Influence of Lactase on the *In Vitro* and *In Vivo* Antiglycaemic Effects of Onion Flavonoids

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Abstract

Introduction
Lactase in addition to its role in the digestion of lactose available in milk and dairy products is implicated in the metabolism of a range of phenolic phytochemicals in the gut. Experiments with Caco-2 cells have shown that these cells which mimic the intestinal mucosa indicate that quercetin glucosides and quercetin aglycone (widely consumed in onions and apples) block glucose uptake from the gut by competing with glucose for the sodium-dependent SGLT-1 and sodium-independent GLUT-2 transporters respectively (Johnston et al., 2005a, Schulze et al., 2015). It has been suggested that dietary phenolics that block glucose uptake from the gut may reduce the risk of type 2 diabetes. However, the ability of quercetin glucosides to block SGLT-1 is lost or reduced when the glucoside moiety is cleaved off during lactase hydrolysis. It is currently unknown if lactose-tolerant individuals deglycosylate quercetin to a greater extent than lactose-intolerant individuals and therefore are less able to reduce glucose uptake from the intestine. The aim of *in vitro* study was to model human gut condition for glucose transport by using Caco-2 cell models and to model role of human intestinal LPH by incubation of Caco-2 cells with quercetin flavonoids and purified β-galactosidases and *in vivo* was to investigate whether lactose-tolerant and lactose-intolerant subjects show differences in the uptake of glucose.
Methods
Caco-2 cells were cultured in DMEM full medium in 24 well plates. Thereafter, glucose uptake assay was conducted by using ^{3}H-glucose in the presence and absence of sodium, to assess the effect of flavonoids such as phloridzin, quercetin 4′-glucoside, quercetin 3,4′-diglucoside, quercetin 3′-glucoside, and quercetin aglycone on glucose uptake. Transwell inserts were also used to demonstrate the bidirectional permeability through Caco-2 monolayers, transport of glucose from apical (SGLT-1) to basolateral side (GLUT-2). β-galactosidase enzyme assay was conducted by using β-galactosidase from *Aspergillus oryzae,* Caco-2 cells were treated with 100 µM quercetin glucosides, 25% w/v onion extract and β-galactosidase in order to model the hydrolysis of flavonoids by lactase in the small intestine. HPLC was carried out to determine if quercetin glucosides are found in onion extract and test whether β-galactosidase is active and result in deglycosylation of substrates such as individual quercetin glucosides and quercetin glucosides in onion extract. For the clinical study, lactose intolerance was identified by the hydrogen breath test (Gastrolyzer), and blood glucose levels were measured by taking finger-prick blood samples in several intervals (0, 15, 30, 60, 90, 120) minutes using an EKF glucose analyser.
Results
Findings from the current *in vitro* research confirm that phloridzin is an inhibitor of sodium-dependent conditions (SGLT-1) transporter with 80% reduction, this therefore was used as a positive control. Quercetin 4′-glucoside and quercetin 3,4′-diglucoside at (100µM) significantly decreased the uptake of glucose in the presence of sodium with up to 75% reduction compared to control $p < 0.01$. However, no significant glucose inhibition was found from these quercetin glucosides in the absence of sodium condition ($p > 0.5$), whilst quercetin aglycone significantly inhibited the glucose uptake with 50% reduction compared to control at significance levels of ($p = 0.02$). HPLC data identified quercetin 3,4′-diglucoside and quercetin 4′-glucoside with RT = 4.082 min and 11.392 min in the onion extract by showing peaks at similar ranges with RT= 4.114 min and 11.385 min with their standards, and the concentration of quercetin 4′-glucoside was measured as the highest level (42µg/ml) in onion extract compared to 3,4′-glucoside and quercetin 3-glucoside. Further HPLC illustrated that, after incubation of quercetin glucosides and onion extract with β-galactosidase *Aspergillus oryzae* for 20, 40 and 60 minutes, the peaks occurred at similar RT =16.453 min and 16.441 min respectively in accordance with standard quercetin (RT=16.239 min), suggesting the deglycosylation of these compounds with β-galactosidase from *Aspergillus oryzae.* According to findings from the clinical study, reduction of peak glucose levels by an onion meal was higher in lactose-intolerant people than lactose-tolerant people (44.2% versus 19.3%, $p = 0.042$). Also, the area under the blood glucose curve was reduced more in lactose-intolerant people compared to lactose-tolerant people, however was not statistically significant (54.5% versus 42.1%, $p = 0.425$).
Discussion
Our result suggests that quercetin 4′-glucoside and quercetin 3,4′-diglucosides and onion extract were the main inhibitors of glucose uptake in sodium-dependent conditions (SGLT-1). Whereas, quercetin aglycone inhibited GLUT-2 glucose transport on Caco-2 cell monolayers under sodium independent conditions. Our findings were in accordance to several previous studies (Boyer et al., 2005, Kwon et al., 2007, Schulze et al., 2015). Notably, in vitro studies were conducted to model whether the *in vivo* study is likely to succeed or not. Findings from our human study showed that glucose uptake was blocked by the onion solution and a diet containing quercetin glucosides (onion meal) may be of greater benefit for glycaemic control in lactose-intolerant people than in lactose-tolerant people.

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Publications

Papers

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Abstracts

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Table of Contents

Abstract 1

Acknowledgments 2

Publications 3

List of Figures 8

List of Tables 16

List of Abbreviations 18

1. CHAPTER ONE – GENERAL INTRODUCTION 20

1.1. Diabetes, Type 1 and Type 2 20

1.1.1. Management of Diabetes 21

1.2. Intestinal Glucose Transport and Absorption 22

1.2.1. The classical model of glucose transport via SGLT-1 and GLUT-2 23

1.2.2. Other mechanisms involved in the transport of glucose 28

1.2.3. SGLT-1 and GLUT-2 role on Diabetes and Hyperglycaemia 28

1.3. Dietary Polyphenols 30

1.3.1. Structure 30

1.3.2. Dietary sources 33

1.3.3. Flavonoid intake 34

1.4. Intestinal Metabolism of Flavonoids 35

1.5. Health Benefits of Flavonoids and their role on Glycaemic Control 40

1.5.1. In vitro studies 41

1.5.2. In vivo animal studies 41

1.5.3. Human studies 42

1.5.4. Onions (phenolic and non-phenolic) compounds effects on glycaemic control 43

1.6. Lactose Intolerance 47

1.6.1. Role of LPH in relation to flavonoid metabolism 48

1.7. Hypothesis and aims of this thesis 51

2. CHAPTER TWO - IN VITRO STUDIES 52

2.1. Introduction 52

2.1.1. Caco-2 cell line as a model of intestinal barrier 52

2.1.2. β-galactosidase as a model of human intestinal LPH 54

2.1.3. Aims 55

2.2 Materials 55

2.2.1. Materials used in vitro studies 55
2.2.2. Consumables and equipment used in Caco-2 Cell-culture 56
2.2.3 Composition of HEPES-buffered salt solution (HBSS) used in glucose uptake studies 57
2.3. Methods (Cell Culture Studies) 58
2.3.1. Cell culture medium and seeding 58
2.3.2. Aseptic Techniques 58
2.3.3. Cell passaging 58
2.3.4. Counting cells with Trypan Blue 59
2.3.5. Freezing the Caco-2 cells (Cryopreservation) 59
2.3.6. Preparation of Flavonoids and Inulin 59
2.3.7. Onion sample preparation 60
2.3.8. Glucose Uptake Assay 60
2.3.9. Method for detection of radioactivity (3H-glucose)-Liquid Scintillation Counting 62
2.3.10. Wipe testing for the use of 3H glucose (Regular monitoring before and after each experiment) 63
2.3.11. Bidirectional transport assay (Caco-2 cell models) 63
2.3.12. Statistical analysis (cell culture studies) 65
2.3.13 Dialysis Procedure 65
2.4 Methods (β-Galactosidase Assay) 66
2.4.1. Properties and activity of β-galactosidase (LPH) 66
2.4.2. β-galactosidase Assay 67
2.4.3. Enzyme hydrolysis 68
2.5 Methods (HPLC) 69
2.5.1. Identification and quantification of flavonoid in onions 69
2.5.2. Identification and quantification of flavonoids 70
2.5.3. Statistical analysis (Flavonoids quantification-HPLC) 71
2.6. Results 72
2.6.1. Caco-2 cells morphology 72
2.6.2. Glucose Uptake Assay 73
2.7. Validation of HPLC -Limit of Detection 92
2.8. Identification of flavonoids in onion by RP-HPLC-DAD 95
2.8.1. Standard curves of quercetin and quercetin glucosides 100
..... 100
2.9. Effect of Quercetin 3,4′-diglucoside, Quercetin 4′-glucoside, onion extract and dialysed onion extract on Caco-2 cells glucose uptake 101
2.10. Modelling of lactase with β-galactosidase 105

2.10.1. Effect of β-galactosidase from Aspergillus oryzae on flavonoids in red onion extract in methanol 115
2.10.3. Effect of β-galactosidase enzyme from Aspergillus oryzae on onion extract and quercetin 3,4′-diglucoside 118
2.11. Discussion 120
2.11.1. Inhibitory effect of dietary flavonoids in onion and onion extract on glucose uptake via SGLT-1 and GLUT-2 on Caco-2 cells 120
2.11.2. Glucose transport via Apical and Basolateral compartments (Transwell inserts) 125
2.11.3. Effect of Inulin (fructan) on Caco-2 cells glucose uptake 126
2.11.4. Overall flavonoids (phenolic compounds) effects on Caco-2 cells glucose uptake 127
2.11.5. Effect of dialysis on flavonoids content in onion extract 128
2.11.6. Effect of dialysed onion extract on Caco-2 cells glucose uptake 128
2.11.7. Modelling of lactase (LPH) with β-galactosidase and its effects on hydrolysis of flavonoid glucosides in onions 129
2.11.8. Identification and quantification of flavonoids in onion extract before and after incubation with β-galactosidase from Aspergillus oryzae 131
2.11.9. LPH effects on hydrolysis of flavonoids on glucose uptake in intestine 134
2.11.10. Possible factors affecting the bioavailability and absorption of flavonoids 135
2.11.11. Effect of hydroxylation and localisation of flavonoids on intestinal metabolism 135
2.11.12. Other mechanisms for the biological effects of polyphenol consumption 137
2.11.13. Common Pitfalls in Caco-2 Assays 139
3. CHAPTER THREE - IN VIVO STUDY 142
3.1 Introduction 142
3.1.1. Clinical Study 142
3.1.2. Aims 142
3.2. Materials used in vivo study 143
3.2. Methods 143
3.2.1. Participants 143
3.2.2. Onion meal preparation 144
3.2.3. Hydrogen breath test (Lactose-Intolerance Diagnosis) 144
3.2.4. Blood Glucose measurement (finger prick) 145
3.2.5. Glucose tolerance test 146
3.2.6. Statistical analysis (Clinical studies) 147
3.3. Results 148
3.4. Discussion 150
4. CHAPTER FOUR - GENERAL DISCUSSION 156

4.3. Conclusion 159
4.4. Future work 160
5. CHAPTER FIVE – REFERNECES 162
6. CHAPTER SIX – APPENDICES 184
6.1. Appendix 1 184
6.2. Appendix 2 204
6.3. Appendix 3 205
6.4. Appendix 4 206
6.5. Appendix 5 207
6.6. Appendix 6 208
6.7. Appendix 7 209

List of Figures

Figure 1.1: Demonstration of glucose and fructose absorption in the small intestine (Shirazi-Beechay et al., 2010).....24

Figure 1.2: SGLT-1 and GLUT-2 mediated glucose transport at low luminal glucose concentrations (30-50 mmol/L). 25

Figure 1.3: Activation of the apical GLUT-2 pathway of glucose transport across enterocyte at high concentrations (200 mmol/L).....26

Figure 1.4: Chemical structures of sub-classes of flavonoids (Pandey and Rizvi, 2009).....31

Figure 1.5: Structure of the poly-hydroxylated flavonoid quercetin representing glycosylated derivatives that are found in plants such as onion. **A**= Quercetin 4′-glucoside, **B**= Quercetin 3-glucoside, **C**= Quercetin 3-rutinoside and **D**= Quercetin 3,4′-diglucoside (Nemeth et al., 2003). 32

Figure 1.6: Structure of Phloridzin and its aglycone Phloretin (Jugdé et al., 2008).....32

Figure 1.7: Mechanism of flavonoid glucosides metabolism in mammalian small intestine (Day et al., 2000b). 36

Figure 1.8: Metabolism of flavonoids inside enterocyte, from various sources (Lotito et al., 2011, Walle, 2004).....38

Figure 1.9: Role of lactase (LPH) in the metabolism of phenolic glucosides. 50

Figure 2.1: Glucose uptake assay conducted on a 24-well plate. 61

Figure 2.2: Picture taken from the 24-well plate containing DMEM medium, consequently removed and HBSS sodium-dependent and independent containing test compounds with ^{3}H-glucose were added. 61

Figure 2.3: Picture taken from liquid scintillation counter including data storage system, monitor and printer 62

Figure 2.4: Transwell insert system shows the compartmentalisation of apical and basal sides of glucose transporters 64

Figure 2.5: Picture taken from a 24-well plate with transwell inserts. 64

Figure 2.6: Picture taken from 6 test tubes representing β-galactosidase assay after a noticeable yellow colour developed 68

Figure 2.7: Demonstration of RP-HPLC system. 70

Figure 2.8: 20-50% Non-confluent Caco-2 cells (10x and 20x magnification) 72

Figure 2.9: 80-90% confluent Caco-2 cells (30x and 40x magnification).....72

Figure 2.10: Effect of DMSO at different concentrations on Caco-2 cells glucose uptake under sodium-dependent condition within 2 minutes, results are expressed as mean ± SEM, n=3 with $p > 0.05$ 73

Figure 2.11: Effect of DMSO at different concentrations on Caco-2 cells glucose uptake under sodium-independent condition within 2 minutes, results are expressed as mean ± SEM, n=3 with $p > 0.05$ 74

Figure 2.12: Effect of methanol at different concentrations on Caco-2 cells glucose uptake under sodium-dependent condition within 2 minutes, results are expressed as mean ± SEM, n=3 with $p > 0.05$ 75

Figure 2.13: Effect of methanol at different concentrations on glucose uptake under sodium-independent condition within 2 minutes, results are expressed as mean ± SEM, n=3 with $p > 0.05$ 75

Figure 2.14: Glucose uptake in Caco-2 cells in the presence of phloridzin under sodium-dependent condition with n=3 ± SEM, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.....76

Figure 2.15: Glucose uptake in Caco-2 cells in the presence of phloridzin under sodium-independent condition with n=3 ± SEM, $p > 0.05$ 77

Figure 2.16: Glucose uptake in Caco-2 cells in the presence of quercetin 4′-glucoside under sodium-dependent condition with n=3 ± SEM, *** $p < 0.001$, * $p < 0.05$ and ns (not significant) = $p > 0.05$ 78

Figure 2.17: Glucose uptake in Caco-2 cells in the presence of quercetin 4′-glucoside under sodium-independent condition with n=3 ± SEM, $p > 0.05$ 79

Figure 2.18: Glucose uptake in Caco-2 cells in the presence of quercetin 3,4′-diglucoside under sodium-dependent condition with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$ 79

Figure 2.19: Glucose uptake in Caco-2 cells in the presence of quercetin 3,4′-diglucoside under sodium-independent condition with n=3 ± SEM, $p > 0.05$ 80

Figure 2.20: Glucose uptake in Caco-2 cells in the presence of quercetin 3-glucoside under sodium-dependent condition with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$ 81

Figure 2.21: Glucose uptake in Caco-2 cells in the presence of quercetin 3-glucoside under sodium-independent condition with n=3 ± SEM, $p < 0.05$ 82

Figure 2.22: Glucose uptake in Caco-2 cells in the presence of quercetin under sodium-dependent condition with n=3 ± SEM, $p > 0.05$ 83

Figure 2.23: Glucose uptake in Caco-2 cells in the presence of quercetin under sodium-independent condition with n=3 ± SEM, ** $p < 0.01$ and $p > 0.05$ 84

Figure 2.24: Glucose uptake in Caco-2 cells in the presence of 100 µM quercetin, 100 µM quercetin 3-glucoside and 25% (w/v) red onion extract under sodium-dependent and sodium-independent conditions with n=3 ± SEM, ** $p < 0.01$ and * $p < 0.05$, from two-way ANOVA multiple comparisons. 85

Figure 2.25: Glucose inhibition in Caco-2 cells in the presence of 100µM quercetin 4′-glucoside and 100µM quercetin 3,4′-diglucoside under sodium-dependent condition at apical side with n=3 ± SEM, * $p < 0.05$ and ** $p < 0.01$ 86

Figure 2.26: Glucose inhibition in Caco-2 cells in the presence of 100µM quercetin 4′-glucoside and 100µM quercetin 3,4'-diglucoside under sodium-dependent condition at the basolateral side with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$ 87

Figure 2.27: Glucose inhibition in Caco-2 cells in the presence of 100 µM quercetin 4′-glucoside and quercetin 3,4′-diglucoside under sodium-independent condition at apical side with n=3 ± SEM, $p > 0.05$ 87

Figure 2.28: Glucose inhibition in Caco-2 cells in the presence of 100 µM quercetin 4'-glucoside and quercetin 3,4′-diglucoside under sodium-independent condition at the basolateral side with n=3 ± SEM, * $p < 0.05$ and $p > 0.0\ 5$ 88

Figure 2.29: Glucose uptake in Caco-2 cells in the presence of 25% w/v onion extract and 5% w/v, inulin under sodium-dependent condition with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$ 89

Figure 2.30: Glucose uptake in Caco-2 cells in the presence of quercetin 4′-glucoside, quercetin 3,4′-diglucoside and 5% w/v inulin under sodium-dependent conditions with n=3 ± SEM, * $p < 0.05$, ** $p < 0.01$ and $p > 0.05$ 90

Figure 2.31: Glucose uptake in Caco-2 cells in the presence of 100 µM quercetin 4′-glucoside, 100 µM quercetin 3,4′-diglucoside and 5% w/v inulin under sodium-independent condition with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$ 91

Figure 2.32: Limit of detection (LOD) of 1ng/ml of quercetin 4′-glucoside in 80% v/v methanol (RT= 11.23 min) 92

Figure 2.33: Limit of detection (LOD) of 2ng/ml of quercetin-4′glucoside in 80% v/v methanol (RT= 11.34 min) 93

Figure 2.34: Limit of detection (LOD) of 1ng/ml of quercetin 3,4′-diglucoside in 80% v/v methanol (RT=3.75 min) 93

Figure 2.35: Limit of detection (LOD) of 2 ng/ml of quercetin 3,4′-diglucoside in 80% v/v methanol (RT= 4 min) 94

Figure 2.36: UV-scan of onion extract, peak at 3.83min appears at Wavelength: 218nm 283nm and 347nm.....95

Figure 2.37: UV-scan of quercetin 3,4′-diglucoside peak at 3.77min appears at wavelength: 219nm, 263nm and 348nm. 96

Figure 2.38: UV-scan of onion peak at 7.31min appears at wavelength= 213, 257 and 355nm. 96

Figure 2.39: UV-scan of quercetin 3-glucoside peak at 7.27min appears at wavelength= 220, 260 and 355nm.....97

Figure 2.40: UV-scan of onion peak at 10.31min appears at wavelength= 221, 253 and 265nm. 97

Figure 2.41: UV-scan of quercetin 4′-glucoside peak at 10.34min appears at wavelength= 218, 263 and 265nm.....98

Figure 2.42: UV-scan of onion extract peak at 15.2 min appears at wavelength= 217, 255 and 379nm 98

Figure 2.43: UV-Scan of quercetin peak at 15.5 min appears at wavelength= 217, 255 and 370nm. 99

Figure 2.44: Quercetin standard curve (10-50 µg/ml) 100

Figure 2.45:Quercetin 4′-glucoside standard curve (10-50 µg/ml) 100

Figure 2.46: Quercetin 3,4′-diglucoside standard curve (10-50 µg/ml) 101

Figure 2.47: 25% w/v onion extract, absorbance against time (RT= 4.021 and 11.201 min) 101

Figure 2.48: Dialysed 25% w/v onion extract after 8 hours, absorbance against time (RT= 4.063 and 11.259 min) 102

Figure 2.49: Dialysed 25% w/v onion extract after 24 hours, absorbance against time (RT= 4.027, 11.167 min).....102

Figure 2.50: The effect of dialysis on the concentration of quercetin glucosides in 25%w/v onion extract (n=3 ± SEM) through standard curve of both quercetin glucosides (Figures 2.45 & 2.46).....103

Figure 2.51: Glucose uptake in Caco-2 cells in the presence of 100µM quercetin 3,4′-diglucoside, 100µM quercetin 4′-glucoside, 25% w/v non-dialysed onion and dialysed onion under sodium-dependent condition with n=3 ± SEM, *** $p < 0.001$,** $p < 0.01$ and * $p < 0.05$ and $p > 0.05$ 104

Figure 2.52: Glucose uptake in Caco-2 cells in the presence of 100 µM quercetin 3,4′-diglucoside, 100 µM quercetin 4′-glucoside, 25% w/v non-dialysed onion and dialysed onion under sodium-independent condition with n=3 ± SEM, ** $p < 0.01$ and $p > 0.05$ 104

Figure 2.53 Standard curve of ONP (μmol/ml) vs. absorbance, n=3 ± SD 105

Figure 2.54: pH dependent β-galactosidase activity (*Aspergillus oryzae*), n=3 ± SD.....106

Figure 2.55: Temperature dependent β-galactosidase activity (*Aspergillus oryzae*), n= 3± SD 107

Figure 2.56: Time dependent β-galactosidase activity (*Aspergillus oryzae*) from 0 to 120 min, n=3± SD 107

Figure 2.57: Quercetin in 80% v/v methanol , peak area against retention time (RT= 2.036, 16.239 min).....108

Figure 2.58: Quercetin 4′-glucoside in 80% v/v methanol, absorbance against time (RT= 11.121 min).....109

Figure 2.59: Quercetin 3,4′-diglucoside in 80% v/v methanol, peak area against retention time (RT= 3.968 min) 109

Figure 2.60: High-performance liquid chromatography of flavonoids in red onion extract. Compounds quantified were quercetin 3,4′-diglucoside, quercetin 4′-glucoside and quercetin aglycone. 25% w/v fresh onion extract (red) in methanol, absorbance versus time (RT= 2.244, 4.122, 8.56, 11.417, 16.373 min) 110

Figure 2.61: Quercetin 4′-glucoside in 80% methanol incubated with β-galactosidase enzyme from *Aspergillus oryzae*, for 60 minutes, absorbance against time (RT= 10.896 and 16.647 min).....110

Figure 2.62: Concentration of quercetin and quercetin 4′-glucoside after incubation of quercetin 4′-glucoside with β-galactosidase from *Aspergillus oryzae* for 60 minutes with n=3 ±SD 111

Figure 2.63: Quercetin 3,4′-diglucoside in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 20 min (RT= 3.172, 16.373 min).....111

Figure 2.64: Quercetin 3,4′-diglucoside in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 40 min (RT= 3.984, 16.479 min).....112

Figure 2.65: Quercetin 3,4′-diglucoside in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 60 min (RT= 3.962, 16. 396 min).....112

Figure 2.66: Concentration of quercetin and quercetin 3,4′-diglucoside after quercetin 3,4'-diglucoside were treated with β-galactosidase from *Aspergillus oryzae* for 20, 40 and 60 minutes with (n=3 ± SD).....113

Figure 2.67: Isorhamnetin in 80% v/v methanol, peak area against retention time (RT= 20.027 min).....114

Figure 2.68: Quercetin 3-glucoside in 80%v/v methanol, peak area against retention time (RT= 8.585 min).....114

Figure 2.69: High-performance liquid chromatography of flavonoids in red onion extract. The compounds quantified were quercetin 3,4′-diglucoside, quercetin 4'-monoglucoside and quercetin aglycone. Red onion extract (25% w/v) in methanol (80% v/v), absorbance against time (RT= 4.218, 4.554, 11.480, 11.778, 12.077 min).....115

Figure 2.70: Onion extract in 80% methanol incubated with β-galactosidase from *Aspergillus oryzae* for 20 min, absorbance against time (RT= 4.243, 4.613, 8.415, 11.487, 16.401 min) 115

Figure 2.71: Onion extract in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 40 min, absorbance against time (RT= 4.209, 4.594, 8.372, 16.441, 20.008 min).....116

Figure 2.72: Onion extract in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 60 min, absorbance against retention time (RT= 4.217, 4.593, 8.405, 16.479, 20.021 min).....116

Figure 2.73: Concentration of Quercetin 3,4′-diglucoside, Quercetin 4′-glucoside and Quercetin aglycone after incubation of 25% onion extract with β-galactosidase from *Aspergillus oryzae* for 20, 40 and 60 min with (n=3 ±SD).....117

Figure 2.74: 25% w/v onion extract incubated with β-galactosidase (β-gal) from *Aspergillus oryzae* for 20, 40 and 60 minutes at sodium-dependent condition with n=3±SEM, *** $p < 0.001$, ** $p < 0.01$ and $p > 0.05$ 118

Figure 2.75: Quercetin 3,4′-diglucoside (DIQG 3,4′ at 100 μM) incubated with β-galactosidase (β-gal) from *Aspergillus oryzae* for 5, 10 and 20 minutes at sodium-dependent condition with n=3 ±SEM, ** $p < 0.01$, * $p < 0.05$ and $p > 0.05$ 119

Figure 3.1: Hydrogen breath analyser (Gastrolyzer). 145

Figure 3.2: EKF glucose analyser and steps involved in finger prick blood collection (EKF, 2010). 146

Figure 3.3: Blood glucose concentrations after consumption of glucose (●) or glucose plus an onion meal (■) in (a) lactose-tolerant adults and (b) lactose-intolerant adults. Data are means ± SEM (n = 12) 149

List of Tables

Table 1.1: Dietary sources of flavonols in fruits, vegetables and tea (Slimestad et al., 2007, Phenol-explorer, 2016). ND: Not Detected 34

Table 1.2: Summary of reports investigating consumption of dietary flavonoids.....35

Table 1.3: Content of polyphenols and fructan in onion varieties, mean (mg/100mg) (Phenol-explorer, 2016).....45

Table 1.4: Effect of LPH (Lactase) from different sources on Quercetin Glucosides.....49

Table 2.1: Material used in cell culture and HPLC labs 56

Table 2.2: Equipment and consumables used in cell culture lab 57

Table 2.3: Concentration of reagents used for HBSS sodium-dependent and sodium-independent (Wang et al., 2008).....57

Table 2.4: Properties of microbial β-galactosidases 66

Table 2.5: Test tubes containing enzyme and substrates for β-galactosidase enzyme assay ..68

Table 2.6: RT (min) and UV scans (nm) of red onion extract and quercetin 3,4′-diglucoside 96

Table 2.7: RT (min) and UV scans (nm) of red onion extract and quercetin 3-glucoside.....97

Table 2.8: RT (min) and UV scans (nm) of red onion extract and quercetin 4′-glucoside.....98

Table 2.9: RT (min) and UV scans (nm) of red onion extract and quercetin 99

Table 3.1: Materials used in human physiology lab 143

Table 3.2: The mean and SD of Age and BMI (kg/m^2) for 12 lactose-tolerant and 12 lactose-intolerant individuals at significance level of $p < 0.05$.....143

Table 3.3: Effects of an onion meal on peak rise in blood glucose and incremental area under the blood glucose – time curve (AUC) in lactose-tolerant and lactose-intolerant adults. Values are expressed as means and s.d. [a] Peak glucose at 30 min minus fasting glucose. [b] Lactose-tolerant versus lactose-intolerant.....148

List of Abbreviations

Abbreviation	Full phrase
3-OMG	3-O-methylglucose
ABC	ATP-binding cassette
AM	Apical Membrane
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ATP	Adenosine triphosphate
BLM	Basolateral membrane
BSA	Bovine serum albumin
CBG	Cytosolic β-glucosidase
CPM	Counts per minute
CVD	Cardiovascular disease
CYP	Cytochrome P450
DAD	Diode array detector
DMSO	Dimethyl Sulfoxide
DPM	Disintegrations per minute
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FFQ	Food-frequency questionnaire
G6P	Glucose-6-phosphate
GI	Glycaemic Index
GIP	Glucose-dependent insulinotropic peptide
GL	Glycaemic Load
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GOS	Galacto-oligosaccharide
HbA1C	Glycated Haemoglobin

HBSS	Hank's Balance Salt Solution
HDL	High-density lipoprotein
HPLC	High Performance Liquid Chromatography
HR	Hazard Ratio
IGF-1	Insulin-like growth factor-I
IGT	Impaired glucose tolerance
IMS	Industrial methylated spirit
LOD	Limit of detection
LPH	Lactase Phloridzin Hydrolase
MRP	Multidrug resistance associated protein
NB-DJG	N-butyldeoxygalactonojirimycin
OGTT	Oral glucose tolerance test
ONP	Ortho-nitrophenol
ONPG	O-Nitrophenyl-β-D galactopyranoside
Ox-LDL	Oxidized low-density lipoprotein
PBS	Phosphate Buffer Saline
P-gp	P-glycoprotein
PMT	Photomultiplier tube
ROS	Reactive Oxygen Species
RP	Reverse Phase
RR	Relative Risk
RS1	Regulatory subunit
RYGB	Roux-en Y gastric bypass
SGLT-1	Sodium dependent glucose transporter
SLC	Solute Carrier
SULT	Sulfotransferase
TEER	Transepithelial electrical resistance
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
α-MDG	Methyl-D-glucopyranoside

1. CHAPTER ONE – GENERAL INTRODUCTION

1.1. Diabetes, Type 1 and Type 2

There are two major types of diabetes, which are dependent upon the secretion and sensitivity to insulin. Type 1 diabetes mellitus is an autoimmune disease, in which the immune system starts destroying the β-cells of the pancreas, and therefore exogenous insulin is required due to the lack of insulin level. The second type of diabetes is non-insulin dependent diabetes in which adequate amounts of insulin may be available. However, cells cannot use it effectively because of insulin resistance (Chang-Chen et al., 2008).

Type 1 diabetes accounts for only 5 to 10% of all diabetic cases, whereas type 2 diabetes, which is the most common type of diabetes, accounts for more than 90% of all diabetic patients (Yach et al., 2006). The main factors contributing to the risk of type 2 diabetes could be genetic factors, peripheral insulin resistance, ageing, and also possible changes in the source of food, quantity of carbohydrates, as well as lack of physical activity (Schulze and Hu, 2005). For instance, the key factor in the development of hyperglycaemia could be the result of a high intake of carbohydrates in the form of beverages and snacks that contain a great amount of sugar. Therefore, high glucose absorption will result in permanent stimulation of insulin secretion (Yach et al., 2006).

In 2014, the estimated number of people with diabetes mellitus was almost 422 million adults worldwide and it is anticipated to reach 552 million cases in 2030 (WHO, 2016). According to the new figures released by Diabetes UK 2016, the population of people living with diabetes in the UK has reached over 4 million.

According to a report, 709,000 deaths from stroke and 1,490,000 deaths from ischaemic heart diseases were linked to high blood glucose levels (above optimum), and an indication that 13% and 21% of all deaths resulted from these conditions (Danaei et al., 2006). It is noteworthy that the rate of mortality of diabetics is over 4 million, which is comparable with the mortality rate from smoking (4.8 million), high cholesterol (3.9 million) and obesity (2.4 million). This is based on data from 52 countries provided by investigators, systematic reviews and individual-level records in population health surveys (Danaei et al., 2006). Moreover, several studies have also found that association of isolated post-challenge hyperglycaemia with the risk of cardiovascular and atherosclerosis in type 2 diabetic patients is much greater than in isolated

fasting hyperglycaemia (Balkau et al., 1999, Bonora, 2002). The postprandial hyperglycaemia causes a 50% greater risk of CVD in impaired glucose-tolerance (IGT) patients who have not undergone therapy, compared to those who have (Esposito et al., 2004).

Metabolic syndrome is highly prevalent among the patients with type 2 diabetes and is reported as a major risk factor for development of cardiovascular diseases and dyslipidaemia as a result of function of increasing BMI. Insulin resistance is present in majority of individuals with metabolic syndrome, it is correlated with the risk of type 2 diabetes and cardiovascular diseases. Moreover, elevated blood pressure associates with obesity and glucose intolerance and usually occurs in insulin resistance individuals (Alberti et al., 2006). Adiponectin is an adipokine that is modulated through expression of AdipoR1 and AdipoR2 receptors, leading to the activation of AMPK pathway which is positively associated with insulin sensitivity and altered by various environmental, genetic factors and pathological conditions. Thus, monitoring levels of adiponectin is a good predictable marker for type 2 diabetes and metabolic syndrome (Kadowaki et al., 2006).

Increased oxidative stress has been found as one of the major concerns of diabetes. Thus, elevated levels of free radicals or reactive oxygen species (ROS) may cause stimulation of oxidised low-density lipoprotein (Ox-LDL). Consequently, sustained hyperglycaemia and high levels of oxidative stress are the major contributors in the development of diabetes. Retaining a balance between antioxidants and ROS is a key mechanism in avoiding damage from oxidative stress (Khaki et al., 2010).

1.1.1. Management of Diabetes

The recommended major therapy for patients with type 2 diabetes are lifestyle changes, such as dietary changes, weight reduction and physical activity (Lindahl et al., 2009, Roumen et al., 2008). For maintaining blood glucose levels, lifestyle intervention may not be enough; therefore, patients with type 2 diabetes will additionally need antihyperglycaemic medications such as metformin in combination with or without subcutaneous insulin injections. The possible limitations regarding the use of antidiabetic drugs have resulted in undesirable side effects, as well as contraindications due to the presence of co-morbid diseases including hypertension and dyslipidaemia in patients (Tschope et al., 2013). Therefore, the demand for antihyperglycaemic agents with minor side effects is required.

Food can be powerful in preventing and reversing diabetes. However, dietary approaches have changed as we have learned more about the disease (Roumen et al., 2008). The traditional approach to diabetes focuses on limiting refined sugars and foods that release sugars during digestion-starches, breads and fruits. With carbohydrates reduced, the diet may contain an unhealthful amount of fat and protein. Therefore, diabetes experts have taken care to limit fats, especially saturated fats that can raise cholesterol levels, and to limit protein for people with impaired kidney function (Asif, 2014).

A recent report has concluded that there is strong evidence that a low glycaemic index (GI) diet decreases the risk of type 2 diabetes and coronary heart disease, and also is most likely to reduce the risk of obesity and several cancers (Augustin et al., 2015a). There is growing evidence that secondary plant metabolites, particularly polyphenols (flavonoids), control postprandial increase in levels of blood glucose. For instance, phloridzin, a dihydrochalcone, which is highly found in apples and onions, has long been known as a very effective natural SGLT-1 inhibitor (Ehrenkranz et al., 2005). Moreover, several pharmaceutical companies have made SGLT-2 (glucose transporter) inhibitors that stop reabsorption of glucose from the kidney, thus improving the hyperglycaemia in type 2 diabetic patients. The approved SGLT-2 inhibitor in Europe is called dapagliflozin, which is used for type 2 diabetes treatment (Kilov et al., 2013).

Fruits and vegetables are high in insoluble fibre. Diets that are high in fibre may be able to help in the management of diabetes. Soluble fibre delays glucose absorption from the small intestine, and thus may help prevent the spike in blood glucose levels that follow a meal or snack (Asif, 2014).

1.2. Intestinal Glucose Transport and Absorption

Glucose is the prime energy source for the majority of energy-dependent processes in living organisms. Glucose is an essential metabolic substrate of all mammalian cells. It is not only a precursor of glycoproteins, triglycerides, and glycogen, glucose metabolism governs many functions, because the oxidation of glucose generates a major source of metabolic energy in eukaryotic cells (Boyer et al., 2005). These functions are secondary to glucose uptake. Glucose is a hydrophilic compound; it cannot pass through the lipid bilayer by passive diffusion, and therefore requires specific carrier proteins to mediate its specific transport into the cytosol (Gray, 2010). When glucose is metabolised in the presence of oxygen, the process leads to

mitochondrial citric acid cycle, cytoplasmic glycolysis and oxidative phosphorylation. Following digestion of glucose, it enters the blood stream and then into cells which mostly take up glucose in an insulin dependent manner. Insulin is produced by the pancreas as a result of increased glucose levels (Song et al., 2005).

There are two types of membrane proteins for the transport of glucose and other monosaccharides across cell membranes through the body. The glucose uptake is regulated by the action of facilitative glucose transporters (GLUTs), coded by the solute carrier (SLC) 2 gene family, on the cell surface. There are three classes of GLUT isoforms: class I consists of GLUT1-4; class II, GLUT6, 8, 10 and 12; and class III, GLUT5, 9 and 11. Several studies with knockout or transgenic mice established the main role of these transporters in the regulation of glucose uptake and storage (Hanhineva et al., 2010b, Bauche et al., 2007).

GLUT-1 and GLUT-3 control the glucose uptake at the basal membrane. GLUT-4 is expressed through kidney and muscle cells and remains stored in insulin-responsive compartments within the cells until insulin has facilitated its localisation on the cell surface (Hanhineva et al., 2010b). The second glucose membrane proteins are sodium-coupled glucose cotransporters (SGLTS) encoded by SLC5 gene family (Scheepers et al., 2004). While GLUTs passively and selectively allow monosaccharides to transport a concentration gradient, the uptake of glucose by SGLTs is mediated in a secondary active manner together with Na^+-ions (Joost and Thorens, 2001, Uldry and Thorens, 2004). The SGLT family of transporters transport sugars against the concentration gradient utilising the sodium-electrochemical gradient (Roder et al., 2014). The GLUT family are facilitative transporters that transport sugars along the concentration gradient. After glucose enters normal cells, it is converted into pyruvate through glycolysis. Subsequently, pyruvate is transformed into acetyl-CoA, which is used as substrate in mitochondria to generate ATP (Szablewski, 2013).

1.2.1. The classical model of glucose transport via SGLT-1 and GLUT-2

The classical model of glucose transport is mediated by two classes of hexose transporters: SGLT-1 expressed at apical membrane (AM) and GLUT-2 expressed at basolateral membrane (BLM). Colon cells like Caco-2 are the most recognised cell line on presenting the two main transporters of glucose; one is the sodium dependent (in the presence of sodium) glucose transporter (SGLT-1), which is located in the apical membrane of the enterocyte. It is believed that it is a high affinity, low capacity active transport protein. Whereas, GLUT-2 transporter is not a sodium dependent glucose transporter and has a low affinity, high capacity and facilitated

protein transport. GLUT-2 is located on the basolateral membrane, and thus mediates glucose transport into the portal venous system (Zheng et al., 2011).

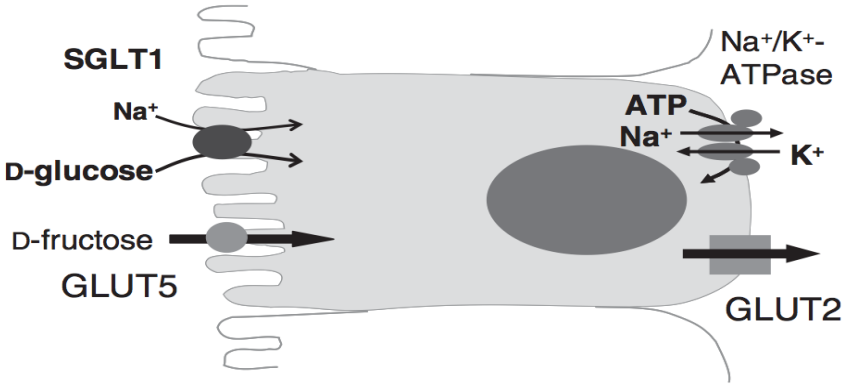


Figure 1.1: Demonstration of glucose and fructose absorption in the small intestine (Shirazi-Beechay et al., 2010).

In enterocytes, the SGLT-1 is responsible for the transport of Na^+ (two) coupled with glucose or galactose and water (Drozdowski and Thomson, 2006). This transporter is facilitated through Na^+/K^+ ATPase activity in the basal membrane. The facilitative transporter GLUT-2 transports sugars across the basolateral membrane. Besides SGLT-1, the facilitative glucose transporter GLUT-5 is expressed at the apical membrane (Gray, 2010) (Figure 1.1). GLUT-5 proteins exhibit no transport activity for glucose; however, it carries fructose into the enterocytes (Corpe et al., 2002). The exit of glucose, galactose and fructose from the enterocytes cytosol into the blood circulation is mediated by facilitative diffusion through GLUT-2 in the basolateral membrane (Miyamoto et al., 1992).

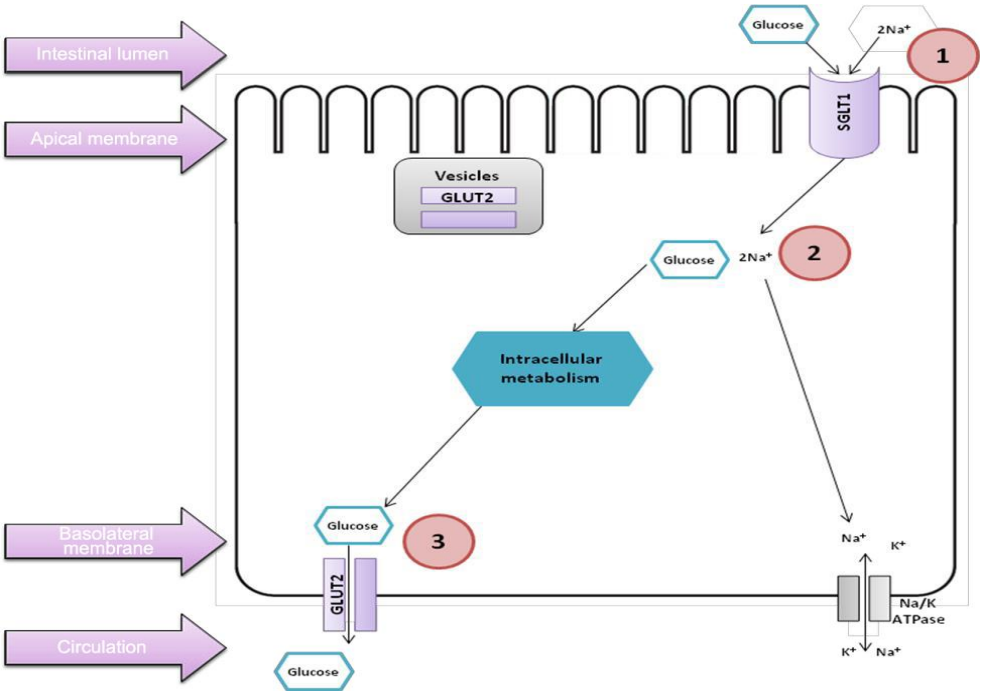


Figure 1.2: SGLT-1 and GLUT-2 mediated glucose transport at low luminal glucose concentrations (30-50 mmol/L).

Figure 1.2 shows 1) SGLT-1 transported the glucose along with two sodium ions. 2) The SGLT-1 is maintained by a Na^+/K^+ ATPase at the enterocytes basolateral membrane. 3) Glucose at intracellular level can be utilised for metabolism or is effluxed through basal GLUT-2, in the presence of sodium conditions, apical GLUT-2 is located in subapical vesicles and does not transport glucose; notably, at low levels of glucose, no GLUT-2 is translocated into the apical membrane (Diez-Sampedro et al., 2004, Williamson, 2013).

Glucose is transported through the apical enterocyte membrane by SGLT-1; facilitated glucose transport through the basolateral membrane is entirely mediated by GLUT-2 (Kellet et al., 2008). Interestingly, GLUT-2 could be associated with the transport of glucose across the apical membrane at the high luminal glucose levels (Kellet and Brot-Laroche, 2005). However, the initial glucose transport via SGLT-1 is required for the GLUT-2 translocation into the apical membrane. Therefore it is evident that SGLT-1 is the prime component in the absorption of glucose and a target in hyperglycaemia management (Gouyon et al., 2003, Mace et al., 2009).

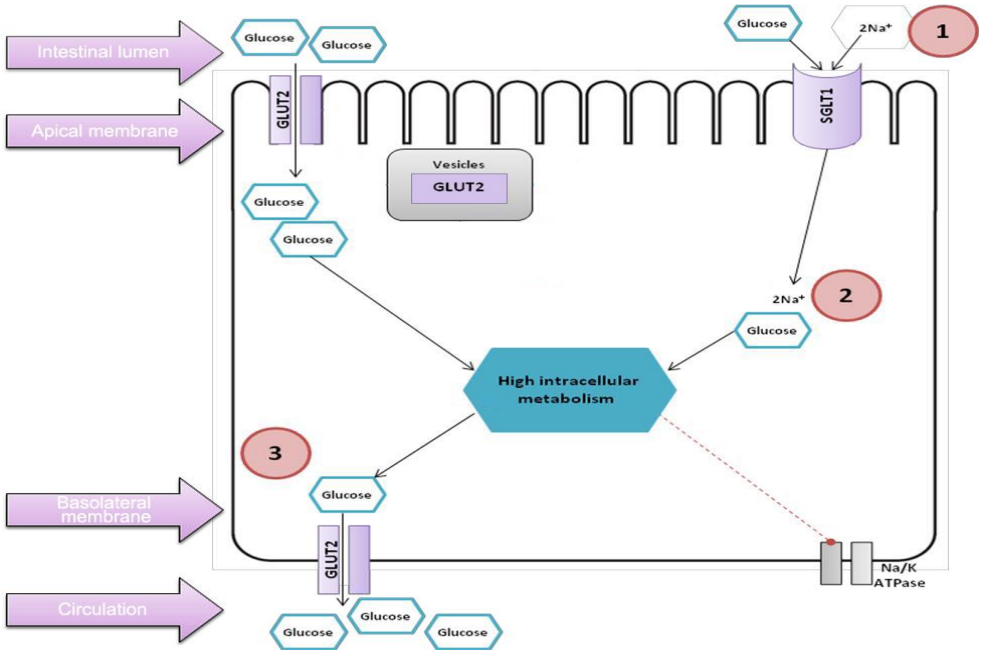


Figure 1.3: Activation of the apical GLUT-2 pathway of glucose transport across enterocyte at high concentrations (200 mmol/L).

Figure 1.3 demonstrates 1) SGLT-1 mediated glucose and sodium influx increased. 2) The high concentrations of glucose and sodium content at intracellular level inhibits the basolateral Na^+/K^+ ATPase. The high intracellular content results in the rearrangement of cytoskeleton, leading to protein trafficking, followed by depolarisation of apical membrane, and is ready for GLUT-2 insertion from the subapical vesicles. 3) High influx of glucose derived from apical GLUT-2 activity, and therefore there is a greater release of glucose into circulation from basolateral membrane GLUT-2 (Goestemeyer et al., 2007, Williamson, 2013).

GLUT-2 is rapidly transported to the apical membrane within a few minutes at elevated glucose levels. As the glucose concentration drops due to transport or at the end of the meal, the GLUT-2 transporters moves away from the apical membrane ready for the next incidence of high glucose concentration (Williamson, 2013). After absorption of glucose into the blood from the intestine, it enters β-cells followed by phosphorylation of glucose and insulin secretion. Insulin reaches muscle and adipose cells, which thus leads to relocation of GLUT-4 to the cell membrane, and therefore stimulates glucose uptake into these tissues and consequential clearance from blood. In support of this, a study conducted by Kellet et al., 2008 showed that GLUT-2 was the main transporter of glucose mass absorption across the apical membrane at high luminal glucose concentrations (Kellet et al., 2008). It is suggested that GLUT-2 was translocated from an internal vesicle pool into the apical membrane in relation to an activation

of protein kinase $C_{\beta II}$, initiated by SGLT-1 mediated transport across the apical membrane (Gouyon et al., 2003, Mace et al., 2009).

Although the exact mechanisms of glucose absorption at high luminal levels is still uncertain, it is noteworthy that SGLT-1 also acts as an important pathway in the uptake of glucose levels in the intestine, since SGLT-1 deficient mice developed a severe malabsorption when fed galactose and glucose (Powell et al., 2013). This is in agreement with the glucose and galactose malabsorption syndrome available in humans with mutations in SLCA1-gene (Wright et al., 2007). Whereas, mice heterozygous for SGLT-1 illustrated no adverse effects of growth retardation on a glucose containing diet (Powell et al., 2013), suggesting that a partial decrease in glucose uptake levels via SGLT-1 may be a potential therapeutic agent to prevent high concentrations of postprandial blood glucose in patients with impaired glucose tolerance and type 2 diabetes (Zheng et al., 2011).

SGLT-1 transporter can transport the glucose and galactose with greatest abundance in the apical membrane of the proximal part of the small intestine (duodenum and proximal jejunum) (Zhou et al., 2003, Yoshikawa et al., 2011). In addition to glucose and galactose, other hexoses such as α-methyl-glucopyranoside (α-MDG) and 3-O-methlyglucose (3-OMG) could be used as substrates for SGLT-1 (Hediger and Rhoads, 1994). Also, SGLT-1 is found in the renal tubes at the proximal S3-segment (Lee et al., 1994), as well as in other tissues such as the brain and heart (Yu et al., 2013, Zhou et al., 2003). It is suggested that the apparent Km which is defined as the substrate concentration at ½ the maximum velocity for glucose through SGLT-1 *in vivo* studies, is between 6-23 mM, whereas it is only 2-6 mM for *in vitro* studies (Hirayama et al., 1996, Ferraris et al., 1990).

On the other hand, GLUT-2 with Km of approximately 17 mM (Thorens, 1996), in addition to being expressed in the small intestine, is also expressed in the pancreatic β-cells, kidney and liver, that need low affinity systems to determine accurate influx at physiological glucose levels (Thorens, 2001). According to the research conducted by (Wright and Turk, 2004), in the intestinal transport cells and kidney, GLUT-2 mediated absorption or reabsorption of glucose. Moreover, GLUT-2 is required for stimulation of insulin secretion from pancreatic β-cells, thus playing as a glucose sensor. This was followed by low levels of GLUT-2 expression in the brain, suggesting that GLUT-2 in the brain may cause a similar sensing function (Arluison et al., 2004).

1.2.2. Other mechanisms involved in the transport of glucose

Several studies have shown that GLUT-2 deficient mice had no changes in the kinetic of glucose absorption in the intestine after an oral glucose tolerance test (OGTT) (Stumpel et al., 2001), similar to subjects carrying mutations in SLC2A2 gene at GLUT-2, the Fanconi-Bickel syndrome condition (Santer et al., 2003). Moreover, another report failed to detect any activity of GLUT-2 in apical membrane vesicles from healthy and/or diabetic cases showing that GLUT-2 is not permanently expressed at the luminal side of the small intestine of diabetic patients (Dyer et al., 2002). Interestingly, Stumpel (et al., 2001) suggested another possible transcellular route as a crucial pathway for transport of glucose across the basolateral membrane that does not need functional GLUT-2. In this pathway the glucose is moved to the apical membrane through SGLT-1 into the enterocyte, followed by phosphorylation to glucose-6-phosphate (G6P) and access into the endoplasmic reticulum (ER). Then, G6P is hydrolysed into glucose and phosphate, and free glucose is realised across the basolateral membrane through exocytosis (Stumpel et al., 2001).

There are several other theories, which have been suggested to demonstrate transepithelial transport of glucose at high luminal glucose concentrations. For example, the solvent drag theory suggested that passive paracellular flux, which involves absorption of glucose predominantly by a passive diffusional process, results in bulk glucose absorption (Mullen et al., 1985). This idea supports the possible passive component of glucose transport through an increase in paracellular permeability, because of widening of tight junctions activated either by SGLT-1, or by the high luminal glucose level, thus allowing great quantities of solvent or other nutrients moving to the paracellular space, followed by dragging glucose with them and thus diffusing into the portal system (Pappenheimer and Reiss, 1987). On the other hand, animal studies conducted on non-membrane permeable substrates, such as L-glucose in the presence of high luminal sugar levels, showed a slight increase in the levels of these substrates in the blood, illustrating that paracellular transport does not contribute to glucose bulk absorption at high luminal concentrations (Schwartz et al., 1995).

1.2.3. SGLT-1 and GLUT-2 role on Diabetes and Hyperglycaemia

It should be noted that high levels of glucose transporters in type 2 diabetes and obesity could develop a greater absorption rate of glucose in relation to a sugar-rich meal, thus resulting in greater postprandial blood glucose peak compared to non-diabetic cases. In support of this, a study observed that the rate of glucose absorption was approximately threefold faster in type 2

diabetics tissues as compared to control subjects (Dyer et al., 1997). Several studies have investigated the effect of different dietary monosaccharides on expression of SGLT-1 in the small intestine. They have illustrated an upregulation when different sugars were present in the small intestine, suggesting that neither transport through SGLT-1 nor metabolism is essential to induce gene expression of SGLT-1 (Dyer et al., 2003, Shirazi-Beechey et al., 1994). Moreover, in a study on diabetic mice, they showed that upregulation of SGLT-1 is activated through sweet taste receptors (T1R2 + T1R3) in the intestine through G-protein α-gustducin or the sweet receptor subunit TR13, and determined no upregulation of SGLT-1 expression in relation to dietary sugars and artificial sweeteners as found in wild-type mice (Margolskee et al., 2007).

Co-overexpression of RS1 (Regulatory subunit 1) and SGLT-1 in *Xenopus* oocytes, in the renal epithelial cell line LLC-PK1 reduced SGLT-1 mRNA, SGLT-1 protein in addition to the amount of SGLT-1-mediated glucose uptake (Korn et al., 2001). In fact, in mice lacking RS1, the expression level of SGLT-1 in addition to postprandial glucose absorption in luminal intestine highly increased; therefore, there was a greater peak in blood glucose concentrations in the portal glucose sensing compared to wild-type mice (Osswald et al., 2005).

Reduction in the protein abundance of SGLT-1 caused a significant decrease in the absorption levels of intestinal hexose for the gastrointestinal peptide cholecystokinin (Hirsh and Cheeseman, 1998), similarly for leptin that reaches the intestinal lumen after its release by mucosal gastric cells (Ducroc et al., 2005). Therefore, both these hormones could act as main targets for changes in expression of SGLT-1 detected in type 2 diabetes patients (Dyer et al., 1997) as well as obesity cases in animal models (Huang et al., 2012, Jurowich et al., 2013).

Downregulation of SGLT-1 expression in changing the levels of GLP-1 and GIP, contributes to the fast improvement of glycaemic control obtained post Roux-en Y gastric bypass surgery (RYGB) in patients with type 2 diabetes and obesity (Jurowich et al., 2013, Stearns et al., 2009). Moreover, another study showed that, in mice with RS1, which is a membrane-associated intracellular protein that inhibits trafficking of intracellular vesicles possessing SGLT-1 from the trans-Golgi network to the plasma membrane, have regulated the postprandial hyperglycaemia (Kroiss et al., 2006).

In diabetes, several transporters and enzymes responsible for glucose uptake in the intestine are upregulated. For example, SGLT-1 levels increased up to fourfold in the gut of type 2 diabetics, and GLUT-2 mRNA also increased threefold. It is believed that brush border vesicles from duodenal biopsies from diabetic subjects take up glucose almost three fold faster than the equivalent from the control subjects (Williamson, 2013).

1.3. Dietary Polyphenols

1.3.1. Structure

Polyphenol is a compound possessing more than one phenolic hydroxyl group (Hoffman and Gerber, 2012). More than 8000 polyphenolic compounds have been recognised in literature, ranging from simple molecules, such as phenolic acids, to highly polymerised structures, like tannins (Crozier et al., 2009). Polyphenols can be divided into several types, dependent on the number of phenolic rings and the structural elements binding these rings. The two main subclasses include flavonoids and non-flavonoids (D'Archivio et al., 2007). Flavonoids, which are the most known types of polyphenols, depending on the variation in the type of heterocycle involved, are divided into six major subclasses: flavonols, flavanones, flavanols, flavones, anthocyanins and isoflavones (Pandey and Rizvi, 2009), and non-flavonoids such as phenolic acids, cinnamic acid derivatives, stilbenes and lignans (Hoffman and Gerber, 2012).

Flavonoids in their structure have phenolic rings substituted either with alcoholic, carboxylic acid or aldehydic groups which can be further deglycosylated in the case of arbutin or salidroside (El-Seedi et al., 2012).

Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation. Flavonoids are found in plants mainly as β-glycosides. Mostly, the processing procedures used in food industries do not result in cleavage of the glycosidic linkage, and hence flavonoids in foods largely exist as glycosides. The amount of glycosylation, position and nature depend on the plant species (Nemeth et al., 2003).The biggest group of polyphenols is characterised by flavonoids. The basic structure involves a 15-carbon basic skeleton (flavan) which, is oriented by two aromatic rings (ring A and ring B) linked by a 3-carbon bridge forming a closed heterocyclic ring (ring C) with the aromatic ring A (figure 1.4).

