucose 6 phosphate to fructose 6 phosphate and then by phosphofructokinase (PFK1) to fructose 1,6 biphosphate and ADP. The latter is important for glycolysis as it is an irreversible step. This enzyme is an allosteric enzyme, controlled by some activators (AMP, fructose 2-6 biphosphate) and inhibitors (ATP, glucagon, low pH). Deoxyglucose is also an

inhibitor of PFK as there is no signaling involved with deoxyglucose inside the cell while glucose signals to PFK. It may be concluded that PFS extracts play an important role in glucose metabolism leading to a decrease in the cellular uptake of glucose.

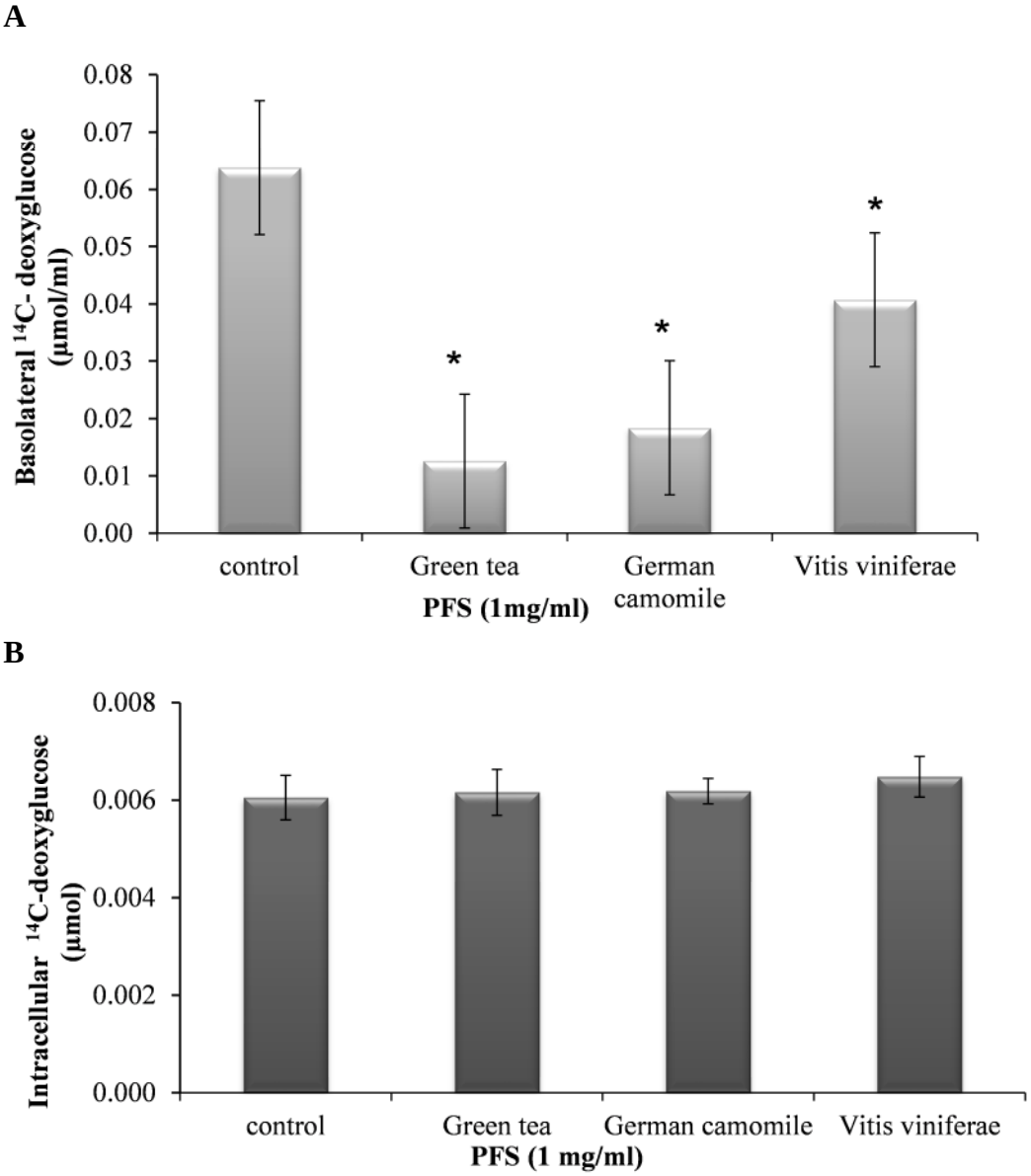
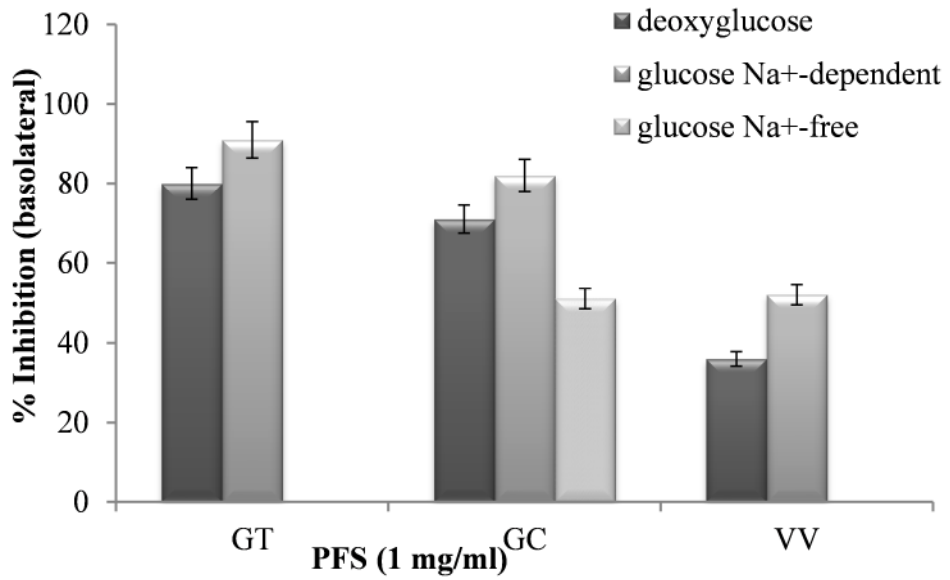



**Figure 51** A: Inhibition of deoxyglucose (1 mM) transport across the Caco-2 differentiated monolayers by 1 mg/ml PFS extracts. Asterisks denote significantly different glucose transport relative to the control (*$p<0.01$). B: Cellular uptake of deoxyglucose in the presence of 1 mg/ml PFS extracts. Mean ± SD (n=6 per measurment). Tukey HSD test applied.

**A**




**B**

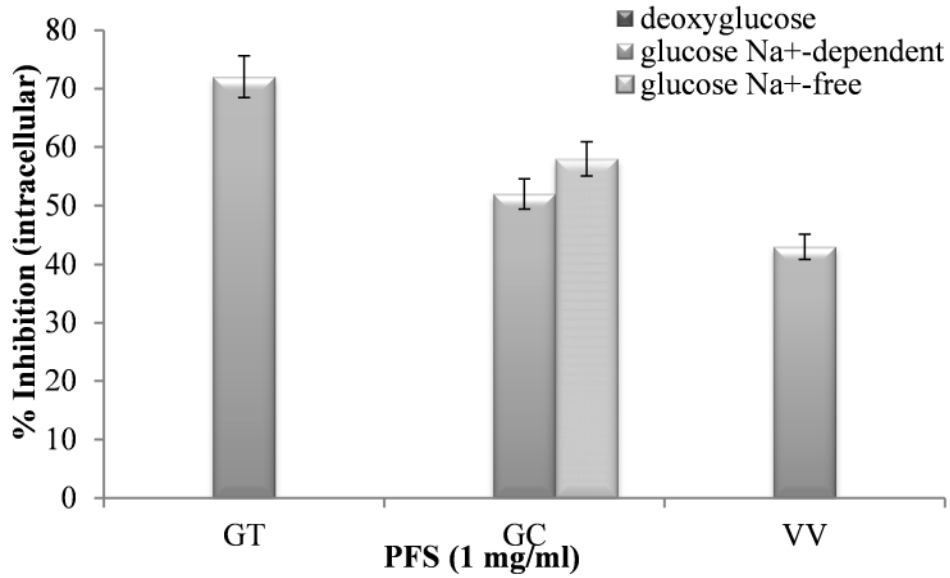



**Figure 52** A: Percentage inhibition of deoxyglucose (1 mM) and glucose (1 mM) (with/without $Na^+$) transport across the Caco-2 differentiated monolayers by 1 mg/ml PFS extracts.

B: Percentage inhibition of intracellular uptake of deoxyglucose and glucose in the presence of 1 mg/ml PFS extracts. Mean ± SD (n=6 per measurment). Tukey HSD test applied. Inhibition of intracellular deoxyglucose was not observed with extracts.

### 4.6.5 Glucose transport inhibition by German chamomile active components in Caco-2 cells

Inhibition of D-[$^{14}$C]-glucose uptake and transport was tested with some of the available compounds, which are present in the German chamomile extract.

Luteolin was the most effective inhibitor of D-[$^{14}$C]-glucose transport and luteolin-7-*O*-glucoside was the most effective inhibitor for cellular uptake of glucose. Following this, apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside also inhibited D-[$^{14}$C]-glucose uptake and transport. Quercetin and apigenin also demonstrated some inhibition (Figure 53 and Figure 54).

Apigenin, luteolin and quercetin did not dissolve completely, and so we assume that they were saturated in the transport buffer. Apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, diCQAs and umbelliferone were soluble under all the assay conditions.

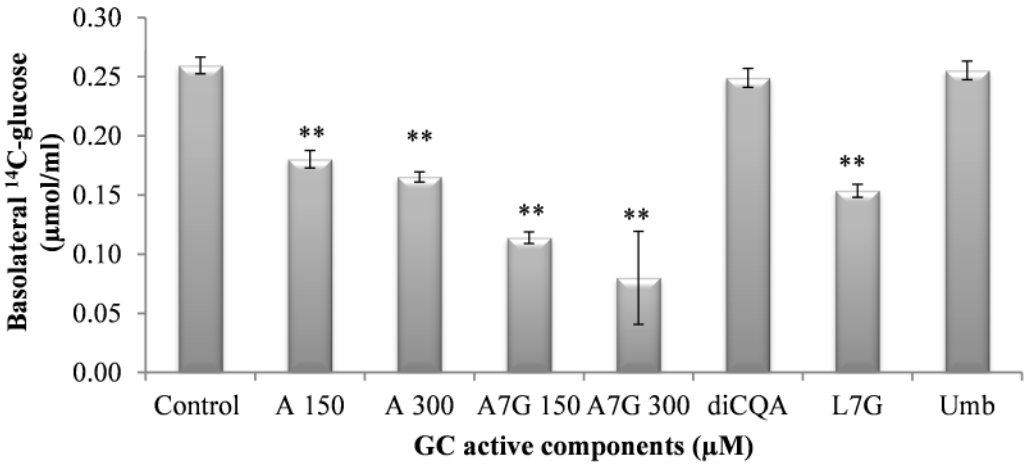



**Figure 53** Inhibition of glucose uptake by German chamomile (GC) active components (control represent the sample without any inhibitor).

A: Apigenin, A7G: Apigenin-7-*O*-glucoside, L: Luteolin L7G: Luteolin-7-*O*-glucoside, diCQA: 100 µM of each 3,4-dicaffeoylquinic acid, 3,5 diCQA and 4,5 diCQA, Umb: Umbelliferone. The asterisk denotes a significant difference from the corresponding control value, *$p<0.01$, **$p<0.05$. Mean ± SD (n=6 per measurment). Tukey HSD test applied.

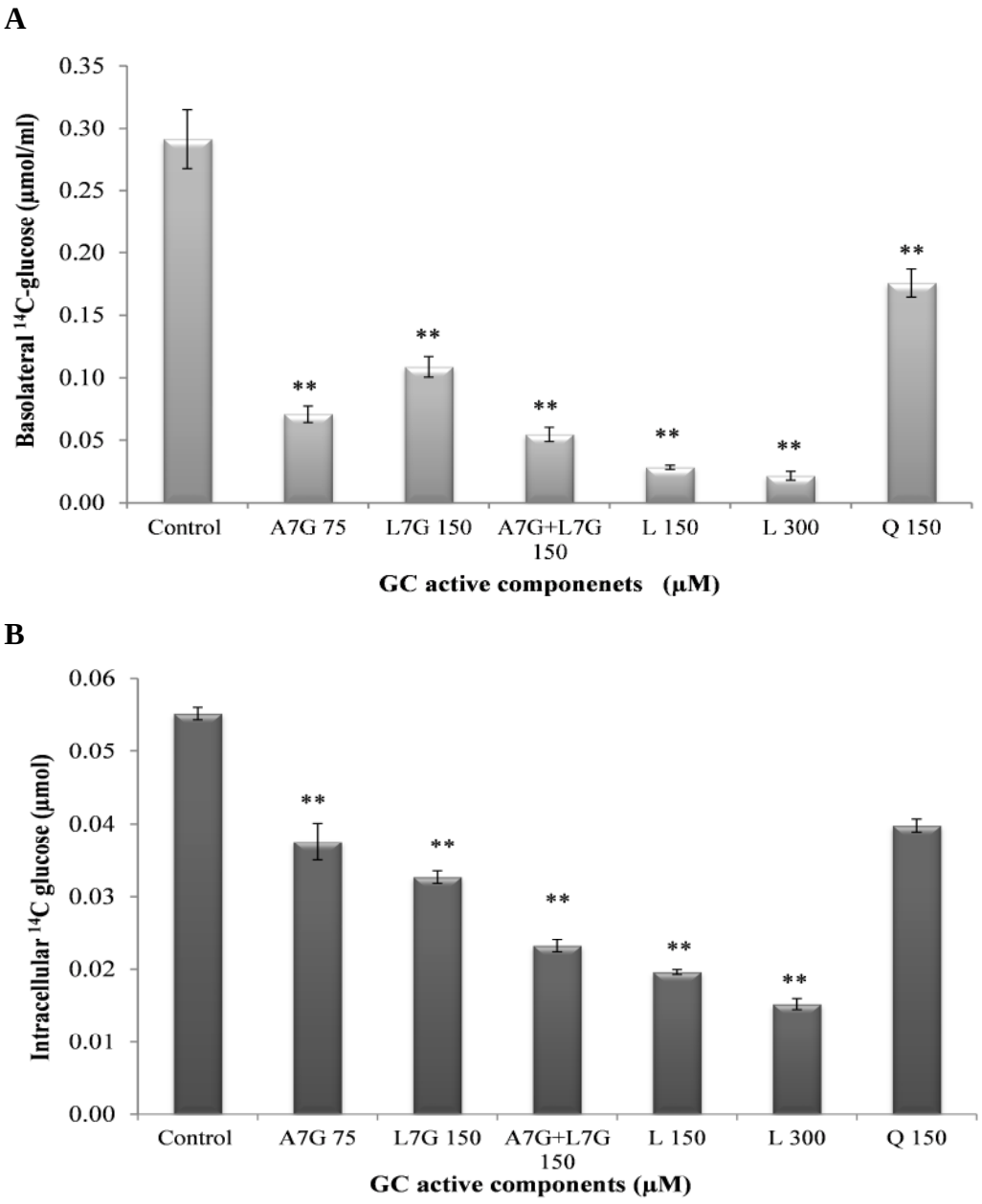



**Figure 54** Inhibition of glucose transport to the basolateral side by the German chamomile (GC) active components (control represents the sample without any inhibitor compound).

A: Apigenin, A7G: Apigenin-7-*O*-glucoside, L: Luteolin L7G: Luteolin-7-*O*-glucoside, Q: Quercetin diCQA: 100 µM of each 3,4-dicaffeoylquinic acid, 3,5 diCQA and 4,5 diCQA, Umb: Umbelliferone. The asterisks denotes a significant difference from the corresponding control value, *p<0.05, **p<0.01. Mean ± SD (n=6 per measurement). Tukey HSD test applied.

### 4.6.6 Glucose transport after overnight FBS starvation

FBS contains important components such as hormones and growth factors. Therefore, the addition of FBS to the cell medium promotes cell adhesion and proliferation (epidermal growth factor, fibronectin, and thyroxine). To create starvation stress in the cells, FBS was removed from the cell culture medium 12 h before the experiment.

According to these results, $^{14}$C-glucose transport was not-significantly decreased with FBS starvation compared to the presence of FBS ($p<0.05$) and cellular uptake was also not affected. Addition of German chamomile during the transport experiment after overnight FBS starvation had an 89 % inhibition of glucose transport (not different from FBS-treated cells in the presence of PFS extract). The cellular uptake of glucose was also significantly decreased when cells were treated with German chamomile after FBS starvation (12 h). By contrast, pre-treatment of cells with German chamomile and without FBS to analyse glucose retention was investigated, and transport and cellular uptake of glucose was not affected. Therefore, it may be assumed that relevant transcript regulation was not occurred (

Table 21). Addition of extra glucose (1 mM) to the feeding medium overnight (12 h) did not have any effect on the transport and cellular uptake of D-[$^{14}$C]-glucose ($p<0.05$).

Cells deprived from FBS 12 h before the experiment did not show any differences in glucose transport and cellular uptake.

**Table 21** Inhibition of glucose uptake and transport compared between FBS starvation overnight and/or treatment with German chamomile and glucose (1 mM) overnight with FBS treatment overnight.

| **Pre-treatment (12 h)** | **Transport (25 min)** | **FBS (%)** | **GC (mg/ml)** | **Glucose in pre-treatment (mM)** | **Transport (a to b) (µmol glucose)** | **Uptake into cell (µmol glucose)** |
|---|---|---|---|---|---|---|
| **no FBS** | Glucose | 0 | 0 | 0 | 0.269±0.005 | 0.008±0.0003 |
| **no FBS+ GC** | Glucose+ GC | 0 | 1 | 0 | 0.029±0.001* | 0.006±0.0003** |
| **no FBS+ GC** | Glucose | 0 | 1 | 0 | 0.286±0.007 | 0.008±0.0002 |
| **FBS+ Glucose** | Glucose | 10 | 0 | 1 | 0.313±0.008 | 0.008±0.0001 |
| **FBS** | Glucose | 10 | 0 | 0 | 0.296±0.005 | 0.009±0.0004 |

The asterisks denote a significantly different transport and cellular uptake concentration relative to the control (with FBS 12h to look at glucose) (*$p<0.01$, **$p<0.05$). Mean ± SD (n=6 per measurement per treatment).

## 4.7 Discussion

In the literature there is growing evidence regarding the effect of polyphenols from green tea or its components on glucose metabolism (interactions of polyphenols with enzymes or transporters) and their promising benefits for the management of type-2 diabetes. There are also some animal model studies and limited human intervention studies regarding the chamomile and grape extracts or their active components on glucose metabolism. However, the literature evidence for the inhibition of cellular glucose uptake and transport by polyphenols across Caco-2 cells is limited. The objective of this study was to make a preliminary assessment of the capacity of PFS extracts to reduce glucose transport across the Caco-2 cell monolayers.

The current study suggested that polyphenols from the PFS extracts were able to decrease the transport of glucose across the cell monolayer. Structural changes may result in altered biological activity (Kellett and Brot-Laroche, 2005). Therefore, the impact of metabolism on the efficacy of inhibition was analysed after enzymatic hydrolysis of the German chamomile. These results suggested that after ingestion of the German chamomile extract, the intact and hydrolysed polyphenols (in the gut lumen) have an important role in the attenuation of glucose absorption. Farrell and colleagues (2013) investigated the effect of hydrolyzed and unhydrolyzed herbal extracts on glucose transport and uptake using the Caco-2 cell line and reported that herbal extract mixtures decreased glucose transport up to 54 %. After enzymatic hydrolysis of herbal extract mixture with glucosidase and esterase activity, polyphenolic fractions were collected using EtAC. This hydrolyzed extract reduced the transport of glucose by 70 %. In the current study, the effect of enzymatic hydrolysis assay constituents (without substrate) on glucose transport was also analysed (40 % inhibition- due to enzymatic hydrolysis there are compounds which were unknown appear and may have an influence on cellular uptake and transport of $^{14}$C-glucose) and this result was subtracted from the hydrolyzed extract sample (72 % inhibition).

Kamiyama *et al.,* 2010 investigated the inhibition of maltase by EGCG using maltase-expressed Caco-2 cells, with an $IC_{50}$ value of 27 µM. The gallated catechins showed significant inhibition while the ungallated catechins did not. Alzaid *et al.,* 2013 observed that the glucose transport in Caco-2 cells in the presence of berry extract was significantly decreased (58 % of total glucose uptake). They also investigated the effect of cyanidin, cyanidin glucoside and cyanidin rutinoside on glucose uptake (approximately 21, 20, and 21 % of total glucose uptake) and found that all these compounds significantly inhibited glucose uptake.

Johnston *et al.,* 2005 analysed the effect of different classes of dietary polyphenols on glucose uptake with Caco-2 cells (cells were seeded to plates therefore there were not any monolayer). They investigated $Na^+$-dependent and $Na^+$-free conditions for glucose uptake in the presence of flavonoid glycosides, non-glycosylated polyphenols, aglycones and phenolic acids. They found that aglycones inhibited GLUT2 and glycosides inhibited SGLT1. Under $Na^+$-dependent conditions, the catechins, EC, EGCG, EGC and ECG, significantly decreased glucose uptake. Under $Na^+$-free conditions, EGCG, EGC and ECG reduced glucose uptake by 37, 60 and 65 %, respectively; however, catechin and EC were ineffective. Under $Na^+$-free conditions, quercetin, apigenin and myricetin also inhibited glucose uptake significantly. In the current study, under $Na^+$-dependent conditions, polyphenols from the German chamomile extract were analysed to discover their individual effect on glucose transport and uptake. As was reported by Johnston and colleagues (2005), our data also represented that flavonoid glycosides inhibited both transport and uptake of glucose, and while cellular uptake and transport of glucose was not affected by phenolic acids. In contrast to the Johnston *et al.,* (2005) study, apigenin significantly inhibited both transport and cellular uptake of glucose under $Na^+$-dependent conditions. Aglycone flavonoids (especially luteolin) also inhibited both transport and cellular uptake of glucose conversely to the Johnston and colleagues (2005) study.

Under $Na^+$-free and $Na^+$-dependent conditions, the transport of glucose was analysed in the presence of the polyphenol-rich herbal extract using Caco-2 cells

and showed 54 and 35 % of inhibition, respectively (Farrell *et al.*, 2013). While the uptake of glucose was not affected under $Na^+$-dependent conditions, it was significantly decreased under $Na^+$-free conditions. In contrast to this, cellular uptake of glucose with/without $Na^+$ did not change in the current study, but they were both lower than control (without PFS) samples. The transport of glucose was also lower in the presence of $Na^+$ compared to $Na^+$-free conditions.

It was reported that flavonoid glycosides interact with SGLT1 (Day *et al.*, 2003), and therefore they may compete with glucose for absorption. Kottra and Daniel, (2007) reported that flavonoids were not absorbed through SGLT1 using SGLT1 expressed in *Xenopus laevis* oocytes. Kobayashi *et al.* (2000) reported that tea polyphenols inhibited SGLT1 competitively, whereas Hossain *et al.* (2002) reported that SGLT1 was inhibited by tea polyphenols non-competitively. Kwon *et al.* (2007) analysed glucose, 2-deoxyglucose and fructose transport using GLUT2 expressed *Xenopus laevis* oocytes in the presence of quercetin, myricetin and isoquercetin. They reported that GLUT2 was non-competitively inhibited by those compounds, whereas SGLT1 was not affected. At a concentration of 50 µM quercetin, the transport of glucose was inhibited completely. Quercetin, myricetin, luteolin and apigenin inhibited 2-deoxyglucose transport with an $IC_{50}$ value of 12.7, 17.2, 30.4 and 65.7 µM, respectively. Our study showed that the inhibition of GLUT2 is greater than SGLT1 as the absence of $Na^+$ did not greatly affect the pattern of inhibition. The transport of deoxyglucose was also decreased in the presence of PFS extracts and the transport of glucose in the absence of $Na^+$ was slightly lower than deoxyglucose transport (in both analyses SGLT1 is inactive). In addition, cellular uptake of deoxyglucose was analysed and PFS did not affect the cellular uptake. The reason for this may be that PFS has an effect on glucose metabolism inside the cell. By contrast, PFS may not have an effect on cellular uptake of deoxyglucose as it is not further metabolised inside the cell.

