Evaluation of excipients for enhanced intestinal absorption of Rooibos flavonoids

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A thesis submitted in the fulfilment of the requirements for the degree of Master of Science in Biochemistry

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November 2016

Declaration

I, Nokulunga Hlengwa hereby declare that the work on which this thesis is based on is my original work (except where acknowledgements are indicated otherwise), and that neither the whole work or part of it has been, is being, or is to be submitted for another degree in this or any other university.

Signature……. Date…….

Nokulunga Hlengwa (201125793)

Abstract

Introduction

Aspalathin, a dihydrochalcone *C*-glucoside, and a major flavonoid unique to Rooibos, has been shown to ameliorate insulin resistance, improve glucose uptake *in vitro* and improve glycaemia in animal models. However, the poor bioavailability of aspalathin limits its potential as a nutraceutical. This study investigated the intestinal absorption of aspalathin, its flavone derivatives, orientin and isoorientin, and nothofagin, its 3-deoxy-derivative, from different green Rooibos extracts in an *in vitro* intestinal epithelial model, the Caco-2 cell model. Addition of excipients and a nanosome preparation of green Rooibos extract to improve absorption of these flavonoid *C*-glucosides was also investigated.

Methods

Seven green Rooibos extracts, prepared using different solvents (60% ethanol, 80% ethanol and an aqueous extracts), were assessed for the intestinal absorption of their major flavonoids, i.e. aspalathin and its flavone derivatives, orientin and isoorientin, as well as nothofagin, the 3-deoxy-derivative of aspalathin. For testing, two samples per extract type were selected from a large set, based on their bioactivity and chemical dissimilarity. These samples were prepared from different batches of plant material. Caco-2 cells were used as an *in vitro* intestinal epithelial model to assess absorption of the compounds. Intestinal permeability of the compounds was quantified by analysis of the apical and basolateral samples by HPLC-DAD. The relative transport rate (P_{app} values) was calculated from the detected concentrations of the compounds crossing the Caco-2 monolayer from the apical to the basolateral compartment. The effect of different extract types, addition of excipients (β-cyclodextrin and inulin) and encapsulation of green Rooibos extract in a nanosome formulation on the absorption of the flavonoids was also explored.

Results

At the highest soluble and non-toxic concentration of 1 mg/mL, the transport of aspalathin, nothofagin, orientin and isoorientin across the Caco-2 cell monolayer was confirmed, as these compounds were detected in the basolateral compartment by HPLC-DAD analysis. Standardization of the different extracts (n = 7) to their aspalathin equivalent concentration of 150 µM demonstrated that the transport rate of aspalathin (±1.72 x 10^{-06} cm/s) was not markedly altered by extract composition as determined by the extraction solvent and plant material. The rate transport was not increased by the addition of β-cyclodextrin (6.99 x 10^{-07} cm/s) and inulin (1.21977 x 10^{-06} cm/s), nor by using a nanosome (3.020 X 10^{-07} cm/s), implying that the strategies used in this study did not enhance intestinal absorption of aspalathin.

Conclusion

This study has provided novel information about the intestinal absorption of aspalathin which was not significantly altered by extract composition as determined by extract type and plant material variation. Neither the addition of excipients nor nanosome formulation of the extract could enhance the absorption of aspalathin.

Acknowledgements

I would like to express great gratitude to the following people and institutions:

My supervisor Prof C.J.F. Muller and co-supervisors Dr S. Bowles, Prof E. Joubert, and Prof A.K Basson for their assistance and guidance throughout this project.

Dr J. Louw, the Director of the Biomedical Research and Innovation Platform (BRIP), South African Medical Research Council for giving me the opportunity to study in his platform.

Dr R. Johnson for assistance with Western blot experiments; Dr D. de Beer for conducting HPLC-DAD analysis; Dr N. Chellan and Dr S. Ridiel for always availing themselves for emergencies in the Tissue culture laboratory.

My colleagues at BRIP Ms Y. Ntamo, Dr P. Dludla and Dr B. Jack for their technical assistance and sharing their knowledge.

My colleagues (Charity Masilela, Ridwaan Milase, Samukelisiwe Shabalala, Namani Ngema and Amsha Viraragavan), the laughter and memories we have shared have made the journey easier.

My family for their love and support. Special thanks to my mom and grandmother forever inspiring the power of education in me, may their souls rest in peace.

The National Research Fund (93438), the SAMRC Research Internship Scholarship Programme (Masters and PhD Scholarships) and the Biomedical Research and Innovation Platform of the South African Medical Research Council for funding.

Last but certainly not least, my Father God from which all things are made possible.

Table of Contents

Abbreviations

Act b	Beta actin
ATCC	American Type Culture collection
ATP	Adenosine-5 triphosphate
AP-BL	Apical to basolateral
Caco-2	Adenoma colorectal carcinoma epithelial cells
CO_2	Carbon dioxide
CYP	Cytochromes
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EMEM	Eagles minimum essential medium
EtOH	Ethanol
GI tract	Gastrointestinal tract
GLUT	Glucose transporters
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GRE	Green Rooibos extract
HBSS	Hanks' balanced salt solution
HEPES	N-[-2-hydroxyethyl] piperazine-N''-[-2-ethanesufonic acid]
HPLC	High performance liquid chromatography
MDR1	Multidrug resistance protein
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium
P_{app}	Apparent permeability
TEER	Transepithelial electrical resistance
SGLT	Sodium-glucose transporter
WHO	World Health Organization

List of Tables

List of Figures

Chapter 1

1. Introduction

Advancement of technology is increasingly making life more convenient and consequentially people are becoming less and less physically active. The resultant sedentary lifestyle, coupled to an unhealthy diet typically high in sugar, refined carbohydrates and saturated fats are major risk factors in the development of chronic diseases such as diabetes, heart diseases, obesity and cancer (Who and Consultation, 2003). A change in lifestyle including regular exercise and a healthier diet with increased consumption of fibre, fruit and vegetables are recommended to help combat the development of these diseases (Li *et al*., 2014). An increased intake of phenolic compounds commonly found in tea, fruit and vegetables has been shown to have various health benefits. Increased consumption of these compounds are particularly helpful in the management of metabolic disorders such as insulin resistance, a major causal factor in the development of metabolic diseases (Landete, 2012, Muller *et al*., 2012). Phenolic compounds are produced by plants as secondary metabolites, primarily as protectants against plant pathogens, herbivores, and are associated with sensory attributes such as colour, bitterness and astringency (Shi *et al*., 2003). These phenolic compounds, sequestered from the plant in the form of an enriched extract, can act as potent dietary supplements. The identification and characterization of specific bioactive phenolic compounds responsible for exhibiting health-promoting properties is essential for the development of products that are safe and effective.

Rooibos (*Aspalathus linearis)* is a fynbos plant indigenous to South Africa. Research into the health properties associated with Rooibos consumption is gathering momentum both locally and internationally. Most of the health benefits associated with Rooibos are linked to its phenolic composition (Joubert *et al*., 2008, Kamakura *et al*., 2015, Ku *et al*., 2015b, Lee and Bae, 2015, Mikami *et al*., 2015, Muller *et al*., 2016). Aspalathin, a dihydrochalcone *C*-glucoside uniquely present in Rooibos, is the most abundant flavonoid. Aspalathin has gathered a lot of interest due to its hypoglycaemic (Son *et al*., 2013), antidyslipidaemic (Beltran-Debon *et al*., 2011), anti-inflammatory (Baba *et al*., 2009) and anti-cancer effects (Snijman *et al*., 2007). Aspalathin has also been found to reduce insulin resistance (Mazibuko *et al*., 2013), stimulate insulin secretion (Kawano *et al*., 2009) and lower cardiovascular disease risk (Dludla *et al*.,

2014). However, aspalathin has a known low bioavailability (Breiter *et al*., 2011, Stalmach *et al*., 2009) and in this study we investigate the effect of extract composition on the intestinal absorption of the major Rooibos flavonoid *C*-glucosides, aspalathin, nothofagin, orientin and isoorientin, as well as excipients and a nanosome formulation of green Rooibos extract (GRE) to increase the absorption of these compounds. For this study excipients tested were inulin and beta-cyclodextrin. NovaSOL® a nanosome formulation, prepared with an aspalathin-enriched GRE and a polysorbate-type nonionic surfactant, was also tested.

Chapter 2

2. Literature review

2.1. Development and treatment of metabolic disease

Current reports on metabolic diseases such as obesity, diabetes and cardiovascular disease show that these diseases affect a concerning percentage of the human population worldwide (WHO, 2016). The socio-economic burden of metabolic diseases is increasing at an alarming rate emphasizing the importance of management of these diseases (WHO, 2016). Although genetics and aging play a role in the incidence of these diseases, global changes in lifestyle habits together with urbanization are largely responsible for their rapid development and incidence (WHO, 2016).

A Body Mass Index (BMI) of 18.5 to 25 is normal, 25 to 30 overweight and >30 clinically obese (Bray, 2014). According to the World Health Organization (2016) more than 1.9 billion adults worldwide are overweight and 600 million of these are obese. The accumulation of excess body fat (>20%) to the extent that an individual's health is negatively affected is a key determinant of obesity. Obesity is thus often a co-morbidity to other metabolic conditions such as insulin resistance, hyperlipidaemia and hypertension, all major casual factors for the development of type 2 diabetes (WHO, 2016). Statistics suggest that by 2030, type 2 diabetes will be the 7th leading cause of death worldwide (WHO, 2016). Type 2 diabetes, predominantly associated with lifestyle, specifically diet and lack of physical activity, accounts for 90% of diagnosed diabetics (WHO, 2016). Impaired glucose tolerance and a high fasting glycaemia are indicative transitional conditions from a healthy individual to a type 2 diabetic (Sarwar *et al*., 2010). The progression of the disease ultimately leads to other vital organs such as the heart, blood vessels, eyes, kidneys and nerves being damaged (Sarwar *et al*., 2010). The number one leading cause of death globally (17.1 million deaths in 2012) is cardiovascular disease. Cardiovascular diseases are classified as a combination of disorders that affect the heart and blood vessels (WHO, 2016). Type 2 diabetics have a two-fold higher risk of having a stroke or a heart attack (da Rocha Fernandes *et al*., 2016).

Available therapies to treat type 2 diabetes, although effective at controlling the clinical symptoms, i.e. glucose and lipid levels, do not prevent their progression (Nathan *et al*., 2009). Therefore, it is imperative that new therapeutic agents are discovered that

can be used alone or in conjunction with current therapeutics to provide treatment that prevent or reverse the disease progression. Apart from many third world countries where plant-based medicine is the mainstay in the treatment of chronic diseases, the use of natural products as therapeutics, due to their perceived efficacy and safety, is also gaining popularity in developed countries (Mishra and Tiwari, 2011, Weidner *et al*., 2012). Recent studies have shown that some of these herbal-based products have health-promoting properties (Craig, 1999, Dubey *et al*., 2004, Hoareau and DaSilva, 1999) attributed to their polyphenol content that could benefit metabolic dysfunction (Kawano *et al*., 2009, Snijman *et al*., 2007). Therefore, a market exists for the use of plant-based therapeutics in the treatment of metabolic diseases (Beltrán-Debón *et al*., 2011, Joubert *et al*., 2005, Kamakura *et al*., 2015, Ku *et al*., 2015b, Lee and Bae, 2015, Muller *et al*., 2016, Smith and Swart, 2016, Snijman *et al*., 2009).

2.2. Rooibos

Aspalathus linearis, better known as Rooibos, is an indigenous fynbos leguminous plant (Family: Fabaceae) that grows naturally in the Cederberg area of Western Cape, South Africa (Joubert *et al*., 2008). The most common and wide spread use for Rooibos is in the form of an herbal tea that has gained worldwide popularity since the 1990s. Even in the early 1960s, before it became known to alleviate colic in babies Rooibos was considered a healthy alternative to other related commonly consumed beverages such as coffee and black tea, because of its caffeine-free status and comparatively low tannin content (Cheney and Scholtz, 1963). Although fermented Rooibos tea is most commonly consumed, unfermented Rooibos, also known as green Rooibos has received much attention by the scientific community due to its higher polyphenolic content, specifically the dihydrochalcone *C*-glucoside, aspalathin, to which some of its health promoting effects including antioxidant activity have been attributed. The leaves of the Rooibos plant contain the highest levels of aspalathin. Although fermented Rooibos tea is considered a healthy beverage, the low levels of aspalathin remaining after processing and variations in its chemical composition limits its usefulness as a nutraceutical (Joubert and de Beer, 2011). Processing has been reported to significantly affect the antioxidant properties of Rooibos (von Gadow *et al*., 1997), with unfermented Rooibos demonstrating heighten mutagenesis protection, hydrogen donating ability and superoxide scavenging ability (Standley *et al*., 2001). Standardized extract preparations from unfermented Rooibos have been reported to

have more predictable biological effects (Ninfali *et al*., 2009). Enriched extracts prepared from unfermented Rooibos, with increased concentrations of flavonoids, in particular dihydrochalcones, have been shown to increase the bioactivity of the plant (Joubert and de Beer, 2011, Kawano *et al*., 2009, Muller *et al*., 2012, Son *et al*., 2013,) further supporting its potential use as a nutraceutical.

Figure 1: Photos of a flowering Rooibos plant showing needle-like leaves (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch provided the photo of the plantation).

2.3. Polyphenols

Polyphenols are an interesting group of phytochemicals that are abundantly present in plants. These phytochemicals are a group of diversified biologically active compounds formed as secondary metabolites (Babbar *et al*., 2015). Polyphenols are found in accumulated amounts in the dermal tissue of plants (Haslam, 1988). Polyphenols can be classified into different classes according to the number of phenolic rings in their structure, the elements that bind these rings to one another and the substituents linked to the rings. The known phenolic compounds of Rooibos are grouped into different classes according to their basic chemical structure. Of interest for this study are the flavonoids (Figure 2), a highly diversified group of polyphenols,

sharing a common three ring skeleton structure (C6–C3–C6) consisting of two aromatic rings (A and B) and a heterocyclic ring (C) containing one oxygen atom, whereas chalcones/dihydrochalcones, as the intermediates in the formation of flavonoids, have not yet established the heterocyclic C-ring (Halbwirth, 2010).