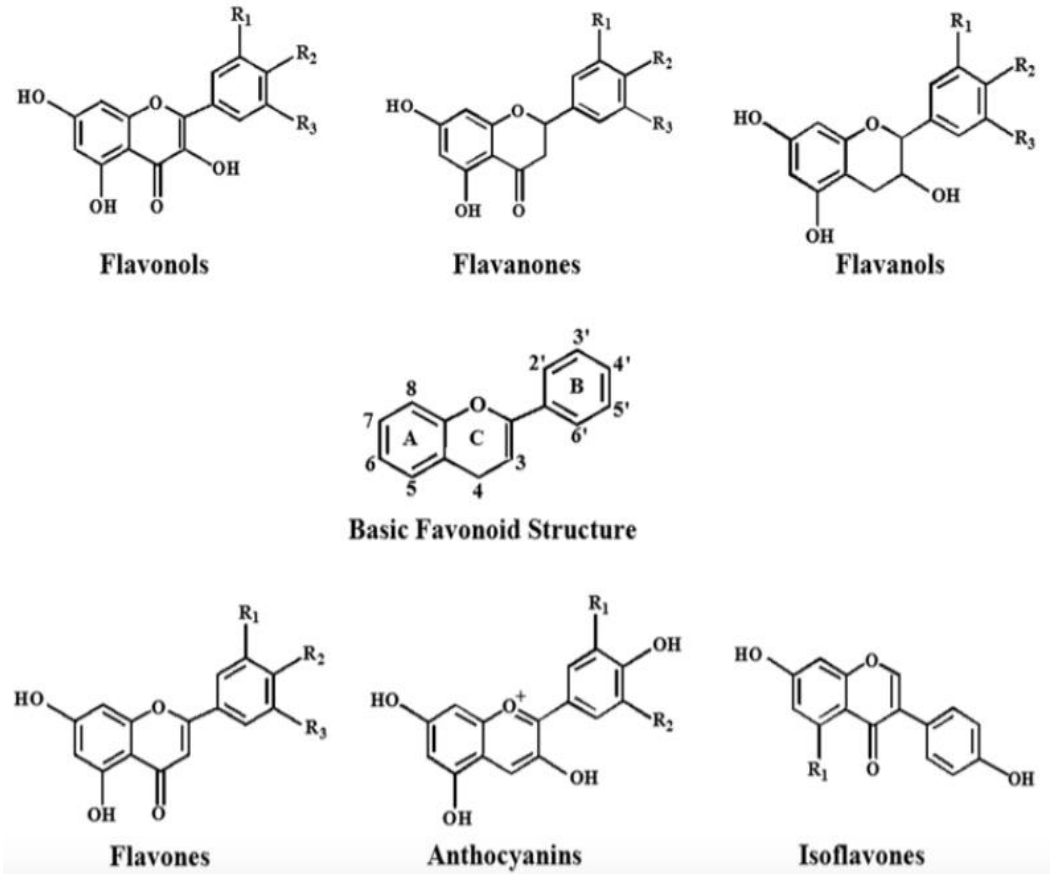


Figure 1.4: Chemical structures of sub-classes of flavonoids (Pandey and Rizvi, 2009).

Figure 1.4 shows, flavonols (such as quercetin) have a 3-hydroxy pyran-4-one group on the C ring. Flavanones (naringenin) possess an unsaturated carbon-carbon bond in the C ring. Flavanols (catechins) lack both a 3-hydroxyl group and the 4-one structure in the C ring. Flavones (luteolin) lack a hydroxyl group in the 3-position on the C ring. Anthocyanins (cyanidin) are characterised by the presence of an oxonium ion on the C ring and in isoflavones (for example genistein), the B ring is attached to the C ring in the 3-position, rather than the 2-position as is the case with the other flavonoids (Pandey and Rizvi, 2009).

Quercetin flavonoid is found as glycosylated forms, mainly as β-glycosides. The nature of glycosylation has considerable impacts on the efficiency of quercetin absorption. The dominant type of glycoside differs between foods. For instance, apples contain mostly galactosides, rhamnosides and arabinosides, whereas onions contain mainly glucosides (Arts et al., 2004). In all onion cultivars, quercetin derivatives of glycosylase moieties are mainly glucose, which is attached to the 4′,3 and/or 7-positions of the aglycones (Slimestad et al., 2007). Whereas in apple mainly occurs in O-linkage to glucose and galactose at position 3 of the flavan backbone (Manach et al., 2004).

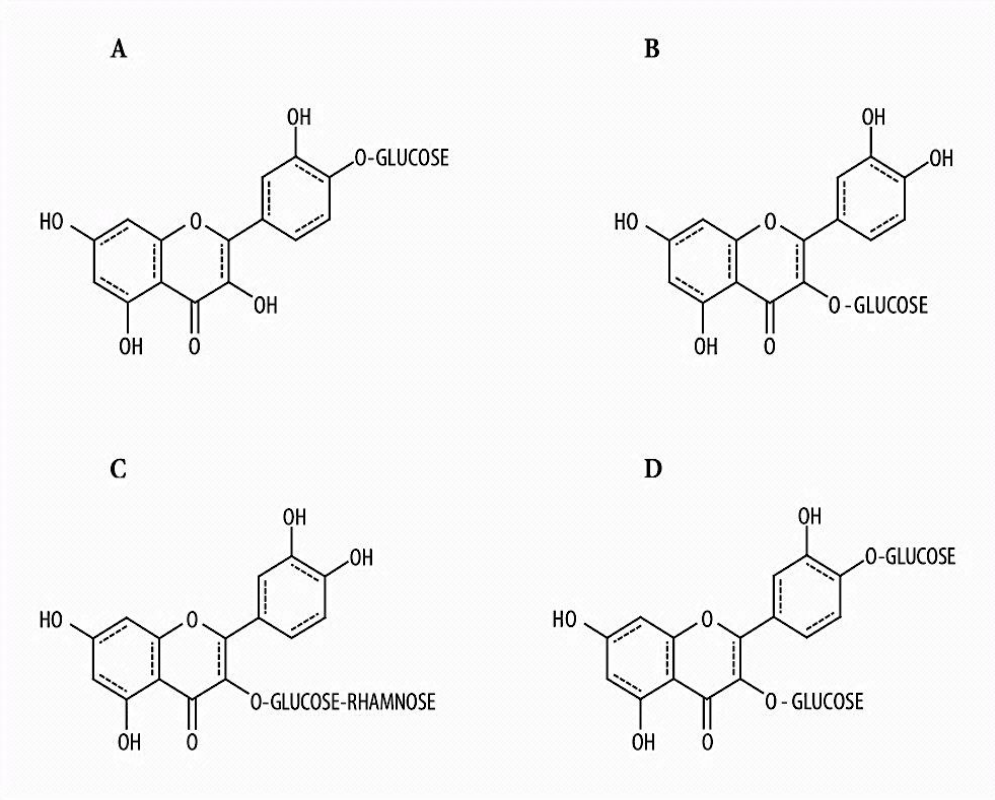


Figure 1.5: Structure of the poly-hydroxylated flavonoid quercetin representing glycosylated derivatives that are found in plants such as onion. **A**= Quercetin 4′-glucoside, **B**= Quercetin 3-glucoside, **C**= Quercetin 3-rutinoside and **D**= Quercetin 3,4′-diglucoside (Nemeth et al., 2003).

These phenolic compounds have aromatic rings in common which contain one or more hydroxyl substituents (figure 1.5). Mostly, they are combined with sugar glucoside and are positioned in cell vacuole (figure 1.5). Intervention studies have shown that the primary site for the absorption of several flavonoid glucosides is the small intestine. Since quercetin glucosides were found to be more bioavailable than quercetin aglycones, they might also absorb earlier in the small intestine and result in a greater plasma peak concentration (Nemeth et al., 2003).

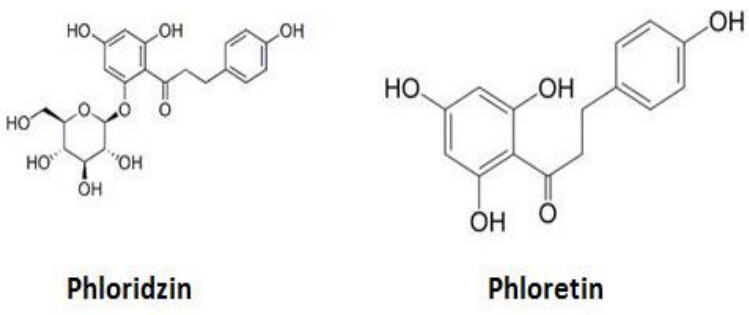


Figure 1.6: Structure of Phloridzin and its aglycone Phloretin (Jugdé et al., 2008).

The phenolic compound phloridzin (phloretin 2′-*O*-glucoside) belongs to the dihydrochalcone flavonoid sub-class and consists of two aromatic rings joined by an alkyl ester (Gosch et al., 2010) (figure 1.6).

Moreover, another group of polyphenolic compounds that are derived from cinnamic acid derivatives are the stilbenes. They are found in plants as cis- or trans- isomers with trans-configuration as the most abundant type; they occur in monomeric form as well as dimeric, trimeric and polymeric stilbenes, called viniferins (Cassidy et al., 2000). They are widespread in red wine from species named *Vitis Vinifera* L (Riviere et al., 2012). The resveratrol is the most known form of stilbene group (Maier-Salamon et al., 2013).

1.3.2. Dietary sources

Polyphenols are present in fruits, vegetables, tea, coffee, cereals, red wine and herbs (Kwon et al., 2007, Tsao, 2010). The most widely found flavonol in diet is quercetin and its glycosylated derivatives, present in onions, apple, berries and tea (Williamson and Manach, 2005). Onions have the highest content of quercetin glucosides, it ranges between 28.4 to 48.6 mg/100g, while the amount in apples range between 2.1 to 7.2 mg/100g fresh weight (Lee and Mitchell, 2012). Yellow onions contain 270-1187 mg of flavonols per kilogram of fresh weight, while red onions contain 415-1917 mg of flavonols per kilogram of fresh weight (Slimestad et al., 2007).

The concentration of phloridzin and phloretin is mainly dependent on the variety of apples and initially found in the apple peel and at lower levels in the pulp. For instance, the concentration of phloridzin and phloretin in the peel of the apple variety *Reineta* ranges between 83-418 and 53-100 mg per 100 gram of fresh weight, respectively, whereas the amount of phloridzin in the pulp is between 16 and 20 mg per 100 gram of fresh sample (Escarpa and Gonzalez, 1998). The amount of chlorogenic acid in apple varies between 30 and 60 mg/kg in fresh weight and 208 mg/l in apple juice. Moreover, according to a study, Portuguese red wine contained 50.8 mg/l of trans-piceid which is the 3-β-glucoside of resveratrol (Ribeiro de Lima et al., 1999).

Table 1.1, demonstrates the widely distributed fruits and vegetables containing quercetin aglycone, quercetin 4′-glucoside and quercetin 3,4′-diglucosides.

Flavonols(Mean) (mg/100g) / Source	Quercetin	Quercetin 4′-glucoside	Quercetin 3,4′-diglucoside
Apple	0.13	0.64	ND
Blueberry	4	0.50	ND
Grape	3.54	2.17	ND
Red Onion	1.31	38.80	77.08
Yellow Onion	0.28	21.55	26.58
Shallot	2	74.62	35.60
Kale	7.71	ND	ND
Black Tea	3.64	1.31	ND

Table 1.1: Dietary sources of flavonols in fruits, vegetables and tea (Slimestad et al., 2007, Phenol-explorer, 2016). ND: Not Detected

1.3.3. Flavonoid intake

As the main dietary sources of flavonoids in the western world are fruits, vegetables, red wine and tea (Johannot and Somerset, 2006), their content varies and it is highly dependent on cultivar, skin to volume ratio of the fruit and other factors (Nyman and Kumpulainen, 2001). According to nutritional data, consumption of flavonoids is mostly dependent on measuring the flavonols and flavones. However, there is a high possibility for the underestimation of rate of intake results derived from diverse flavonoid species in the diet (Aherne and O'Brien, 2002). The estimated daily consumption of polyphenols with a diet rich in vegetables and fruit is approximately 2 g (Ovaskainen et al., 2008). For individuals with a low intake of fruits and vegetables, the other polyphenols sources such as coffee, tea and red wine are considered as their main source (Tagliazucchi et al., 2012). It has been estimated that the non-flavonoid intake in diets account for one third of total phenolic consumption, whereas flavonoids accounts for two thirds (Scalbert and Williamson, 2000).

There have been many studies estimating varied intake levels of flavonoids in several countries. In these studies, they used a variety of methods for flavonoid quantification such as food frequency questionnaires and food balance sheets, which may provide different results (Mullie et al., 2008). Table 1.2, represents the summary of studies conducted in different parts of the world, investigating flavonoid intake from various food sources.

Country	Population	Flavonoid Average Intake (mg/day)	Main sources collected by 24-Hour Recall, Food Balance Sheet &FFQ	References
Australia	Male & Female	454	Onions, Apples, wine, & black tea	(Johannot and Somerset, 2006)
Denmark	Male Female	1786 1626	Coffee, tea, fruits	(Zamora-Ros et al., 2016)
Greece	Male Female	744 584	Coffee, tea, fruits	(Zamora-Ros et al., 2016)
Ireland	Male & Female	177	Wine, onion, tea	(Beking and Vieira, 2011)
Italy	Male & Female	137	Tea, red wine, grapes, onion garlic & citrus fruits	(Tavani et al., 2006)
Spain	Male Female	313	Apples, Red wine, unspecified fruits & oranges	(Zamora-Ros et al., 2010)
UK	Female	808	Tea, coffee, vegetables (onion, broccoli)	(Yahya et al., 2016)
USA	Male & female	190	Tea, Wine Citrus fruit	(Song and Chun, 2008, Chun et al., 2007)
USA	Male& Female	200.1	Tea, berries & wine	(Kim et al., 2016)

Table 1.2: Summary of reports investigating consumption of dietary flavonoids.

Notably, very few flavonoid types are included for quantification, 5-10 most common flavonoids are included in intake estimates; these include flavonols quercetin and quercetin glycosides, kaempferol and myricetin and flavone, such as luteolin and apigenin, while there more than 5000 flavonoids that are concurrently distributed within the human diet (Ovaskainen et al., 2008).

1.4. Intestinal Metabolism of Flavonoids

It is believed that dietary polyphenols are metabolised by colonic bacteria. Indeed, the microbial mechanism is essential for their absorption. Therefore, intestinal bacteria modulate polyphenols by various mechanisms, such as hydrolysis and decarboxylation (Boyer et al., 2005). The crucial step in the metabolism of flavonoids involves deglycosylation of glycosidic forms. Lactase-phlorizin hydrolase (LPH) and 1-β-glucosidase are two enzymes at the brush borders of the intestines that have been shown to deglycosylate flavonoids within the gut lumen (Day et al., 1998).

The metabolism of flavonoids has been broadly investigated both *in vivo* and *in vitro*. The uptake of flavonoids through intestinal epithelium has been illustrated by previous studies (Manach et al., 1998, Aziz et al., 1998). However, there is still uncertainty on whether there is preferential transport of aglycone or glycosidic forms. In a study conducted by Hollman et al. (1995), they found that flavonoid glucosides rate of absorption was dependent on the nature of sugar moiety. For instance, quercetin rutinoside had just 20% of the quercetin glucoside bioavailability (Hollman et al., 1995). Notably, conjugation to a monosaccharide (i.e. glucose) indicates the role of active glucose transporters in the movement or cotransport of flavonoid glycosides (Johnston et al., 2005a). However, aglycones are able to be absorbed by facilitative diffusion, but conjugation to disaccharides will need further metabolism, such as deglycosylation and glucuronidation, prior to complete absorption. Consequently, because of occurrence of several steps in preabsorptive metabolism, the bioavailability of conjugated forms of flavonoids may be hindered (Spencer, 2003, Johnston et al., 2005a).

Hydrolysis of glucoside moiety represents the initial step on intestinal metabolism of the glucosylated forms. Quercetin aglycones passively diffuse throughout the apical membrane and are then glucuronidated. It is suggested that quercetin glucosides are first hydrolysed by the lactase site of lactase phloridzin hydrolase (LPH) prior to diffusion across the apical membrane (Schwanck et al., 2011). Thus, quercetin glucosides are transported into the cells by the SGLT-1 transporters and then hydrolysed by the cytosolic β-glucosidase (Boyer et al., 2005).

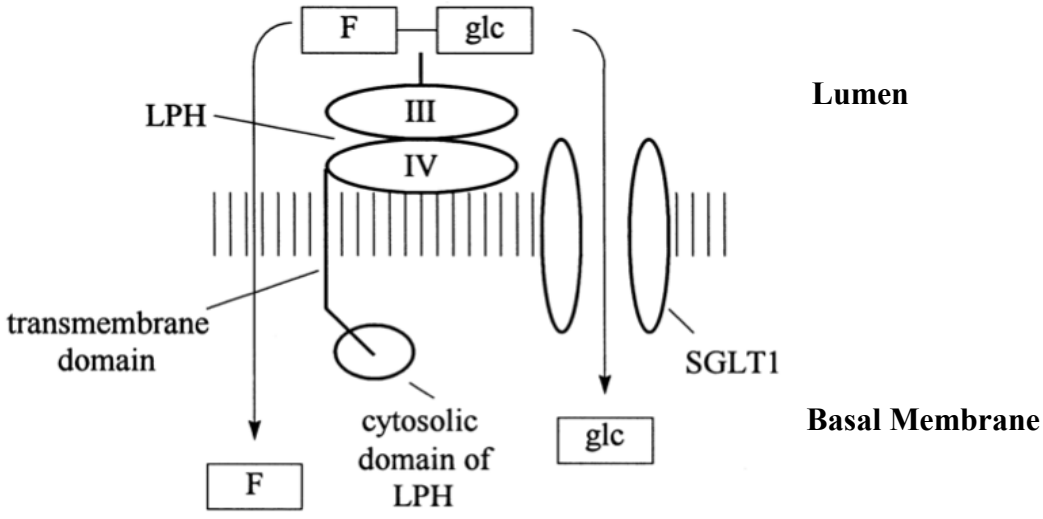


Figure 1.7: Mechanism of flavonoid glucosides metabolism in mammalian small intestine (Day et al., 2000b).

Domain III and IV of LPH are demonstrated as transmembrane protein with release of aglycone and glucosides (F: Flavonoid, glc: glucose), (Day et al., 2000b). Figure 1.7 shows the SGLT-1 glucose transporter will carry the glucose released from the LPH action to the small intestine. And quercetin glucosides interaction with SGLT-1 results in glucose transport inhibition (Day et al., 2000b).

Phenolic glucosides are relatively hydrophilic and do not diffuse passively throughout the biological membrane. It is demonstrated that phenolic glucosides remain intact in the colon until they become deglycosylated in the presence of enzymes such as LPH (Nemeth et al., 2003).

It has been suggested that glycosides or aglycones transport will occur via different pathways, like active or passive mechanisms. Indeed, the hydrophobic status of flavonoid aglycones helps them to be transported passively through diffusion or facilitated diffusion across cell membranes (Walle, 2004). On the other hand, for flavonoid glucosides which are more polar, an active transport is required. Followed by the sodium-dependent glucose cotransporter (SGLT-1) and bilitranslocase, which is a plasma membrane protein, has been identified in the transport of flavonoid glucosides (Walgren et al., 2000). In addition to this, flavonoid aglycones are made readily available in enzymatic action in the intestine. Therefore, the impacts of glycosylation on flavonoid aglycone absorption may be negligible (Day et al., 2000a).

The tissues in the jejunum of intestine have been observed to have the greatest transport efficiencies for flavonoids (Matuschek et al., 2006). Flavonoids that have not been absorbed nor metabolised by the luminal enzymes, such as LPH in the small intestine, undergo bacterial degradation in the colon (Talavera et al., 2004). In fact, colonic bacteria metabolise flavonoid glucosides to aglycones and phenolic acids are easily reabsorbed (Hollman et al., 1995). The last step in the metabolism of flavonoids is the excretory pathway. When in polar forms, flavonoids are excreted in urine or secreted in bile. Flavonoids secreted in bile will go through further intestinal metabolism (Prior and Wu, 2008).

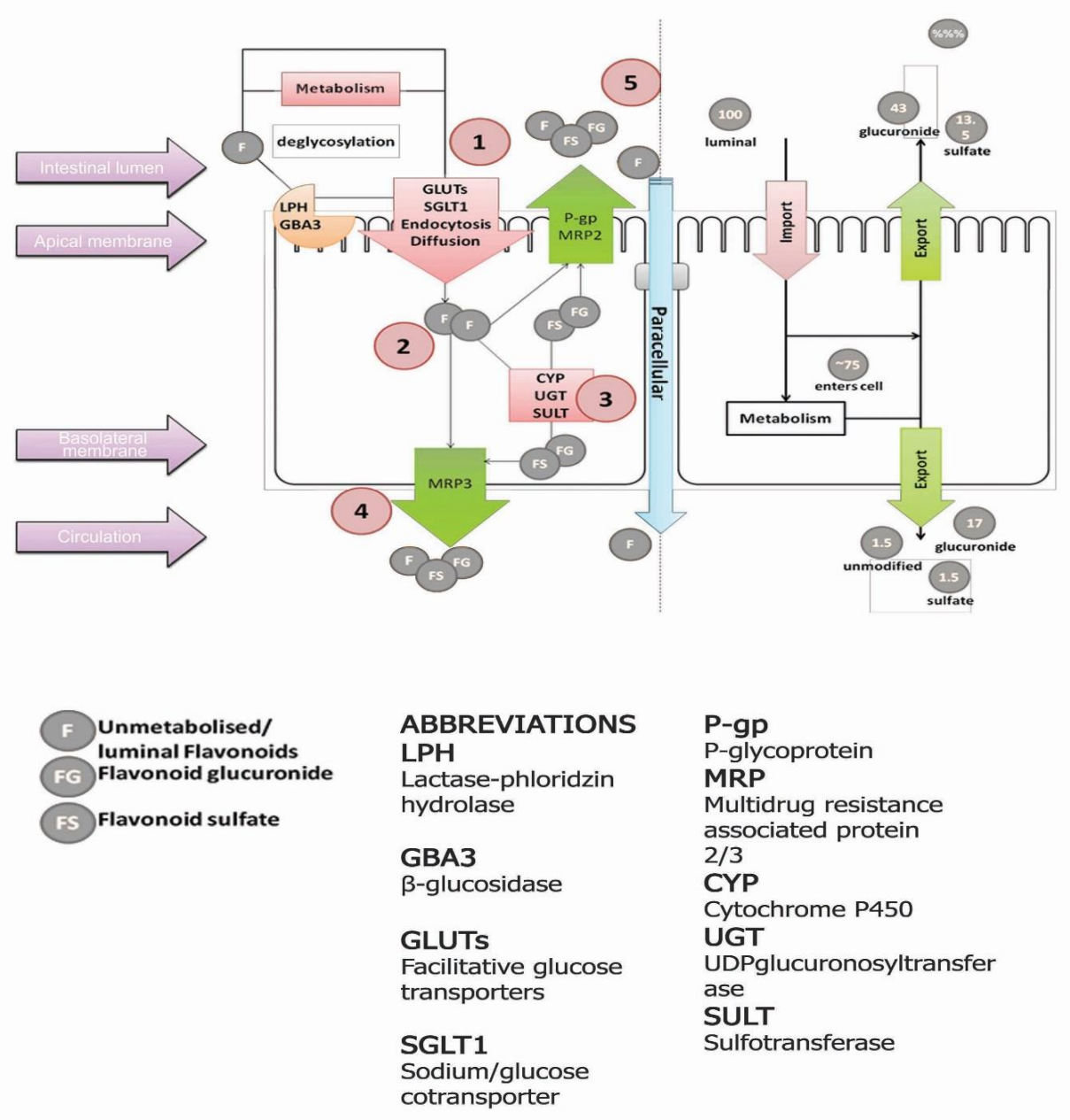


Figure 1.8: Metabolism of flavonoids inside enterocyte, from various sources (Lotito et al., 2011, Walle, 2004).

Figure 1.8 illustrates 1) Flavonoids enter the cells unaltered or go through deglycosylation by bacterial or endogenous enzymes. 2) The metabolised flavonoids are then moved to efflux. 3) Metabolism is dependent on hydroxylation, sulfonylation or glucuronidation. 4) Flavonoids reach the circulation by paracellular diffusion or are released from basolateral membrane 5) and can also be released back into the lumen through an apical transporter (Andlauer and Furst, 2003, Planas et al., 2012). It is previously found that almost 75% of flavonoids in the lumen will be absorbed, more than 50% of the initial dose will be metabolised and rereleased back into intestinal lumen and up to 20% will be sulfonated and glucuronidated and released into the circulation (Lotito et al., 2011, Walle, 2004).

Moreover, the secretion of the intestinal hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) in response to the active absorption of nutrients might also be modified by flavonoid phloridzin. These hormones have a significant effect on the metabolism of glucose by stimulating insulin secretion (Johnston et al., 2005a).

Deglycosylation is one of the major steps in the transfer of flavonoids from the intestinal lumen into the circulation, as designated by the activity of endogenous β-glucosidases *in vitro* studies. The place of deglycosylation and transport across the intestinal enterocytes depends on the nature of the flavonoid aglycone moiety and nature and position of the attached sugar. For example, the contradictory pharmacokinetics of quercetin-3-glucoside and quercetin-3-rhamnoglucoside reveal the importance of structural dissimilarities on the site of absorption. Quercetin 3-glucoside is mainly deglycosylated in the small intestine, while intestinal β-glucosidases appear inactive against quercetin-3-rhamnoglucoside (Day et al., 2000b).

Moreover, parent compounds may also be absorbed unaltered, and the metabolism at cellular level was associated with hydroxylation, conjugation and methylation. Consequently, these reactions could result in the efflux of flavonoids back into the lumen or released into the circulation (Spencer, 2003). A study by Williamson and Manach, (2005) demonstrated that flavonoids are metabolised during absorption and appeared as glucuronide, sulfate and methyl conjugates, which are the most common metabolites in the human plasma. Methylation improves the solubility of lipids and majority of flavonoids in plasma, also conjugation with sulfate or glucuronic acid greatly develops hydrophilicity. It should be noted that the conjugated forms of flavonoids (quercetin glucosides) possess different physiochemical properties compared to the aglycone forms (quercetin) (Williamson and Manach, 2005).

A study conducted by (Wen and Walle, 2006) investigated the effect of structure on the bioactivity of dietary flavonoids in comparison with methylated and unmethylated flavonoids using Caco-2 cell line and human liver S9 fraction. They showed that both Caco-2 cells and S9 fraction of liver possess promising results on the oral bioavailability of dietary polyphenols. The methylated version was more bioavailable for absorption at intestinal tissues and metabolic activity (Wen and Walle, 2006).

1.5. Health Benefits of Flavonoids and their role on Glycaemic Control

There have been many studies on protective effects and health promoting importance of flavonoids on glycaemic control, cancer cell proliferation, cardiovascular complications and neurodegenerative decline (Mursu et al., 2008, Renis et al., 2008). This section will cover the research conducted on the effects of flavonoids *in vitro* and *in vivo* in relation to glycaemic control.

It is noteworthy to investigate the plant extracts in addition to individual polyphenolic compounds that can potentially decrease postprandial increase in blood glucose level by inhibition of intestinal monosaccharide transporters with focus on SGLT-1 and GLUT-2. These include quercetin glucosides, which have been shown to inhibit the active transport of glucose from the luminal side of the brush border into the small intestine via the sodium-dependent glucose transporter SGLT-1. By contrast, it is suggested that quercetin aglycone does not inhibit SGLT-1 (Cermak et al., 2004, Johnston et al., 2005b). Moreover, phloridzin has been established as a known inhibitor of glucose in the sodium-dependent SGLT-1 transporter (Johnston et al., 2005a). Phloridzin results in glycosuria through inhibition of the renal reabsorption of glucose (Kobori et al., 2012). Moreover, previous research has shown the antihyperglycaemic effects of some polyphenolic plant-based foods can be related additionally to α-amylase and α-glucosidase inhibitory activity (Adisakwattana et al., 2012).

In order to overcome complications derived from high levels of glucose and high expression of SGLT-1 transporter in diabetes, the intestinal glucose absorption could be supressed by inhibitors of SGLT-1. This could decrease the uptake of glucose and prevent postprandial portal glycaemic rates, and hence may be supportive in improvement of the metabolic condition in type 2 diabetes. There is recent *in vitro* evidence that polyphenols (flavonoids) are the most important group of phytochemicals, which can alter the pattern of intestinal glucose uptake through their interactions with certain glucose transporters (Gray, 2010). For instance, inhibition of sodium dependent SGLT-1 transporter in which glucose is co-transported with sodium ion or by inhibition of GLUT-2 sodium independent glucose transporter (Gray, 2010). Notably, the flavonoid- quercetin glucoside ability to block SGLT-1 is reduced when the glucoside moiety is cleaved off during lactase hydrolysis (Kottra and Daniel, 2007). Therefore, it is still unknown if deglycosylation of quercetin glucosides in lactose tolerant individuals is higher than lactose intolerant individuals, and thus are less able to decrease the uptake of glucose from the intestine.

1.5.1. In vitro studies

Inhibition of glucose transporters by the interaction of quercetin and quercetin glucosides such as quercetin 3-glucoside, quercetin 4′-glucosides and quercetin 3,4′-diglucoside with SGLT-1 and GLUT-2 has been investigated in several *in vitro* studies (Ader et al., 2001, Cermak et al., 2004, Kwon et al., 2007, Johnston et al., 2005a). An *in vitro* study on Caco-2 cells have examined the effect of quercetin and its derivatives: quercetin 4′-glucoside and quercetin 3,4′-diglucosides. They demonstrated that transport is associated with the position and nature of glucosidic moiety and hypothesised that a glucose moiety in the 3-position could promote absorption and vice versa with respect to glycosylation at the 4′-position (Walgren et al., 1998).

The effect of quercetin on carbohydrate homeostasis has been measured by *in vitro* studies, through inhibition of α-glucosidase activity, inhibition of glucose absorption from the gut, beta cells protection in cell culture and abolishing damage from free radicals in the pancreas (Tadera et al., 2006).Though inhibition of glucose transporters by the onion extract has been studied in few studies, they also showed the onion extract and flavonoids from onion inhibit the SGLT-1 transporter (Schulze et al., 2015). Moreover, absorption of glucose by Caco-2 cells was significantly inhibited by aqueous extract of Kuding tea and purple sweet potato stem under both sodium-dependent and sodium-independent conditions suggesting the effects of SGLT-1 and GLUT-2 transporters (Wang et al., 2008). The flavonoid quercetin has been also found as inhibitors of GLUT-2 overexpression and prevents absorption of mediated glucose and fructose in GLUT-2 transport (Kwon et al., 2007).

1.5.2. In vivo animal studies

According to a study conducted on a rat model of obesity based on high-fat feeding, they showed that a purified anthocyanins addition to their diet decreased the body fat and fasting glucose levels compared to high-fat fed controls (Prior and Wu, 2008). Moreover, three other studies demonstrated that anthocyanins induced expression of adiponectin, which involved inhibition of adipocyte differentiation and an increase in energy expenditure, therefore leading to reduction in weight gain (Bauche et al., 2007, Tsuda et al., 2006, Hsu et al., 2009). In the study carried out by Hsu et al. (2009), they demonstrated in mice intake a high-fat diet and supplemented with o-coumaric acid and rutin containing phenolic compounds, that levels of serum lipids were reduced, followed by inulin and leptin concentrations (Hsu et al., 2009).

In other animal models, orally administered phloridzin has been found to decrease the absorption of glucose in rodents (Kobori et al., 2012). Moreover, mice models with cancer had

up to a 50% increase in their life span when treated with quercetin glucosides (Lamson and and Brignall, 2000). Additionally, the administration of quercetin and its derivatives to Zucker diabetic rats considerably reduced postprandial hyperglycaemia. Quercetin, quercetin-3-gucoside and rutin have shown inhibitory activities on α-glucosidase from the rat intestine (Song et al., 2005). Thus, quercetin and quercetin glucosides could characterise a therapeutic method for diminishing absorption of sugar in type 2 diabetes and metabolic syndrome (Mooradian and Thurman, 1999).

Quercetin has been recognised as an extremely safe compound to be consumed; carcinogenic research in rats have presented that it has no evident side effects, even when consumed in large quantities (2000 mg in per Kg of body weight) (Tadera et al., 2006).

1.5.3. Human studies

A prospective study in type 2 diabetic case-control pairs in the Nurses' Health Study I and II (NHS and NHSII), investigating urinary excretion of eight polyphenol metabolites, found that flavanones (naringenin and hesperetin) and flavonols (quercetin and isorhamnetin), as well as phenolic acid and caffeic acid, were associated with a 39%-48% lower type 2 diabetes risk during early follow-up (median of 4.6 years since urine sample collection) (Sun et al., 2015). Apple consumption of ≥ 1 apple/day showed a 28% lower type 2 diabetes risk compared with no apple consumption (RR: 0.72; 95% CI 0.56–0.92; p = 0.006) (Song et al., 2005). In another study, they found that higher intakes of anthocyanins were significantly associated with a lower risk of type 2 diabetes (HR 0.85; 95% CI 0.80–0.91; p < 0.001) (Wedick et al., 2012). Apples(HR 0.77; ≥ 5 servings/week vs. < 0.001) and blueberries (HR 0.77; ≥ 2 servings/week vs. < 0.001) were inversely associated with type 2 diabetes (Wedick et al., 2012), but total flavonoid intake or other flavonoid subclasses were not associated with a lower risk of type 2 diabetes.

Coffee intake which contain flavonoids, such as quercetin, quercetin glucosides and phloridzin, demonstrated that they were helpful in decreasing the prevalence of type 2 diabetes and obesity, The RR of type 2 diabetes was 0.65 (95% CI, 0.54-0.78) for the highest (≥6 or ≥7 cups per day) and 0.72 (95% CI, 0.62-0.83) for the second highest (4-6 cups per day) category of coffee consumption compared with the lowest consumption category (0 or ≤2 cups per day) (Van Dam and Hu, 2005). According to the research conducted by (Song et al., 2005), quercetin, kaempferol, myricetin, apigenin and luteolin was significantly associated with risk of type 2 diabetes. Among flavonoid-rich foods, apple and tea consumption were associated with

diabetes risk. Women consuming ≥ apple/day showed a significant 28% reduced risk of type 2 diabetes compared with those who consumed no apples (RR = 0.72, 95% CI: 0.56, 0.92; p = 0.006). Tea consumption was also inversely associated with diabetes risk but with a borderline significant trend (> or =4 cups/d vs. none: RR 0.73, 95% CI: 0.52-1.01; p = 0.06) (Van Dam and Hu, 2005).

According to a research conducted by Taj Eldin, et al. (2010), intake of crude *Allium cepa* (red onion) in type 1 and type 2 diabetic patients significantly decreased the blood glucose levels by about 89 mg/dl in relation to insulin (145 mg/dl) in type 1 diabetic subjects and it decreased fasting blood glucose concentrations by 40 mg/dl compared to glibenclamide (81 mg/dl) in type 2 diabetic patients after 4 hours of intake (Taj Eldin et al., 2010). Moreover, an oral administration of a single dose of quercetin simultaneously with starch or maltose solution to normal and diabetic rats as we as all participants with type 2 diabetes resulted in a great inhibition of postprandial plasma levels (Kim et al., 2011), whereas quercetin in combination with glucose did not affect postprandial blood glucose concentrations (Hussain et al., 2012).

Moreover, human studies conducted by Johnston et al. (2002) illustrated that intake of 25g of glucose in commercial apple juice compared with control (water) has significantly delayed the absorption of glucose on plasma levels. In fact, apple juice contains polyphenols such as chlorogenic acid, phloridzin and quercetin glucosides (Johnston et al., 2002). Clear apple juice compared with the control significantly reduced plasma glucose concentrations at 15 and 30min ($p < 0.001$ and $p < 0.05$) respectively (Johnston et al., 2002).

1.5.4. Onions (phenolic and non-phenolic) compounds effects on glycaemic control

Onions (*Allium cepa*) are the second most produced vegetable crop after tomatoes in the world. Onions have a high level of phenolics, which act as protective compounds against different diseases such as cardiovascular, neurological, cancer and diabetes. Flavonoids are the major phenolics in onions (Perez-Gregorio et al., 2010). The strong evidence in support of use of onion in our research is the hypoglycaemic activity of *Allium cepa* (onion) which has been found in several clinical trials showing that, the addition of raw onions to the diet for non-insulin dependent diabetic individuals reduced the dose of antidiabetic medication required to control the disease (Taj Eldin et al., 2010). *Allium cepa* acts as a hypoglycaemic agent by mechanisms rather than increasing insulin levels having extra pancreatic effects; acting directly on tissues as liver, muscles and small intestine and alter the activities of the regulatory enzymes of glycolysis and gluconeogenesis (Taj Eldin et al., 2010).

In a recent study which was in accordance with antiglycaemic significance of onion, they focused on the polyherbal formulation of five different medicinal plants, mango (6g) guava (10g), amla (3g) onion (50g) and garlic (1g) with proven antidiabetic effects were selected for treatment of type 2 diabetic patients (Sukalingam et al., 2015). Reduction in hyperglycaemia by polyherbal may reduce the risk of developing micro vascular complications and most possibly lower the risk of macro vascular complications (Latha et al., 2004). The antidiabetic activity of the polyherbal formulation which mainly contained onion used the glucose induced hyperglycaemic model. High level of glucose in the blood induced the insulin secretion. This secreted insulin can stimulate peripheral glucose intake and control the production of glucose through different mechanisms (Obatomi et al., 1994).

Moreover, it is noteworthy that oral administration of *Allium cepa* crude hydroalcoholic extract in animal models (alloxan-induced diabetic rats) formed a great hypoglycaemic activity and favourable good health effects which may be most probably attributed to improvement and/or regeneration of pancreatic beta-cells (Taj Eldin et al., 2009). Also*, Allium cepa* acts as a hypoglycaemic agent by mechanisms rather than elevating insulin concentrations having extra pancreatic effects; acting directly on tissues such as liver (Shukia et al., 2000, Taj Eldin et al., 2008).Flavonols and anthocyanins are the most available types of flavonoids in onions. They are usually found as glycosylated forms. For instance, quercetin 4′-glucoside and quercetin 3,4′-diglucoside are commonly ingested flavonoids. In addition to flavonoids, Inulin-type fructans (inulin) contain fructose monomers linked by β (1-2) bonds) are present in significant amounts in onion, wheat, chicory, banana and garlic root (Liu et al., 2017). Average daily consumption has been estimated to be 1 to 4 g in the U.S. and 3 to 11 g in Europe. Chicory inulin and oligofructose are officially recognised as natural food ingredients and classified as dietary fibre in almost all European countries (Flamm et al., 2010). Inulin can be found in onions (1–5% on a fresh weight basis), garlic (4–12%), banana (0.2%), and chicory roots (15–20%). Inulin type fructans are consist of 2–60 fructose units linked by a glycosidic linkage often with a terminal glucose unit (Liu et al., 2017). These fructans are not hydrolysed by the digestive enzymes in the small intestine; they reach the colon unabsorbed and are utilised selectively as a substrate for the growth of bifidobacteria (Fedewa and Rao, 2014). Indeed, it is considered as a soluble fibre that can be incorporated into various food products (Reimer and Russle, 2008). In the food industry, inulin is used as a fat or sugar replacement and soluble dietary fibre (Barclay et al., 2010). Table 1.3, demonstrates the content of phenolic compounds and fructans found in different types of onions.

Phenolic Compounds	Red Onion, raw Mean (mg/100g)	Yellow Onion, raw Mean (mg/100g)	White Onion, raw Mean (mg/100g)
		Flavonols	
Quercetin	1.31	0.28	0.03
Quercetin 3-O-glucoside	1.80	0.70	ND
Quercetin 3-O-rutinoside	0.21	0.68	ND
Quercetin 3,4′-O-Diglucosides	77.08	26.58	3.12
Isorhamnetin	1.51	9.31	ND
Quercetin 7,4′- O-diglucoside	1.80	0.36	ND
Quercetin 4′-O-glucoside	38.80	21.55	2.25
Isorhamnetin 4′-O-glucoside	6	2.89	ND
Kaempferol-3-O-glucoside	1	0.80	ND
		Anthocyanins	
Cyanidin 3-O-glucoside	1.50	1	ND
Cyanidin 3-O-glucosyl-glucoside	6.5	ND	ND
Delphinidin 3-O-glucosyl-glucoside	1	ND	ND
		Phenolic acids	
Protocatechuic acid	2	1	ND
		Non-phenolic	
Fructan	2.1	1.2	1.8

Table 1.3: Content of polyphenols and fructan in onion varieties, mean (mg/100mg) (Phenol-explorer, 2016).

In a recent meta-analysis conducted by (Liu et al., 2017), from the 15 trials, in general there was no significant difference in the glucose level (CI: 0.18, 0.08; p = 0.44) with no significant heterogeneity between the individual study results, following inulin supplementation. However, in another study, data reported that inulin-type fructans extracted from chicory roots regulated glucose and lipid homeostasis by enhancing colon production of GLP-1. It is reported that fructans from any botanical origin initiated the production of GLP-1 from the colon, and it is responsible for the amendment of glucose and lipid metabolism (Urias-Silvas et al., 2008).

In the colon, inulin is degraded by gut microbiota into short-chain fatty acid, such as acetate, propionate and butyrate (Kimura et al., 2011). Both propionate and butyrate are metabolised in the colon and liver, which mainly affect their functions. They also induce intestinal gluconeogenesis and sympathetic activity, which thereby improves glucose homeostasis (Mithieux and Gautier-Stein, 2014). Evidence from animal studies has illustrated that inulin or oligofructose supplementation improves weight loss and promotes regulation of glucose in diabetic models (Cani et al., 2005). The mechanism by which inulin acts on glucose

metabolism is still unclear. Several studies have demonstrated increased plasma PYY and GLP-1 and upregulation of proglucagon mRNA with inulin-type fructans in rodents (Delzenne et al., 2005, Urias-Silvas et al., 2008). GLP-1 has many antidiabetic characteristics, such as food intake inhibition, delayed gastric emptying, induction of β-cell proliferation and insulin secretion stimulation (Drucker, 2006).

It is shown in previous studies that a high fibre diet, onion solution significantly reduced plasma glucose area under the curve (AUC) during oral glucose tolerance test (OGTT) in obese rats, which reflected both an increased GLP-1 AUC and higher fasting insulin (Reimer and Russle, 2008). Due to the body's limited ability to process fructans, inulin has minimal increasing impact on blood glucose levels. It is considered suitable for diabetics and potentially helpful in managing blood sugar-related illnesses (Flamm et al., 2010).

In another study it was shown that the fibrous root of the Welsh onion decreased fasting and postprandial hyperglycaemia by inhibiting α-glucosidase in an animal model of diabetes. Thus, the fibrous root of the Welsh onion may be helpful in controlling blood glucose (Kang et al., 2010).

In a study conducted by (Campos et al., 2003), a normal control (group A), and a non-diabetic group (group B), were treated daily with 1 ml onion solution (0.4 g onion/rat). Groups C and D were made diabetic by an intraperitoneal injection of streptozotocin (STZ) (60 mg/kg body weight) in citrate buffer (pH 6.3). These animals (groups C and D) were the STZ diabetic control and STZ diabetic rats with onion intake, respectively. Onion increased the fasting serum high-density lipoprotein levels, and demonstrated alleviation of hyperglycaemia in STZ diabetic rats. The hypoglycaemic and hypolipidaemic actions of onion were associated with antioxidant activity, since onion decreased superoxide dismutase activities while no increased lipid hydroperoxide and lipoperoxide concentrations were observed in diabetic rats treated with onion (Campos et al., 2003).

According to a study on acute effect of onion on blood glucose levels of diabetic patients, when the glucose and onion were administered, the plasma glucose levels were found to be decreased when compared to those levels after giving glucose only: (225.60 ± 27.25 mg/dl vs 214.40 ± 33.39 mg/dl at 30 min; $p = 0.099$), (282.55 ± 31.67 mg/dl vs 229.40 ± 37.61 mg/dl at 60 min; $p = 0.0001$), (270.20 ± 22.48 mg/dl vs 194.45 ± 37.26 mg/dl at 90 min; $p = 0.0001$) and (248.75 ± 20.13 mg/dl vs 161.65 ± 30.50 mg/dl at 120 min; $p = 0.0001$), respectively. This study

showed that onion has an acute effect of lowering the plasma glucose levels which could be useful in the management of patients with diabetes mellitus (Myint et al., 2009).

1.6. Lactose Intolerance

The term lactose intolerance determines the inability to hydrolyse dietary lactose and derives from adult or congenital lactase deficiency. Two-third of the world's population have lactose intolerance condition (Lomer et al., 2007). NDDK (The National Institute of Diabetes and Digestive and Kidney Disorders) estimated that up to 90% of Asian Americans and 75% of all adult Native Americans and African Americans and of North Europeans are lactose intolerant (Devesh and Kayastha, 2012). Lactose is available in the mammalian milk and it is hydrolysed by the enzyme lactase-phloridzin hydrolase (LPH), which is mostly known as lactase. Lactase is a type of β-galactosidase that mediates the hydrolysis of lactose to glucose and galactose in the small intestine (Lomer et al., 2007).

Common symptoms of lactose intolerance include: abdominal bloating, abdominal pain, gas and diarrhoea. These symptoms range from mild to severe according to the amount of lactose the person intakes or the amount an individual can tolerate (Lomer et al., 2007). Thus, decreasing the amount of lactose in milk could possibly reduce gastrointestinal symptoms in lactose intolerant subjects. There are also other several possible ways to overcome this problem, either by adding lactase enzyme in dairy products that hydrolyse lactose available in food or using complementary prebiotics microorganisms (Lomer et al., 2007).

It is found in a human study (Meloni et al., 2001), that patients with diabetes type 1 and 2 have elevated lactase activity; consequently, those with a high intake of lactose-based foods (dairy products) would be exposed to lager amounts of monosaccharides galactose and glucose, and this may lead to a greater blood glucose concentration. High prevalence of lactase persistence has been found in the diabetic patients from Sardinia (Italy), where the prevalence of lactase deficiency is very high (Meloni et al., 2001). This supports the idea that high intestinal lactase activity in diabetic subjects contributes to the high level of blood glucose, while less intestinal lactase activity could be associated with healthy subjects who are lactose intolerant.

According to a recent study conducted by Ji et al. (2014), lactose intolerant subjects had decreased risk of lung and breast cancer in a population-based study in Sweden, which is suggested to be linked to their specific dietary pattern, low consumption of lactose-containing

food (Ji et al., 2014). Milk can contain high amounts of saturated fat, and some growth factors, such as insulin like growth factor-I (IGF-I), and these dietary components have been suggested to be linked with the development of various cancer types (Ji et al., 2014). Moreover, consumption of milk in individuals with lactase persistence has been associated with an increased risk of cataract. Therefore, it has been suggested that hypolactasia would protect an individual against cataract. Another disease, which is suggested as being linked with the ability to digest lactose, is ovarian cancer, the background to this study is suggested as toxin to oocyte (Vesa et al., 2000). Nolan et al. (2010) also found that lactase persistence as evident by presence of the T allele of rs4988235 was associated with the risk of Crohn's disease in the Caucasian population of New Zealand (Nolan et al., 2010).

1.6.1. Role of LPH in relation to flavonoid metabolism

LPH is mainly responsible for metabolism of lactose from milk during infant years. This enzyme is genetically regulated with lower levels in adolescents and most adults in the world are lactose intolerant (Tribolo et al., 2007). Different types of β-galactosidase are found in the small intestine. These include glucocerebrosidase, lactase phloridzin hydrolase (LPH), broad-specificity cytosolic β-glucosidase and pyridoxine glucoside (Day et al., 2000b). All of these enzymes, apart from LPH, act intracellularly and would need transport of intact flavonoid aglycones if they were to act as an important factor in the metabolism of these compounds. Whereas LPH is found in the brush border of the small intestine and is able to act on dietary glucosides prior to the absorption (Tribolo et al., 2007). Thus, LPH enzyme acts as a crucial factor for the hydrolysis of flavonoid glucosides, which consequently alters blood glucose level in the small intestine.

Notably, in addition to the role of lactase in the metabolism of lactose, it is also associated in the intestinal metabolism of several phenolic compounds. Therefore, those individuals who do not express lactase may act differently in the metabolism of some phenolics present in plant-based foods, such as onions or apples, compared to those who continue to express lactase. Therefore, it is possible that this may influence particular diseases risks such as type 2 diabetes on which these phenolic phytochemicals are associated (Shrier et al., 2008). Table 1.4, demonstrates the effect of LPH (lactase) enzyme purified from different sources on three most widely found quercetin glucosides in onion as substrates.

3 most widely found Quercetin-glucosides in Onion		Quercetin-3′-glucoside	Quercetin-4′-glucoside	Quercetin 3,4′-diglucoside
Lactase Source	**Lactase present in rat small intestine cell free-extract**	Deglycosylation (Ader et al., 2001)	Deglycosylation (Ader et al., 2001)	Not examined
	Lactase present in Rat liver cell free-extract	No-Deglycosylation (Day et al., 2003)	No-Deglycosylation (Day et al., 2003)	Not examined
	Lactase purified from sheep small intestine	Deglycosylation (Nemeth et al., 2003)	Deglycosylation (Nemeth et al., 2003)	Deglycosylation (Nemeth et al., 2003)
	Lactase in small intestine and liver of (patients undergoing gastrointestinal Surgery)	No-Deglycosylation (Day et al., 2000c)	Deglycosylation (Day et al., 2000c)	No-Deglycosylation (Day et al., 2000c)
	Lactase from the Fungus *Penicilium Decumbent*	Deglycosylation (Mamma et al., 2004)	Not examined	Not examined
	Lactase purified from pig liver	No-Deglycosylation (Lambert et al., 1999)	Deglycosylation (Lambert et al., 1999)	No-Deglycosylation (Lambert et al., 1999)

Table 1.4: Effect of LPH (Lactase) from different sources on Quercetin Glucosides.

Since lactase (LPH) can cleave several phenolic glucosides, it is possible that people with lactase insufficiency also have a reduced ability to hydrolyse phenolic glucosides in the gut (Nemeth et al., 2003). Hence, there is the possibility that lactose intolerant subjects retain more intact phenolic glycosides in the gut, and that this is associated with a greater inhibition of glucose uptake than occurs in lactase persistent individuals.

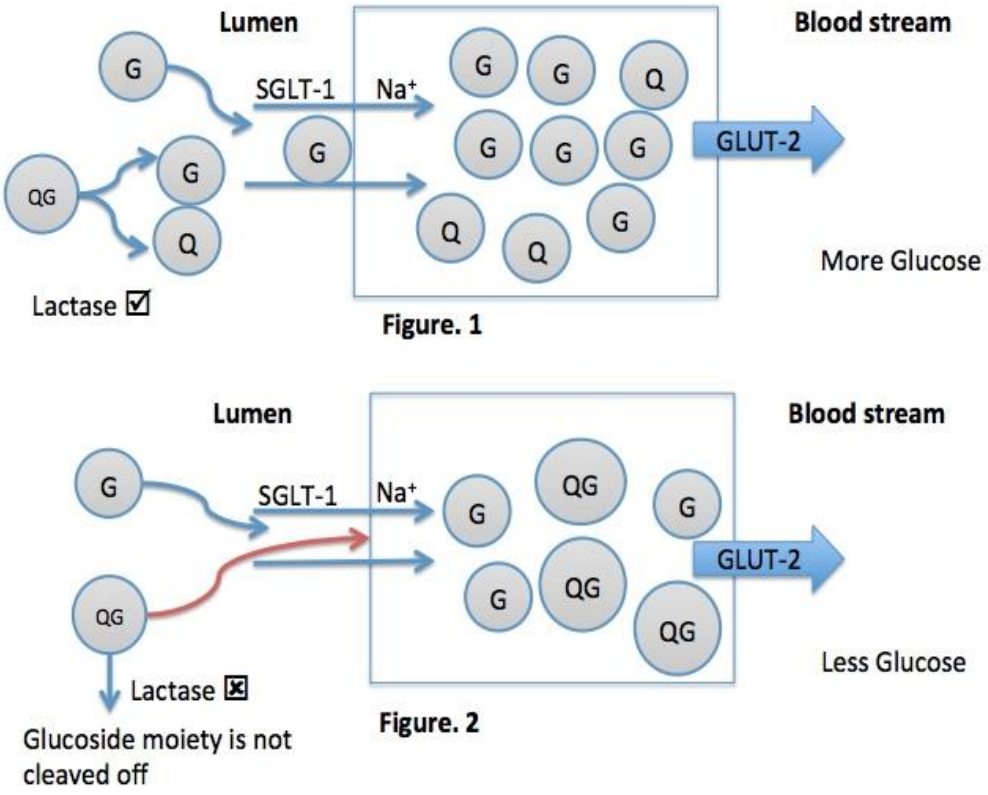


Figure 1.9: Role of lactase (LPH) in the metabolism of phenolic glucosides.

The above figure is a brief summary of our hypothesis, demonstrating the possible role of lactase in the metabolism of phenolic glucosides such as quercetin glucosides in onion.

The evidence from epidemiological studies on the health benefits of polyphenol rich foods are helpful; however, only controlled human studies confirm the positive results obtained through *in vitro* assays and animal models. It is believed that controlled human studies are required to achieve conclusive results in respect to the role of polyphenols on maintaining blood glucose levels. Thus, a pilot clinical study was planned to be conducted in the later stages of the current research project.

1.7. Hypothesis and aims of this thesis

We hypothesised that dietary onion flavonoids are capable of reducing intestinal glucose transport. To address this, we investigated the effects of dietary flavonoids in purified forms and flavonoid-rich onion extract on intestinal epithelial Caco-2 cells, followed by pre-treatment of flavonoids with β-galactosidase enzyme from *Aspergillus oryzae*. In support of *in vitro* study, an *in vivo* study was conducted, and we hypothesised that quercetin glucosides may be less effective at blocking glucose uptake in lactose-tolerant people, because they can hydrolyse quercetin glucosides to the aglycone with lactase compared to lactose-intolerant people.

- To model human gut condition for glucose transport by conducting *in vitro* Caco-2 cell models to assess the role of two main glucose transporters, i.e. SGLT-1 (sodium-dependent) and GLUT-2 (sodium-independent) on glucose uptake.

- To evaluate the effect of onion flavonoids (mainly quercetin 4′-glucoside and quercetin 3,4′-diglucoside) and onion extract on glucose uptake in human intestinal epithelial Caco-2 cells through bidirectional transport model, illustrating both apical uptake and basolateral release.

- To model the role of human intestinal LPH by incubating Caco-2 cells with purified β-galactosidases

- To compare inhibition of sodium-dependent and sodium-independent glucose uptake in Caco-2 cells by flavonoid glucosides and onion extract before and after β-galactosidase treatment.

- A human study to compare inhibition of the glycaemic response to glucose by a dietary source of quercetin glucosides (onion solution) in lactose tolerant adults (n=12) and lactose intolerant adults (n=12).

2. CHAPTER TWO - IN VITRO STUDIES

2.1. Introduction

2.1.1. Caco-2 cell line as a model of intestinal barrier

The human intestinal Caco-2 cell line has been widely used as a model of the intestinal barrier. The parent cell line, originally derived from a human colon carcinoma, forms monolayers of cell through spontaneous cellular differentiation, leading to expression of several morphological and functional characteristics of mature enterocyte (Sambuy et al., 2005). The conditions related to culture is suggested to affect these characteristics, partly because of the intrinsic heterogeneity of parental cell line, resulting in selection of sub-populations of cells becoming prominent in the culture. Caco-2 cells differentiate spontaneously in culture without supplementation of differentiating factors and, usually, standard protocols recommend to subculture cell when culture reaches at least 80% of confluence (Natoli et al., 2011).

The Caco-2 cell line can be used for the assessment of absorption of drugs via the intestinal membrane enterocytes (Artursson and Karlsson, 1991, Rubas et al., 1996). It was developed through research by Jorgen Fogh (1975) at Sloan-Kettering Institute for Cancer Research (Fogh and Trempe, 1975). The Caco-2 cells have the intrinsic ability to initiate spontaneous differentiation on reaching confluence in the presence of normal culture conditions (Rubas et al., 1996). At confluence, there is progressive development of brush border. The surface occupied by each cell gradually reduces from 5 to 21 days, post-confluence and intimate intercellular junctions are developed. During the same period, the length and density of microvilli increase.

Most cells develop complete brush borders with tall and regular microvilli when the full structural polarisation is achieved after 30 days (Artursson et al., 2001). The complete polarised Caco-2 cells resemble human small intestinal mucosa cells expressing brush borders, tight junctions and efflux and uptake transporters at both apical and basolateral compartments. The Caco-2 cell line represent a biochemical barrier in the small intestine due to expression of membrane efflux proteins (P-gp, MRP 1–3), CYP450 isoenzymes and phase II conjugating enzymes such as sulfotransferase, UDP-glucuronyltransferase, glutathione S-transferase (Awortwe et al., 2014).

For differentiation of human intestinal Caco-2 cell models, it is suggested to seed them on culture inserts made with polycarbonate filters, and allowing spontaneous differentiation to process for 2-3 weeks in culture medium in both the apical and basolateral compartments (symmetric protocol) (Ferruzza et al., 2012). Concurrently, *in vivo*, the basolateral side of enterocyte is in direct contact with fluids found in the intestine, whereas the apical side is in association with rapid change of intestinal fluid, which is highly affected by rate of food digestion (Ferruzza et al., 2012).

In the current study, Caco-2 cell line was used as it has been greatly employed as a model of intestinal barrier in literature, it provides a quick and inexpensive screening model, standard Caco-2 model lack morphological and physiological features of complete intestinal tissues. For example, Caco-2 cells show differences with complete intestinal tissue with regard to mucus production, passive diffusion, carrier-mediated uptake and excretion, paracellular transport via tight junctions and intestinal metabolism (Rozehnal et al., 2012).

In fact, Caco-2 cell model is the most suitable choice to investigate sugar transport inhibition due to several morphological and functional characteristics of the mature enterocyte can be expressed by Caco-2 (Angelis and Turco, 2011). The Caco-2 cell line is the most extensively characterised cell-based model for the assessment of absorption of quercetin and its glycosides via the intestinal membrane enterocytes (Boyer et al., 2005, Johnston et al., 2005b). Several quercetin glucosides inhibit glucose uptake into brush border membrane vesicles of porcine jejunum and also are capable of interacting with sodium-dependent glucose transporters in the mucosal epithelium, as a consequence, this may even be the route by which these flavonol glucosides are absorbed by the small intestine *in vivo* (Cermak et al., 2004).

The intestinal absorption of compounds across the intestinal epithelium depends on their chemical characteristics, and compounds can be substrates for numerous transporter proteins and metabolising enzymes in the currently used model Caco-2 cell. Although, there is some literature available on the gene expression of drug transporter genes in Caco-2 cells and human intestine (Hilgendorf et al., 2007, Englund et al., 2006), mRNA expression levels of transporter proteins are shown not to correlate well with protein abundance levels (Ohtsuki et al., 2012).

However, there are possible limitations associated with the use of Caco-2 cell line, since it is not applicable to mimic all the biological activity of the human intestine (Sambuy et al., 2005). Also, problems of reproducibility occur in use of this cell line, due to variability in different laboratories as well as different culture conditions, like the type of animal serum used, the passage number, the source of clones (Natoli et al., 2012).