These results may in part explain the inhibitory effect of PFS extracts on glucose uptake and transport in the current study.

The effect of polyphenols on sugar metabolism has also been investigated in several human intervention studies. Toolsee *et al.* (2013) studied pre-diabetic subjects who consumed 3 cups of green tea or water as a control daily for 14

weeks. They found that green tea consumption did not affect fasting glucose but prevented impaired fasting glucose from a significant increase as compared to the control. Therefore, they concluded that green tea is a potent glycaemic regulator. Park *et al.* (2009) found that circulating green tea catechins elevated blood glucose level by preventing normal glucose uptake into tissues resulting in secondary hyperinsulinemia. Zunino (2009) reviewed several reports and concluded that grapes or grape products could be used in the management of type-2 diabetes due to their abundant polyphenol content and low glycaemic index. Banini *et al*, (2006) observed the effect of muscadine grape juice (MJ), muscadine grape wine (MW) and dealcoholized muscadine grape wine (dMW) on blood glucose levels of healthy and type-2 diabetic subjects over 28 days. Daily intake of 150 ml of MW and dMW consumption with meals decreased blood glucose levels compared to diabetics administered MJ. The effect of the ethanolic chamomile flower extract was analysed for the treatment and prevention of type-2 diabetes with both *in vivo* and *in vitro* studies (Weidner *et al.*, 2013). The *in vitro* study showed that chamomile flower extracts activate PPARγ which regulates insulin sensitivity. Their *in vivo* study showed the reduction of insulin resistance and glucose intolerance with C57BL/6 mice that were fed with chamomile flower extract (200 mg/kg body weight) for 6 weeks. Suksomboon *et al.* (2011) identified 9 randomised placebo-controlled trials and consumed plant supplements studies. They reported that *lpomoea batatas, silybum marianum* and *trigonella foenum-graecum* were shown to improve glycaemic control in diabetic patients.

The effect of polyphenols on sugar metabolism was also investigated with animal intervention studies. Cemek *et al.* (2008) observed the effect of chamomile ethanolic extract (70mg/kg body weight) on hyperglycaemia using streptoztocin (STZ)-induced diabetic rats. They found that treatment with the chamomile extract significantly reduced postprandial hyperglycaemia. The fasting and postprandial blood glucose level of alloxan-induced diabetic rats was observed in the presence of chamomile tea and glibenclamide (standard drug for diabetic treatment) in their diet for 60 days. The measurement of fasting and postprandial blood glucose level was performed on days 7, 30 and 60. Both

fasting and postprandial glucose levels were significantly reduced compared to controls (chamomile replaced with water) for all three days; and was significantly lower than glibenclamide on day 30 only (Khan *et al.*, 2014). Another study investigated the blood glucose levels of STZ-induced diabetic rats and healthy controls when fed with a polyphenol extract of French red wine, or ethanol, or both together (Al-Awwadi *et al.*, 2004). They reported that when control and diabetic rats were fed with only the red wine extract, their blood glucose level decreased. When diabetic rats received only the ethanol or red wine extract, with ethanol there was a reduction in hyperglycaemia compared with the untreated groups. Orhan *et al.* (2006) analysed the effect of aqueous extract from the leaves of *Vitis Vinifera L.* on hyperglycaemia and antihyperglycaemia of healthy, glucose hyperglycaemic and STZ-induced diabetic rats. Treatment with the ethylacetate fraction (polyphenol-enriched extract) reduced blood glucose in the diabetic rats compared to the control animals. Pinent *et al.*, 2004 reported the effect of a grape seed procyanidins extract on glucose metabolism using STZ-induced diabetic rats. A single oral dose of extract (250 mg/kg body weight) significantly decreased the blood glucose level in diabetic rats.

Human and animal studies support the theory of inhibition of glucose transport in the small intestine may prevent hyperglycaemia. As some PFS were very effective on decreasing glucose transport and uptake, they may replace the present management of diabetes treatment (such as acarbose) avoiding some of their side effects. In summary, green tea and German chamomile extracts were very effective at decreasing the glucose absorption across the intestine. The *Vitis Viniferae* extract was less effective compared to green tea and German chamomile extracts. By contrast, PFS of the Pelargonium and Echinacea extracts were ineffective for both transport and uptake of glucose. In the light of these present results, PFS may have a major role in mediating intestinal glucose transport across the brush border membrane of enterocytes; therefore, due to their biological activity, they may improve the management of diabetes.

# CHAPTER 5 TRANSPORT AND METABOLISM OF FRUCTOSE IN CACO-2 CELLS IN THE PRESENCE OF PLANT FOOD SUPPLEMENTS

## Abstract

Fructose transport across the Caco-2 cell monolayer was significantly reduced in the presence of PFS, which may have a major role in mediating intestinal fructose transport across the brush border membrane of enterocytes. Green tea, German chamomile and *Vitis viniferae* extracts were effective on both uptake and transport of fructose dose dependently. Although, 3 mg/ml *Vitis viniferae* extract was not able to achieve 50% inhibition of the transport. The uptake and transport was also tested at different time points. It was observed that both uptake and transport of fructose was time dependent. After FBS overnight starvation, it was observed that the cellular uptake and transport of fructose was not significant compared to the normal condition FBS treatment.

Different concentrations of fructose were also analysed in the presence of 1 mg/ml German chamomile extract to determine the kinetic mechanism of inhibition. $^{14}$C-fructose uptake and transport significantly decreased at all various fructose concentrations with 1 mg/ml German chamomile ($p<0.01$). To analyse the most active inhibitor in the German chamomile extract, some of the components were analysed. Some of those compounds inhibited both uptake and transport of $^{14}$C-fructose. However, none showed high inhibition that could affect both uptake and transport of fructose. Umbelliferone and diCQA (3,4diCQA, 3,5 diCQA and 4,5 diCQA) were ineffective on the cellular uptake and transport of fructose. The response of Caco-2 cells to the stress (FBS starvation or fructose load overnight) was assessed after treatment with German chamomile or additional fructose. Both transport and uptake of fructose were tested. FBS starvation or the addition of German chamomile overnight (12 h) were ineffective on both cellular uptake and transport of fructose, however,

addition of German chamomile extract without FBS overnight treatment and during the transport experiment, fructose significantly decreased ($p<0.01$). Fructose intake is increasing dramatically, especially with the consumption of processed foods. Due to the inhibitory effect of PFS on fructose metabolism, they may have a role in managing metabolic disorders.

## 5.1 Introduction

### 5.1.1 Transport of Fructose Metabolism in Caco-2 cell model

Fructose has been a part of the human diet for thousands of years but its consumption has been increasing dramatically. Fruits have the highest concentration of fructose and it is also widely used as a sweetener in processed foods, such as high fructose corn syrup in beverages. Cereals and beverages are rich in dietary fructose. The American diet has taken 17-20 % of the daily energy intake (330-380 kcal/day) from fructose (Elliot *et al.,* 2002). High dietary levels of fructose intake could lead to the development of type-2 diabetes, through fat storage leading to insulin resistance (Törrönen *et al.,* 2010; Hansen *et al.,* 2012; Halschou *et al.,* 2012).

The absorption rate of fructose in the small intestine is slower than glucose. This difference comes from the absorption process of these monosaccharides. Fructose is absorbed from the lower part of the duodenum and jejunum by the facilitated glucose transporter (GLUT5) at a slower rate. Following this, fructose is transported into the blood by GLUT2 (Riby *et al.,* 1993; Ferraris, 2001). Fructose is transported to the blood from the intestinal lumen by facilitated sodium-independent transport via the glucose transporters GLUT5 and GLUT2. GLUT5 is in the apical and GLUT2 is in the basolateral membrane of enterocytes. The $K_m$ of GLUT5 for fructose is 6 mM and GLUT2 is 11 mM (Manolescu *et al.,* 2007). From the lumen, fructose is transported to enterocytes by GLUT5 (Figure 55). Inside the enterocytes, part of the fructose is converted into lactate (12% of absorbed fructose) and released into the portal circulation (Tappy and Le, 2010), and the other part is diffused into the blood vessels by GLUT2 (Figure 55). Fructose present in the blood is extracted by the liver. Some studies have shown that fructose absorption is quantitatively limited compared to glucose absorption, for example fructose loading without glucose may cause diarrhoea and flatulence in humans due to the different degree of absorption of fructose (Tappy and Le, 2010).

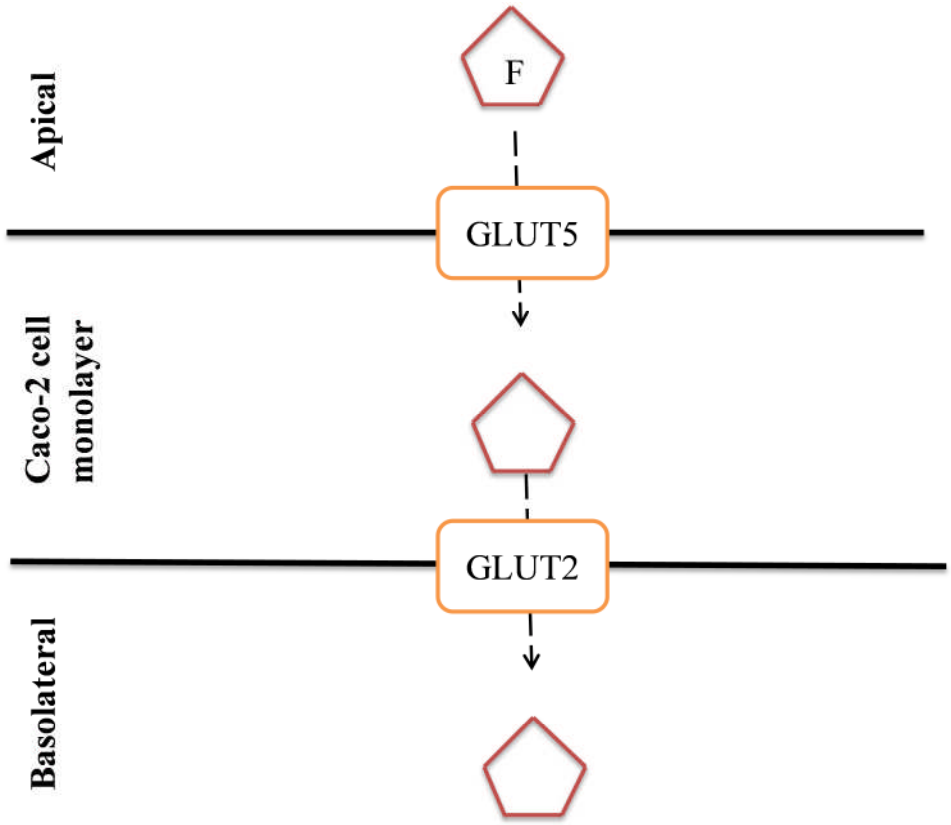



**Figure 55** Mechanism of fructose absorption in to basolateral side in a Caco-2 cell model (F: Fructose).

Numerous studies here tried to link high fructose intake and GLUT5 expression levels with the risk of type-2 diabetes and obesity (Pagliasotti and Horton, 2004; Bizeau and Pagliasotti, 2005; Havel, 2005; Montenen *et al.*, 2007). There are inconsistencies between those studies' in the correlation of diabetes with serum fructose concentration. The serum fructose concentration and urinary fructose excretion were measured for 2 weeks in Japanese healthy volunteers (diabetic and non-diabetic patients). Kawasaki and colleagues reported that the serum fructose concentration and urinary fructose excretion increased significantly in diabetic Japanese patients (Kawasaki *et al.*, 2002). By contrast, Pitkanen, 1996, reported that serum fructose concentrations did not show any difference among healthy and type 1 or 2 diabetes Finnish volunteers. Only a few of the studies investigated the link between diabetes and GLUT5. These studies also have some contradictory findings. In the light of these studies, fructose may have an effect on the pathogenesis of diabetic complication (Gaby, 2005; Gross *et al.*,

2004; Le and Tappy, 2006; Pagliasotti and Horton, 2005). An inconsistent correlation between GLUT5 and diabetes may come from two reasons: diabetes may have an effect on GLUT5 (age- or insulin-dependent) or different levels of dietary fructose in diabetic patients may affect GLUT5 (Douard and Ferraris, 2008). Dyer *et al.* (2002), reported that GLUT5 expression is affected by diabetes in the small intestine. They found that patients with type 2 diabetes had three to four-fold more duodenal GLUT5 mRNA and protein levels. Also, they reported that lowering hyperglycaemia in certain patients down regulated GLUT5. They concluded that the level of fructose in the blood has an effect on GLUT5 expression in the intestine of diabetic patients. In contrast to this study, Zucker diabetic fatty rats and lean control rats were fed with troglitazone (drug improving insulin action) and their mucosa of the small intestine extracted. It was observed that the protein and mRNA levels of GLUT5, GLUT2 and SGLT1 were the same in both types of rats.

There are a number of studies regarding the fructose transporters and diabetes association in human or rats, however, there are few studies for the inhibition of fructose transport by polyphenols. There is one previous study that analysed the inhibition of fructose transport with myricetin, quercetin and isoquercitrin using GLUT2-expressed *Xenopus laevis* oocytes (Kwon *et al.*, 2007) and the half inhibitory concentration was 42.2, 15.9 and 38 µmol respectively.

In this chapter, substantial inhibition on fructose transport and uptake was observed by PFS extracts from green tea, German chamomile and *Vitis Viniferae*.

## 5.2 Material and methods

### 5.2.1 Standards and reagents

D-[$^{14}$C]-fructose 0.1 µCi/ml (ARC 0116A) was purchased from American Radiolabelled Chemicals (St Louis, USA). Fructose was purchased from Fisher Scientific (F/1950/50; Loughborough, UK). PFS extracts were supplied by PhytoLab Co. KG (Vestenbergsgreuth, Germany). Dulbecco's Modified Eagle's Medium (DMEM; including 1000 mg glucose/L, L-glutamine, $NaHCO_3$ and pyridoxine HCl) were purchased from Sigma (Dorset, UK; D6046). Caco-2 cells

(HTB-37) were obtained from the American Type Culture Collection (LGC Promochem, Middlesex, UK).

### 5.2.2 Cell cultures

Chapter 4 section 4.2.2.

### 5.2.3 Fructose transport measurements in Caco-2 cells

#### 5.2.3.1 Preparation of reagents

All the reagents were prepared freshly on the day of the experiment.

#### 5.2.3.2 Transport buffer solution (TBS)

Chapter 4, Section 4.3.1.

#### 5.2.3.3 Fructose

A stock solution of 10 mM fructose was prepared. Briefly, 180 mg of fructose (Fisher Scientific; F/1950/50) was dissolved in 100 ml of TBS. 1 ml of stock solution with/without PFS was added to TBS to a final volume of 10 ml containing 9 µl of 0.1 µCi/µl $^{14}$C-fructose. The pH was adjusted between 7.2 to 7.6 at 37 $^{0}$C for each sample with 1 M HCl or NaOH.

#### 5.2.3.4 PFS extracts preparation

Chapter 4, Section 4.3.1.

#### 5.2.3.5 Preparation of German chamomile active components

Chapter 4, Section 4.3.4.

### 5.2.4 Inhibition assay protocol

Cells between passage 36 and 50 were utilised in the investigation of fructose transport and cellular uptake. A minimum of six replicates was allocated per experimental condition.

On or after 21 days, transport studies were initiated by replacement of growth medium with TBS and cells were washed three times with it. After washing cells, 0.5 ml TBS was added to the apical and 1 ml added to the basolateral, which were pre-incubated for 30 minutes at 37 °C under a humidified atmosphere.

Differentiation of the monolayer was assessed by TEER of the cell monolayers. Only Transwell® inserts with a resistance exceeding a blank membrane by 300Ω were utilised in the experiment. If it is a low value it may increase the paracellular transport of fructose across the membrane due to less well-formed tight junctions. After equilibration, TEER values were recorded from three positions per insert, and subsequently the compartments were carefully aspirated to waste. For experiments, 0.5 ml TBS containing 5 mM fructose solution and 0.045 µCi/ml [$^{14}$C]-fructose with or without PFS extracts (pH 7.4) was added to the apical compartment and 1 ml TBS was added to the basal receiver compartment. Cells were then incubated for 60 min at 37°C. Following incubation, TEER values were measured from three positions per insert again and solutions from the apical and basolateral compartments were removed to scintillation vials immediately. To wash cells for radioactivity assessment, 0.5 ml TBS was added to the apical and 1 ml added to the basolateral compartment, and then transferred into the scintillation vials. Following this, 0.5 ml of 1 M NaOH solution was added to detach the cells from the membrane. The plates were placed on a plate shaker for 30 minutes. Subsequently, the suspended cells were transferred to 1.5 ml centrifuge tubes and 0.4 ml was moved to the scintillation vials to neutralize the cell suspension with 0.4 ml of 1 M HCl. The remaining cell suspension was stored in the freezer. The scintillation cocktail was added to the apical, basolateral and cell suspension samples at a volume of 10, 5 and 5 ml, respectively. All the vials were mixed. Radioactivity was determined using a Packard 1600 TR Liquid Scintillation Analyser. Light emission was counted for 10 min per vial and the number of counts per minute (CPM) was calculated by the equipment software (for the liquid scintillation analysis method, see chapter 4 (section 4.4.1).

### 5.2.5 Statistical analysis

IBM SPSS Statistics 22 was used for the analysis of the data. The Levene test was used to evaluate the homogeneity of the means groups. If the criterion was met, the Tukey HSD post hoc test was applied; otherwise, the Dunnett C followed the one way Anova. The values shown represent the mean values and

the error bars indicate the standard deviation (SD). Unless otherwise stated, differences were considered as statistically significant when $p \leq 0.05$.

## 5.3 Results

### 5.3.1 Set up and validation of the D-[$^{14}$ C]-fructose transport across Caco-2 cells

A calibration curve of radioactivity as a function of [$^{14}$C]-fructose quantity was prepared. With the y-intercept fixed at zero the gradient was calculated as 40030 CPM/µmol and the adjusted $R^2$ was equal to 0.999, suggesting good proportionality of radioactivity and molar quantity (Figure 56). Counting precision of the equipment was assessed by comparing the reference calibration solution of 123000 CPM/KBq (the typical average count was 118679 CPM). The results were corrected for the counter efficiency based on background absorbance standard signals (the typical average count was 29.7 CPM, the solutions involved in the experiment (HCl + NaOH mixture) had a typical count of 37.2 CPM and for TBS this was 35.9 CPM) (n=8).

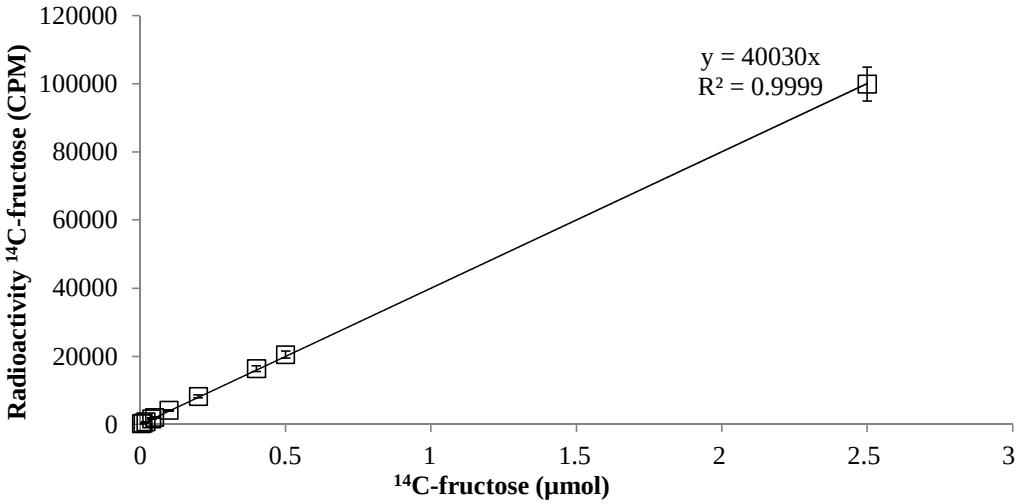



**Figure 56** Fructose standard curve for radioactivity measurements.

(CPM = [$^{14}$C]-fructose counts per minute). Mean ± SD (n=6 per concentration for each of 8 experiment).

Caco-2 cells were plated on 6-well plates at a density of 6.43 x $10^4$ cells/$cm^2$. The experiments were performed after the cell monolayer reached full differentiation. The differentiation of the monolayer was assessed by measurement of TEER values of the cell monolayer. Only Transwell® inserts with a resistance exceeding a blank membrane by 300 Ω were utilised in the experiment. TEER values <300 Ω may be demonstrating the less well-formed tight junctions between the cells. The TEER value of the cells was measured after the pre- and post-incubation process. There was no significance in the pre-incubation TEER values of each well. However, after exposing cells with fructose and PFS extracts (post-incubation) the TEER values were significantly different for some PFS extracts (Table 22). It appears that higher concentrations of Green tea and *Vitis Viniferae* extracts may improve the electrical resistance between the cells.

**Table 22** Blank corrected TEER of Caco-2 monolayers measured after pre-incubation (25 min) and post-incubation (60 min) in the absence (control) and presence of PFS extracts.

| **PFS extracts** | **concentration (mg/ml)** | **TEER (pre-incubation 30 min)** | **TEER (post-incubation 60 min)** | **% difference (pre-post incubation)** |
|---|---|---|---|---|
| **Control** | 0 | 822 | 643 | 22 |
| **German chamomile** | 1 | 1075 | 930 | 14 |
| **Green tea** | 1 | 807 * | 836 * | 0 |
| ***Vitis Viniferae*** | 1 | 973 * | 956 * | 0 |

% difference demonstrates the difference between pre- and post-incubation TEER values. Asterisks denote significantly different cellular concentrations relative to the control ($p<0.01$). Mean ± SD (n=6 measurement per sample and 18 times readings from each sample). Tukey HSD test applied.

The effect of PFS extracts on the transport of fructose from the apical to basolateral compartment and cellular uptake of fructose was investigated. The

rate of basolateral transport from cell culture medium was 1.7 nmol/min and cellular uptake of D-[$^{14}$C]-fructose (5 mM) was 0.6 nmol/min. Addition of different concentrations of German chamomile, green tea and *Vitis viniferae* extracts decreased the transport of D-[$^{14}$C]-fructose to the basolateral side.

Basolateral transport of D-[$^{14}$C]-fructose (5 mM) was analysed at different time points with and without overnight FBS starvation to analyse the starvation stress on Caco-2 cells. There was a linear increase of fructose transport with time (Figure 57). Starvation of FBS for 12 h had no effect on D-[$^{14}$C]-fructose transport from the apical to basolateral side at any of the time points. Cellular uptake of fructose increased linearly with the time (Figure 58). By contrast, cellular uptake of fructose decreased with overnight FBS starvation at both the 10 and 30 min time points (Figure 58).