Demonstration of anti-oxidant properties of polyphenols have generated interest in their potential health-promoting effects. The demonstration of the protective effects of polyphenols against neurodegeneration, chronic diseases including type 2 diabetes, cardiovascular diseases and cancer (Mishra and Tiwari, 2011, Weidner *et al*., 2012), provides substantive evidence that long term intake of polyphenols could have beneficial effects. Polyphenols are shown to help reverse neurodegeneration pathology and neurocognitive decline associated with age (Vauzour *et al*., 2008), although the mechanism(s) whereby polyphenols ameliorate the disorder still requires further study. Their ability to improve the neurological health and prevent neuronal damage and losses is related to their interaction with intracellular neuronal and glial signalling, and their influence on the peripheral and cerebrovascular blood flow (Fraga and Oteiza, 2011, Hajipour *et al*., 2014, Schaffer and Halliwell, 2012, Williams *et al*., 2004). *In vitro* studies on the activity of polyphenols suggest that the health effect elicited extends beyond antioxidant properties (Kim *et al*., 2014). Polyphenols have been shown to modulate nuclear receptor gene expression and multiple signalling pathways related to oxidative stress, inflammation and lipid and glucose metabolism (Hanhineva *et al*., 2010). Polyphenols are able to protect pancreatic β-cells from glucotoxicity (Kim *et al*., 2007) and improve insulin secretion by increasing hepatic glucokinase activity, which enhances glucose utilization (Mazibuko *et al*., 2015) to promote energy storage in the form of glycogen and supressing hepatic glucose output (Hanhineva *et al*., 2010). Polyphenols exert numerous anti-cancer effects. Polyphenols available from food sources such as red wine and tea have been shown to influence carcinogenesis and tumour expansion (Middleton *et al*., 2000). Several possible mechanisms have been suggested including elimination of carcinogenic agents (Duthie and Dobson, 1999, Owen *et al*., 2000), intonation of cancer signalling pathways (Corona *et al*., 2007, Khan *et al*., 2006), promotion of apoptosis (Fini *et al*., 2008, Mantena *et al*., 2006) and cell cycle progression (Adam *et al*., 2009). Tumour formation is associated with changes in the metabolism of the cell as a result of increased cell growth requiring more energy and as a result increasing glucose uptake

and the rate of glycolysis. This may lead to the non-enzymatic glycation of proteins or lipids that can generate advanced glycation end products (AGEs) (Vauzour *et al*., 2008). Bioactive polyphenols have been documented to inhibit AGE receptors shown to play a role in regulating cancer cell invasion and metastasis (Kuniyasu *et al*., 2002, Sparvero *et al*., 2009). Polyphenols have been suggested to provide cardioprotection through the stimulation of antioxidant defences (Rein *et al*., 2000), enhancing endothelial function (Grassi *et al*., 2005, Habauzit and Morand., 2011, Heiss *et al*., 2007) reducing blood pressure (Habauzit and Morand, 2011, Vauzour *et al*., 2008), inhibiting platelet aggregation (Erlund *et al*., 2008, Keevil *et al*., 2000, Pearson *et al*., 2002) and through the inhibition of oxidation of low density lipoprotein (Mathur *et al*., 2002, Wan *et al*., 2001). The biological function of polyphenols is immense and polyphenolic compounds all display different therapeutic effects, also referred to as multi-component therapeutics which are linked to their chemical structure.

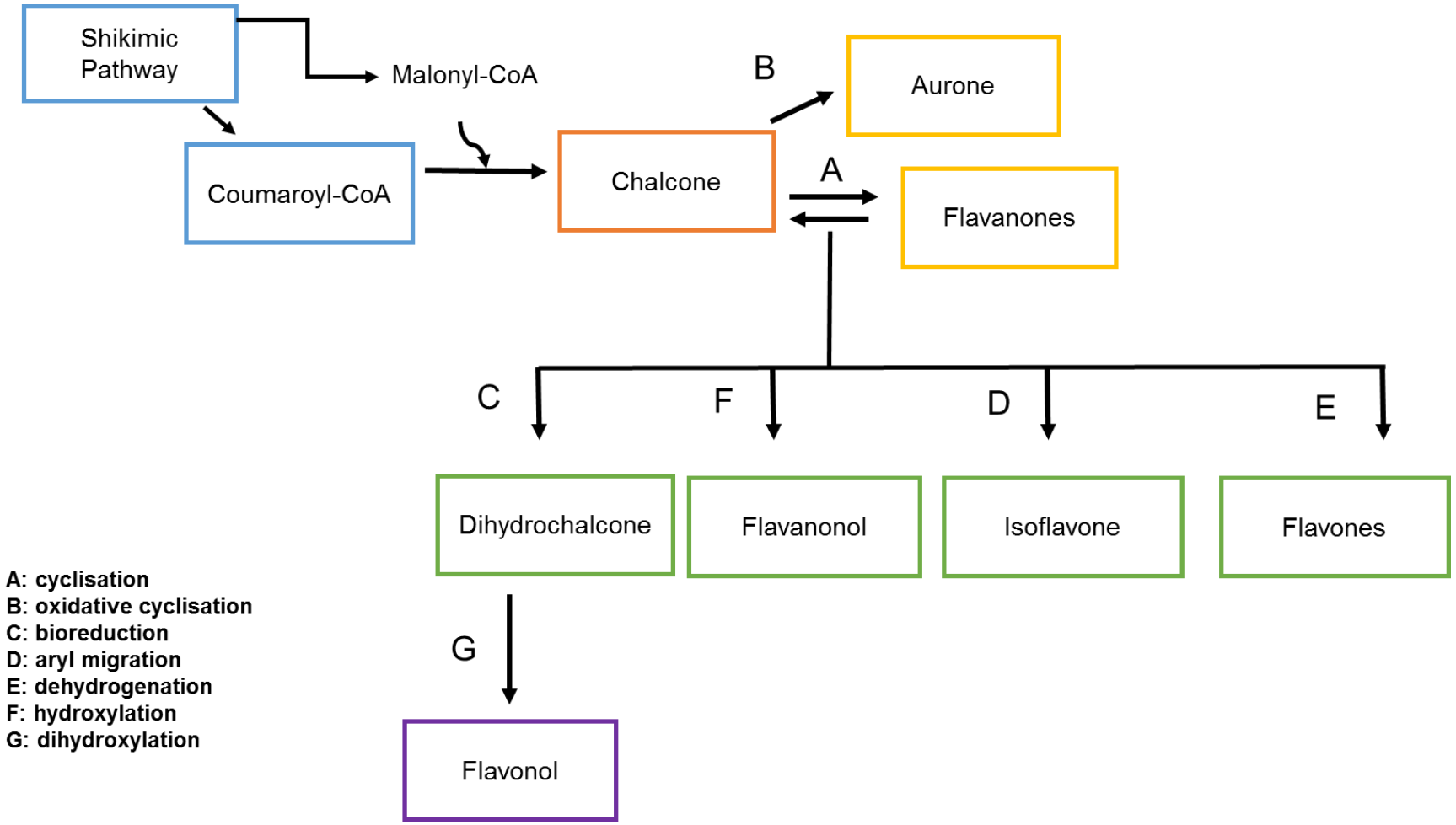


Figure 2: Schematic representation of the biosynthetic pathway of some flavonoids (adapted from Harborne and Baxter, 1999). Coumaroyl-CoA originates from phenylalanine which is produced from the shikimate pathway. Coumaroyl-CoA is a precursor for naringenin chalcone, an intermediate in the biosynthesis of dihydrochalcones such as aspalathin.

2.3.1. Flavonoids

The most abundant flavonoids are flavones and flavonols (Courts and Williamson, 2015). The Rooibos composition of monomeric flavonoids is unique in that it contains aspalathin, novel to Rooibos. Other major flavonoids are nothofagin, the 3-deoxy analogue of aspalathin and the flavone derivatives orientin and isoorientin (Joubert *et al*., 2008). Aspalathin and nothofagin belong to the dihydrochalcone subclass (Bramati *et al*., 2002). The high redox potential of these phenolic compounds makes them important anti-oxidants (Snijman *et al*., 2009, van der Merwe *et al*., 2010). Aspalathin is one of the most effective anti-oxidants present in Rooibos (Snijman *et al*., 2009). Additionally, proof of its glucose lowering properties and amelioration of palmitate insulin-resistance (Mazibuko *et al*., 2013, Muller *et al*., 2012, Snijman *et al*., 2009, Son *et al*., 2013) has increased interest in this compound. Although aspalathin is the most abundant bioactive flavonoid of Rooibos, the presence of other bioactive flavonoids have been identified in Rooibos extracts. These include the flavonol glycosides, rutin (quercetin-3-rhamnoglucoside), isoquercitrin (quercetin-3-β-D-glucoside) hyperoside (quercetin-3-β-D-galactoside) and phenylpyruvic acid-2-O-glucoside (PPAG) Z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid) (Bramati *et al*., 2002). Rutin has the ability to increase intracellular ascorbic acid levels, decrease capillary permeability and fragility, scavenge oxidants and free radicals, inhibit the destruction of bones, as well as lower the risk of heart diseases (Van Duyn and Pivonka, 2000). Several studies (Kamalakkannan and Prince, 2006a, Kamalakkannan and Prince, 2006b, Lin *et al*., 2012, Singh *et al*., 2008) have shown that rutin has anti-cancer, anti-fungal, anti-inflammatory and anti-hyperglycaemic effects. Isoquercitrin has anti-oxidant and oxidative damage protective activities (Dhiman *et al*., 2012). It exerts anti-oxidant effects by inhibiting lipid peroxidation, chelating redox-active metals, increasing absorption of vitamin C and direct scavenging of ROS (Dhiman *et al*., 2012). Hence, isoquercitrin displayed bioactivity against several types of cancers (Ran *et al*., 2016, Sudan and Rupasinghe, 2015). Hyperoside has been shown to display anti-mutagenic effects (Snijman *et al*., 2007) and has potent apoptotic and autophagic properties against pancreatic cancer cell lines (Boukes and van de Venter, 2016). Phenylpropenoic acid glucoside is not a phenolic compound, but it is a biosynthetic precursor of phenolic compounds. To date, it has only been found in Rooibos and a few other plant species. This compound displays hypoglycaemic activity in Chang cells

(Muller *et al*., 2013), protects against pancreatic beta cell death (Himpe *et al*., 2016) and prevents the development of the metabolic syndrome (Dludla *et al*., 2014).

2.3.2. Flavones

Flavones are abundantly found in celery, parsley and some herbs like Rooibos (Harborne and Williams, 1988). Flavones are similar to flavonols though they lack oxygenation at the third carbon. Generally known flavones include apigenin, luteolin, tangeritin, chrysin, 6-hydroxyflavone and baicaleine. The compounds are able to be structurally altered through methylation, *O*-and *C*-glycosylation and alkylation (Škerget *et al*., 2005).

2.3.3. Isoflavone

Bountifully found in leguminous plants such as Rooibos, isoflavones structurally have a B-ring attached to the third carbon (Cederroth and Nef, 2009). This distinct feature allows the binding to oestrogen receptors due to isoflavones similarity to oestrogen, hence they are classified as phytoestrogens. These are commonly found in soybean as conjugates i.e. diaidzein and gestein, but are also detected at lower concentrations as aglycone derivatives (Cederroth and Nef, 2009).

2.3.4. Flavanones

The most common flavanones include narigenin and hesperidin, found in fruits such as lemons and grapefruit, and occur as colourless compounds (Fabre *et al*., 2001). Flavanones are characterized by the absence of a double bond at the second carbon and the presence of a chiral centre. Flavanones occur as conjugated hydroxyl, glycosylate, *O*-methylate derivatives (Fabre *et al*., 2001).

2.3.5. Flavonol

Flavanols exist as a complex subclass of flavonoids. They are one of the few subclasses that are not found as glucosides (Murphy *et al*., 2003). Flavanols range from simple monomers to complex oligomers and polymeric proanthocyanidins. Tannis is one of the commonly known oligomeric proanthocyanidins. The addition of an epicatechin unit to proanthocyanidins produce procyanidins which are the most abundant proanthocyanidins (Del Rio *et al*., 2013).

2.4. Therapeutic effects of dihydrochalcone *C*-glucosides and flavone *C*-glucosides present in Rooibos

A common protective effect is observed amongst the flavonoids of Rooibos irrespective of structural differences. Current research is focused on revealing the specific mechanisms by which these flavonoids elicit their bioactivity. *In vitro* dihydrochalcones, such as aspalathin and its flavone derivatives, act as reducing agents and therefore exhibit anti-oxidant properties by decreasing the rate or preventing the oxidation of molecules, as well as participation in the regeneration of other anti-oxidants (Snijman *et al*., 2009). The ability to act as an anti-oxidant is linked to the chemical structure of the compounds (Landete, 2013). Certain structural features of flavonoids *C*2' and *C*6' hydroxyl groups, as well as the keto-enol tautomerism of the carbonyl and alpha-methylene groups, are important contributing factors to the anti-oxidant activity of dihydrochalcones (Nakamura *et al*., 2003, Rezk *et al*., 2002). *In vivo* the contribution of these exogenous anti-oxidants in comparison to the endogenous anti-oxidant system is small. However increased ingestion of various flavonoids, including aspalathin, have been shown to enhance the endogenous responses, particularly under increased oxidative stress (Breiter *et al*., 2011).

Aspalathin and nothofagin have been reported to display anticoagulant activities (Ku *et al*., 2015a). The dihydrochalcones are shown to actively prolong activated partial thromboplastin time and prothrombin time and inhibited the activity of thrombin activated factor X. Additionally, aspalathin and nothofagin inhibited thrombin catalysed fibrin polymerization and platelet aggregation (Ku *et al*., 2015a).

The anti-inflammatory activity of the dihydrochalcones is suggested to prevent DNA damage and inflammation by reducing oxidative stress (Baba *et al*., 2009). The dihydrochalcones are shown to actively inhibit lipopolysaccharide induced blood-brain barrier disruption, expression of cell adhesion molecules, transendothelial migration of neutrophils to human endothelial cell and the suppression of tumor necrosis factor-α and the activation of nuclear factor-kB (Lee and Bae, 2015).

An aspalathin-enriched GRE (ARC 2; ca. 18%) showed α-glucosidase inhibitory effects *in vitro* which demonstrates the anti-diabetic potential of Rooibos (Muller *et al*.,

2012). Enhanced glucose uptake was also demonstrated for ARC 2 in C2C12 skeletal myotubules and Chang liver cells. *In vivo* the extract improved glycaemia in STZ-induced diabetic rats. The study showed that ARC 2 was effective in increasing glucose uptake independent of insulin stimulation. This efficacy of the extract was further confirmed in a rat study where ARC 2 lowered glucose levels over a broad therapeutic range (5-50 mg/kg) (Muller *et al*., 2012). A study conducted by Mazibuko *et al*. (2015) on insulin resistance and adipocyte inflammation, showed that the initiation of these conditions is strongly linked with the activation of nuclear factor-kβ. Palmitate-induced insulin resistant 3T3-L1 adipocyte cells which, when treated with either the GRE or pure aspalathin suppressed activation of nuclear factor-kB and IRS1 (Ser ^{307}) phosphorylation (Mazibuko *et al*., 2015). The GRE increased insulin stimulated protein kinase B (AKT) activation which was reduced by palmitate, increasing glucose transporter4 (GLUT4) translocation to the cell membrane and enhanced glucose uptake. A concomitant decrease in activated protein kinase (AMPK) phosphorylation was also demonstrated (Figure 3). This effect was observed for the green Rooibos extract only and not for aspalathin. Pure aspalathin was able to effectively increase peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor gamma (PPARγ) protein expression**.** Although the dihydrochalcones prove to be effective potential therapeutics, their low bioavailability and rapid metabolism restricts their bioactivity.