2.1.2. β-galactosidase as a model of human intestinal LPH

Lactase (LPH), a part of the β-galactosidase family of enzymes, catalyses the hydrolysis of lactose to glucose and galactose, it is found in plants, microorganism and animals and is widely used in the food industry due to its hydrolytic activity on lactose and galactooligosaccharides production (Ansari and Husain, 2012). The active site of the enzyme has one sulfhydryl and one imidazole group, and the reaction corresponds to a S_N2-like displacement mechanism (Meera et al., 2013). Factors that can influence enzyme activity include substrate concentrations, pH and temperature.

It is indicated that LPH plays an important role in the metabolism of glycosylated phytochemicals, and that the expression and activity of this enzyme in the small intestine can modify the profile of metabolites appearing in the circulation (Wilkinson et al., 2003). Hydrolysis of LPH is the first step in the uptake of daidzein glucosides as rats' small intestines exhibit a broad substrate specificity for quercetin glucosides, daidzein glucosides as well as kaempferol and luteolin glucosides (Wilkinson et al., 2003). In this study, we investigated the deglycosylation of individual quercetin glucosides and onion containing quercetin glucosides as substrates by β-galactosidase from *Aspergillus oryzae* (isolated enzyme) to model the condition in human intestine in the presence of LPH, and evaluated the concentration of quercetin aglycone after deglycosylation of quercetin glucosides by β-galactosidase.

β-galactosidase from *Aspergillus oryzae* has several advantages in comparison with other microbial sources. It is extensively accepted as the source of enzyme used for food and has been recorded as GRAS (generally regarded as safe) status (Godfrey and Reichelt, 1983). Since this natural isolate produced very low levels of β-galactosidase, attempts were made to increase the productivity by optimising following parameters pH, and temperature (Nizamuddin et al., 2008). Several possible bacterial species, which are currently used by the dairy industries, produce β-galactosidase enzyme and are derived from *Lactobacillus* and *Bifidobacterium.* Moreover, the yeast *Kluyveromyces lactis* and fungi *Aspergillus oryzae* are

considered as the main commercial source of lactase due to their dairy environmental habitat. However, β-galactosidase *Kluyveromyces lactis* has low thermo-stability, which is a major disadvantage (Dagbagli and Goksungur, 2008).

2.1.3. Aims

- To assess the inhibitory effects of polyphenols on Caco-2 cells under sodium-dependent and sodium-independent conditions.
- To evaluate the effects of β-galactosidases on inhibitory role of phenolic glucosides on glucose uptake.

2.2 Materials

2.2.1. Materials used in vitro studies

Chemicals/Samples & Purity Grade	Product code	Supplier
Caco2-cell lines	86010202	Life and Medical Sciences division, University of Hertfordshire, UK
D-[6-3H] Glucose	NET100C	Perkin Elmer (Boston, USA)
Liquid Scintillation Counting Cocktail (≥99.5% HPLC grade) 4000ml	SC/9205/21	Fisher Scientific, UK
Cell culture medium and plastic ware	CLS290, C6231	Sigma Aldrich (UK) and Merck Millipore (UK)
Foetal Bovine Serum (FBS) 500ml	F2442	Sigma Aldrich, UK
Trypsin-EDTA solution 10x 20ml	T4174	Sigma Aldrich, UK
HEPES (≥99.5% HPLC grade) 25g	H3375	Sigma Aldrich, UK
Phloridzin (99% HPLC grade) 100mg	274313	Sigma Aldrich, UK
Quercetin (≥95% HPLC grade) 10g	Q4951	Sigma Aldrich, UK
Quercetin 3-β-D-glucoside (≥90% HPLC grade) 10mg	17793	Sigma Aldrich, UK
Quercetin-3,4′-*O*-diglucoside (≥98.5% HPLC grade) 5mg	1023	Extrasynthese, France
Quercetin-4′-*O*-glucoside (≥95% HPLC grade) 10mg	1201	Extrasynthese, France

Industrial Methylated Spirits (IMS) (99% HPLC grade) 2500ml	MESP-1485-22	ReAgent, UK
Methanol (99.8% HPLC grade) 2000ml	322415	Sigma Aldrich, UK
2-Nitrophenyl-β-galactopyranoside ONPG (≥98% HPLC grade) 500mg	N1127	Sigma Aldrich, UK
Ortho-Nitrophenyl ONP (98% HPLC grade) 5g	N19702	Sigma Aldrich, UK
β-galactosidase from *Aspergillus oryzae* (≥8.0 units/mg)	G5160	Sigma Aldrich, UK
Inulin from chicory (10g)	12255	Sigma Aldrich, UK
Trypan blue solution (0.4%) 20ml	T8154	Sigma Aldrich, UK
DMSO (≥99.5% HPLC grade) 100ml	D4540	Sigma Aldrich, UK
Non-essential amino acid solution (100x) 100ml	M7145	Sigma Aldrich, UK
DMEM media 500ml	D5796	Sigma Aldrich, UK
Tetrahydrofuran (≥99.9% HPLC grade) 100ml	401757	Sigma Aldrich, UK
Trifluoroacetic acid (≥99% HPLC grade) 100ml	T1647	Sigma Aldrich, UK
PBS tablets (1x)	BR0014G	ThermoFisher Scientific, UK
Penicillin-Streptomycin (100x) 100ml	15140122	ThermoFisher, UK Scientific
Red onion (500g)	921002	Asda, Hatfield, UK

Table 2.1: Material used in cell culture and HPLC labs

2.2.2. Consumables and equipment used in Caco-2 Cell-culture

Consumables/Equipment	Product details	Supplier
Air Cabinet	Airtech Class2	Howorth, Bolton, UK
Glass pipettes (10ml)	CLS4487	Sigma Aldrich, UK
Pipette tips (10µl, 100µl, 1000µl, 5000µl)	CLS4809	Sigma Aldrich, UK
Eppendorf (1ml, 1.5ml, 2ml)	BR780546	Sigma Aldrich, UK
Freezing vials, 2m sterile	V9380	Sigma Aldrich, UK
Centrifuge	5810R	Eppendorf, Cambridge
CO_2 Incubator	MCO-36A	Sanyo

Cover slips	631-1566	VWR, PA
Haemocytometer	CM175-20	Sigma Aldrich, UK
Disposable filters (0.2µM)	Z259942	Sigma Aldrich, UK
Falcon tubes (15-50 ml)	210261	Grenier Bio One
Flasks (T-25/T75)	690/658175	Grenier Bio One
Waterbath	TSGP10	ThermoFisher Scientific
Upright light microscope	TS100	Nikon
Scintillation counter	LS6500	Beckman-Coulter
Sterile 24- transwell inserts	TKT-526-010M	Fisher Scientific
Sterile 24-well plates	CLS3527	Sigma Aldrich, UK
Spectrophotometer	DU530	Beckman model, CA
UV quartz cuvette	Z276693	Sigma Aldrich, UK
Hand Blender	DDF644	Moulinex, UK

Table 2.2: Equipment and consumables used in cell culture lab

2.2.3 Composition of HEPES-buffered salt solution (HBSS) used in glucose uptake studies

Reagent	Concentration (mM) Sodium Dependent	Concentration (mM) Sodium-Independent
Sodium chloride	140	5
Potassium chloride	2.5	140
Sodium phosphate	1	0
Potassium phosphate	0	1
Calcium chloride	1	1
Magnesium chloride	0.5	0.5
HEPES	10	10
BSA	0.2% (w/v)	0.2% (w/v)

Table 2.3: Concentration of reagents used for HBSS sodium-dependent and sodium-independent (Wang et al., 2008).

2.3. Methods (Cell Culture Studies)

2.3.1. Cell culture medium and seeding

Stock cultures of Caco-2 cells were maintained in T75 plastic flasks and cultured in 95% air and 5% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 20% FBS, 1% penicillin/streptomycin, 1% non-essential amino acids and 1% L-glutamine. All experiments were carried out on cells between passage numbers 40 to 80 from the initial cell line stock with passage number. For experiments, cells were seeded at density of 10,000 cells/cm^2 into 24 well plates and grown for 5-20 days. The expression of sugar transporter SGLT-1 increases from 12-20 days (Johnston et al., 2005a); therefore, we chose 14 days as the most appropriate time duration for the growth of cells prior to the glucose uptake assays.

2.3.2. Aseptic Techniques

Disposable gloves were worn at all times. Separate lab coats were used in the cell culture laboratory. Fume hood and suction were cleaned thoroughly with 70% IMS to minimise contamination of the cells.

2.3.3. Cell passaging

For the detachment of cells from the walls once cells were adherent, trypsinisation of cells were required. For this, cells were washed twice with 5 ml phosphate buffered saline (5mM PBS) which maintain pH and osmotic balance of the cells. Following this, cells were then treated with 2 ml of 1% trypsin for 40 seconds with vigorous taping on the sides of the flask to detach cells from the walls. Floating cells were seen under the microscope at 10X magnification, and at this point, 8 ml of complete culture medium were added to the flask. Afterwards, 2 ml of floated cells was transferred to new T-75 flasks and 13 ml of fresh medium was added to make a final volume of 15 ml. Cells were then checked every couple of days for their confluency and their medium was changed, thus kept in the cell culture incubator gassed with 5% CO_2 at 37°C. Stock cells were subcultured once a week at a 1:10 ratio; media for all cells was changed too. Cells were seeded at a density of 10,000 cells/cm^2 in growth media and left to differentiate for 10 days after reaching confluence.

2.3.4. Counting cells with Trypan Blue

Trypan Blue is one of the several stains suggested for counting of viable cells; staining enables the visualisation of cell morphology (Boyer et al., 2005).This method is based on the principle that viable cells do not take up certain dyes, whereas non-viable cells do. The procedure for cell counting is shown below:

1. Resuspend the cell pellet and mix to have a homogenous suspension; therefore, mix 50µl of the cell suspension with 50µl of 4% (*v/v*) Trypan Blue solution, and allow to stand for 5-15 minutes.
2. Transfer the cell suspension: Trypan Blue mixture to a haemocytometer and leave under a microscope (10x objective) with use of coverslip.
3. Each square of the haemocytometer shows total volume of 0.1 mm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the following cell concentration per ml (and total number of cells) is measured by using the following calculations
4. Cell concentration (cells/ml) = total number of cells in 1 x 0.1mm^3 square x (1 x 10^4) x 2.
5. Total number of cells = total number of cells (viable and non-viable) x original volume of cell suspension.
6. Cell viability % = total viable cells (unstained) / total cells (stained and unstained) x 100.

2.3.5. Freezing the Caco-2 cells (Cryopreservation)

After passaging the cell line, followed by centrifugation of cells within DMEM media, we resuspended the cell pellet in 1ml 10% DMSO and 90% complete DMEM medium, and then transferred the cell suspension to a sterile cryovial, labelled with cell type, passage number, date and name. This was followed by transfer of cryovials to a polystyrene box where it was stored at -80ºC for 2-3 days. Thereafter, the cryovial were moved into the liquid nitrogen tank at -196ºC and the details were added to the cell culture lab log book for future use.

2.3.6. Preparation of Flavonoids and Inulin

Quercetin and quercetin glucosides are soluble in dimethyl sulfoxide (DMSO) and are stable in this form when stored at 4°C, immediately prior to the reaction: an aliquot was diluted in 1% (v/v) methanol at pH 6 to give a final DMSO concentration of 0.1% (v/v) in the reaction mixture. Thus, 20µM, 50µM and 100µM of the Phloridzin, Quercetin, Quercetin 3-β-glucoside, Quercetin 4′-glucoside and Quercetin 3,4′-diglucoside were prepared for the

enzyme assay and analysis by HPLC. 5% (w/v) Inulin from chicory was also dissolved in 0.1% DMSO.

2.3.7. Onion sample preparation

Red onions were washed with tap water to remove dirt and dried thoroughly with absorbent paper. The outer layer was then removed and onion was cut into small pieces for extraction; 20 g was weighed and 80 ml of 80% v/v methanol was added, immediately after the solution was grounded with an electric blender to a homogeneous reddish puree. Thus, the solution was filtered twice by using suction filtration and then filter paper (P8 Fisher Scientific), then 0.22 µm syringe filter (Sigma-Aldrich Z290807). Thereafter, 25% w/v red onion extract filtrate was collected in Eppendorf tubes and placed into storage at -20C^{o} until analysis.

2.3.8. Glucose Uptake Assay

Glucose uptake assay were performed with 3 D-[^{3}H]-glucose to demonstrate the effects of flavonoid and onion extract on glucose uptake in the presence and absence of sodium which allowed differentiation between effects of sodium-dependent (mediated by SGLT-1) and sodium-independent (mediated by GLUT-2) pathways.

Caco-2 cells in the 24-well plates were treated with serum free medium (1 ml each well) and were kept in the incubator 24 hours before uptake studies. After 24 hours, serum free media was aspirated and HBSS with sodium (NaCl &Na_2HPO_4) and without sodium (KCL &K_2HPO_4) were used to run the experiments. Caco-2 cells were first treated with HBSS, containing both sodium and sodium free, for 15 minutes followed by the test compounds 20µM, 50µM and 100µM flavonoids (phloridzin, quercetin glucosides and quercetin aglycone). 1mM glucose was added to each solution as well as ^{3}H-glucose. The concentration of radioactive glucose in either the test or the control compound was 12.5 kBq per ml according to the known glucose uptake assay method used in a study conducted by (Johnston et al., 2005a). The addition of 1 mL of HBSS solution containing glucose, tested compounds and ^{3}H-glucose initiated the uptake both in the presence and absence of sodium. The effect of phloridzin (positive control), quercetin and quercetin derivatives and onion extract on glucose uptake were measured over 2 minutes. For time-dependent experiments, cells were incubated at various time points with test compounds in sodium dependent and sodium free solutions. Followed by removal of radioactive glucose first, ice-cold PBS was added twice to wash the cells. After washing of the cells, PBS was aspirated and 1 ml 200 mM NaOH was added at each well and then kept overnight prior to scintillation counting. The solution

from wells were removed and placed in scintillation vials. In the last step, 4 ml of scintillation fluid in scintillation vials were added and mixed properly. Vials were taken to the scintillation counter for counting and the uptake values shown by radiolabelled glucose. The counting time for activity measurement was 2 minutes for all the vials (Johnston et al., 2005a).

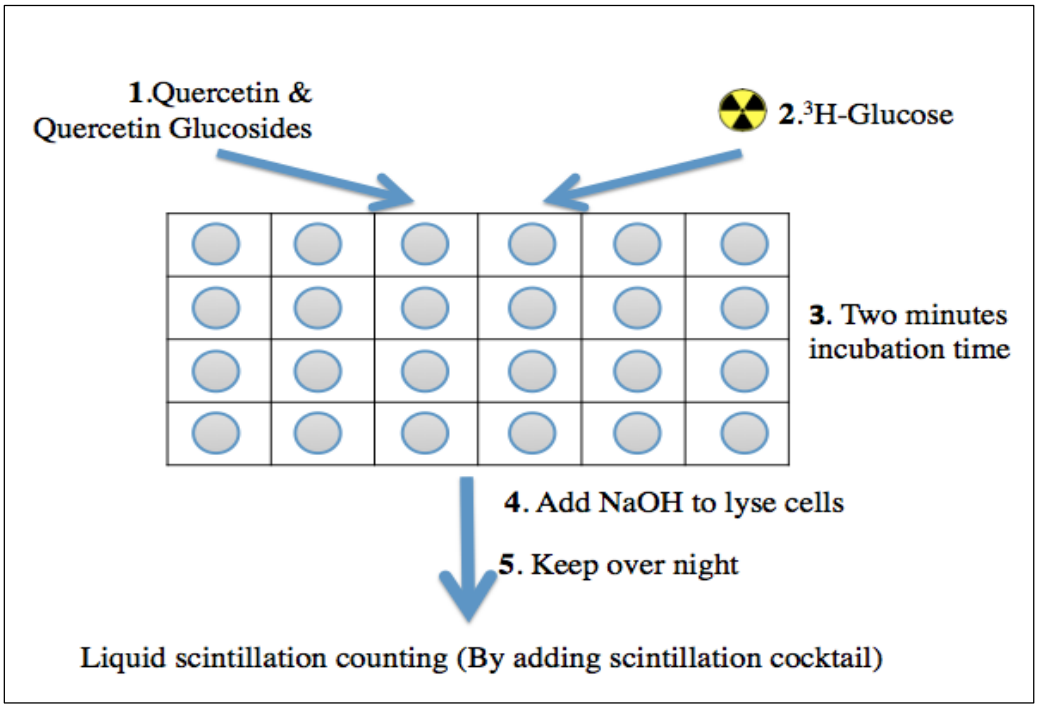


Figure 2.1: Glucose uptake assay conducted on a 24-well plate.

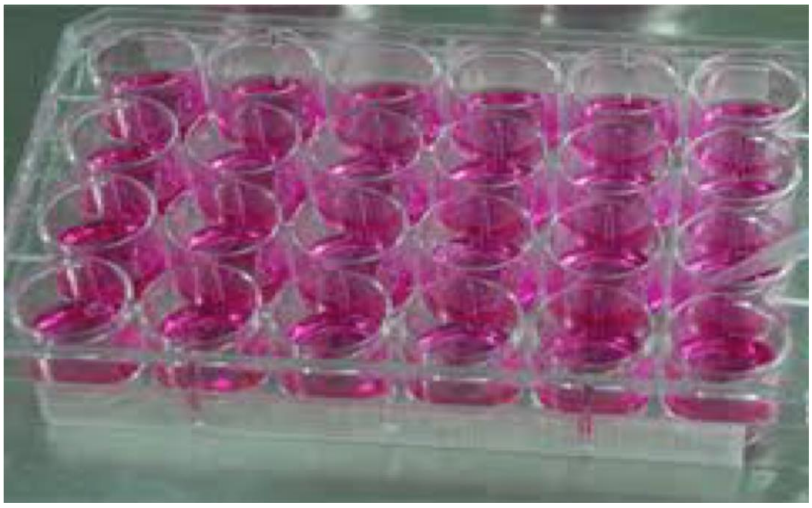


Figure 2.2: Picture taken from the 24-well plate containing DMEM medium, consequently removed and HBSS sodium-dependent and independent containing test compounds with ^{3}H-glucose were added.

2.3.9. Method for detection of radioactivity (3H-glucose)-Liquid Scintillation Counting

A scintillation counter is the most widely used method for detection of radioactivity, and therefore we used it in this research project for measurement of radiation from β-emitting nuclides like tritium ^{3}H (a low energy β-emitter). Radioactive samples for counting were prepared by combining with a liquid scintillation cocktail. An ionising particle is produced by the decay of radionuclide. Part of the kinetic energy of this ionising particle is transferred to the 'scintillator', which converts this energy into light photons, and these light photons are detected by the liquid scintillation system. These photons are directed towards either of two photomultiplier tubes (PMTs). Thus, DPM (disintegrations per minute) values were shown by tritiated glucose after the uptake study (Wang et al., 2008). Units of radioactivity involves 1 Becquerel (Bq) = 1 disintegration per second (dps) or 60 disintegrations per minutes (dpm) and 1 Curie (Ci) = 3.7 x 10^{10} disintegrations per second or 2.2 X 10^{12} disintegrations per minute. NaOH from all the wells were mixed with 4 mL of scintillation fluid in translucent vials and mixed uniformly for each vial before running on the liquid scintillation counter 6500.

Figure 2.3: Picture taken from liquid scintillation counter including data storage system, monitor and printer

2.3.10. Wipe testing for the use of 3H glucose (Regular monitoring before and after each experiment)

Wipe testing was required for detection of possible radioactive contamination in all the areas where liquid isotopes were used. First, the end of the cotton bud was moistened with 70% IMS. Then, the areas which were used regularly (100 cm^2) and equipment such as Gilson pipettes, stock container of ^3H glucose and background (unused area) as control were wiped. Then, the bud was placed into a scintillation vial and scissors were used to cut off the unused end. Then, 4mL scintillation fluid was added to each vial containing wipes, it was ensured that the cap was fully in place and then shaken well. The activity was calculated as CPM values (counts per minute) in the liquid scintillation counter.

2.3.11. Bidirectional transport assay (Caco-2 cell models)

Millicell inserts for (24-well plates) or Transwell-24 insert permeable plates with a treated polycarbonate membrane and 0.4 µm pore size were used, which provided an excellent substrate for Caco-2 cell attachment, growth and differentiation. An open culture reservoir plate was used to reduce liquid handling during cell feeding, and a medium insert was transferred to a standard Corning® 24 well microplate for running experiments. This allowed easy access to both the apical and basolateral sides of cells. The volume of medium added to per plate well and transwell inserts were 0.6ml and 0.1ml respectively (Transwell®, 2010).

Caco-2 cells were placed in serum-free media for 24 hours prior to uptake studies and were incubated for 15 minutes at room temperature in HBSS (with sodium and without sodium) prior to experiments. Uptake was initiated by the addition of 1% methanol as control in the presence or absence of the test substrate (100 µM quercetin 4′-glucoside and 100 µM quercetin 3,4′-diglucoside) pretreated with ^3H-glucose at the apical for apical to basolateral transport side of monolayer, (Transport from inserts to wells) (Awortwe et al., 2014). The reaction was terminated after 2 minutes by aspiration of the uptake buffer followed by the addition of ice-cold PBS. Cells were washed twice in ice-cold PBS and solubilised overnight in 200 mM NaOH prior to scintillation counting (Johnston et al., 2005a).

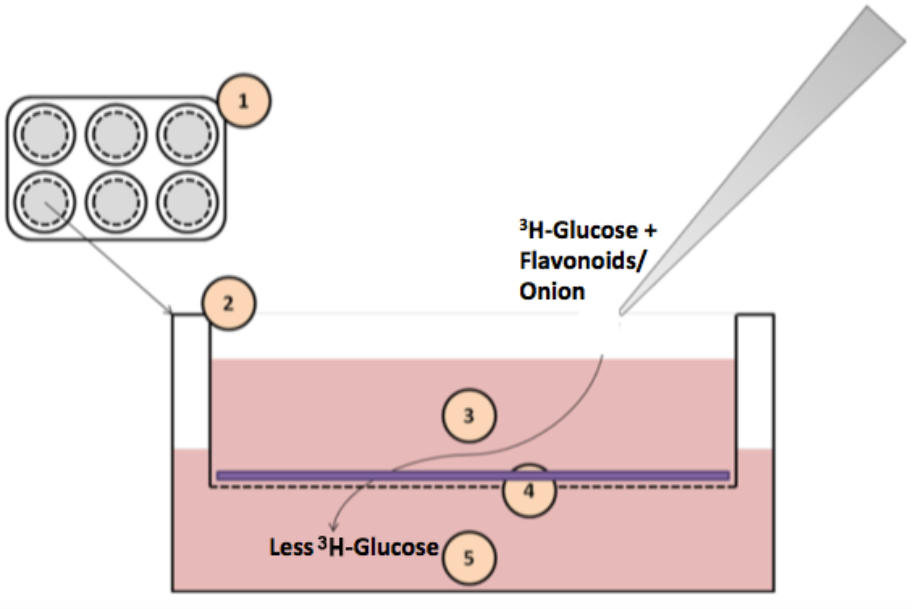


Figure 2.4: Transwell insert system shows the compartmentalisation of apical and basal sides of glucose transporters

In Figure 2.4 above, number (1) indicates the transwell insert system by the compartmentalisation of two main glucose transporters (both apical uptake and basolateral release); number (2) inserts allow cells to become highly confluent and polarise on a semipermeable membrane, where both apical and basolateral sides of cells are exposed to media; (3) shows the media on the apical side is in contact with the apical surface of the Caco-2 monolayers and models the intestinal lumen conditions; this is followed by number (4), which depicts the semipermeable membrane that allows a physical barrier for cells to grow on, as well as separating the apical media from the basolateral media, therefore resulting in quantification of apical to basolateral transport in contact with the basolateral side of the Caco-2 cells; and finally, (5) shows the basolateral chamber carries media in contact with the basal side of Caco-2 and mimics the conditions in circulation.

Figure 2.5: Picture taken from a 24-well plate with transwell inserts.

2.3.12. Statistical analysis (cell culture studies)

Values are presented as mean ± SEM. For each analysis, at least three independent experiments with nine replicates (control and treated wells) were carried out. Comparisons of only two mean values were performed by Student's t-test or one-way ANOVA. If more than two means were statistically evaluated, a two-way ANOVA with subsequent multiple comparisons, according to Bonferroni, was performed. In order to correct for multiple comparisons, the level of significance (x-value) was reduced to $p < 0.001$. However, the mean difference was statistically significant at the 5% level. In all experiments, a probability of $p < 0.05$ (*) was significant, $p < 0.01$ (**) and $p < 0.001$ (***) were determined as statistically highly significant and $p > 0.05$ was not statistically significant (ns) (GraphPad Prism 6.01, Version Date: 19/10/2012, GraphPad Software, San Diego, USA).

2.3.13 Dialysis Procedure

Dialysis involves removal of a very small molecular weight solutes from a solution. It contains “dialysis tubing” which was used and has a semi-permeable membrane, made of cellulose acetate. Dialysis tubing was cut into desired length. Then, the dialysis tubing was rinsed inside and outside with distilled water, as there was glycerol inside. Dialysis tubing was immersed into 1L of 2% sodium biocarbonate/1Mm EDTA in 2L glass beaker. Then, a knot was tied at one end of the tubing, checked for leakage and then samples were loaded into the tubing using a pasteur pipette, and added 50% Ethanol/1Mm EDTA and submerge completely. Then, a stirrer bar was placed at the bottom of the beaker and the beaker was placed on a magnetic stirring plate in cold room. After dialysis proceeded to two hours, the buffer was replaced with a fresh dialysis buffer. Subsequently, the dialysis buffer was removed after 8 and 24 hours respectively from the beaker.

2.4 Methods (β-Galactosidase Assay)

2.4.1. Properties and activity of β-galactosidase (LPH)

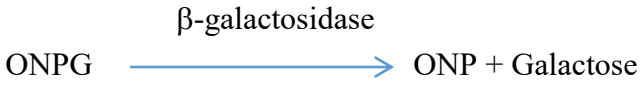
The properties, specificity and structure of β-galactosidase significantly differ on the microbial source of the enzyme, e.g. different molecular weight, amino-acids chain length, position of the active site, pH- and thermal-optimum and stability (Table 2.4). The choice of suitable β-galactosidase source depends on reaction conditions of lactose hydrolysis. For example, dairy yeasts with a pH optimum 6.5–7.0 are habitually used for the hydrolysis of lactose in milk or sweet whey. On the other hand, the fungal β-galactosidases with optimum pH 3.0–5.0 are more suitable for acidic whey hydrolysis. The activity of different β-galactosidases also depends on the presence of ions. The fungal β-galactosidase *Aspergillus oryzae* is active without ions as cofactors, but the yeast β-galactosidase isolated from *Kluyveromyces lactis* requires ions, such as Mn^{2+}, Na^{+}. Interestingly, Ca^{2+} and heavy metals inhibit the enzyme activity of all β-galactosidases (Mlichova and Rosenberg, 2006).

Microorganism	Production of enzyme	pH-optimum	Temperature ºC-optimum	References
Fungi ***Aspergillus niger***	Extracellular	3.0-4.0	55-60	(Greenberg and Mahoney, 1981)
Aspergillus oryzae		4.5-5.0	50-55	(Boon et al., 2000, Tanaka et al., 1975, Tanriseven and Dogan, 2002)
Yeasts **Kluyveromyces Lactis**	Intracellular	6.5-7.0	30-35	(Boon et al., 2000, Roy and Gupta, 2003, Jurado et al., 2004)
Kluyveromyces fragilis		6.6	30-35	(Jurado et al., 2004, Boon et al., 2000)
Bacteria **Escherichia coli**	Intracellular	7.2	40	(Greenberg and Mahoney, 1981, Giacomini et al., 2001)
Bacillus circulans		6	65	(Boon et al., 2000)

Table 2.4: Properties of microbial β-galactosidases

2.4.2. β-galactosidase Assay

The hydrolysing activity of β- galactosidase was determined by measuring the release of *o*-nitrophenol (ONP) from *o*-nitrophenyl β-D-galactopyranoside (ONPG) at 450 nm. One unit of enzyme β-galactosidase from fungi *Aspergillus oryzae* will hydrolyse 1 µmole of o-Nitrophenyl β-D-Galactoside to o-Nitrophenol and D-galactose per minute at pH 4.5-5 at 37 °C (Kaur et al., 2007). For thermal stability, enzymes were incubated at the temperature range of 24°C-60°C for 20 minutes and optimum pH was studied by the same method in the pH range of 3.0-7.0. Physicochemical properties (pH and temperature) can play an important role on rate of products formation through β-galactosidase hydrolysis (Meera et al., 2013).



ONPG, which is the substrate, was hydrolysed by β-galactosidase into galactose and ortho-nitrophenol (ONP). Thus, yellow colour appeared, indicating the enzyme activity by means of using a colorimetric assay (at 450nm wavelength).

The reaction was carried out with continuous shaking in an assay volume of 2.0 ml containing 1 ml of 100 mM PBS buffer, pH 4.5, 0.1 ml *β*-galactosidase (2 U), and 0.9 ml of reaction mixture (5.6 ml 0.1 M PO_4 pH 5.5 + 1.4 ml of 20 mM ONPG). The reaction was stopped after 20 minutes by adding 1 ml potassium carbonate solution (4% K_2CO_3) and *o*- nitrophenol (ONP) formation was measured spectrophotometrically at 450 nm (Ansari and Husain, 2012). The activity of β-galactosidase was determined at different temperatures (24, 37, 45 and 60 ºC at pH 4.5 using ONPG as the substrate. The reaction mixture contained 20 mM phosphate buffer at pH (5.5) and 1.6 mM ONPG (Mlichova and Rosenberg, 2006). One unit of β-galactosidase activity (EU) was determined as the amount of enzyme hydrolysing one micromole of the ONPG to o-nitrophenol and D-galactose per minute under different conditions as stated above (Jin et al., 2015). The effective pH for the activity of β-galactosidase from *Aspergillus oryzae* is 4.5-6.0 and the optimum temperature is up to 55 °C (Ansari and Husain, 2012).

Test Tube	PBS (ml)	Rxn Mix (ml)	Enzyme (µl)	Final Volume (ml)
1 (Blank)	1	1	0	2
2	0.975	1	25	2
3	0.95	1	50	2
4	0.90	1	100	2
5	0.80	1	200	2
6	0.70	1	300	2

Table 2.5: Test tubes containing enzyme and substrates for β-galactosidase enzyme assay

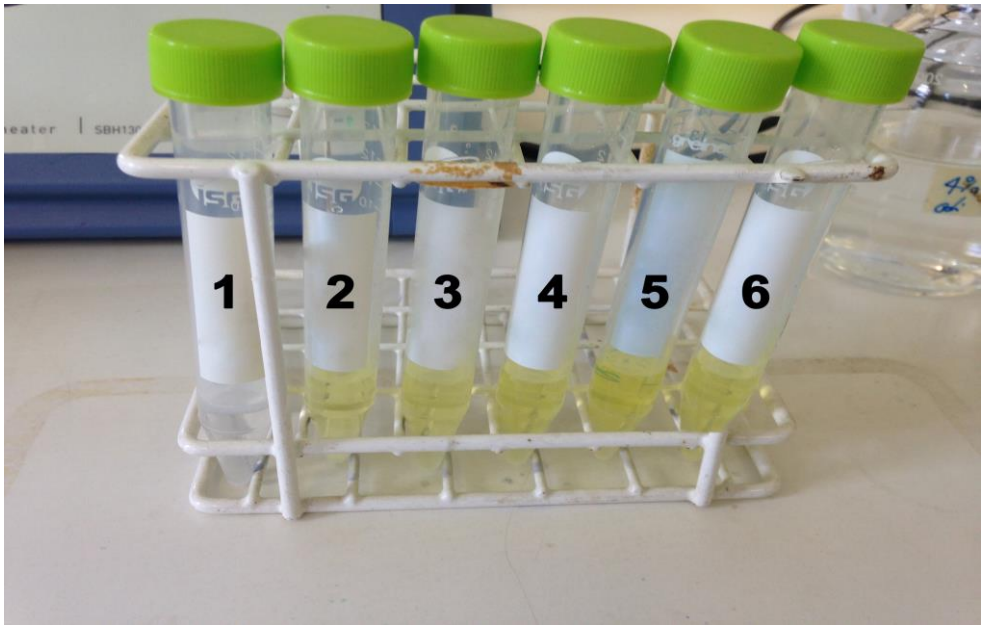


Figure 2.6: Picture taken from 6 test tubes representing β-galactosidase assay after a noticeable yellow colour developed

Results were presented as means ± standard deviations (SD) for three replicates for each sample. The buffers used were glycine-HCl (pH 3.0), sodium acetate (pH 4.0, 4.5, and 5.0), and sodium phosphate (6.0-7.0). The standard calibration curve of ONP versus OD absorbance (450nm) was demonstrated in order to calculate the concentration of formed ONP (µmol/ml/min) products by hydrolysis of ONPG with β-galactosidase from *Aspergillus oryzae.*

2.4.3. Enzyme hydrolysis

One millilitre of purified quercetin 3,4′-diglucoside or methanol extract of onions was dried under vacuum at 45 ºC. One millilitre of β-galactosidase from *Aspergillus oryzae* solution (2 mg/ml) was added and the solution was kept at 45 ºC for 2 hours to achieve complete hydrolysis. The hydrolysate was suspended in 1 ml 80% methanol, and analysed by the analytical HPLC system. Completion of hydrolysis was confirmed by disappearance of quercetin glucosides peaks in the chromatogram.

2.5 Methods (HPLC)

2.5.1. Identification and quantification of flavonoid in onions

High performance liquid chromatography (HPLC) is used widely and is a powerful technique in separation and analysis of various natural compounds such as flavonoids (aglycones and glycosides). Its advantages include simplicity, sensitivity, specificity, high resolution, speed, precision, good reproducibility, recovery, accuracy and sample preservation (Amorim et al., 2014). This technique also allows simultaneous separation and quantification of flavonoids. To obtain adequate resolution, a wide range of stationary and mobile phase combinations have been used for the separation of flavonoids in a mixture. Silica gel columns are used in normal phases for appropriate separation of nonpolar or weakly polar flavonoid aglycones. Usually no gradient is employed and different classes of flavonoids in plants are separated by using reversed phase HPLC (RP-HPLC) (Lombard et al., 2002). The common HPLC instrument includes C18 column, a solvent delivery system, sample injection, detector and recorder. The column is selected based on the class of flavonoids to be separated and the characteristics of the stationary phase that is capable of receiving satisfactory retention, peak shapes and selectivity. Guard columns are used to extend the life time and to protect the analytical column from sample impurities (Amorim et al., 2014).

HPLC was used to identify and quantify the quercetin aglycone and main quercetin glucosides in onions, and this technique was then used to study the effects of β-galactosidase enzyme on the hydrolysis of quercetin glucosides to free quercetin aglycone. HPLC (Model 200, Perkin Elmer) at wavelength = 370 nm was used. HPLC includes pumps, which pass pressurised liquid and a sample mixture through a column, leading to the separation of sample components. Solvents A (water-tetrahydrofuran-trifluoroacetic acid 98:2:0.1 v/v) and B (acetonitrile) were run at a flow rate of 1 ml/min, using a gradient of 17% B (2 min), increasing to 25% B (5 min), 35% B (8 min), 50% B (5 min) and then to 100% B (5 min). A column clean-up stage maintained B at 100% (5 min) followed by a re-equilibration 17% B (15 min). Diode array detection monitored the eluent at 370 nm for 25 minutes (Lombard et al., 2006). The appropriate flavonoid glycoside for each enzyme analysis was used as an external standard.

The product in each of the reactions will be confirmed by co-elution of peak with standard compounds and by matching UV spectra (Day et al., 2000c). Identification of quercetin glucosides in onion will be determined by comparing the relative position of the peaks in

onion extract with standards. Moreover, 0.1% is the maximum concentration of DMSO, which was used as the solvent for each of these flavonoids for enzyme assay and HPLC analysis.

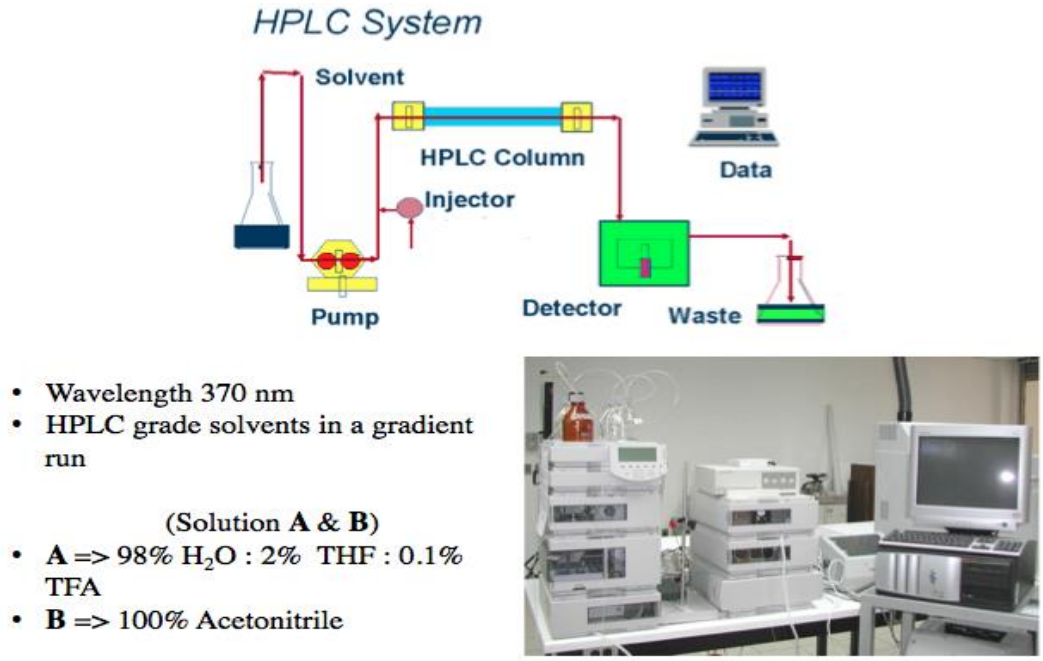


Figure 2.7: Demonstration of RP-HPLC system.

2.5.2. Identification and quantification of flavonoids

Identical standard curves of flavonoids quercetin 3′-glucoside, quercetin 4′-glucoside and quercetin 3,4′-diglucoside, were made for quercetin quantification by HPLC methods. Absorbance values and peak areas (RP-HPLC) for each level of the standard curve were recorded every 25 minutes for calibration purposes. Quercetin dihydrate was also used as standards for identification of other flavonol compounds (quercetin glucosides) during HPLC analysis.

Concentrations of quercetin conjugates in red onion extract were calculated by linear regression onto the standard curve of known samples. The relationship of peak area versus concentration for HPLC analyses were used to express results. The standard curve of quercetin and quercetin 4′-glucoside, quercetin 3,4′-diglucoside in 80% methanol, ranged between 0 and 50 µg/ml. Data analysis was performed in Excel using general linear model and correlation procedure.

For quantification of quercetin 4′-glucoside and quercetin 3,4′-diglucoside, paired series of quercetin glucosides in methanol and 1 millilitre of *Aspergillus oryzae* β-galactosidase solution (2 mg/ml) was added for enzyme hydrolysis. A total of 25% w/v onion extract in 80% methanol and onion solution boiled in water for 15 minutes were used, which showed differences in the levels of individual quercetin and quercetin glucosides in the onion extract during enzyme hydrolysis monitored over time. One millilitre of onion extract was mixed with 1 ml of β-galactosidase solution and incubated at pH 4.5 and 45 ºC for 20, 40 and 60 minutes. After the incubation, samples were placed on ice to stop the enzyme activity (Yoo et al., 2010).

2.5.3. Statistical analysis (Flavonoids quantification-HPLC)

Results are presented as means ± standard deviations (SD) for three replicates for each sample. In chromatographic assays, each replicate solution was injected two or three times, and the averaged peak areas were used to calculate analyte concentrations. Differences between mean values were assessed by Student's t-test with a significance level of $p < 0.05$. The calibration curve was constructed by plotting the measurements of mean absorbance versus the concentration of the standard solutions. The results were analysed by linear regression and the correlation coefficient (R^2) was calculated (GraphPad Prism 6.01 from GraphPad Software, San Diego USA). One-way ANOVA was calculated to compare the replicates of the calibration curve. All statistical calculations were performed using GraphPad Prism 6.01, V2.01, Version Date: 19/10/2012, GraphPad Software, San Diego USA).

2.6. Results

2.6.1. Caco-2 cells morphology

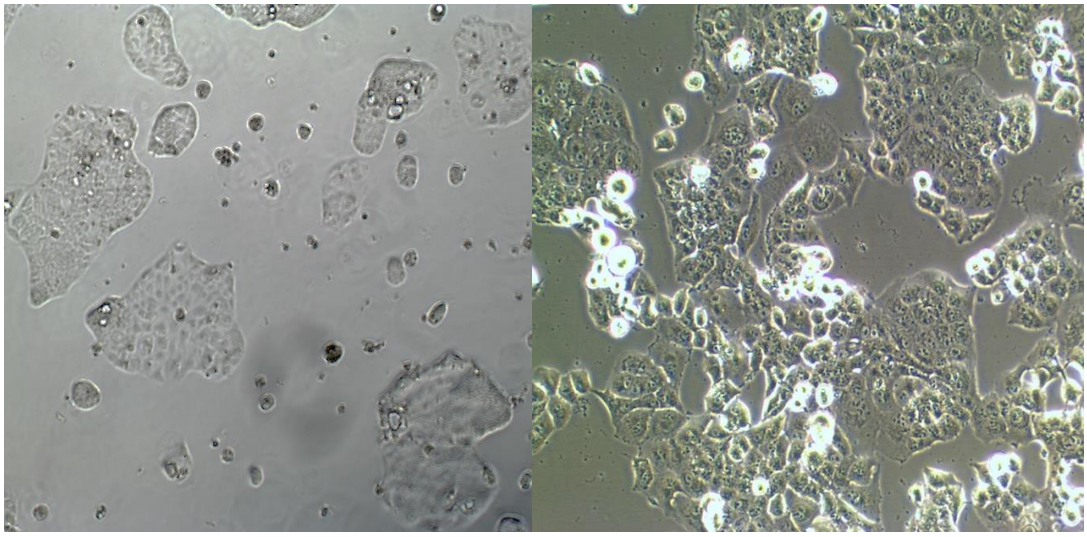


Figure 2.8: 20-50% Non-confluent Caco-2 cells (10x and 20x magnification)

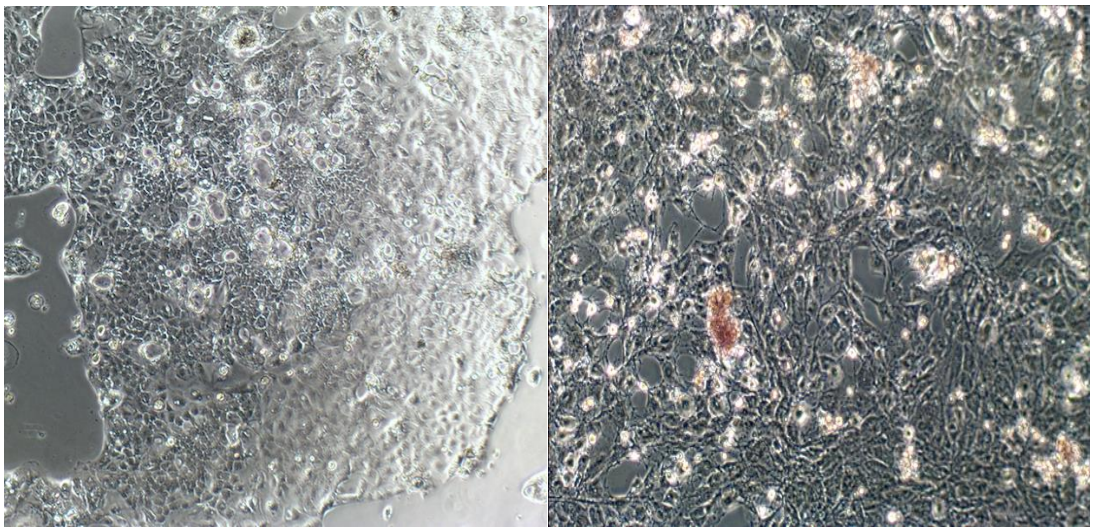


Figure 2.9: 80-90% confluent Caco-2 cells (30x and 40x magnification)

Trypsinisation (passage) of the Caco-2 cells were required for detachment of the Caco-2 cells. For this, cells were washed twice with 5 ml phosphate buffered saline (PBS) which maintained pH and osmotic balance of the cells. Following this, cells were then treated with 2 ml of 1% trypsin for 40 seconds with vigorous taping on the sides of the flask to detach cells from the walls.

Floating cells were seen under the microscope at 10X magnification, and at this point, 8 ml of complete culture medium were added to the flask. Afterwards, 2 ml of floated cells was transferred to new T-75 flasks and 13 ml of fresh medium was added to make a final volume of 15 ml.

Cells were divided into 3 to 4 T-75 flasks and then checked every couple of days for their confluency; and their DMEM medium was changed, and thus kept in the cell culture incubator gassed with 5% CO_2 at 37 °C. According to figure 2.8, Caco-2 cells were less than 50% confluent, whereas figure 2.9 presents up to 90% confluency which was essential for cell passage stage. The growth of these cells involved formation of monolayers, which showed a cylindrical polarised morphology, with microvilli on the apical membrane, tight junction between adjacent cells and express small intestinal hydrolase enzyme activities on the apical membrane.

2.6.2. Glucose Uptake Assay

2.6.2.1. The Effect of DMSO and Methanol on Caco-2 cells- Dose Response Experiments

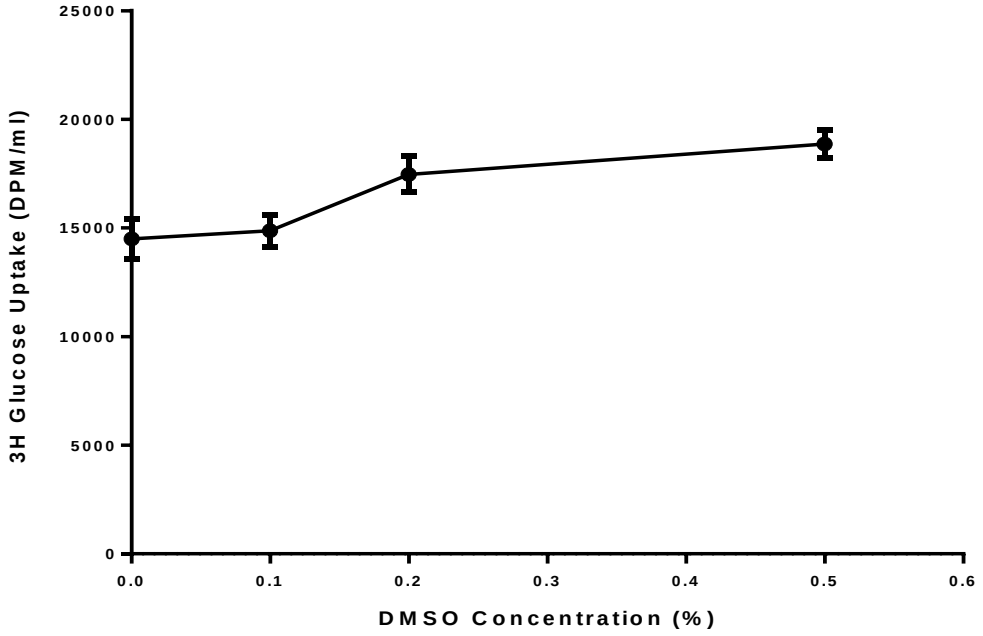


Figure 2.10: Effect of DMSO at different concentrations on Caco-2 cells glucose uptake under sodium-dependent condition within 2 minutes, results are expressed as mean ± SEM, n=3 with $p > 0.05$

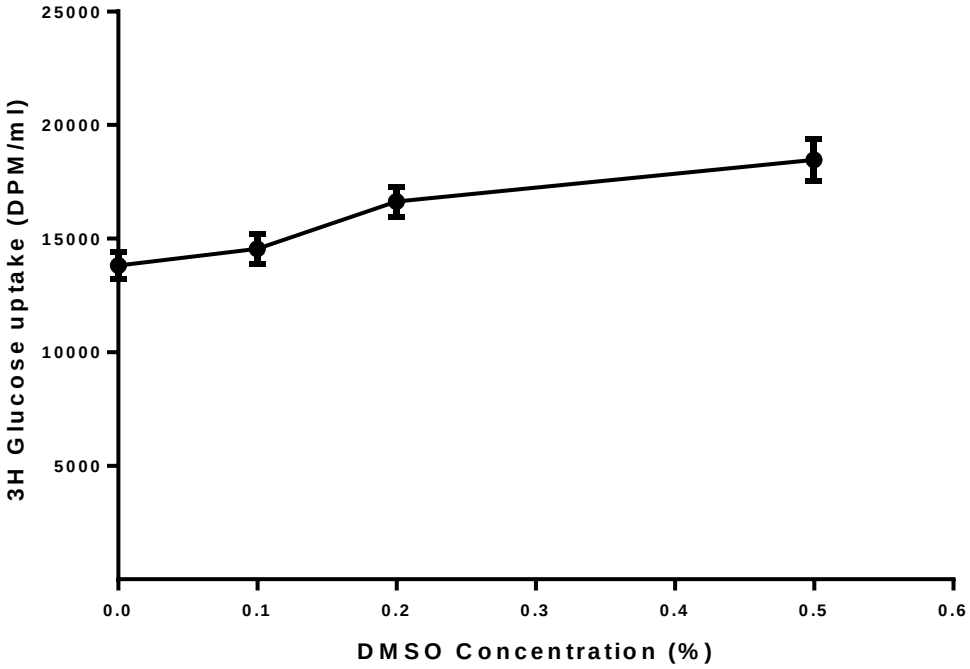


Figure 2.11: Effect of DMSO at different concentrations on Caco-2 cells glucose uptake under sodium-independent condition within 2 minutes, results are expressed as mean ± SEM, n=3 with $p > 0.05$

Both figures illustrate that 0.1% concentration of DMSO caused no effect on glucose uptake levels ($p > 0.05$) under sodium-dependent and sodium-independent conditions, thus 0.1% is the maximum concentration of DMSO to be used as the solvent for flavonoids.

Cytotoxicity of methanol was assessed on the Caco-2 cells by using different concentrations of methanol. 1% has been found as the maximum concentration mostly used in previous studies (Salucci et al., 2002).

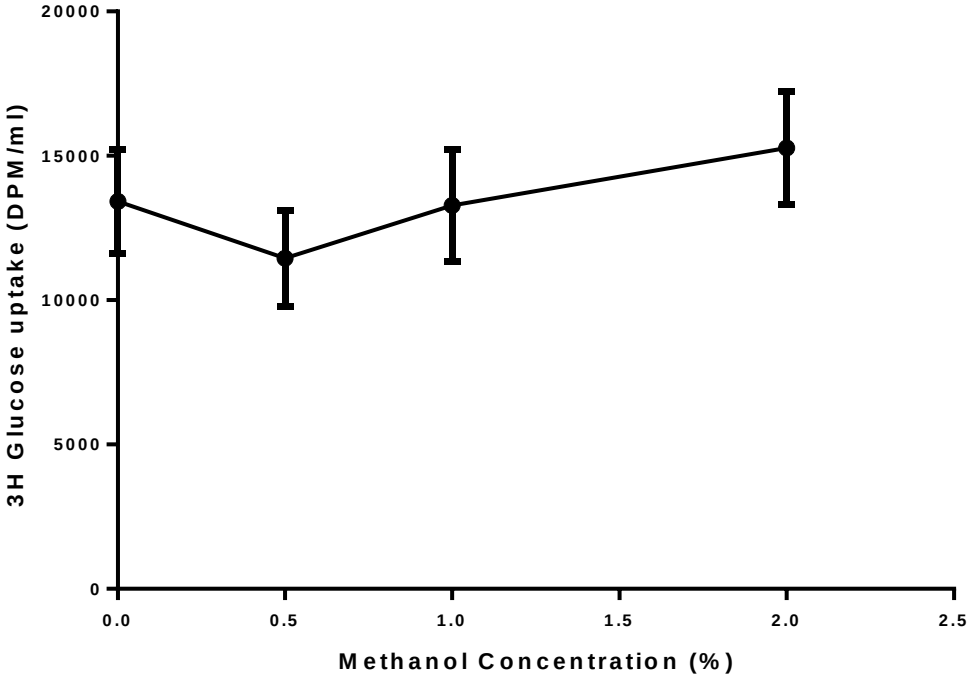


Figure 2.12: Effect of methanol at different concentrations on Caco-2 cells glucose uptake under sodium-dependent condition within 2 minutes, results are expressed as mean ± SEM, n=3 with $p > 0.05$

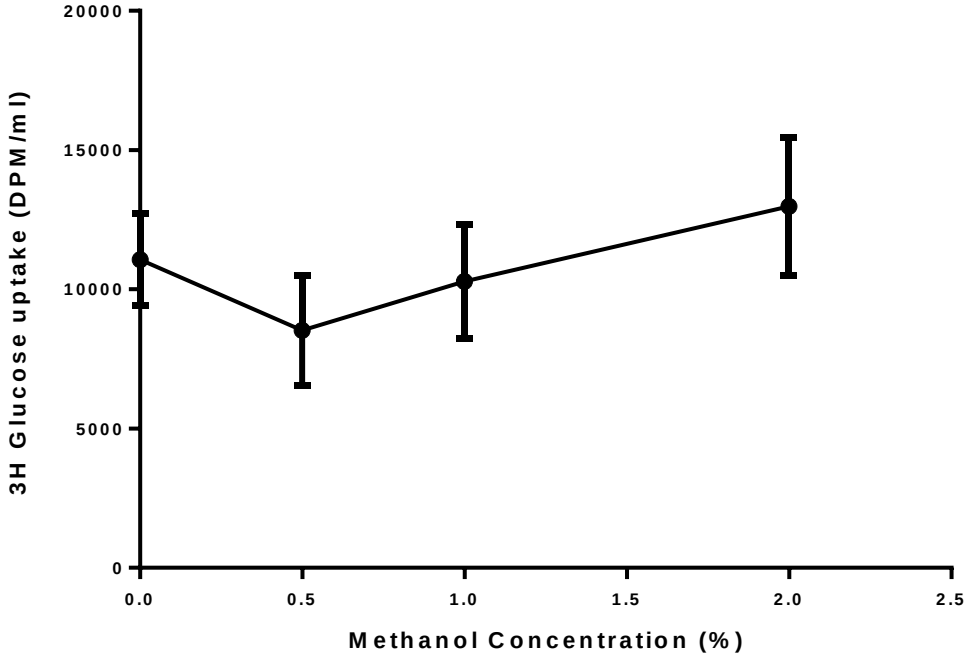


Figure 2.13: Effect of methanol at different concentrations on glucose uptake under sodium-independent condition within 2 minutes, results are expressed as mean ± SEM, n=3 with $p > 0.05$

Figure 2.12 and 2.13: compare different concentrations 0.5%, 1% and 2% of methanol to control in the presence and absence of sodium conditions respectively. According to

statistical t-test, it is shown that methanol at 1% concentration had p = 0.55 (p > 0.05), confirmed no statistical difference on the uptake of glucose at 1% concentration compared to the control. Therefore, 1% methanol caused no cellular toxicity and it was chosen as the top concentration of methanol in our experiments. Following this, 20µM, 50µM and 100µM phloridzin, quercetin 3-glucoside, quercetin 4′-glucoside and quercetin 3,4′-diglucoside were dissolved in DMSO, the final concentration of DMSO in treatment media was 0.1 w/v%.

In vitro studies were conducted to compare the inhibition of sodium-dependent and independent glucose uptake in Caco-2 cells by flavonoids in onion which are mainly phloridzin, quercetin 4′-glucoside and quercetin 3,4′-diglucoside (Day et al., 2000b). These experiments were conducted at different µM concentrations of flavonoids (aglycone and glycosides) and 25% w/v red onion extract, to measure the optimum glucose uptake in the presence and absence of sodium conditions.

2.6.2.2. Phloridzin as a positive control

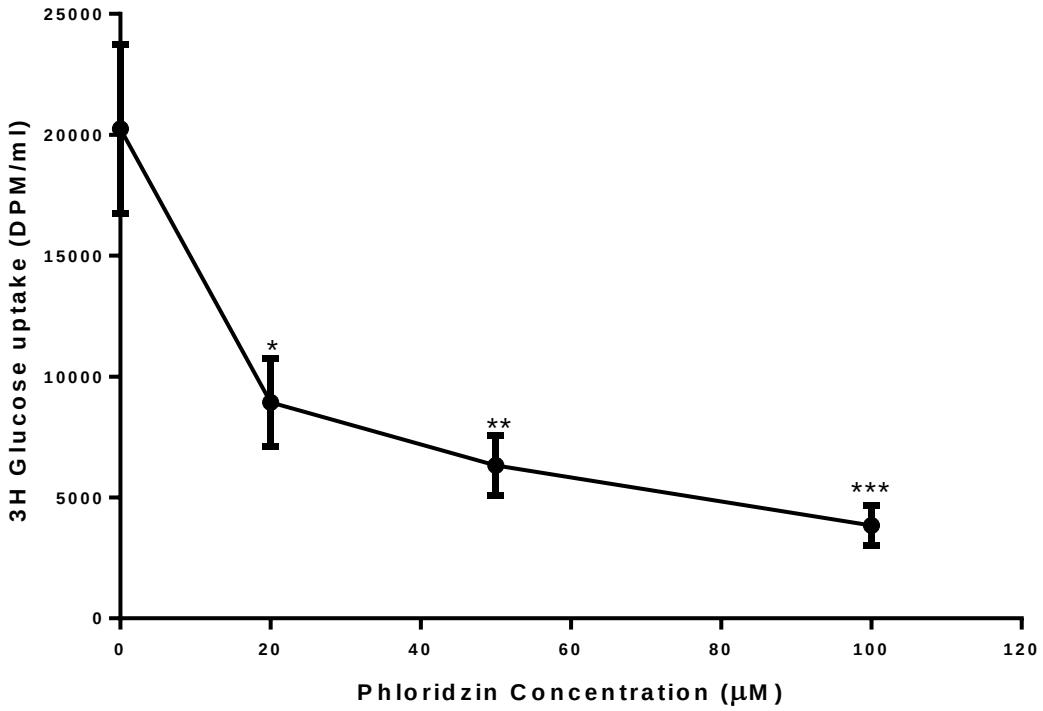


Figure 2.14: Glucose uptake in Caco-2 cells in the presence of phloridzin under sodium-dependent condition with n=3 ± SEM, *** p < 0.001, ** p < 0.01 and * p < 0.05

Figure 2.14 illustrates the significant inhibitory influence of phloridzin as a positive control on Caco-2 cellular glucose uptake under sodium dependent condition. There was a higher glucose inhibition as the concentration of phloridzin increased, showing up to 80% inhibition

of glucose at 100µM at significance level of $p < 0.0001$ ($p = 0.0004$) and 65% and 50% were determined at 50µM and 20 µM with $p < 0.05$ ($p = 0.01$) and $p < 0.05$ ($p = 0.045$) respectively.