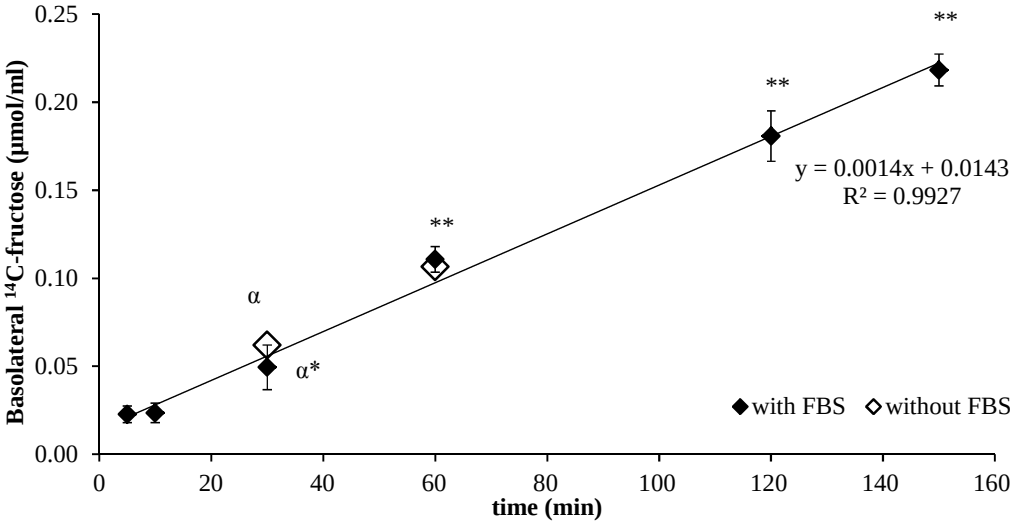



**Figure 57** Transport of 5 mM $^{14}$C-fructose from the apical to basolateral side with and without overnight FBS pre-treatment.

The Greek letter denotes the significance difference from the treatment with FBS and pre-treatment without FBS for 30 min and the asterisks denote the significance differences of the time points compared to the 5 min time point (*$p<0.05$, **$p<0.01$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per time point). Tukey HSD test applied.

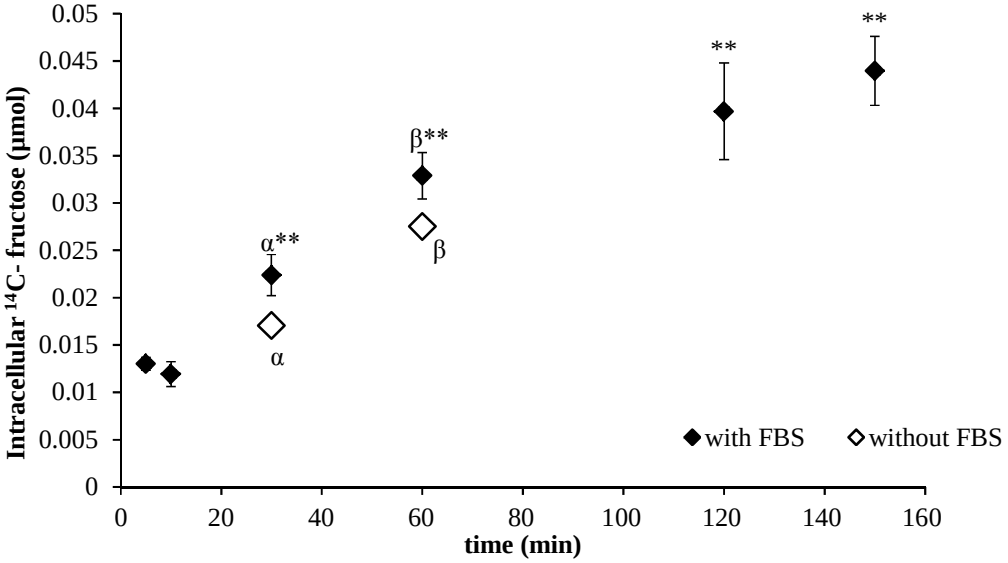
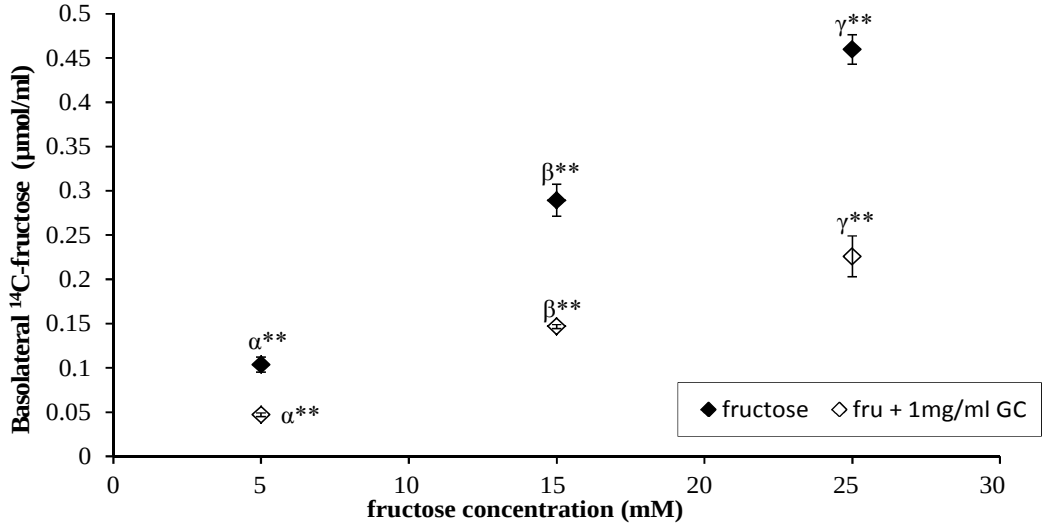



**Figure 58** Intracellular concentration of $^{14}$C-fructose after incubation with apical 5 mM fructose with and without overnight FBS pre-treatment.

The Latin letters denote the significance differences from the treatment with FBS and pre-treatment without FBS (30 and 60 min) and the asterisks denote the significance differences of the time points (*$p<0.05$, **$p<0.01$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per time point). Tukey HSD test applied.

Figure 59 shows the effect of various concentrations of fructose on the rate of $^{14}$C-fructose transport. The rate increases significantly with substrate concentration. In the presence of 1 mg/ml German chamomile extract, the transport of $^{14}$C-fructose from the apical to basolateral compartment decreased significantly for various fructose concentrations. Cellular uptake of $^{14}$C-fructose was inhibited in the presence of 1 mg/ml German chamomile at all fructose concentrations (Figure 60).

**A**




**B**

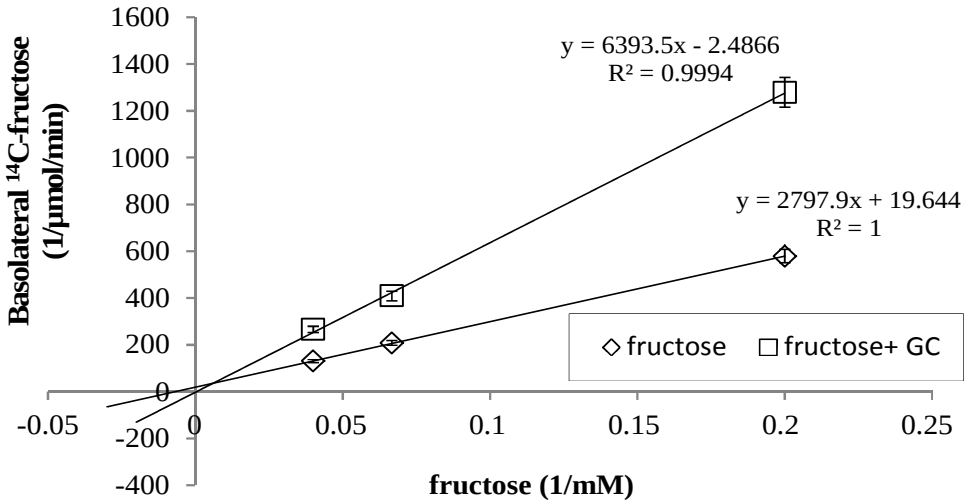
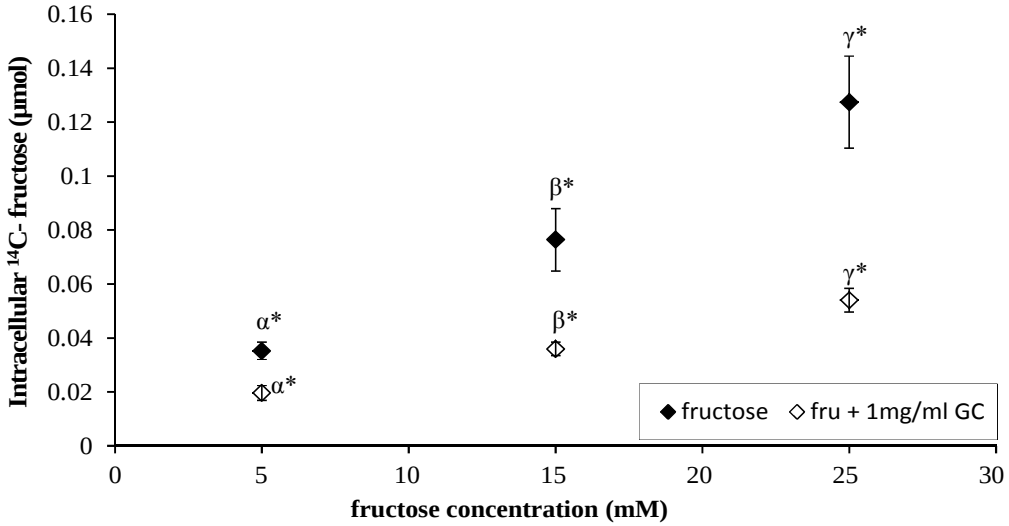



**Figure 59** A: Inhibition of $^{14}$C-fructose transport by GC at different concentrations of apical fructose. The Latin letters denote the significant differences between specific fructose concentration and treatments with/without German chamomile (1 mg/ml) and the asterisks denote the significant differences of the concentration (*$p<0.05$). B: Lineweaver-Burk plot for cellular uptake of fructose with/without 1 mg/ml German chamomile. From this, $K_m$ = 142 mM fructose and $V_{max}$ = 0.05 µmol substrate hydrolyzed/min. Plot indicative of competitive inhibition. Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

**A**




**B**

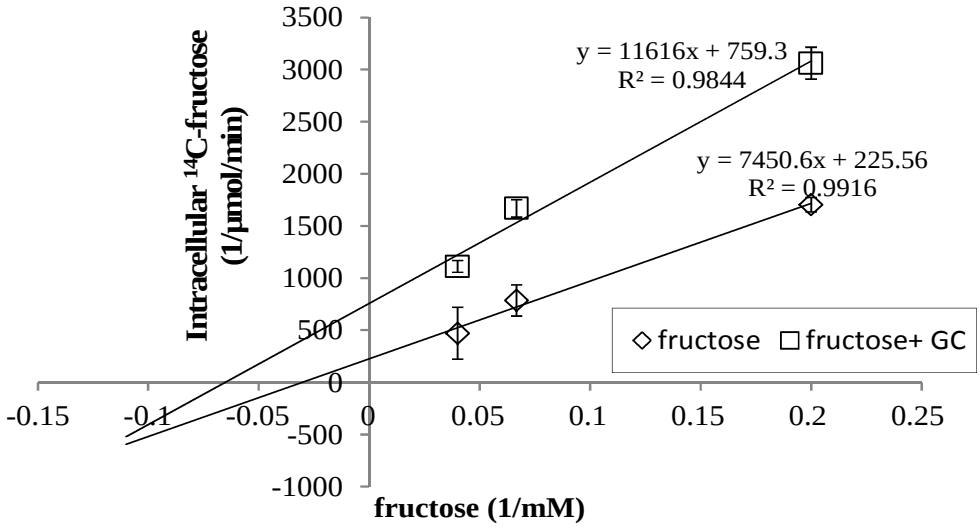
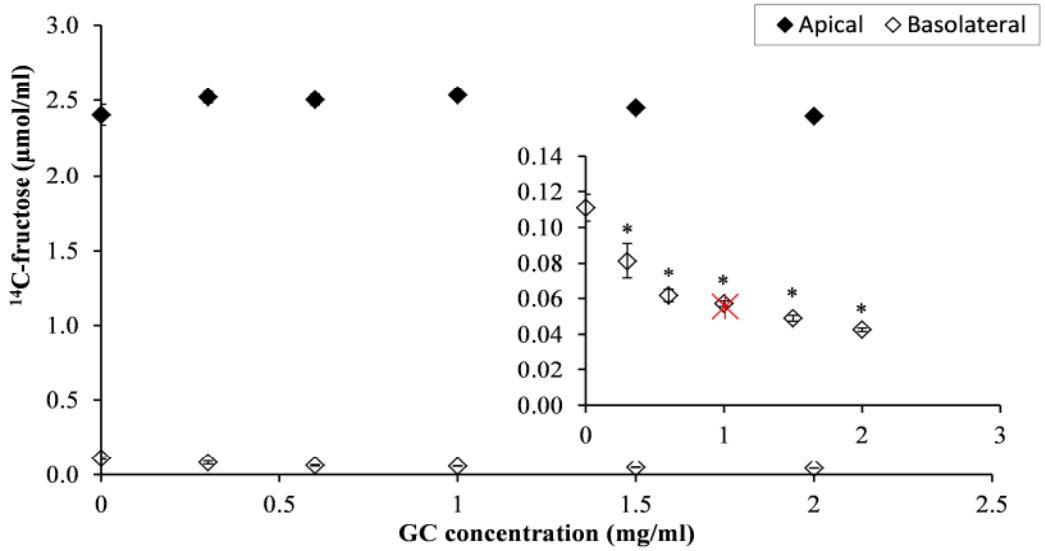



**Figure 60** A: Inhibition of cellular $^{14}$C-fructose uptake by German chamomile at different fructose concentrations. The Latin letters denote the significant differences between specific fructose concentration and treatments with/without German chamomile (1 mg/ml) and the asterisks denote the significant differences of the concentration (**p<0.01). B: Lineweaver-Burk plot for cellular uptake of fructose with/without 1 mg/ml German chamomile. From this, $K_m$ = 33 mM fructose and $V_{max}$ = 0.004 µmol substrate hydrolyzed/min. Plot indicative of uncompetitive inhibition. Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

### 5.3.2 Inhibition of fructose transport and uptake by PFS

Co-incubation of 5 mM fructose with green tea, German chamomile and *Vitis Viniferae* extracts attenuated both uptake and transport of D-[$^{14}$C]-fructose dose dependently. However, the highest concentration of *Vitis Viniferae* extract (3 mg/ml) was not able to achieve 50% inhibition for transport of D-[$^{14}$C]-fructose. Each concentration of green tea, German chamomile and *Vitis Viniferae* extracts significantly reduced the transport of fructose, $p<0.01$.

**A**




**B**

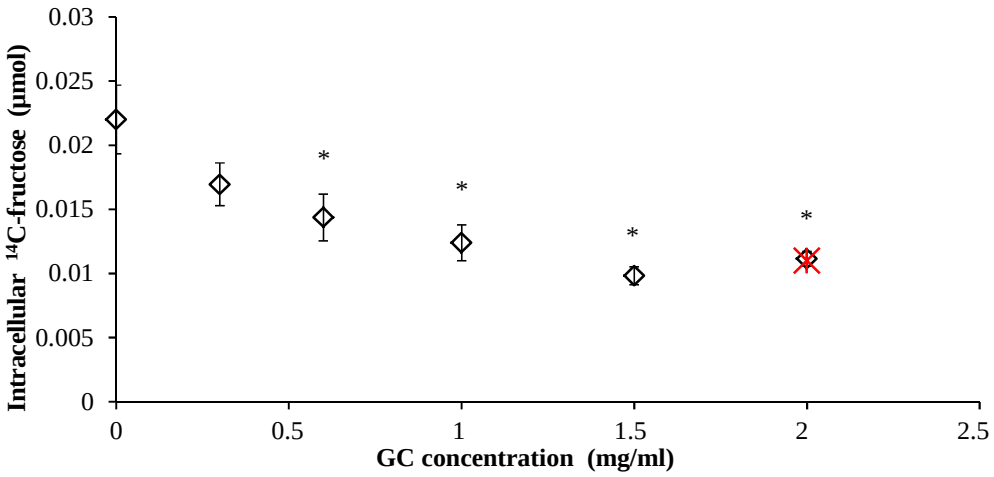



**Figure 61** A: Inhibition of $^{14}$C-fructose transport across Caco-2 differentiated monolayers by different concentrations of German chamomile (GC); inset shows magnified section with estimation of $IC_{50}$. B: Cellular uptake of $^{14}$C-fructose at different concentrations of GC. Asterisks denote significantly different cellular concentrations relative to the control (*$p<0.01$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

The rate of transport from the apical to basolateral compartment was 1.7 nmol/min and cellular uptake of D-[$^{14}$C]-fructose was 0.6 nmol/min without

extracts. Table 23 indicates that extracts from green tea and German chamomile inhibited both the cellular uptake and basolateral transport of D-[$^{14}$C]-fructose. *Vitis viniferae* extract also inhibited the cellular uptake of D-[$^{14}$C]-fructose but the highest concentration of the extract was not able to achieve 50% inhibition of D-[$^{14}$C]-fructose transport. In the presence of 1 mg/ml German chamomile, green tea and *Vitis viniferae* extracts, cellular uptake of D-[$^{14}$C]-fructose (5 mM) was at a rate of 0.2, 0.3 and 0.26 nmol/min, respectively. Addition of those extracts at different concentrations to the apical side led to a dose-dependent inhibition of cellular uptake and basolateral transport of D-[$^{14}$C]-fructose (Figure 61, Figure 62, Figure 63). In the presence of 1 mg/ml German chamomile, green tea and *Vitis viniferae* extracts, the rate of basolateral transport from the apical compartment was 1, 0.8 and 0.3 nmol/min, respectively.

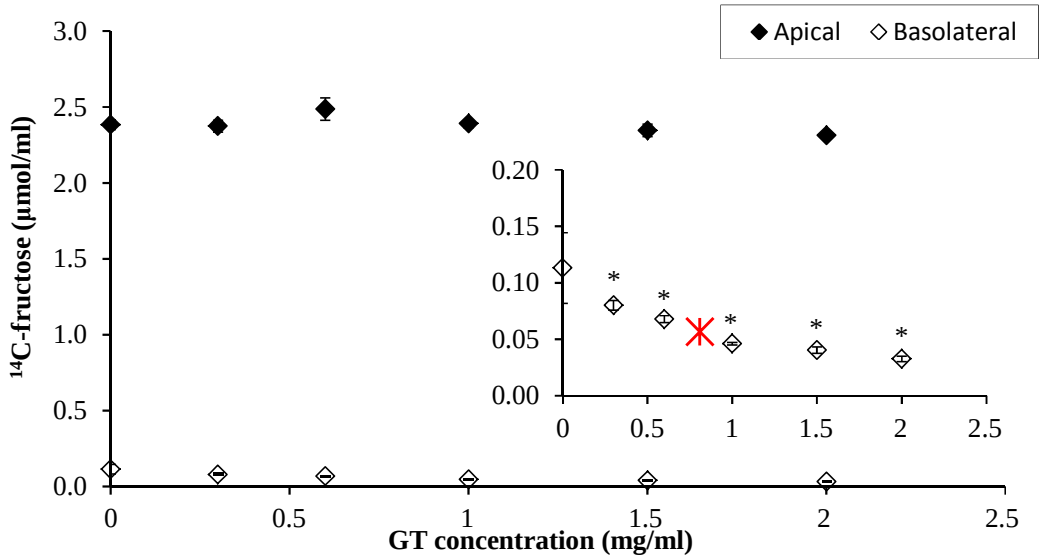
The concentration required for $IC_{50}$ was calculated for cellular uptake and transport of D-[$^{14}$C]-fructose (Table 23). Green tea extract had the highest impact on D-[$^{14}$C]-fructose. This table explains that there is a substantial capacity to inhibit D-[$^{14}$C]-fructose transport with green tea and German chamomile extracts. The highest inhibition of cellular uptake of D-[$^{14}$C]-fructose was with the green tea extract. Besides this, the *Vitis Viniferae* extract inhibited cellular uptake of D-[$^{14}$C]-fructose better than the German chamomile extract.

**Table 23** Effect of PFS on fructose transport across the intestinal monolayer model, and on cellular uptake.

| Supplement extract | $IC_{50}$ (mg/ml) transport | $IC_{50}$ (mg/ml) uptake |
|---|---|---|
| **German chamomile** | 1 ± 0.12 | $2.0 \pm 0.17^{\beta}$ |
| **Green tea** | 0.8 ± 0.09 | $0.7 \pm 0.08^{\alpha}$ |
| ***Vitis Viniferae*** | Not achievable | $1.5 \pm 0.12^{\alpha\beta}$ |

For cellular uptake, means of same row followed by Greek letters α denotes the significant difference than German chamomile and β represent the significant difference from green tea ($p<0.05$). Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

**A**




**B**

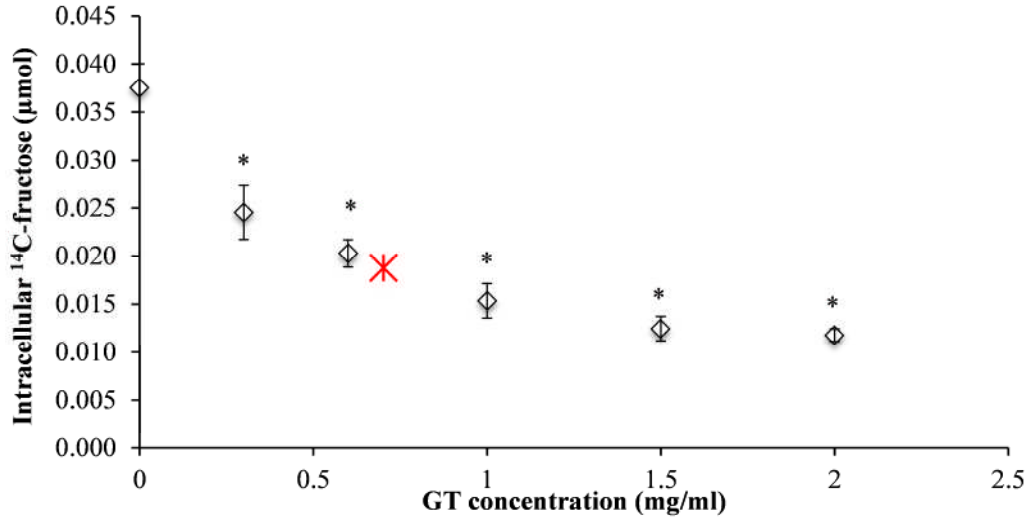
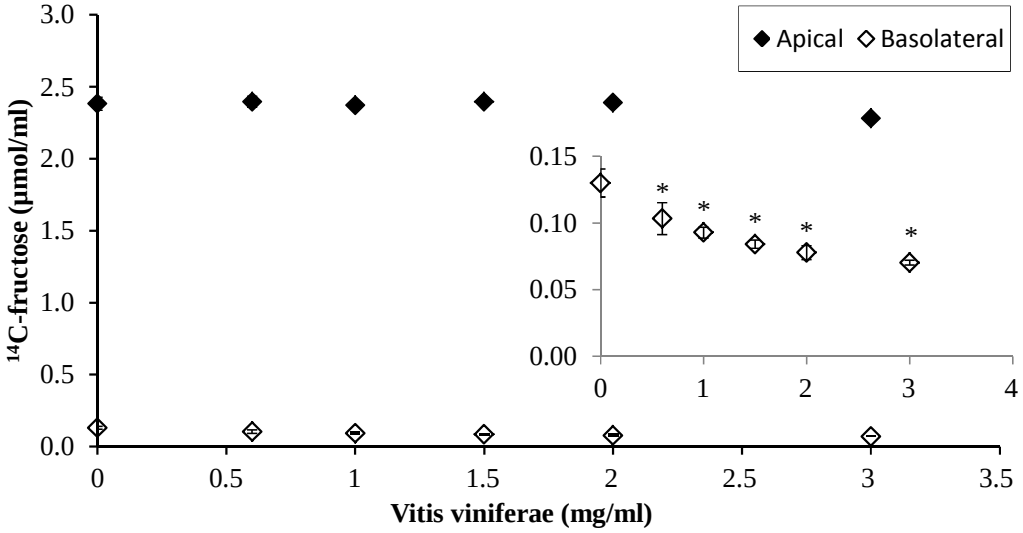



**Figure 62** A: Inhibition of $^{14}$C-fructose transport across the Caco-2 differentiated monolayers by different concentrations of green tea (GT); inset shows magnified section with estimation of $IC_{50}$.