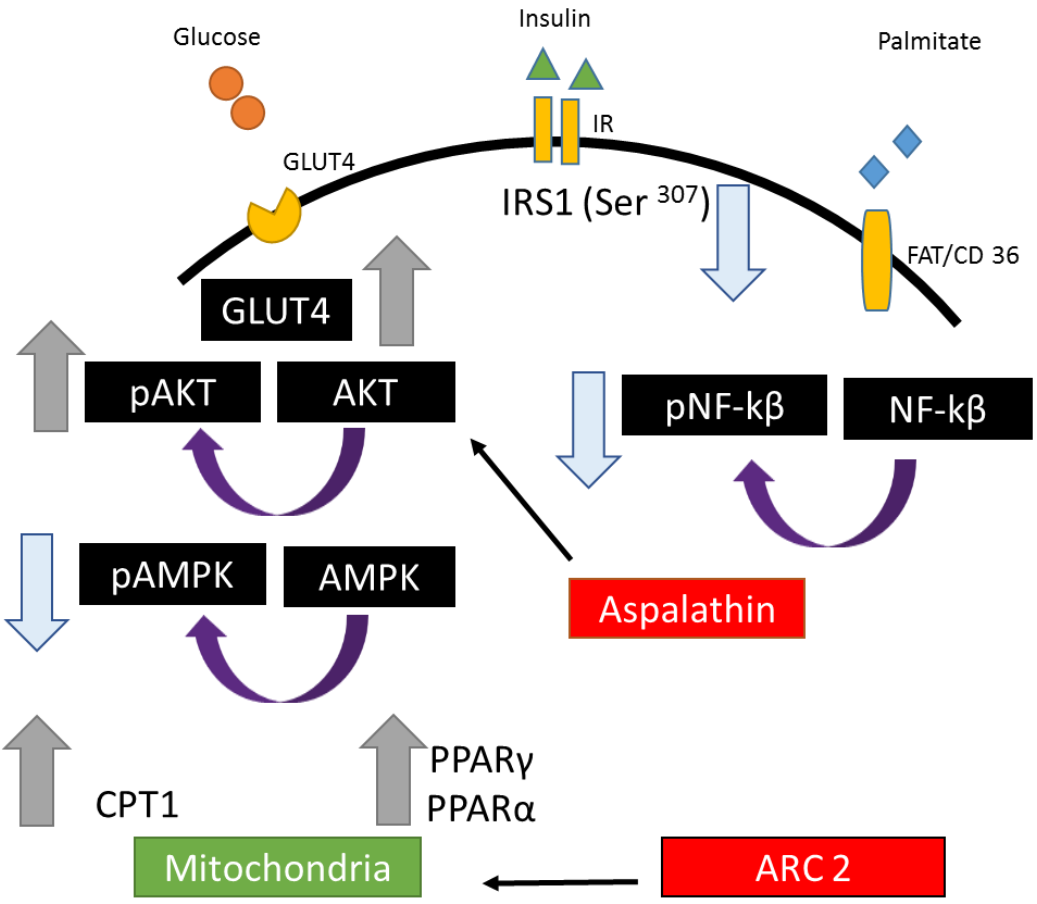


Figure 3: Schematic diagram illustrating the signal transduction pathways affected by ARC 2 and aspalathin inhibiting insulin resistance in 3T3-L1 adipocytes (adapted from Mazibuko *et al*., 2015). Molecular mechanisms whereby ARC 2 and aspalathin ameliorated palmitate-induced insulin resistance include suppression of IRS 1(Ser ^{307}) phosphorylation and activation of NF- kB, activators of cellular insulin resistance. The extract and aspalathin enhanced insulin signaling via AKT and GLUT4 expression, and activation of AMPK culminating in increased glucose uptake and lipid metabolism by increasing PPARα, PPARγ and CPT1.

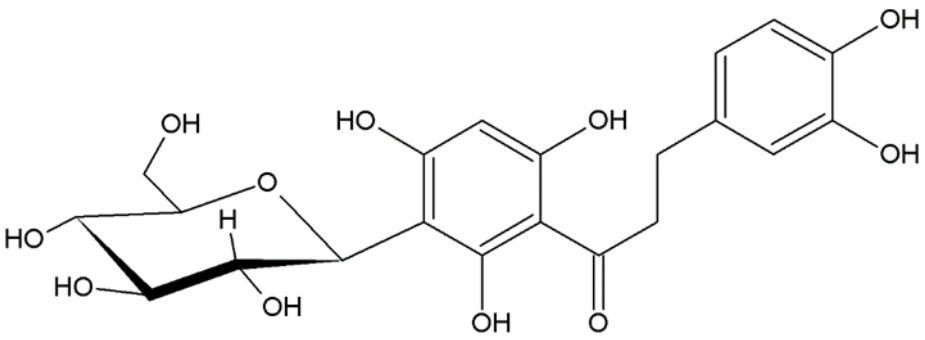


Figure 4: Chemical structure of aspalathin, a dihydrochalcone *C*-glucoside novel to Rooibos (Chemsketch).

2.5. Intestinal absorption, distribution, metabolism and excretion of dihydrochalcone *C*-glucosides (ADME)

Phenolic compounds, although generally associated with health benefits, are treated by the body as xenobiotics, similar to many pharmaceutical products. In general, polyphenols are extensively metabolized by the gut enzymes and microflora (Mosele *et al*., 2015, Scalbert *et al*., 2002). Further metabolism occurs once the metabolites reach the liver and the subsequent conjugated derivatives *O*-glucuronides, sulphate esters or *O*-methyl ethers are then excreted either by the urinary or biliary routes (Scalbert *et al*., 2002).

A number of studies have been conducted on the metabolism and absorption of aspalathin, identifying the presence of unmetabolised aspalathin, together with the presence of some major metabolites (mostly methylated, glucuronidated and sulphated) (Breiter *et al*., 2011, Courts and Williamson, 2009, Kreuz *et al*., 2008, Stalmach *et al*., 2009). Pharmacokinetic studies showed low or undetectable levels of aspalathin in the blood plasma, however, detection of aspalathin and its metabolites in the urine confirmed its absorption albeit at very low levels (Breiter *et al*., 2011, Courts and Williamson, 2009, Kreuz *et al*., 2008, Stalmach *et al*., 2009). Aspalathin is poorly absorbed across the intestinal barrier. The hydrophilic nature of aspalathin is due largely to the presence of the attached glucose molecule and it is suggested that the stable *C*-*C* bond between the glucose moiety and the dihydrochalcone structure may hinder the absorption of aspalathin (Courts and Williamson, 2009).

Huang *et al* (2008) evaluated the transport of aspalathin from green Rooibos extracts and synthetic aspalathin across human abdominal skin vertical Franz diffusion cells and Caco-2 cell monolayers. Transport across percutaneous cells showed that only a minute percentage (0.01%) of pure aspalathin and aspalathin present in the extract were transported. P_{app} values obtained from the transport of aspalathin across the Caco-2 cell monolayer reached almost 100% and were shown to be concentration dependent. Aspalathin present in the extract showed a lower transport rate (79.03%). However, our findings are not in agreement with Huang *et al* (2008). The metabolic characteristic and transport of the flavonoid *C*-glucosides orientin and isoorientin were investigated by Shi and colleagues (2016). The *C*-glucosides were metabolized and generated two metabolites which were monoglucoronides. The route of transport used for the passage of orientin and isoorientin across the Caco-2 cell monolayer was passive diffusion (Shi *et al*., 2016). The flavone luteolin is the aglycone of aspalathin and it is abundantly found in *Artemisia afra*. The flavone is found as an aglycone and *O*-glucoside (luteolin-7-*O*-β-glucoside) in the plant (Mukinda *et al*., 2010). The aglycone of luteolin is absorbed and extensively metabolized by the enterocytes to produce monoglucoronide conjugates (Mukinda *et al*., 2010). Luteolin-7-*O*-β-glucoside is deglycosylated into the aglycone form prior to absorption (Shimoi *et al*., 1998).

Polyphenols that are not absorbed in the small intestines accumulate in the colon to be further metabolized by the gut microflora to yield a wide array of metabolites (Mosele *et al*., 2015). Quercitin and rutin are biotransformed by the intestinal microbiota into protocatechuic acid and quercetin-3-glucoside for quercetin (Rao and Weisner, 1981) and 3,4-dihydrophenylacetic acid (DOPAC) for rutin (Aura *et al*., 2002) respectively, and these metabolites are shown to have anti-cancer, anti-inflammatory, cardioprotective and neuroprotective properties (Nunes *et al*., 2009). The bacterial fermentation products serve as nutrients as well as growth signals for the intestinal epithelium (Nunes *et al*., 2009). Polyphenols are reported to be inhibitors of several digestive enzymes including lipases and α-glucosidases (Muller *et al*., 2012, Nishibori *et al*., 2012). Polyphenols may act by decreasing the lipid absorption (Nishibori *et al*., 2012). This strongly suggest that some polyphenols are effective inhibitors of lipases and α-glucosidases and display potential therapeutic properties against hyperlipidaemia. However, the biotransformation of polyphenols by the gut microbiota

is shown to have beneficial effects to the host (Klinder *et al*., 2016). Non-digestible food ingredients that improve health and selectively stimulate growth and activity of a group of beneficial gut bacteria is termed a prebiotic (Manning and Gibson, 2004). The interaction between gut microbiota and polyphenols has two fates, either the polyphenols are broken down by the microbial and digestive enzymes, absorbed and excreted as metabolites and catabolic products of the parent polyphenols or gut microbial population could be altered by polyphenols (Van Duynhoven *et al*., 2011). Polyphenols and their respective metabolites influence and induce the modulation of gut microbiota and inhibit pathogenic bacteria (Laparra and Sanz, 2010). The complex polyphenols are converted into many metabolites that in turn elicit an array of health benefits (Klinder *et al*., 2016). The hepatic biotransformation of aspalathin and nothofagin by sulfation is one of the major conjugation pathways for aspalathin (van der Merwe *et al*., 2010). However, methylation diverts the conjugation pathway from sulfation as more *O*-methyl glucuronide metabolites are detected in urine (Stalmach *et al*., 2009).

There have been numerous challenges in understanding the specific mechanism of absorption of aspalathin. Liu *et al*. (2015) suggested glucose transporter 2 (GLUT2) and the sodium-glucose linked transporter 1 (SGLT1) as a possible mechanism of transport. On the other hand, a number of studies (Stalmach *et al*., 2009, Zhang 2007, Walgren *et al*., 1998) have suggested passive diffusion or alternatively the movement of flavonoids through the tight junctions of intestinal cells as possible modes of transport. The uncertainty of the specific mechanism of transport requires further investigation.

Carrier-mediated transport involves facilitated and active mechanisms of transport, responsible for both the uptake and efflux of drugs, which are located on the apical and basolateral side of the enterocytes, kidney, liver, placenta, blood-brain barrier, testes, and choroid (Dobson and Kell, 2008). The most commonly expressed efflux transporters belong to the ATP–binding cassette which include P-glycoprotein, multidrug-resistant protein and the breast cancer resistant protein. The P-glycoprotein is a well-recognized efflux transporter which influences absorption and secretion of several drugs due to its widely reactive spectrum (Dobson and Kell, 2008). The breast cancer resistant protein is also capable of extrusion of glucuronides and sulfate conjugates formed in the enterocytes (Dobson and Kell, 2008).

Active transport involves the use of energy (ATP) and the translocation of transporters to the cell membrane for active uptake of drugs. The human body has phase 1 and phase 2 defensive barriers against foreign substances. Phase 1 reactions consist of oxidation, reduction and hydrolysis (Dobson and Kell, 2008). The aim of these reactions is to render a molecule more hydrophilic with the addition of a functional group to facilitate phase 2 reactions carried out by groups of closely related isozymes called cytochrome P450-depedent mixed function oxidases (Jancova *et al*., 2010). Metabolism of bulk xenobiotics, that enter the body through the oral route, are carried out by CYP1A, CYP2C, CYP2D and CYP3A (Jancova *et al*., 2010, Patel *et al*., 2016) which are cytochrome P450 enzymes involved in a variety of metabolic and biosynthetic processes. CYP3A4 have broad spectrum and specificity and hence are important in xenobiotic metabolism. Glucoronidated, sulphated and methylated metabolites of aspalathin have been detected in urine samples (Kreuz *et al*., 2008). In the liver substantial modification of polyphenols takes place and the metabolites formed are then exported back out of the liver and into the blood stream and carried out into the kidneys where they are excreted in urine. Generally, flavonoid metabolites may be exported back into the bile and released back into the gut lumen. These metabolites have several fates; either being excreted in the faeces, that undergo microbial enzyme metabolism forming less polar metabolites or being reabsorbed and further metabolized (Spencer *et al*., 2004).

The absorption of polyphenolic compounds is largely dependent on the modifications that occur during metabolism and absorption in the gastrointestinal tract. Polyphenols are partially released from food matrixes in the oral cavity (Ghosh and Bandyopadhyay, 2012) via the interaction with salivary protein that is rich in proline (Ghosh and Bandyopadhyay, 2012). This occurs either through hydrogen bonds or hydrophobic interactions. The polyphenol-protein complex improves solubility but does not affect its absorption. The conditions in the stomach such as pH are known to affect polyphenols. As such, phenolic compounds are modified and some phenolic derivatives are absorbed in the stomach (Tagliazucchi *et al*., 2010). Flavonoids are extensively metabolized in the small intestine, deglycosylated into their aglycone derivatives releasing the sugar unit. The metabolism is facilitated by two active enzymes lactase phloridzin hydrolase and cytosolic β-glucosidase (Lewandowska *et al*., 2013). Lactase phloridzin hydrolase is abundantly found in the brush borders of

the small intestines (Lewandowska *et al*., 2013). Dihydrochalcones *C*-glucosides are shown to be resistant to lactase phloridzin hydrolase and cytosolic β-glucosidase as the *C*-glycosidic linkage appears to remain intact in the upper and mid- gastrointestinal tract (Courts and Williamson, 2015).