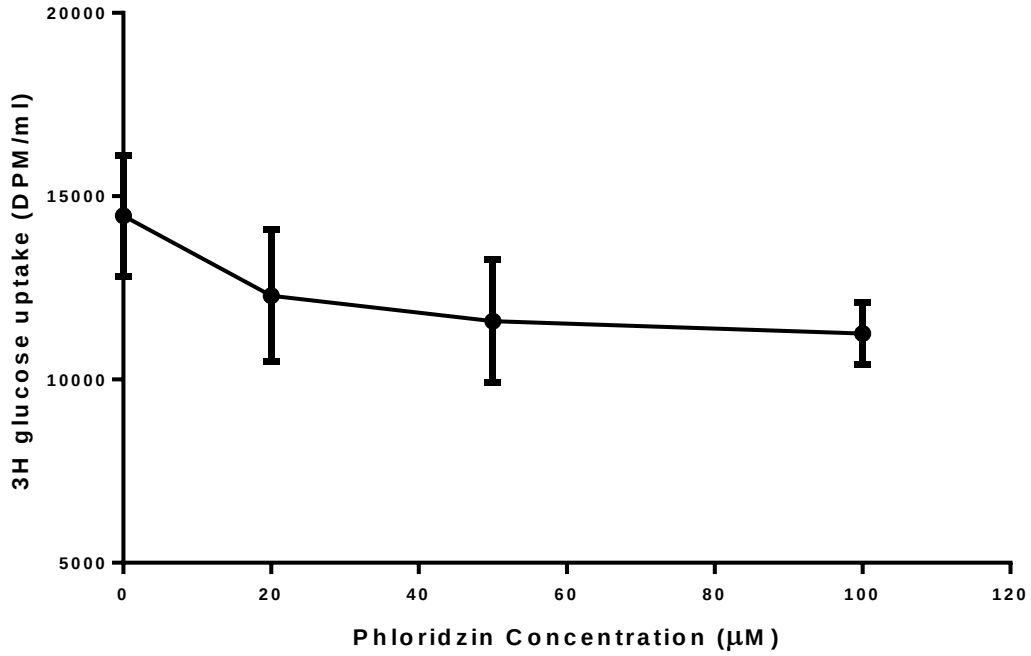


Figure 2.15: Glucose uptake in Caco-2 cells in the presence of phloridzin under sodium-independent condition with n=3 ± SEM, $p > 0.05$

Figure 2.15 shows under sodium-independent conditions, there was not a significant reduction in cellular glucose uptake when Caco-2 cells were treated with phloridzin. Even at the highest concentrations, 100µM only 30% inhibition was found at a statistical significant level of $p > 0.05$ ($p = 0.17$). Subsequently, lower levels, 50µM and 20µM, demonstrated 20% and 15% glucose inhibition with $p > 0.05$ respectively.

2.6.2.3. Effects of Flavonoids (glycosides and aglycone) on Caco-2 cells glucose uptake

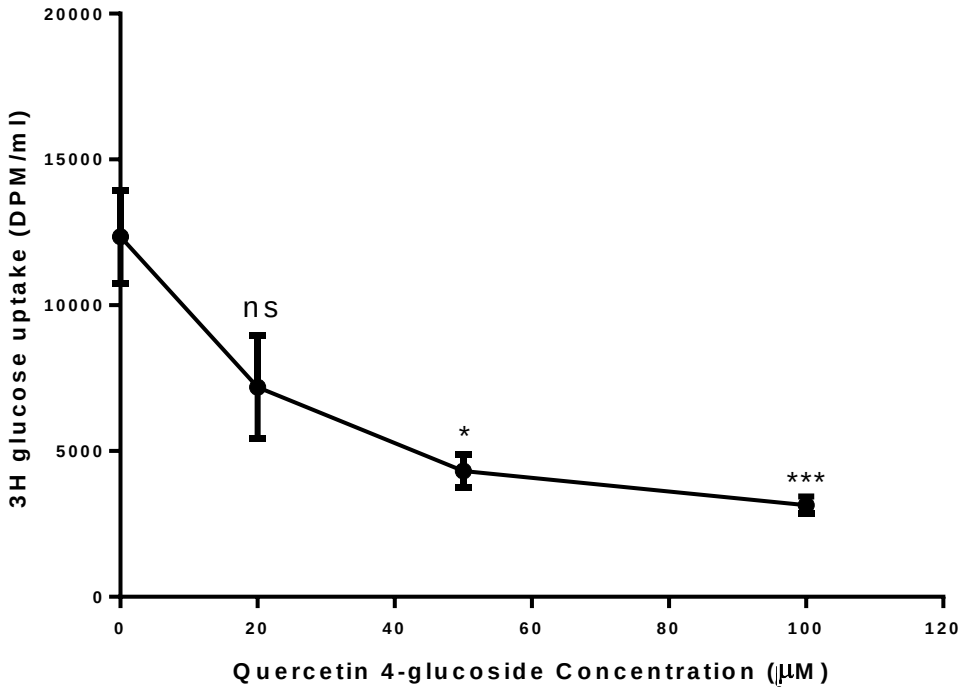


Figure 2.16: Glucose uptake in Caco-2 cells in the presence of quercetin 4′-glucoside under sodium-dependent condition with n=3 ± SEM, *** $p < 0.001$, * $p < 0.05$ and ns (not significant) = $p > 0.05$

Figure 2.16 illustrates that 100µM quercetin 4′-glucoside significantly reduced the uptake of glucose compared to control by 75% at significance level of ($p < 0.001$) with $p = 0.0007$ under sodium-dependent condition. This result demonstrated the concentration-dependent inhibitory effect of quercetin 4′-glucoside on Caco-2 cells glucose uptake with 65% and 42% reduction at 50µM and 20µM respectively. The glucose inhibition at 50µM was statistically significant with $p = 0.037$ ($p < 0.05$), while no statistical significance was found at 20 µM with $p = 0.3$ ($p > 0.5$).

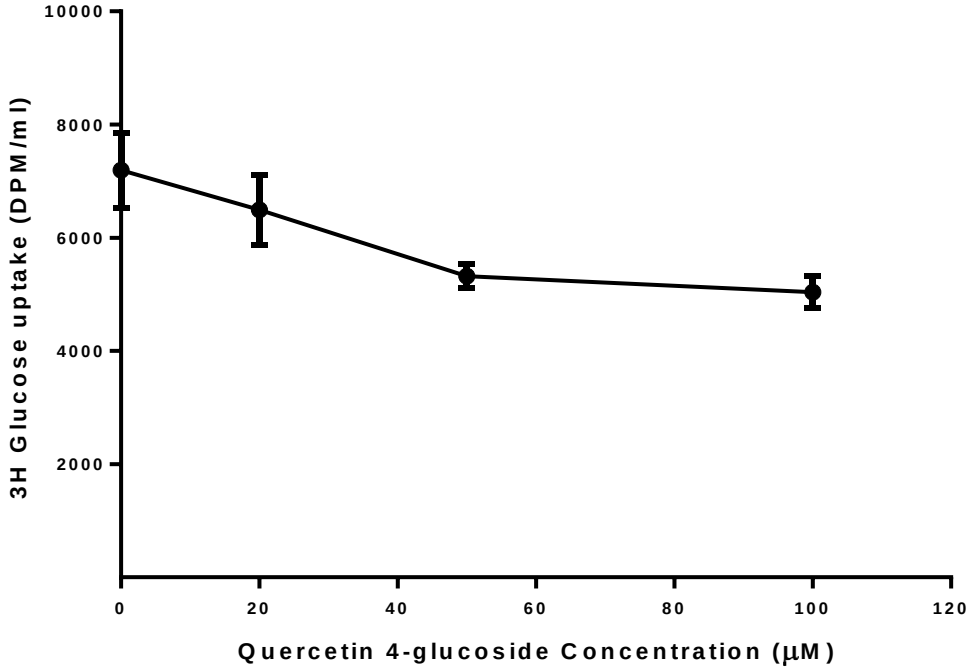


Figure 2.17: Glucose uptake in Caco-2 cells in the presence of quercetin 4′-glucoside under sodium-independent condition with n=3 ± SEM, $p > 0.05$

Figure 2.17 indicates that under sodium-independent condition, quercetin 4′-glucoside did not significantly inhibit the glucose uptake in Caco-2 cells, only 30% reduction was found at the presence of 100 µM quercetin 4′-glucoside with $p = 0.065$ ($p > 0.05$).

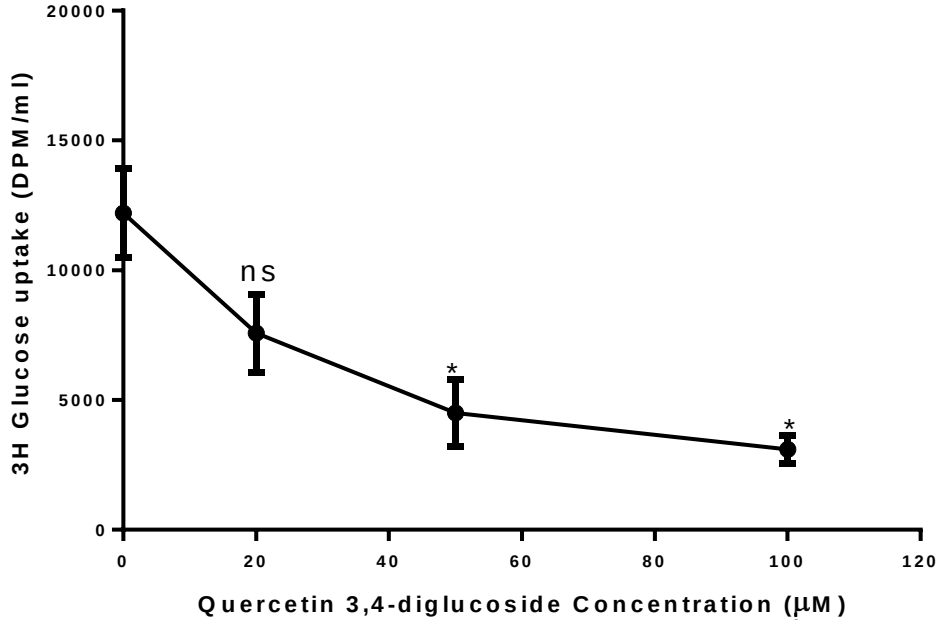


Figure 2.18: Glucose uptake in Caco-2 cells in the presence of quercetin 3,4′-diglucoside under sodium-dependent condition with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$

Figure 2.18 shows quercetin 3,4′-diglucoside at 50 µM and 100µM significantly reduced the glucose uptake by 64% and 75% at significance level of $P < 0.05$ ($p = 0.04$) and $p \leq 0.01$ ($p = 0.01$) respectively under sodium-dependent condition. Our finding suggests that quercetin 3,4′-diglucoside, similar to quercetin 4′-glucoside, compete with the glucose present at the SGLT-1 transporters at the apical membrane, and thus inhibits the uptake of glucose at sodium-dependent condition.

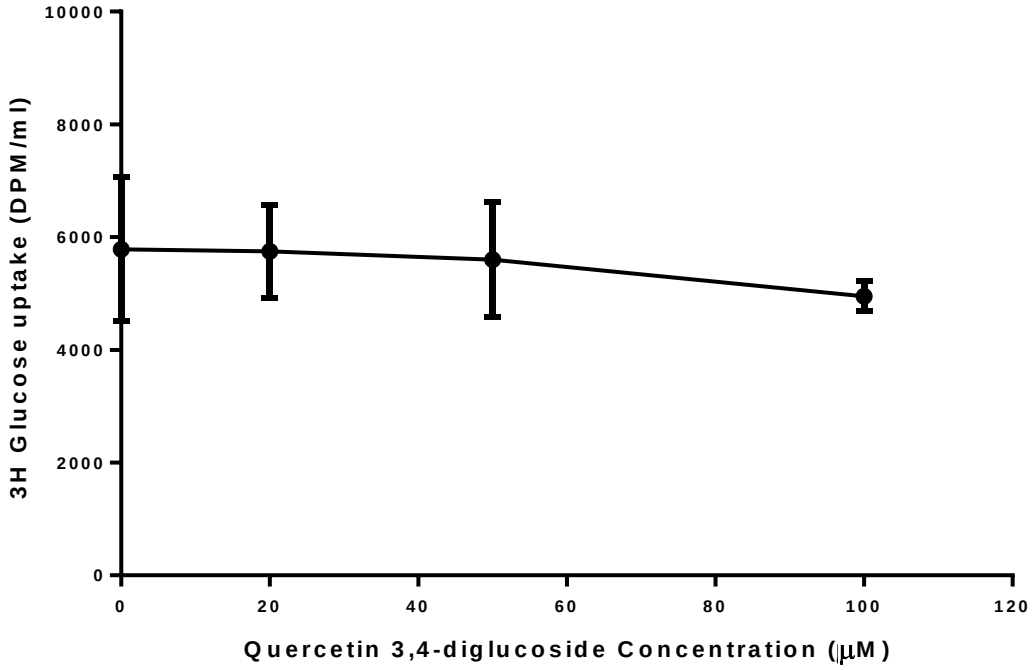


Figure 2.19: Glucose uptake in Caco-2 cells in the presence of quercetin 3,4′-diglucoside under sodium-independent condition with n=3 ± SEM, $p > 0.05$

Figure 2.19 shows under sodium-independent condition quercetin 3,4′-diglucoside caused only 5%-10% reduction in cellular glucose uptake ($p > 0.05$). It is suggested that SGLT-1 will be inactive in the sodium-independent conditions and the uptake of glucose is carried out by the GLUT-2 facilitative component.

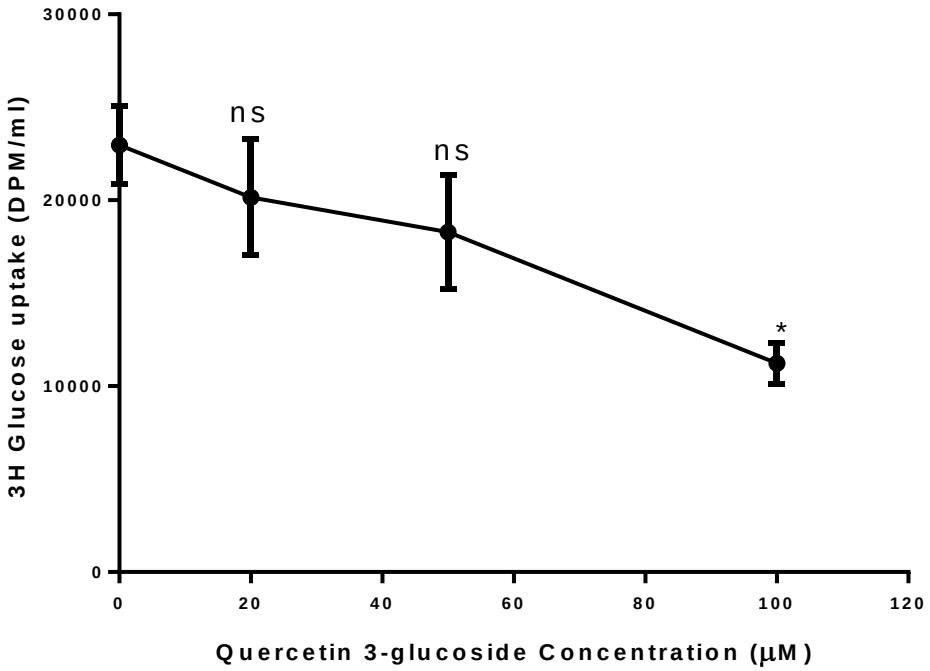


Figure 2.20: Glucose uptake in Caco-2 cells in the presence of quercetin 3-glucoside under sodium-dependent condition with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$

Figure 2.20 illustrates that 100µM of the compound is resulting in significant inhibition (52%) compared to 50µM (21%) and 20µM (13%) in the sodium dependent condition at significance level of $p < 0.05$, $p = 0.034$. Therefore, under the sodium-dependent condition, 100µM concentration of quercetin 3-glucoside caused the highest inhibition concentration compared to 50µM and 20µM; therefore, the higher the concentration, the more inhibition was determined.

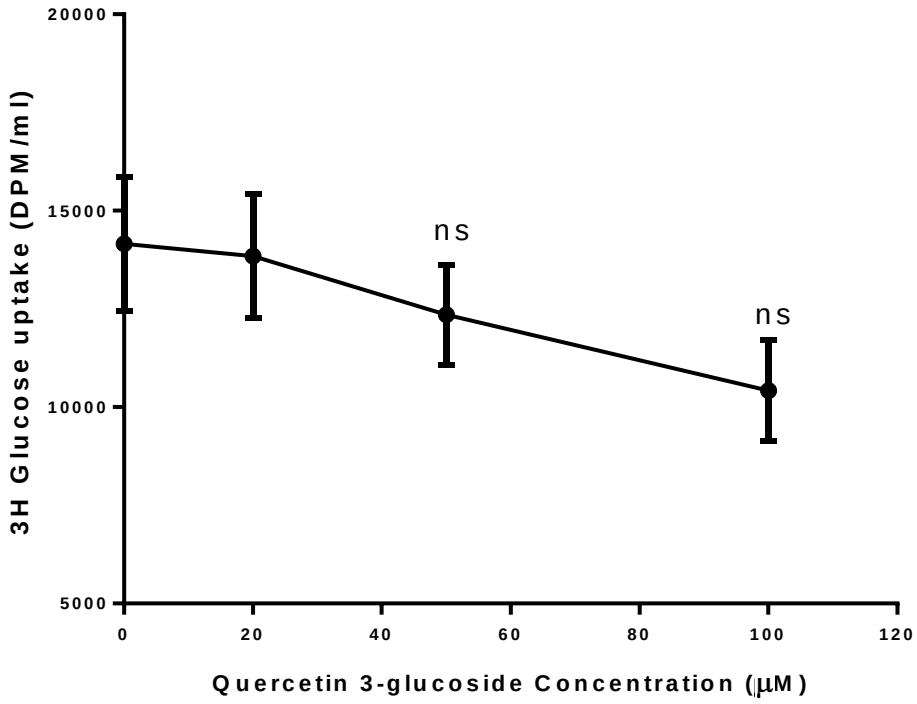


Figure 2.21: Glucose uptake in Caco-2 cells in the presence of quercetin 3-glucoside under sodium-independent condition with n=3 ± SEM, $p < 0.05$

Figure 2.21, illustrates a slight inhibitory effect in the sodium independent condition. At sodium free condition, the highest glucose inhibition was 20% at 100µM, which was not statistically significant with $p > 0.05$.

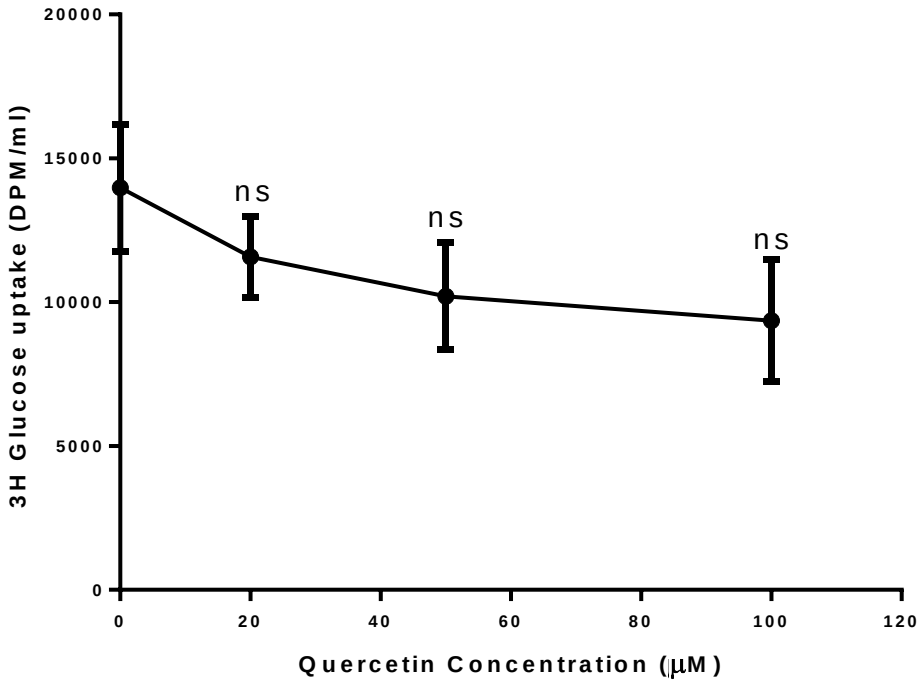


Figure 2.22: Glucose uptake in Caco-2 cells in the presence of quercetin under sodium-dependent condition with n=3 ± SEM, $p > 0.05$

Figure 2.22 shows quercetin aglycone caused no statistically significant inhibition on the cellular glucose uptake under sodium-dependent condition with $p > 0.05$ ($p = 0.1$) at 100μM concentration. This result suggests that quercetin can possibly compete with glucose molecules at the GLUT-2 transporter; therefore, under sodium-dependent conditions, quercetin had no effect of the accumulation of glucose by Caco-2 cells compared to control. Thus, SGLT-1 was inactive and glucose was mediated solely by GLUT-2 facilitative component.

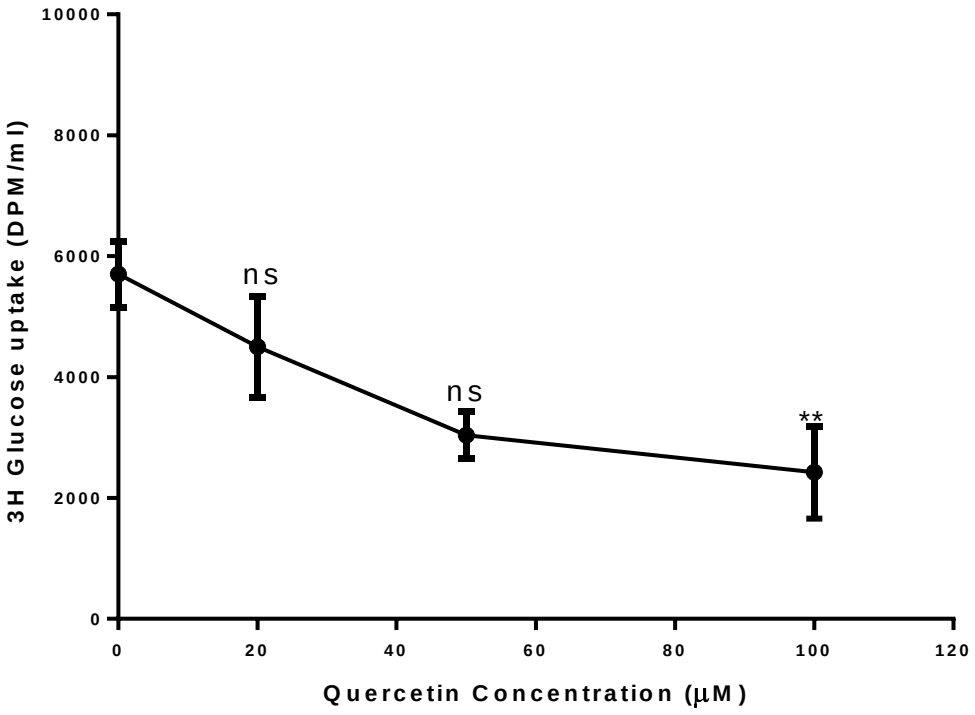


Figure 2.23: Glucose uptake in Caco-2 cells in the presence of quercetin under sodium-independent condition with n=3 ± SEM, ** $p < 0.01$ and $p > 0.05$

Figure 2.23 demonstrates a significant inhibitory effect of quercetin on glucose uptake in the sodium free condition at 100 µM with 58% reduction compared to control, with $p < 0.01$ ($p = 0.008$). Therefore, sodium-dependent glucose absorption appears to be rather specific to quercetin mono and diglucosides such as quercetin 4′-glucoside, quercetin 3,4′-diglucoside and quercetin 3-glucoside (figures 2.16, 2.18 and 2.20).

Since quercetin 4′-glucoside and quercetin 3,4′-diglucosides and quercetin 3-glucoside are the main flavonoids in onions, we were interested to conduct further experiments with the same conditions to demonstrate the effect of onion and these quercetin glucosides on Caco-2 cells glucose uptake.

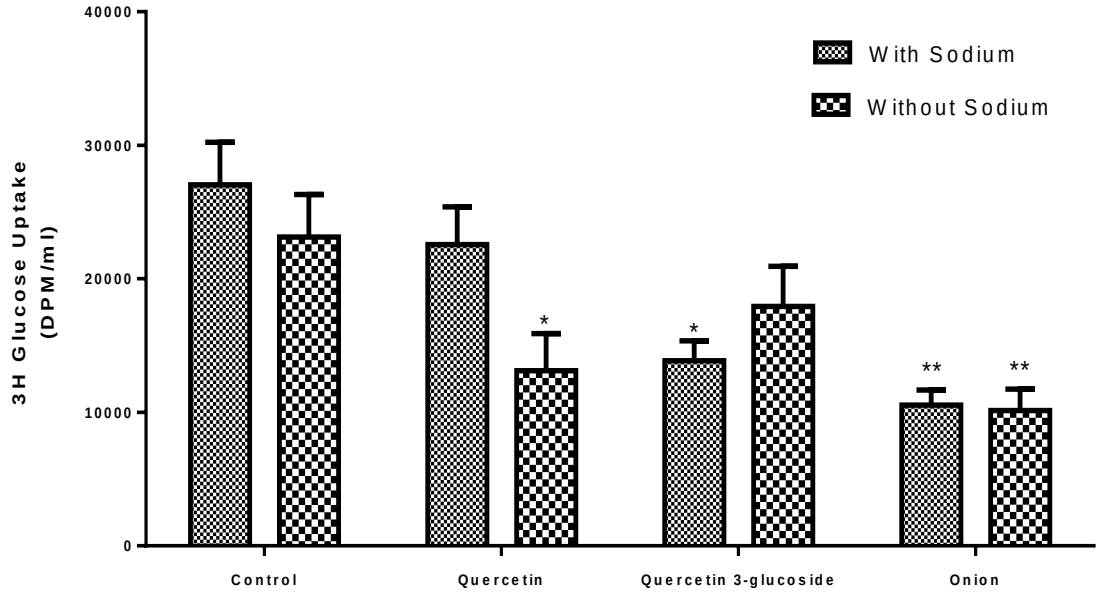


Figure 2.24: Glucose uptake in Caco-2 cells in the presence of 100 μM quercetin, 100 μM quercetin 3-glucoside and 25% (w/v) red onion extract under sodium-dependent and sodium-independent conditions with n=3 ± SEM, ** $p < 0.01$ and * $p < 0.05$, from two-way ANOVA multiple comparisons.

Figure 2.24 illustrates in the sodium dependent condition, there was a significant reduction of glucose levels in the presence of quercetin 3-glucoside and onion, with up to 42% and 55% inhibitory effect respectively. Therefore, this data illustrates that both may act as inhibitors of glucose in SGLT-1 and GLUT-2 transporters. However, in the absence of sodium, quercetin 3-glucoside did not significantly inhibit the glucose uptake compared to control 27% at significance levels with $p = 0.12$ (figure 2.24). 100 μM quercetin demonstrated a significant inhibitory effect in the sodium free condition with 56% reduction compared to control, and $p = 0.02$ ($p < 0.05$).

From our result, it is evident that quercetin 3-glucoside is most likely to cause inhibition of glucose uptake in the presence of sodium, while quercetin blocks glucose uptake in the absence of sodium which is most likely through GLUT-2. Our results suggest that inhibition of SGLT-1 by onion extract is mainly caused by quercetin glucosides, while GLUT-2 inhibition by onion extract could be caused by quercetin aglycone (Boyer, et al. 2005).

2.6.2.4. Effect of quercetin glucosides on glucose uptake at both Apical and Basolateral membrane

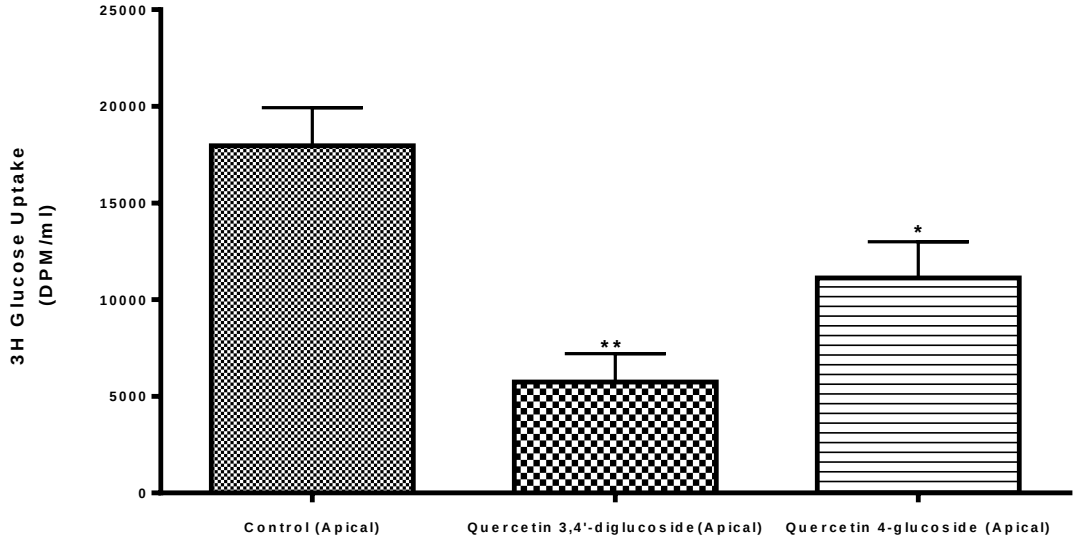


Figure 2.25: Glucose inhibition in Caco-2 cells in the presence of 100µM quercetin 4′-glucoside and 100µM quercetin 3,4′-diglucoside under sodium-dependent condition at apical side with n=3 ± SEM, * $p < 0.05$ and ** $p < 0.01$

Figure 2.25 shows under sodium dependent condition, there was a significant reduction in the cellular glucose apical uptake treated with 100µM quercetin 3,4′-diglucoside and 100µM quercetin 4′-glucoside, 70% and 60% with $p < 0.01$ and $p < 0.05$ respectively. The significant decrease in glucose levels by quercetin glucosides are possibly due to the competitive inhibition of sodium-dependent glucose transport across the intestinal brush-border membrane, which was subsequently mediated by sodium-dependent glucose transporter SGLT-1.

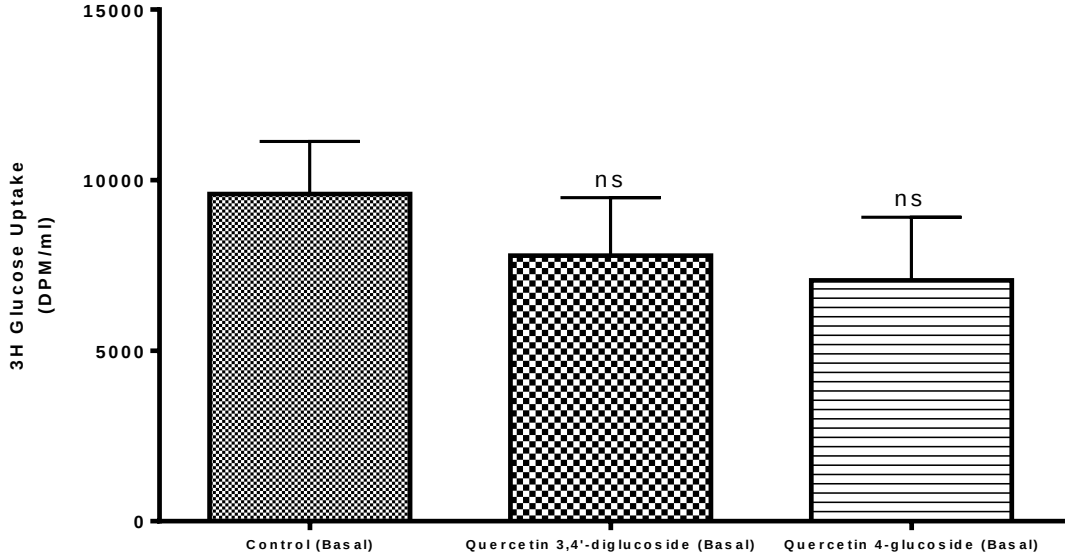


Figure 2.26: Glucose inhibition in Caco-2 cells in the presence of 100µM quercetin 4′-glucoside and 100µM quercetin 3,4'-diglucoside under sodium-dependent condition at the basolateral side with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$

Figure 2.26 illustrates under sodium dependent condition at the basal side, both quercetin 3,4′-diglucoside and quercetin 4′-glucoside showed a slight inhibitory effect on glucose transport; it was not statistically significant $p > 0.05$. Our result is in support of previous findings where quercetin glucosides were sodium-dependent; therefore, they played through SGLT-1 transporters available at the apical membrane of Caco-2 models (Johnston et al., 2002). Thus, our finding characterises that Caco-2 cells in transwell inserts represent the apical membrane of enterocytes where glucose is transported by SGLT-1 transporters.

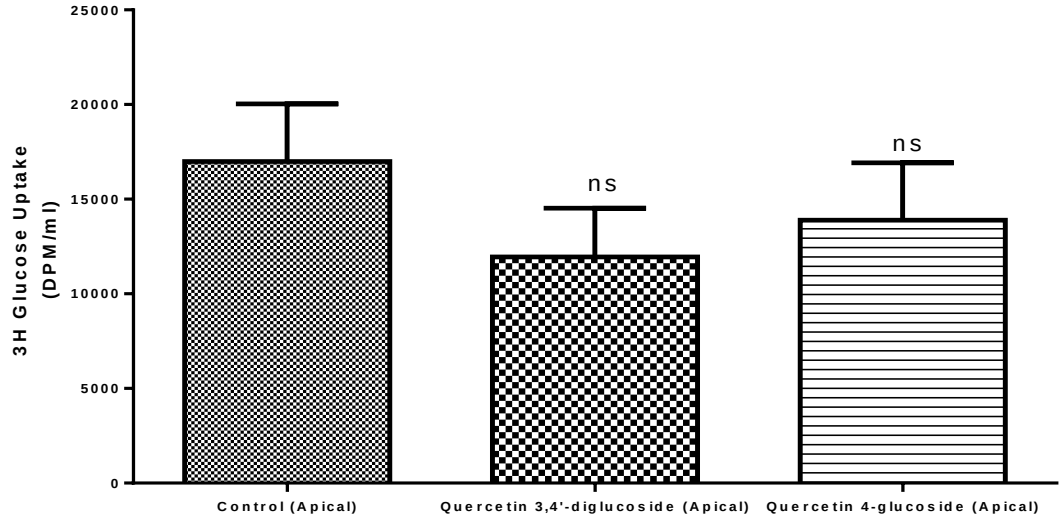


Figure 2.27: Glucose inhibition in Caco-2 cells in the presence of 100 µM quercetin 4′-glucoside and quercetin 3,4′-diglucoside under sodium-independent condition at apical side with n=3 ± SEM, $p > 0.05$

Figure 2.27 shows under sodium independent conditions, none of the quercetin glucosides reduced the transport of glucose at cellular levels with $p > 0.05$. This is in accordance with findings from (Lombard et al., 2002), where quercetin 4′-glucoside and quercetin 3,4′-diglucoside did not decrease glucose levels in Caco-2 cells, as they mostly function at sodium-dependent condition through SGLT-1 glucose transports.

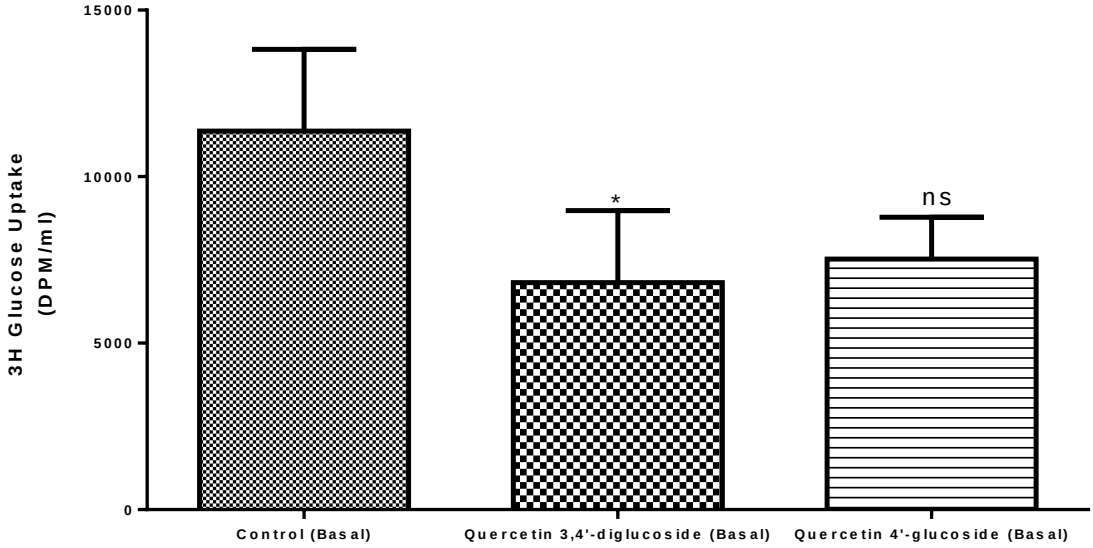


Figure 2.28: Glucose inhibition in Caco-2 cells in the presence of 100 µM quercetin 4'-glucoside and quercetin 3,4′-diglucoside under sodium-independent condition at the basolateral side with n=3 ± SEM, * $p < 0.05$ and $p > 0.0$ 5

Reduction of glucose uptake at the basolateral membrane of enterocytes, which in the current Caco-2 model was presented by the transport of glucose from the upper to lower side of transwell inserts (wells), may be related to inhibition of GLUT-2 by quercetin produced from the hydrolysis of quercetin glucoside by α-glucosidase. It is important to note that quercetin has been shown to inhibit GLUT-2, and transport of glucose from the gut to the blood stream requires not only luminal glucose uptake into enterocytes via SGLT-1, but also release from the basal membrane of enterocytes into the blood stream via GLUT-2 (Zheng et al., 2011).

2.6.2.5. Effect of Inulin and onion extract on Caco-2 cells glucose uptake

In addition to flavonoids, there is another important compound, such as Inulin, which belongs to a class of compounds known as Fructans. This compound is widely found in onions (Mcclearly and Murphy, 2000). Inulin is a soluble fibre, some soluble fibres may help lower blood glucose levels (Kaur and Gupta, 2002).

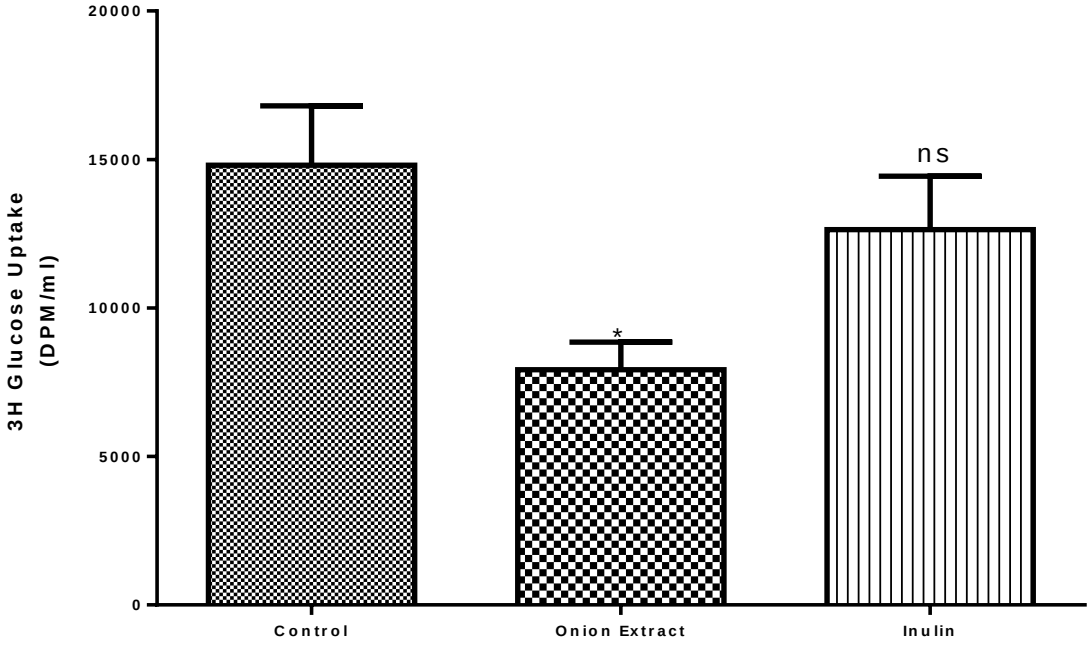


Figure 2.29: Glucose uptake in Caco-2 cells in the presence of 25% w/v onion extract and 5% w/v, inulin under sodium-dependent condition with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$

Figure 2.29 shows 5% w/v inulin did not result in a significant inhibition of glucose uptake compared with control, 15% reduction was found at sodium dependent condition with $p > 0.05$ ($p = 0.6$). However, again the onion extract significantly reduced glucose uptake with 47% inhibition $p < 0.05$ ($p = 0.01$). Our result indicates that the inhibitory effect of onion on lowering glucose levels could mainly be due to the presence of flavonoids. It is believed that more than 95% of the flavonoids in onion is quercetin, and its derivatives, 1-5% inulin (Perez-Gregorio et al., 2010). Therefore, inulin is suggested to have a lower anti-glycaemic effect in our Caco-2 cells models.

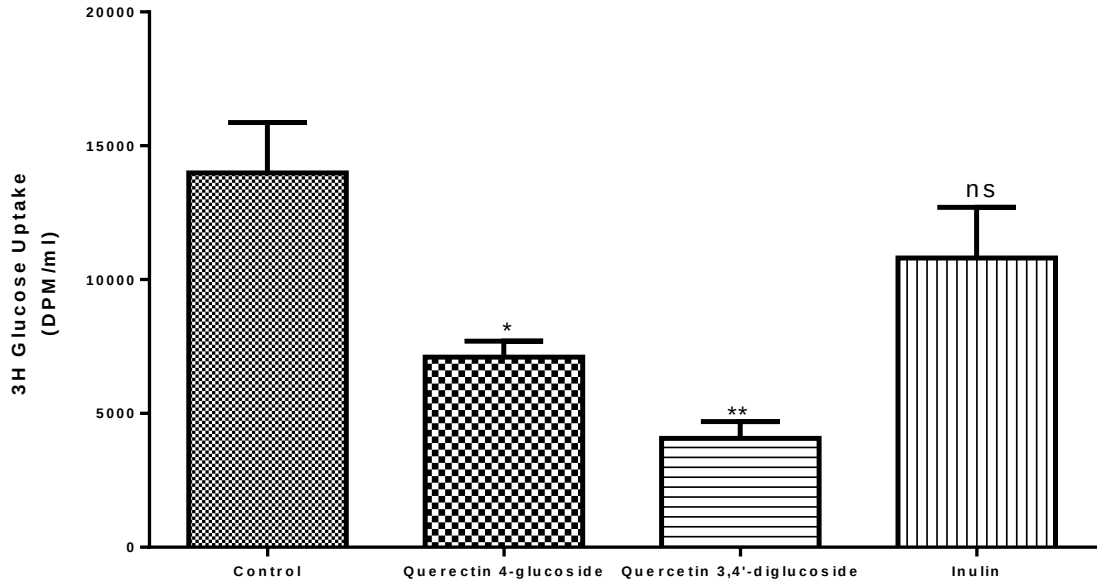


Figure 2.30: Glucose uptake in Caco-2 cells in the presence of quercetin 4′-glucoside, quercetin 3,4′-diglucoside and 5% w/v inulin under sodium-dependent conditions with n=3 ± SEM, * $p < 0.05$, ** $p < 0.01$ and $p > 0.05$

Quercetin 3,4′-diglucoside shows 70% reduction at Caco-2 glucose concentrations, followed by 50% reduction through quercetin 4′-glucoside with $P = 0.01$ and $p = 0.03$ $p < 0.05$ respectively. Whereas, no statistically significant inhibition was caused by inulin $p > 0.05$. Under a sodium-dependent condition, both quercetin 4′-glucoside and 3,4′-diglucoside caused a drastic reduction on glucose absorption, through the SGLT-1 transporter (figure 2.30).

Our result is in accordance with findings from most of the previous studies and our earlier findings; therefore, quercetin monoglucosides and quercetin diglucosides were found as the main inhibitors of glucose uptake through sodium-dependent conditions on the apical side (Figures 2.16, 2.18 and 2.20).

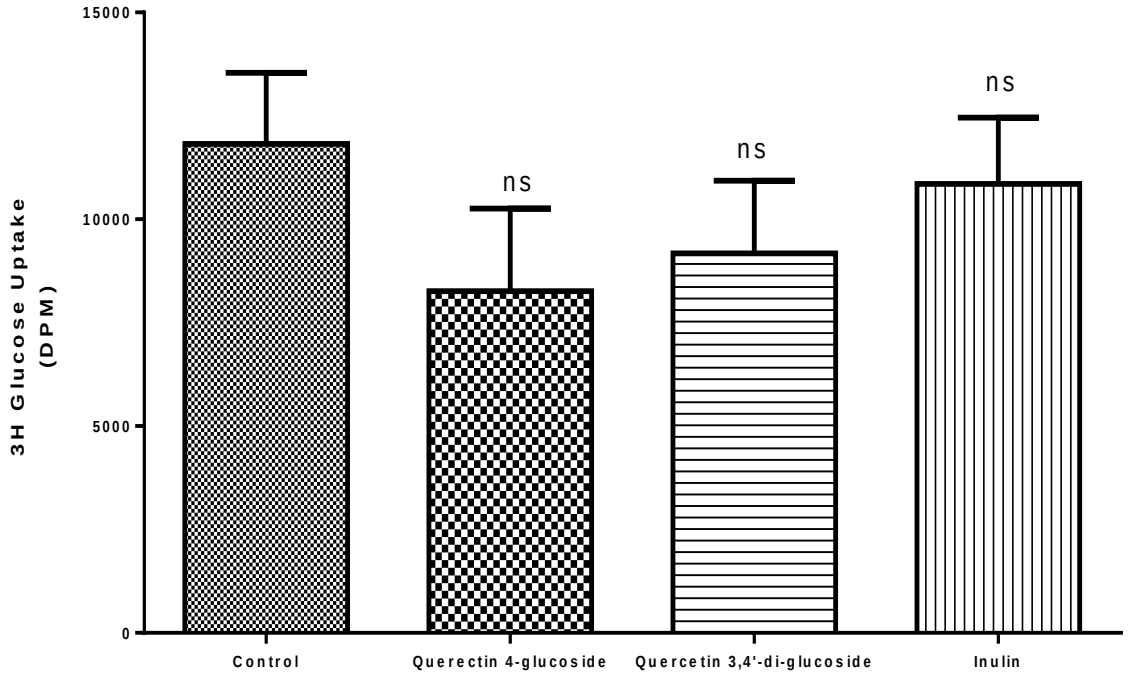


Figure 2.31: Glucose uptake in Caco-2 cells in the presence of 100 µM quercetin 4′-glucoside, 100 µM quercetin 3,4′-diglucoside and 5% w/v inulin under sodium-independent condition with n=3 ± SEM, * $p < 0.05$ and $p > 0.05$

Comparing the effect of quercetin 4′-glucoside, quercetin 3,4′-diglucoside and inulin with control on Caco-2 cells glucose uptake, Inulin showed a non-significant glucose uptake inhibition 22% and 10% at both sodium dependent and independent conditions with $p > 0.05$ (Figure 2.30 & 2.31). Also, none of the quercetin glucosides reduce the glucose uptake at a significant level in the absence of sodium condition, since they found to be mostly active at sodium-dependent conditions (figure 2.30). Therefore, our result proposes that inhibition in glucose uptake by onion extract is mainly by flavonoids quercetin 4′-glucoside and quercetin 3,4′-diglucoside, not by inulin. It is important to note that in our research we used inulin from chicory (not onion); therefore, our result is not defining a consistent effect of inulin (from chicory not onion) on Caco-2 cells glucose uptake under the same conditions compared with onion extract and its flavonoid glycosides.

2.7. Validation of HPLC -Limit of Detection

A validation study was carried out to demonstrate the applicability of this analytical approach. Therefore, limit of detection (LOD) of several samples were detected. LOD of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified (Tuszynska, 2014). Based on signal-to-noise approach, determination of signal-to-noise ratio was performed by comparing measured signal from quercetin 4′-glucoside and quercetin 3,4′-diglucoside with 1ng/ml and 2ng/ml concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise-ratio between 3 or 2:1 is considered acceptable for estimating detection limit (Chen et al., 2012). The test samples with signal-to-noise-ratio rates are shown as below.

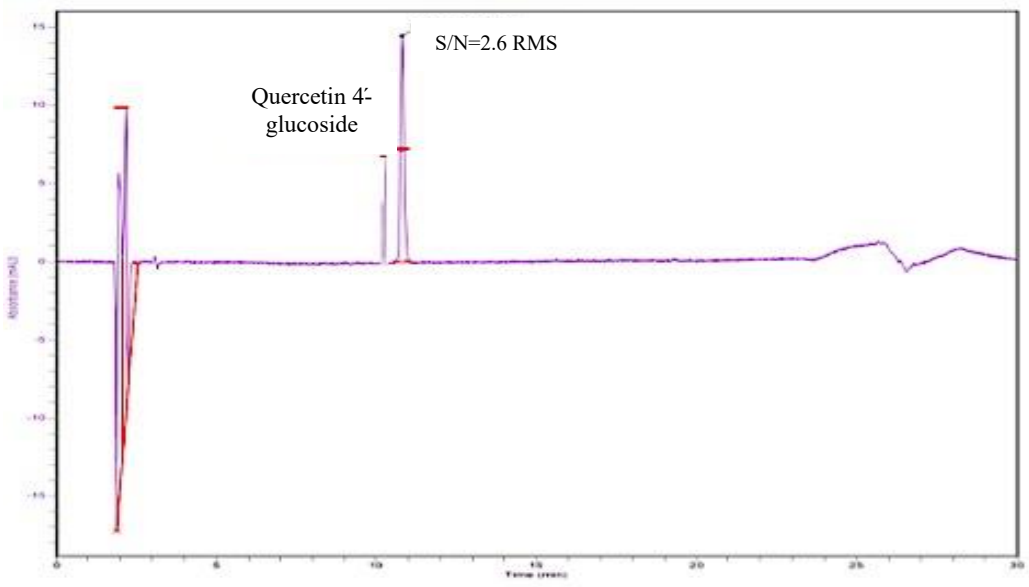


Figure 2.32: Limit of detection (LOD) of 1ng/ml of quercetin 4′-glucoside in 80% v/v methanol (RT= 11.23 min)

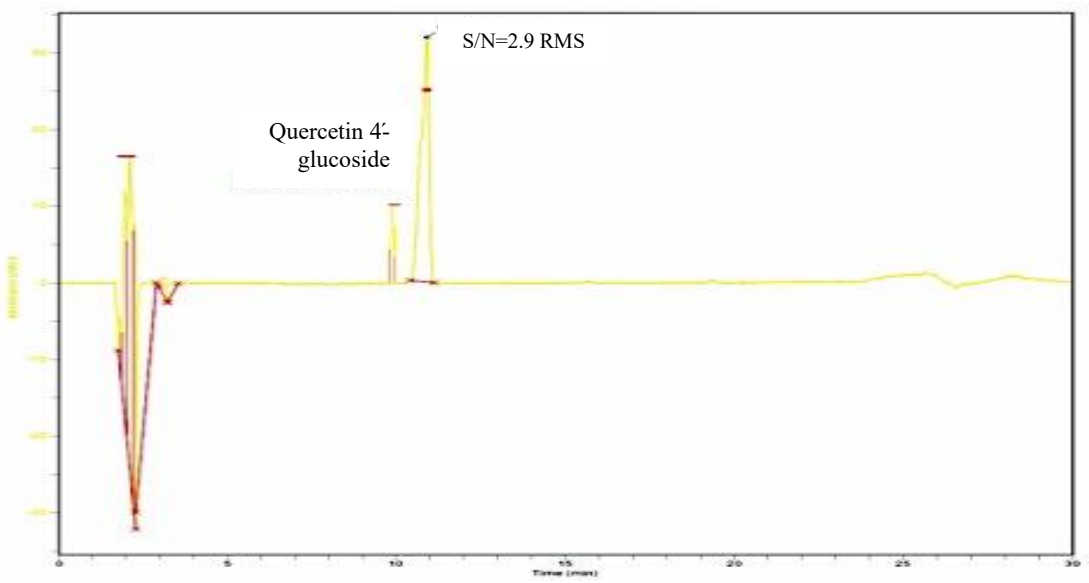


Figure 2.33: Limit of detection (LOD) of 2ng/ml of quercetin-4′glucoside in 80% v/v methanol (RT= 11.34 min)

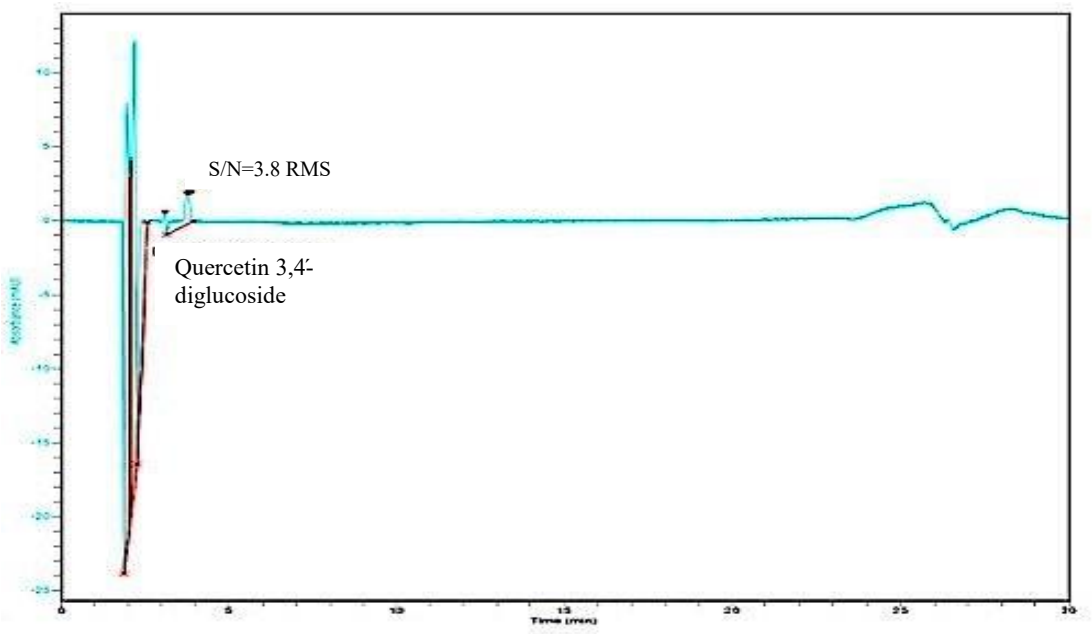


Figure 2.34: Limit of detection (LOD) of 1ng/ml of quercetin 3,4′-diglucoside in 80% v/v methanol (RT=3.75 min)

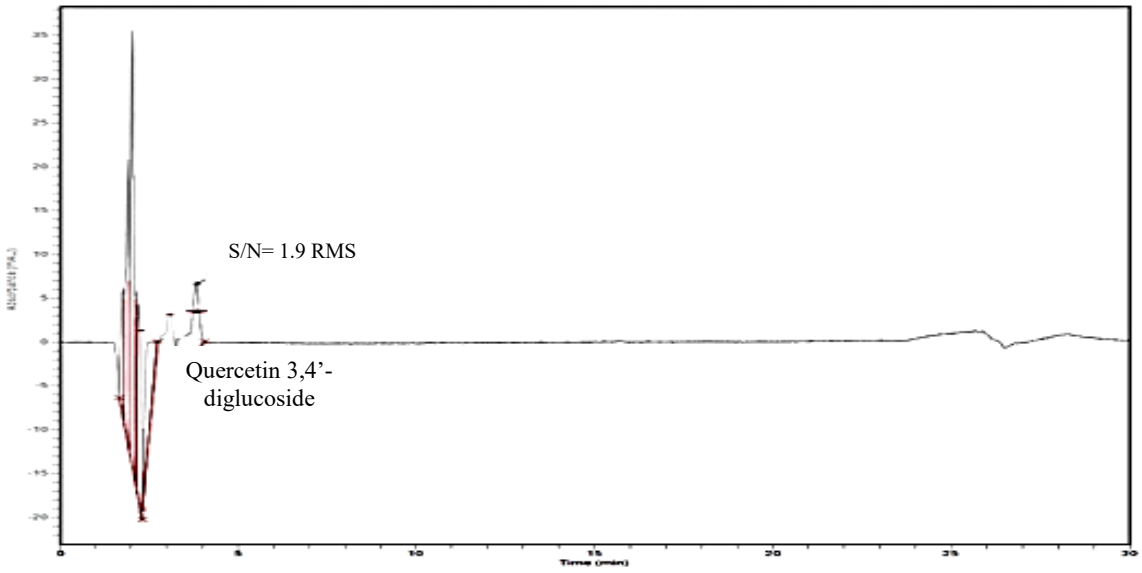


Figure 2.35: Limit of detection (LOD) of 2 ng/ml of quercetin 3,4′-diglucoside in 80% v/v methanol (RT= 4 min)

According to figure 2.32-2.35, no other significant peaks with a signal-to-noise of three or more were observed at specific retention times of quercetin 4′-glucoside and quercetin 3,4'-diglucoside. LOD was detected at 1ng/ml and 2 ng/ml of quercetin 4′-glucoside with UV-chromatogram at RT= 11.23 and 11.34 min, which showed 2.6 and 2.9 times more signal than the noise at RT= 10.78 and 10.88 min respectively. Moreover, 3,4′-diglucoside at 1ng/ml and 2ng/ml with UV-chromatogram at RT=3.75 and 4 min, demonstrated 3.8 and 1.9 times more signal than the noise at 3.05 to 3.15 min.