B: Cellular uptake of $^{14}$C-fructose at different concentrations of GT. Asterisks denote significantly different cellular concentrations relative to the control (*$p<0.01$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

**A**




**B**

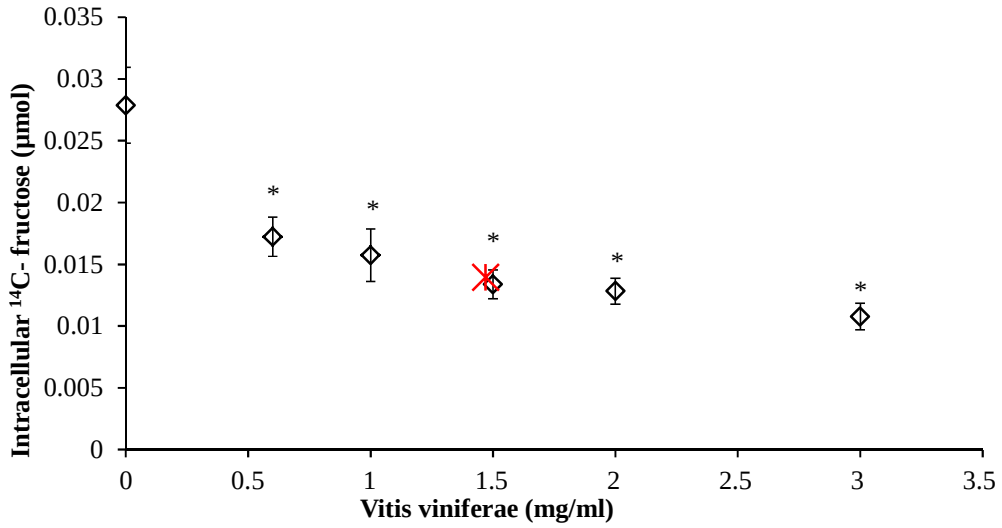



**Figure 63** A: Inhibition of $^{14}$C-fructose transport across the Caco-2 differentiated monolayers by different concentrations of *Vitis Viniferae* extract; inset shows magnified section with estimation of $IC_{50}$.

B: Cellular uptake of $^{14}$C-fructose at different concentrations of *Vitis Viniferae* extract. Asterisks denote significantly different cellular concentrations relative to the control (*$p<0.05$, **$p<0.01$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

According to the experiments, PFS extracts can inhibit $^{14}$C-fructose transport across the Caco-2 cell monolayer. It was considered that they have a major role to inhibit GLUT2 activity, as the $IC_{50}$ values of German chamomile extract for glucose transport without $Na^+$ and fructose transport experiments were similar (chapter 4 section 4.6.2). In the first case, SGLT1 was not active and the extract could inhibit only GLUT2, and in the second case it could inhibit GLUT5 or GLUT2. The $IC_{50}$ values of these two experiments were not significantly different so this may indicate that the extract inhibited only GLUT2 and not GLUT5 for the transport of $^{14}$C-fructose to the basolateral compartment.

### 5.3.3 $^{14}$C-Fructose transport inhibition by German chamomile active components in Caco-2 cells

Some of the active components of German chamomile were also individually assayed to explore the possibility that they were able to moderate fructose uptake and transport at concentrations relevant to the German chamomile extract. German chamomile is rich in polyphenols, containing predominantly apigenin-7-*O*-glucoside (A7G) and ferulic acid hexosides.

None of the analysed compounds were particularly effective on both uptake and transport of $^{14}$C-fructose. The inhibition of transport from the apical to basolateral compartment for apigenin, A7G and L7G was 39, 38 and 34%, respectively. However, the inhibition for the cellular uptake of D-[$^{14}$C]-fructose was 4, 35 and 27%, respectively. diCQA and umbelliferone were ineffective for the transport and cellular uptake of D-[$^{14}$C]-fructose (Figure 64 and Figure 65).

The experiment discussed above was limited by the solubility of the components within the transport solution. Apigenin was not fully dissolved and it was saturated in the transport buffer immediately after adding into TBS. A7G (300 µM) partly precipitated after the incubation step. However, A7G (150 µM), L7G, diCQAs and umbelliferone were dissolved and analysed without any precipitation.

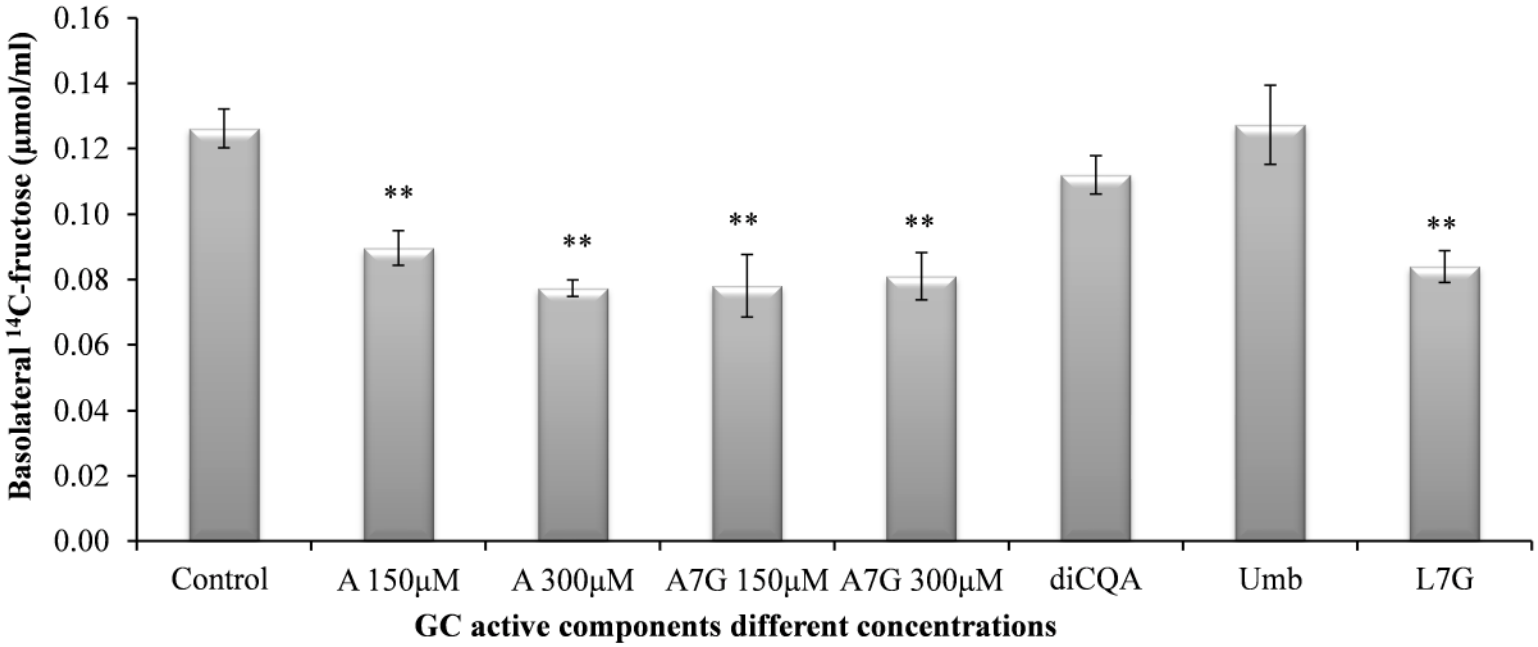



**Figure 64** Inhibition of $^{14}$C-fructose transport to the basolateral side by German chamomile active components (control represent the sample without any inhibitor compound.

A: Apigenin, A7G1: Apigenin-7-*O*-glucoside, diCQA: 100 µM each of 3,4dicaffeoylquinic acid, 3,5 diCQA and 4,5 diCQA, Umb: Umbellifeone 300 µM, L7G: Luteolin-7-*O*-glucoside 300 µM. Asterisk denotes a significant difference from the corresponding control value, **p<0.01, *p<0.05. Mean ± SD (n=6 per sample). Tukey HSD test applied.

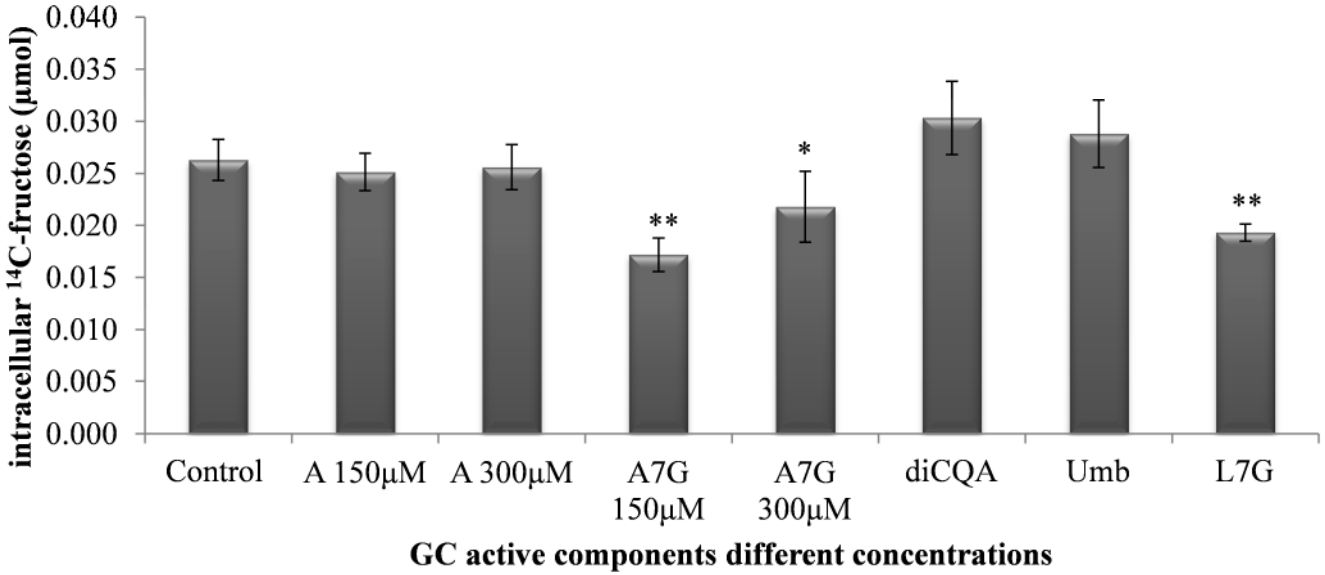



**Figure 65** Inhibition of cellular $^{14}$C-fructose uptake by German chamomile active components (control represent the sample without any inhibitor compound.

A: Apigenin, A7G1: Apigenin-7-*O*-glucoside, diCQA: 100 µM each of 3,4dicaffeoylquinic acid, 3,5 diCQA and 4,5 diCQA, Umb: Umbellifeone 300 µM, L7G: Luteolin-7-*O*-glucoside 300 µM. Asterisk denotes a significant difference from the corresponding control value, **$p<0.01$, *$p<0.05$. Mean ± SD (n=6 per sample). Tukey HSD test applied.

### 5.3.4 Fructose transport under overnight FBS starvation

The FBS in the cell medium promotes cell adhesion and proliferation since it contains hormones and growth factors (epidermal growth factor, fibronectin, and thyroxine). Therefore, there will be a starvation stress in the cells in the absence of FBS for 12 h. The effect of FBS starvation on Caco-2 cells was observed in FBS-deprived cells 12 h before the fructose transport experiment. Also, to analyse the effect of German chamomile extract, the cells were treated with 1 mg/ml extract 12 h before the experiment.

When the cells were deprived of FBS overnight, uptake into cells and transport of $^{14}$C-fructose to the basolateral side was hardly affected (not significant of the control). Therefore, starving the cells from FBS had no affect on cellular uptake of fructose. Addition of German chamomile (1 mg/ml) overnight (12 h) without FBS pre-treatment did not affect the transport of $^{14}$C-fructose, but cellular uptake of D-[$^{14}$C]-fructose was significantly lower than with FBS pre-treatment ($p<0.05$). Addition of German chamomile extract during pre- and post-treatment represented the same inhibition as the usual treatment with German chamomile extract, and cellular uptake and transport of D-[$^{14}$C]-fructose was significantly decreased (Table 24).

Cells deprived from FBS 12 h before the experiment did not show any change in fructose transport and cellular uptake. However, the German chamomile pre-treated cells significantly decreased the cellular uptake of fructose therefore it may be concluded that German chamomile may interact with signaling pathways or transcription.

**Table 24** Effect of FBS starvation and with/without German chamomile (1 mg/ml) overnight treatment on $^{14}$C-fructose cellular uptake and transport across the Caco-2 cell monolayer.

| **Pre-treatment (12h)** | **Transport (1h)** | **FBS (%)** | **GC (mg/ml)** | **Transport (a to b) (µmol)** | **Uptake into cell (µmol)** |
|---|---|---|---|---|---|
| **FBS** | Fructose | 10 | 0 | 0.113±0.003 | 0.0025±0.0006 |
| **non FBS+ GC** | Fructose | 0 | 1 | 0.110±0.002 | 0.0022±0.0006 |
| **non FBS+ GC** | Fructose+ GC | 0 | 1 | 0.072±0.002** | 0.0017±0.0001* |
| **Non FBS** | Fructose | 0 | 0 | 0.112±0.003 | 0.0023±0.0009 |

Asterisks denote significantly different transport and cellular uptake concentrations relative to the control (with FBS overnight to assess fructose) (*$p<0.05$, **$p<0.01$). Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

## 5.4 Discussion

The intake of fructose causes an increase of portal and peripheral blood fructose concentrations of up to 1 and 0.1 mM, respectively (Douard and Ferraris, 2013). Sweetened beverages are the dominant source of fructose in our diet, and high consumption of these beverages may increase the risk of diabetes. It is reported that high dietary intake of fructose increased serious adverse metabolic effects in both humans and rodents (increased plasma triglyceride concentration, impaired glucose tolerance and insulin resistance) (Gaby, 2005; Gross *et al.,* 2004; Le and Tappy, 2006; Pagliasotti and Horton, 2005; Tappy and Le 2012; Simopoulos, 2013; DiNicolantonio *et al.,* 2015).

There are only few studies that have investigated the link between fructose transport and diabetes. The results of these studies were inconsistent, although GLUT5 may be affected by diabetes. In this study, it was observed that fructose uptake and transport to the basolateral compartment were decreased in the presence of PFS, using Caco-2 cells. This is the first report regarding the analysis of fructose transport and metabolism in Caco-2 cells with polyphenol-containing extracts.

Diabetes significantly affects the expression of GLUT5 and fructose uptake rates. Duodenal GLUT5 mRNA and protein levels were three to four-fold higher in type 2 diabetic subjects (Douard and Ferraris, 2008). GLUT5 is a high-affinity facilitative transporter in the basolateral membrane with 12 transmembrane domains and intracellular N and C terminals; and GLUT2 is a low affinity, high-capacity facilitative transporter in the basolateral membrane with 12 transmembrane domains and intracellular N and C terminals for transport of fructose (Drozdowski and Thomson, 2006). GLUT5 is the main transporter for fructose absorption. Gouyon *et al,* (2003) reported that if the fructose concentration in the lumen is low, GLUT5 may be sufficient for transport of fructose to enterocytes. By contrast, a large intake of fructose in GLUT2-null mice showed that GLUT5 was not able to compensate the missing transport capacity fully. Therefore GLUT2 could be upregulated for fructose absorption. They analysed the fructose uptake using isolated brush border membrane

vesicles from high fructose diet fed mice and found that the uptake was ~ 6-fold higher compared to low carbohydrate diet mice. In the current study it was discovered that the presence of some PFS extracts had an influence on fructose uptake and transport to the basolateral compartment by inhibiting the activities of glucose transporters. The active inhibitor compounds in PFS influence either the glucose transporters in the apical side (GLUT5 /GLUT2) and/or the transporters in the basolateral side (GLUT2). The rate of $^{14}$C-fructose transport was lower than $^{14}$C-glucose transport. Riby *et al,* (1993), and Ferraris, (2001) also reported that the rate of fructose absorption is slower than glucose in the small intestine.

Using GLUT2 expressed in *Xenopus oocytes*, myricetin, quercetin, isoquercetrin, luteolin and apigenin were shown to inhibit the transport of fructose with $IC_{50}$ values of 12, 16, 38, 23 and 65 µM, respectively (Kwon *et al.*, 2008). In the current study, apigenin, A7G and L7G inhibited the transport of $^{14}$C-fructose at a percentage inhibition of 39, 38 and 34% for transport. Also, the cellular uptake of $^{14}$C-fructose was inhibited by apigenin, A7G and L7G with a percentage inhibition of 4, 35 and 27%, respectively. Cellular uptake and transport of fructose were not inhibited by diCQA and umbelliferone.

There are several human and animal intervention studies reported in the literature regarding to relationship of fructose and diabetes. The Finnish Mobile Clinic Health Examination Survey collected dietary and life style information from 51,522 subjects, aged 40-60 years during the period of 1966-1972. Montonen and colleagues analysed this cohort study with regards to several conditions (Montonen *et al.,* 2007). They assessed 4,304 non-diabetic men and women at the beginning of the study. They reported that a combined intake of glucose and fructose, consumption of sweetened fruit juices and soft drinks could increase the risk of diabetes. Therefore, the daily sugar intake should be limited. The American Health Association reported this limit for women and men as 100 and 140 kcal/d, respectively (Tappy *et al.,* 2010).

Some other promising animal studies reported that myricetin, cinnamon, fenugreek seed extract or quercetin and longan flower extract were able to balance the glucose-insulin metabolism with fructose-rich diet fed rats (Liu *et al.*, 2007; Kannappan and Anuradha, 2009; Tsai *et al.,* 2008; Qin *et al.,* 2004).

Shrestha *et al.* (2009) reported on a fructose fed ovariectomized rats diet supplemented with 0.5 and 1 % of green tea for 6 weeks and they found that the liver triglycerides concentrations were reduced 27-30% when compared to fructose and starch fed control diets. Cinnamon extracts enhanced the *in vivo* insulin-regulated glucose utilisation with high fructose diet fed rats (Qin *et al.,* 2003; Qin *et al.,* 2004). Another animal study observed the effect of dietary EGCG (3.2 g/kg diet) supplementation on high fat diet fed mice for 16 weeks (Bose *et al.,* 2008). They reported that an EGCG supplemented diet reduced the blood glucose levels and liver triglyceride contents. Girniene and colleagues observed the effect of conformationally locked carbohydrates, 1,3- oxazolidin-2-thione or 1,3-oxazolidin-2-one (OZO)-derived sugars for the fructose uptake using GLUT5-expressed Chinese hamster ovary cells (Girniene *et al.,* 2003). L-sorbose-Bn-OZO showed the greatest inhibitory activity of fructose uptake with a $K_i$ value of 3.1 mM.

In summary, the compounds in the green tea, German chamomile and *Vitis viniferae* extracts crossed the cell membrane and inhibited the basolateral transport and cellular uptake of $^{14}$C-fructose due to the inhibitory effect on GLUT5/GLUT2. Comparison of $IC_{50}$ values of the $Na^+$-free German chamomile extract in the glucose transport (0.89 mg/ml) (only GLUT2 is active on the apical part) and transport of fructose in the presence of same extract (1 mg/ml) could indicate that the extracts inhibited the activity of GLUT2 more than GLUT5. Therefore, PFS may have a major role in mediating intestinal fructose transport across the brush border membrane of enterocyte. As it is already reported that high intake of fructose is a potential trigger for diabetes, the biological activity of the PFS extracts may improve the management of diabetes.

# CHAPTER 6 TRANSPORT AND METABOLISM OF SUCROSE IN THE PRESENCE OF PLANT FOOD SUPPLEMENTS IN CACO-2 CELLS

## Abstract

Sucrose transport across the Caco-2 cell monolayer was significantly reduced in the presence of green tea, German chamomile and *Vitis Viniferae* extracts. These extracts were effective on the transport of D-[$^{14}$C]-sucrose significantly ($p<0.01$). German chamomile, green tea and *Vitis Viniferae* extracts did not show any inhibitory activity on the cellular uptake of sucrose. Green tea and German chamomile extracts inhibited sucrose transport with $IC_{50}$ values of 0.98, and 1.12 mg/ml, respectively. In contrast to this situation, Pelargonium and Echinacea were ineffective.

The response of the Caco-2 cell line to stress (FBS starvation or sucrose load) overnight (12 h) was assessed after treatment with German chamomile or additional sucrose (5 mM). FBS starvation and the addition of German chamomile overnight (12 h) significantly increased the cellular uptake of D-[$^{14}$C]-sucrose ($p<0.05$). However, the addition of German chamomile extract during pre-treatment and treatment steps brought sucrose retention in cells back to the normal concentration (same as control sample). Overnight (12 h) incubation with sucrose (5 mM) increased the transport of sucrose significantly ($p<0.05$) but cellular uptake remained the same. A repeated dose of German chamomile (incubation with extract overnight and during the experiment) reduced the transport of D-[$^{14}$C]-sucrose in the absence of FBS but did not affect cellular uptake. Inhibition of sucrose transport was also analysed with TC7 cells (subclone of Caco-2). It was reported that TC7 cells have higher sucrase activity than Caco-2 cells. Transport of radiolabelledsucrose or its products to the basolateral side with German chamomile and *Vitis viniferae* extract show a similar degree of inhibition with the Caco-2 cell transport study; however, the results for the inhibition activity of green tea extract were different in the two cell lines. More radiolabelled sucrose or its products were taking by TC7 cells compare to Caco-2 cells results in the higher activity of sucrase. For the three

PFS extracts, cellular uptake of sucrose was reduced significantly ($p<0.01$). Acarbose (0.1 mM) also inhibited 48% of sucrose transport across TC7 cells. Due to their inhibitory activity (acarbose-like activity) on liberated glucose transport or hydrolysis of sucrose by sucrase activity, green tea, German chamomile and *Vitis viniferae* extracts may improve the management of diabetes in response to sucrose intake.

## 6.1 Introduction

Consumption of carbohydrate-rich food, meals or beverages increases the postprandial blood glucose level. Sucrose is a disaccharide consisting of one glucose and one fructose unit linked with a α-1,4 glycosidic bond. As sucrose can cause an increase of the postprandial glucose concentration (liberated glucose from sucrose) and liberated fructose is utilized in the liver to make products like glucose, glycogen, lactate and pyruvate, and also might affect insulin resistance and type 2 diabetes (Törrönen *et al.,* 2010; Hansen *et al.,* 2012; Halschou *et al.,* 2012). Sucrose is hydrolysed by sucrase/isomaltase located in the small intestine**.** Monosaccharide products are then transported by active transport SGLT1 and by facilitated transport GLUT2. After two $Na^+$ ions bind to SGLT1 on the luminal side of the intestinal brush border membrane, a conformational change occurs which allows glucose binding. A second conformational change allows glucose and $Na^+$ to enter the enterocyte. Glucose is released from the enterocyte via the GLUT2 passive transporter at the basal surface and enters the circulation. The process of uptake and expulsion is simultaneous (Goodman, 2010; Nelson and Cox, 2000). Fructose enters (GLUT5) and exits (GLUT2) the cells via facilitated diffusion (Figure 66). The digestion and absorption of carbohydrates in the intestinal tract can be retarded using a strong inhibitor of α-glucosidase. Therefore, whether PFS extracts may have a role to inhibit sucrose transport and/or hydrolysis of sucrose by sucrase was analysed.