Glucuronidation occurs primarily in the small intestines mediated by a family of enzymes called uridine diphosphate glucuronosyltransferase (Kay, 2006). After extensive metabolism some phenolic compounds are absorbed into the interior of enterocytes transported by the portal vein to the liver to undergo phase two metabolism (Kay, 2006). The role of dihydrochalcones on the oxidative status of the liver and observed effects on the serum biochemical parameters relates to their bioavailability. The presence of aspalathin and its flavone derivatives (orientin and isoorientin) respectively, has been reported in plasma of humans. In humans Breiter (2011) reported a concentration of up to 1.97 nmol aspalathin in the plasma, 3 h after consumption of 500 mL (containing 287 mg aspalathin) of Rooibos. The measurement of the dihydrochalcone concentration in the plasma and urine gives a predictable concentration of the compounds at the receptor site of target tissues to confer its therapeutic effect (Scalbert *et al*., 2002). In addition, the concentration of the compounds in the sampled fluids reflect the concentration at the receptor site as well as in other tissues allowing therapeutic plasma concentrations of the compound to be detected (Scalbert *et al*., 2002).

2.6. Pharmacological agents for improving absorption

Improving the bioavailability of aspalathin and its flavone derivatives will overcome one of the major limiting factors in their use as therapeutic agents. Bile salts present themselves as suitable substrates in aiding in the absorption of compounds. Bile salts are emulsifying agents that assist the absorption of dietary fats and other compounds (Youngson, 2005). A study conducted by Meaney and colleagues (2000) evaluated the membrane permeation enhancement potential of simple bile salt and mixed bile salts on mannitol and polyethylene glycol using Caco-2 cells. The results from the study showed that the mixed bile salts (mixed micellar system with linoleic acid) had a superior enhancement effect without increasing toxicity. However, the mechanism appeared complex and possible combination effect of paracellular and transcellular routes may be involved.

The use of chemical compounds that act as mediums to transport compounds and render them more soluble is extensively used in food and pharmaceutical industries. These include excipients which are defined as natural or synthetic compounds used to enhance the bioavailability of bioactive pharmaceutical and nutraceutical compounds (McClements, 2015). Efficacy of active compounds, whose bioavailability is limited by factors such as gastrointestinal transformation, poor absorption and efflux mechanisms, can be improved by excipients (McClements, 2015). The selection of an excipient is guided by the existing physiochemical properties of each compound i.e. factors need to be considered for aspalathin are its hydrophilic nature, strong affinity for proteins, anti-oxidant properties and its resistance to digestive enzymes.

2.6.1. Excipients

Cyclodextrins are a commonly used class of excipients in the pharmaceutical industry. They possess the characteristic feature of a toroidal shape consisting of cyclic oligosaccharides (Hamman and Steenekamp, 2012), resulting in a hydrophilic exterior and a hydrophobic interior character (Bindu *et al*., 2010). Cyclodextrins are able to solubilize drugs by forming inclusion complexes incorporating lipophilic drugs or part of the drug into the hydrophobic interior of the cyclodextrin molecule (Bindu *et al*., 2010). Inclusion of the drug improves the solubility and dissolution rate of poorly soluble drugs (Arun *et al*., 2008). Beta-cyclodextrin-compound inclusion complex is

transported across the Caco-2 cell monolayer using endocytosis (Fenyvesi *et al*., 2014).

Inulin is classified as a storage polysaccharide naturally occurring in plants that belong to the *Asteracea* family. Industrially produced from chicory root (*Cichorium intybus*), inulin is a widely used food ingredient (Madrigal and Sangronis, 2007). It is present as mixtures of oligosaccharides and polysaccharides, consisting of fructose units with beta-configuration of the anomeric C2 (Apolinario *et al*., 2014). This characteristic feature makes inulin resistant to hydrolysis by the human digestive system (Roberfroid, 2007), thus exhibiting a very low caloric value. As a result, inulin has extensive food and pharmaceutical applications (Roberfroid, 2007). Inulin as an excipient is an ideal transport vehicle, encapsulating sensitive ingredients and allowing their slow release into the circulatory system (Barclay *et al*., 2010). Compounds encapsulated with inulin are transported across the monolayer using either passive diffusion or paracellular transport (Ma *et al*., 1991).

Table 1: Industrially used excipients.

Function	Excipient	Derivatives	Properties	Disadvantages
Specialized delivery systems	Cyclodextrins	β-cyclodextrin, 2-Hydroxypropyl-β-cyclodextrin, Sulfobutylether β-cyclodextrin sodium, Randomly methylated β-cyclodextrin and Branched β-cyclodextrin	Complexation ability, reduce gastrointestinal drug irritation, convert liquid drugs into microcrystalline, prevent drug-drug interaction and drug-excipient interactions	Relatively large
Specialized delivery systems	Inulin	Oligofructose and fructooligosaccharides	Prebiotic, humectant, emulsifier, gel forming and sugar and fat substitute	Increased consumptions could lead to gut bacterial over growth, flatulence and diarrhea.
Specialized delivery systems	Hypromellose acetate succinate	2-hydroxy propyl ether and methylhydroxyprop(ox)yl cellulose	High drug dissolution levels, temperature and humidity stable and able to modulate drug release	Polymer made matrix will swell in presence of media
Specialized delivery systems	Methyl methacrylate diethylaminoethyl methoacrylate	Poly-methyl methacrylate or acrylic glas	Monofunctional acrylate monomer with both methacrylic and amine functional groups, offers excellent adhesion to metallic and plastic substrates, prevent stomach dissolution	An allergic response to skin exposure may develop, and respiratory symptoms such as chest tightness, shortness of breath, coughing, and wheezing may occur.

Summary of specialized delivery excipients extensively used in the food and pharmaceutical industries.

2.6.2. Nanoemulsion

Nanotechnology deals with the use and application of nanoscale materials (1 to 100 nanometres) for industrial use. Nanotechnology is applied to enhance drug delivery *in vivo* and *in vitro*. Its uses aim to improve drug and nutraceutical bioavailability (Duncan, 2003, Tomellini *et al*., 2005). The application of nanotechnology in drug delivery is increasingly being applied in food and pharmaceutical industries to make compounds more stable, improving functionality, aid in the delivery of compounds and addition of taste (De Jong and Borm, 2008). Nanomedicine has been shown to be able to decrease toxicity and ameliorate damaging side effects of particular drugs by regulating their release into the circulatory system (De Jong and Borm, 2008). The use of nanotechnology in the development of functional foods such as plant extracts, aims to improve stability and efficacy. Formulation of solubilisates on nanoscale, known as liposomes or nanosomes, have an advantage of high intestinal absorption and dermal penetration (Castor, 2005). Nanosomes are very small liposomes. They are created by down-sizing liposomes with ultrasonic energy until they are less than 100 nm in diameter. Nanosomes display other favourable characteristics such as thermal and pH stability as well as stability to microbial damage (Castor, 2005).

An example of a nanosome product is the NovaSOL® nanosome used to encapsulate both hydrophilic and hydrophobic compounds and plant extracts into a nano "solubilisate" that are soluble in both water and fat (Blasco and Pico, 2011). The nanoscale size of the micelles improves the bioavailability of bioactive compounds. *Reseda luteola* extract containing 40% flavonoids was solubilized with polysorbate, resulting in product micelles with a diameter of 10 nm (Casetti *et al*., 2009). Several functional food ingredients and additives were encapsulated in this nanomicelle-based carrier system (NovaSOL from Aquanova, Germany) for introduction of anti-oxidants and supplements in food and beverages (Chaudhry *et al*., 2008). An aspalathin-enriched green Rooibos extract encapsulated in this manner, with ascorbic acid as anti-oxidant, was available for testing and thus was included in the current study. Previously it was used to produce a clear Rooibos iced tea and to improve stability of aspalathin when subjected to heat when in solution (Joubert *et al*., 2010).

2.7. Caco-2 cell model

Gastrointestinal permeability of drugs is commonly assessed using the Caco-2 model, which is employed frequently in pre-clinical investigations as an integral component of the Biopharmaceutics Classification system (BCS) recommended by the Food and Drug Administration (FDA) (Bergstrom *et al*., 2014, Wu and Benet, 2005). Caco-2 cells express cytochrome P450 enzymes, transporters, microvilli and enterocytes with identical characteristics to the human small intestine. This model aids in the prediction of solubility, bioavailability and the possibility of drug-drug or herb-drug interactions in the gut lumen (Awortwe *et al*., 2014). Laboratories use the Caco-2 cell line to screen for gastrointestinal absorption of new chemical entities (Awortwe *et al*., 2014). Caco-2 cells are derived from human epithelial colorectal adenocarcinoma cells and are used as an *in vitro* cell model representing the enterocytes barrier of the gastrointestinal tract (Hu *et al*., 2004). To study absorption and transport of drugs and chemical compounds, Caco-2 cells are grown on polycarbonate membrane inserts where they undergo spontaneous enterocytic differentiation under appropriate culture conditions. These cells become polarized adopting a columnar appearance and displaying similar characteristics to the large and small intestinal epithelial cells (enterocytes), expressing functional brush border enzymes (Hu *et al*., 2004).

The Caco-2 cells form intact monolayers with junctional complexes between adjacent cells. These junctional complexes comprise of several structures (Hu *et al*., 2004), including desmosomes, that provide strong cell-cell attachment via intermediate filaments, adjacent cell attachment via calcium-dependent cell-cell adhesions linked to actin and myosin filaments and tight junctions facilitated by adherent junctions. Gap junctions facilitate communication between cells. The tight junctions are responsible for sealing the intracellular space on the apical portion of the enterocyte (Tsukita *et al*., 2001).

The gastrointestinal epithelium is composed of a variety of cells i.e. enterocytes, goblet cells, Paneth cells and enteroendocrine cells (Buddington and Sangild, 2011). The enteroendocrine cells constitute 1% of the entire epithelium and are responsible for numerous processes such as the production of secretin hormones involved in gastric motility, blood flow and food intake (Buddington and Sangild, 2011). There are two types of enteroendocrine cells, divided based on their location in the gastrointestinal

epithelium (Sternini *et al*., 2008). The closed type cells are not in direct contact with the lumen while the open type cells are in direct contact with lumen and are suggested to be able to detect the chemical changes of luminal nutrients (Liou *et al*., 2011). L cells, a sub-group of the open type entero-endocrine, are responsible for the production and secretion of proglucagon derived peptides such as glincentin, oxyntomodulin, glucagon-like peptide 1 (GLP-1) and glucagon-like peptide (GLP-2) (Pedersen *et al*., 2008). K-cells, a sub-population of enteroendocrines, are responsible for the production and secretion of the incretin hormone, glucose-dependent insulinotropic polypeptide (GIP). During the consumption of food, incretin hormones are released by enteroendocrine L and K cells. The incretin hormones GLP and GIP act as receptors for the pancreatic beta cells to stimulate insulin production and secretion (Lim and Brubaker, 2006). Insulin then stimulates glucose uptake in the liver, muscle and adipose tissue (Foryst-Ludwig *et al*., 2008). The restoration of glucose levels to the basal concentrations stops incretin-mediated insulin secretion minimizing hypoglycaemia (Ranganath, 2008). The Caco-2 cell monolayer has been documented to have limitations. Caco-2 cells are heterogeneous and the parental population has been shown to express numerous clones that are morphologically and biochemically different (Delie and Rubas, 1997, Sun *et al*., 2008). The cells are unable to produce mucin which functions to provide protection against hydrolysis from gastric and pancreatic secretions. They also lack certain P-450 metabolizing enzyme activity (Blaser, 2007). Despite these limitations, Caco-2 monolayers are able to yield extremely useful information with regard to the apparent rate of transport of compounds across their monolayer that can be directly comparable to the human gastrointestinal tract and is an excellent and well used model to predict compound bioavailability.

The transport of drugs across the intestinal epithelium occurs through four possible routes: passive transcellular, paracellular, carrier mediated and transcytosis (Figure 5). The transcellular pathway is the most common route of transport due to the cellular absorbing surface, which is greater than the area of the paracellular spaces (Pappenheimer and Reiss, 1987). A paracellular permeating compound is normally small and possesses the physiochemical properties of being polar and/or water soluble. The carrier mediated route is facilitated by active transporters such as the glucose transporter2 (GLUT2) and sodium gradient linked transporter1 (SGLT1) which

is abundantly found in the gastrointestinal tract (Hayashi *et al*., 2003). The passive diffusion of compounds requires bidirectional transport rate to be uniform. Compounds that are actively transported should display transport rates that are significantly higher from the apical-basolateral (A-B) direction when compared to the basolateral-apical direction (B-A). The active efflux of compounds is determined by the transport rate of the A-B directional transport experiments being significantly lower when compared to the B-A (Hu *et al*., 2004).