According to a signal-to-noise approach, detection of signal-to-noise ratio was evaluated through comparison of measured signal from quercetin 4′-glucoside and quercetin 3,4′-diglucoside with 1ng/ml and 2ng/ml concentrations with those of blank samples. Both quercetin 4′-glucoside and quercetin 3,4′-diglucoside showed a signal-to-noise-ratio between 2 to 3, which indicated 1ng/ml or 2ng/ml are accepted as their limits of detection (Chen et al., 2012).

2.8. Identification of flavonoids in onion by RP-HPLC-DAD

HPLC-DAD was used to identify components by the comparison of their spectrum. The spectrum was recorded from 200-600nm; therefore, flavonoids were identified by maximum UV absorption wavelength. In the current research based on the spectral analysis from RP-HPLC DAD, the UV spectrum of red onion extract was compared with each control standard (Quercetin and Quercetin glucosides) from 200nm-600nm; therefore, the main four flavonoids present in the onion were identified as follows quercetin 4′-glucoside, quercetin 3,4′-diglucoside, quercetin 3-glucoside and quercetin (Figures 2.36-2.43).

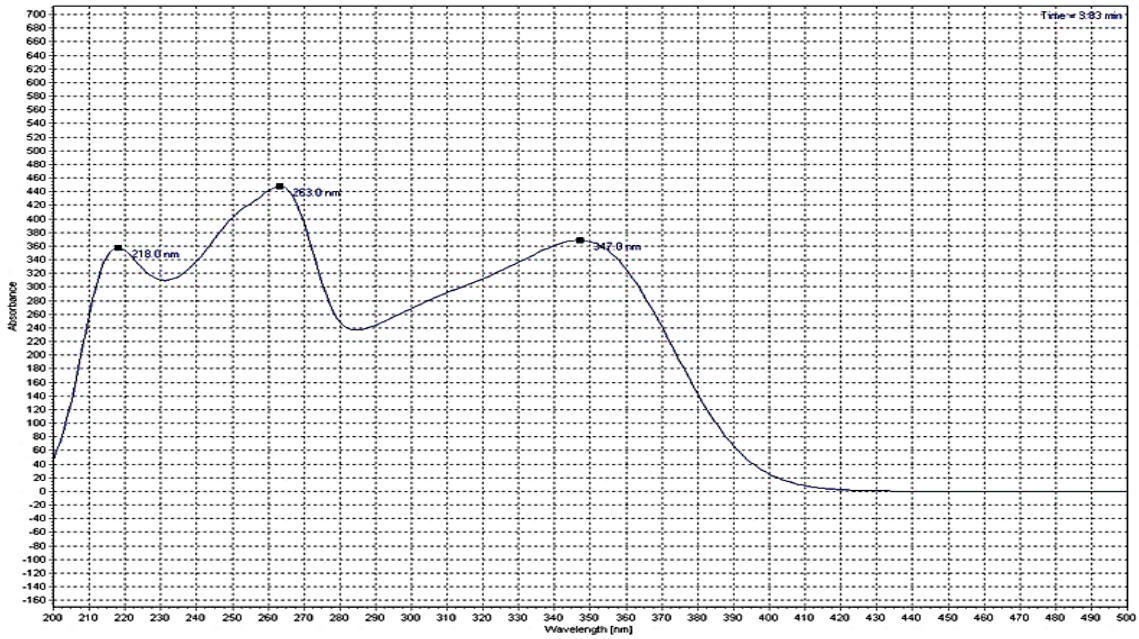


Figure 2.36: UV-scan of onion extract, peak at 3.83min appears at Wavelength: 218nm 283nm and 347nm.

Based on the UV spectrum and by comparison with the standards, four flavonoids were identified as follows: quercetin, quercetin 3-glucoside, quercetin 4′-glucoside and quercetin 3,4′-diglucoside in the red onion extract (25% w/v), which supported our previous findings. Peaks were assigned to quercetin and quercetin glucosides when monitoring the chromatographic runs at 370nm.

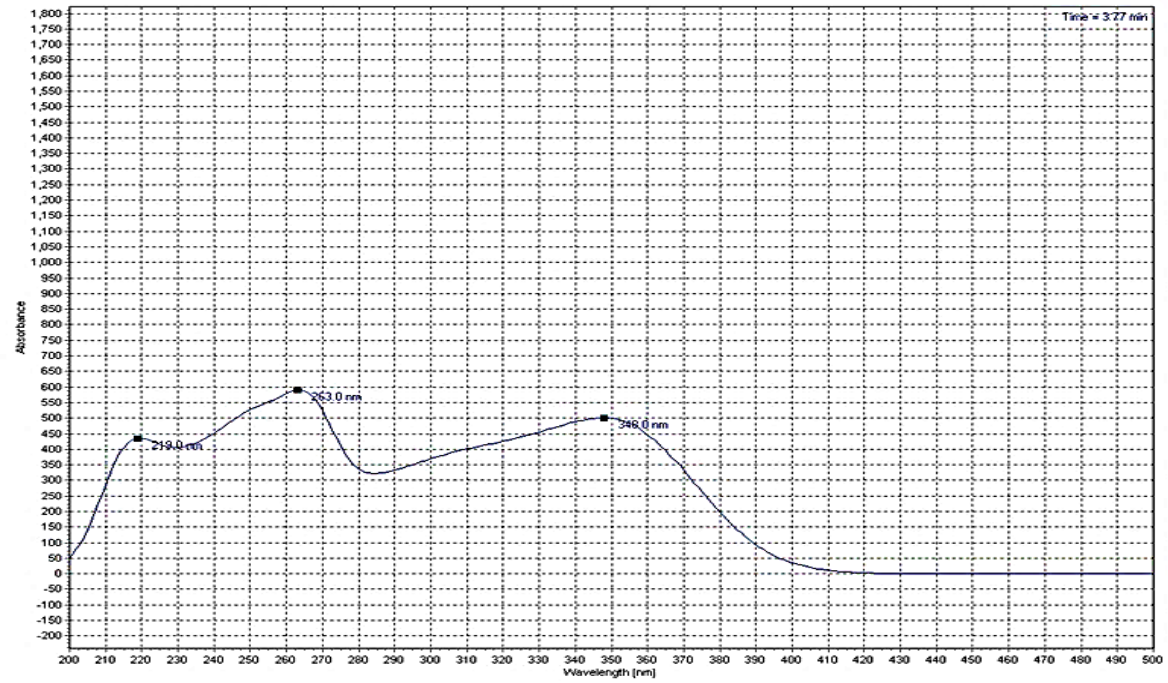


Figure 2.37: UV-scan of quercetin 3,4′-diglucoside peak at 3.77min appears at wavelength: 219nm, 263nm and 348nm.

Samples	Red Onion Extract	Quercetin 3,4′-Diglucoside
Retention Time- RT (min)	3.8	3.8
UV Scans (wavelengths nm)	218, 283, 347	219, 263, 348

Table 2.6: RT (min) and UV scans (nm) of red onion extract and quercetin 3,4′-diglucoside

The similar UV-scans from quercetin 3,4′-diglucoside to onion extract at RT=3.8min, confirmed the presence of this compound in the onion extract.

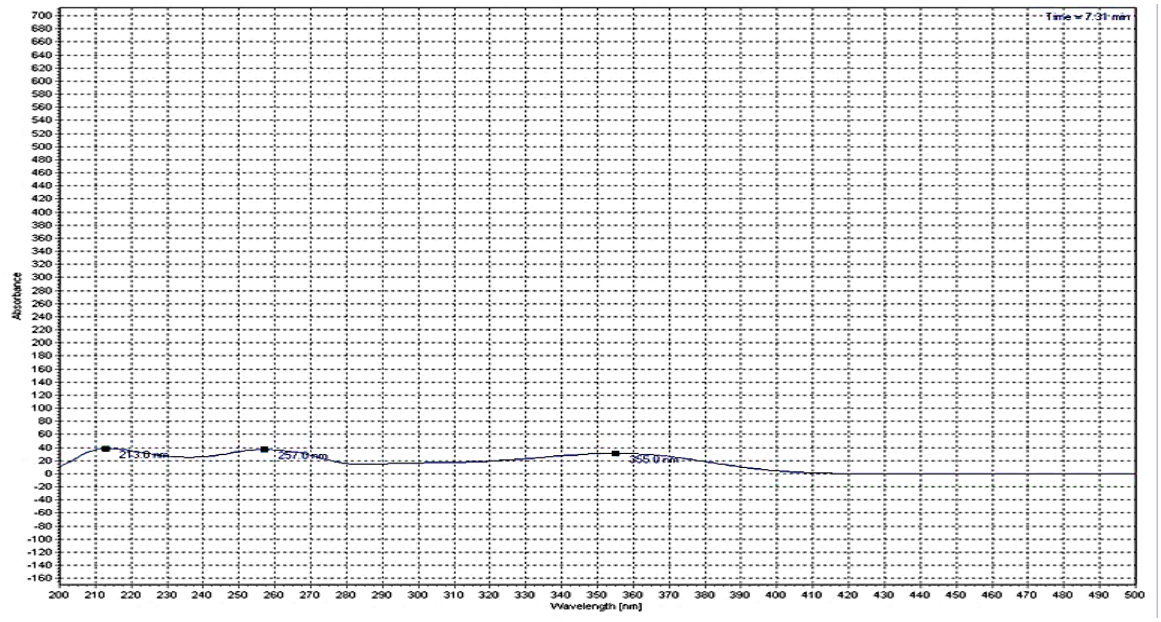


Figure 2.38: UV-scan of onion peak at 7.31min appears at wavelength= 213, 257 and 355nm.

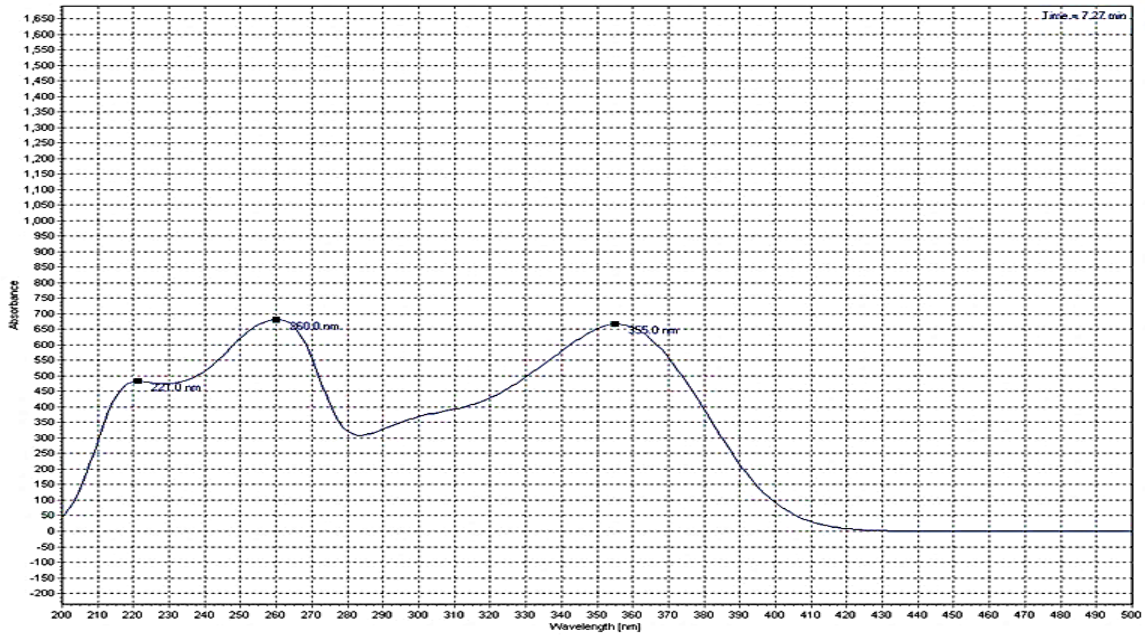


Figure 2.39: UV-scan of quercetin 3-glucoside peak at 7.27min appears at wavelength= 220, 260 and 355nm.

Samples	Red Onion Extract	Quercetin 3-Glucoside
Retention Time-RT (min)	7.3	7.3
UV Scans (Wavelengths nm) (wavelengths nm)	213,257, 355	220, 260, 355

Table 2.7: RT (min) and UV scans (nm) of red onion extract and quercetin 3-glucoside

UV-scans from quercetin 3-glucoside is similar to the onion extract at RT= 7.3 min, which determined the presence of this compound in the onion extract.

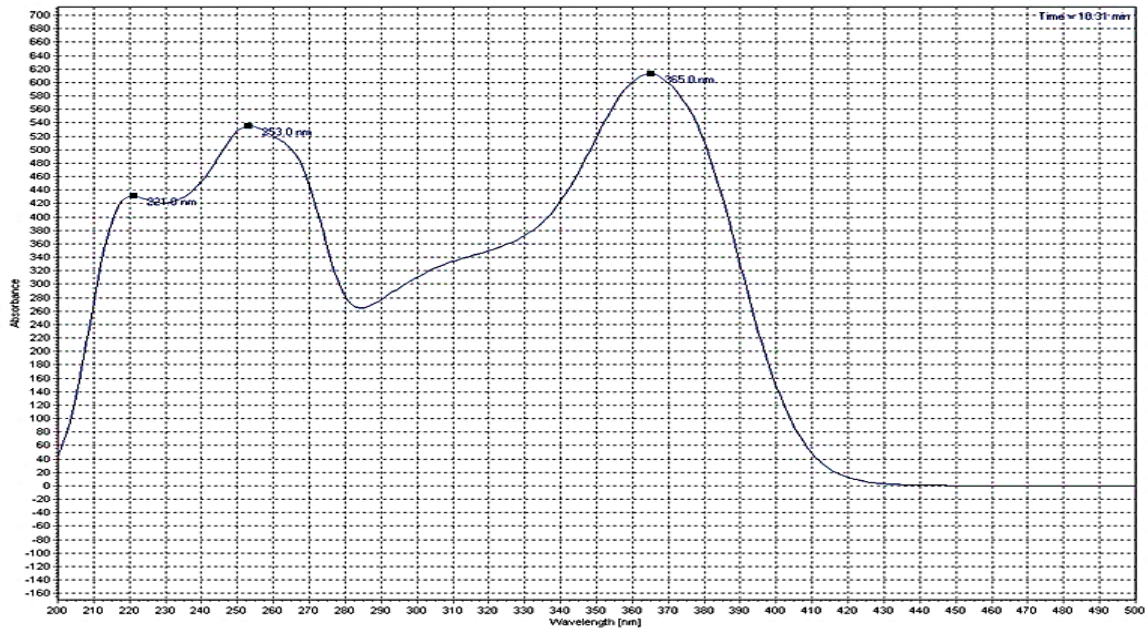


Figure 2.40: UV-scan of onion peak at 10.31min appears at wavelength= 221, 253 and 265nm.

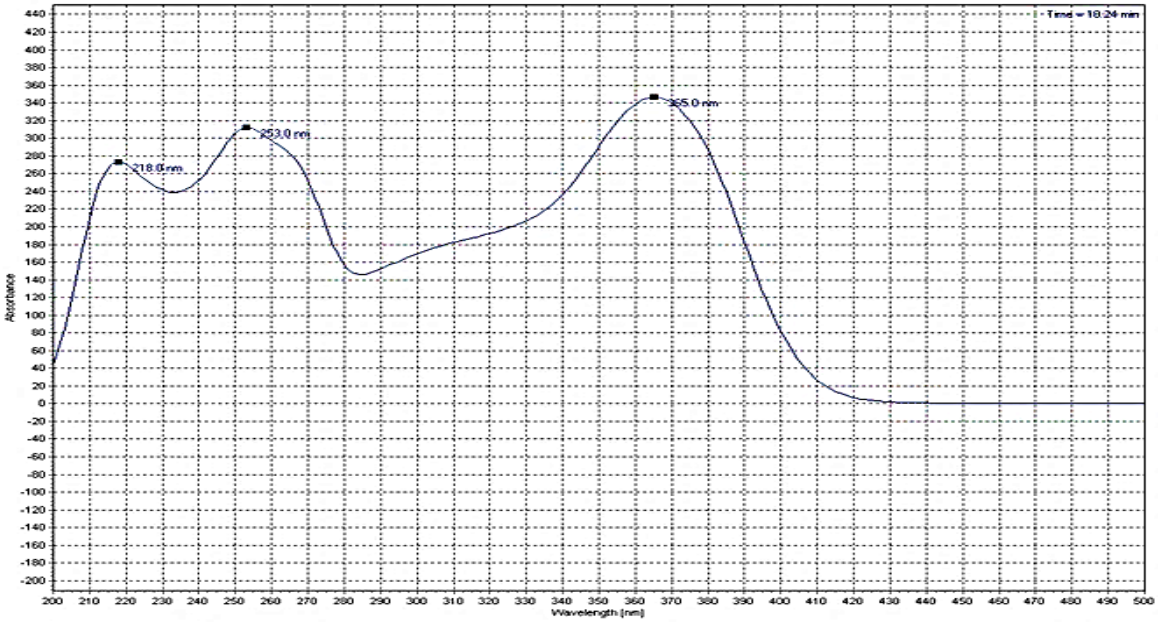


Figure 2.41: UV-scan of quercetin 4′-glucoside peak at 10.34min appears at wavelength= 218, 263 and 265nm.

Samples	Red Onion Extract	Quercetin 4′-Glucoside
Retention Time-RT (min)	10.3	10.3
UV Scans (wavelengths nm)	221, 253, 265	218, 253, 265

Table 2.8: RT (min) and UV scans (nm) of red onion extract and quercetin 4′-glucoside

UV-scans from quercetin 4-glucoside is in accordance to the onion extract at RT=10.3min which showed this compound is found in the onion extract.

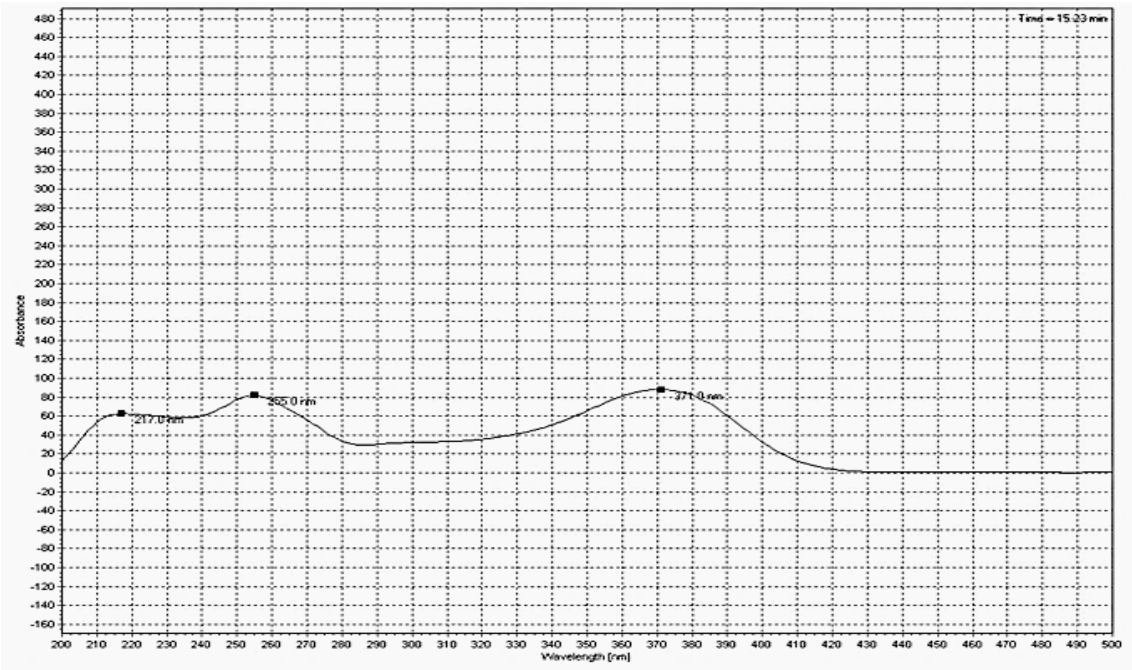


Figure 2.42: UV-scan of onion extract peak at 15.2 min appears at wavelength= 217, 255 and 379nm

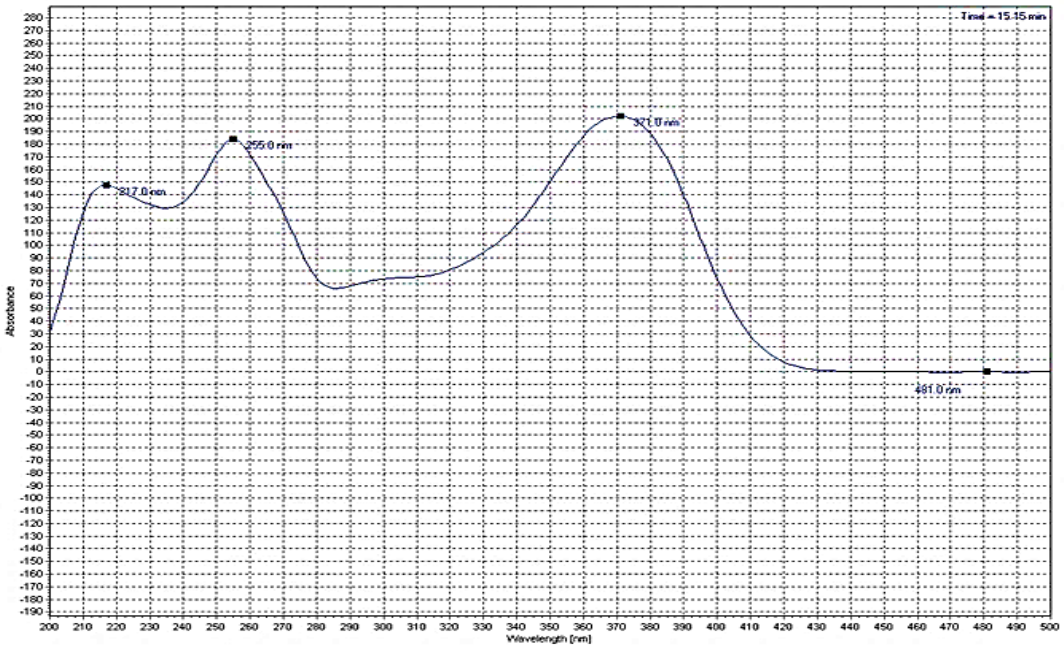


Figure 2.43: UV-Scan of quercetin peak at 15.5 min appears at wavelength= 217, 255 and 370nm.

Samples	Red Onion Extract	Quercetin
Retention Time - RT (min)	15.2	15.5
UV Scans (wavelengths nm)	217, 255, 379	217, 255, 370

Table 2.9: RT (min) and UV scans (nm) of red onion extract and quercetin

The similar UV-scans from quercetin to the onion extract at RT=15 min also confirmed our previous findings, thus it was found in the onion extract. Based on the current spectral analysis, the four flavonols present in the onion were identified as follows: quercetin 3,4′-diglucoside, quercetin 4′-glucoside, quercetin 3-glucoside and quercetin.

2.8.1. Standard curves of quercetin and quercetin glucosides

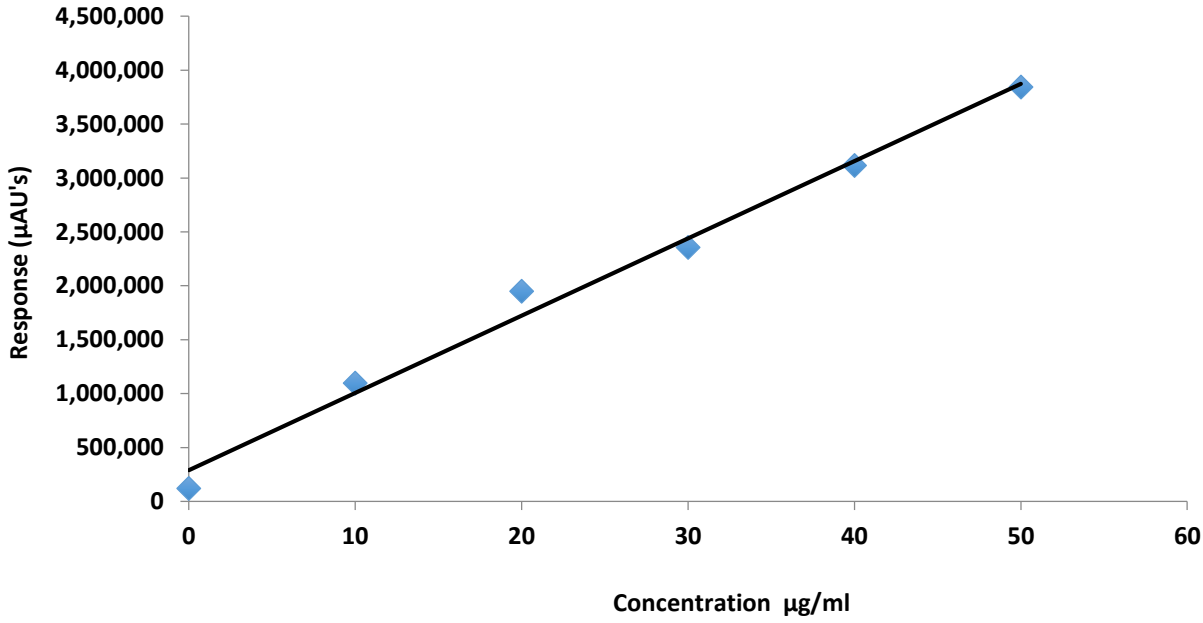


Figure 2.44: Quercetin standard curve (10-50 µg/ml)

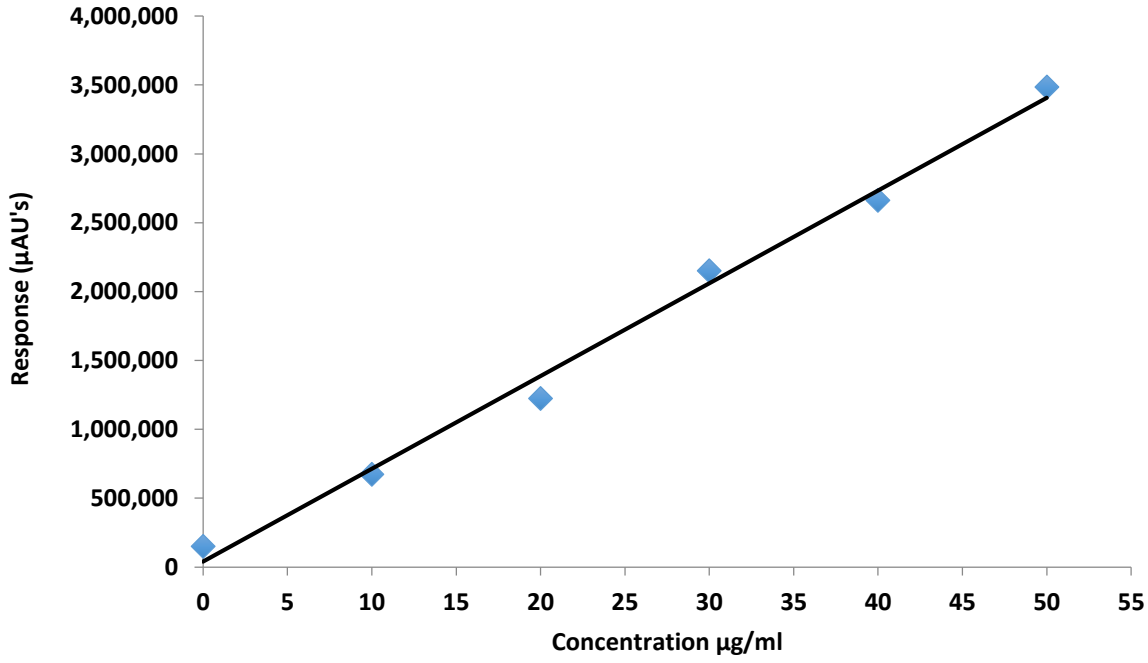


Figure 2.45:Quercetin 4′-glucoside standard curve (10-50 µg/ml)

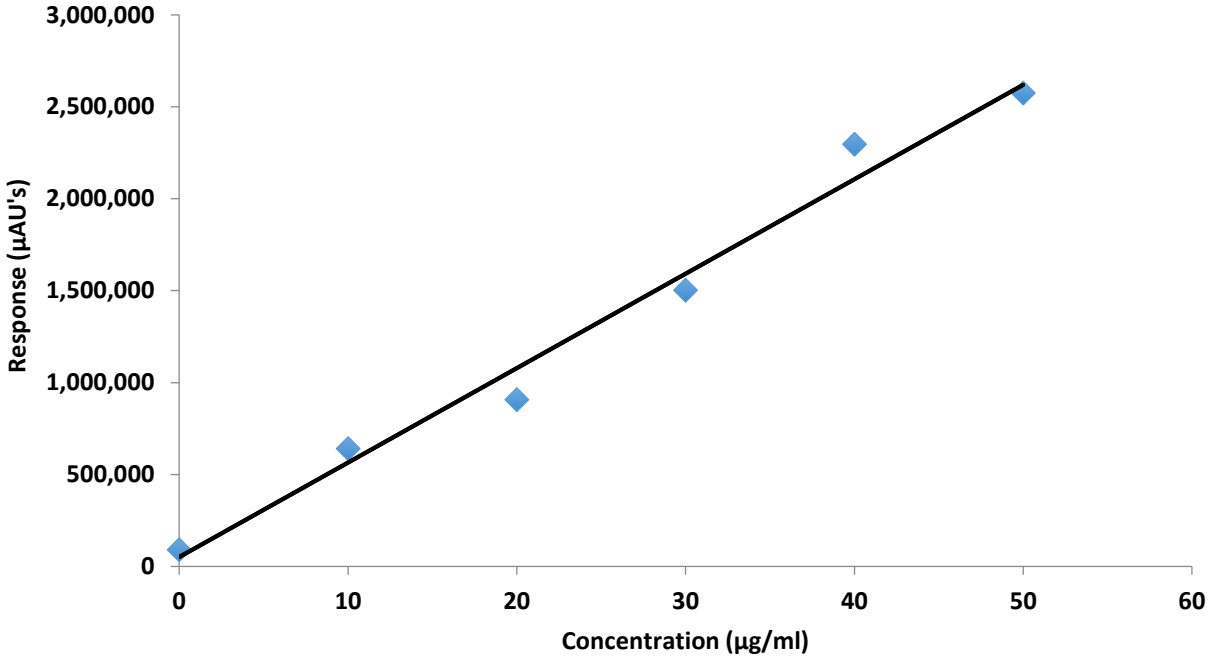


Figure 2.46: Quercetin 3,4′-diglucoside standard curve (10-50 µg/ml)

2.9. Effect of Quercetin 3,4′-diglucoside, Quercetin 4′-glucoside, onion extract and dialysed onion extract on Caco-2 cells glucose uptake

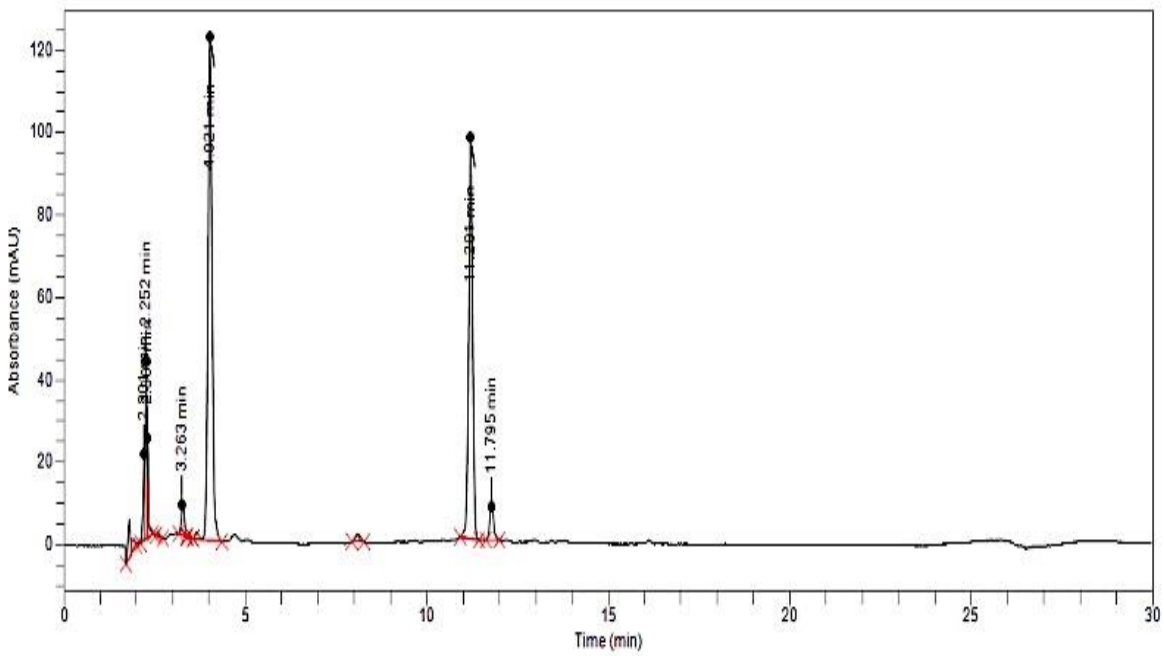


Figure 2.47: 25% w/v onion extract, absorbance against time (RT= 4.021 and 11.201 min)

According to figure 2.47, 25% onion extract indicates two large peaks occurring at RT 4.021 and 11.201min, which are identified as quercetin 3,4′-diglucoside and quercetin 4′-glucoside respectively by relative position of these peaks compared to standards (3,4′-diglucoside and 4′-glucoside.

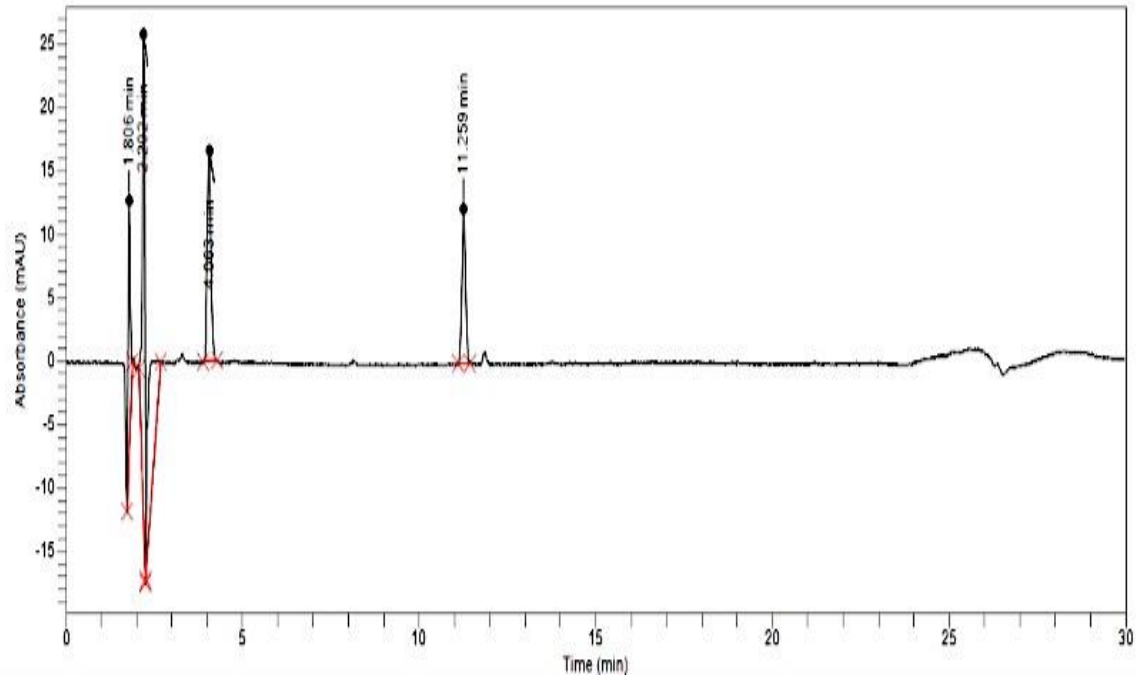


Figure 2.48: Dialysed 25% w/v onion extract after 8 hours, absorbance against time (RT= 4.063 and 11.259 min)

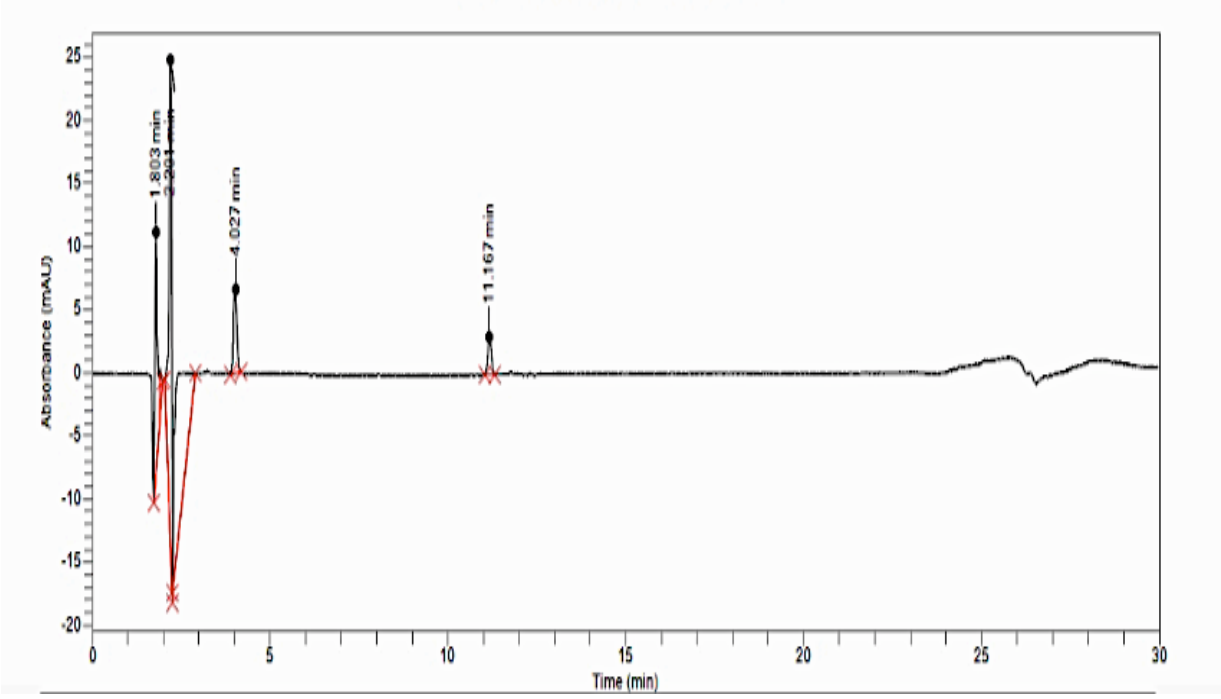


Figure 2.49: Dialysed 25% w/v onion extract after 24 hours, absorbance against time (RT= 4.027, 11.167 min)

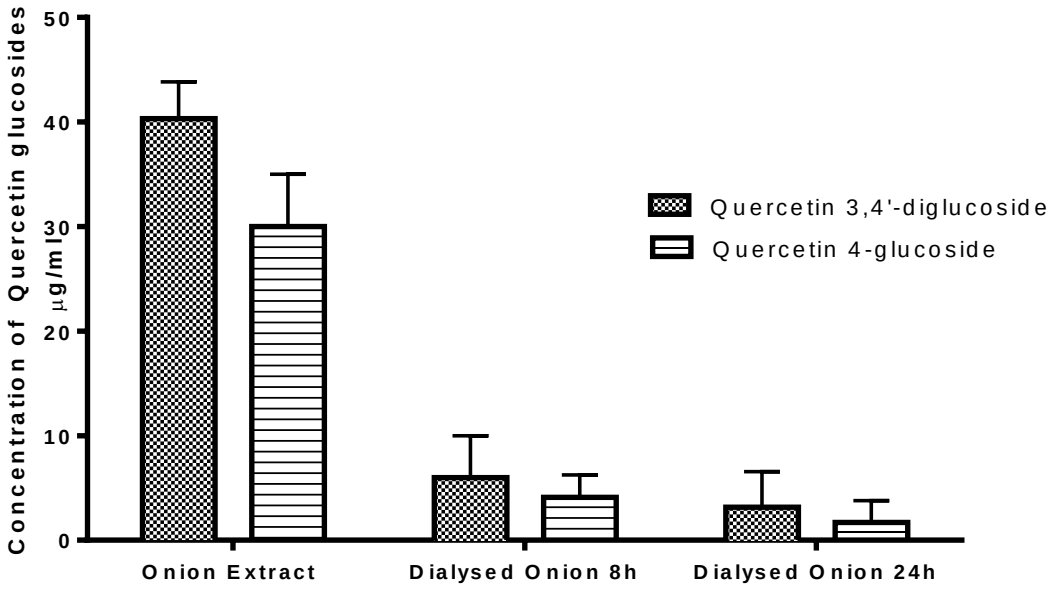


Figure 2.50: The effect of dialysis on the concentration of quercetin glucosides in 25%w/v onion extract (n=3 ± SEM) through standard curve of both quercetin glucosides (Figures 2.45 & 2.46)

Figure 2.50 shows that after 8 hours of dialysis, the concentration of both quercetin 3,4′-diglucoside and quercetin 4′-glucoside significantly reduced from 40.1 µg/ml and 30.4µg/ml to 6.5µg/ml and 4.4µg/ml respectively ($p < 0.01$). Moreover, after 24 hours of dialysis, quercetin 3,4′-diglucoside and quercetin 4′-glucoside concentrations significantly decreased to 1.7 µg/ml and 1.1 µg/ml respectively with $p < 0.001$. This could be due to the low molecular weight of quercetin glucosides which results in their loss in the 25% w/v onion extract after dialysis. Also, dialysis buffer was assessed to check if quercetin glucosides were still present; therefore, the presence of both quercetin 4′-glucoside and quercetin 3,4′-diglucoside with RT= 11.167 and 4.027 min were obtained which were in accordance with their standards.

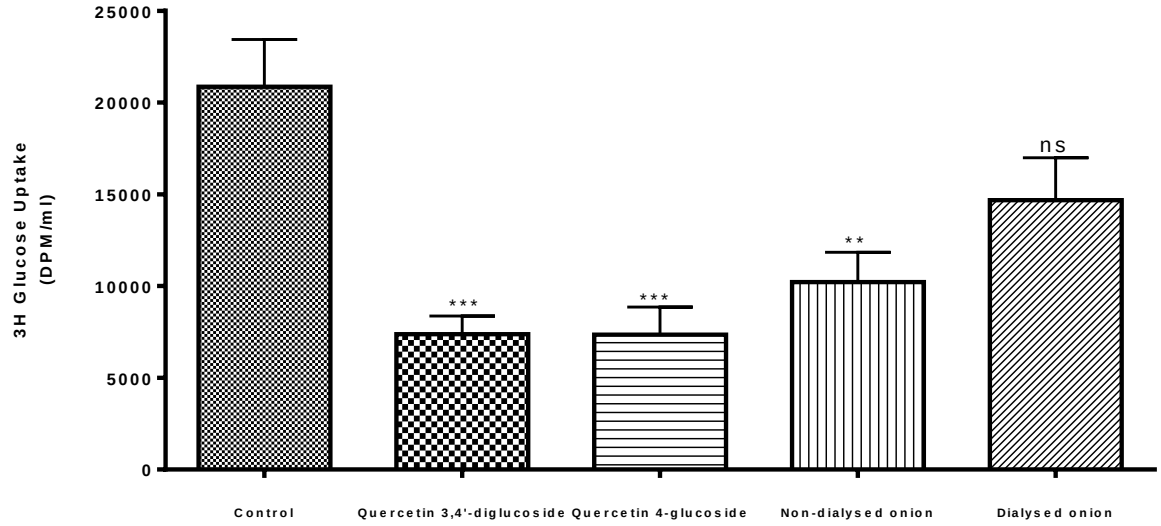


Figure 2.51: Glucose uptake in Caco-2 cells in the presence of 100µM quercetin 3,4′-diglucoside, 100µM quercetin 4′-glucoside, 25% w/v non-dialysed onion and dialysed onion under sodium-dependent condition with n=3 ± SEM, *** $p < 0.001$,** $p < 0.01$ and * $p < 0.05$ and $p > 0.05$

Figure 2.51 illustrates a significant reduction in cellular glucose uptake in the wells treated with quercetin 3,4′-diglucoside and quercetin 4′-glucoside by 65% at a statistical significant level of $p < 0.001$; followed by inhibitory influence of non-dialysed onion by 51% with $p < 0.01$ ($p = 0.007$), whereas after 8 hours of dialysis of onion, the inhibitory effect significantly reduced to 29% with $p > 0.5$ ($p = 0.2$).

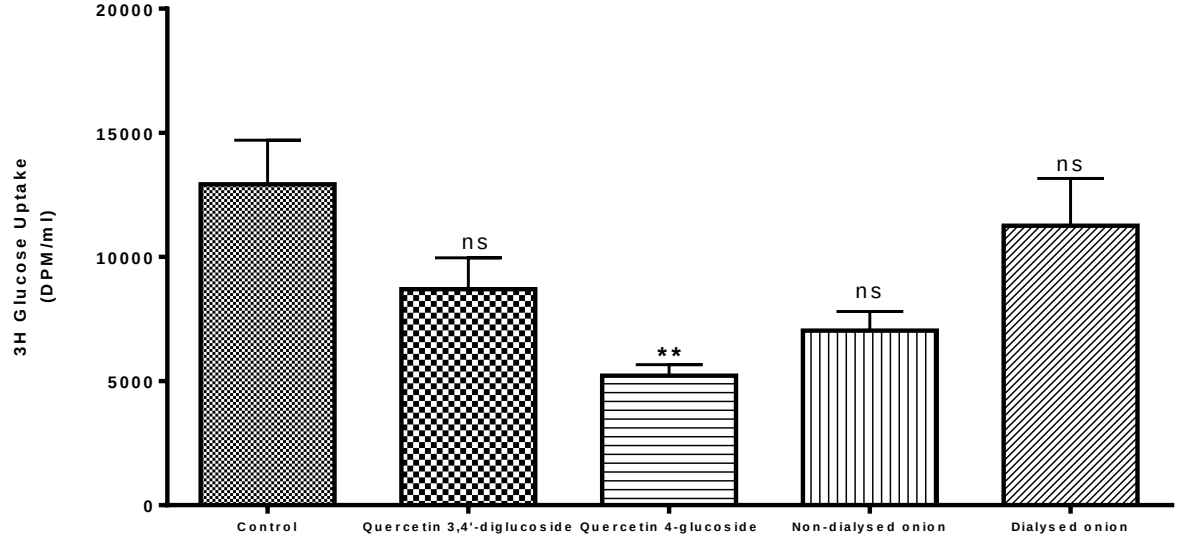


Figure 2.52: Glucose uptake in Caco-2 cells in the presence of 100 µM quercetin 3,4′-diglucoside, 100 µM quercetin 4′-glucoside, 25% w/v non-dialysed onion and dialysed onion under sodium-independent condition with n=3 ± SEM, ** $p < 0.01$ and $p > 0.05$

Figure 2.52 demonstrates that there was a significant reduction in cellular glucose uptake in the wells treated with 100µM quercetin 4′-glucoside by 60% at a statistical significant level of $p < 0.01$ ($p = 0.0008$). Followed by lower inhibitory influence of 25% w/v non-dialysed onion, 100 µM quercetin 3,4′-diglucoside and dialysed onion by 42%, 32% and 10% with $p > 0.05$ respectively.

Dialysed onion did not have a significant inhibitory effect in glucose uptake at both sodium dependent and sodium free conditions respectively (figure 2.51 & 2.52). This could be due to the low molecular weight of quercetin glucosides, which leads to their loss after dialysis of onion extract. Our finding suggests that some of the inhibitory activity in onion is dialysable.

2.10. Modelling of lactase with β-galactosidase

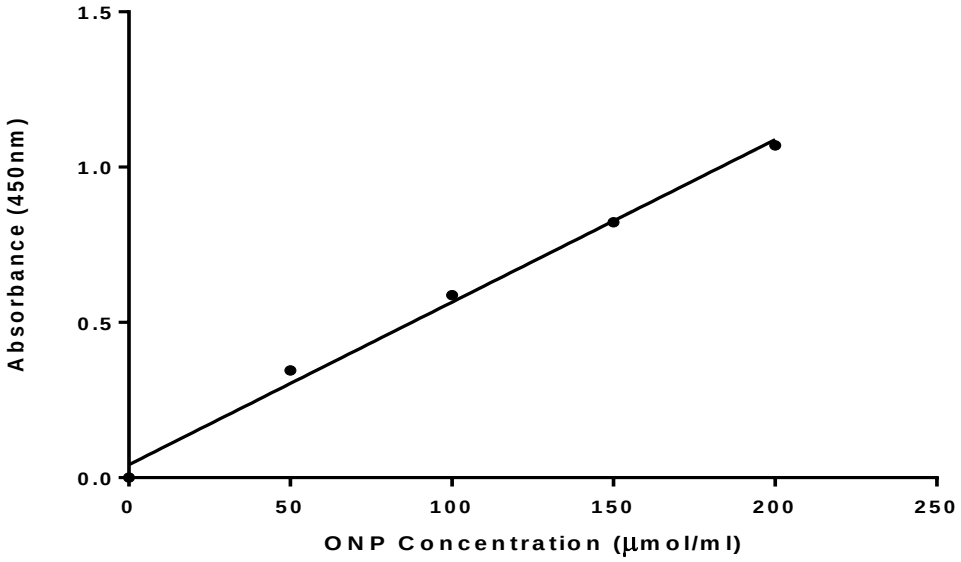


Figure 2.53 Standard curve of ONP (µmol/ml) vs. absorbance, n=3 ± SD

The substrate used in the β-galactosidase assay from *Aspergillus oryzae* is Ortho-Nitrophenyl β-galactoside, which upon hydrolysis of β-galactosidic bond, yields galactose and nitrophenol, a yellow compound (absorption maximum = 450nm). Figure 2.53, illustrates the linear relationship between the rate of OD A_{450} and ONP product formed (µmol/ml/min), and therefore was used to demonstrate the rate of conversion of 1µmol substrate (ONPG) into product in one minute. The rate at which product can be formed is limited by the concentration of substrate, pH and temperature (Mlichova and Rosenberg, 2006).

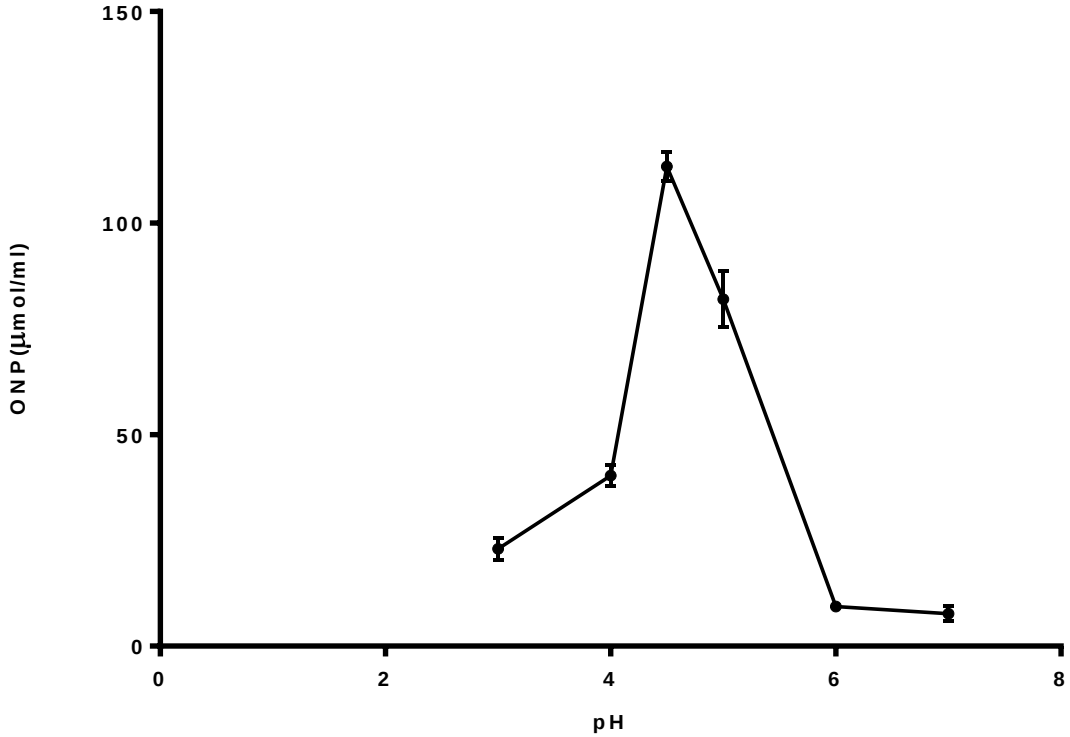


Figure 2.54: pH dependent β-galactosidase activity (*Aspergillus oryzae*), n=3 ± SD

Figure 2.54 illustrates that at 37 °C, the effect of pH was measured by the rate of product formed within the range of pH 3:0-7:0. The maximum (ONP) µmol/ml/min product was formed at pH 4.5. Therefore 4.5 is the optimum pH for β-galactosidase from *Aspergillus oryzae* activity. β-galactosidase enzyme activity drastically decreased after pH 4.5-5.0. Notably, pH-dependent enzyme activity is proportional to the increase in absorbance of A_{450} during a timed incubation due to ONPG. Therefore, the maximum concentration of product formed was determined through ONP (µmol/ml) standard curve (figure 2.53).

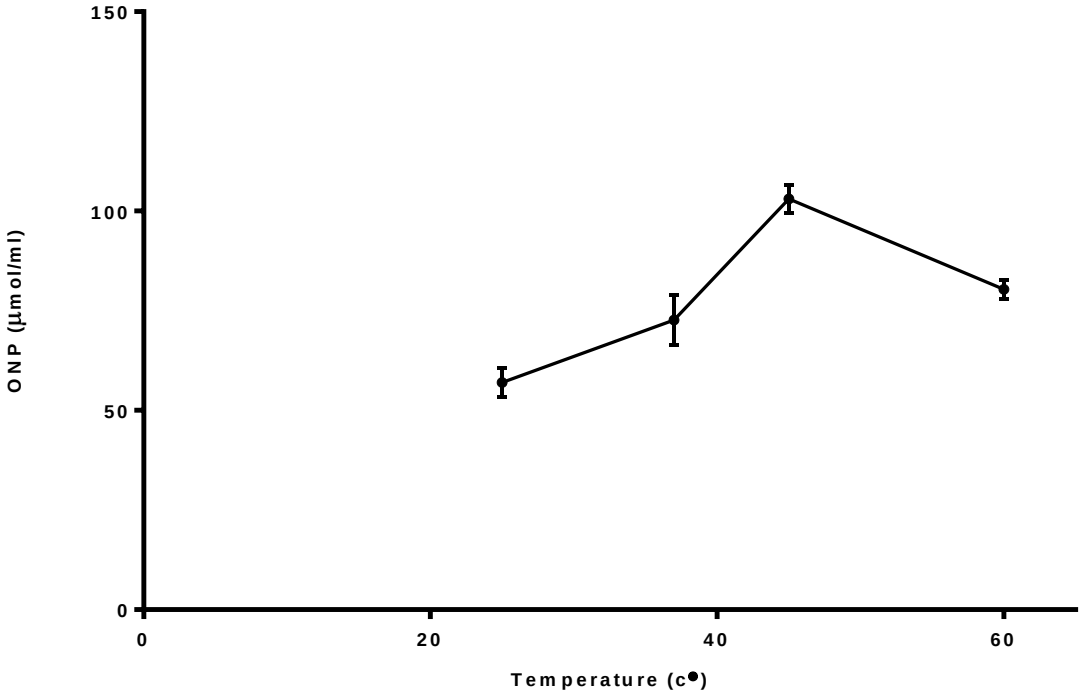


Figure 2.55: Temperature dependent β-galactosidase activity (*Aspergillus oryzae*), n= 3± SD

Figure 2.55 illustrates the optimum temperature at (45 °C). To assess the optimum temperature for enzyme activity, lactase was incubated at the temperature range of 24 ºC, 37 ºC, 45 ºC and 60 ºC at pH 4.5 for 20 minutes. Maximum activity of enzyme was obtained at 45°C; therefore, high temperature reduced the rate of product ONP (µmol/m) formation (figure 2.53).