There is a large number of studies on carbohydrate-metabolising enzymes that are present in the small intestine, and these studies are generally focused on α-amylase and α- glucosidase (sucrase and maltase) (Hansawasdi and Kawabata, 2006; Laurent *et al.,* 2007; Zhong, *et al.,* 2006; Trugnen *et al.,* 1986; Kano *et al.,* 1996; Gupta *et al*. 2007; Adisakwattana and Chanathong 2011; Pereira *et al.* 2011; Minai-Tehrani *et al.,* 2010). The majority of these studies showed inhibition of the enzyme by phenolic compounds.

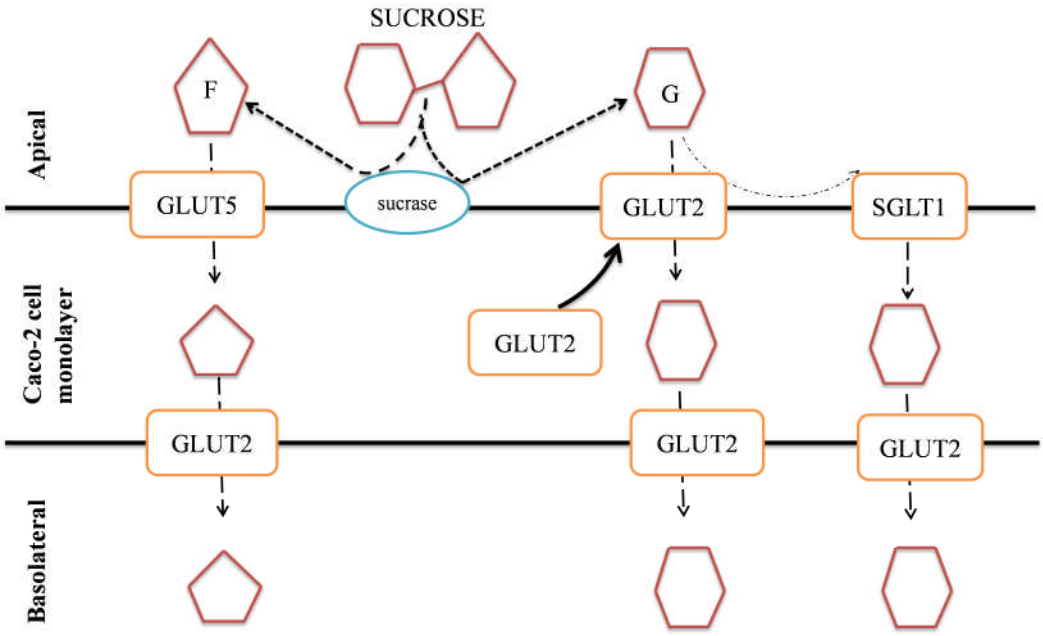



**Figure 66** Absorption of sucrose into the blood. G: glucose and F: fructose.

Grape seed extract (GSE) contains a high concentration of flavanols (54%) which reduced sucrase activity by 61% in the Caco-2 cell line at a concentration of 0.3 g/l, with a degree of polymerisation greater than the dimer (Laurent *et al.*, 2007). Hansawasdi and Kawabata (2006) also investigated the effect of mulberry tea on sucrose transport on α-glucosidase-expressing Caco-2 cells. The liberated glucose concentration in both apical and basolateral compartments was measured using the glucose oxidase method and reported that mulberry tea inhibited sucrase activity and transportation at a lower concentration ($IC_{50}$ was not achievable). There are several animal and human intervention studies reported in the literature regarding the effect of polyphenols on sucrose metabolism. Wilson *et al.* (2008) studied twelve healthy participants fed with sucrose (35 g) and berries (150 g puree consisting of bilberries, blackcurrants, cranberries and strawberries providing nearly 800 mg polyphenols). Berries are rich sources of anthocyanins, flavonols, proanthocyanidins and phenolic acids. Participants who consumed sucrose without berries produced a different postprandial glycemic response compared to the subject who ate with berries**.** Törrönen *et al*. (2010) fed their volunteers, who had a normal plasma glucose level, with 150 g berry puree containing 35 g sucrose or a control sucrose load in a randomized, controlled

cross-over design and showed that the plasma glucose concentration was significantly lower at 15 and 30 min and significantly higher at 150 min compared to the control meal.

Zhong *et al*. (2006) reported that 100 mg tea extract (black, green and mulberry) is able to induce carbohydrate malabsorption in healthy human volunteers following the consumption of a carbohydrate-rich meal, suggesting that tea extracts are potentially able to reduce post-prandial blood glucose. One previous study regarding the inhibition of purified human sucrase-isomaltase by ethanolamine derivatives including tris showed that tris is a strong inhibitor of both sucrase and isomaltase but bis-tris inhibits sucrase more than isomaltase (Kano *et al.*, 1996). Recently, there have been studies on sucrase activity with therapeutic drugs. Codeine has been reported to inhibit yeast sucrase activity (Minai-Tehrani *et al.*, 2010) and castanospermine inhibits sucrase in the Caco-2 cell line (Trugnan *et al.*, 1986).

The effect of polyphenols from different plant food supplements (green tea, German chamomile, *Vitis viniferae*, Pelargonium and Echinacea) on the transport of sucrose from apical to basolateral and cellular uptake was investigated using Caco-2 intestinal cell monolayers. Substantial inhibition on sucrose transport was observed by plant food supplement extracts from green tea and German chamomile.

## 6.2 Material and methods

### 6.2.1 Standards and reagents

Sucrose was purchased from Sigma (Dorset, UK - S0389). PFS was supplied by PhytoLab Co. KG (Vestenbergsgreuth, Germany). Dulbecco's Modified Eagle's Medium (DMEM; including 1000 mg glucose/L, L-glutamine, $NaHCO_3$ and pyridoxine HCL) was purchased from Sigma (D6046). Caco-2 cells (HTB-37) were obtained from the American Type Culture Collection (LGC Promochem, Middlesex, UK).

### 6.2.2 Cell Cultures

Please see chapter 4 section 4.2.2.

## 6.3 Sucrose transport measurements in Caco-2 cells

### 6.3.1 Preparation of Reagents

All the reagents were prepared freshly on the day of the experiment.

#### 6.3.1.1 Transport buffer solution (TBS)

Please see chapter 4 section 4.3.1.

#### 6.3.1.2 Sucrose:

D-[$^{14}$C] sucrose 50 µCi (1.85 MBq) (NEC100X050UC) was purchased from Perkin Elmer (Boston, USA). D-[$^{14}$C] sucrose is universally labelled $^{14}$-C and the basolateral activity measured was a combination of glucose and fructose liberated from sucrose hydrolysis, and the apical compartment included radioactive-labelled sucrose, glucose and fructose.

A stock solution of 100 mM sucrose was prepared. Briefly, 342.3 mg of sucrose was dissolved in 10 ml of transport buffer solution. 0.5 ml of stock sucrose solution with/without PFS was added to TBS to a final volume of 10 ml containing 9 µl of 0.1 µCi/µl $^{14}$C-sucrose. To provide the best activity for the enzymes the pH was adjusted to 7.4 at 37 $^{0}$C for each sample with 1 M HCl or NaOH.

#### 6.3.1.3 Plant Food Supplement extracts preparation

Please see chapter 4 section 4.3.4.

### 6.3.2 Inhibition assay protocol

Cells between passage 36 and 50 were utilised in the investigation of sucrose transport and cellular uptake. A minimum of six replicates was allocated per experimental condition.

On or after 21 days, transport studies were initiated by replacement of growth medium with TBS and cells were washed three times with it. After washing cells, 2 ml TBS was added to the apical and basolateral sides and pre-incubated for 30

minutes at 37 °C under a humidified atmosphere. Differentiation of the monolayer was assessed by trans-epithelial electrical resistance (TEER) of the cell monolayers. Only Transwell® inserts with a resistance exceeding a blank membrane by 300Ω were utilised in the experiment. A low value may indicate increased paracellular transport of glucose across the membrane due to less well-formed tight junctions. After equilibration, TEER values were recorded from three positions per insert and subsequently the compartments were carefully aspirated to waste. For the experiments, 2 ml TBS containing 5 mM sucrose solution and 0.045 µCi/ml [$^{14}$C]-sucrose with or without PFS extracts (pH 7.4) was added to the apical compartment and 2 ml TBS in the basal receiver compartment. Cells were incubated for 60 min at 37°C. Following incubation, the TEER values were measured at three positions per insert again and the solutions from apical and basolateral compartments were removed to scintillation vials immediately. To wash cells for radioactivity assessment, 2 ml TBS was added to the apical and basolateral compartments, which was removed to the scintillation vials. Following this, 1 ml of 1 M NaOH solution was added to detach the cells from the membrane and the plates were placed on a plate shaker for 30 minutes. The suspended cells were removed to 1.5 ml centrifuge tubes. Subsequently, 0.7 ml of NaOH was removed to the scintillation vials and to neutralize the pH, 0.7 ml of 1 M HCl was added to tube. The remaining NaOH sample was stored at -20ºC. The scintillation cocktail was added to the apical, basolateral and cell suspension samples at a volume of 10, 5 and 5 ml, respectively, and all the vials were mixed. Radioactivity was determined using a Packard 1600 TR Liquid Scintillation Analyser (see chapter 4 section 4.4.1). Light emission was counted for 10 min per vial and the number of counts per minute (CPM) was calculated by the equipment software.

### 6.3.3 Statistical analysis

IBM SPSS Statistics 22 was used for the analysis of the data. The Levene test was used to evaluate the homogeneity of the means groups. If the criterion was met, the Tukey HSD post hoc test was applied; otherwise, the Dunnett C followed the one way Anova. The values shown represent the mean values and

the error bars indicate the standard deviation (SD). Unless otherwise stated, differences were considered as statistically significant when $p \leq 0.05$.

## 6.4 Results

### 6.4.1 Set up and validation of D-[$^{14}$C]-sucrose transport across Caco-2 cells

A calibration curve of radioactivity as a function of [$^{14}$C]-sucrose quantity was prepared. With the y-intercept fixed at zero, the gradient was calculated as 21967 CPM/µmol and the adjusted $R^2$ was equal to 0.999, suggesting good proportionality of radioactivity and molar quantity (Figure 67). Counting precision of the equipment was assessed by comparing the reference calibration solution of 123000 CPM/KBq and background noise (n=14).

The results were corrected for the counter efficiency based on background absorbance standard signals (the typical count averaged 34.9 CPM, and the solutions involved in the experiment (HCl + NaOH mixture and TBS) had a typical average count of 42.9 and 39.7 CPM, respectively) (n=3 measurement for 14 times experiment).

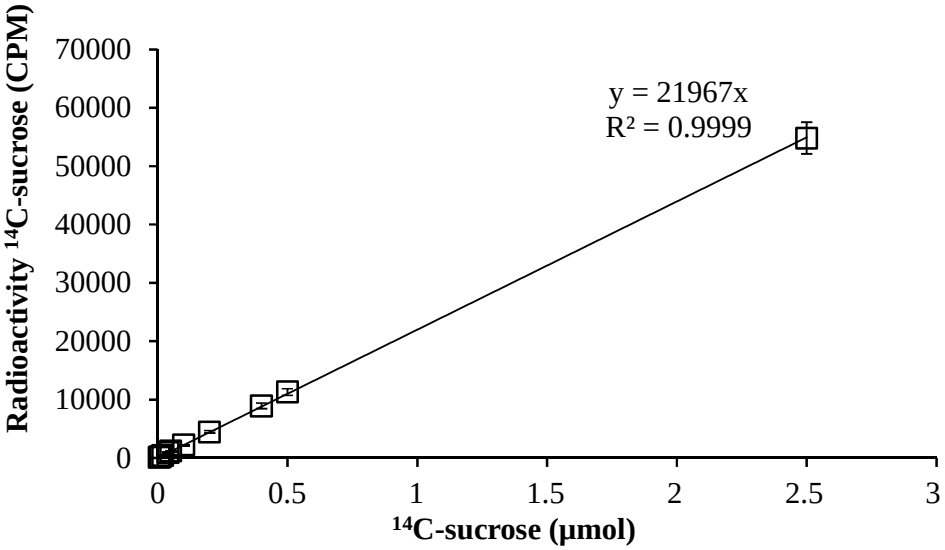



**Figure 67** Sucrose standard curve for radioactivity measurements (CPM = [$^{14}$C]-sucrose counts per minute). Mean ± SD (n=6 per concentration point, total 11 samples).

The effect of PFS extracts on apical to basolateral transport and cellular uptake of sucrose was investigated using the Caco-2 and Caco-2 /TC7 cell lines. Caco-2 and TC7 cells were plated on 6-well and 12-well plates, respectively, at a density of 6.43 x $10^4$ cells/$cm^2$. The experiments were performed after the cell monolayer reached the full differentiation. The differentiation of the monolayer was assessed by measurement of the TEER values of the cell monolayer. Only Transwell® inserts with a resistance exceeding a blank membrane by 300 Ω were utilized in the experiment. TEER values <300 Ω may be demonstrating less well-formed tight junctions between the cells. The TEER value of the cells was measured after the pre- and post-incubation process. There was no significance in the pre and post-incubation TEER values of each well (Table 25). Therefore, PFS extracts did not affect the electrical resistance between the cells.

**Table 25** Blank corrected TEER of Caco-2 monolayers measured after pre-incubation (30 min) and post-incubation (60 min) in the absence (control) and presence of PFS extracts.

| PFS extracts | Concentration of PFS (mg/ml) | TEER post-incubation | % difference |
|---|---|---|---|
| **Control** | 0 | 378.3 ± 40 | 12 |
| **German chamomile** | 1 | 305 ± 12* | 9 |
| **Green tea** | 1 | 390 ± 32 | 2 |
| ***Vitis Viniferae*** | 3 | 333 ± 11* | 8 |

% difference demonstrates the difference between pre and post-incubation TEER values. The asterisk denotes a significantly different TEER measurement relative to control TEER measurement ($p<0.05$). Mean ± SD (n=6 measurement per sample and 18 times readings from each sample). Tukey HSD test applied.

The rate of transport from apical to basolateral cell culture medium was 2.4 nmol/min and cellular uptake of D-[$^{14}$C]-sucrose (5 mM) was 0.15 nmol/min without PFS (control sample).

Basolateral transport of D-[$^{14}$C]-sucrose (5 mM) was analysed at different time points with and without 12 h FBS treatment to analyse the effect of starvation

stress on Caco-2 cells. There was a linear increase on sucrose transport at different time points (Figure 68). Transport of D-[$^{14}$C]-sucrose to the basolateral side significantly decreased slightly under FBS starvation only at 30 min. Cellular uptake of sucrose increased over time. Sucrose uptake also significantly increased with 12 h FBS starvation compared to FBS-treated cells ($p<0.01$) (Figure 69).

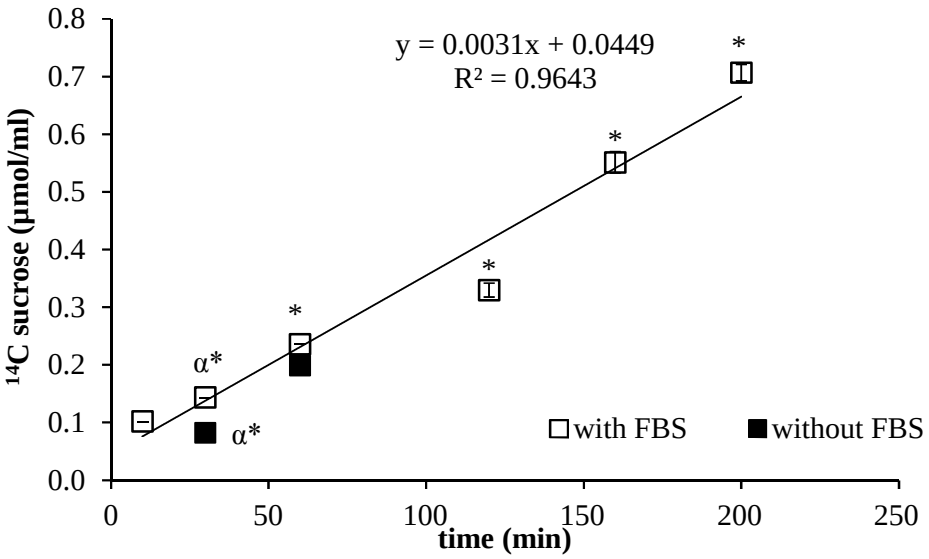



**Figure 68** Transport of 5 mM [$^{14}$C]-sucrose from the apical to basolateral side with and without overnight FBS pre-treatment.

Asterisks denote a significant difference from control samples and Greek letter denotes a significant difference from the related time point (FBS treated and FBS starvation 12h) ($p<0.01$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per time point). Tukey HSD test applied.

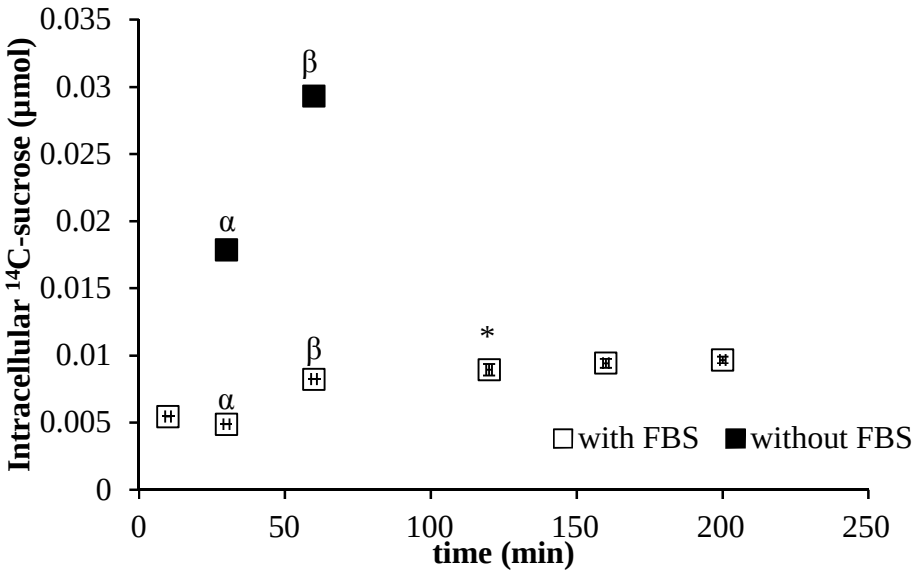
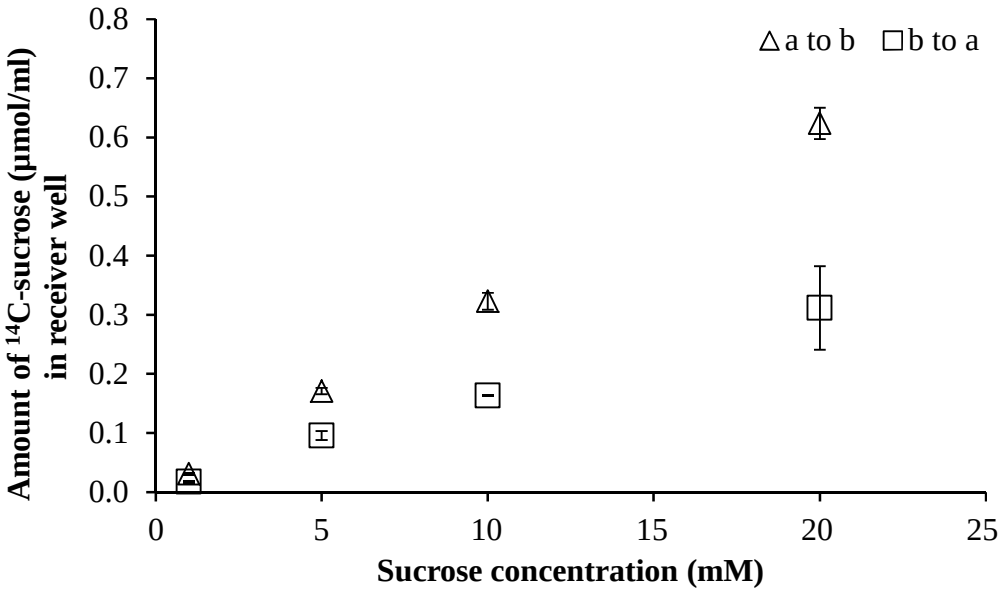



**Figure 69** Intracellular sucrose after apical incubation with 5 mM sucrose with and without overnight (12 h) FBS pre-treatment.

Asterisk denotes a significant difference from control samples ($p<0.05$) and Greek letters denote significant differences from the related time point (FBS treated and FBS starvation 12h) ($p<0.01$). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per time point). Tukey HSD test applied.

Figure 70 shows the effect of various concentrations of sucrose on the rate of transport from apical to basolateral (a to b) and basolateral to apical (b to a). Sucrose is transported to the blood in two ways: i) hydrolysis to glucose and fructose by sucrase and then transport to blood via glucose transporters or ii) paracellular transport. It is possible that sucrose may also be transported back to the lumen paracellularly. Approximately half of the amount of sucrose was transported back to lumen (Figure 70). In both conditions, the rate of transport increased with substrate concentration. Also, cellular uptake of D-[$^{14}$C]-sucrose remained constant from a to b but it was slightly increased from b to a. Sucrase has an activity only on the luminal side, therefore it may have an effect on cellular up of D-[$^{14}$C]-sucrose from a to b (Figure 70).

**A**




**B**

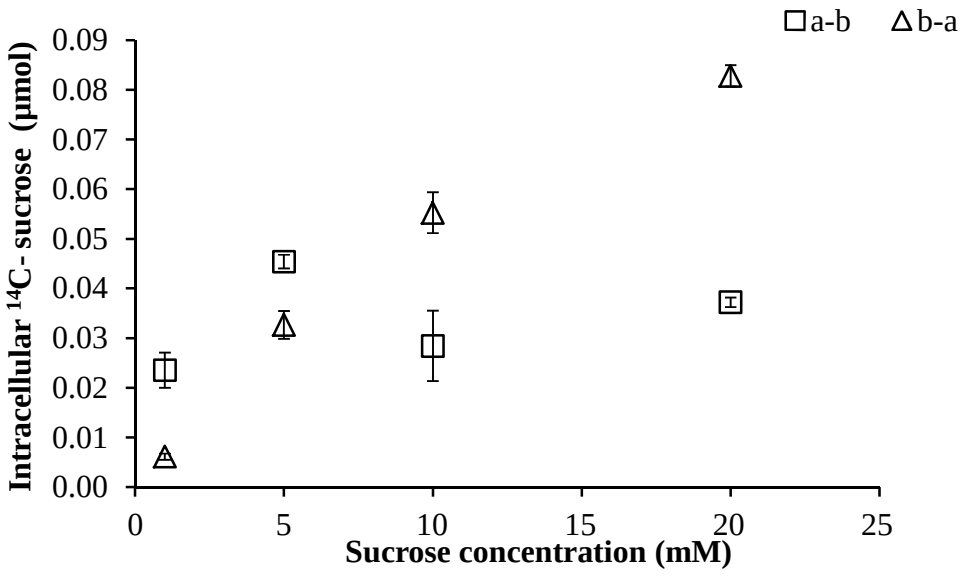


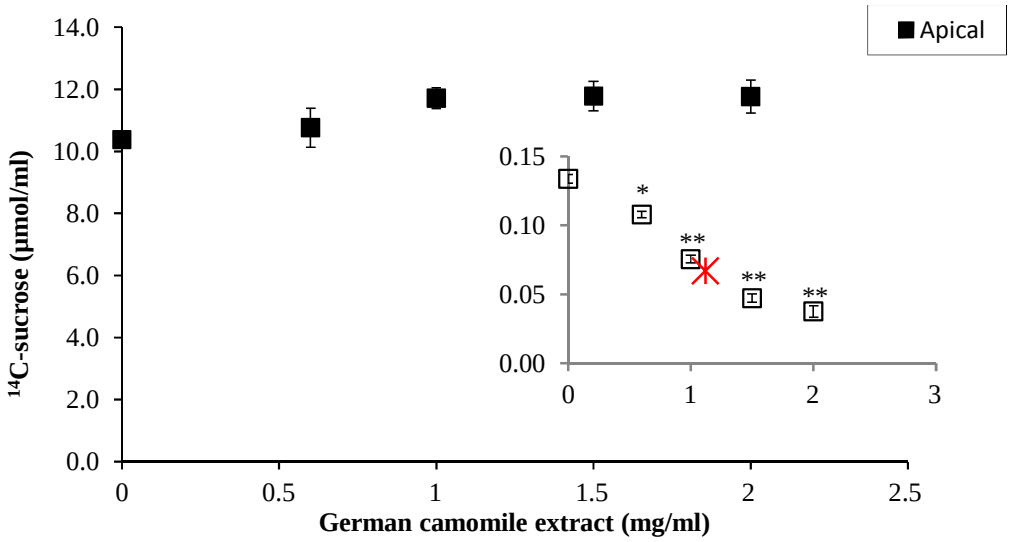

**Figure 70** A: Transport of [$^{14}$C]-sucrose from apical to basolateral (a to b) and basolateral to apical (b to a) at different concentrations of sucrose.