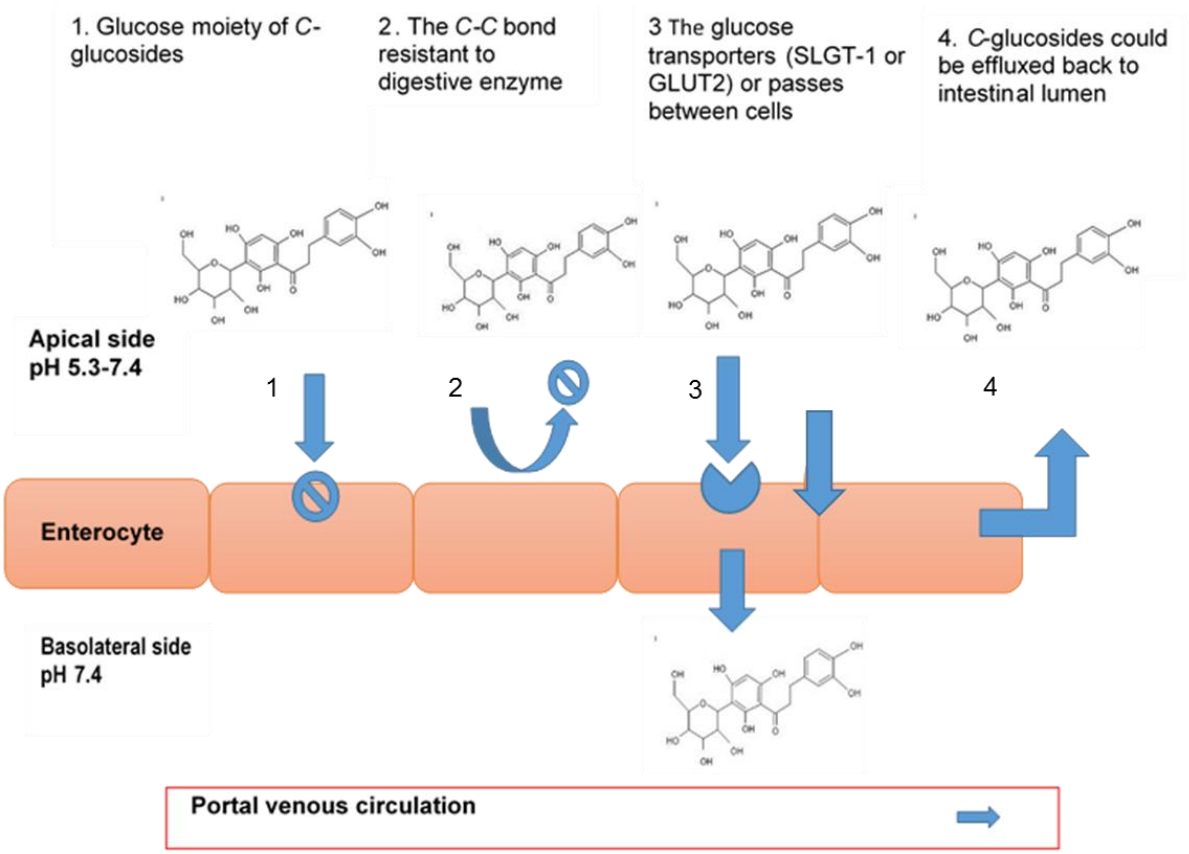


Figure 5: Theoretical representation of the potential intestinal transport mechanisms used for the absorption of flavone *C*-glucosides across the gut intestinal epithelial (adapted from Courts and Williamson, 2009). 1-Passive diffusion unlikely due to the presence of glucose moiety, 2-Resistant to digestive hydrolysis due to the strong *C-C* attached to the nucleus of the dihydrochalcone *C*-glucosides, 3-Possible use of glucose transporters due to the presence of the glucose moiety or the passage of the dihydrochalcone *C*-glucosides via the paracellular route and 4- Possible efflux of the compounds due to being identified as foreign substances.

Chapter 3

3. Aim

The proposed aim of the study was to evaluate the absorption and metabolism of aspalathin, nothofagin, orientin and isoorientin from green Rooibos extracts (prepared using different extraction methods) using Caco-2 cells as a model representing digestive tract absorption as well as to identify excipients that enhance intestinal absorption of these flavonoids.

3.1. Research hypothesis

3.1.1. Hypothesis

The use of polysaccharide excipients will increase the absorption of Rooibos flavonoid *C*-glucosides.

3.1.2. Null hypothesis

Polysaccharides excipients do not affect the absorption of Rooibos flavonoid *C*-glucosides.

3.2. Materials

The materials used for the purpose of this study together with suppliers and product numbers are listed in Appendix 1. Prof. E. Joubert from the Plant Bioactive Group of the Post-Harvest and Wine Technology Division, Agricultural Research Council, Infruitec-Nietvoorbij kindly supplied GREs selected from extracts which formed part of a larger study. For each type of solvent extract two samples, prepared from different plant material batches, were selected for testing based on (i) bioactivity and (ii) quantitative chemical dissimilarity in polyphenol content. Additionally, ARC 2, a green Rooibos extract previously tested in several models for bioactivity, was included in the sample series: [ARC 2 (80% ethanol extract with an ethyl acetate clean-up step), ARC 1022 (60% ethanol), ARC 1023 (80% ethanol) and ARC 1026 (aqueous extracts)]. ARC 1022, ARC 1023 and ARC 1026 were produced from the same plant batches i.e. batches 5 and 9 (Table 2).

Table 2: Polyphenolic profile of GREs.

Extracts	Batch no.	Extraction method	Content (g/100 g SS)			
			Asp	Isoo	Ori	Not
ARC 1022	1	60% ethanol	12.33	1.92	1.23	1.22
ARC 1022	2	60% ethanol	13.45	1.47	0.98	1.40
ARC 1022	3	60% ethanol	11.16	1.52	1.01	0.77
ARC 1022	4	60% ethanol	9.57	1.61	1.17	0.66
ARC 1022	5	60% ethanol	15.12	1.60	1.05	1.78
ARC 1022	6	60% ethanol	14.10	1.53	1.01	1.47
ARC 1022	7	60% ethanol	15.01	1.54	1.03	0.97
ARC 1022	8	60% ethanol	17.18	1.62	1.06	1.30
ARC 1022	9	60% ethanol	12.86	1.31	0.87	1.01
ARC 1022	10	60% ethanol	14.50	1.56	1.04	1.13
ARC 1023	1	80% ethanol	16.71	1.98	1.39	1.71
ARC 1023	2	80% ethanol	17.92	1.58	1.12	1.88
ARC 1023	3	80% ethanol	16.19	2.05	1.53	1.22
ARC 1023	4	80% ethanol	13.77	1.69	1.24	1.02
ARC 1023	5	80% ethanol	19.75	1.76	1.22	2.35
ARC 1023	6	80% ethanol	20.86	1.76	1.25	2.19
ARC 1023	7	80% ethanol	18.40	1.59	1.12	1.21
ARC 1023	8	80% ethanol	21.63	1.73	1.19	1.64
ARC 1023	9	80% ethanol	20.54	1.71	1.19	1.66
ARC 1023	10	80% ethanol	19.57	1.80	1.25	1.55
ARC 1026	1	aqueous	8.07	1.14	0.93	0.79
ARC 1026	2	aqueous	9.86	1.10	0.87	0.99
ARC 1026	3	aqueous	7.93	1.00	0.80	0.54
ARC 1026	4	aqueous	6.75	1.01	0.84	0.48
ARC 1026	5	aqueous	10.78	1.10	0.86	1.19
ARC 1026	6	aqueous	9.84	0.98	0.79	0.97
ARC 1026	7	aqueous	11.00	1.15	0.91	0.69
ARC 1026	8	aqueous	10.93	1.05	0.81	0.84
ARC 1026	9	aqueous	10.40	1.06	0.83	0.79
ARC 1026	10	aqueous	10.41	1.13	0.89	0.77

Highlighted extracts were selected from a larger study, for transport experiments from dissimilar plant batches (4, 5 and 9) and different extraction solvents which were aqueous and ethanol (60% and 80%). Asp-aspalathin, Isoo-isoorientin, Ori-orientin and Not-nothofagin.

3.3. Caco-2 cells

Caco-2 cells (human colon adenocarcinoma cell line) (ECACC# 86010202), originating from the European Collection of Cell Cultures, UK, were purchased from Sigma (St. Louis, MO, USA) and supplied at an original stock of passage 45.

3.4. Cell culture

3.4.1. Thawing of cells

Cryopreserved cells, stored in freezing EMEM (containing 100 mM sodium pyruvate, 1% non-essential amino acids (NEAA), 10% fetal bovine serum (FBS), 2 mM L-glutamine and 7% (v/v) dimethyl sulfoxide (DMSO) as cryoprotectant), were thawed by placing the cryopreservation tube in a water bath at 37˚C. Immediately after the cells had thawed, the cell suspension was transferred gently into a 75 cm^2 flask containing 18 mL of complete EMEM (containing 100 mM sodium pyruvate, 1% non-essential amino acids (NEAA), 10% fetal bovine serum (FBS), and 2 mM L-glutamine). The cells were thoroughly mixed in the medium by gently pipetting up and down several times. The flask was incubated at standard culture conditions (37°C, 5% CO_2 in humidified air). Sub-culturing of cells was limited to within 15 passages in order to prevent phenotypic drift.

3.4.2. Counting of cells

The cell suspension was stained with 0.4% (w/v) trypan blue (Cat No.: T93595; Sigma, Stanheim, Germany) in Dulbecco's phosphate buffered saline (DPBS) (Cat No.: 17-513, Lonza, MD, USA) solution at a 1:1 ratio (20 µL of cell suspension and 20 µL of trypan blue). The suspension was mixed gently by pipetting up and down several times in order to ensure that cells were evenly dispersed, whereafter 10 µL of the suspension was pipetted into the counting chamber of a hemocytometer. The cells were counted using a 100X magnification lens of an inverted light microscope (Olympus ck x31, Tokyo, Japan) phase contrast and an improved Neubauer hemocytometer. The cell concentration per mL was calculated using the following formula:

$$\text{Cells/mL} = \frac{\text{Average count per mm}^2 \text{ x 2 (1:1 cell suspension and trypan blue solution)}}{\text{Volume counted (1x1x0.1 mm=0.1 µL)}}$$

3.4.3. Sub-culturing of cells

Caco-2 cells were seeded in 75 cm^2 culture flasks at a density of 720 000 cells per flask in 18 mL pre-warmed complete EMEM. Cells were incubated at standard culture conditions for three days until they were approximately 70-80% confluent. The medium was refreshed every 2-3 days.

Caco-2 cells were sub-cultured twice after cryopreservation before being seeded for experimentation. For seeding, the medium was removed from the 75 cm^2 flask and the Caco-2 cells were briefly washed with 8 mL pre-warmed Dulbecco's phosphate buffered saline (DPBS) (Cat No.: 17-513, Lonza, MD, USA) before adding 2 mL of trypsin (Cat No.: 17-161F Lonza, MD, USA). The cells were incubated for 4 min at 37˚C to detach the cells from the flask surface. Cells were mechanically dislodged after incubation by gently, but firmly tapping the flask with the palm of the hand. Detachment of cells was confirmed by microscopic observation. Trypsin was deactivated by adding 6 mL complete EMEM. The cells were resuspended in complete EMEM, transferred to a 50 mL tube and centrifuged at 200 x g for 5 min. The cell pellet was resuspended in complete EMEM and transferred into a 1 mL Eppendorf tube, and counted as described (section 3.4.2). Cell were routinely seeded at a 1:3-1:5 split ratio.

3.4.4. Seeding into 96 well plate

Caco-2 cells were first trypsinized as described (section 3.4.2), the supernatant discarded and the cell pellet suspended in 1% penicillin-streptomycin (pen-strep) (100 units/mL of penicillin and 100 µg/mL of streptomycin) complete EMEM. Cells were counted as described (section 3.4.2) and seeded into a 96-well plate at 10 000 cells per well. The medium was refreshed every 2-3 days and cells were grown at standard culture conditions for 12 days.

3.4.5. Seeding into 6-well transwell plates

Prior to seeding the Caco-2 cells in 6-well transwell plates, the plate membrane inserts were allowed to equilibrate in 1% penicillin-streptomycin complete EMEM for 1 hour.

For seeding, Caco-2 cells were first trypsinized as described (section 3.4.3), the supernatant discarded and the cell pellet suspended in 1% penicillin-streptomycin complete EMEM. The cells were seeded at 180 000 cells per well of the 6-well trans-well plate. Complete EMEM supplemented with penicillin-streptomycin was added to the basolateral side (2.5 mL) first to provide basal support and 1.5 mL of cell suspension was added into each trans-well insert on the apical side. The seeded cells were incubated at standard culture conditions for 5 h. After incubation, the medium was refreshed in both compartments, and thereafter refreshed every 2-3 days using 1% pen-strep complete EMEM media. Cells were grown to maturity as a robust, fully differentiated monolayer and were used between days 21-25 for transport experiments.

3.5. Validation of Caco-2 cell model

3.5.1. Electron microscopy

This method was modified from Kay and Cosslett (1965). Cell pellets were fixed in 2.5% glutaraldehyde-0.1 M phosphate buffer pH 7.4 at 4^{o}C for 4 – 6 h and cut into small blocks of no more than 1-2 mm thickness. Following fixation, the tissue was rinsed in phosphate buffer to remove unbound glutaraldehyde. The tissue was post-fixed using 1.5% osmium tetroxide in Palade's buffer pH 7.4 for one hour and then rinsed with two changes of distilled water to remove excess unbound osmium. The tissue was further processed (Leica Microsystems, Wetzlar, Germany) using the tissue processor in 2% uranyl acetate dissolved in 70% ethyl alcohol and further dehydrated in ascending concentrations of ethyl alcohol (70% for 5 min, 2 x 96% for 5 min each, 3 x 100% for 10, 15 and 20 min respectively). Following dehydration, the tissue was impregnated, initially with a 50/50 mixture of Spurr's resin (NSA x 13 mL, ERL 4206 x 5 mL, DER x 3 mL and DMAE x 0.2 mL; all reagents from Agar Scientific, Stansted, UK) and 100% ethyl alcohol followed by two changes of clean Spurr's resin. Resin impregnated tissue was embedded into resin filled gelatin capsules and the blocks allowed to polymerise overnight at 60 ^{o}C. The tips of the capsules were trimmed using a Reichert TM60® block trimmer (Reichert, Vienna, Austria) in order to expose the

embedded tissue. Semi-thin sections (1 µm) were cut on a Leica EM UC7 (Leica Microsystems) ultra-microtome using a glass knife fitted with a water trough. Sections were removed from the trough and placed on a drop of water and the sections were allowed to dry on a hot plate. Once the drop of water had evaporated and the sections adhered to the slide, the resin was removed from the sections using sodium methoxide. Sodium methoxide was prepared by reacting 1% metal sodium in absolute methanol. The slides were washed in tap water to remove the sodium methoxide, and then stained with warm 1% toluidine blue in 1% sodium tetraborate for 1 min. Slides were washed in distilled water, blotted on filter paper and dried on a hot plate. Dry sections were mounted with DPX and studied under the light microscope. The area of particular interest was identified light microscopically and matched with the corresponding area in the resin block. The resin block was re-trimmed into a small (maximum 2 x 1 mm) trapezium shape containing the area of interest using a Reichert TM60® block trimmer fitted with a diamond tip trimming blade. Gold sections, thickness 90 –120 nm, were cut on the Leica EM UC7 (Leica Microsystems) using the heat feeding mechanism and a glass knife with fitted water trough. A ribbon of sections was then picked up from the water bath, placed onto a G200 copper grid and allowed to dry on a filter paper. The sections were then stained for 5 min with 2% acetyl acetate in 50% ethyl alcohol, rinsed in clean 50% ethyl alcohol and double stained with Reynold's lead citrate (Kay *et al*. 1967) for a further 5 min. Reynold's lead citrate was prepared by mixing lead nitrate (1.33 g) with sodium citrate (1.76 g) in distilled water (30 mL). After thorough mixing the solution was allowed to react for at least one hour for the lead nitrate to convert to lead citrate. Lead citrate was dissolved with 1 N sodium hydroxide (8 mL) and distilled water added to a final volume of 50 mL. After staining the grids were washed in two changes of distilled water and replaced onto a filter paper in a closed petri dish. Once the grids had dried the sections were viewed in a JEOL JEM-1011 (JEOL Ltd., Tokyo, Japan) transmission electron microscope operated at 75 kV.