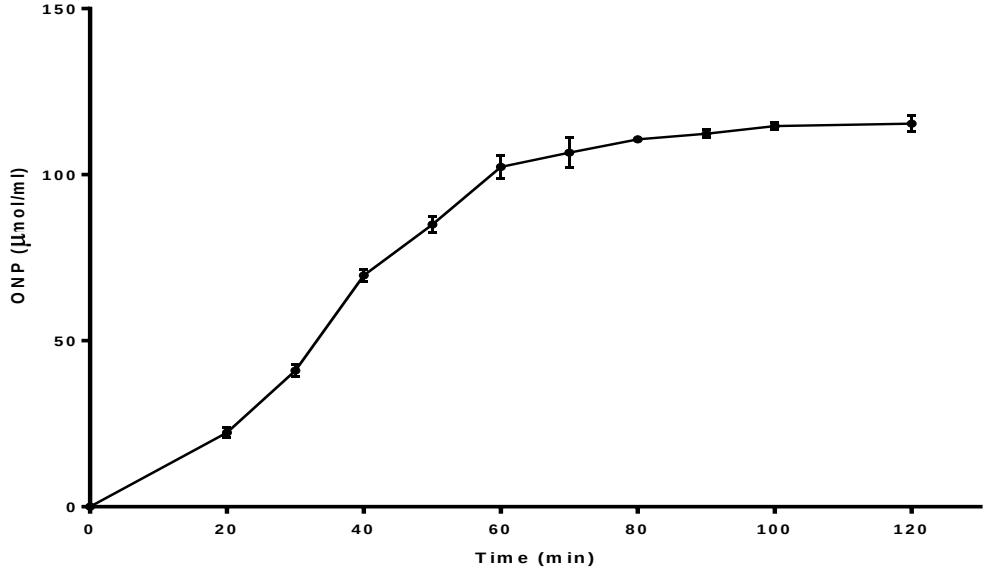


Figure 2.56: Time dependent β-galactosidase activity (*Aspergillus oryzae*) from 0 to 120 min, n=3± SD

Figure 2.56 demonstrates as the time increased from 0 to 60 minutes, due to enzyme hydrolysis, more substrates were converted to products ONP (µmol/ml). It shows the maximum activity of β-galactosidase enzyme occurring at (60 minutes), 60 minutes is the time point where enzyme activity reduced/stopped, thus less product ONP is produced after β-galactosidase incubation. However, at longer incubation time, such as after 60 minutes there was not a big increase on the product formation, and the linear range went off from the graph. The rate of formation of product now depends on the activity of the enzyme itself, and increasing β-galactosidase incubation time did not affect the rate of the reaction to any significant effect after 60 minutes.

Following the current β-galactosidase assay, quercetin glucosides and onion extract/onion solution as the substrates were incubated with β-galactosidase for 20, 40 and 60 minutes to evaluate the concentration of free quercetin (products) after hydrolysis by β-galactosidase from *Aspergillus oryzae*.

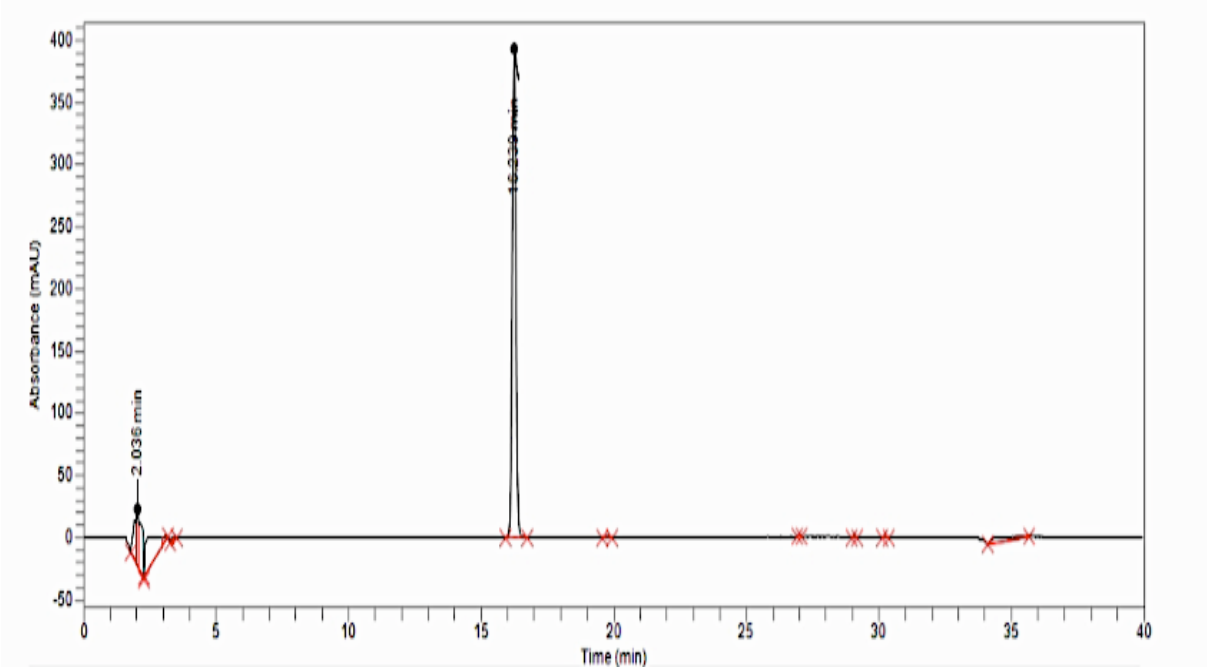


Figure 2.57: Quercetin in 80% v/v methanol , peak area against retention time (RT= 2.036, 16.239 min)

In figure 2.57 quercetin illustrates one large peak at RT 16.239 min, which is in accordance with the peak in the onion extract occurring at RT 16.373 min (figure 2.60).

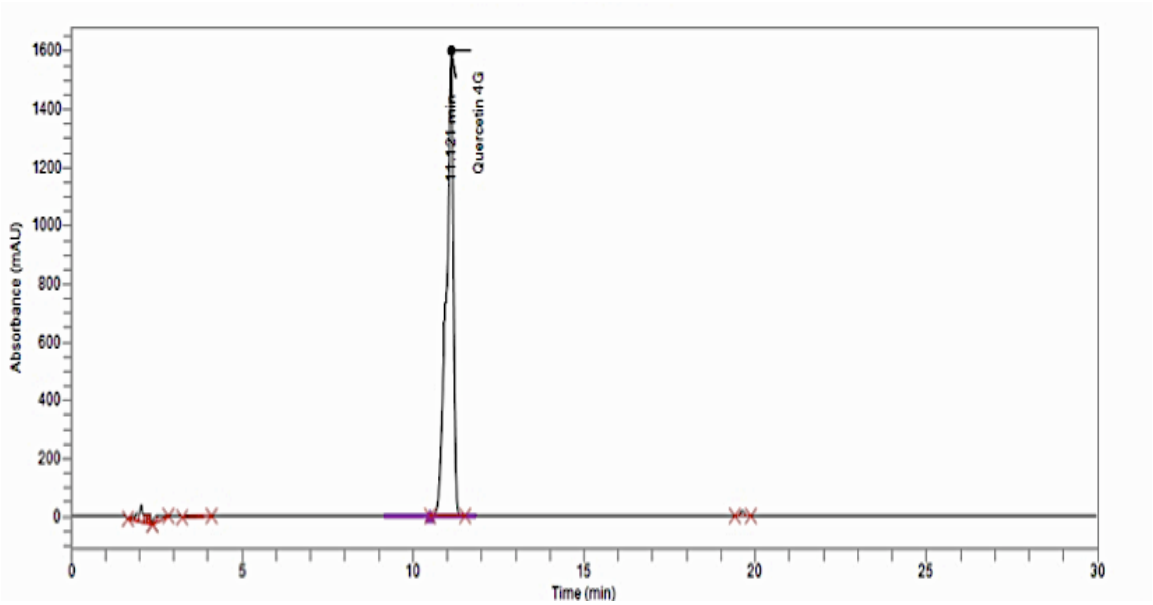


Figure 2.58: Quercetin 4′-glucoside in 80% v/v methanol, absorbance against time (RT= 11.121 min)

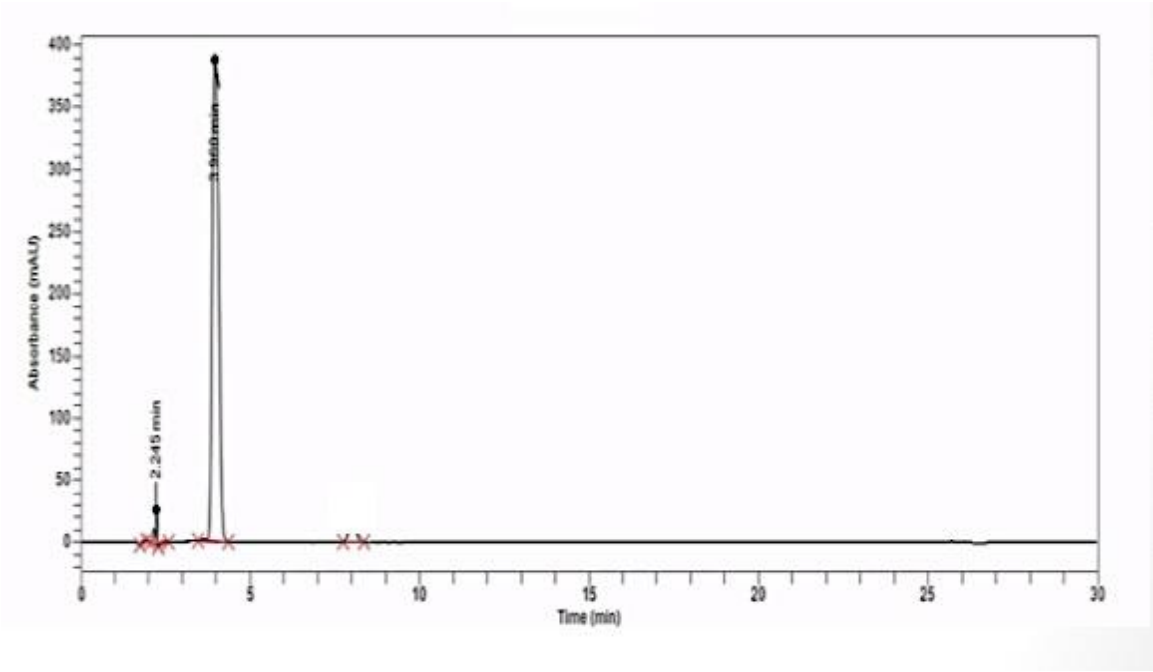


Figure 2.59: Quercetin 3,4′-diglucoside in 80% v/v methanol, peak area against retention time (RT= 3.968 min)

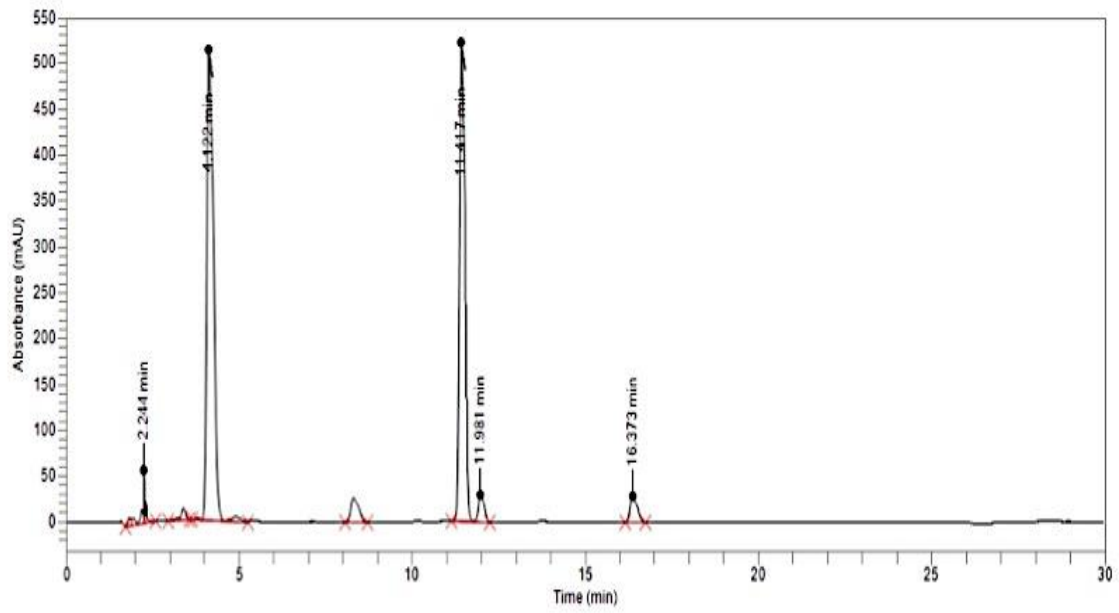


Figure 2.60: High-performance liquid chromatography of flavonoids in red onion extract. Compounds quantified were quercetin 3,4′-diglucoside, quercetin 4′-glucoside and quercetin aglycone. 25% w/v fresh onion extract (red) in methanol, absorbance versus time (RT= 2.244, 4.122, 8.56, 11.417, 16.373 min)

Onion extract indicates two large peaks occurring at RT 4.122 min and 11.417 min, which were identified as quercetin 3,4′-diglucoside and quercetin 4′-glucoside respectively by relative position of these peaks compared to standards (3,4′-diglucoside and 4′-glucoside with RT 3.968 and 11.121 respectively (figures 2.58 and 2.59).

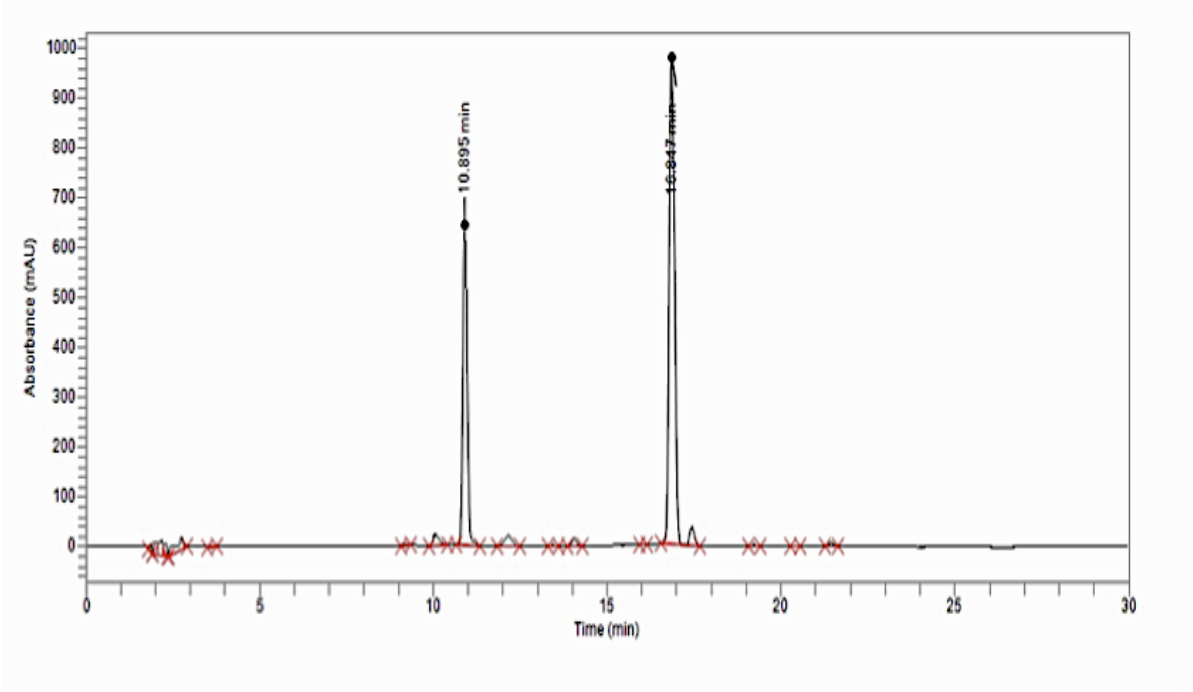


Figure 2.61: Quercetin 4′-glucoside in 80% methanol incubated with β-galactosidase enzyme from *Aspergillus oryzae*, for 60 minutes, absorbance against time (RT= 10.896 and 16.647 min)

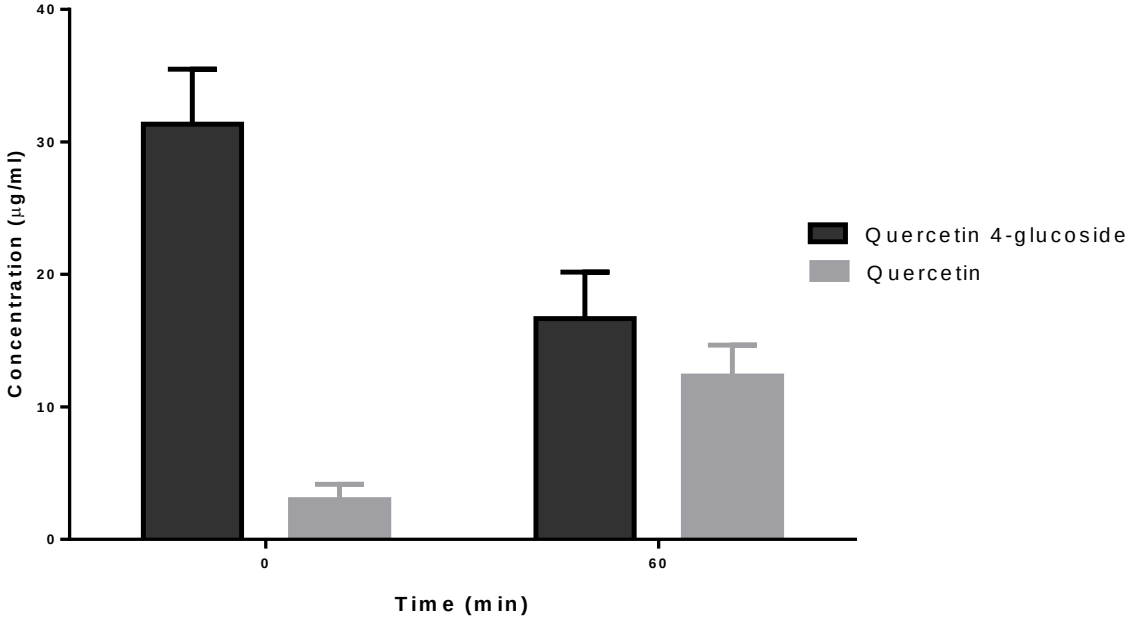


Figure 2.62: Concentration of quercetin and quercetin 4′-glucoside after incubation of quercetin 4′-glucoside with β-galactosidase from *Aspergillus oryzae* for 60 minutes with n=3 ±SD

Figure 2.62 shows the concentration of both quercetin and quercetin 4′-glucoside calculated from calibration curves (figures 2.44 & 2.45). Quercetin concentration increased from 2.2 µg/ml to 12.1 µg/ml, whereas quercetin 4′-glucoside reduced from 31.2 µg/ml to 17.1 µg/ml after 60 minutes pre-treatment with β-galactosidase from *Aspergillus oryzae*.

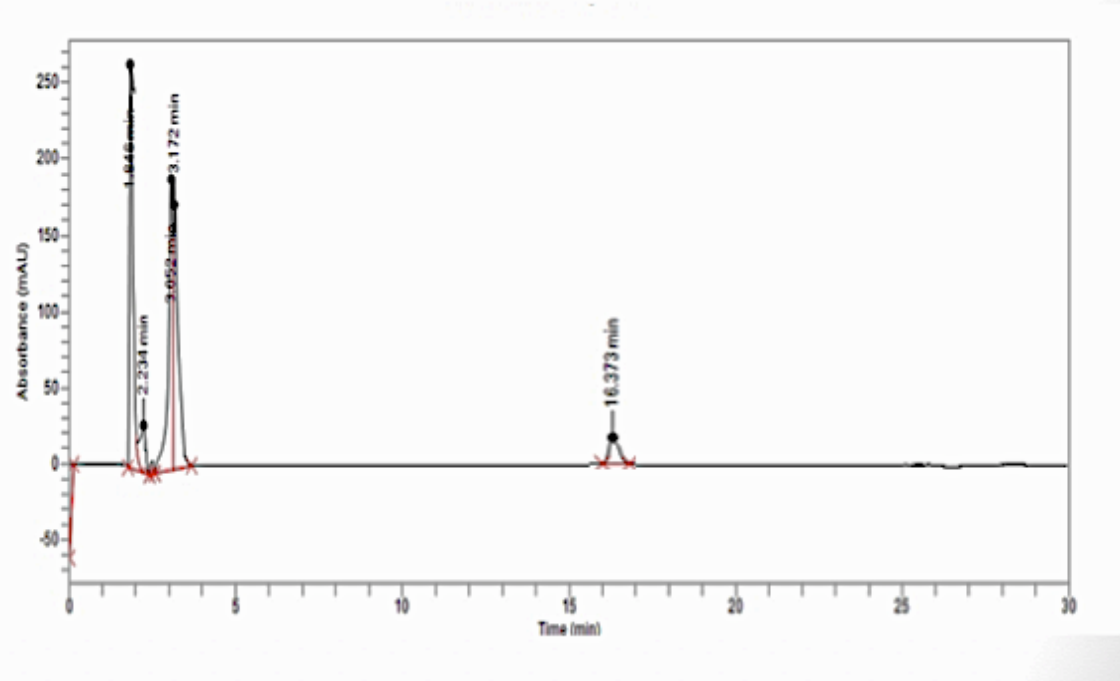


Figure 2.63: Quercetin 3,4′-diglucoside in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 20 min (RT= 3.172, 16.373 min)

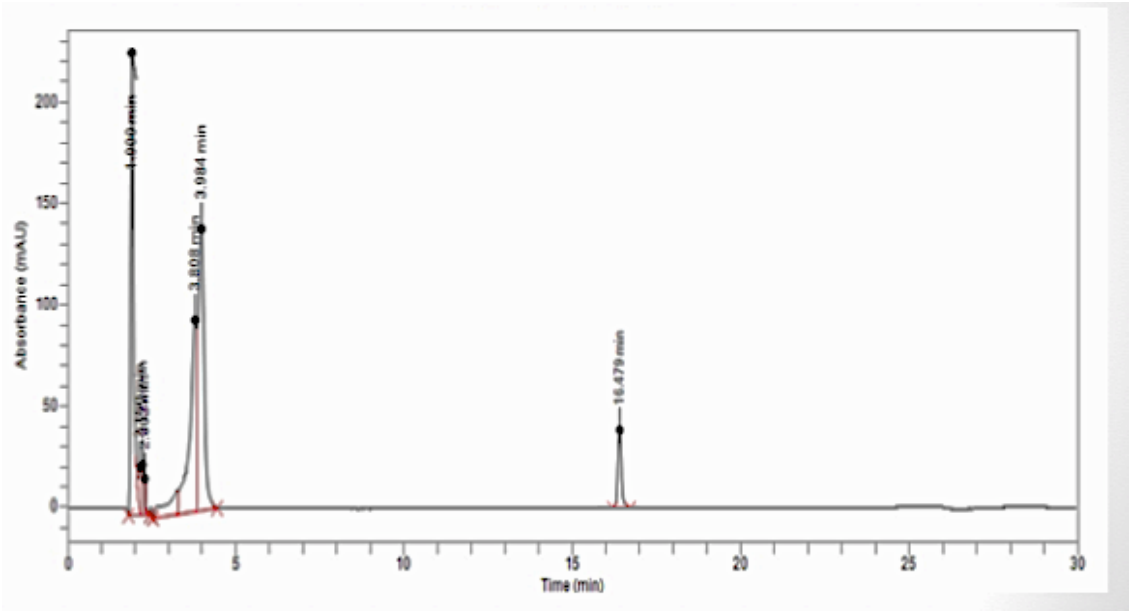


Figure 2.64: Quercetin 3,4′-diglucoside in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 40 min (RT= 3.984, 16.479 min)

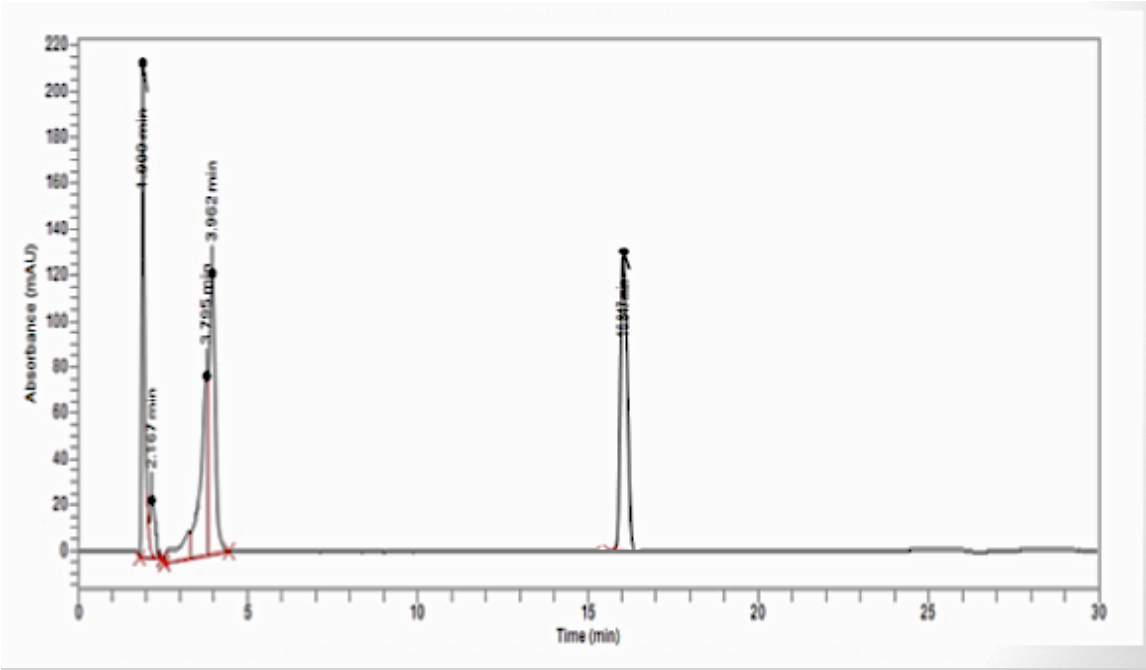


Figure 2.65: Quercetin 3,4′-diglucoside in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 60 min (RT= 3.962, 16. 396 min)

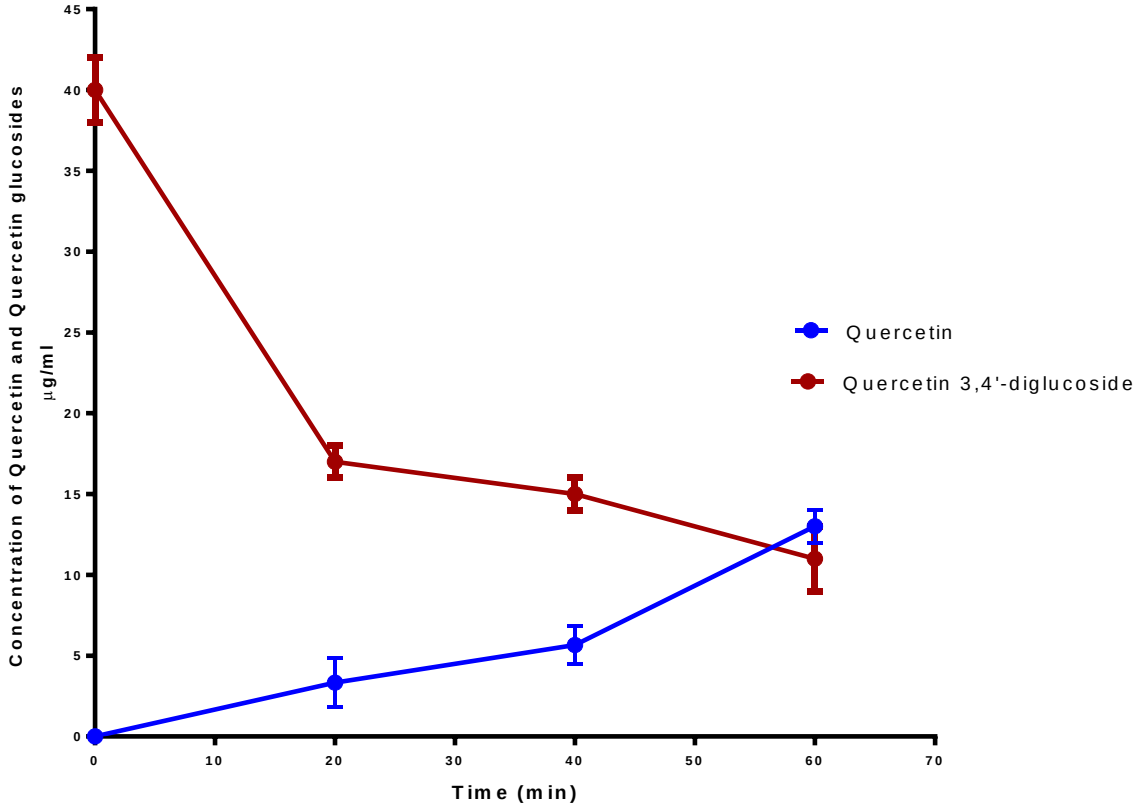


Figure 2.66: Concentration of quercetin and quercetin 3,4′-diglucoside after quercetin 3,4'-diglucoside were treated with β-galactosidase from *Aspergillus oryzae* for 20, 40 and 60 minutes with (n=3 ± SD)

Figure 2.66 demonstrates after 20 minutes' concentration of quercetin 3,4′-diglucoside significantly decreased by 58% after incubation with the β-galactosidase enzyme from *Aspergillus oryzae* at a significant level of $p = 0.022$ ($p < 0.05$), whereas the concentration of quercetin increased by 15%. There was a steady decrease and increase in quercetin 3,4′-diglucoside and quercetin respectively after 40 minutes of incubation with the β-galactosidase. Quercetin 3,4′-diglucoside concentration decreased from 40.1 µg/ml to 11.2 µg/ml, suggesting its break down by β-galactosidase which results in quercetin concentration from 0.7 to 13.3 µg/ml after 60 minutes of incubation.

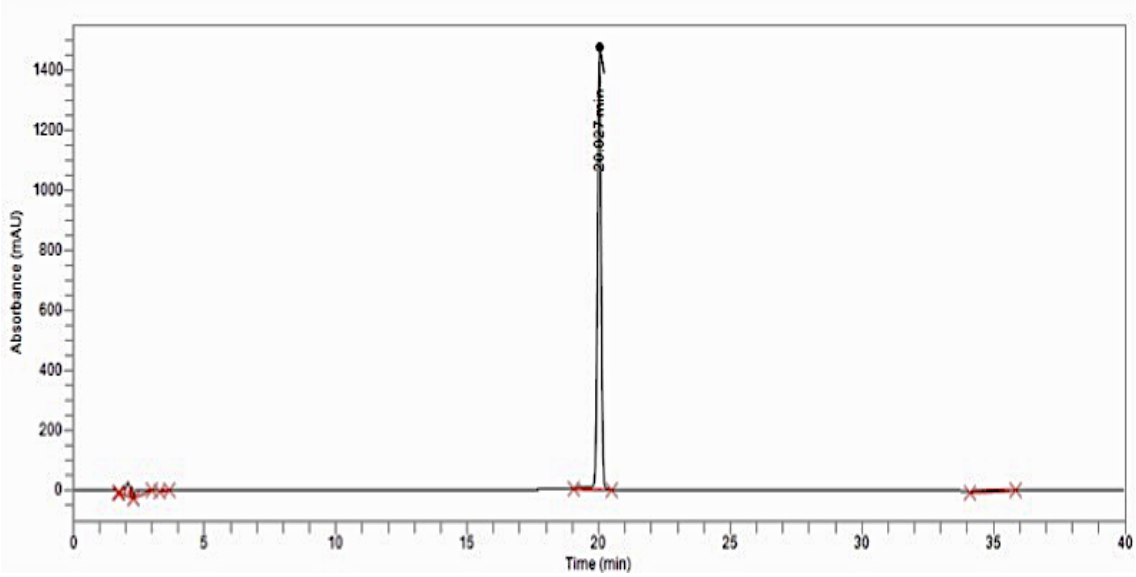


Figure 2.67: Isorhamnetin in 80% v/v methanol, peak area against retention time (RT= 20.027 min)

It is shown that red onion extract did not show a peak at similar RT=20.027 with Isorhamnetin (figure 2.60), therefore Isorhamnetin was not identified in the current onion extract.

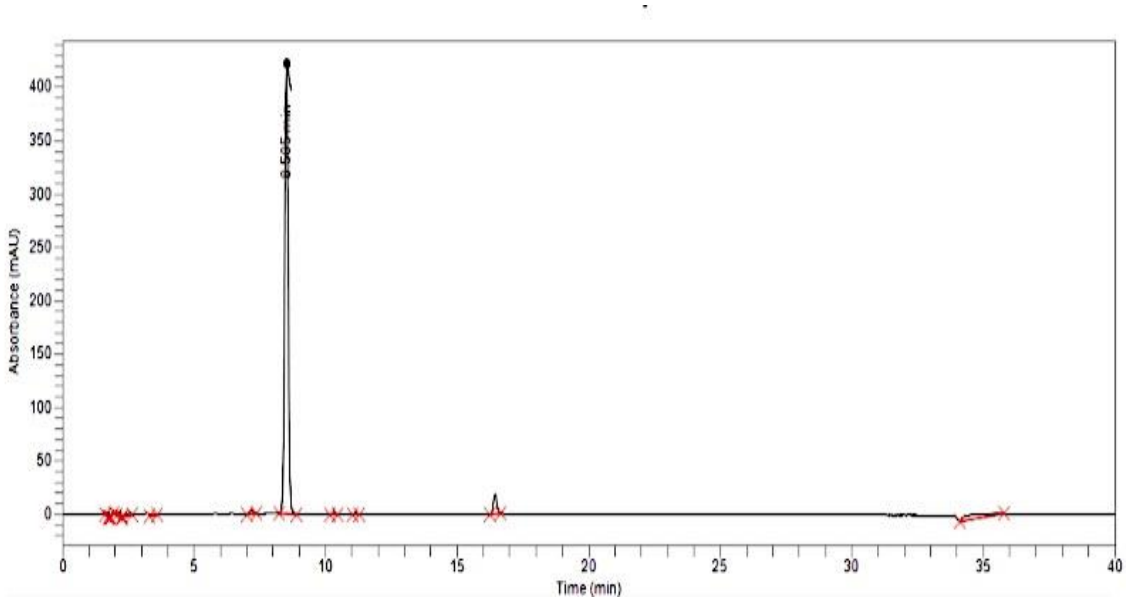


Figure 2.68: Quercetin 3-glucoside in 80%v/v methanol, peak area against retention time (RT= 8.585 min)

Quercetin 3-glucoside at figure 2.68 illustrates a small peak at RT 8.585min, which is in accordance with the peak in the onion extract occurring at RT=8.56 min (figure 2.60). Therefore, quercetin 3-glucoside also existed in the onion extract, but only consisted 6% of total flavonoid AUC in the onion extract.

2.10.1. Effect of β-galactosidase from Aspergillus oryzae on flavonoids in red onion extract in methanol

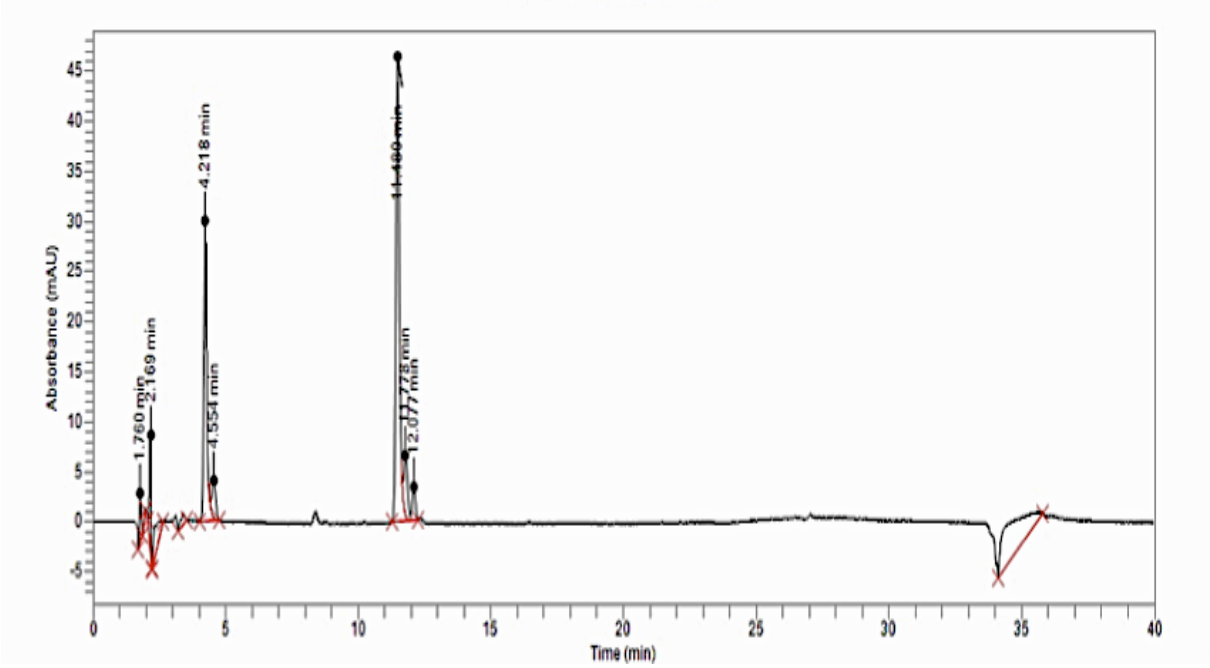


Figure 2.69: High-performance liquid chromatography of flavonoids in red onion extract. The compounds quantified were quercetin 3,4′-diglucoside, quercetin 4'-monoglucoside and quercetin aglycone. Red onion extract (25% w/v) in methanol (80% v/v), absorbance against time (RT= 4.218, 4.554, 11.480, 11.778, 12.077 min)

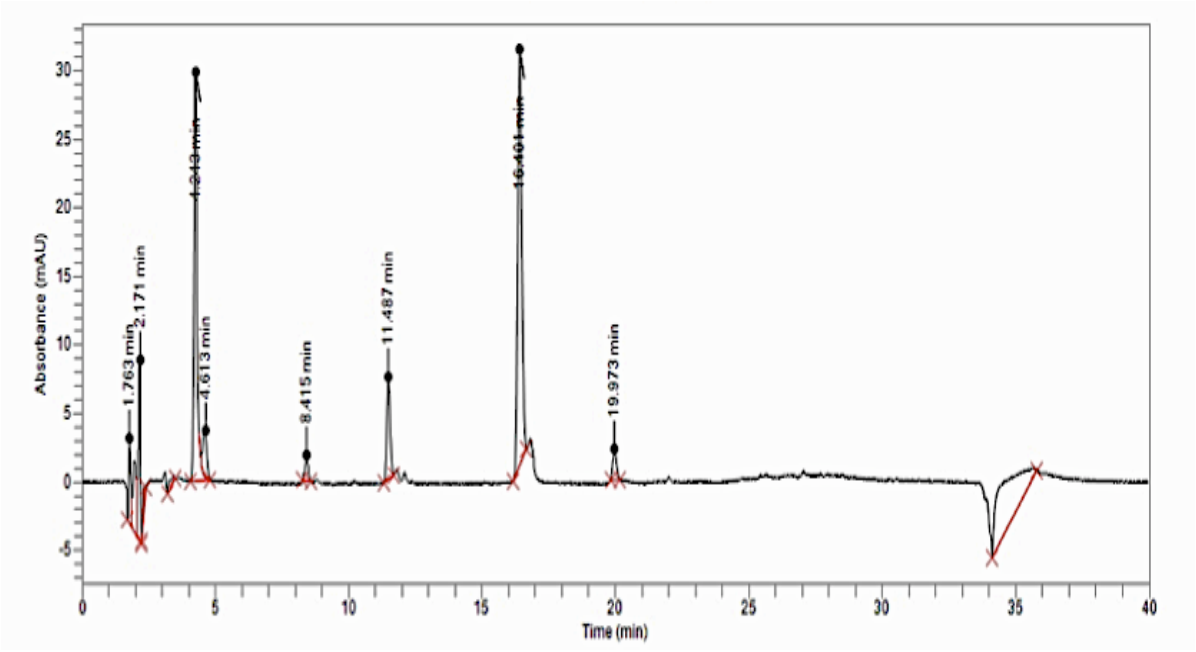


Figure 2.70: Onion extract in 80% methanol incubated with β-galactosidase from *Aspergillus oryzae* for 20 min, absorbance against time (RT= 4.243, 4.613, 8.415, 11.487, 16.401 min)

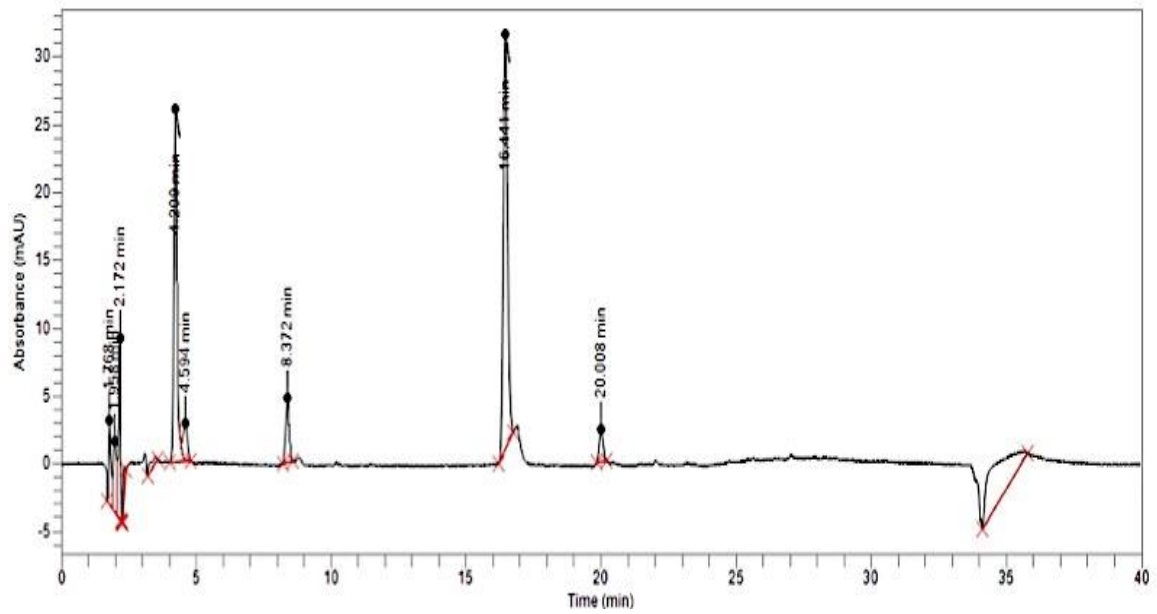


Figure 2.71: Onion extract in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 40 min, absorbance against time (RT= 4.209, 4.594, 8.372, 16.441, 20.008 min)

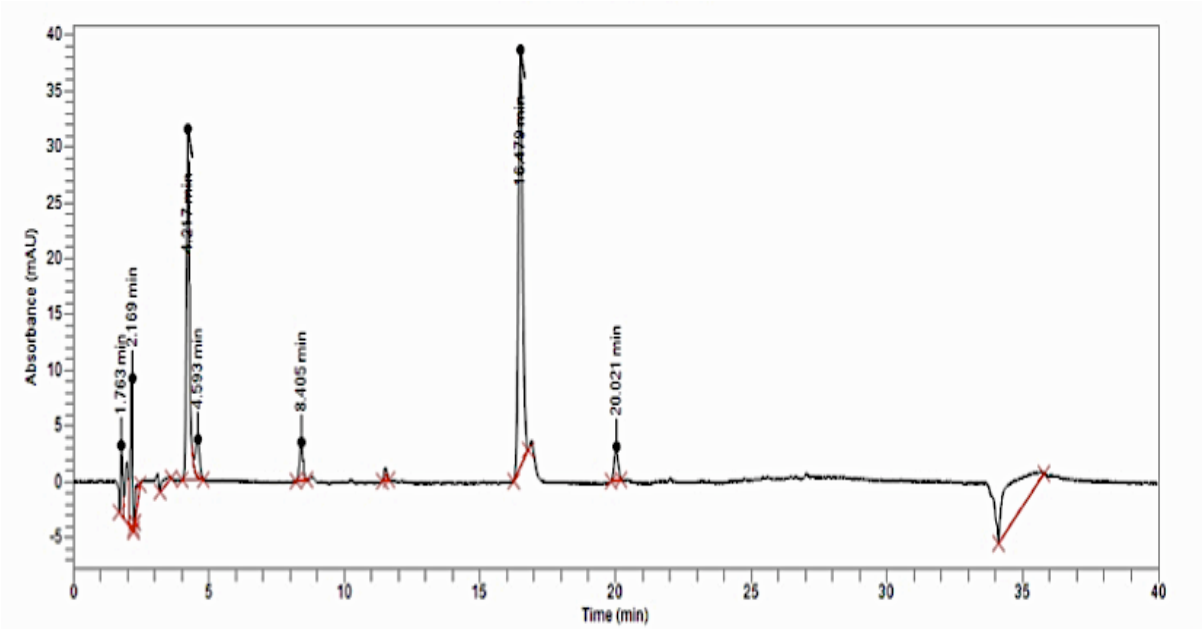


Figure 2.72: Onion extract in 80% v/v methanol incubated with β-galactosidase from *Aspergillus oryzae* for 60 min, absorbance against retention time (RT= 4.217, 4.593, 8.405, 16.479, 20.021 min)

Figure 2.69 confirms the presence of quercetin 3,4′-diglucoside and quercetin 4′-glucoside in onion extract. Figure 2.70, 2.71 and 2.72 illustrate that onion in the presence of β-galactosidase is no longer showing quercetin 4′-glucoside peak as the incubation time with β-galactosidase increased, while free quercetin peak appeared, suggesting the hydrolysis of quercetin 4′-glucoside to free quercetin.

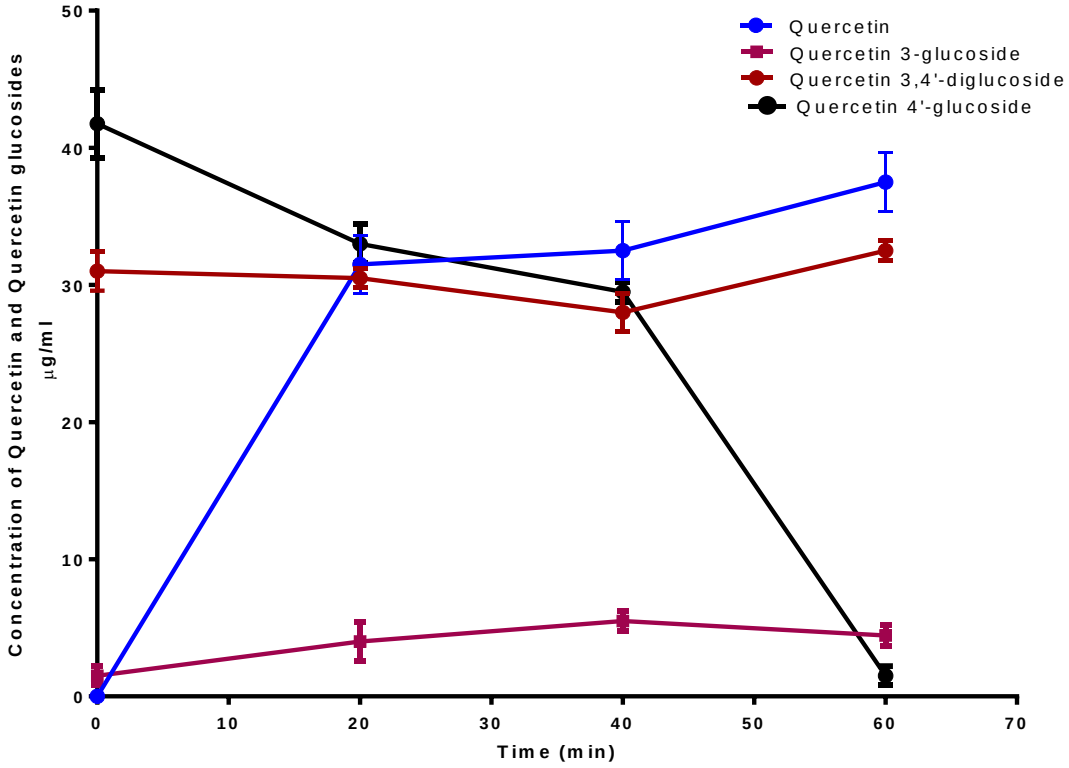


Figure 2.73: Concentration of Quercetin 3,4′-diglucoside, Quercetin 4′-glucoside and Quercetin aglycone after incubation of 25% onion extract with β-galactosidase from *Aspergillus oryzae* for 20, 40 and 60 min with (n=3 ±SD)

Figure 2.73 illustrates pre-treated red onion extract with β-galactosidase for 20, 40 and 60 min from *Aspergillus oryzae*, showed 96% decrease in the concentration of quercetin 4′-glucoside from 42.2 µg/ml to 2.3 µg/ml with $p = 0.0004$ ($p > 0.001$). However, quercetin 3,4′-diglucoside concentration 31 µg/ml remained almost the same in onion after incubation with β-galactosidase at various time points.

Our data is in accordance with previous findings from Schwanck et al. 2011, about 40% of quercetin 4′-glucoside. The main component of onion extract was deglycosylated with LPH which demonstrated its importance in the metabolism of biologically active compounds (Schwanck et al., 2011).

2.10.3. Effect of β-galactosidase enzyme from Aspergillus oryzae on onion extract and quercetin 3,4′-diglucoside

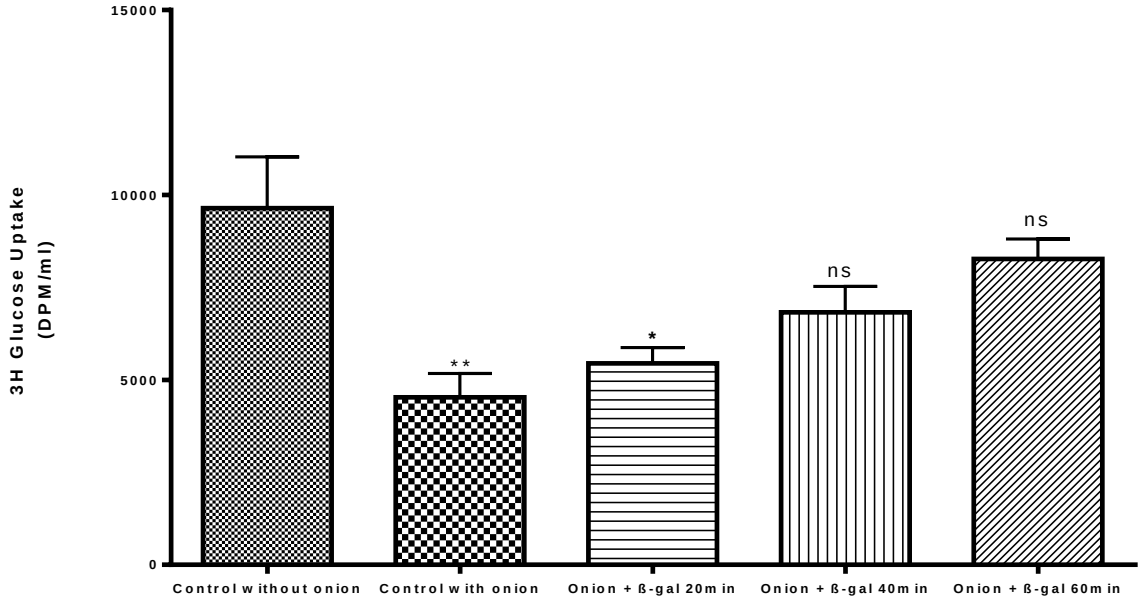


Figure 2.74: 25% w/v onion extract incubated with β-galactosidase (β-gal) from *Aspergillus oryzae* for 20, 40 and 60 minutes at sodium-dependent condition with n=3±SEM, *** $p < 0.001$, ** $p < 0.01$ and $p > 0.05$

Figure 2.74 illustrates onion extract (25% w/v) incubated with β-galactosidase from *Aspergillus oryzae* for various times, Caco-2 cells treated with onion no added β-galactosidase showed a significant reduction on glucose levels, with 53% inhibition $p = 0.001$ ($p < 0.001$). However, the pre-treated onion solution with the β-galactosidase had a no inhibitory effect, illustrating only 15% reduction after 60 minutes of incubation with β-galactosidase at a significance level of $p = 0.8$ ($p > 0.05$) (figure 2.74). This suggests that quercetin 4'-glucoside and quercetin 3,4′-diglucoside may be contributing to the inhibition of glucose uptake in Caco-2 cells. Notably, the breakdown of quercetin glucosides in onion extract to free quercetin by β-galactosidase caused less inhibition at glucose levels on Caco-2 cell lines in the presence of sodium. Therefore, β-galactosidase pre-treated onion solution caused slight inhibition of glucose uptake than un-pretreated onion extract on Caco2-cells.

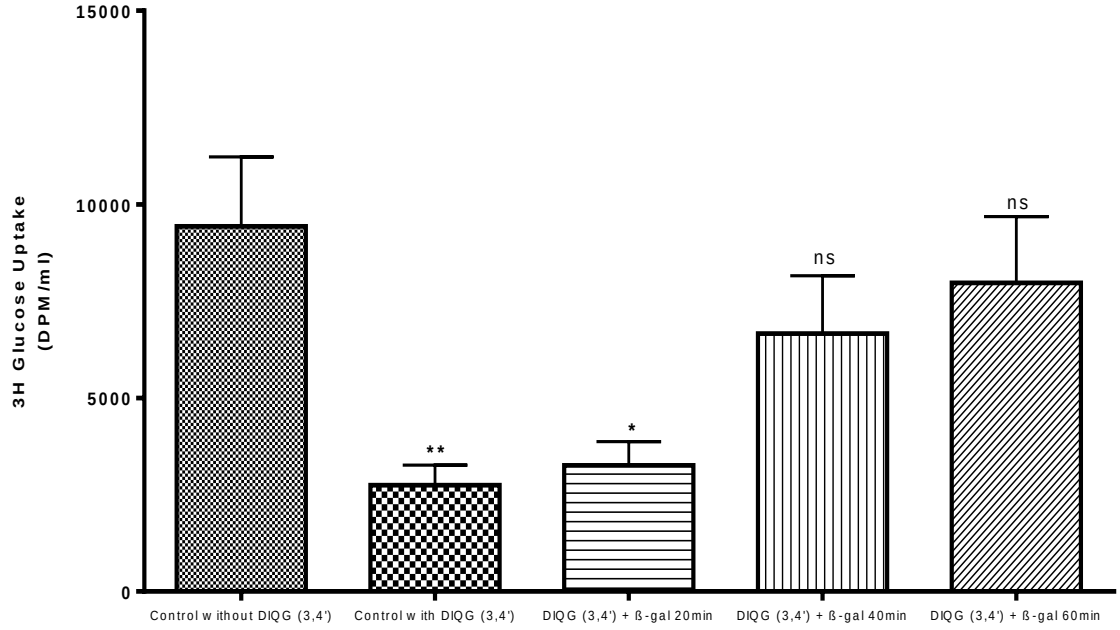


Figure 2.75: Quercetin 3,4′-diglucoside (DIQG 3,4′ at 100 µM) incubated with β-galactosidase (β-gal) from *Aspergillus oryzae* for 5, 10 and 20 minutes at sodium-dependent condition with n=3 ±SEM, ** $p < 0.01$, * $p < 0.05$ and $p > 0.05$

Figure 2.75 elucidates that pre-treated quercetin 3,4′-diglucosides for 60 minutes' incubation with β-galactosidase, caused no statistically significant inhibition $p = 0.8$ ($p > 0.05$). Whereas at control with quercetin 3,4′-diglucoside and 20 minutes' incubation with β-galactosidase, 72% and 65% significant inhibition were found with $p = 0.001$ and $p = 0.02$ $p < 0.05$ respectively.

Both figures 2.74 and 2.75 demonstrating that β-galactosidase from *Aspergillus oryzae* greatly reduced the inhibitory effect of onion and quercetin 3,4′-diglucoside after 60 minutes of incubation. Consequently, there was not a significant inhibition of glucose uptake on Caco-2 cells by both pre-treated onion and quercetin 3,4′-diglucoside with *Aspergillus oryzae* β-galactosidase for 60 minutes, only 15% reduction was found with $p > 0.05$. Our finding indicates that onion inhibited glucose uptake on the Caco-2 cells, and pre-treatment of onion extract with *Aspergillus oryzae* β-galactosidase possibly broke the intact quercetin 3,4′-diglucoside to free quercetin aglycones in onion, which therefore reduced the ability of onion to inhibit glucose uptake.

2.11. Discussion

2.11.1. Inhibitory effect of dietary flavonoids in onion and onion extract on glucose uptake via SGLT-1 and GLUT-2 on Caco-2 cells

Significant data are available which link the effect of dietary flavonoids in plants and herbs on lowering uptake of glucose and regulating the metabolism of carbohydrates. These phenolic compounds may include mechanisms for development of insulin release and improved insulin sensitivity. Therefore, there is a potential for these compounds to act as protective agents against development of type 2 diabetes and metabolic syndrome (Song et al., 2005). Also, polyphenols could decrease the postprandial increase in blood glucose levels through their interaction with glucose transporters and intestinal enzyme that are effective in the dietary glucose absorption and release (Williamson, 2013).

Polyphenols are mostly consumed in plant rich diets, fruits (apple and strawberry), vegetables (onion) and green tea. They may exist at high levels in lumen of human gut, and therefore could be transported to the intestinal glucose transporters and cause their inhibition by competing with glucose levels (Johnston et al., 2005a). Monolayers of a well differentiated human intestinal epithelial cell line, Caco-2 cells were used as a model to investigate effects of dietary polyphenols-flavonoids in onion and onion extract on transport of glucose levels across the intestinal epithelium.