Related concentration points of two data significantly different than each other. B: Cellular uptake of [$^{14}$C]-sucrose from apical to basolateral (a to b) and basolateral to apical (b to a) at different concentrations of sucrose. Mean ± SD (n=6 per concentration point).

### 6.4.2 Inhibition of sucrose transport and uptake by PFS

The addition of different concentrations of PFS extracts reduced the transport of D-[$^{14}$C]-sucrose to the basolateral side. The $IC_{50}$ of cellular uptake and transport of D-[$^{14}$C]-sucrose was calculated (Table 26). Without PFS extracts (control sample), the rate of basolateral transport from cell culture medium was 2.4 nmol/min and cellular uptake of D-[$^{14}$C]-sucrose (5 mM) was 0.15 nmol/min. For co-incubation of 5 mM sucrose with green tea and German chamomile, the extracts dose dependently attenuated the transport of D-[$^{14}$C]-sucrose to the basolateral side. These $IC_{50}$ values showed that green tea extract had the highest impact on D-[$^{14}$C]-sucrose transport. *Vitis Viniferae* extract also dose dependently attenuated the transport of D-[$^{14}$C]-sucrose but its highest concentration did not reach the $IC_{50}$ value. 1 mg/ml Pelargonium and Echinacea extracts were ineffective for both uptake and transport of D-[$^{14}$C]-sucrose. The compounds in green tea and German chamomile extracts can cross the cell membrane and inhibit the basolateral transport of sucrose more effectively than other PFS extracts. The results indicated that cellular uptake of sucrose was not affected by the PFS extracts (Figure 71, Figure 72, and Figure 73 B).

**A**




**B**

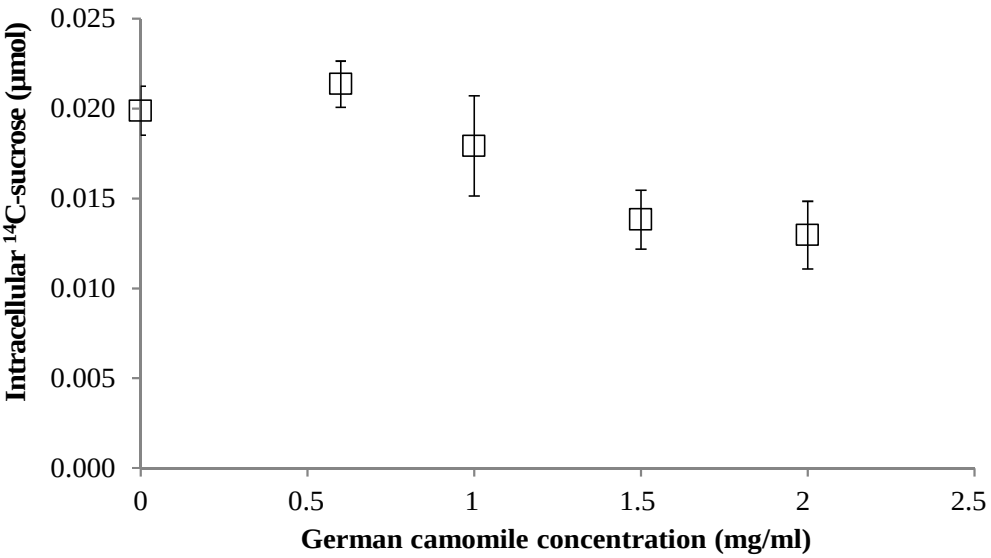



**Figure 71** A: Sucrose uptake is significantly inhibited by different concentrations of German chamomile across Caco-2 differentiated monolayers The inset shows the magnified section with estimation of IC50 for transport of radiolabelled sucrose and its products to basolateral compartment. B: Cellular uptake of sucrose in Caco-2 cells at different concentrations of German chamomile (**p<0.01, *p<0.05). Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per concentartion point). Tukey HSD test applied.

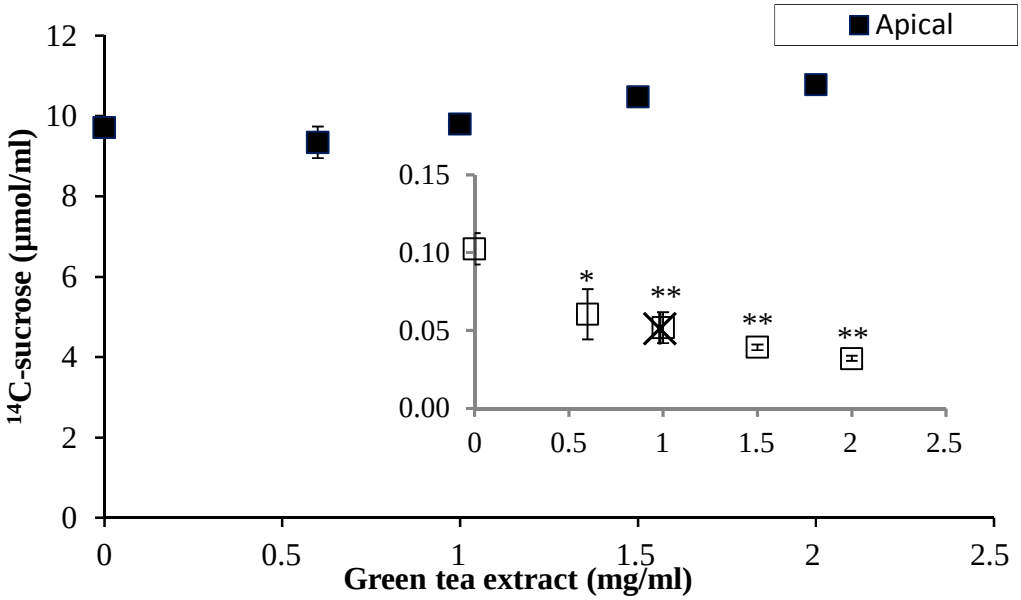
Addition of PFS extracts to the apical side at different concentrations led to a dose-dependent inhibition of basolateral transport of D-[$^{14}$C]-sucrose (Figure 71, Figure 72 and Figure 73). In the presence of 1 mg/ml German chamomile, green tea and *Vitis Viniferae* extract, the rate of basolateral transport from the apical compartment was 0.8, 0.9 and 0.5 nmol/min, respectively. In the presence of 1 mg/ml German chamomile, green tea and *Vitis Viniferae* extracts, cellular uptake of D-[$^{14}$C]-sucrose (5 mM) was at a rate of 0.09, 0.11 and 0.13 nmol/min well, respectively.

**Table 26** Effect of PFS on sucrose transport across the intestinal monolayer model, and on cellular uptake.

| **Supplement extract** | **$IC_{50}$ (mg/ml) transport** | **$IC_{50}$ (mg/ml) uptake** |
|---|---|---|
| **German chamomile** | 1.12 ± 0.17 | None |
| **Green tea** | 0.98 ± 0.12 | None |
| ***Vitis Viniferae*** | Not achievable | None |
| ***Pelargonium*** | None | None |
| ***Echinacea*** | None | None |

Mean± SD (n=3) Tukey HSD test applied.

**A**




**B**

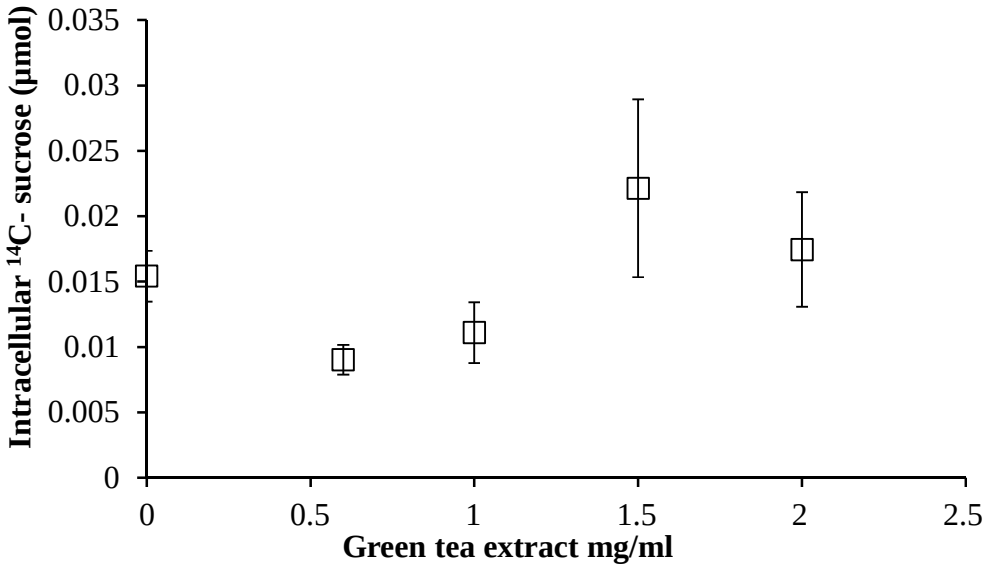
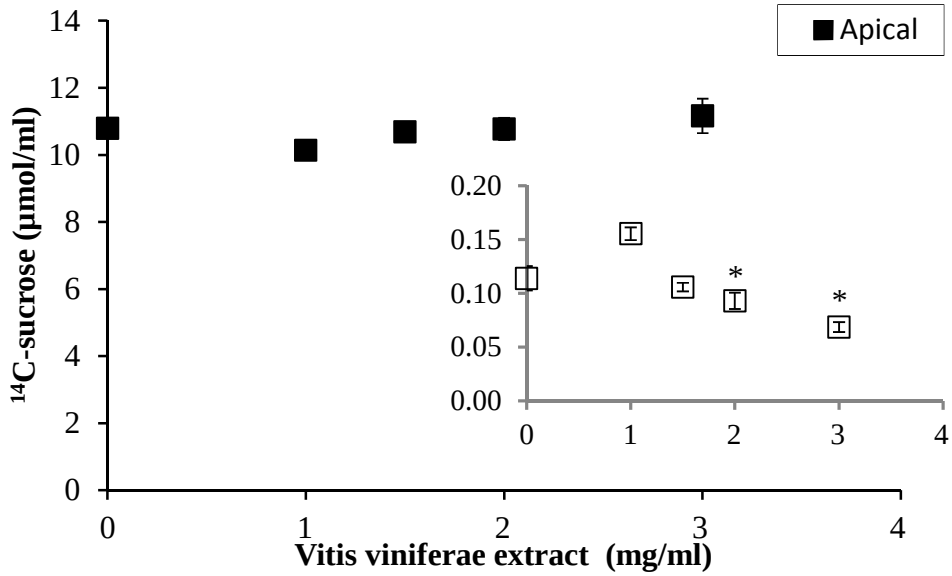



**Figure 72** Sucrose transport and uptake were significantly inhibited by different concentrations of green tea extract across Caco-2 differentiated monolayers.

B: Cellular uptake of sucrose in Caco-2 cells at different concentrations of green tea extract. Inset shows a magnified section with estimation of $IC_{50.}$ Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

**A**




**B**

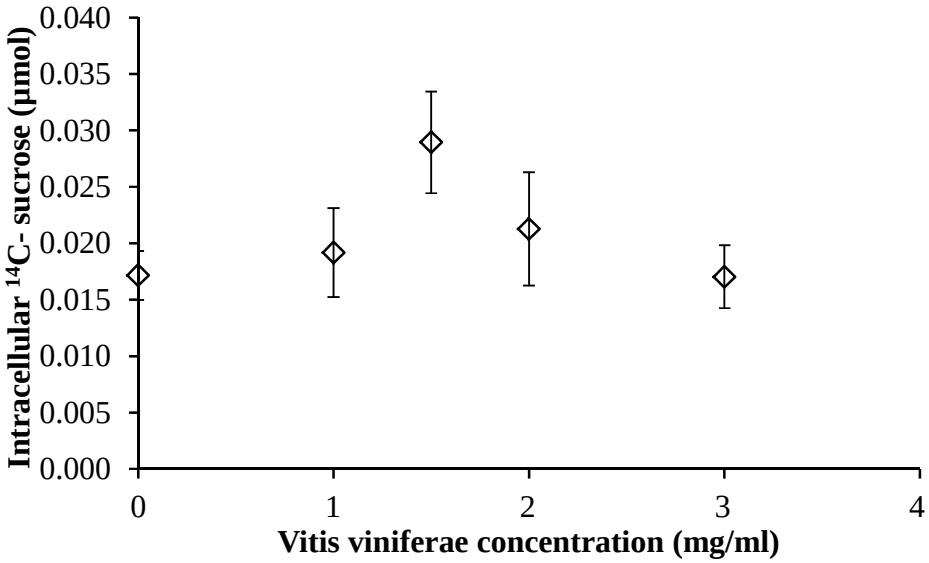



**Figure 73** Sucrose uptake and transport were not significantly inhibited by different concentrations of *Vitis viniferae* extract across Caco-2 differentiated monolayers ($p<0.01$).

B: Cellular uptake of sucrose in Caco-2 cells at different concentrations of *Vitis viniferae* extract. *Vitis viniferae* extract was unable to achieve 50% inhibition of sucrose transport at concentrations <3 mg/ml. Where not visible, error bars are smaller than the size of the data point. Mean ± SD (n=6 per concentration point). Tukey HSD test applied.

### 6.4.3 Sucrose transport under overnight FBS starvation

FBS contains important components such as hormones and growth factors. Therefore, the addition of FBS to cell medium promotes cell adhesion and proliferation (epidermal growth factor, fibronectin, and thyroxine). To create starvation stress in the cells, FBS was removed from the cell culture medium 12h before the experiment. Beside, cells were treated with 1 mg/ml German chamomile extract and sucrose (5 mM) 12 h before the experiment.

When the cells were deprived of FBS overnight, uptake of D-[$^{14}$C]-sucrose into the cells was 0.016 µmol/well. Cellular uptake of D-[$^{14}$C]-sucrose was 0.012 µmol/well in the presence of FBS. This shows that FBS starvation significantly increases the cellular uptake of D-[$^{14}$C]-sucrose ($p<0.05$). On the other hand, when the Caco-2 cells were treated with German chamomile for 12 h in the absence of FBS, cellular uptake of D-[$^{14}$C]-sucrose was increased significantly ($p<0.05$). However, the addition of German chamomile extract (1 mg/ml) overnight (12 h) and during the experiment decreased the cellular uptake of sucrose to the same extent as the control sample without FBS treatment (

Table **27**). Pre-treated cells with sucrose (5 mM) without FBS starvation did not effect the cellular uptake of D-[$^{14}$C]-sucrose.

German chamomile extract inhibited the cellular uptake of D-[$^{14}$C]-sucrose with an $IC_{50}$ value of 0.309 mg/ml. By contrast, the cells (deprived of FBS 12 h) that were exposed to German chamomile extract overnight and during the transport experiment did not inhibit cellular uptake of D-[$^{14}$C]-sucrose. FBS starvation and German chamomile extract affected the amount of cellular sucrose therefore it may conclude that relevant transcript regulation occurred and could be further analysed. When the cells were deprived of FBS for 12 h, transport of D-[$^{14}$C]-sucrose to the basolateral compartment increased significantly ($p<0.05$), the same as cellular uptake. Under FBS starvation, pre-treatment with German chamomile (12 h) increased the transport of D-[$^{14}$C]-sucrose further, similar to the retention inside the cells. Pre-treatment with sucrose (12 h) significantly increased the D-[$^{14}$C]-sucrose transport across compared to the control sample

($p<0.05$). This may be due to the transporters evolving because of sucrose overnight (12 h) in the presence of sucrose.

**Table 27** Effect of 12 h treatment of FBS starvation and with/without German chamomile (1 mg/ml) on sucrose cellular uptake and transport across the Caco-2 cell monolayer.

| **Pre-treatment (12h)** | **Transport (1h)** | **FBS (%)** | **GC (mg/ml)** | **Sucrose in pre-treatment (mM)** | **Transport (a to b) (µmol sucrose)** | **Uptake into cell (µmol sucrose)** |
|---|---|---|---|---|---|---|
| **No FBS** | Sucrose | 0 | 0 | 0 | 0.160±0.005* | 0.016±0.0001 |
| **No FBS+ GC** | Sucrose+ GC | 0 | 1 | 0 | 0.094±0.006** | 0.012±0.0001 |
| **No FBS+ GC** | Sucrose | 0 | 1 | 0 | 0.145±0.009* | 0.022±0.0002* |
| **FBS+ Sucrose** | Sucrose | 10 | 0 | 5 | 0.129±0.004* | 0.012±0.0001 |
| **FBS** | Sucrose | 10 | 0 | 0 | 0.114±0.002 | 0.012±0.0001 |

Asterisk denotes significantly different transport and cellular uptake concentration relative to the control (with FBS 12 h to assess sucrose) (*$p<0.05$, **$p<0.01$). Mean ± SD (n=6 measurement). Tukey HSD test applied.

### 6.4.4 Inhibition of sucrose uptake and transport to the basolateral side using the TC7 cell line

Caco-2 cells are commonly used in research as they are able to differentiate in long-term culture. They are also able to mimic the intestinal enterocyte with a continuous monolayer of cells linked by tight junctions. However, it consists of a heterogeneous cell population and highly depends on the culture conditions (Delie and Rubas, 1997; Turco *et al.,* 2011). To reduce the heterogeneity of the Caco-2 cells, the TC7 clone obtained was isolated from a late passage of the parental Caco-2 line (Turco *et al.,* 2011; Cheng *et al.,* 2014). This clone is characteristic of more developed intercellular junctions and more representative functions of the small intestinal enterocyte and more homogeneous population (Turco *et al.,* 2010). Caro *et al.* (1995) reported that TC7 cells show higher sucrase/isomaltase activity. Another study also observed that the TC7 clone population is more homogeneous and the intercellular junctions are more developed than the Caco-2 cell line (Zucco *et al.,* 2005). The clone is used as an alternative model for permeability assessments. In this experiment, transport and uptake of D-[$^{14}$C]-sucrose was compared between TC7 and the Caco-2 cell line.

The TEER value of the TC7 cells was measured after the pre- and post-incubation process. There was no significance between the pre- and post-incubation TEER values of each well, except for the green tea extract (Table 28). Higher concentrations of green tea extracts may improve the electrical resistance of the TC7 cell monolayers. TEER values between TC7 and Caco-2 cells were somewhat different when compared between themselves (Table 18 and Table 28). The orientation of tight junction proteins (claudin and occludin) is different between the two cell lines and this has been offered as a possible explanation in the literature. Growth conditions, e.g. the absence of FBS in the basolateral compartment after 7 days of attachment with TC7 cells, can also affect TEER values.

German chamomile and *Vitis viniferae* extracts inhibited D-[$^{14}$C]-sucrose transport in both cell lines. For the green tea extract, the transport of D-[$^{14}$C]-sucrose from the apical to basolateral side was 0.143 µmol in Caco-2 cells and

0.060 µmol in TC7 cells (the surface area of the two cell lines is different; transport experiments with TC7 cells were carried out transwells with a surface area that is 4 times larger than transwells used for Caco-2 cell experiment, and therefore, the result for transport was corrected based on this difference. Delie and Rubas, 1997 reported that pore size influences cell growth and transport. However for both cell lines pore size was same (0.4 µM) in the current study. These results indicate that the activity of sucrase is higher in TC7 cells, and therefore, the transport of D-[$^{14}$C]-sucrose or its products from apical to basolateral side was higher than for the Caco-2 cells (cells are breaking sucrose to glucose and fructose and therefore more glucose is transported from the apical to basolateral compartment). The uptake in Caco-2 cells was 0.009 µmol of D-[$^{14}$C]-sucrose and for TC7 cells it was 0.026 µmol D-[$^{14}$C]-sucrose. In the presence of green tea and German chamomile extracts, cellular uptake of D-[$^{14}$C]-sucrose was reduced significantly for the concentration points of 0.125 and 0.25 mg/ml ($p<0.01$) but it was not possible to reach $IC_{50}$. By contrast, the presence of higher concentrations of green tea and German chamomile extracts improved the cellular uptake of D-[$^{14}$C]-sucrose (Figure 74 and Figure 75). *Vitis viniferae* extract did not affect the cellular uptake (Figure 76). Table 29 shows the $IC_{50}$ values of D-[$^{14}$C]-sucrose transport and uptake with PFS extracts. Transport of D-[$^{14}$C]-sucrose to the basolateral side was significantly different for all the concentrations of green tea and German chamomile extracts compared to the control samples ($p<0.01$) and only the highest concentration of the *Vitis Viniferae* extract was significantly different to the control sample ($p<0.01$). Acarbose (0.1 mM) also inhibited 48 % of sucrose transport across the TC7 cells.

**Table 28** Blank corrected TEER of TC7 monolayers measured after pre-incubation (30 min) and post-incubation (60 min) in the absence (control) and presence of PFS extracts.

| PFS extracts | PFS (mg/ml) | TEER pre-incubation | TEER post-incubation | % difference |
|---|---|---|---|---|
| **Control** | 0 | 333 | 323 ± 9 | 3 |
| **German chamomile** | 1 | 311.5 | 311.3 ± 5 | 0 |
| **Green tea** | 1 | 330 | 391 ± 30 * | 118 |
| ***Vitis Viniferae*** | 1 | 343 | 326 ± 12 | 5 |

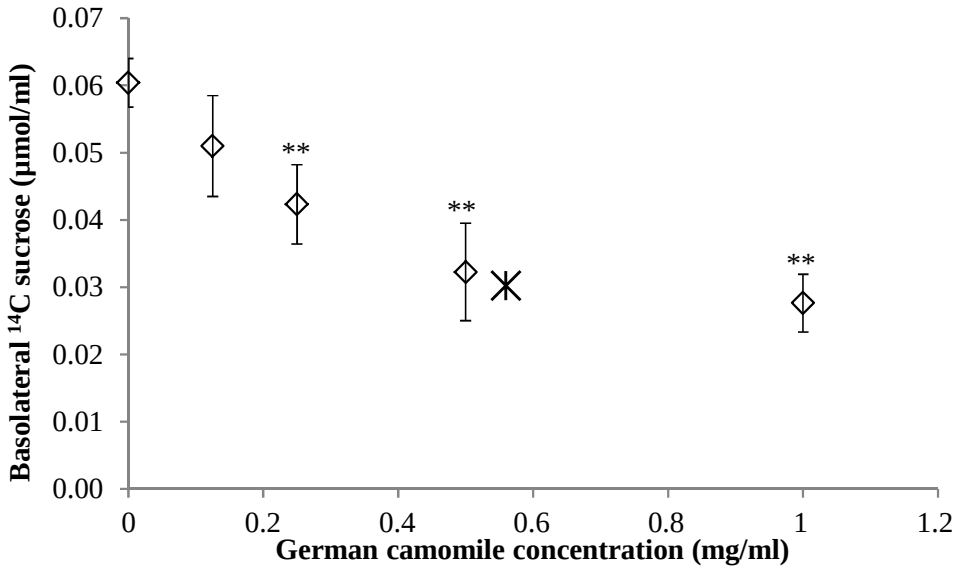
% difference demonstrates the difference between pre and post-incubation TEER values. The asterisk denotes a significantly different TEER measurement relative to control TEER measurement ($p<0.05$). Mean ± SD (n=6 per sample and 18 values for each sample). Tukey HSD test applied.