3.5.2. Protein isolation

Fully differentiated monolayers of Caco-2 cells, grown in 6-well transwell plates, were washed with 400 µL of DBPS/well (to removes excess media). Three wells were used for the purpose of this experiment. Ripa buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 50 mM NaF 100 µM Na_3VO_4, 1% NP40 and 1% Triton X 114. 25 µg/mL RNase

was added at 400 µL/well onto the cells. Cells were collected by scraping and transferred into a 2 mL Eppendorf tube. A stainless steel ball was inserted into the 2 mL tube and the tube was placed in a precooled tissue lysis block. Cells were homogenised in a tissue lyser (Qiagen, Hilden, Germany) at 25 Hrtz for 60 sec, with 60 sec ice-recovery in between homogenization (this sequence was repeated 5 times). The homogenised sample was centrifuged for 15 min at 15 890 x g at 4˚C whereafter the supernatant was carefully removed into a clean 2 mL tube. The protein concentration was determined using the RC DC protein assay and bovine serum albumin (BSA) as the standard.

3.6. Protein quantification

3.6.1. (RC DC protein assay)

The RC DC Protein assay is a colorimetric assay for protein quantification. The assay is based on the Lowry assay, however, modified to be reducing agent compatible (RC) as well as detergent compatible (DC). Briefly, a BSA standard series was prepared as follows 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2 mg/mL. Thereafter, 5 µL of the BSA standards and lysate were pipetted in duplicate into separate wells of a 96-well plate. Reagent A prime (A') was prepared by adding 20 µL of reagent S into 1000 µL of reagent A. Twenty-five microliters of reagent A' and 200 µL of reagent B were added to each of the standards and samples. Plates were incubated for 10 min in the dark while covered with aluminium foil and absorbance was read at 630 nm on a BioTek® ELX 800 plate reader (BioTek Instruments Inc., Winooski, VT, USA). The actual protein concentration was calculated by plotting the absorbance measurement of the sample on the BSA standard curve. Protein concentration was expressed as mg/mL.

3.6.2. Western blot

Electrophoresis was conducted using a 12% sodium dodecylsulfate-polyacrylamide gel, loaded with 20 µg lysate protein. Briefly, a polyvinylidene fluoride (PVDF) membrane was pre-wetted with methanol for 5 min. Prior to the transfer, the SDS-gel and PVDF membrane were equilibrated with transfer buffer for 20 min. Electroblotting (Tank transfer method) was used, whereby the protein gel was electrophoretically transferred to the PVDF membrane and the transfer sandwich was prepared in the

following order: [pad-filter paper-gel-membrane-filter paper-pad] (Figure 6). The tank was filled with transfer buffer and an ice pack was placed in the tank to mitigate the heat produced during transfer.

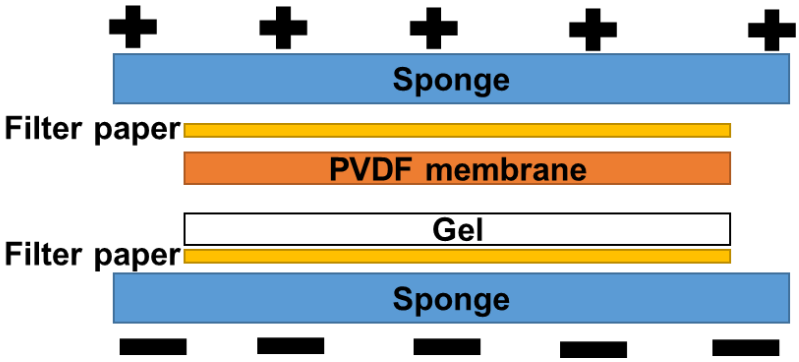


Figure 6: Western blot transfer sandwich diagrammatic representation.

3.6.3. Ponceau S stain protein determination

The blotted membrane was immersed in a sufficient amount of Ponceau S staining solution covering the membrane for 5 min. The stain was reversed by washing the stained membrane with distilled water for another 5 min and the bands were clearly visible.

3.6.4. Staining nitrocellulose membrane with antibody

The PVDF membrane containing the proteins of interest was blocked in 5% (w/v) non-fat milk dissolved in 1 x trisaminomethane (Tris)-buffered saline and Tween 20 (1 x TBST) at room temperature for 2 h on an orbital shaker. Subsequently, the membrane was labelled with the following antibodies: anti-sodium-glucose linked transporter (SGLT1) polyclonal antibody at 1:1000 dilution (abcam, Cambridgeshire, United Kingdom CAT no ab14686), anti-glucose transporter2 (GLUT2) polyclonal antibody at 1:1000 dilution (abcam, Cambridgeshire, United Kingdom, CAT no ab54460) and multi-drug resistant protein1 (MDR1) monoclonal antibody at 1:1000 dilution (Santa Cruz, Heidelberg, Germany CAT no.SC5510), overnight at 4˚C (diluted in 1 x TBST). The membranes were washed 3 x 10 min in 1 x TBST at room temperature on an orbital shaker. The membranes were labelled with the relevant horseradish peroxidase (HRP) conjugated secondary antibody (donkey anti-mouse monoclonal) at 1:4000 dilution (Santa Cruz, Heidelberg, Germany CAT no. SC2318) and streptavidin (Bio-Rad, Hercules, CA, USA) at a ratio of 1:10 in 1x TBST at room temperature for 90 min

for the detection of the marker in 2.5% non-fat milk. After 90 min, the membrane was washed 3 x 10 min in 1 x TBST. Labelled proteins were detected and quantified using a Chemidoc-XRS imager and Quantity One software (Bio-Rad, Hercules, CA, USA).

3.7. Transepithelial electrical resistance (TEER) readings

TEER readings were taken regularly to assess the integrity of the monolayer once a visible monolayer was formed (from day 11 until the monolayer was fully differentiated at day 25). During transport experiments, TEER readings were initially taken before the cells were washed. Cells were allowed to adjust to room temperature, after which the TEER readings were measured using a STX 2 probe (as illustrated in Figure 7 below).

After this initial reading the medium was removed first from the basolateral side followed by the apical side. Cells were washed for 1 min with 1.5 mL Hank´s balanced salt solution (HBSS) buffer containing 10 mM MES (pH 6.0) on the apical side and 2.5 mL HBSS buffer containing 25 mM HEPES (pH7.4) on the basolateral side. This was followed by a 15 min wash step with the plate placed on a shaker at 75 rpm at 37˚C. Subsequent to this wash step, the buffer was removed and inserts were transferred into a new 6-well plate. TEER readings were also recorded after incubation with treatment in the transport experiment. TEER readings above 300 Ω were indicative of an intact Caco-2 cell monolayer (Chen *et al*., 2015).

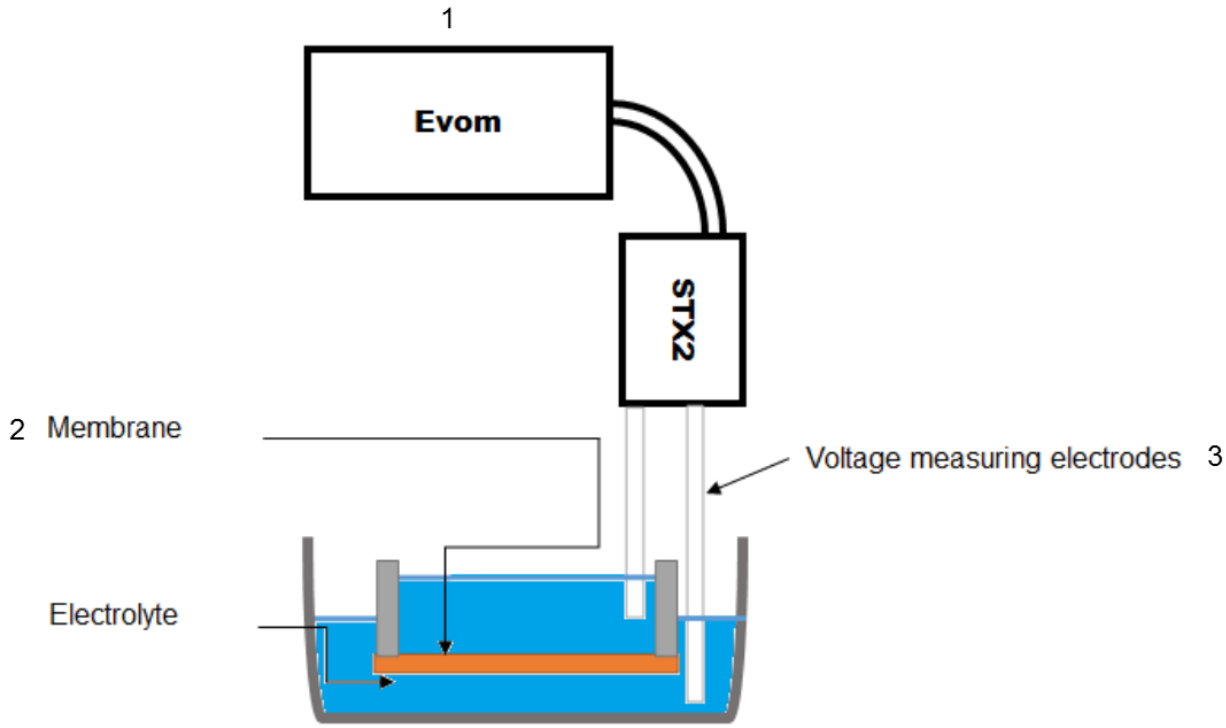


Figure 7: Representative image illustrating 1-TEER apparatus, 2- insert membrane and 3-STX2 probe. TEER readings were performed by inserting electrodes into the apical and basolateral compartments and resistance was measured across the Caco-2 cell monolayer and insert membrane.

3.8. Lucifer yellow permeability Assay

Lucifer yellow (final concentration of 0.1 mM), used as an indicator molecule to confirm the integrity of the monolayer (Pourmirjafari Firoozabadi *et al*., 2015), was added at the same time as the extracts. In order to verify an intact, differentiated monolayer, with tight junctions comparable with that of the small intestine *in vivo,* the percentage of Lucifer yellow passing across the membrane would have to be less than 3% (Volpe, 2010). The concentration of Lucifer yellow in the transported fractionation was determined by reading the fluorescence of a 50 µL sample in a black, solid bottom 384 well plate. The fluorescence was corrected with a blank and the percentage of Lucifer yellow was determined using the standard curve (100 µM serial diluted to 0.05 µM with 12 standards). Fluorescence was read using a BioTek® ELX 800 microplate plate reader using Gen 5® software (ex: 485/20nm, em: 528/20nm).

3.9. Cytotoxicity

3.9.1. ATP assay

The ATP assay (Cat No.: LT27-008 ViaLight, Whitehead Scientific, JHB, SA) is a highly sensitive ATP bioluminescence assay used for cell proliferation and cytotoxicity. The ATP assay was used to assess the functional integrity of living cells since ATP is essential for cell function. Caco-2 cells were used for cytotoxicity once a visible monolayer was formed (12 days). Initially the GREs were tested at 2 mg/mL for cytotoxicity prior to standardization. The GREs and the Rooibos nanosome formulation (NovaSOL®) were tested at aspalathin equivalent concentrations of 150 µM for ARC 2 and Rooibos nanosome formulation (NovaSOL®), and 300 µM for ARC 1022, ARC 1023 and ARC 1026 which was the highest concentration used for cytotoxicity testing. Extracts were reconstituted in 10% DMSO as a concentrated stock solution and diluted in transport medium to working concentrations (300 µM, 150 µM, 75 µM, 37.5 µM and 18.75 µM) to establish the highest non-toxic dose. The final concentration of DMSO in all working concentrations used was adjusted to 0.125% which was also used as a vehicle control. Cells were incubated for 2 h at 37˚C under agitation (75 rpm).

After incubation, 50 µL of cell lysis reagent was added for 10 min to extract ATP from the cells. Due to the large number of cells in the monolayer, and subsequent high ATP concentrations, the cell suspension was diluted (1:1) in 50 µL of DPBS and transferred into a clear bottom ATP plate. One hundred microliters of ATP monitoring reagent plus (AMR plus) was added for 2 min to generate luminescence signal. Plates were read using a fluorescence plate reader BioTek® plate reader FLX 800 equipped with Gen 5® software from BioTek Instruments Inc., Winooski, VT, USA and were directly compared to that of the vehicle control to obtain the percentage of viable cells.

Table 3: GREs standardized to aspalathin equivalent concentration of 150 µM.

Extracts	Batch no.	Extraction method	Asp%	Conc. (mg/mL)	Asp equivalent concentrations (µM)
ARC 2	n/a	80% ethanol	18.44	0.38	150
ARC 1022	5	60% ethanol	15.12	0.45	150
ARC 1022	9	60% ethanol	12.86	0.53	150
ARC 1023	5	80% ethanol	19.75	0.35	150
ARC 1023	9	80% ethanol	20.54	0.33	150
ARC 1026	5	Aqueous	10.78	0.64	150
ARC 1026	9	Aqueous	10.40	0.66	150
NovaSOL®	n/a	n/a	2%	9	150

Standardization of GREs to 150 µM aspalathin equivalent concentration. Asp-aspalathin.

3.9.2. MTT (3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide) assay

The MTT (3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide) assay (Cat No.: M5655; Sigma, Stanheim, Germany) was conducted as a secondary assay to confirm the results obtained from the ATP assay. Cytotoxicity of the GREs and NovaSOL® in Caco-2 cells was assessed using the MTT at concentrations previously described in the ATP assay. Briefly, after incubation, treatments were removed and the cells were washed with 50 µL of DBPS followed by the addition of 50 µL of MTT solution to each well and then incubated for 30 min at 37˚C. Formazan crystal formation was observed, and the MTT solution was removed. Two hundred microliters of 100% DMSO and 25 µL of Sorenson's Glycine Buffer (pH 10.5) was added to each well and the suspension was mixed gently. Absorbance was read at 570 nm on a BioTek® plate reader ELX 800 using Gen 5® software (BioTek Instruments Inc., Winooski, VT, USA).

3.10. Transport experiment

The method for the transport experiment was adapted from Van Breemen *et al* (2005). The experiments were performed in the apical to basolateral (AP-BL) direction.