Phloridzin flavonoid has been established as a known inhibitor of glucose in the sodium-dependent SGLT-1 transporter (Johnston et al., 2005a). According to figures 2.14, there was a statistically greater cellular glucose reduction as phloridzin concentration increased, 80% glucose inhibition at 100µM at significance level of $p < 0.001$ ($p = 0.0004$), followed by 65% and 50% significant glucose inhibition at 50µM and 20 µM with $p < 0.05$ ($p = 0.01$) and $p < 0.05$ ($p = 0.045$) respectively under sodium-dependent conditions (figure 2.14). On the other hand, according to figure 2.15, in the absence of sodium, there was not a drastic reduction in glucose uptake when Caco-2 cells were treated with phloridzin, even at the highest concentration 100µM, only 30% glucose reduction was observed with $p > 0.05$ ($P = 0.17$). Consequently, lower levels, 50µM and 20µM, established 20% and 15% glucose inhibition with $p > 0.05$. This result confirms that our findings were in agreement with previous research by (Johnston et al., 2005a). Also, a previous study demonstrated that the phloridzin-sensitive SGLT-1 transport, accounted for saturable glucose uptake, the diffusive component, however, it is suggested to be mediated via GLUT-2 at an apical localisation (Helliwell et al., 2000).

In accordance with that, there has been an *in vivo* study on mice, where a significant inhibition of glucose absorption was obtained after an acute administration of 15 mM phloridzin together with a 10% glucose solution to normoglycaemic mice. It is suggested that the main reduction of glucose levels occurred through SGLT-1 transporters in the presence of phloridzin flavonoids (Takki et al., 1997).

Phloridzin is also a substrate for the phloridzin domain of LPH. Phloretin, released from the hydrolytic step, will compete with other phenolics for both UDP-glucuronosyl transferase (UGT) during process of conjugation to apical and basolateral transporters (Day et al., 2003). While phloridzin is a competitive inhibitor of SGLT-1 (Mariappan and Singh, 2004), its aglycone phloretin inhibits SGLT-1 non-competitively and 800-fold less potent than phloridzin (Hirayama et al., 2001).This illustrates the crucial role of glucose moiety at 2′-position for binding to SGLT-1. It has been suggested that phloridzin binding to SGLT-1 occurs at both aglycone-binding site as well as the sugar binding site (Sala-Rabanal et al., 2012). Besides, past research has established that aglycone flavonoids, such as phloretin, can target GLUT-2; therefore, the inhibitory effect is expected to be carried out in the sodium-independent condition (Kwon et al., 2007). Moreover, according to an *in vivo* study, phloridzin has reduced over expression of SGLT-1 mRNA that occurs in diabetes. The decreased SGLT-1 mRNA is possibly a response to changes in cellular oxidative balance with the increase with an antioxidant capacity with the phloridzin treatments (Li et al., 2004).

Quercetin and its glucosides are the major flavonoids present in several vegetables, in particular fresh onions and fruits (Yahya et al., 2016) . According to figures 2.16, 2.18 and 2.20 under sodium-dependent conditions, quercetin glycosides resulted in a great Caco-2 cells glucose inhibition at 100µM with $p < 0.01$ and $p < 0.001$. As the concentration of these compounds elevated from 20µM to 100µM, further glucose inhibition was found. For example, according to figures 2.16 and 2.18, 100µM quercetin 4′-glucoside and quercetin 3,4′-diglucoside significantly reduced the uptake of glucose compared to control by 75% at significance level of ($p < 0.001$) with $p = 0.0007$ under sodium-dependent condition, followed by almost similar but less effective inhibitory role of quercetin 3-glucoside on Caco-2 glucose uptake under sodium-dependent conditions, 52% inhibition was observed by 100µM at significance level of $p = 0.012$ (figure 2.20).

However, under sodium-independent conditions, less than 30% inhibition ($p = 0.065$) was observed by 100µM of quercetin 4′-glucoside (figure 2.17), notably only 10% glucose inhibition was determined by quercetin 3,4′-diglucoside $p > 0.05$ (figure 2.19). Our finding was in accordance with a study conducted by Ader et al. (2001), where they found no significant inhibitory effect of quercetin 4′-glucoside on glucose uptake of rat-jejunum under sodium-independent conditions (Ader et al., 2001). Also, only 20% reduction in glucose uptake was found at 100µM of quercetin 3-glucoside in the absence of sodium condition ($p > 0.05$) (figure 2.21). However, there is evidence that quercetin 3-glucoside inhibited SGLT-1-independent, non-saturable uptake of glucose, this is possibly because of passive diffusion or to glucose transport via a sodium-independent glucose transporter. It is rather unlikely that quercetin 3-glucoside changed the permeability of the BBM, thus inhibiting glucose diffusion (Day et al., 2003).

Under sodium-independent conditions, it is suggested that GLUT-2 was the main pathway of glucose mass absorption across the apical membrane at high luminal glucose concentrations (Helliwell et al., 2000). This is through a rapid translocation of GLUT-2 from an internal vesicle pool into the apical membrane in response to an activation of protein kinase, initiated by SGLT-1 (sodium-dependent) mediated transport across the apical membrane (Kellet et al., 2008). The majority of studies that propose transport of polyphenolic glucoside through SGLT-1 were performed in isolated rat intestinal segments as well as Caco-2 cell models that express, in addition to SGLT-1 other carriers that could contribute to transport of polyphenolic compounds (Day et al., 2000a).

From the current *in vitro* results shown in figures 2.16, 2.18 and 2.20, it is evident that quercetin glycosides are most likely to cause inhibition of glucose uptake in the presence of sodium conditions. In several studies such as Day et al. (2003), Walgren et al. (2000) and Cermak et al. (2004), they also showed that quercetin glucosides such as quercetin 3-glucoside and quercetin 4′-glucoside caused inhibition of glucose uptake in Caco2- cell line through sodium-dependent SGLT-1 transporter. This result can be explained by the weak inhibition of GLUT-2 expressed on the apical membrane of Caco-2 cells under sodium dependent conditions. Moreover, it is demonstrated that quercetin 4′-glucoside is effluxed from Caco-2 monolayers through apically expressed multidrug resistance-associated-protein, MRP2 (Walgren et al., 2000). Thus, quercetin 4′-glucoside absorption in intestinal apical

membranes appears to be controlled by the balance between SGLT-1 uptake and MRP2 efflux (Walgren et al., 2000).

In a study conducted by (Kwon et al., 2007), they demonstrated that flavonoid glucosides such as quercetin 4′-glucoside and quercetin 3-glucoside were not transported by SGLT-1. They failed to detect high transport of either glycosylated or non-glycosylated flavonoids; however, they detected several compounds that potentially reduced electrogenic SGLT-1-mediated α-MDG uptake. Moreover, in another study conducted by Murota et al. (2000), they observed no transport of quercetin glucosides, such as quercetin 4′-glucoside and quercetin 3,4′-diglucoside across the apical membrane of Caco-2 cells (Murota et al., 2000). It should be noted that most of the studies that suggested the transport of flavonoid glucosides through SGLT-1 were conducted in isolated rat intestinal segments along with cell culture models (Caco-2 cells) which also express other carriers such as GLUT-2 and multi-drug resistance-associated protein 2 (MRP2) could help in the transport and bioavailability of polyphenolic compounds in onion.

According to figure 2.22, in the presence of quercetin aglycone, there was no statistically significant inhibition on the cellular glucose uptake under sodium-dependent conditions at 100μM concentration with $p > 0.05$ ($p = 0.1$); quercetin is an aglycone, which according to previous studies does not inhibit the sodium-dependent SGLT-1 glucose uptake pathway (Kwon et al., 2007). However, figure 2.23 demonstrated a significant inhibitory effect of quercetin on glucose uptake in the sodium free condition at 100 μM with 58% reduction compared to control ($p = 0.008$) $p < 0.01$. Quercetin aglycone blocks glucose uptake in the absence of sodium which is most likely through GLUT-2 (Kwon et al., 2007). It is suggested that this could be because GLUT-2 translocates to the apical membrane of enterocytes when exposed to high concentrations of glucose (Zheng et al., 2011). Interestingly, Barranetxe et al. (2006) found that the uptake of maltose and galactose, but not glucose, was decreased by quercetin through SGLT-1. Also, Kwon et al. (2007) and Johnston et al. (2005) illustrated that quercetin was unable to block the uptake of glucose in the sodium dependent condition, which is through SGLT-1. Also, in a previous research performed on mouse intestine, there was no uptake of glucose through GLUT-2 in the apical membrane, even at high concentrations of glucose (Roder et al., 2014). These results were in agreement with findings from a study conducted on isolated rat everted gut sleeves that did not show any GLUT-2 in the apical membrane (Scow et al., 2011).

However, GLUT-2 mediated glucose uptake into *Xenopus* oocytes injected with cRNA encoding human GLUT-2 greatly reduced in the presence of onion extract containing quercetin and quercetin glycosides (Kottra and Daniel, 2007). Our result is in agreement with findings from a previous study Zheng et al. (2011), where quercetin aglycone caused significant GLUT-2 inhibition (figure 2.22). In the absence of sodium, quercetin 4′-glucoside and quercetin 3,4′-diglucoside did not compete with glucose transport through GLUT-2 sodium-independent; therefore, no inhibition was found (figures 2.17 & 2.19) (Zheng et al., 2011).

Under sodium-independent conditions, lower glucose levels were transported by the Caco-2 cells in most of our cell culture models. This may suggest that when quercetin and its derivatives, coupled with two sodium molecules under sodium dependent conditions, there was possibly a lower transport ability for glucose levels from the apical to the basolateral side. It is suggested that inhibition of Caco-2 cells apical uptake was of mixed type, with GLUT-2 inhibited non-competitively and SGLT-1 inhibited competitively, whereas basolateral release of glucose inhibited in a non-competitive manner, indicated an effect on GLUT-2 only (Manzano and Williamson, 2010).

The high content and great inhibition of blood glucose levels by quercetin 4′-glucoside and quercetin 3,4′-diglucoside in onion solution would have suggested for their effect on SGLT-1 and GLUT-2 transporters in the subject's small intestine. Both quercetin glucosides could have hydrolysed by LPH in the small intestine, which is far less active in inhibition of SGLT-1 but highly active in GLUT-2 inhibition. This also holds true for phloridzin which can quickly cleaved to phloretin and glucose by LPH with phloretin much less effective in SGLT-1 inhibition.

In the current *in vitro* study, Caco-2 cell monolayer has been used because they mimic intestinal absorptive epithelium. However, due to their cancerous origin, they may have some limitations; for instance, several proteins are significantly different from that found in healthy animal models and human tissue (Nemeth et al., 2003).

2.11.2. Glucose transport via Apical and Basolateral compartments (Transwell inserts)

According to figure 2.25, in the presence of sodium conditions, there was a significant inhibition in Caco-2 cells glucose uptake on transwell inserts treated with 100µM quercetin 3,4′-diglucoside and 100µM quercetin 4′-glucoside, 70% and 60% with $p < 0.01$ and $p < 0.05$ respectively. The great inhibitory effects on glucose levels by quercetin glucosides are possibly caused by competitive inhibition of sodium-dependent glucose transport across the intestinal brush-border membrane by transporter SGLT-1 and MRP2. On the other hand, according to figure 2.26, under sodium dependent condition at the basal side (wells), both quercetin 3,4′-diglucoside and quercetin 4′-glucoside indicated a slight inhibitory effect on glucose uptake, it was not statistically significant $p > 0.05$. Our result are in support of previous findings where quercetin glucosides were sodium-dependent; therefore, they played through SGLT-1 transporters available at the apical membrane of Caco-2 models (Johnston et al., 2002). There is also a possibility of reduced abundance of GLUT-2 protein in response with quercetin 4′-glucoside and quercetin 3,4′-diglucoside. This could be the result of either protein degradation and/or reduced translation. The reduction in translation would arise from the decrease in GLUT-2 mRNA expression (Salucci et al., 2002).

However, stating that there is less inhibitory effect on glucose levels through GLUT-2, it is possible that degradation may be site-specific. In other words, GLUT-2 that is not localised for apical uptake is subjected to degradation. Rather, the basolaterally GLUT-2 would have been targeted. Otherwise, another glucose uptake transport may have been involved, or the apical presence of GLUT-2 is not the rate-limiting factor for intestinal glucose uptake. Therefore, more *in vitro* research is required to demonstrate the degradation and migration of GLUT-2 in Caco-2 cells.

According to figure 2.27, under sodium independent conditions, quercetin 4′-glucoside and quercetin 3,4′-diglucoside did not decrease the uptake of glucose at cellular levels with $p > 0.05$. Our data are in agreement with findings from (Lombard et al., 2002), where quercetin 4′-glucoside and quercetin 3,4′-diglucoside in the absence of sodium condition caused no glucose inhibition on Caco-2 cells, as they are mainly active at sodium-dependent condition through SGLT-1 glucose transporters. Reduction of glucose levels at the basolateral membrane of enterocytes, which in the current Caco-2 model was illustrated by the transport of glucose from the upper to lower side of transwell inserts (wells), could be associated with

inhibition of GLUT-2 by quercetin aglycone from the breakdown of quercetin glucoside through glucosidase activity (figure 2.28).

Notably, flavonoids have been identified to retain insulin-like properties (Andersom et al., 2004), and insulin is known to have impacts on the presence of GLUT-2 at apical membranes (Tobin et al., 2008). Subsequently, SGLT-1 is known to traffic between intracellular pools and the apical membrane (Khoursandi et al., 2004) and it is likely that polyphenols in onion extract effect the distribution of transporter protein without changing the overal levels of SGLT-1 proteins. However, the roles of other flavonoid importers, and their impacts on the release of glucose on basolateral membrane, followed by the effects of GLUT-2 in flavonoid efflux, need more investigation.

The source of Caco-2 cells and inter-laboratory discrepancies in protocol design contribute to differences in paracellular permeability of compounds in the monolayer. For instance, previous studies have shown that the source of Caco-2 cells could deliver 20-fold differences in TransEpithelial Electrical Resistance (TEER) because of various culture conditions and composition of cell subpopulation (Jonker et al., 2000). TEER could be used as indicators for determination of monolayer integrity and how much cells were confluent (Srinivasan et al., 2015). Change in TEER across confluent Caco-2 monolayers over time could demonstrate the level of confluency.

2.11.3. Effect of Inulin (fructan) on Caco-2 cells glucose uptake

In addition to quercetin and quercetin derivatives, inulin is a soluble fibre, which is widely found in onions. Soluble fibres may help lower blood cholesterol and glucose levels, because normal digestion does not break inulin down into monosaccharides, or elevate blood sugar levels and may, therefore, be helpful in the management of diabetes. Inulin also stimulates the growth of bacteria in the gut. Inulin passes through the stomach and duodenum undigested and is highly available to the gut bacterial flora (Reimer and Russle, 2008).

According to figures 2.29 and 2.30, on Caco-2 cells treated with 5% w/v inulin from chicory, there was no statistically significant inhibition of glucose uptake compared with control; 15% and 13% reduction were found at sodium dependent conditions with ($p = 0.6$ and $p = 0.65$) respectively. However, according to figure 2.30, the onion extract significantly decreased glucose absorption compared to control with 47% inhibition $p < 0.05$ ($p = 0.01$). Our data demonstrated the inhibitory effect of onion extract on lowering glucose levels could possibly

be due to the high presence of flavonoids. It is believed that more than 95% of the flavonoids in onion is quercetin and its derivatives and only 1-5% inulin (Perez-Gregorio et al., 2010). Hence, inulin is proposed to have a lower antiglycaemic effect in the current Caco-2 cell models. It should be noted that in this *in vitro* study, the source of inulin was chicory not onion; therefore, it initiated possibility for inconsistency in our data.

Figure 2.30 showed that under sodium-dependent condition both quercetin 4′-glucoside and quercetin 3,4′-diglucoside significantly reduced Caco-2 glucose levels ($p < 0.01$), through SGLT-1 transporter. Comparing the effect of quercetin 4′-glucoside, quercetin 3,4′-diglucoside and inulin with control on Caco-2 cells glucose uptake, inulin illustrated a non-significant inhibition on radiolabelled glucose levels by 22% and 10% at both sodium dependent and independent conditions with $p > 0.05$ (figures 2.30 and 2.31). Also, figure 2.31 showed none of the quercetin glucosides reduce the glucose uptake at a significant level in the absence of sodium condition, since they are found to be mostly active at SGLT-1 sodium-dependent transporters.

2.11.4. Overall flavonoids (phenolic compounds) effects on Caco-2 cells glucose uptake

In addition to quercetin glucosides and phloridzin in onions, some phenolic acids like p-coumaric acid and ferulic acid were found to inhibit the glucose uptake in Caco-2 cells (Manzano and Williamson, 2010). However in another study, they showed no effect of phenolic acids including chlorogenic acid, ferulic acid and caffeic acid on the levels of glucose uptake in Caco-2 cells under sodium-dependent (SGLT-1) and sodium-independent (GLUT-2) conditions (Johnston et al., 2005a). Interestingly, other research on rat intestinal membrane vesicles showed that 1mM chlorogenic acid inhibited the absorption of glucose up to 80%, while ferulic acid and caffeic acid with similar concentrations decreased the glucose uptake by only 38% and 35% respectively (Welsch et al., 1989). Therefore, the inhibitory effects of chlorogenic acids, ferulic acid and caffeic acid on glucose levels were inconsistent. However, their effect could interfere with the inhibitory effects of flavonoids in onions, and be considered as cofactors on overal glucose inhibition levels.

In the current *in vivo* study, quercetin illustrated the strongest inhibition of radiolabelled glucose uptake in Caco-2 cells through sodium-independent condition whereas, quercetin 4′-glucoside, quercetin 3,4′-diglucoside and quercetin 3-glucoside revealed strong inhibition at sodium-dependent condition. These findings propose the inhibition of glucose transport under sodium-independent condition by onion extracts were mostly due to quercetin and

phloretin and sodium-dependent glucose inhibition was caused mainly by quercetin glycosides and phloridzin.

We investigated the functional effects of flavonoids and onion extract on Caco-2 cells in order to characterise the competitive or interactive effects of flavonoids, i.e. co-incubation of flavonoids and radiolabelled glucose. The treatment with onion extract and quercetin glucosides reduced glucose uptake mediated through sodium-dependent SGLT-1 and sodium-independent GLUT-2 transporters. The acute inhibitory effects of phloridzin as well as quercetin glucosides and quercetin aglycone confirmed the function of both glucose transporters in the Caco-2 cell line as phloridzin and quercetin are specific inhibitors of SGLT-1 and GLUT-2 glucose uptake, respectively. It seems that flavonoids must be present to inhibit the uptake of glucose, as we have obtained in current *in vitro* experiments.

2.11.5. Effect of dialysis on flavonoids content in onion extract

Dialysis procedure was also conducted on onion extracts, in order to illustrate the effect of dialysis procedure on the presence of quercetin glucosides in the onion. Therefore, figure 2.50, illustrated the change in the concentrations of quercetin 3,4′-diglucosides and quercetin 4′-glucoside after 8 hours of dialysis, and the concentration of both compounds significantly reduced from 40.1µg/ml and 30.4µg/ml to 6.5µg/ml and 4.4µg/ml respectively ($p < 0.01$) (figure 2.48). Followed by greater loss of their concentrations after 24 hours of dialysis, quercetin 3,4′-diglucoside and quercetin 4′-glucoside concentrations drastically decreased to 1.7µg/ml and 1.1µg/ml respectively with $p < 0.001$ (figure 2.49). This could be due to the low molecular weight of quercetin glucosides, which result in their loss in the onion extract after dialysis.

2.11.6. Effect of dialysed onion extract on Caco-2 cells glucose uptake

Figure 2.51 demonstrated a statistically significant inhibition in Caco-2 cells glucose uptake under sodium dependent condition in the wells treated with 100 µM quercetin 3,4-diglucoside and 100 µM quercetin 4′-glucoside by 65%, $p < 0.001$. This was followed by an inhibitory influence of 25% w/v non-dialysed onion extract by 51% with $p = 0.007$, though after dialysis of onion extract, the inhibitory effect considerably decreased to 29% with $p = 0.2$.

Figure 2.52 demonstrated under sodium-independent conditions, a great inhibition of glucose levels in Caco-2 cells were observed in the wells treated with 100µM quercetin 4′-glucoside by 60% at a statistical significant level of $p < 0.01$ ($p = 0.0008$). This was followed by lower inhibitory influence of 25% w/v non-dialysed onion, 100 µM quercetin 3,4′-diglucoside and 25% w/v dialysed onion by 42%, 32% and 10% with $P > 0.05$ respectively. In onion extract, the enzymatic activity of quercetin 4′-glucosidase and quercetin 4-glucosyltransferase could increase during dialysis, therefore resulted in deglycosylation of quercetin glucosides into quercetin aglycone. Also, a less inhibitory effect was found as sodium-dependent conditions through SGLT-1 (Griffiths et al., 2002). Whereas, the slight reduction on glucose uptake levels could possibly be due to the hydrolysis of quercetin glucosides to free quercetin during dialysis procedure; therefore, GLUT-2 transporters were inhibited on the basal side.

After dialysis of the onion for 8 hours and changing the buffer every 2-3 hours, the dialysed onion resulted in about 40% loss of quercetin glucosides (figure 2.50). This could be due to low molecular weight of quercetin glucoside, which led to their loss. However, dialysed onion still caused 35% glucose inhibition on Caco2-cells. This could be due to their high concentration of sulfur compounds, such as allyl propyl disulfide and fructans (Griffiths et al., 2002).

2.11.7. Modelling of lactase (LPH) with β-galactosidase and its effects on hydrolysis of flavonoid glucosides in onions

β-galactosidase was used as its optimum pH and temperature to model human intestinal LPH. It is highly distributed in several biological systems; for instance, microorganism, plants and animal tissues. In our study, we have used β-galactosidase from *Aspergillus oryzae* (fungi) as compared to plant or animal sources of enzyme, and produce enzyme at higher yields, thus resulting in a decrease price of β-galactosidase (Santos et al., 1998). In this study, β-galactosidase from *Aspergillus oryzae* was used to model the lactase and its effect on the hydrolysis of flavonoid glucosides in onion extract. During absorption, quercetin glucosides are hydrolysed by cytosolic β-glucosidase to quercetin aglycone. It has been suggested that the main factor for the absorption of quercetin glucoside starts with hydrolysis by lactase (LPH) enzyme (Boyer et al., 2005).Therefore, deglycosylation and availability of lactase enzyme are also important factors for the absorption and transfer of flavonoids from the intestinal lumen into the circulation (Day et al., 2003). In order to model the hydrolysis of

flavonoids with intestinal lactase, β-galactosidase from *Aspergillus oryzae* was used. The role of β-galactosidase from *Aspergillus oryzae* in the deglycosylation of onion and quercetin 3,4′-diglucoside has been previously shown (Takahama and Hirota, 2000). Deglycosylation of quercetin glucosides at the brush border membrane places the resulting aglycone in a prime position for diffusion across the brush border. The deglycosylation of the quercetin glucoside would lead to a greater concentration of aglycone at the apical enterocyte membrane and eventually elevate the absorption rates (Boyer et al., 2005).

Properties, specificity and structure of β-galactosidase is dependent on the length of amino-acid chains, various molecular weight, active site position, pH and thermal optimum and stability (Zhou and Chen, 2001). These enzymes have two major properties: the removal of lactose from milk and dairy products for lactose-intolerant people and production of galactosylated products (Neri et al., 2008). β-galactosidases function in broad pH range: enzymes from fungi *Aspergillus oryzae* are active between pH 2.5-5.4 (Jacob et al., 2002). Based on our results, the β-galactosidase from *Aspergillus oryzae* displayed its optimum activity at pH 4.5 with little activity outside pH 3.0 and pH 7.0 (figure 2.54).

It may be noted that different pH optimum is the result of the presence of several enzymes in crude preparation or by the presence of several substrates on which the enzyme can act. The effect of temperature on β-galactosidase from *Aspergillus oryzae* illustrated maximum enzyme activity at temperature 45°C (figure 2.55). A similar result has also been established in a study conducted by (Ansari and Husain, 2012), in which β-galactosidase from *Aspergillus oryzae* were useful in milk hydrolysis at temperature 45°C.

According to figure 2.56, ONPG as the substrate was hydrolysed to β-galactose and ONP through activity of β-galactosidase; therefore, from 0 to 60 minutes the formation rate of ONP µmol/ml product increased. Whereas, at longer incubation times, such as after 60 minutes, there was no increase on the product formation (figure 2.56). Therefore, β-galactosidase has the highest activity at 60 minutes, while as the time increased after 60 minutes its activity was stopped, hence resulting in lower ONP (µmol/ml) formation.

2.11.8. Identification and quantification of flavonoids in onion extract before and after incubation with β-galactosidase **from** *Aspergillus oryzae*

For the quantitative determination of individual flavonoid glycosides in onion extract, the glycosides were hydrolysed by β-galactosidase from *Aspergillus oryzae*, and thus aglycones were identified and quantified by using the HPLC-DAD approach. Therefore, on the basis of the results obtained with standards, the concentration of quercetin glycosides and free quercetin were determined.

HPLC technique was conducted for identification and separation of flavonoids such as quercetin and quercetin glucosides in onion extract and to model the effect of LPH on phenolic glucosides by assessing the activity of β-galactosidase from *Aspergillus oryzae* on the hydrolysis of these phenolic glycosides in onion extract. Figure 2.60, onion extract indicated two large peaks occurring at RT 4.122 and 11.417min, which are identified as quercetin 3,4′-diglucoside and quercetin 4′-glucoside respectively by relative position of these peaks compared to standards (3,4-diglucoside and 4′-glucoside) in figures 2.58 and 2.59. Quercetin aglycone illustrated one peak at RT = 16.239min (figure 2.57), which was in accordance with the peak in the onion extract occurred at RT = 16.373 min (figure 2.60).

The incubation of quercetin 4′-glucoside with β-galactosidase *Aspergillus oryzae* for 60 minutes illustrated a UV peak at RT = 16.647 (figure 2.61), which occurred at a similar position to standard quercetin with RT = 16.239min (figure 2.57). Concentration of both quercetin and quercetin 4′-glucoside were calculated by using their standard curves (figures 2.44 and 2.45). Quercetin concentration increased from 2.2µg/ml to 12.1µg/ml, whereas quercetin 4′-glucoside reduced from 31.2µg/ml to 17.1µg/ml after 60 minutes pre-treatment with *Aspergillus oryzae* β-galactosidase (figure 2.62). Our findings were in accordance with findings from the study conducted by Nemeth et al. (2003).

Moreover, quercetin 3,4′-diglucoside was incubated with β-galactosidase from *Aspergillus oryzae*, for 20, 40 and 60 minutes. The standard quercetin 3,4′-diglucoside showed a peak at RT = 3.969min (figure 2.59), after 20 minutes with β-galactosidase incubation, two UV peaks appeared at RT 3.172 and 16.373min (figure 2.63), followed by appearance of bigger peaks occurring after 40 and 60 minutes at RT 16.479min and 16.396min respectively (figures 2.64 and 2.65), which were in accordance with quercetin standard peak with RT = 16.239 (figure 2.57).

Figure 2.63 showed hydrolysis of quercetin 3,4′-diglucoside with β-galactosidase for 20 minutes of incubation significantly reduced the concentration of quercetin 3,4′-diglucoside by 58% at significance level of $p = 0.022$ ($p < 0.05$), while quercetin aglycone levels increased by 15%. There was a steady reduction and increase in quercetin 3,4′-diglucoside and quercetin respectively after 40 minutes of incubation with β-galactosidase (figure 2.64). Quercetin 3,4′-diglucoside concentration decreased from 40.1µg/ml to 11.2µg/ml (figure 2.65) indicated its break down by β-galactosidase which caused an increase in quercetin concentration from 0.7 to 13.3µg/ml after 60 minutes of incubation (figure 2.66).

Figure 2.67, Isorhamnetin showed a peak at RT = 20.027 which was not in a similar position compared to onion extract peaks (figure 2.60). Thus, this compound was not found in this particular onion type in the current research. UV peaks from quercetin 3-glucoside appeared at RT = 8.585 (figure 2.68) which was in a similar position compared to the chromatogram of onion extract at RT = 8.612 (figure 2.60); notably, quercetin 3-glucoside was counted for only 6% of total flavonoids AUC in the onion extract.

Figure 2.69 showed the main flavonoids in onion such as quercetin 3,4′-diglucoside and quercetin 4′-glucoside in 25% w/v red onion extract. Figures 2.70, 2.71 and 2.72 demonstrated that onion extract in the presence of β-galactosidase no longer possessed quercetin 4′-glucoside peak as the incubation time with β-galactosidase increased, while free quercetin peak occurred at RT = 16.479. Figure 2.73 illustrated the change in concentration of these quercetin glucoside in an onion extract after incubation with β-galactosidase. Hence, pre-treated red onion extract (25% w/v) with β-galactosidase for 20, 40 and 60 minutes from *Aspergillus oryzae* presented a statistically significant 96% reduction in the concentration of quercetin 4′-glucoside from 42.2µg/ml to 2.3µg/ml with $p = 0.0004$ ($p > 0.001$). Whereas, quercetin 3,4′-diglucoside concentration 31µg/ml stayed with almost no change in onion after incubation with β-galactosidase from 0 to 60 minutes (figure 2.73).

Our data supports the idea that β-galactosidase causes deglycosylation of quercetin glucosides (quercetin 4′-glucoside) which suggests that quercetin glucoside can no longer stay intact in the presence of β-galactosidase; thus, free quercetin will be released. Nemeth et al. (2003) have already published the substrate specificity of purified sheep LPH for the above quercetin glucosides, which is in line with our present findings that quercetin 4′-glucoside and onion are suitable substrates for β-galactosidase hydrolysis. Also, our data is

in agreement with previous research from Schwanck et al. (2011), as about 40% of quercetin 4′-glucoside was hydrolysed with LPH which showed its importance in the metabolism of biologically active compounds (Schwanck et al., 2011).

Figures 2.74 and 2.75 illustrated that on Caco-2 cells under sodium dependent condition, both onion extract and quercetin 3,4′-diglucoside were hydrolysed by β-galactosidase enzyme, suggesting the release of free aglycone, thus lower inhibition was found in glucose uptake in Caco-2 cell models. β-galactosidase from *Aspergillus oryzae*, prominently decreased the inhibitory effect of 25% w/v onion extract and 100µM quercetin 3,4′-diglucoside after 60 minutes of incubation. Therefore, there was not a significant inhibition of glucose uptake on Caco-2 cells under sodium dependent conditions by both pre-treated onion extract and quercetin 3,4′-diglucoside with *Aspergillus oryzae* β-galactosidase for 60 minutes, only 15% and 17% reduction were observed with $p > 0.05$ (figure 2.74 & 2.75). Our result suggests that onion extract inhibited Caco-2 cell glucose uptake as pre-treatment of onion extract with *Aspergillus oryzae* β-galactosidase possibly deglycosylated the intact quercetin 3,4′-diglucoside to free quercetin aglycones, and consequently diminished the ability of onion on inhibition of glucose levels, although such deglycosylation has been previously shown (Takahama and Hirota, 2000).

We demonstrated that the principal quercetin compounds in onion are the β-glycosides quercetin 3,4′-diglucoside and quercetin 4′-glucoside. We found that β-galactosidase (*Aspergillus oryzae*) increased the levels of quercetin aglycone, with concomitant reductions in quercetin 3,4′-diglucoside and mainly quercetin 4′-glucoside, and thus confirmed that *Aspergillus oryzae* β-galactosidase may regioselectively deglycosylate the 4′-O-β linked glycosyl moiety of quercetin 3,4′-diglucoside and quercetin 4′-glucoside of onion (Chung et al., 2011). Furthermore, the loss of quercetin 3,4′-diglucoside and quercetin 4′-glucoside after 60 minutes could also be due to the conversion of quercetin 3,4′-diglucoside into quercetin 4′-glucoside by the activity of quercetin 3-*O*-β-glucosidase, and to the conversion of quercetin 4′-glucoside in quercetin by the activity of quercetin 4′-*O*-β-glucosidase (Perez-Gregorio et al., 2010).

The β-galactosidase-pretreated onion solution showed lower levels of quercetin glucosides than un-pretreated onion solution. This also supports our previous findings where intact quercetin 4′-glucoside and quercetin 3,4′-diglucoside in onion may be contributing to a decrease in glucose levels of Caco-2 cells. Notably, according to our results, the content of

total flavonoids is higher in fresh red onion extract compared to the red onion solution boiled for 15 minutes. The loss of quercetin and quercetin derivatives after boiling condition could be due to the high temperature or filtration procedure.

2.11.9. LPH effects on hydrolysis of flavonoids on glucose uptake in intestine

In vitro studies with purified LPH from sheep intestine demonstrated that several flavonoid glucosides were substrates for LPH enzyme (Day et al., 2000c). In a study conducted by Art et al., 2004, they showed that during perfusion of the small intestine of rat with quercetin-3-glucoside, inhibition of LPH caused up to 67% decrease in hydrolysis of quercetin-3-glucoside. Studies with isolated human LPH demonstrated high deglycosylation of phloridzin. Moreover, both quercetin 4′-glucoside and quercetin 3-glucoside were observed as LPH substrates, but determined lower affinities than phloridzin (Nemeth et al., 2003). Therefore, LPH may have substrate specificity for hydrolysis of different quercetin glucosides, which leads to their varying bioavailability (Arts et al., 2004).

According to two other studies on rat intestine tissues conducted by Sesink et al. (2003) and Day et al. (2003), there was a reduction in the deglycosylation of quercetin 3-glucoside and quercetin 4′-glucoside when treated with LPH inhibitor *N*-butyldeoxygalactonojirimycin (NB-DJG). Therefore, this overlapped with slight appearance of quercetin on intestinal serosal side, indicating the role of LPH on quercetin 3-glucoside and quercetin 4′-glucoside at intestinal levels (Day et al., 2003, Sesink et al., 2003). It was suggested that NB-DJG LPH inhibitor decreased the hydrolysis of quercetin 4′-glucoside, and therefore transport of quercetin aglycone was reduced in intestinal tissues, meaning SGLT-1 played a minor role in intestinal uptake of quercetin glucoside *in vivo*.

It is noteworthy that LPH can also cause deglycosylation of daidzein-7-O-glucoside in the rat small intestine (Wilkinson et al., 2003). In addition to the effect of LPH, cytosolic β-glucosidase (CBG) was also found to be effective in deglycosylation of flavonoid glucosides; the deglycosylation of trans-piceid in the presence of purified rat small intestine with both LPH and CBG resulted in the release of trans-resveratrol from trans-piceid (Wilkinson et al., 2003). Although several other apical transporters (MCT1, PEPT1, SMCT) of intestinal epithelial cells are likely to be involved in the absorption of these polyphenolic compounds from onions, by which they cross the intestinal brush border membrane (Wilkinson et al., 2003).

2.11.10. Possible factors affecting the bioavailability and absorption of flavonoids

There are several important factors which could affect the bioavailability and rate of absorption of quercetin glucosides by intestine. Indeed, quercetin is present as conjugates with the flavonoid aglycone linked to variable sugar moieties by β-glyosidic bond, dietary flavonoids are attached to sugar residues which affect the mechanism of absorption by altering their physicochemical properties and ability to enter cells, or to interact with transporters and cellular lipoproteins (Day et al., 2003). Moreover, deglycosylation and availability of lactase enzyme (LPH) are also important factors for the absorption and transfer of flavonoids from the intestinal lumen into circulation (Day et al., 2003). The deglycosylation of the quercetin glucoside would lead to a greater concentration of aglycone at the apical enterocyte membrane and eventually elevate the absorption rates (Boyer et al., 2005). Moreover, it is important to consider the conjugation reactions, such as sulfation or glucuronidation, which result in modification of quercetin and quercetin glucosides in liver and intestinal cells (Day et al., 2000c).

Caco-2 cells, due to their cancerous origin, may have some limitations; for instance, several proteins are significantly different from that found in healthy animal models and human tissue (Nemeth et al., 2003). In terms of bioavailability of flavonoids, it is suggested that quercetin glucosides are more bioavailable in humans than the quercetin aglycone (Boyer et al., 2005). Quercetin glucoside is more stable than quercetin and is consequently more likely to stay intact in the gut. For instance, quercetin-glucoside bioavailability in Caco-2 cells increased up to 2-fold, while there was no change in the absorption of quercetin (Boyer et al., 2005). Also, Hollman et al (1995) showed that 52% of the quercetin glucosides from onions was absorbed, whereas only 24% of free quercetin was absorbed in human intestinal model. It would therefore appear that the quercetin glucosides are better absorbed than the free aglycone (Salucci et al., 2002).

2.11.11. Effect of hydroxylation and localisation of flavonoids on intestinal metabolism

Onion extract is also rich in anthocyanins such as cyanidin, petunidin and delphinidin (Park, 1999). These anthocyanins are all hydroxylated at the C-5 and C-7 positions. Hydroxylation at these positions has been previously discussed as a structural feature that elevates inhibitory effects of flavonoids on Caco-2 cells glucose absorption (Park, 1999). The research conducted by Park (1999) is of high importance as he evaluated 10 different flavonoid species, the structural classes of which covered flavones, flavonols, flavanones, isoflavones,

anthocyanidins and catechins. He also investigated various hydroxylation patterns, orientation of the B-ring, availability of a ketone group, presence of double bonds on the 6-member rings and moiety effect on inhibitory effects of these compounds.

Notably, Park (1999) found that hydroxylation at specific positions was effective for glucose inhibition; for instance, hydroxylation at C-5 and C-7 are necessary, while at C-3 and C-3', it had no effect. Moreover, positioning of the B-ring is crucial, as flavonoids have a greater inhibitory property than isoflavonoids that contain the B-ring conjugated at C-3 rather than C-2. Also, presence of a double bond among C-2 and C-3 raised the inhibitory capacity (Park, 1999). Other studies have shown that glucose moieties will bind to the similar transporter binding site as hexoses; therefore, competitively delaying the transport of monosaccharide through steric interference preventing transport of glucose alone (Johnston et al., 2005a). Alternatively, both glycoside and aglycone flavonoids may bind to a separate binding site, affecting both transporter function and membrane integrity (Nakayama et al., 2000). However, regardless of the possibility of non-specific polyphenol interactions with any membrane or any glucose binding site, in support of our current findings, this inhibition has been previously shown in Caco-2 and *Xenopus* oocytes, the effect seems to be glucose transporter specific (Kwon et al., 2007, Salucci et al., 2002).

As GLUT-2 translocates in accordance to its substrate; therefore, it is crucial to detect its localisation at the suitable area to be inhibited (Helliwell et al., 2000). In our research, we have shown the inhibition of GLUT-2 under sodium-independent condition through function of GLUT-2 on basolateral compartment. Other studies have also illustrated appearance of GLUT-2 on the basolateral membrane (Kwon et al., 2007). Here we have demonstrated on Caco-2 cell models that, mimicking human gastrointestinal enterocyte, flavonoids could inhibit both glucose-transporters: i.e. apical uptake from the lumen and glucose into blood circulation through basolateral membrane. This is important because metabolism of flavonoid will involve the apical reflux of flavonoids as well as biliary excretion into intestinal lumen (Lotito et al., 2011). Thus, the presence of flavonoids or their reabsorption before arrival of dietary glucose into lumen of intestine could lead to a great inhibition of glucose transport at intracellular levels.

Moreover, it is suggested that membrane proteins can interact with lipid bilayers on the C-13 terminal loop of SGLT-1, therefore that interactions of quercetin glycosides with phospholipids prevent proper conformational changes of membrane proteins essential for substrate binding, thus leads to glucose transport inhibition (Raja and Kinne, 2005).

In the current *in vitro* study, the effects of polyphenols on glucose transport across the intestine were demonstrated by using intact cellular models, Caco-2 cells. Here, two parameters were measured, the amount of glucose that appeared inside the cells, as a result of apical uptake, and the amount that appears in the basolateral side, indicated the amount that passed through the apical and basolateral sides. According to our findings, quercetin 4′-+glucoside and quercetin 3,4′-diglucoside, quercetin 3-glucoside and phloridzin and onion extract all inhibited this transfer from the apical to the basolateral side, mainly by inhibition of glucose transport under sodium-dependent conditions with less inhibition under sodium-independent conditions. In support of our findings, one study showed that strawberry and apple extracts also inhibited this apical to basolateral transfer (Manzano and Williamson, 2010).

2.11.12.Other mechanisms for the biological effects of polyphenol consumption

The metabolism of polyphenols is expected to be a key factor determining whether a normal diet can deliver effective concentrations of polyphenols to target tissues. The bioavailability of different polyphenols is highly variable, containing diverse and complex physiological processes. There are between subject variations, which are partially explained by heterogeneity in enzymatic activity and gut flora in human beings (de Bock, et al. 2012).

It is found that concentration of polyphenol metabolite in humans after normal dietary exposure is mostly below 1 µmol/L. *In vitro* studies use media mostly in excess of 1 µmol/L for sustained period of time (Manach et al., 2004). Blood concentrations may provide information on polyphenol absorption but they do not necessarily correspond to target tissue levels. Therefore, measuring polyphenol levels in plasma may not accurately evaluate exposure (Hanhineva et al., 2010). Once absorbed, polyphenols undergo rapid reconjugation and excretion through similar pathways as pharmaceutical drugs. Whereas, pharmaceutical drugs manage to saturate these pathways, polyphenols in normal dietary levels do not. The rate of reconjugation of polyphenols is associated with genetic polymorphism for example catechol-O-methyltransferase and environmental factors such as smoking (Lotito et al., 2011). Nevertheless, the net effect of diverse bioavailability, failure to saturate metabolic

pathways, and rapid clearance determines why plasma levels of polyphenols in most human studies do not reach the required levels as per *in vitro* and animal studies (Scalbert and Williamson, 2000). However, the intestinal absorption can be high, measurement of the parent polyphenol in blood rarely exceeds 1% of the raw product (Scalbert and Williamson, 2000). The half-life values of antioxidant and anti-inflammatory polyphenol properties obtained *in vitro* are 5 to 100 µmol/L, a target hardly found in human studies. For instance, eating five apples let to no detectable antioxidant effects, even though just 1% polyphenol bioavailability would likely necessary to yield the observe *in vitro* effects (Lotito et al., 2011). In order to mimic an *in vitro* environment, several intakes per day of polyphenol-rich diet would be required to ingest the amount and mix of polyphenols necessary to apply the observed beneficial effects on glucose homeostasis.

2.11.12.1. Calorie restricted diet

In mammals calorie restriction (CR) repeatedly delay disease onset, thereby prolonging healthspan. As diet consumption also seems to influence health, like preventing CVD and type 2 diabetes. Interestingly, polyphenols intake and calorie restriction seem to have similar effects on metabolism in humans, and supplementation with polyphenols induce may decrease negative effects from a high fat diet in mice (Pallauf et al., 2013). Thus, polyphenols are also referred to as CR mimetics. An important mechanism by which polyphenols induce CR-like signaling pathways suggested to function through sirtuin activation. Sirtuins are NAD-dependent deacetylases that were shown to be involved in the metabolism in different organisms (Pallauf et al., 2013). More evidence exists in support of the polyphenols role in gene regulation through gamma coactivator 1-alpha (PGC-1α) and transcription factors such as a nuclear factor-erythroid 2 (Nrf2), all of which are also implicated in CR-mediated effects (Shai, et al. 2008).

Mediterranean diet, which involves restricted-calorie diet and meals rich in vegetables with high amounts of secondary plant bioactives such as polyphenols, appears to be a safe strategy to benefit from potentially healthspan-improving CR mimetics (Amiot et al., 2016). According to a study, mice with diets rich in olive oil phenolics had reduced oxidative damage markers such as lipid peroxides, and improved expression of Nrf2-dependentt gene encoding antioxidant proteins (Pallauf et al., 2013). Resveratrol in red wine and quercetin in onion highly consumed in Mediterranean diet could both induce Sirtuin, results in endothelium protection and inhibition of inflammation via NF*k*B downregulation (Shai, et

al. 2008).

2.11.12.2. Epigenetics

Epigenetics is the study of heritable changes in gene expression caused by mechansims other than changes in the underlying DNA sequence. During epigenetic changes, the nucleotide sequence of a gene does not change, however other alterations occur in the DNA, like DNA methylation or histone acetylation that affect gene expression (Korzus, 2010). Changes in the pattern of DNA methylation has been associated with a range of activities in prevention and alleviation of various diseases such as cancer, type 2 diabetes and nueroinflammation (Ayissi et al., 2014). It has been suggested that many polyphenols exert some of their beneficial effects through chromatin remodeling and epigenetic modifications. Initial evidence indicates that dietary polyphenols change epigenetic state and accordingly might reverse abnormal gene expression (Rahman and Chung, 2010). These compounds are able to alter DNA methylation and histone modifications, leading to gene activation or silencing. Many polyphenols, such as resveratrol and catechins are reported to modulate nuclear factor kappa B (NF kappaB) expression and chromatin remodeling through modulation of histone deacethylase (HDAC) and DNA methyl transferase (DNMI) activities (Ayissi et al., 2014).

2.11.13. Common Pitfalls in Caco-2 Assays

Some of the current *in vitro* results were unexpected due to possible limitations associated with cell culture studies. Therefore, it is important to note how accurately cell culture techniques are performed. For instance, improper distribution of cells in the wells and inserts or low viability rate of Caco-2 cells in the wells, could cause inconsistency in the radiolabelled glucose uptake levels. Moreover, the high likelihood of contamination or over-growth of cells within 5-20 days need to be considered. Also, inaccurate use of Gilson pipettes may determine a great possibility on incorrect volume of the sample solutions to be taken, and lead to discrepancy of our findings.

At cellular level, it has been shown that there is a significant difference in mRNA expression levels of efflux transporters in Caco-2 cell monolayer compared to small and large intestines in humans. The variations in transporter proteins expression in Caco-2 cell monolayer create false negative results that may not associate with human or animal studies (Awortwe et al., 2014).Moreover, the expression of CYP3A4-intestinal Cytochrome plays a vital role in the metabolism of flavonoids and herbs. An ideal cell-based model for intestinal permeability studies should mimic the human gastrointestinal enterocytes by expressing metabolic

enzymes together with the lipid bilayer combined with transporters and tight junctions to give a consistent representation of the interaction between CYP3A4 and (P-glycoprotein) P-gp. However, there is a low expression of CYP3A4 on Caco-2 cell monolayer which fails to simulate *in vivo* intestinal environment, and is considered the core flavonoid-metabolising enzyme in the human epithelial cells (Rege et al., 2002).

Several factors that affect the permeability of flavonoid compounds on Caco-2 monolayers include: the amount of flavonoid compounds that appeared in the receiver (wells) compartment, the initial concentration in the donor section (inserts), and the surface area of the physical barrier (for example, lipid bilayers and cell monolayer) affect the permeability. It is also possible that these polyphenolic compounds non-specifically bind to plastic devices (transwell plates), changing the concentration in the donor and receiver compartments during incubation period and therefore leading to underestimation of permeability estimates and false negatives. Non-specific binding can also occur during transfer of cultured sample together with the quercetin glucosides or onion into another container.

Another important factor could be the lack of adequate solubility; interestingly, the permeability evaluation of test compounds in Caco-2 is underestimated due to poor aqueous solubility. In our study, organic co-solvent such as DMSO was added to the tests samples at a concentration less than1% v/v. Above this concentration, the cell tight junction is compromised and reduces the monolayer viability (Bogman et al., 2003).

Intestinal pH variations from the upper small intestine to the distal large intestine define the nature of compounds absorbed at a specific region. For instance, weak acidic compounds remain unchanged in the upper small intestine because of high pH. Thus, passive transcellular transport becomes the main absorption route into the circulation (Rege et al., 2001). Caco-2 studies are incapable of simulating the changes of intestinal pH system since it is performed at constant pH conditions (apical pH of 6.5 and basolateral pH of 7.4). Any changes in pH of Caco-2 cell protocols may influence its cytoarchitecture and underestimate the permeability characteristics (Awortwe et al., 2014).

Other experimental variability, for example, different laboratories work under diverse experimental conditions such as culturing, passage number and culture duration, could account for differences in permeability rates. Also, the inter-laboratory variations affect the

expression levels of glucose transporters which depend on the age of the cell culturing, culture duration and passage number (Balimane and Chong, 2005).

3. CHAPTER THREE - IN VIVO STUDY

3.1 Introduction

3.1.1. Clinical Study

Some dietary polyphenols can modify the apparent GI of foods by reducing glucose absorption (Hanhineva et al., 2010a). Onions are a major dietary source of polyphenols, such as quercetin glucosides, and the glucosides in an onion meal can be completely hydrolysed in the human small intestine to quercetin, as demonstrated in ileostomy patients (Walle et al., 2000).

According to a previous study conducted by Walgren, et al. 2000, both quercetin 4′-glucoside and quercetin 3,4′-diglucoside in an onion solution were effectively hydrolysed in the human small intestine to the quercetin aglycone. It was determined that almost none or only trace amounts of both quercetin glucosides appeared in the ileostomy fluid, after the hydrolysis of onion flavonoids components with LPH (Walgren et al., 2000). In support of that, in the current *in vitro* study, quercetin 4′-glucoside and quercetin 3,4′-diglucoside were hydrolysed to free quercetin aglycone by β-galactosidase from *Aspergillus oryzae*. This was followed by incubation with Caco-2 cells to show the changes on glucose uptake, which modelled the effect of intestinal LPH on quercetin glucosides hydrolysis and glucose uptake.

The β-glycosidase lactase phlorizin hydrolase (LPH) is responsible for hydrolysis on the luminal side of enterocytes (Day et al., 2000a). LPH is expressed in lactose-tolerant people but not in lactose-intolerant people. Hence, we hypothesised that quercetin glucosides may be less effective at blocking glucose uptake in lactose-tolerant people (because they can hydrolyse quercetin glucosides to the aglycone with LPH) compared to lactose-intolerant people. We tested this hypothesis using an onion meal, since onions are a rich source of quercetin glucosides (mainly quercetin 4′-glucoside and quercetin 3,4′-diglucoside), but contain very little free quercetin (Lombard et al., 2002).

3.1.2. Aims

- To investigate whether a food extract (onion solution) acts as inhibitors of the uptake of glucose in human subjects
- To compare the glucose levels in lactose tolerant and intolerant subjects after intake of onion solution

3.2. Materials used in vivo study

Consumables/Equipment
Hydrogen breath analyser (Bedfont)
Blood glucose analyser (EKF)
Box of test strips
Lancet (bunzlehealthcare)
Non-sterile gloves
Sharps container
Multi standard 12mmol/L ready for use-2ml (EKF diagnostic)
Biosen sensor test solution Glucose/Lactate 1ml tubes
Glucose/Lactase haem solution tubes-20µl Capillary
Biosen glucose/Lactate system solution 2.5L Can

Table 3.1: Materials used in human physiology lab

3.2. Methods

3.2.1. Participants

All 24 participants gave written consent and participant information was coded. Exclusion criteria: under 18 years old; history of blood disorder; pregnant; diabetic; data on contraceptive pill use was not collected. The protocol was approved by University of Hertfordshire Ethics Committee (Protocol cumber LMS/PG/UH/00187, Appendix 1 & 2) and all participants gave written consent (Appendix 3).

24-Participants	Age ± SD	Female / Male	P-value (Age) LT vs. LINT	BMI (kg/m^2)±SD	P-value (BMI kg/m^2) LT vs. LINT
12-Lactose Tolerant (LT)	27.6± 4.6	8 / 4	0.4	22±3.4	0.18
12-Lactose Intolerant (LINT)	29.8±7.4	7 / 5		24.3±4.3	

Table 3.2: The mean and SD of Age and BMI (kg/m^2) for 12 lactose-tolerant and 12 lactose-intolerant individuals at significance level of $p < 0.05$

3.2.2. Onion meal preparation

Fresh red onions were purchased from Asda super market (10 fresh organic red onions in total, 500 grams). Onion bulbs had their dry skin peeled off to the first whole fresh scale, cut into 4-6 mm cubes and then 18-20g aliquots were left as raw. Afterwards, they were homogenised in water (25% w/v), boiled for 15 minutes, cooled to room temperature and filtered twice through a handheld baking sieve. Quercetin and quercetin glucosides were analysed by reverse phase HPLC (Lombard et al., 2002).

3.2.3. Hydrogen breath test (Lactose-Intolerance Diagnosis)

The Gastro^{+} Gastrolyzer (Bedfont product number GP0221400913) is a breath hydrogen monitor used to measure hydrogen levels in expired breath (Bedfont, 2016). It is intended for multi-patient use by healthcare professionals in a clinical environment. It can be used to indicate the following disorders, carbohydrates breakdown deficiency, carbohydrate malabsorption, lactose intolerance and bacterial overgrowth (Ghoshal, 2011).

A Breath test was performed after an overnight fast. At the start of the test, fasting breath hydrogen was estimated 3 to 4 times and the average of these values was taken as the basal breath hydrogen.

Operation of the Gastrolyzer is straightforward. A D-piece sampling system enabled end-expired breath to be sampled easily and hygienically, using single-use disposable cardboard Faltpak mouthpieces and screen to ensure ease of operation. Also, the users can view results in a tabular or graphical format. Temperature may affect the accuracy of the Gastrolyzer. The instrument was calibrated at 21 °C (±4 °C). If the Gastrolyzer is used at a lower temperature than when it was calibrated, readings may be lower. When using at a higher temperature than calibrated, reading may be higher (Eisenmann et al., 2008).

The participants were asked to inhale as deeply as possible and hold their breath throughout the on-screen sound during the last three seconds of the countdown. Then they were asked to exhale slowly but gently into the mouthpiece, aiming to empty the lungs as far as possible. Lactose intolerance was measured by using the hydrogen breath test analyser. Lactose (25g dissolved in 220 ml water) was given to subjects and breath hydrogen was measured over 2 hours. Hydrogen levels 20 ppm above baseline were classified as lactose intolerance (Eisenmann et al., 2008).

Figure 3.1: Hydrogen breath analyser (Gastrolyzer).

3.2.4. Blood Glucose measurement (finger prick)

A self-monitoring or blood glucose monitoring by finger prick is a quick and easy method of identifying problems with blood sugar levels. EKF Diagnostics is a worldwide manufacturer of point of care equipment for the measurement of glucose, lactate, haemoglobin and glycated haemoglobin (HbA1C). EKF (Biosen S-Line Lab^{+} with catalogue number 5222-0123-6200) glucose analyser can test blood, plasma or serum to provide glucose and lactase values with excellent precision (less than >2% CV) over a wide measurement range. The figure below demonstrates the steps taken in the finger prick collection of blood and measurement of glucose levels (EKF, 2010).

Figure 3.2: EKF glucose analyser and steps involved in finger prick blood collection (EKF, 2010).

3.2.5. Glucose tolerance test

The participants were asked to avoid strenuous exercise and not intake alcohol or caffeine at night before the study day (Johnston et al., 2003). Finger prick capillary blood samples were obtained after an overnight fast and at 15, 30, 60, 90 and 120 minutes after drinking a glucose solution (50 g glucose dissolved in 220 ml water). The same blood collection regime was then repeated on a subsequent day after participants had consumed 220 ml of an onion meal (25% w/v) containing glucose (50 g). Glucose was measured with an EKF glucose analyser. The incremental area under the time glucose curve (AUC) was calculated using a linear trapezoidal method in Excel, taking the fasting blood glucose concentration as the baseline. The mean changes for peak glucose and blood glucose AUC were then compared between lactose-tolerant and lactose-intolerant groups in Excel by two-tailed t-tests and using two-sample unequal variance. Quality of variance was tested for by an F test.

3.2.6. Statistical analysis (Clinical studies)

Data are given as mean ± standard deviations (SD) from 24 participants. Changes in blood glucose concentrations from the baseline concentrations were measured by subtracting the fasting value from the highest value and were demonstrated as incremental (**Δ**) concentrations. The incremental area under the curve (iAUC) for blood glucose was calculated from the incremental concentrations by using the trapezoid rule. Percentage changes in peak glucose and blood glucose AUC values were calculated for glucose control versus glucose plus onion with each participant acting as their own control. The mean changes for peak glucose and blood glucose AUC were then compared between lactose-tolerant and lactose-intolerant groups in Excel by two-tailed t-tests and using two-sample unequal variance. Quality of variance was tested for by an F test. Analysis of variance (ANOVA) can determine whether the means of three or more groups are different. ANOVA uses F-tests to statistically test the equality of means.

3.3. Results

The onion meal contained 1.7 µg/ml quercetin, 42.2 µg/ml quercetin 4'-glucoside and 31 µg/ml quercetin 3,4'-diglucoside. Consuming an onion meal reduced the glycaemic response in both lactose-tolerant and lactose-intolerant people as determined by glucose AUC and peak blood glucose concentration.

	AUC (mM x min)			Reduction in AUC by onion		**Δ** Blood glucose (mM)[a]			Reduction in **Δ** glucose by onion	
	Glucose Control	Glucose + onion	P	%	p[b]	Glucose control	Glucose + onion	p*	%	p[b]
Lactose-tolerant	186.4 (58.05)	109.3 (58.15)	0.0037	42.06 (25.98)	0.425	3.06 (1.18)	2.24 (0.93)	0.083	19.28	0.042
Lactose-intolerant	130.2 (49.22)	57.1 (55.48)	0.0034	54.53 (44.05)		3.03 (0.90)	1.77 (0.93)	0.0038	44.19	

Table 3.3: Effects of an onion meal on peak rise in blood glucose and incremental area under the blood glucose – time curve (AUC) in lactose-tolerant and lactose-intolerant adults. Values are expressed as means and s.d. [a] Peak glucose at 30 min minus fasting glucose. [b] Lactose-tolerant versus lactose-intolerant.

There was a statistically significant greater reduction in glycaemic response, as measured by changes in peak blood glucose, by the onion meal in lactose-intolerant people compared to lactose-tolerant people (44.19% and 19.28% respectively, p = 0.042) (Table 3.3). The onion meal also caused a greater reduction in blood glucose AUC in lactose-intolerant people compared to lactose-tolerant people, but this was not statistically significant (54.53% versus 42.06% respectively, p = 0.425) (Table 3.3).