**Table 29** Effect of PFS on sucrose transport across the TC7 intestinal monolayer model, and on cellular uptake.

| Supplement extract | $IC_{50}$ (mg/ml) transport | $IC_{50}$ (mg/ml) uptake |
|---|---|---|
| **German chamomile** | 0.56 ± 0.035* | None |
| **Green tea** | 0.32 ± 0.05 | None |
| ***Vitis Viniferae*** | Non detectable | None |

Asterisk denotes significant difference of German chamomile compare to green tea extract ($p<0.01$). Mean ± SD (n=6 per inhibitor concentration). Tukey HSD test applied.

**A**




**B**

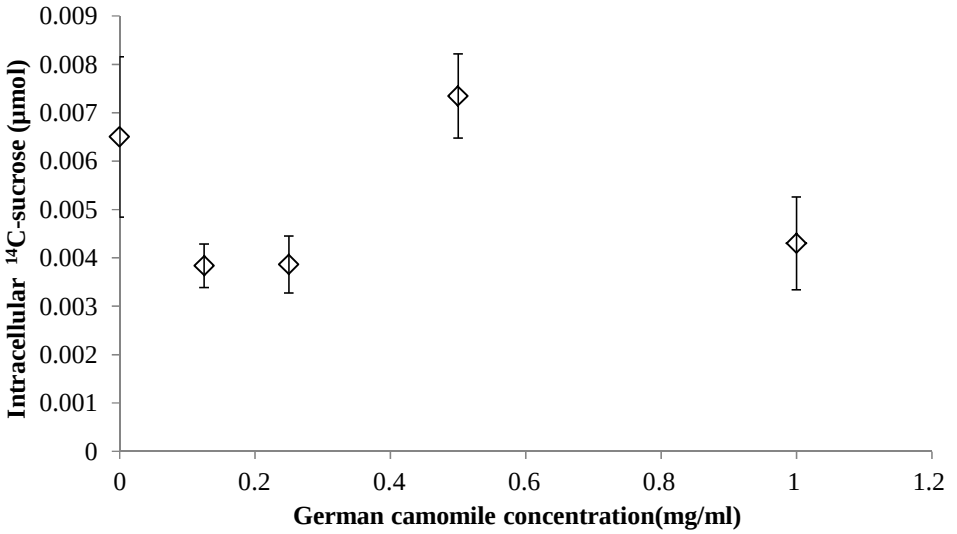
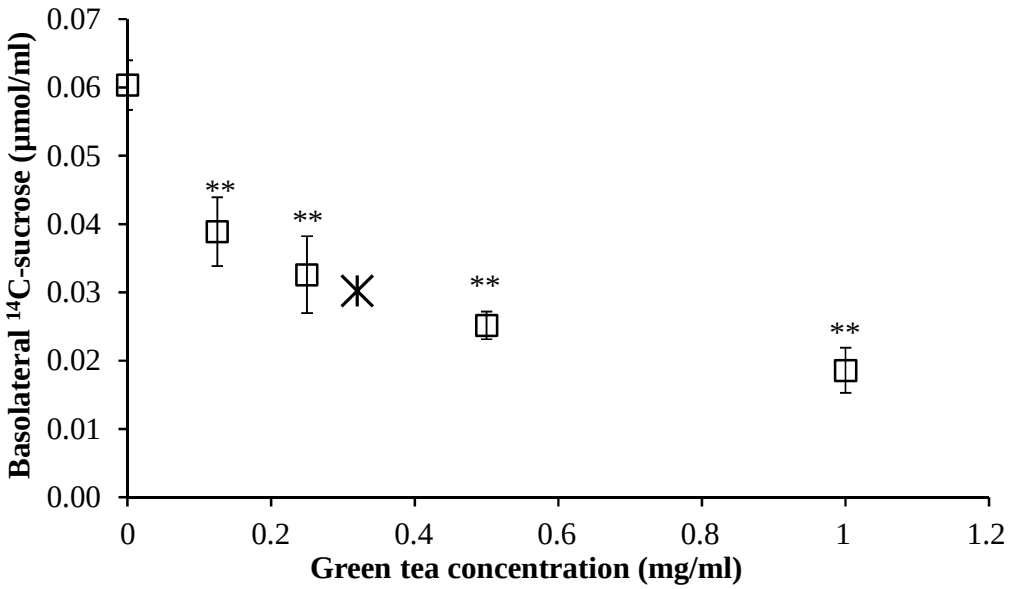



**Figure 74** A: Transport of radiolabelled sucrose and its products at different concentrations of German chamomile extract using TC7 differentiated monolayers.

B: Intracellular uptake of sucrose and its products in to TC7 cells at different concentrations of German chamomile extract. Inset show the magnified section with estimation of $IC_{50.}$ The asterisks denotes the significantly different cellular concentration relative to control (*$p<0.05$, **$p<0.01$). Mean ± SD (n=6 per concentration). Tukey HSD test applied.

**A**




**B**

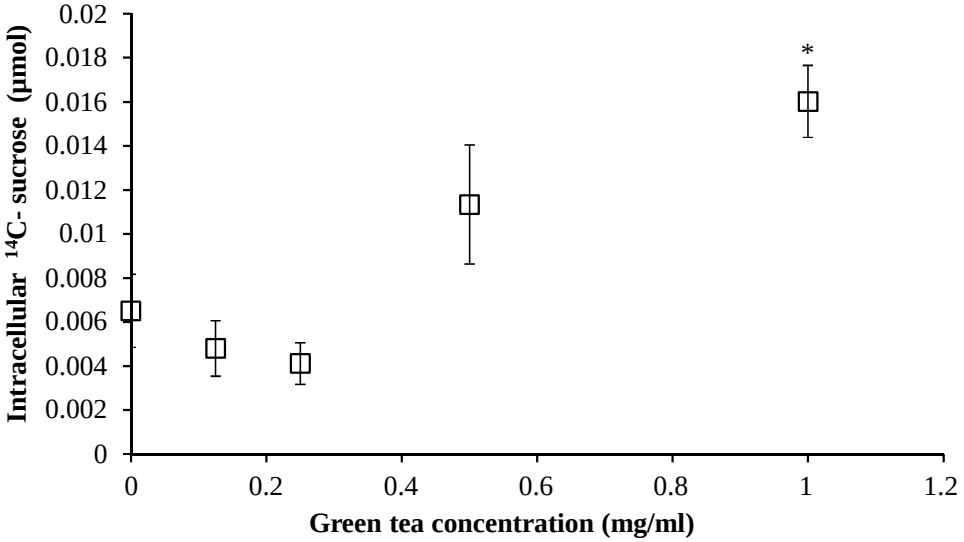
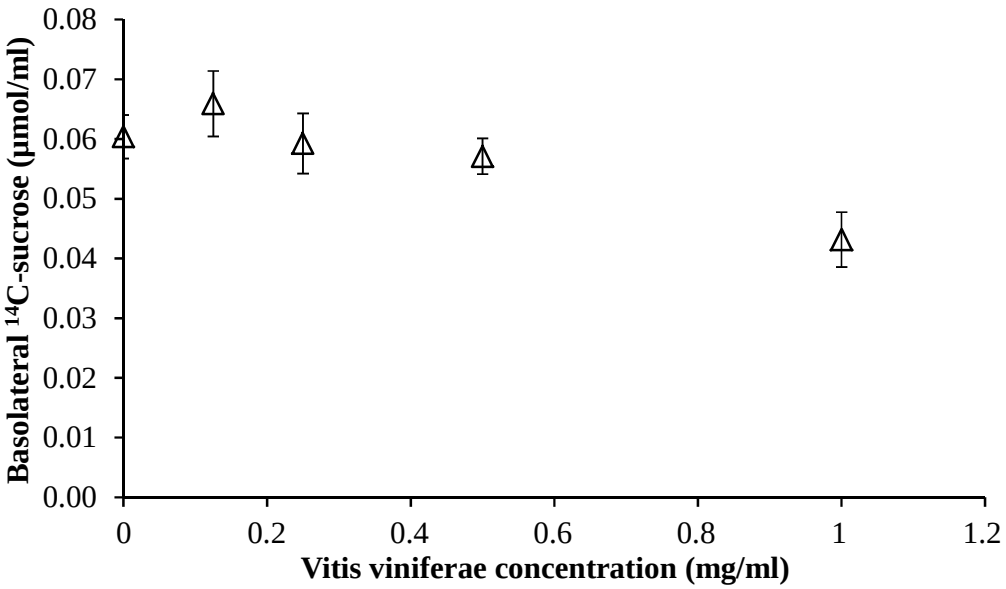



**Figure 75** A: Transport of radiolabelled sucrose and its products at different concentrations of green tea extract using TC7 differentiated monolayers.

B: Intracellular uptake of sucrose and its products in to TC7 cells at different concentrations of green tea extract. Inset show the magnified section with estimation of $IC_{50.}$ The asterisks denotes the significantly different cellular concentration relative to control (*$p<0.05$, **$p<0.01$). Mean ± SD (n=6 per concentration). Tukey HSD test applied.

**A**




**B**

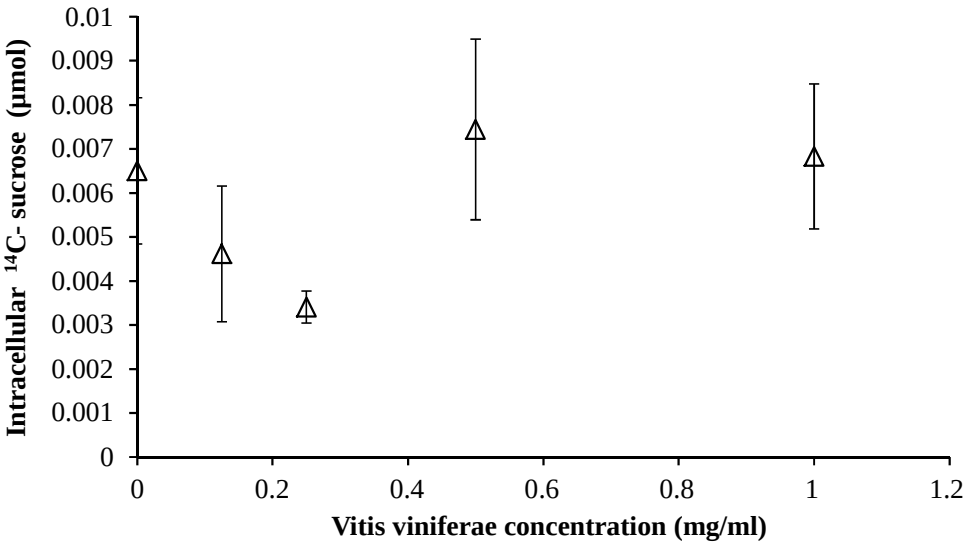



**Figure 76** A: Transport of radiolabelled sucrose and its products at different concentrations of *Vitis viniferae* extract using TC7 differentiated monolayers.

B: Intracellular uptake of sucrose and its products in to TC7 cells at different concentrations of *Vitis viniferae* extract. Mean ± SD (n=6 per concentration). Tukey HSD test applied.

## 6.5 Discussion

Sucrose contains one glucose and one fructose molecule. After hydrolysis of sucrose to glucose and fructose the absorbed glucose is used by primarily peripheral tissues in an insulin-dependent manner. Fructose is transported to liver and due to its lipogenic activity, it may contribute to the development of cardiovascular diseases, insulin resistance and type-2 (Hansen *et al.*, 2012).

In the literature, there is no study regarding radiolabelled sucrose transport to the basolateral side and cellular uptake analysis using the Caco-2 cell line. The presence of some PFS extracts has an influence on sucrose uptake and transport to the basolateral compartment by inhibiting the activities of either glucose transporters or sucrase. The active inhibitor compounds in the PFS influence either the glucose transporters on the apical side (GLUT5, GLUT2 or SGLT1) and/or the transporters on the basolateral side, GLUT2 or GLUT5. They may also have an effect on the villous membrane-bound sucrase activity in Caco-2 cells. Sucrose was transported from the basolateral to apical side paracellularly and transport of sucrose (after hydrolysis by sucrase to glucose and fructose) across the basolateral side was 2.4 nmol/min (approximately half of the transported radiolabelled sucrose and its products concentration was transported back to apical compartment) (Figure 77). Therefore, it may be concluded that the activity of the sucrase enzyme is not high. While green tea and *Vitis Viniferae* extracts were ineffective on the cellular uptake of sucrose, German chamomile extract inhibited it with an $IC_{50}$ value of 0.309 mg/ml. It may be concluded that German chamomile extracts have the ability to inhibit sucrase activity more effectively than other PFS extracts.

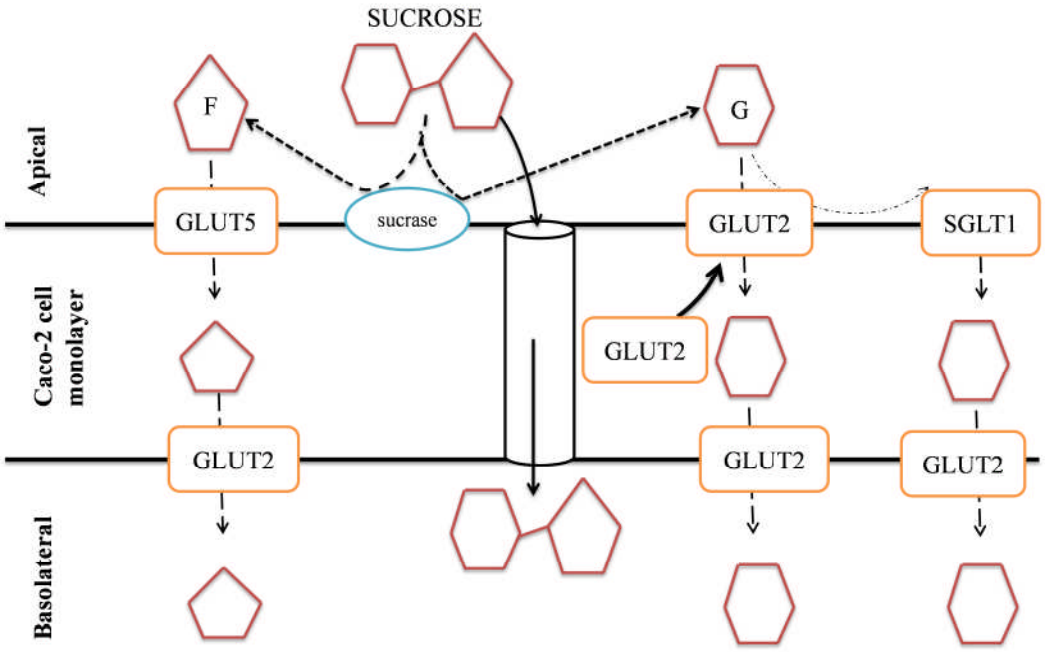



**Figure 77** Absorption of sucrose in to the blood. G: glucose F: fructose.

Hansawasdi and Kawabata (2006) reported, using Caco-2 cells, that Mulberry (*Morus Alba*) tea inhibited sucrase activity and glucose transport to the basolateral side. They analysed the glucose concentration of the apical and basolateral compartment media using the glucose oxidase assay. They also reported the effect of the commercial α-glucosidase inhibitor drug known as 1-deoxynojirimycin as a positive control with an $IC_{50}$ value of $7.7x10^{-5}$ mM. The villous membrane-bound sucrase activity in Caco-2 cells and the transport of glucose to the basolateral media were inhibited by a hot water extract of mulberry tea. Another study regarding grape seed extract (GSE), rich in flavanols (54%), analysed its inhibitory effect on sucrase activity (Laurent *et al.*, 2007). GSE reduced the sucrase activity by 61% in the Caco-2 cell monolayer at a concentration of 0.3 g/l (Laurent *et al.*, 2007). Here, the transport of sucrose was analysed with radiolabelled sucrose using the Caco-2 cell monolayer. Further studies should be performed to observe the inhibitory mechanism of PFS extracts for sucrose transport. There are two possible mechanisms for the inhibition of sucrose transport, but in this study the type of inhibition could not be observed since radiolabelled sucrose, and its products, all contribute to the

measured total counts. As the level of sucrose in the apical compartment was not analysed chemically, the degree of sucrase inhibition could not be observed but it could be concluded that the overall system were affected by the PFS extracts.

In the literature there are several animal studies regarding the inhibition of sucrase activity with polyphenols. It is reported that gallic acid and tannic acid were potent inhibitors of sucrase activity in mice and rats (Welsch *et al.,* 1989; Gupta *et al.,* 2010; Gupta *et al.,* 2007). Eucalyptus leaf is traditionally used to treat diabetes in South America and Africa. Sugimoto *et al.* (2005) observed the effect of Eucalyptus leaf extract (ELE) in high sucrose diet-fed rats for 5 weeks. The intestinal sucrase activity was analysed in the presence of ELE using *in vitro* tests. The inhibition of sucrase was concentration dependent with $IC_{50}$ value = 3 mg/ml. The extract was found to inhibit sucrase activity, suggesting the influence of the isolated polyphenols from the ELE, 1,2,3,4,6-penta-O-galloyl-b-glucose (hydrolysable tannins). Ishikawa *et al.* (2007) reported that a leaf from Pakistan called *Nerium Indicum* was able to reduce the postprandial glucose level of rats after they were orally fed with sucrose. Another study analysed acerola fruit and it was also able to inhibit intestinal sucrase of mice that were orally fed with sucrose (Hanamura *et al.,* 2006). Hosaka and colleagues (2011) analysed the effect of buckwheat on diabetes. They found that mice fed with buckwheat bran extracts (BBE) had a lower blood glucose level compared to control mice after 60 min of oral sucrose administration. However, rutin (one of the major components of BBE) did not affect the blood glucose level. Therefore, it was concluded that BBE inhibits sucrase activity but rutin did not have any inhibitory activity on sucrase.

There are also human studies regarding the effect of polyphenols on the blood glucose level. Wilson *et al.* (2008) studied twelve healthy participants fed with sucrose (35 g) and berries (150 g puree made of bilberries, blackcurrants, cranberries and strawberries providing nearly 800 mg polyphenols). Berries are a rich source of anthocyanins, flavonols, proanthocyanidins and phenolic acids. Participants fed without berries produced a different postprandial glycemic response compared to the subjects who were fed with berries. Törrönen *et al.* (2009) observed the effect of berry puree (containing bilberries, cranberries,

strawberries and blackcurrant) on the blood glucose level of 12 healthy subjects. The subjects were fed with 150 g of a berry meal that also contained 35 g sucrose or a control sucrose load in a randomised, controlled cross-over study. The blood glucose level was significantly lower at 15 and 30 min after the consumption of the berry meal and significantly higher at 150 min. The peak glucose concentration reached was at 45 min in subjects who consumed berry meal, whereas it was 30 min for subjects who consumed the control meal. They concluded that berries were able to decrease the postprandial glucose level in healthy subjects, and the delayed and reduced glycaemic response showed the decrease of sucrose digestion or absorption from the berry meal. Therefore, they (bilberries, cranberries, strawberries and blackcurrant) may be used to improve diabetes (Törrönen *et al.*, 2009). Hoggard *et al.* (2013) studied 8 male diabetic subjects. After a 2-week washout period, the subjects were fed with 50 g bilberries (equal to standardised 0.47 g bilberry extract, 36% (w/w) anthocyanins) or placebo (75 g glucose drink) in a double-blinded cross-over study design. The bilberry extract was able to reduce the postprandial glycaemic response and insulin in type-2 diabetic subjects. They assumed that this effect comes from the attenuated carbohydrate absorption or digestion.

In conclusion, polyphenol-rich products may help to manage diabetes. PFS extracts (green tea and German chamomile) reduced the transport of radiolabelled sucrose to the basolateral compartment. As the sucrose concentration in the apical compartment was not analysed, the degree of sucrase inhibition was not observed, but it could be concluded that the transporters were affected by PFS extracts more than sucrase as half of the transported sucrose was transported from the basolateral to the apical compartment. Therefore, due to their inhibitory activity (acarbose-like activity) on liberated glucose transport or hydrolysis of sucrose by sucrase activity, green tea and German chamomile extracts have potential for the management of type-2 diabetes.

# CHAPTER 7 DISCUSSION AND FUTURE WORK

## 7.1 Method development and novelty

The International Diabetes Federation reported that >371 million people around the world have diabetes. This is predicted to rise to >550 million by 2030, with 90 % of all people having type-2 diabetes. In the UK, it is assumed that ~750,000 people have type-2 diabetes, but have not been diagnosed, and ~2.7 million have been diagnosed (Diabetes, UK, 2012). Due to the side effects of some common drugs (flatulence, diarrhoea, tiredness and upset stomach) in the treatments of diabetes, use of herbal remedies has increased; 2 to 3.6 million diabetic patients in the USA use herbal remedies for management of diabetes (Dabaghian *et al.*, 2012). The World Health Organization (WHO) (2003) reported that 65 to 80 % of the world population uses traditional medicines for health care.

Glucose is an important carbohydrate that plays a role in the development of diabetes. Sucrose can lead to an increase of post-prandial blood glucose (liberated glucose from sucrose) and liberated fructose is utilised in the liver to generate products such as glucose, glycogen, lactate and pyruvate, and affect lipid metabolism, which also might affect insulin resistance and type-2 diabetes (Törrönen *et al.*, 2010; Hansen *et al.*, 2012; Halschou *et al.*, 2012). Type-2 diabetes is caused when there is deficient or ineffective insulin for blood glucose absorption resulting in a high blood glucose level. To prevent or delay type-2 diabetes, the blood glucose level needs to be controlled. Diabetes increases the risk of heart disease and complications that damage the kidney, retina and peripheral nerves (Liu *et al.*, 2000; Liese *et al.*, 2005; Flynn, *et al.*, 2005; Manzano and Williamson, 2010; Hauner *et al.*, 2012).Another recent study analysed 3 cohort study (1984-2008 Nurses' health study I, 1991-2009 Nurses' health study II and 1986-2008 Health professional follow up study (started with healthy men and women)) and observed that higher intake of GI and GL increased the risk of type-2 diabetes. Therefore, controlling the blood glucose level will decrease the risk of those diseases and may assist in the prevention of

progression to type-2 diabetes. There are several *in vitro* and *in vivo* studies regarding this.

Polyphenols and their effect on sugar metabolism was first reported ~46 years ago. A report from 1969 indicated that tannins from ripe and green carobs inhibited α-amylase activity by ~45 % (Tamir and Alumot, 1969). Another report from 1984 observed the effect of polyphenol intake on blood glucose level of diabetic and non-diabetic patients and observed a negative correlation between the glycaemic index and the total intake of polyphenols (Thompson and Yoon 1984). Following those studies, Welsch *et al.* (1989) reported that SGLT1 activity was inhibited by chlorogenic, ferulic, caffeic and tannic acids using rat intestinal brush border membrane vesicles and indicated an 80 % reduction of glucose transport with chlorogenic acid and 30-40 % reduction observed in the presence of ferulic, caffeic and tannic acid.