Media was changed 24 h prior to the transport experiment. On the day of the experiment TEER readings were recorded as described (section 3.7) to confirm the readiness of the Caco-2 cell monolayer. An intact monolayer was confirmed by readings > 300 Ω. For transport experiments the treatments (extracts and controls) were prepared as concentrated stock solutions in 10% DMSO and diluted to contain a final concentration of 0.125% DMSO. Caffeine (C0750, Sigma, St Louise, MO, USA) (50 µg/mL) was used as a positive control. The GREs and Rooibos nanosome formulation (NovaSOL®) were all standardized to contain a final concentration 150 µM aspalathin as per Table 3. Lucifer yellow was reconstituted in HBSS pH 6.0 and added to the treatment at a final concentration of 0.1 mM. Transport experiments using ARC 2 were conducted in the presence and absence of the excipients (1:1 ratio of ARC 2: excipients) at a final concentration of 1 mg/mL. The solution was vortexed, sonicated and incubated for 30 min. The cell monolayer was washed briefly. After the inserts were transferred into a new 6-well plate, 1.5 mL of the treatment was added to the apical side and 2.4 mL of HBSS buffer (pH 7) was added to the basolateral compartment. Transport experiments were under sink conditions while maintaining the plates at standard culture conditions. Samples (1.2 mL) were withdrawn from the basolateral side; at 0, 0.5, 1, 1.5 and 2 h for future bioanalysis (Lucifer yellow and HPLC-DAD analysis) and replaced with equal volume of relative transport buffer. Samples were frozen after the addition of 1% vitamin C in liquid nitrogen and stored thereafter at -80˚C until bioanalysis was performed.

The apparent permeability coefficient, P_{app} (cm/s), for unidirectional transport studies was calculated using the following formula:

$$P_{app} = \frac{(dQ/dt)}{C_0.A}$$

Where dQ/dt is the cumulative transport rate (nmol/s) defined as the slope obtained by linear regression of cumulative transport amount as a function of time (s); A is the surface area of inserts in the transwells; C_o is the initial concentration of the treatment on the donor side (µM). The concentration of the treatment compounds in receiver side (basolateral) was determined to calculate the P_{app} value for the apical-to-basolateral transport was measured using HPLC as described in the bioanalysis section (3.11).

3.11. Analysis of samples

HPLC-DAD analysis was performed on an Agilent 1200 system (Agilent Technologies, Inc., Santa Clara, CA) equipped with an in-line degasser, quaternary pump, autosampler, column thermostat and diode-array detector (DAD), controlled by Chemstation software (Agilent Technologies, Waldbronn, Germany). Briefly, chromatographic conditions described in detail by de Beer *et al* (2016) were as follows: Separation was performed at 30˚C on a Poroshell SB-C18 column (50 × 4.6 mm, 2.7 µm particle size; Agilent Technologies, Inc.) protected by an Acquity UPLC in-line filter (Waters; 0.2 µm) and an Acquity UPLC Van Guard pre-column (Waters; stationary phase: BEH C18 1.7 µm). The flow rate was 1.0 mL/min and a multilinear gradient was performed as follows: 0–10 min, 12.4–16.6%B; 10–10.5 min, 16.6–80% B; 10.5–11.5 min, 80% B; 11.5–12 min, 80–12.4% B; 12–16 min, 12.4% B, with solvents A and B being acetonitrile and 0.1% aqueous formic acid, respectively. UV-Vis spectra were recorded between 220 and 450 nm.

Stock solutions of the phenolic standards were prepared in dimethyl sulfoxide (DMSO) at concentrations of approximately 1 mg/mL and diluted with water according to experimental requirements. All diluted solutions were filtered through 0.22 µm polyvinylidene difluoride (PVDF) filters (Merck Millipore) prior to use.

Six-point calibration curves were set up for all standards. The calibration mixtures were injected at different injections volumes (1, 5, 10, 15 and 20 µL), leading to levels of 0.02 - 1.2 µg on-column for aspalathin and nothofagin, 0.04 - 0.8 µg on-column for caffeine and 0.01 - 0.2 µg on-column for isoorientin and orientin. Linear regression, using the least-squares method (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA), was performed on the calibration curve data for each compound to determine the slope, y intercept and correlation coefficients (R2). Dihydrochalcones (aspalathin and nothofagin) and caffeine were quantified using the peak areas at 288 nm, while the flavones (isoorientin and orientin) were quantified at 350 nm. The slope obtained by linear regression of cumulative transport amount as a function of time (s); A is the surface area of insert in the transwells; C0 is the initial concentration of treatment on the donor side (µM). Results were corrected for dilution and expressed by means of the cumulative transport (% of initial dose) as a function of time.

3.12. Bile salts and linoleic acid experiments

Caco-2 cells were used 25 days post culture. The cells were seeded into a 6-well transwell plate with inserts. The medium was changed 24 h before the assay was conducted. Hank´s balanced salt solution (HBSS) buffers containing either 10 mM MES (HBSS pH 6) or 25 mM HEPES (HBSS pH 7.4) were prepared and thereafter the bile salt (20 mM) and linoleic acid (1 mM) treatment was prepared using HBSS buffer (pH 6). Cells treated with complete media and transport buffer were experimental controls to enable comparative effects on TEER readings and cell recovery.

Prior to treatment, the plate was allowed to calibrate to room temperature and subsequently TEER readings were taken. Cells were washed with 1.5 mL HBSS buffer pH 6 on the apical side and 2.4 mL HBSS pH7.4 on the basolateral side. After the cells were washed, they were incubated with bile salt (20 mM) and linoleic acid (1 mM) (a 20:1 ratio) by adding 1.5 mL on the apical side and 2.4 mL HBSS pH7.4 on the basolateral and incubating for 10 min at 37º C. Bile salt-linoleic acid treatment was removed, cells were washed as previously described, and HBSS buffer was added to the cells, followed by recording of TEER readings. After 2 h, HBSS buffer was removed and replaced with fresh penicillin-streptomycin complete media. TEER readings were taken at 3, 4, 5, 6 and 24 h to assess cell recovery.

3.13. Statistical analysis

Microsoft Excel was used to analyse the data. Three independent experiments were done and each experimental condition was performed in triplicate (n = 9). The data are expressed as mean ± standard deviation (SD). For statistical analysis Prism (GraphPad Prism® version 5.04, GraphPad Software Inc., La Jolla, USA) was used, differences between mean values were analysed by means of a student's paired two tailed t-test and were considered to be significant if $p < 0.05$.

Chapter 4

4. Results

4.1. Validation of Caco-2 cell model

4.1.1. Microscopy and Western blots

The Caco-2 cells spontaneously differentiated and formed an intact monolayer after 25 days of culture (Figure 8). The differentiated cells were shown to express several of the ultrastructural specializations of intestinal epithelial cells (enterocytes) including apical microvilli (MV) and basolateral membrane interdigitations (I) known to be sites for Na+ and K+ ATPase activity and apical membrane junctional complexes including tight junctions (TJ) and desmosomes (D). Several transport vesicles were present including micropinocytotic vesicles. Mitochondria (M) and glycogen were present throughout the cytoplasm (Figure 9). Western blot analysis confirmed the presence of the transport proteins SGLT1, GLUT2 and the efflux transporter MDR1 (Figure 10).

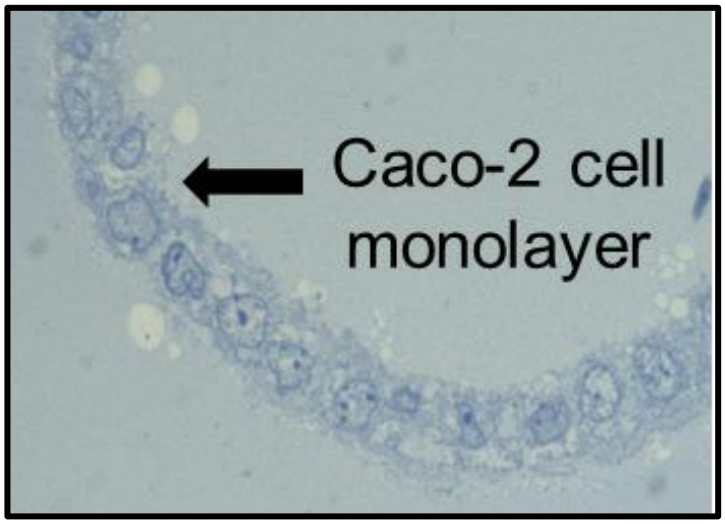


Figure 8: Toluidine blue stained 1 µm semi thin resin section demonstrating a differentiated intact 25-day Caco-2 cell monolayer in cross sectional view.

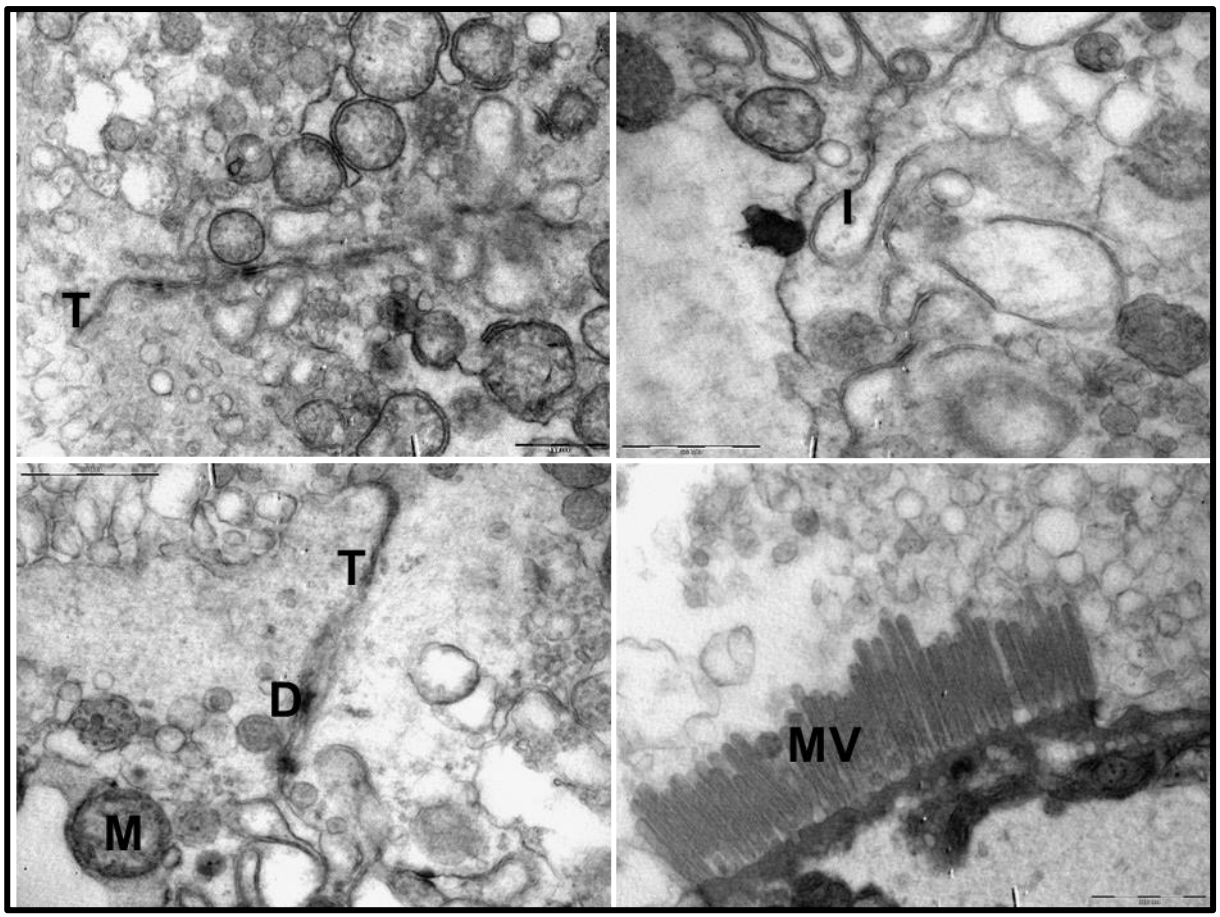


Figure 9: Transmission electronmicrograph of differentiated Caco-2 monolayer cells at day 25 demonstrating absorption and transport specializations including microvilli (**MV**) with micropinocytotic vesicles at the apical surface, junctional complexes (**T**) including tight junctions and desmosomes (**D**) between adjacent cells, indigitations (**I**) on the lateral and basolateral aspects of the cells. Other organelles include transport vesicles and mitochondria (**M**) 500 nm.

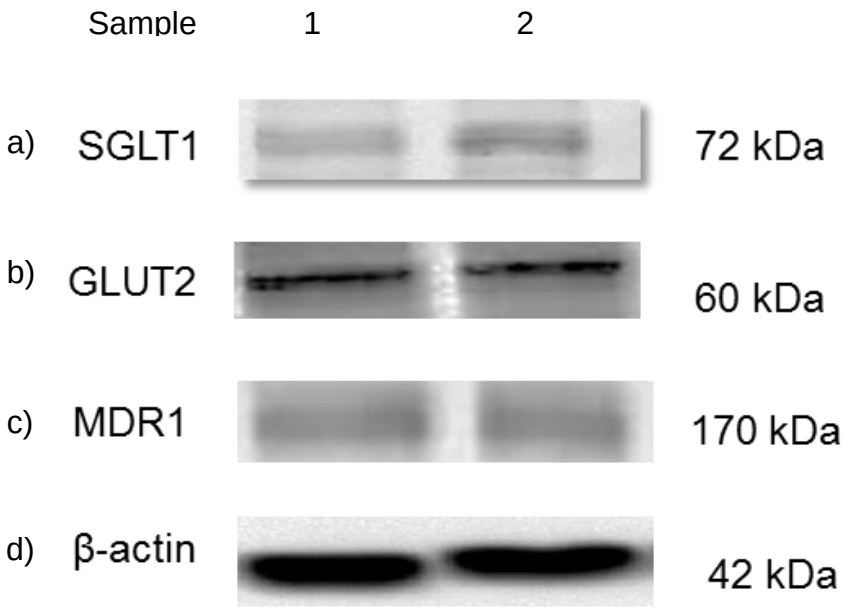


Figure 10: Protein expression demonstrating the presence of transport and efflux proteins in Caco-2 cells harvested on day 25 a) SGLT1 (72 kDa), B) GLUT2 (60 kDa) and c) MDR-1 (170 kDa).

4.2. Integrity of Caco-2 cell monolayer

A Caco-2 cell monolayer two compartmental transwell system was used to study the transport of aspalathin, nothofagin, orientin and isoorientin from different GRE extracts. The use of excipients (inulin and beta-cyclodextrin) and a nanosome solution of ARC2 was also investigated for the enhancement of the transport of these flavonoid *C*-glucosides. The integrity of the Caco-2 monolayer was monitored throughout the transport experiment by assessing Lucifer yellow permeability and TEER readings to ensure the intactness of the monolayer. The theoretical cut off for an intact Caco-2 monolayer was set at a permeable percentage of Lucifer yellow < 3% (Figure 12) and TEER readings > 300 Ω (Figure 11).