Figure 3.3 below shows blood glucose concentrations following consumption of glucose or glucose plus an onion meal in 12 lactose-tolerant and lactose-intolerant adult participants.

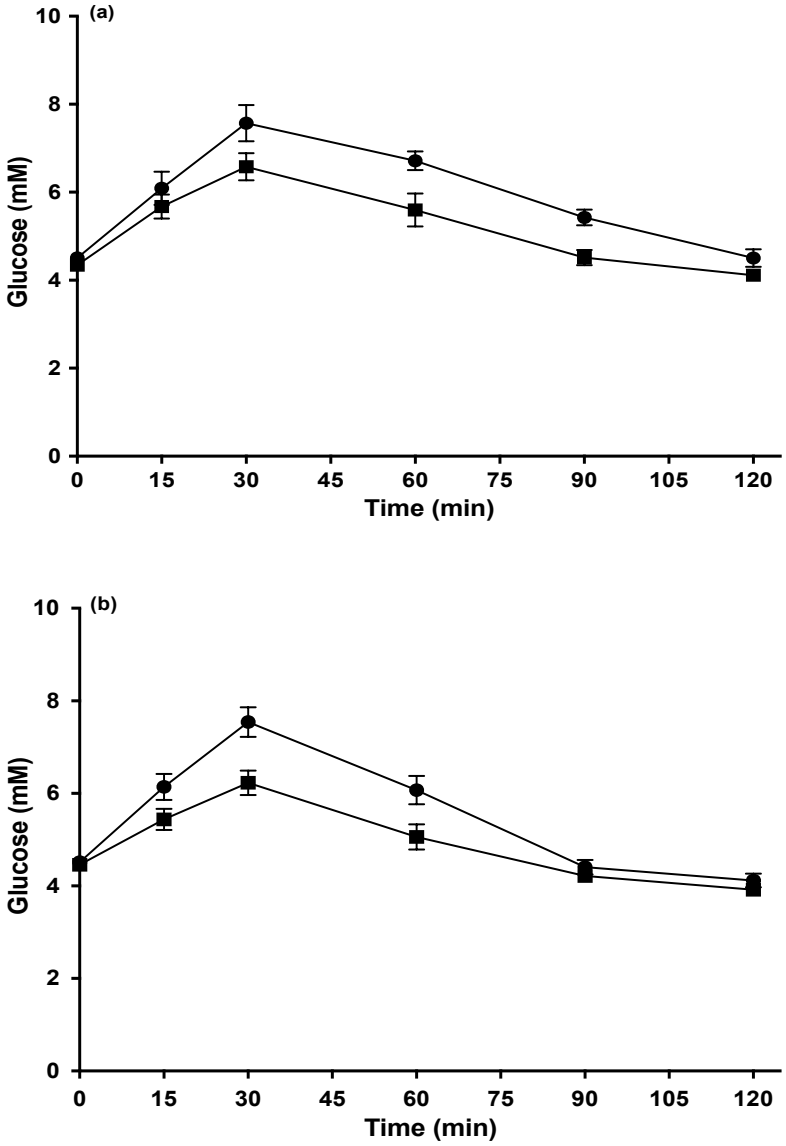


Figure 3.3: Blood glucose concentrations after consumption of glucose (●) or glucose plus an onion meal (■) in (a) lactose-tolerant adults and (b) lactose-intolerant adults. Data are means ± SEM (n = 12)

3.4. Discussion

The results of our pilot study indicate a more statistically significant reduction of peak glucose concentrations by an onion meal (solution) in lactose-intolerant people compared to lactose-tolerant people. This supports our hypothesis that LPH in lactose-tolerant people is hydrolysing quercetin glucosides in the onion meal and that this diminishes the quercetin glucosides ability to inhibit glucose uptake.

In the current *in vivo* study, we have addressed the possible factors, which could have affected the antiglycaemic ability of flavonoids in onion in lactose-tolerant and intolerant people. Firstly, reduced glucose uptake in lactose-tolerant people may be related to inhibition of GLUT-2 by quercetin produced from the hydrolysis of quercetin glucosides by LPH. Quercetin has been shown to inhibit GLUT-2, and transport of glucose from the gut to the blood stream requires not only luminal glucose uptake into enterocytes via SGLT1, but also release from the basal membrane of enterocytes into the blood stream via GLUT- 2 (Johnston et al., 2005b) Secondly, both lactose-tolerant and intolerant people may express other glucosidases able to cleave quercetin glucosides. Thirdly, onions are rich in the soluble fibre inulin, and some types of dietary fibre reduce postprandial glycaemia.

In order to use onions, it is important to consider the phenolic compositions of onion, which vary because of biological variations associated to cultivar, growing season, and environmental and agronomic conditions (Perez-Gregorio et al., 2010). Moreover, change in the content of flavonoids in onion depends on the availability of nutrients and water. It is believed that there is a strong phenotypic and genotypic correlation among the soluble solid contents, flavour and nutritional values of onions. In addition, harvesting at maturity stage is also a vital aspect when considering the chemical content and storage of the onion bulb (Slimestad et al., 2007).

The flavonoids from onions have been described to have antidiabetic properties. These include mechanisms for improvement of insulin release and improved insulin sensitivity (Williamson, 2013). It is suggested that these compounds can modulate the postprandial rise in blood glucose levels due to an interaction with intestinal enzymes such as LPH and transporters that are responsible for the release and absorption of dietary glucose. Polyphenols are mainly consumed with a diet rich in fruits, vegetables, tea and other plant-based food products and usually represent a versatile mixture of different components. They

can reach concentrations up to several hundred micromolar in the lumen of the human gut (Williamson, 2013); www.phenol-explorer.eu) and therefore may be able to reach intestinal glucose transporters and cause their inhibition. It is important to note that, participants in our pilot study were healthy (non-diabetic), and it has been suggested that GLUT-2 is permanently located at brush border membrane in insulin resistant individuals (but not healthy individuals) reducing higher levels of glucose and fructose absorption (Ait-Omar et al., 2011). Therefore, for the case of diabetic patients, onion could have a great antiglycaemic effect.

Apart from glucose transporters in the intestine, it is important to consider the renal tubule which expresses in addition to SGLT-1, the SGLT-2 transporter with the latter, causing most of the renal glucose reabsorption. Both transporters possess the same affinities for glucose. Since quercetin is bioavailable and is filtered in kidneys, in a human study they tested whether the onion extract given to the human volunteers affected glucose disposal into urine after the OGTT. Such an effect was shown in a study after intake of an apple extract rich in phloridzin (Schulze et al., 2014). However, intake of the onion extract did not result in any changes in urinary glucose levels examined over 24 hours after the OGTT. Therefore, from our findings and these previous findings, further research is required to be carried out on urinary glucose levels after consumption of onion meals, in addition to blood glucose levels. Moreover, a new class of antidiabetic drug with partially similar structure with phloridzin inhibit SGLT-2 in the kidney, and demonstrated reduction in fasting plasma glucose levels and less postprandial glucose rates in patients with type 2 diabetes (List and Whaley, 2011). The effects of SGLT-2 inhibitors are linked with great glucose loss in renal sites by significant reduction of glucose re-uptake in S2 and S3 segments in kidney (Ferrannini et al., 2010).

According to findings from several other human studies, in type 2 diabetic volunteers, quercetin (400 mg) given 30 min prior to an OGTT with 100 g glucose failed to change levels of blood glucose (Hussain et al., 2012), whereas onion extracts were shown to have an impact on the glucose plasma levels in patients with type 2 diabetes (Myint et al., 2009). This indicates more and other effects of the extract than that of quercetin and quercetin derivatives.

Various herbs and dietary supplements, such as quercetin supplements, are known to alter β-cell insulin output or peripheral insulin activity (Yeh et al., 2003). For quercetin, glucose-induced insulin release from isolated rat pancreatic islets revealed an increase (Youl et al.,

2010). As an example for effects in target tissues of insulin may be the recent *in vitro* study that revealed that extracts from vinification byproducts of *Vitis vinifera* with quercetin as the dominant constituent significantly inhibited glycogen phosphorylase (GP), an enzyme that catalyses the first step of intracellular degradation of glycogen to glucose-1-phosphate and the hepatic release of glucose (Kantsadi et al., 2014). GP inhibitors have been suggested as new hypoglycaemic agents for treatment of type 2 diabetes (Treadway et al., 2001) similar to inhibitors of hepatic glucose-6-phosphatase, an enzyme involved in the glucose release from glucose-6-phosphate as the final step in gluconeogenesis and glycogenolysis (Arion et al., 1997). Therefore, onion extracts or onion-derived polyphenols have the potential to affect physiological processes beyond intestinal glucose absorption and their effect will likely depend on dose and time.

The glycaemic index (GI) of onion solution containing glucose can be measured to rank its glycaemic potency (Jenkins et al., 1981). GI is calculated as the incremental area under the curve (AUC) for blood glucose after intake of onion solution containing glucose, divided by the AUC of reference glucose solution. Therefore, for the case of mixed meals, i.e. onion with glucose, it is possible to rank order of the average glycaemic responses produced by onion meal eaten on multiple occasions over a period of time (15, 30, 45, 60, 80 and 120 minutes). Our participants were given an onion solution containing glucose. It is important to note that, because of large between and within person variation of glycaemic responses, the amount cannot be used to predict the absolute glycaemic response of a person on a single occasion.

However, accurate prediction of the relative glycaemic effect of onion meal is only possible in clinical settings if accurate values for GI of the onion and dietary glucose are known (Wolever et al., 2006). It is suggested that the GI of individual foods is lost when combined in a mixed meal (Hollenbeck et al., 1986). However, in another study, they showed the GI of individual foods is a significant determinant of the glycaemic effect of composite mixed meals (Wolever et al., 2006). Moreover, in our clinical study, the effects of protein and fat were not measured in the mean glycaemic response, which was in agreement with a previous study conducted by Wolever, et al. (2006). However, this is inconsistent with findings from other studies (Flint et al., 2004, Alfenas and Mattes, 2005).

Consuming dietary flavonoid glucosides is an interesting approach to reducing the glycaemic response to a meal, and this aligns with a recent conclusion of the International Carbohydrate

Quality Consortium that overall diet, rather than just the GI values of individual foods, is important when evaluating the potential health risks of sugary foods consumed as part of a meal.(Augustin et al., 2015b).

In support of current *in vivo* findings, several studies have reported the antidiabetic effects of various forms of onions, including aqueous onion extracts (El-Demerdash et al., 2005), dietary onions (Jelodar et al., 2005) and isolated or synthesised active compounds in onions (Kumari and Augusti, 2002, Sheela et al., 1995). All of these reports determined a great antihyperglycaemic effects of onions and its compounds in STZ-induced diabetic rats. Interestingly, in another research, they found that drinkable ripe onion juice, had a low hypoglycaemic activity in normal rats, while it caused antidiabetic effect in STZ-induced rats (Lee et al., 2013).

Application of onion extract in mice fed with high fat diet resulted in lowering levels of postprandial blood glucose (Pereira et al., 2011). Similar findings were also detected in a study with type-2 diabetic patients (Myint et al., 2009). Here in our study, intake of 25% w/v onion solution containing 50g fresh red onion with 50g glucose (boiled 15min) resulted in a significant reduction in blood glucose concentrations at 45 minutes after the glucose load in lactose-intolerant individuals. Therefore, this result showed that the onion meal preparation worked on lowering levels of blood glucose *in vivo* study. Notably, in support of our current findings, an *in vitro* study illustrated an increased inhibitory potential for α-glucosidase and α-amylase inhibition in the presence of combinations (onion solution) compared to single compounds (quercetin) (Oboh et al., 2011).

In the current clinical study, participants were given onion meal (boiled solution) rather than raw onion; therefore, the boiling condition could have altered the antiglycaemic effects of onion. Some clinical studies in humans have shown the hypoglycaemic activity of *Allium cepa* by showing that addition of raw onion to the diet for non-insulin-dependent diabetic people reduced the dose of antidiabetic medication needed for disease control (Bhushan, 1984). According to several reports, onion acts as a hypoglycaemic agent through its effects on the small intestine, liver and muscles tissues, and changes the activities of the regulatory enzymes of glycolysis, gluconeogensesis and other pathways, such as reduction of ER stress, rather than increasing insulin levels and generating extra pancreatic effects (Taj Eldin et al., 2010, Eldin et al., 2009). ER stress has been shown to have a crucial effect in the development of insulin resistance and diabetes by impairing insulin signalling (Fonseca et al., 2009,

Kaufman et al., 2010); therefore, effects of onion extracts or its components could retain the properties as strong antidiabetic agents by alleviating ER stress.

It is important to take into consideration that some ingredients, in particular, volatile sulfur compounds including thiosulfonates and polysulfides for hypoglycemic activity might be lost during the preparation of onion meal or by passing over its best distribution period. Also, these sulfur compounds, available in onions in the form of cysteine derivatives, could be degraded during extraction by the enzyme allinase (El-Demerdash et al., 2005). Moreover, another study reported that S-methylcysteine sulfoxide isolated from onion has an antihyperglycaemic effect (Kumari and Augusti, 2002). Our observed increase in fasting blood glucose levels at both lactose-tolerant and lactose-intolerant groups during the first 30 min after oral intake of onion meals containing glucose, in addition to glycaemic effect of glucose, is also thought to be attributed to the glucogenic effects of *Allium cepa*, which might be from the cysteine present in onion (Sheela et al., 1995). These glucogenic effects can counteract the common side effect (hypoglycaemia) of antidiabetic agents currently used if *Allium cepa* is taken concurrently as a food supplement.

Further attention is required on the effect of cooking procedures on the content of flavonoids in onion. In fact, examining the following cooking methods: boiling, frying with oil or butter and microwave cooking. Previously, various cooking methods did not illustrate a consistent result on degradation of quercetin conjugates in onion. For instance, microwave cooking without water retained flavonoids, also frying did not affect flavonoid intake and viability (Loku et al., 2001). However, in agreement with our findings, the boiling of onion (onion solution) caused up to 30% loss of quercetin glycosides, which were transferred to the boiling water. The hydrolysis of quercetin glycosides for daily cooking might occur with the addition of seasonings such as glutamic acid. Additional ferrous ions could also accelerated the loss of flavonoids (Loku et al., 2001, Park, 1999).

Our data indicates that different types of dietary polyphenols affect the intestinal glucose transport. Furthermore, these data suggest that foods rich in these dietary polyphenols might provide a dietary mechanism for the regulation of intestinal sugar absorption that is an important factor in the management of diabetes and in the long-term offer some protection against developing type-2 diabetes. Therefore, physiological and pharmacological health benefits of polyphenols cannot be side-lined. This would be helpful in reducing sudden

elevation in glucose and insulin levels in response. Hence, the need of time is to utilise these naturally occurring compounds for the health benefits.

Although, in the current clinical study, onion meal resulted in reducing peak blood glucose levels in both lactose-tolerant (non-diabetic) and intolerant participants (non-diabetic), lower hypoglycaemic activity of onion meal in lactose-tolerant adults was observed. Further long term studies for the antiglycaemic effect of onion is required for healthy subjects with lactose intolerance condition.

4. CHAPTER FOUR - GENERAL DISCUSSION

The principal quercetin compounds in onion are β-glycosides quercetin 3,4′-diglucoside and quercetin 4′-glucoside. In current study, 3,4'-diglucoside and quercetin 4′-glucoside accounted for 31% and 43% of total concentrations of quercetin glucosides in onion, representing the combined value of up to 74%. Our findings agree with Lombard et al. (2002) and Price et al. (1997) who reported these two main quercetin glucosides conjugated together accounted for up to 80% of total flavonols content in onion. Moreover, in our study, quercetin 4′-glucoside is found as the main quercetin glucoside in onion, agreeing with findings from Lombard et al. (2002) and Leighton et al. (1992).

In our research, phloridzin, which is a known inhibitor of SGLT-1, was used as a positive control, and quercetin aglycone, quercetin 4′-glucoside, quercetin 3,4′-diglucoside, quercetin 3-glucoside were tested on Caco-2 cells radiolabelled ^{3}H-glucose uptake under both sodium-dependent and independent conditions. The three quercetin glucosides caused a significant reduction on Caco-2 radiolabelled glucose levels under sodium-dependent conditions; also their inhibitory effect were found concentration dependent (Boyer et al., 2005). According to several other studies whose results correlate with ours, it is generally considered that flavonoid glucosides in onion such as phloridzin, quercetin 4′-glucoside, quercetin 3,4′-diglucoside, quercetin 3-glucoside and onion extract are causing greater glucose inhibition under sodium dependent condition compared to sodium free condition (Walgren et al., 2000, Walle and Walle, 2003).

However, under sodium-independent conditions, none of these quercetin glucosides showed a significant glucose inhibition, while only their aglycone form quercetin caused a great glucose inhibition. Aglycones such as quercetin, flavone and daidzein or genistein are absorbed by passive diffusion in the intestine through sodium-independent GLUT-2 due to their lipophilic characteristics (Kuo, 1998, Murota et al., 2002).

In addition to flavonoids, inulin which is a soluble fibre is found in onions. According to a previous study several types of dietary fibre reduced postprandial glycaemia (Bonsu et al., 2011), although, in the current study, inulin did not reduce the uptake of radiolabelled glucose in the presence and absence of sodium conditions. Therefore, the main inhibitory effect of the onion extract is suggested to be through flavonoids quercetin and its derivatives on SGLT-1 and GLUT-2 transporters (Cermak et al., 2004). However, in our experiments, the

main source of tested inulin was chicory not onion; therefore, this could be the reason for inconsistency of our data on low inhibitory effect of inulin on glucose uptake.

We found that onion extract treated with *Aspergillus oryzae* β-galactosidase, had an increase on the levels of quercetin aglycone, with concomitant reductions in quercetin 3,4′-diglucoside and quercetin 4′-glucoside, and confirmed that β-galactosidase deglycosylated quercetin 4′-glucoside and quercetin 3,4′-diglucoside to quercetin, respectively. These results were in accordance to research conducted by Cheng et al. (2011), In the current *in vitro* study, lower inhibitory effect of both onion and quercetin 3,4′-diglucoside were found after 60 minutes' incubation with β-galactosidase *Aspergillus oryzae* on Caco-2 cells glucose uptake.

Our data support the idea that β-galactosidase is causing deglycosylation of quercetin glucosides (quercetin 4′-glucoside) which suggests that quercetin glucoside can no longer stay intact in the presence of β-galactosidase, thus free quercetin will be released. Nemeth et al. (2003) have already published the substrate specificity of purified sheep LPH for the above quercetin glucosides, which is in line with our present findings quercetin 3,4′-diglucoside, 4-glucoside and onion are suitable substrates for β-galactosidase hydrolysis.

The scientific evidence on the importance of polyphenol compounds to delay digestion and absorption of carbohydrate and to suppress hyperglycaemia in the postprandial state is promising, although it is mainly established on *in vitro* and animal studies. Evidence from human studies indicates that beverages such as apple juice, red wine and onion soup have glycaemic control effect (Johnston et al., 2002). Current *in vitro* studies were conducted to model or predict whether the *in vivo* study is likely to succeed or not. Thus, the *in vitro* study demonstrated quercetin glucosides and onion extract block glucose uptake, whereas free quercetin did not. Also, the *in vivo* study found that glucose uptake was blocked by the onion solution; therefore, it is possible, based on findings from the *in vitro* studies, that this could be due to the fact that in the absence of lactase, quercetin glucosides are not broken down and thus block the uptake of glucose.

LPH-lactase is a membrane-bound enzyme present on the luminal side of the brush border and can therefore act on flavonoid glucosides before absorption. Another possibility is that enterocytes are constantly shed from the top of the intestinal villi into the lumen at a very high rate (Day et al., 2000a). Whether these enterocytes are viable or not, they may be capable of hydrolysing flavonoid glucosides present in the intestinal lumen β-glucosidase activity

toward quercetin 4′-glucoside and other flavonoid glucosides have been detected in intestinal tissue from rats (Ioku et al., 1998) and humans (Walgren et al., 2000). The finding that ileostomy fluid had β-glucosidase activity supports either mechanism. It is possible that some of the hydrolysis may have occurred in the ileostomy bag since a bacterial contamination of the ileostomy fluid from bacterial β-glucosidase activity cannot be excluded. A variety of bacterial strains have high activity of this hydrolytic enzyme which can also lead to hydrolysis of these flavonoid glucosides (Walgren et al., 2000).

In addition to the LPH expression in the enterocytes, the expression of other glucosidases such as α-glucosidases were possible in both lactose-tolerant and intolerant people, which could lead to deglycosylation of quercetin glycosides in the small intestine (Bonsu et al., 2011). Our small pilot study requires a larger scale study to confirm if this benefit is greater in lactose-intolerant people compared to lactose-tolerant people.

In addition, our study cannot rule out that other components in the onion meal are responsible for the reduced glucose uptake in the presence of onion. Nevertheless, our study does raise the possibility that a diet containing quercetin glucosides (and possibly other flavonoid glucosides hydrolysed by LPH) may be of greater benefit for glycaemic control in lactose-intolerant people than for lactose-tolerant people.

In order to optimise our pilot study, a higher number of participants are required to confirm if health benefits of onion remain greater in lactose-intolerant people compared to lactose-tolerant adults. Moreover, our study illustrated that a diet containing quercetin glucoside may be of greater benefit for glycaemic control in lactose-intolerant people than for lactose-tolerant people. Therefore, lactose tolerance could be a confounding factor in studies that compare glycaemic responses to diets between regions of the world where lactose tolerance is high, such as Northern Europe, with regions where lactose intolerance is high, such as the Mediterranean basin.

4.3. Conclusion

According to our current findings from experimental work, a clear picture of how glucose transporters work and how much glucose uptake was obtained on the Caco-2 cell culture models in the presence of several most widely found flavonoids in onions. Caco-2 cell models demonstrated that phloridzin, quercetin 4′-glucoside, quercetin 3,4′-diglucoside and quercetin 3-glucoside inhibited sodium-dependent glucose uptake, whereas the aglycones quercetin inhibited sodium-independent glucose uptake. Quercetin glycosides inhibited this transfer from the apical to the basolateral side, primarily by inhibition of sodium independent GLUT-2 with less inhibition of sodium-dependent SGLT-1.

Quercetin 4′-glucoside followed by quercetin 3,4′-diglucoside had the highest concentrations in the red onion extract. The assay of β-galactosidase from *Aspergillus oryzae* was conducted to model intestinal LPH, which showed the hydrolysis of quercetin 4′-glucoside and quercetin 3,4′-diglucoside to free aglycones. In vitro experiments were useful on designing clinical studies, but they cannot be used to decisively conclude that the same results will occur in humans. This is because humans possess different enzymes and have different ways that lactase works in the body. This, however, still supports the notion that lactose intolerant people might be less prone to diabetes because they are more able to block dietary glucose.

A human study was conducted in order to investigate, the antiglycaemic effects of flavonoids in onions, when lactose-tolerant and intolerant individuals consumed onion solution. There was a statistically significant higher decrease in glycaemic response, as determined by changes in peak blood glucose, by the onion meal in lactose-intolerant people compared to lactose-tolerant people. Although the number of participants in the human study groups was small, but it showed that onion (*Allium cepa*) in addition to its nutritional values has antiglycaemic effects that could be beneficial in management of blood glucose levels in adults and can possibly be in type 2 diabetic patients, mainly the level of its safety as revealed by its worldwide use as vegetable.

We suggested that taking onion solution may reduce hyperglycaemia in lactose-intolerant participants more than lactose-tolerant people. In conclusion, the current research suggests that onion solution may be able to normalise the levels of blood glucose when doses are continuous for long periods. These findings provide a basis for the use of this drinkable onion product for the prevention of diabetic patients also. Therefore, we conclude that intake of this

product could be beneficial in prevention of type 2 diabetes mellitus, and lactose-intolerant people would benefit more from the antiglycaemic effect of onions compared to lactose-tolerant adults. Of course, further studies for its long term effect for the prevention of diabetes and their link with lactose-intolerance are required.

4.4. Future work

Various cohort studies have shown an inverse relationship between flavonoids intake and diabetes. Flavonoids such as quercetin and its derivatives have received much attention on the literature over the past 10 years. However, most of the studies have been conducted *in vitro*; thus it is difficult to draw definite conclusion about usefulness of flavonoids in the diet. There is a need to improve analytical techniques to allow collection of more data in this aspect.

Regarding the Caco-2 cell models, the efflux of polyphenolic compounds into the basolateral medium could be addressed by adding phloretin, which is a known inhibitor of GLUT-2 mediated glucose transport on the basolateral membrane. Determining the exchange of glucose distribution between flavonoids will also help in showing the cooperation between cellular glucose levels and these compounds. Further research is required to determine the impacts of flavonoids on activation of carbohydrate response elements or protein trafficking.

In addition to Caco-2 cell lines that expresses SGLT-1and GLUT-2 glucose transporters in the small intestine, it will be useful to consider the role of other transporters such as SGLT-2 sodium-dependent glucose cotransporter, that is responsible for majority of glucose transport in kidney (Yi et al., 2013). Therefore, for future work use of CHO cell lines that express the human SGLT-2 transmembrane protein, along with a fluorescent glucose transporter assay that uses 2-NBDG as a glucose analog, a non-radioactive cell-based assay is suggested to be conducted for characterisation of SGLT-2 inhibitors (Blodgett et al., 2011).

To validate the effect of onion intake *in vivo* for future, the efficiency of onion extract is suggested to be compared with acarbose, a well-inhibitor of intestinal disaccharidases (Kang and Kim, 2010). In order to have a more consistent results from our clinical study, more participants and longer duration of the experiment is advised i.e. blood glucose levels are suggested be measured for 2-6 hours. Also, we elaborated that lactose-intolerant people would benefit more from the antiglycaemic effect of onions compared to lactose-tolerant

adults. However, more research for its long term beneficiary effects on type 2 diabetes and their link with lactose intolerance are required.

In addition to Caco-2 cell models and human studies on antiglycaemic effects of flavonoids in onion on glucose levels, further research needs to be conducted on animal models such as rats. Also, a feeding study and oral glucose tolerance test in mice are recommended; which involves mice with 12 weeks of dietary intervention and collection of blood samples 15, 30, 60, 90 and 120 min after glucose load with subsequent blood glucose measurements. Moreover, developing and validating an *in situ* closed loop perfusion method in rat colon is suggested, in order to compare with small intestine and Caco-2 cell models. Also, it is suggested that examination of interaction between onion extract and sodium-dependent glucose absorption, using isolated jejunal vesicles from rats and pigs be conducted by way of future research.

5. CHAPTER FIVE – REFERNECES

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6. CHAPTER SIX – APPENDICES

6.1. Appendix 1

UNIVERSITY OF HERTFORDSHIRE

FORM EC1: APPLICATION FOR ETHICAL APPROVAL OF A STUDY INVOLVING HUMAN PARTICIPANTS

(See Guidance Notes)

Relevant ECDA:	
	☐
Science & Technology	X
	☐

OFFICE USE ONLY

Protocol Number:

DECLARATIONS

DECLARATION BY APPLICANT (See GN 2.1.3)

(i) I undertake, to the best of my ability, to abide by accepted ethical principles in carrying out the study.

(ii) I undertake to explain the nature of the study and all possible risks to potential participants, to the extent required to comply with both the letter and the spirit of my replies to the foregoing questions (including information contained in Appendices 1 & 2).

(iii) Data relating to participants will be handled with great care. No data relating to named or identifiable participants will be passed on to others without the written

consent of the participants concerned, unless they have already consented to such sharing of data when they agreed to take part in the study.

(iv) All participants will be informed **(a)** that they are not obliged to take part in the study, and **(b)** that they may withdraw at any time without disadvantage or having to give a reason.

Where the participant is a minor or is otherwise unable, for any reason, to give full consent on their own, references here to participants being given an explanation or information, or being asked to give their consent, are to be understood as referring to the person giving consent on their behalf. (See Q 19 above; also GN Pt. 3, and especially 3.6 & 3.7

Enter your name here

Golnaz Ranjbar……..Date…19/03/2014……..

If you are a member of staff, please obtain the signature of your line manager to indicate their agreement to this application:

……
……

(Signature) (Name in BLOCK CAPITALS and position within the School)

DECLARATION BY SUPERVISOR (see GN 2.1.3)

I confirm that the proposed study has been appropriately vetted within the School in respect of its aims and methods as a piece of research; that I have discussed this application for Ethics Committee approval with the applicant and approve its submission; and that I accept responsibility for guiding the applicant so as to ensure compliance with the terms of the protocol and with any applicable ethical code(s).

Enter your name here……...Date……

THE STUDY

Q1. Please give the title (or provisional title) of the proposed study. (NB – you will be asked for further details later)

Health Significance of Lactose Intolerance

1. THE APPLICANT

Q2. Please answer **either** **Q2.1** **or** **Q2.2** by providing the information requested. **Q2.1** should be answered by individual applicants, both staff and students, who require protocol approval for work which they themselves intend to carry out. **Q2.2** should be answered by academic staff requiring approval for standard protocols governing classroom practical work (or equivalent work) to be carried out by a specified group of students. (See GN 2.2.1 & 2.2.19)

Q2.1. Name of applicant/(principal) investigator

Status:

(a) undergraduate ☐

(b) postgraduate (taught/research) X

(c) academic staff ☐

(d) other - please give details here ☐

School/Department

School of Life and Medical Sciences

Department of Human and Environmental Sciences

Programme of study or award (e.g. BA/MSc/PhD/Staff research)

PhD

E-mail address

g.ranjbar2@herts.ac.uk

Name of supervisor

Dr Richard Hoffman

Supervisor's contact details (email, extension number)

r.hoffman@herts.ac.uk Extension number: 4526

Q2.2. Class Protocol Applications Only.

Name of applicant/(principal) investigator (member of staff)

School/Department

Programme of study or award (e.g. BSc/MA)

Module Title

Year/group to be governed by the protocol

Number of students conducting the study

Programme Tutor (if different from the applicant)

E-mail address

Please note: Risk Assessment Form EC5 is mandatory for all Class Protocol Applications and must accompany this application.

2. DETAILS OF THE PROPOSED STUDY

Q3. (a) Is it likely that your application will require NHS approval? (See GN 2.2.2)

☐ YES	☒ NO

(If YES, please answer **(b)** & **(c)**) (If NO, please continue on to **Q4**)

(b) Please confirm whether your research involves any of the following:

NHS Patients	☐
Clinical trial of an investigational product	☐
Clinical trial of a medical device	☐
Exposure to any ionising radiation	☐
Adults who lack the capacity to consent	☐
Human Tissue	☐

(c) Please confirm whether this study is considered to be a Clinical Trial of Investigational Medical Products (CTIMP) or Clinical Trial of Investigational Medical Devices. (See GN 2.2.2)

☐ YES	☒ NO

If YES, please indicate if the study involves any of the following categories:

Children under 5 ☐

Pregnant women ☐

A group of more than 5,000 people ☐

Study would be undertaken overseas ☐

If your study is likely to require NHS approval or is a Clinical Trial of Investigational Medical Products or Devices, DO NOT complete this form any further and submit it to your relevant ECDA at this stage. All NHS applications must be made on an IRAS form. If your study is a Clinical Trial of Investigational Medical Products or Devices involving one of the above specified categories, you will be contacted by the relevant ECDA with information on the next steps. Please note, you will be issues with a UH Protocol Number but this will not be valid until you have sent your relevant ECDA a copy of your NHS approval.

Q4. Please give a short synopsis of your proposed study; stating its aims and highlighting, if appropriate, where these aims relate to the use of human participants. (See GN 2.2.3)

Please enter details here.

The aim is to investigate whether there is any difference in the uptake of glucose, when a food extract (onion) or other foods e.g. apple is consumed by lactose-tolerant and lactose-intolerant individuals. In this study, a hydrogen breath test will be

conducted for diagnosis of lactose tolerance and intolerance, and glucose levels will be measured by taking blood samples through finger pricks.
Human participants are needed for part of this study, which cannot be performed in vitro or with animals. Details are described in the answer in Q5.

Q5. Please give a brief explanation of the design of the study and the methods and procedures used, highlighting in particular where these involve the use of human participants. You should clearly state the nature of the involvement the human participants will have in your proposed study and the extent of their commitment. Thus you must complete and attach the Form EC6 (Participant Information Sheet) (see Appendix 2)**.** Be sure to provide sufficient detail for the Committee to be clear what is involved in the proposed study, particularly in relation to the human participants. (See GN 2.2.4)

I will provide a promotional poster in the CP snow building, and will invite students through study net. I will also ask the student union to advertise this study through the media available to them to obtain appropriate number of participants.

In order to measure the glucose level among lactose tolerant and intolerant subjects, 20 subjects will be recruited. Subjects will be excluded if they have had a significant current or previous medical history, such as diabetic patients, those who are/were receiving regular medication (apart from oral contraceptives) that may have affected gastrointestinal function, those with a history of fits, bleeding disorder (e.g. haemophilia) and pregnant women. Inclusion criteria (both groups): both sexes.

The proposed study involves the use of a hydrogen breath test. The Gastrolyzer+ is a portable hand-held hydrogen breath tester, it is designed to aid in detection of gastro-intestinal disorders such as lactose intolerance. The hydrogen breath test involves 50 g of oral consumption of lactose, and then measuring the levels of hydrogen in breath (> 20 ppm) over the following 3-6 hours with levels above 20 ppm showing lactose intolerance. Operation of the monitor is straightforward. A D-piece sampling system enables end-expired breath to be sampled easily and hygienically, using single-use disposable cardboard Flatpak^{TM} mouthpieces.

After distinguishing lactose tolerant and intolerant individuals, will fast overnight for 10-12 hours, a blood samples from human subjects will be drawn from a finger by pricking with a lancet (0 min) and collected into a capillary tube (10 µl samples). Each participant will then be given a solution of 50 g glucose in 220 ml water, then

blood samples will be taken at 15, 30, 60, 90 and 120 minutes. Each participant will then be given a solution of 50 g glucose in 220 ml water plus a portion of onion soup (mixture). Blood samples will again be taken by finger-prick at 0, 15, 30, 60, 90 and 120 minutes. The sample is measured in an EKF glucose analyser by transferring into the EKF safe-lock cup prefilled with 0.5ml haemolysis solution, which reads the blood glucose level (mmol/L).

Q6. Please give the starting date and finishing date. (For meaning of

"starting date" and "finishing date", see GN 2.2.5)

Starting Date: June 2014

Finishing Date: December 2014

Q7. Where will the study take place? (If this is on UH Campus, who will permission be obtained from e.g. your Module Leader, Programme Tutor, Pro-Vice Chancellor (Student Experience) or the Dean of Students. If this is NOT on UH Campus, please attach a copy of the written permission, given by the proprietor, manager or other person with such authority over the premises, to use the premises for the purposes of carrying out this research (see Appendix 2)) (See GN 2.2.6)

Please enter details here.

University of Hertfordshire (College Lane Campus)- Human Physiology Lab and Diet Lab

Permission has been obtained from both lab managers/technical staff (see attached).

Q8. If the location is off campus, have you considered whether a risk assessment is necessary for the proposed location? (in respect of hazards/risks affecting both the participants and researchers) Please see **Form EC5** (see Appendix 2, which is an example of a risk assessment form.) Please use this example if a risk assessment is necessary, and you have not been provided with a subject specific risk assessment form by your School or Supervisor.

(See GN 2.2.7)

Q9. (a) Will anyone other than yourself and the participants be present with you when conducting this study? (See GN 2.2.8)

YES	NO
	X

If YES, please state the relationship between anyone else who is present other than the applicant and/or participants? (e.g. health professional, parent/guardian)

(b) Will the proposed study be conducted in confidence? If NOT, what steps will be taken to ensure confidentiality of the participants' information. (See GN 2.2.8)

Yes

3. HARMS, HAZARDS & RISKS

Q10. Will this study involve invasive procedures on the human participants? (See GN 2.2.9)

[X] Yes [ ] No ARE YOU SURE?

(If YES, please fill out **Appendix 1 – Increased Hazards and Risks.** (If NO, answer **Q11, Q12, Q13 & Q14**)

Once this is complete, move on to **Q15**)

Q11, Q12, Q13 & Q14 - NON INVASIVE STUDIES ONLY

Note: You are advised to read GN 2.2.10, 2.2.11, 2.2.12 & 2.2.13 carefully before you answer the following questions.

Q11. Are there potential hazards to participant(s) and/or investigator(s) from the proposed study? (See 2.2.10)

[ ] YES NO [ ]

If YES,

- Indicate their nature here.
- Indicate here what precautions will be taken to avoid or minimise any adverse effects.

Q12. Will or could the study cause discomfort or distress of a mental or emotional character to participants and/or investigator(s)? (See NG 2.2.11)

[ ] YES NO [ ]

If YES,

- Indicate its nature here.

- Indicate here what precautions will be taken to avoid or minimise such adverse effects.

Q13. Will or could medical or other aftercare and/or support be needed by participants and/or investigator(s) as a result of the study? (See GN 2.2.12)

☐ YES ☐ NO

Q14. (a) If you have answered 'YES' to **Q11, Q12 & Q13**, please state here the previous experience (and/or any relevant training) of the supervisor (or academic member of staff applying for a standard protocol) of investigations involving the hazards, risks, discomfort or distress detailed in those answers. (See GN 2.2.13)

(b) Please describe in appropriate detail what you would do should the adverse effects or events which you believe could arise from your study, and which you have mentioned in your replies to the previous questions, occur.

(See UPR RE01, S 2.3 (ii) and GN 2.2.13)

4. ABOUT YOUR PARTICIPANTS

Q15. Please give a brief description of the kind of people you hope/intend to have as participants, for instance, a sample of the general population, University students, people affected by a particular medical condition, children aged 5 to 7, employees of a particular firm, people who support a particular political party.

Students and staff from the University of Hertfordshire

Q16. Please state here approximately how many participants you hope will participate in your study.

24 participants (12 lactose tolerant and 12 lactose intolerant)

Q17. By completing this form, you are indicating that you are reasonably sure that you will be successful in obtaining the number of participants which you hope/intend to recruit. Please outline here how you intend to recruit them. (See GN 2.2.14)

I will produce a promotional poster and place copies of the poster on notice boards throughout the University of Hertfordshire. I will increase the number of participants through the assistance of the Nutrition and Dietetics' programme tutor, who will inform the undergraduate students of this programme (permission letter attached). I will also seek permission from and the support of the students union to advertise this study through the media available to them in order to obtain appropriate number of participants (Promotional poster).

5. CONFIDENTIALITY AND CONSENT

[For guidance on issues relating to consent, see GN 2.2.15 & Pt. 3.]

Q18. Is it intended to seek informed consent from the participants?

[X]	[ ]
YES	NO

(See UPR RE01, S 2.3 & 2.4 and GN 3.1)

If NO, please explain why it is considered unnecessary or impossible or otherwise inappropriate to seek informed consent.

If YES, please attach a copy of the Consent Form to be used (See Form EC3 & EC4 for reference and GN 3.2), or describe here how consent is to be obtained and recorded. The information you give must be sufficient to enable the Committee to understand exactly what it is that prospective participants are being asked to agree to.

Q19. If the participant is a minor (under 18 years of age), or is otherwise unable for any reason to give full consent on their own, state here whose consent will be obtained and how? (See especially GN 3.6 & 3.7

One of the preconditions of selecting participants is that they should be 18 years old and older. Since the pool of selection for our participants is the campus of the University of Hertfordshire, it is likely that our volunteers will fit this criterion.

Q20. Are personal data of any sort (such as name, age, gender, occupation, contact details or images) to be obtained from or in respect of any participant? (See GN 2.2.16).

[X] YES [ ] NO

If YES,

(a) Give details here of personal data to be gathered, and indicate how it will be stored.

Standard questionnaire will be provided in order to collect participants' names, ages, gender and contact details. Moreover, information regarding participants' current or pervious medical histories, diabetes and those with history of blood disorder (i.e. haemophilia) will be gathered.

(b) State here what steps will be taken to prevent or regulate access to personal data beyond the immediate investigative team?

The data collected will be stored on a password-protected laptop.

(c) Indicate here what assurances will be given to participants about the security of, and access to, personal data

The same level of assurance provided in question (b) above will be provided to the participants in written format before they physically participate in the study. The notice will clearly state that I will be personally responsible for maintaining the security and confidentiality of such data.

(d) State here, as far as you are able to do so, how long personal data collected during the study will be retained, and what arrangements have been made for its secure storage.

The data will be held in a secure manner for at least the duration of my research project

and will be used as part of the findings of my PhD research project. After such period when the PhD research project is completed, all the personal data will be destroyed.

Q21. Is it intended (or possible) that data might be used beyond the present study? (See GN 2.2.16)

☐ YES NO ☒

If YES, please give here an indication of the kind of further use that is intended (or which may be possible).

If NO, will the data be kept for a set period and then destroyed under secure conditions?

☒ YES NO ☐

If NO, please explain here why not.

Q22. If your study involves work with children and/or vulnerable adults you will require a satisfactory Enhanced Criminal Records Bureau Disclosure. (See GN 2.2.17) Please indicate as appropriate:

(a) CRB Disclosure not required ☒

(b) CRB Disclosure required and obtained ☐

If a satisfactory CRB Disclosure is required, a copy of this must be attached to Appendix 2 in order for reviewers to be able to consider your application.

6. REWARDS

Q23. (a) Are you receiving any financial or other reward connected with this study? (See UPR RE01, 2.3)

☐	☒ X
YES	NO

If YES, give details here.

(b) Are participants going to receive any financial or other reward connected with the study?

☐	☒ X
YES	NO

If YES, give details here.

(c) Will anybody else (including any other members of the investigative team) receive any financial or other reward connected with this study?

☐	☒ X
YES	NO

If YES, give details here

7. OTHER RELEVANT MATTERS

Q24. Enter here anything else you want to say in support of your application, or which you believe may assist the Committee in reaching its decision.

This section is to be completed if your answer to Q10 affirms the **USE OF INVASIVE PROCEDURES** in your study.

Note: You are advised to read GN 2.2.10, 2.2.11, 2.2.12, 2.2.13 & 2.2.18 carefully before you answer the following questions.

QA1. Please give details of the procedures to be used (e.g. injection of a substance, insertion of a catheter, taking of a blood or saliva sample), and any harm, discomfort or distress that their use may cause to participants and/or investigator(s). (See GN 2.2.10)

- Clean the finger
- Encourage blood flow (Keep finger warm)
- Hold the lancet firmly against the finger and press the release bottom
- Dispose of lancet into sharps bin
- Wait 6 seconds to allow blood to flow and the bottom of the finger to the puncture site
- Continue this process until a drop of blood is obtained from the puncture site
- Load the blood into a10 µl capillary tube
- Wipe the puncture site with clean swab and apply pressure until the bleeding stops
- Bruising and pain may arise on the side of the fingers
- Bleeding may not stop immediately after collection of blood by capillary tube

Indicate here what precautions will be taken to avoid or minimise any adverse effects.

- The side of finger is generally less sensitive than the tip of a finger, therefore using side of finger will be used
- Make sure that researcher's hands are washed prior to the procedure
- Keep the clean swab on the site of bleeding until it is completely stopped, to minimise the risk of any airborne infections
- Dispose of lancet immediately after use (appropriate bins) to avoid cross contamination
-

QA2. Will the study involve the administration of any substance(s)? (See GN 2.2.10)

[X] YES [ ] NO

If YES,

- Give details here of the substance(s), the dose or amount to be given, likely effects (including duration) and any potential hazards to participant(s) and/or investigator(s).

- Indicate here what precautions will be taken to avoid or minimise any adverse effects.

After measuring the basal hydrogen concentration in the respiratory air of the

participants in the fasting state (which should not be higher than 5 ppm and at most 10 ppm), the participant will be giving 25 g of lactose dissolved in 250 ml of water.

It is recommended to dissolve lactose in warm water, as it dissolves better in warm water. The hydrogen values are measured at 0 minutes (before the load) as well as at 15, 30, 60, 90 and 120 minutes after the lactose load.

Following to the diagnosis of lactose tolerant and lactose intolerant individuals, each participant will be given a solution of 50 g glucose solution in 220ml water in addition to a portion of onion soup or other food products (e.g. apple). All blood samples will be taken immediately: before eating (0 min) and 15, 30, 45, 60, 90 and 120 min after eating. The glucose levels will be measured at each time point by using EKF machine.

QA3. Are there any potential hazards to participant(s) and/or investigator(s) arising from the use of the proposed invasive procedures? (See GN 2.2.10)

YES [X] NO [ ]

If YES,

- Indicate their nature here.

Blood collection from fingertips may lead to:

- Exposure to infectious agents
- Blood cross-contamination
- Bruising at sample size

For hydrogen breath test monitor (Gastrolyzer):

- People with lung disease or chest ailments may not be able to achieve the full breath-hold. Therefore, in such cases, this procedure may cause no harm however it may cause breathing discomfort in participants

- Indicate here what precautions will be taken to avoid or minimise any adverse effects.

For blood sample collection:

- Laboratory coats and gloves should be worn throughout the experiments
- Lancets must not be re-used

- If the blood drop is drawn directly onto a capillary tube, the tube must be removed from the analyser so that there is not potential for cross contamination
- All disposable items should be placed immediately in the appropriate clinical waste containers
- Any blood spills should be wiped up immediately
- The EKF analyser should be cleaned regularly
- Only minimal pressure should be used to encourage the formation of a blood drop

For hydrogen breath test analyser:

- Participants are advised to inhale and hold their breath for as long as possible when the breath test is started, and exhale, if necessary, before the countdown has completed. Alternatively, the user can use the face mask sampling mode
- Under no circumstances should the Gastrolyzer be immersed or splashed with liquid
- When cleaning the monitor, never use alcohol, cleaning agents containing alcohol, or other organic solvents, as these vapors will damage the H2 sensor inside.

QA4. Will or could the study cause discomfort or distress of a mental or emotional character to participants and/or investigator(s)? (See GN 2.2.11)

[X] YES [ ] NO

If YES,

- Indicate its nature here

Lancet stick injury, may lead to infection, however the probability is negligible. Moreover, bruising and painful lancet entry side, will result in participants' discomfort. Incorrect blood collection may cause withdrawal of participants from the study, due to lack of confidence of the investigator. Participants may feel faint at the sight of blood drops.

- Indicate here what precautions will be taken to avoid or minimise such adverse effects.

Follow the detailed blood collection procedures for finger picks.

The lancets for finger pricks are designed in such a way that they can only be used once, thereby minimising the possibility of cross contamination.

If unable to draw blood, withdraw the lancet and apply light pressure to the site. Should not attempt to withdraw blood at the same site again.

QA5. Medical or other aftercare and/or support must be made available for participants and/or investigator(s) who require it where invasive procedures have been used in the study. Please detail what aftercare and/or support will be available and in what circumstances it is intended to be used. (See UPR RE01, S 2.3 (ii) and GN 2.2.12)

There could be a very small chance of fainting of participants, once blood is taken by finger-prick.

If the participant fainted lie her/him down and stay with them until they have recovered. Little sips of water and a wet towel applied to the forehead. If complications arise contact the First Aid Officer.

Plaster should be used on the site of sampling (tips of fingers)

QA6. **(a)** Please state here previous experience (and/or any relevant training) of the supervisor (or academic member of staff applying for a standard protocol) of investigations involving hazards, risks, discomfort or distress as specified. (See GN 2.2.13)

Supervision of many project students

(b) Please describe in appropriate detail what you would do should the adverse effects or events which you believe could arise from your study, and which you have mentioned in your replies to the previous questions, occur.

Pain and bruising may arise while stabbing the lancet, therefore informing the participants prior to the use of lancet is recommended, followed by minimum pressure on the side of fingers to enhance the flow of blood drop.

In the case of fainting, first aid officers will be asked to come for help and immediate treatment steps will be taken:

- Loosen tight clothing around the neck
- Raise person's feet above the level of heart
- Keep the participant lying down for at least 10 minutes, preferably in a cool and quiet space

QA7. In the event that the study reveals that a participant has a pre-existing medical condition (of which they may or may not be aware), and which could affect their present or future health or that of others, they should be informed of this in an appropriate manner and advised of follow-up action that they should take. (See GN 2.2.18) Advice should be sought as to whether information should be passed to their GP and a decision taken whether they should be allowed to continue to take part in the study. If a potential participant is not willing to agree to such action being taken in these circumstances, they should not be allowed to take part in the study. Please indicate here what arrangements have been made for complying with these requirements.

If the participant already knows that she/he is lactose intolerant, no additional action will be taken. If the participant being told she/he is lactose intolerant for the first time, the researcher has a duty of care, relevant information that is available in public domain (NHS website) will be provided for the participant. The researcher is not qualified to give dietary advise about lactose intolerance however if they have any concerns they should make an appointment to see their GP.

In the case of abnormal blood glucose reading, if the blood sugar level is high (hyperglycaemia) or low (hypoglycaemia), the researcher will explain to the participants that it is not good for long-term health.

Please revert to Q15.

Please attach the following documents if you have affirmed possession of them in the relevant questions:

(a)	Permission from the location to be used to carry out this study (Q7) (This includes permission to use a location on UH Campus and any location off of UH Campus which requires permission to use.)	X
(b)	Risk assessment for off campus location (See Form EC5) (Q8)	
(c)		
(d)	Copy of Consent Form (See Form EC3 & Form EC4) (Q18)	X
(e)		
(f)	Copy of Form EC6 (Participant Information Sheet) (Q5)	X
(g)	A copy of the proposed questionnaire and/or interview schedule (if appropriate for	X

6.2. Appendix 2

University of Hertfordshire UH

UNIVERSITY OF HERTFORDSHIRE
HEALTH & HUMAN SCIENCES

ETHICS APPROVAL NOTIFICATION

TO Golnaz Ranjbar

CC Richard Hoffman

FROM Dr Richard Southern, Health and Human Sciences ECDA Chairman

DATE 05/06/14

Protocol number: **LMS/PG/UH/00187**

Title of study: Health Significance of Lactose Intolerance

Your application for ethical approval has been accepted and approved by the ECDA for your school.

This approval is valid:

From: 05/06/14

To: 01/12/14

Please note:

Approval applies specifically to the research study/methodology and timings as detailed in your Form EC1. Should you amend any aspect of your research, or wish to apply for an extension to your study, you will need your supervisor's approval and must complete and submit form EC2. In cases where the amendments to the original study are deemed to be substantial, a new Form EC1 may need to be completed prior to the study being undertaken.

Should adverse circumstances arise during this study such as physical reaction/harm, mental/emotional harm, intrusion of privacy or breach of confidentiality this must be reported to the approving Committee immediately. Failure to report adverse circumstances would be considered misconduct.

Ensure you quote the UH protocol number and the name of the approving Committee on all paperwork, including recruitment advertisements/online requests, for this study.

Students must include this Approval Notification with their submission.

6.3. Appendix 3

University of Hertfordshire

CONSENT FORM FOR STUDIES INVOLVING HUMAN PARTICIPANTS

I, the undersigned *[please give your name here*, *in BLOCK CAPITALS]*

……
.
of *[please give contact details here, sufficient to enable the investigator to get in touch with you, such as a postal or email address]*

…..……
..
hereby freely agree to take part in the study entitled *[insert name of stu*dy *here]*

……
.

1 I confirm that I have been given a Participant Information Sheet (a copy of which is attached to this form) giving particulars of the study, including its aim(s), methods and design, the names and contact details of key people and, as appropriate, the risks and potential benefits

2 I have been assured that I may withdraw from the study at any time without disadvantage or having to give a reason.

3 I have been given information about the risks of my suffering harm or adverse effects.

4 I have been told how information relating to me (data obtained in the course of the study, and data provided by me about myself) will be handled: how it will be kept secure, who will have access to it, and how it will or may be used.

5 I have been told that I may at some time in the future be contacted again in connection with this or another study.

Signature of participant ……
Date ……..

Signature of (principal) investigator ……
Date ……

Name of (principal) investigator *[in BLOCK CAPITALS please]*

……
……...

NAME: Golnaz Ranjbar

DATE: 20/01/2014

6.4. Appendix 4

FORM EC5 – STANDARD RISK ASSESSMENT FORM

1. IDENTIFY HAZARDS	2. WHO COULD BE HARMED & HOW?	3. EVALUATE THE RISKS	4. ACTION NEEDED
Unintended stabbing by lancet	Participants Inappropriate use of lancet	One of the trained staff will be with the researcher on day of experiment	Only retractable lancet devices should be used and lancets should be immediately disposed of in a sharps bin after use
Exposure to infectious agents	Participants Exposure to bacterial or fungous surface	Hands should be washed before start of the experiment Any blood spills should be wiped off immediately	Both subject and experimenter(s) hands should be washed prior to the experiment. Laboratory coats and gloves should be worn throughout the experiment.
Bruising at sampling site	Participants On the site of the injection	Only minimal pressure should be used to encourage the formation of a blood drop	Do not squeeze the site of injection Do not carry heavy bag straight after the blood test
Fainting	Subjects may feel faint at the site of blood	Do not panic at the time of fainting, since this can cause further complications	Lie the patient down and stay with them until they have recovered. Little sips of water and a wet towel applied to the forehead. Verbal communication throughout the procedure will reassure the subject. If complications arise contact the First Aid Officer
Incorrect blood collection procedure	No blood drawn	Lack of training may lead to an incomplete procedure	Adequate training for researcher is essential, in order to be able to collect blood samples correctly

6.5. Appendix 5

Participant Information Sheet

Study Title

Health Significance of Lactose Intolerance

This study will investigate if there is a difference in the effects of onion or a similar food on glucose uptake in lactose tolerant compared to lactose intolerant people.

Background:

The study involves you taking a hydrogen breath test in order to establish if you are lactose tolerant or lactose intolerant. You will blow into the hydrogen breath tester. You will then consume 25 grams of lactose dissolved in 250 ml of water and blow into the hydrogen breath tester 15, 30, 60, 90 and 120 minutes after consuming lactose.

You may then be selected for the next part of the experiment. This involves fasting overnight and then coming to the lab. A very small blood sample (10μl) will be taken by pricking your fingertip with a lancet. You will then drink a solution of 50g glucose dissolved in 220ml water and blood samples will be taken by finger prick at 15, 30, 45, 60, 90 and 120 minutes after drinking the glucose solution. This procedure is then repeated, but the second time you consume the glucose solution and an onion soup or similar food.

Voluntary participation:

There is no obligation for you to have these blood samples taken, which are being done for research purposes only. Your participation in this study is entirely voluntary and you may refuse to participate or discontinue participation at any time without penalty or loss of benefits to which you would normally be entitled.

6.6. Appendix 6

Confidentiality of records:

The samples will be destroyed soon after the results are available. You may have some minor discomfort associated with the collection of blood. For example, you may have pain or bruising due to the needle. Fainting may also occur when blood is taken, although this is rare.

There is no cost to you nor will you receive any payment for taking part. In any medical research study certain benefits may be derived. Such benefits include the possibility that information learned during this study may help others in the future. It is possible that there will be no benefit to you for participating in this study.

By signing this form, you authorize the use and disclosure of your protected information as necessary in order to carry out the research described in this document. Your personal identity will be kept confidential

Your information will remain confidential. Golnaz Ranjbar as the main researcher the ethics committee may have direct access to your information collected. These individuals will do everything possible to protect your identity.

INFORMED CONSENT

I have read the information provided above. I have taken enough time to decide whether or not I would like to give permission to undertake the blood sampling for this pre-screening analysis. I authorize the use and disclosure (sharing) of my personal health information as described in this form. I have retained a signed copy of this form.

Name of Participant: Signature of Participant: Date:

6.7. Appendix 7

Lactose Tolerant (LT)					
Participants	Age yr	Sex	Height (cm)	Weight (kg)	BMI kg/m2
1	26	8-Female (67%) 4-Male (33%)	162	65	24.7
2	29		173	71	20.7
3	28		177	54	17.2
4	29		177	78	24.9
5	29		162	69	26.3
6	32		167	52	18.6
7	34		173	57	19.7
8	31		167	77	27.6
9	21		165	63	23.1
10	21		177	57	18.1
11	20		165	55	20.2
12	31		174	70	23.1
Average	27.583333		169.9167	64	22.01667
SD	4.640892		5.853644	9.04534	3.411167

Lactose Intolerant (LINT)					
Participants	Age yr	Sex	Height (cm)	Weight (kg)	BMI kg/m2
1	34	7-Female (58%) 5-Male (42%)	171	82	28.7
2	30		168	74	26.2
3	32		175	69	22.5
4	23		169	95	33.2
5	27		179	85	26.5
6	28		167	52	19.5
7	37		173	80	26.7
8	25		164	54	20
9	21		169	62	20.7
10	19		168	64	22.7
11	44		172	75	25.3
12	37		183	63	18.8
Average	29.75		171.5	71.25	24.2333
SD	7.39932		5.36826	12.9553	4.30926

Age (LT+LINT)	
p-Value t-test	0.39943
p > 0.05 No statistically significant difference	
BMI (LT+LINT)	
p-Value t-test	0.1763
p > 0.05 No statistically significant difference	

Lactose Tolerant			**Lactose Intolerant**		
Participants	**Peak Gluc**	**Peak (Gluc+On)**	**Participants**	**Peak Gluc**	**Peak (Gluc+On)**
1	2.06	1.35	**1**	2.4	0.42
2	1.99	2.89	**2**	2.9	1.72
3	3.81	2.3	**3**	1.7	0.52
4	3.18	2.1	**4**	3.81	2.3
5	4.33	3.1	**5**	2.24	0.87
6	4.61	3.87	**6**	2.44	1.55
7	3.19	2.22	**7**	3.01	2.01
8	3.92	0.89	**8**	3.57	1.31
9	2.5	1.64	**9**	3.91	3.64
10	2.11	2.36	**10**	5.14	3.23
11	0.6	0.78	**11**	2.4	1.9
12	4.46	3.4	**12**	2.9	1.77
Mean	3.063	2.242	**Mean**	3.035	1.770
SD	1.180	0.927	**SD**	0.899	0.933
SE	0.340	0.260	**SE**	0.250	0.270
p-value	0.083		**p-value**	0.004	
% Peak G+Go (Inhibition p=0.08)			**% peak G+Go (Inhibition p=0.0037)**		
Peak (G+Go)	73.18	26.82	**Peak (G+Go)**	58.32	41.68