The aim of this research was to investigate sucrase, maltase, isomaltase and human salivary α-amylase inhibition by the plant food supplement (PFS) extracts and the effect of PFS on sugar transporters (SGLT1, GLUT2 and GLUT5). The initial steps of the *in vitro* tests development involved determining the activities of sucrase, maltase, isomaltase and human salivary α-amylase in an acetone-extract of rat intestinal tissues, improving a previously published method (for α-glucosidase: Gao *et al.,* 2007 and for α-amylase: Akkarachiyasit *et al.,* 2010) by analysing the glucose concentration via the hexokinase assay, and analysing the effect of PFS on sugar transporters (SGLT1, GLUT2 and GLUT5) using a previously published method (Manzano and Williamson 2010) using the Caco-2 cell monolayer. Using the Caco-2 cell model, uptake and transport of $^{14}$C-radiolabelled glucose, fructose and sucrose has been investigated in the presence of PFS and measured with a scintillation counter. The highest concentration of post-prandial polyphenols is in the gut lumen compared to any other site in the body (Williamson, 2012). The Caco-2 cell line is the best choice to assess the inhibition of sugar transport because several morphological and functional characteristics of the mature enterocyte is able to expressed by Caco-2 cells

(Sambuy *et al.,* 2005; Angelis and Turco, 2011) and it is thought that glucose metabolism may be affected in the gut lumen.

In the literature, the effect of polyphenols on glucose transport has been analysed using brush border membrane vesicles or everted sacs and Caco-2 cells (Welsch *et al.,* 1989; Kobayashi *et al.,* 2000; Shimizu *et al.,* 2000; Song *et al.,* 2002; Cermak *et al.,* 2004; Johnston *et al.,* 2005; Li *et al.,* 2006; Manzano and Williamson 2010; Goto *et al.,* 2012; Farrell *et al.,* 2013). There are some animal model studies and limited human intervention studies regarding green tea, chamomile and grape extracts or their active components on glucose metabolism. However, the literature evidence for the inhibition of cellular glucose uptake and transport by polyphenols across Caco-2 cells is limited. Also, to the best of our knowledge, this research is the first report regarding the analysis of cellular uptake and transport of $^{14}$C-sucrose and $^{14}$C-fructose using the Caco-2 cell monolayer with polyphenol-containing extracts. The objective of this research was to make a preliminary assessment of the capacity of PFS extracts to reduce glucose, fructose and sucrose transport (acarbose-like activity) across the Caco-2 cell monolayers. Additionally, $^{14}$C radioactivity was used due to its easy detection. Using radiolabelled glucose, fructose and sucrose allowed high sensitivity. However, there was a limitation regarding the study; the complete metabolism of the biological intestine does not occur with Caco-2 cells (low level of sucrase expression), and therefore, only the effect of unmodified compounds (extracts' digestion does not occur) on sugar metabolism was analysed.

Present publications regarding radiolabelled glucose uptake using Caco-2 cells are limited. Goto *et al.* (2012) reported that tiliroside inhibited glucose uptake in Caco-2 cells with an $IC_{50}$ value of 94 µM (cells were seeded to plates, therefore there was no monolayer). Johnston *et al.* (2005) analysed the effect of different classes of dietary polyphenols on glucose uptake with Caco-2 cells (cells were seeded to plates, therefore there was no monolayer) and reported that aglycones inhibited GLUT2 and glycosides inhibited SGLT1. Farrell *et al.* (2013) investigated the effect of hydrolysed and unhydrolysed herbal extracts on

glucose transport and uptake using the Caco-2 cell line and reported that herbal extract mixtures decreased glucose transport <54 %. Alzaid *et al.* (2013) observed that the glucose transport in Caco-2 cells in the presence of berry extract was significantly decreased (58 % of total glucose uptake). They also investigated the effect of cyanidin, cyanidin glucoside and cyanidin rutinoside on glucose uptake (~21, 20 and 21 % of total glucose uptake, respectively) and found that all these compounds significantly inhibited glucose uptake. In the current study, using the Caco-2 cell monolayer, the transport and cellular uptake of glucose was analysed in the presence of PFS. PFS extracts were chosen based on the list in Annex 1 (the partner's suggestions) and EFSA.

Previous studies have shown that polyphenols have the potential to inhibit α-glucosidase and α–amylase enzymes, which in turn would slow down the products of sucrose (glucose and fructose α-1,4 glycosidic bonds), maltose (glucose and glucose 1-4α linkage), isomaltose (glucose and glucose 1-6α linkage) and starch (large number of glucose units linked with glycosidic bonds) reaching the blood (Kim *et al.,* 2000; Shimizu *et al.,* 2000; Song *et al.,* 2005; Bhandari *et al.,* 2008; Kamiyama *et al.,* 2010; Boath *et al.,* 2012; Akkarachiyasit *et al.,* 2013; Posedek *et al.,* 2014; Pantidos *et al.,* 2014). The result of inhibition of α-glucosidase and human salivary α-amylase enzymes by PFS extracts appears to be in agreement with the literature. In the current study, each of the PFS extracts was analysed for their possible interference with α-glucosidase and α–amylase assays and assessed by considering critical parameters affecting the final inhibition (maltodextrin content of the extract, polyphenol reduction potential (the number of OH groups in the chemical structure) causing an interference with DNS and hexokinase reagent (Rice-Evans *et al.,* 1996) and the effect of DMSO to enzyme activity). After the enzymatic hydrolysis of substrates the potential reactive inhibitors were removed by SPE and then DNS and/or hexokinase reagent was used to assess the product. Therefore, in many published studies the reducing capacity of polyphenols was ignored and the results of the inhibition may be inconsistent.

## 7.2 Summary of the results

For Caco-2 cells both uptake and transport is important. According to the analysis of different concentrations of sugars, less fructose was transported to the basolateral side and glucose transport was higher than sucrose transport. Therefore, it may be assumed that the transport of glucose is faster than sucrose and fructose in the intestine. By contrast, cellular uptake of fructose was higher than glucose and sucrose uptake was the lowest. GLUT5 is a highly specific transporter for fructose. Cellular uptake of fructose increases with brush border fructose transport and fructose transport increases only with luminal fructose (Cui *et al.,* 2004; Suzuki *et al.,* 2011). Cui *et al.* (2004) reported that cellular uptake of fructose is higher than glucose after 4 h of perfusion of high glucose and high fructose in neonatal rat intestine. To create a starvation stress in the cells, FBS was removed from medium 12 h before the analysis of different incubation time points. It was observed that basolateral transport of glucose was not affected in the absence of FBS; however, cellular uptake of glucose significantly decreased at 30 min. Basolateral transport of fructose was not affected in the absence of FBS; however, cellular uptake significantly decreased at 30 and 60 min ($p<0.05$). Sucrose transport to the basolateral compartment was significantly decreased under FBS starvation at 30 min but it was not affected at 60 min, and cellular uptake of sucrose was significantly increased at 30 and 60 min under FBS starvation.

Glucose, fructose and sucrose transport across the Caco-2 cell monolayer was significantly attenuated in the presence of PFS. Green tea, German chamomile and *Vitis Viniferae* extracts inhibited the transport of glucose, fructose and sucrose when tested independently. However, the *Vitis Viniferae* extracts were not able to achieve 50 % inhibition for the sucrose and fructose transport. While the cellular uptake of glucose and fructose was inhibited by the PFS extracts, they were in effective on the cellular uptake of sucrose. By contrast, Pelargonium and Echinacea were ineffective for both transport and cellular uptake of sugars. Glucose transport to the basolateral compartment was analysed under $Na^+$-containing and $Na^+$-free conditions in the presence of German

chamomile, and we concluded that the inhibition of GLUT2 was greater than SGLT1. Accordingly, the results indicate that apical GLUT2 is the primary target of the compounds from the German chamomile extract. Different concentrations of sucrose transport were observed from the apical to basolateral and from the basolateral to apical compartment. It was found that half of the transported sucrose is transported back to the lumen, which may indicate paracellular transport of sucrose across the intestine in the absence of sucrase. The kinetic mechanism of glucose and fructose transport inhibition and cellular uptake was analysed with 1 mg/ml German chamomile and showed that basolateral transport of fructose and glucose represented the competitive inhibition, whereas cellular uptake of fructose was uncompetitive but glucose uptake was mixed competitive.

To analyse the effect of digestion on the biological activity of the German chamomile extract, the extract was subjected to enzymatic hydrolysis followed by extraction as a model of the first step of digestion in the gut. This digestion affects the biological activity of German chamomile as assessed by glucose transport across the cell monolayer, which was decreased by ~50%. The cellular uptake of glucose was significantly decreased by the German chamomile hydrolysed ACN-treated sample but the cellular uptake of glucose with the EtAC-extracted sample was significantly increased compared to the control sample. However, the cellular uptake of glucose with the ACN-control and ACN-treated samples were the same. Therefore, it was concluded that the background of the hydrolysis process is affecting the cellular uptake. Also, the inhibition of glucose transport across the Caco-2 cell monolayer was 32 and 34 % for the ACN-treated and EtAC-extracted samples, and therefore, it may be concluded that it is mainly aglycones that have a role in inhibiting glucose transport (while the EtAC-extract sample contains aglycone, the ACN-treated samples contain glucosides and aglycones). These results suggested that after ingestion of the PFS, the intact and hydrolysed polyphenols (in the gut lumen) have an important role in the attenuation of glucose absorption. Some of the compounds that are present in the German chamomile extract were also tested to determine their contribution to the inhibition of transport and uptake of glucose

and fructose. Luteolin was the most effective compound to inhibit glucose transport; by contrast, luteolin-7-*O*-glucoside produced the most effective inhibition of cellular glucose uptake (this data also supports the result for the German chamomile hydrolysed data). Some of those compounds inhibited both uptake and transport of fructose. However, none showed high inhibition that could affect both uptake and transport of fructose.

FBS contains important components such as hormones and growth factors. Therefore, the addition of FBS to the cell medium promotes cell adhesion and proliferation (epidermal growth factor, fibronectin and thyroxine). To create starvation stress in the cells, FBS was removed from the cell culture medium 12 h before the experiment. The response of the Caco-2 cell line to stress (FBS starvation or glucose, fructose and sucrose load overnight (12 h)) was assessed after treatment with German chamomile or additional glucose. Both transport and uptake of sugars were tested. FBS starvation overnight and addition of German chamomile (1 mg/ml) (12 h) did not have any effect on the cellular uptake and transport of glucose and fructose. However, transport and cellular uptake of sucrose increased significantly in both conditions ($p<0.05$).

Deoxyglucose cannot be further metabolised in the intestine since the 2-hydroxyl group is replaced by hydrogen; therefore, it is phosphorylated rapidly and completely and is not effluxed following phosphorylation (Olefsky, 1978, Burant and Bell, 1992). Transport of deoxyglucose was significantly inhibited by the same PFS treatments that affected glucose. In contrast to glucose, deoxyglucose uptake was not changed by any PFS treatments. Inside the cell, glucose is converted into pyruvate by glycolysis. The first step is phosphorylation of glucose by hexokinase, which adds a charged phosphate group to glucose (glucose-6-phosphate). Subsequently, glucose phosphate isomerase converts glucose-6-phosphate to fructose-6-phosphate, and subsequently by phosphofructokinase (PFK1) to fructose 1,6 biphosphate and ADP. The latter is important for glycolysis as it is an irreversible step. This enzyme is an allosteric enzyme, controlled by some activators (AMP and fructose 2-6 biphosphate) and inhibitors (ATP, glucagon and low pH). Deoxyglucose is also an inhibitor of

PFK as there is no signaling involved with deoxyglucose inside the cell while glucose signals to PFK. It may be concluded that PFS extracts play an important role in glucose metabolism leading to a decrease in the cellular uptake of glucose.

Inhibition of sucrose transport was also analysed with TC7 cells (subclone of Caco-2). It was reported that TC7 cells have a higher sucrase activity than Caco-2 cells (Caro *et al.,* 1995). Transport of radiolabelled sucrose or its products to the basolateral side with German chamomile and *Vitis viniferae* extracts showed a similar degree of inhibition with the Caco-2 cell transport study; however, the results for the inhibition activity of green tea extract were different in the two cell lines. More radiolabelled sucrose or its products were taken up by TC7 cells compared to Caco-2 cells, which is a result of the higher activity of sucrase. For the three PFS extracts, cellular uptake of sucrose was reduced significantly ($p<0.01$). Acarbose (0.1 mM) also inhibited 48 % of sucrose transport across TC7 cells.

In this present work, it was also investigated whether the presence of PFS extracts can inhibit human salivary α-amylase and rat intestinal sucrase, isomaltase and maltase (acarbose-like activity). There were some limitations regarding the study; it was observed that due to the reducing activity of polyphenols, the measurement of products by DNS and hexokinase reagent could not be analysed. Therefore, using solid phase extraction, polyphenols were removed and analysis completed. As maltodextrin was found to be a potent substrate for α-amylase and α-glucosidase enzymes, the maltodextrin content of German chamomile was removed and this purified extract was used for the analysis. Purified German chamomile and green tea extracts were found to be moderate inhibitors of α-amylase digestion of amylopectin and α-glucosidase enzymes.

## 7.3 Conclusion

Plant food supplements may influence intestinal glucose transport across the brush border membrane of enterocytes. Therefore, they can be seen as beneficial for use by diabetics as part of a nutritional intervention and in combination with

exercise and drug treatment. Avoiding or blunting postprandial spikes of glucose in diabetics is regarded as a first-line strategy to amplify determinable effects related to diabetic complications. This research is a pioneering study regarding the analysis of cellular uptake and transport of $^{14}$C-sucrose and $^{14}$C-fructose using the Caco-2 cell monolayer with polyphenol-containing extracts. However, the evidence in the literature for the inhibition of cellular glucose uptake and transport by polyphenols across Caco-2 cells is limited. In this research, sugar transport was investigated with PFS extracts, using the Caco-2 cell monolayer. The inhibitory activity of the green tea extract is higher than German chamomile. *Vitis viniferae* extracts show the lowest inhibitory effect for the transport of glucose, fructose and sucrose. Due to the acarbose-like activity of the PFS extracts, they may have a potential role to play in reducing the risk of diabetes by inhibiting the hydrolysis of starches and reducing post-prandial blood glucose spikes. PFS may also be beneficial for use by diabetics due to their effect on sugar transport and metabolism.

## 7.4 Future work and overview

In summary, the experimental hypothesis of this thesis is supported by the results. According to the reported results, plant polyphenols definitely have an influence on carbohydrate metabolism; however, it is not clear how much difference it will make to diabetes management. Therefore, human intervention studies are needed to assess the effect of plant food supplements and to confirm the optimum dose of the supplement. In some studies postprandial blood glucose level measured with blood glucose monitor. However, it will be possible to measure the glucose concentration liberated from sucrose but not specific to glucose transport. The measurement of $^{13}$C-D-glucose could be a more precise and sensitive method for blood glucose monitoring in the presence of food matrix and it has been commonly used in the human intervention studies as a metabolic tracer. Using the stable $^{13}$C-D-glucose isotope will enable the specific measurement of $^{13}$C labelled glucose and the calculation of $^{13}$C to$^{12}$C-D-glucose ratio using HPLC separation and spectrophotometry detection of glucose. Healthy adult volunteers will be asked not to consume polyphenol-rich food for three days. The subjects will have fasted for 12 h before the study day. On the day of study, it may be

asked of them to consume a specific amount of glucose drink (including $^{13}$C-D-glucose) and German chamomile supplement. Then, blood would be collected at specific time points up to 2 hours and glucose measurement would be analysed using HPLC-MS.

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# ANNEX 1

Priority plants for benefits as identified by partners and SIAG members based on analytical challenges, market share and market prospects (www.plantlibra.eu)

| **Latin name** | **Common name** | **Used portion** | **Active compounds** | **Main reported benefits** | **Origin** |
|---|---|---|---|---|---|
| *Abies alba Mill.*<br>*Aloe ferox Mill.* | Silver fir<br>Bitter aloe/tap aloe | Buds, barks, leaves, resin Leaves/Leaves Juice | Essential oils<br>Anthroquinones | Balsamic expectorant, antimicrobial, rubefacient Helps to promote intestinal regularity. Ease intestinal transit | Western Europe<br>S. Africa, Central/S. America |
| *Artemisia abrotanum L.*<br>*Calendula officinalis L.*<br>*Carica papaya L. Carum carvi L.* | Southernwood<br>Pot marigold<br>Papaya Caraway | Herb and young shoots<br>Leaves and flowers<br>Fruits, leaves, latex Seeds, roots, leaves | Essential oils<br>Carotenoids and essential oil Enzymes, phenolyc compounds<br>Essential oils, sennosides | Antiseptic, cholagogue<br>Antiphlogistic, vulnerary and soothing of the skin and mucosae<br>Digestive, antimicrobial, Antispasmodic, digestive, carminative, expectorant Helps to promote intestinal regularity. | Southern Europe Europe, Asia, North Africa Central/South America<br>Europe, Asia, North Africa |

| | | | | | |
|---|---|---|---|---|---|
| *Chrysanthemum balsamita Desf.*<br>*Cichorium intybus L.*<br>*Citrus aurantium L. var. dulcis* | Costmary<br>Chicory<br>Sweet orange | Leaves and flowers<br>Flowers, leaves, roots<br>Skin of fruits | Essential oils<br>Inulin<br>Essential oils | Spasmolitic, balsamic, cholagogue<br>Tonic affect upon the liver and digestive tract<br>Ansiolitic, stomachic, carminative, antimicrobial (E.O.) | Europe, Africa<br>Wide distribution<br>Mediterranean |
| *Citrus limon Burm.*<br>*Crataegus monogyna Jacq.* | Lemon<br>Hawthorn | Skin of fruits<br>Flowers, fruits, leaves | Essential oils<br>Flavonoids | Ansiolitic, antimicrobial (E.O.)<br>Mild cardiotonic, hypotensive, antioxidant | Mediterranean<br>Wide distribution<br>Mediterranean |
| *Cuminum cyminum L.* | Cumin | Seeds | Essential oils | Carminative, spasmolitic | Europe, Asia, North Africa |
| *Echinacea pallida Britton*<br>*Eschscholtzia californica Ch.* | Pale purple coneflower<br>California poppy | Herb, roots<br>Herb tops | Echinacosides<br>Eschscholtzine and californidine | Adaptogen, antiseptic, immunostimulant<br>Mild sedative, ansiolitic, antispasmodic | North America, Europe<br>North America, Europe |

| | | | | | |
|---|---|---|---|---|---|
| *Foenicolum vulgare Mill.*<br>*Ginkgo biloba L.*<br>*Glycyrrhiza glabra L.* | Fennel<br>Maidenhair tree<br>Licorice | Seeds, leaves and roots<br>Leaves<br>Roots | Essential oils<br>Ginkgolides<br>Glycyrrhetic acid | Carminative, spasmolitic<br>Memory and concentration enhancer<br>Antiphlogistic, emollient, mildly ease intestinal transit. Adrenergic tonic | Europe, Asia,<br>South America, China<br>Asia, Mediterranean |
| *Grindelia Robusta Nutt.*<br>*Harpagophytum procumbens DC*<br>*Helichrysum italicum G. Don.* | Great valley gumweed<br>Devil's claw<br>Curry plant | Leaves and flowering top<br>Roots<br>Flowering tops | Total phenols, grindelic acid<br>Iridoid glycoside, harpagosid<br>Flavonoids, caffeol quinic acid | Expectorant, broncospasmolitic<br>Antiphlogistic, antirheumatic, bitter tonic<br>Antispasmodic, antiphlogistic, mild choleretic | North America<br>South Africa<br>South Europe |
| *Hibiscus sabdariffa L.*<br>*Hippophae rhamnoides L.*<br>*Lavandula angustifolia Mill.* | Roselle<br>Sea buckthorn<br>Lavender | Flowers<br>Leaves, flowers, fruits<br>Flowers, flowering tops | Antioxidants<br>Carotenoids and essential oil<br>Essential oils | Antioxidant, mildly ease the intestinal transit.<br>Antibacterial and soothing agent of the skin and gastric mucosa. Tonic | Tropical areas<br>Europe<br>East Europe, Mediterranean |

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| *Matricaria recutita L.*<br>*Melissa officinalis L.*<br>*Myrtus communis L.* | Chamomile<br>Lemon balm<br>Myrtle | Flowers and flowering tops<br>Leaves and flowering shoots<br>Leaves and fruits | Bisabolols, flavonoids, coumarins<br>Rosmarinic acid<br>Essential olis | Antimicrobial, mild ansiolitic, tonic Antiphlogistic, spasmolitic, vulnerary, mild ansiolitic<br>Mild sedative, spasmolitic, carminative Antiphlogistic, expectorant, antimicrobial, diuretic, tonic | Wide distribution Europe, Asia, North Africa<br>Europe and Asia |
| *Olea Europea L.*<br>*Panax ginseng C.A. Meyer* | Olive<br>Ginseng | Oil and leaves<br>Roots | Oleuperine<br>Ginsenosides | Antispasmodic, hypotensive<br>Adaptogen, tonic, immunomodulant, cardiotonic, enhance mental faculties | Mediterranean<br>China, Korea |
| *Passiflora incarnata L.* | Passion flower | Herb and leaves | Flavonoids | Ansiolitic | Europe, North America |

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| *Pelargonium sidoides DC* | Umckaloab | Roots | Coumarins, flavonoid and catechins | Mucolitic, immunostimulant, antimicrobial, antispasmodic | South Africa |
| *Peumus boldus Molina*<br>*Plantago lanceolata L.*<br>*Plantago ovata Forsk.* | Boldo<br>Ribwort Plantain<br>Hispagula | Leaves<br>Herb and leaves<br>Seeds and seed husks | Boldine<br>Aucubine<br>Mucillage | Cholagogue, choleretic<br>Antiphlogistic, ease the intestinal transit, helps to promote intestinal regularity<br>Helps to promote intestinal regularity. Ease intestinal transit | Chile<br>Europa Europe,<br>Asia |
| *Pseudowintera colorata*<br>*Dandy Serenoa serrulata Hook f.*<br>*Silybum marianum Gaertn.* | Mountain Horopito<br>Saw palmetto<br>Milk thistle | Leaves<br>Fruits<br>Herb and seeds | Sesquiterpene dialdehyde polygodiali<br>Phytosterols, fatty acid<br>Sylimarin | Antimicotic, gastric spasmolitic<br>Help reducing benign prostatic hyperplasia<br>Epatoprotective, choleretic, antioxidant, bitter tonic | New Zealand<br>Florida<br>Wide distribution |
| *Taraxacum officinale Weber* | Dandelion | Herb and roots | Terpenes, sterols | Diuretic, choleretic<br>Digestive, antimicrobial, | Wide distribution |

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| *Thymus serpillum L. s.l.* | Wild Thyme | Herb and leaves | Essential oils | spasmolitic, carminative, expectorant. | Europe, Asia |
| *Trifolium pratense L.* | Red clover | Flowers, leaves | Isoflavonoids, | Mild estrogenic | Europe, America, Asia |
| *Vaccinium myrtillus L.* | Bilberry | Leaves, fruits | Tannins and anthocyanosides | Astringent, enhance vascular tone, antioxidant, antiphlogistic | Europe, North America |
| *Valeriana officinalis L.* | Valerian | Roots | Monoterpenes, Sesquiterpene, etc | Mild ansiolitic, spasmolitic Anti-inflammatory, antioxidant | Wide distribution Europa, America |
| *Vitis vinifera L.* | Grape | Fruits, leaves, seeds | Favonoids | Laxative, | Africa |