To ensure that the monolayer was representative of the intestinal epithelial barrier layer the Caco-2 cells were allowed to differentiate for 25 days, thereby allowing for an intact monolayer with well-developed junctional complexes and expression of transport proteins relevant to the absorption of chemical substances (Figures 9 and 10). This model allowed for the calculation of the apparent permeability of these flavonoids.

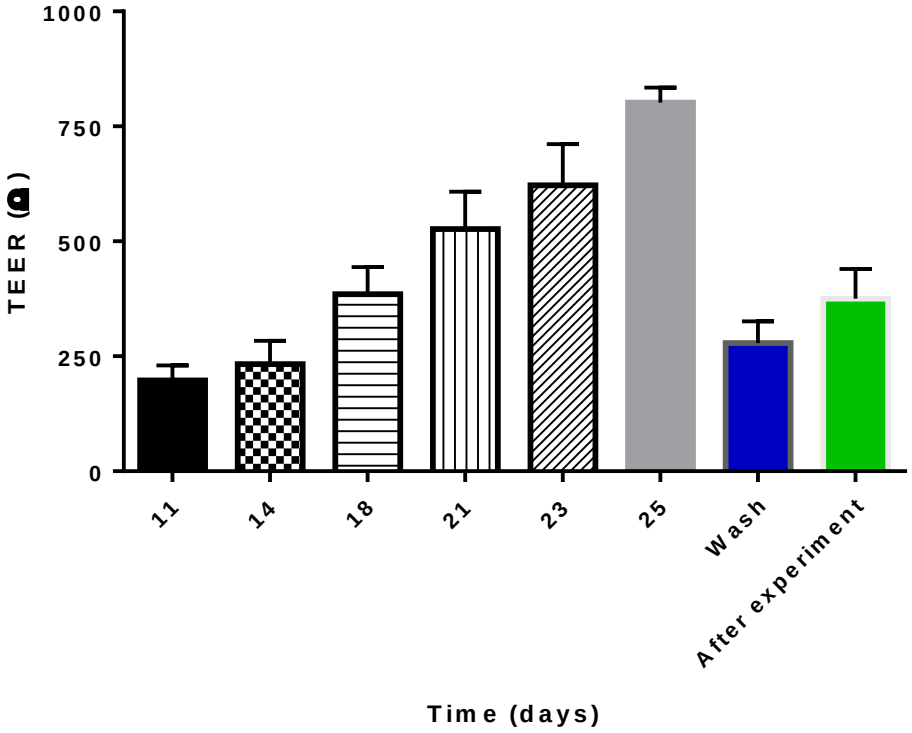


Figure 11: Transepithelial electric resistance readings taken from day 11 to 25, showing increasing resistance as the Caco-2 monolayer integrity improves. TEER readings >300 Ω were indicative of a differentiated intact Caco-2 cell monolayer. During transport experiments TEER readings decreased to approximately 300 Ω and recovered after the experiments. Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean ± SD.

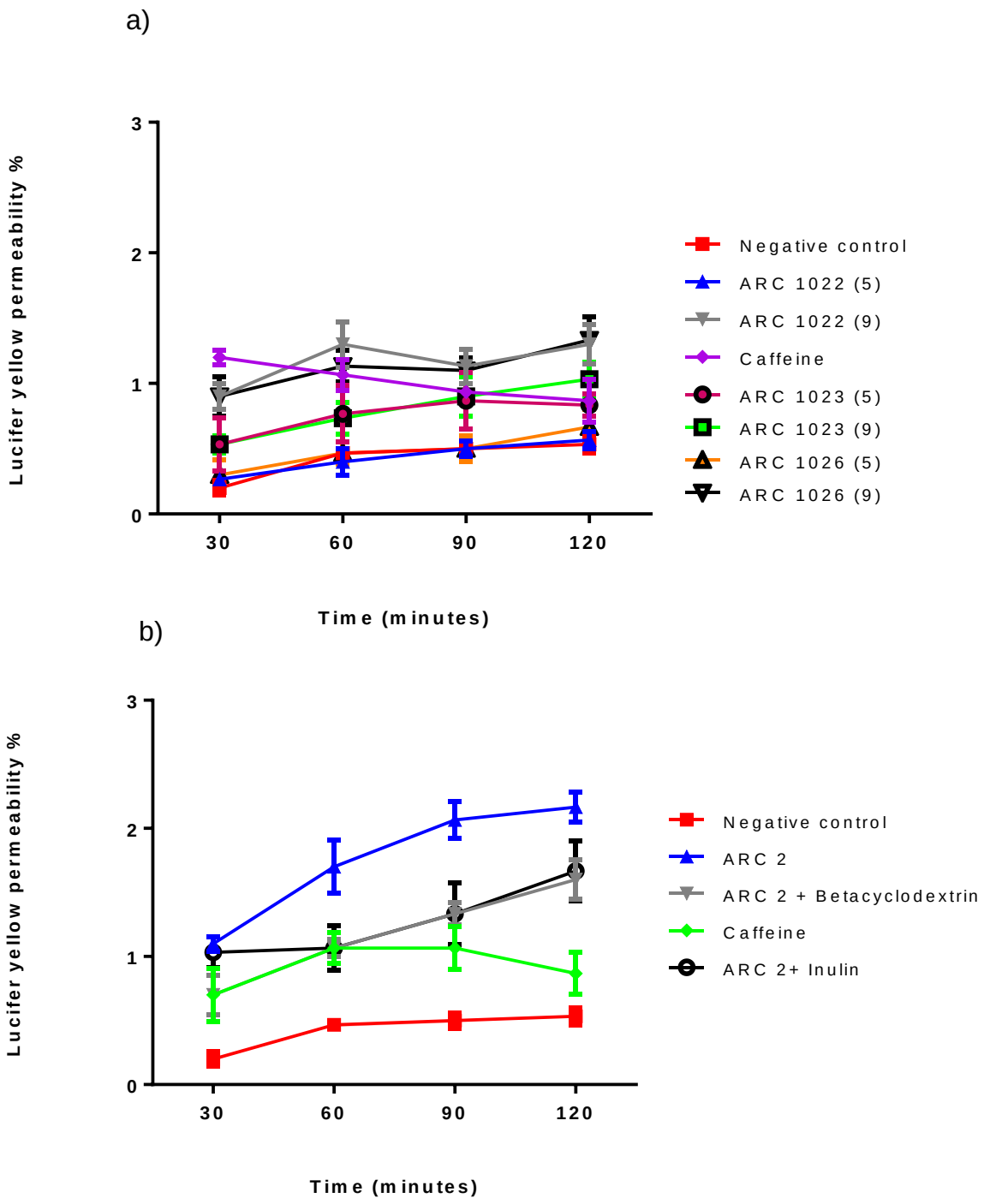


Figure 12: Lucifer yellow permeability assay of Caco-2 cell monolayers grown for 25 days, exposed to a) different GREs and b) ARC 2 in the presence and absence of excipients. The observed permeable percentage of Lucifer yellow was < 3% indicative of a differentiated intact Caco-2 cell monolayers. Results are from three independent experiments done in triplicate (n = 3) and are expressed as the mean ± SD.

4.3. Cytotoxicity

4.3.1. Green Rooibos extracts

Green (unfermented) Rooibos was used for the preparation of the different extracts to obtain a high flavonoid content, especially a high aspalathin content as it has been reported that fermentation reduces the dihydrochalcone content (Joubert, 1996). Potential cytotoxicity was assessed by cellular ATP or MTT activity for the different GREs in Caco-2 cells. The guidelines (Promega Corporation, 2015) state that the reduction in cell viability by more than 30% is indicative of toxicity. To establish the highest concentration of the extracts for the subsequent transport studies, solubility and cytotoxicity studies were conducted. In this study, solubility of the GREs was established to ensure detectability of metabolites in transport experiments. The extracts were soluble up to a concentration of 2 mg/mL and this was the highest concentration tested for cytotoxicity (Figures 13). Cytotoxicity was observed for ARC 1023 batch 9 at 2 mg/mL yielding <75% cell viability, while the other extracts were non-cytotoxic at a concentration of 2 mg/mL.

The tested concentration of the GREs was 150 µM (previously tested by Dr S. Bowles at 300 µM using the MTT assay personal communication) for ARC 2 (Figure 14) and 300 µM for ARC 1022, ARC 1023 and ARC 1026 extracts (Figures 15 and 16) which yielded ± 80% cell viability, respectively. Therefore, the extracts were deemed as non-cytotoxic at these concentrations. In experiments conducted using pure aspalathin cytotoxicity was observed at 300 µM (<75% cell viability) so for further transport experiments a non-toxic aspalathin equivalent concentration of 150 µM was used.

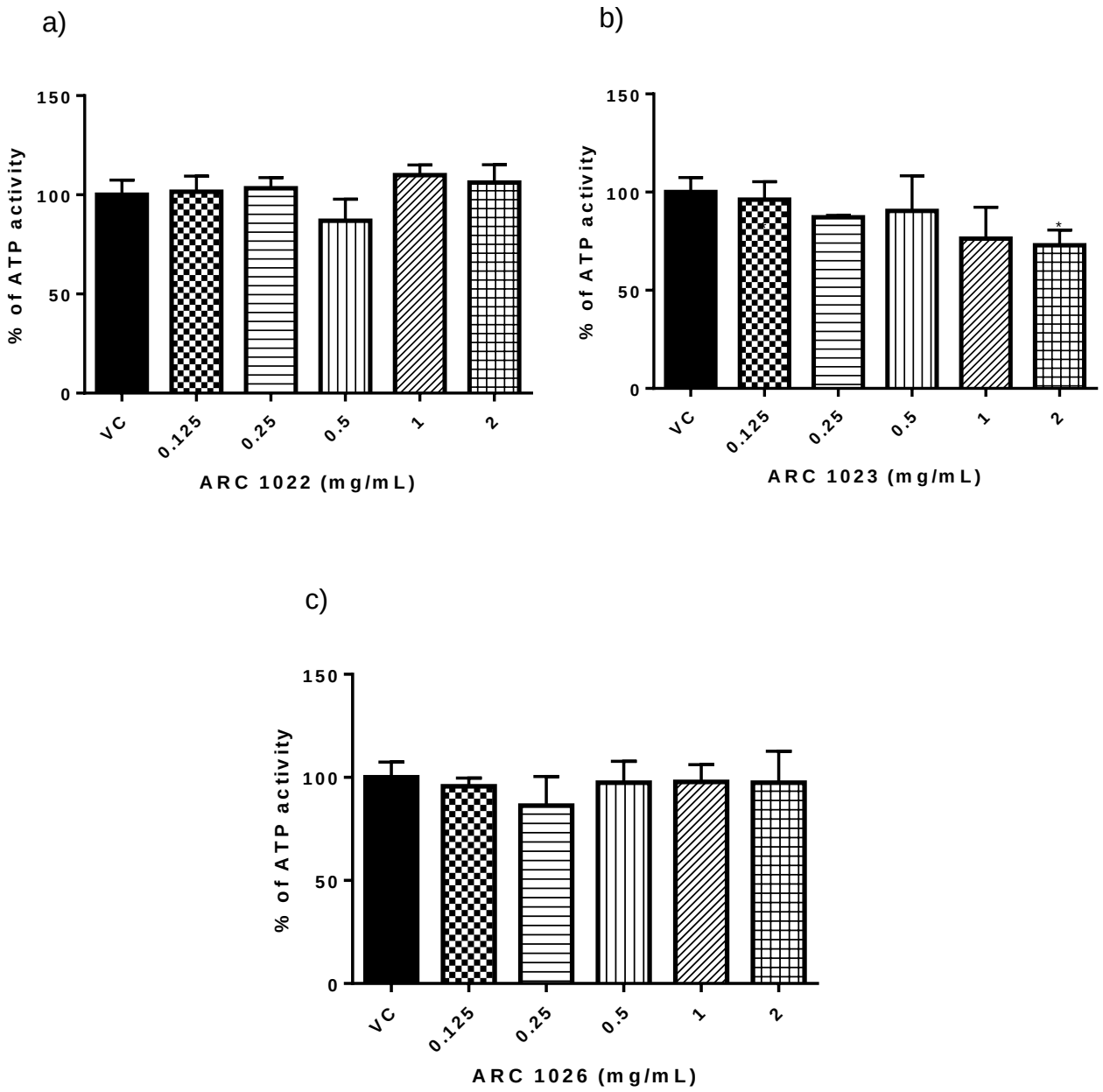


Figure 13: The ATP activity of Caco-2 cells exposed to a) 60% ethanol (ARC 1022 batch 5), b) 80% ethanol (ARC 1023 batch 9) and c) aqueous (ARC 1026 batch 4) GREs. The highest concentration of the extracts was 2 mg/mL. Caco-2 cells, cultured for 12 days were exposed to serial dilutions of the extracts for 2 h and the ATP assessed using chemoluminescence. Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean ± SD and are reflected percentage of the vehicle control set at 100%. * $p < 0.05$ students paired two tailed t-test.

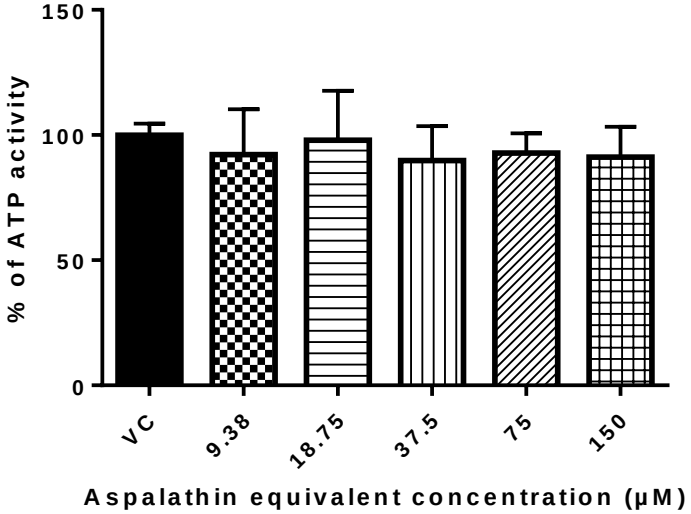


Figure 14: The ATP activity of Caco-2 cells exposed to the reference extract ARC 2. The concentration of the extract was calculated as aspalathin equivalent concentrations with ARC 2 containing 18% aspalathin. Caco-2 cells, cultured for 12 days were exposed to serial dilutions of ARC 2 for 2 h and the ATP assessed using chemoluminescence. Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean ± SD and are reflected percentage of the vehicle control set at 100%.

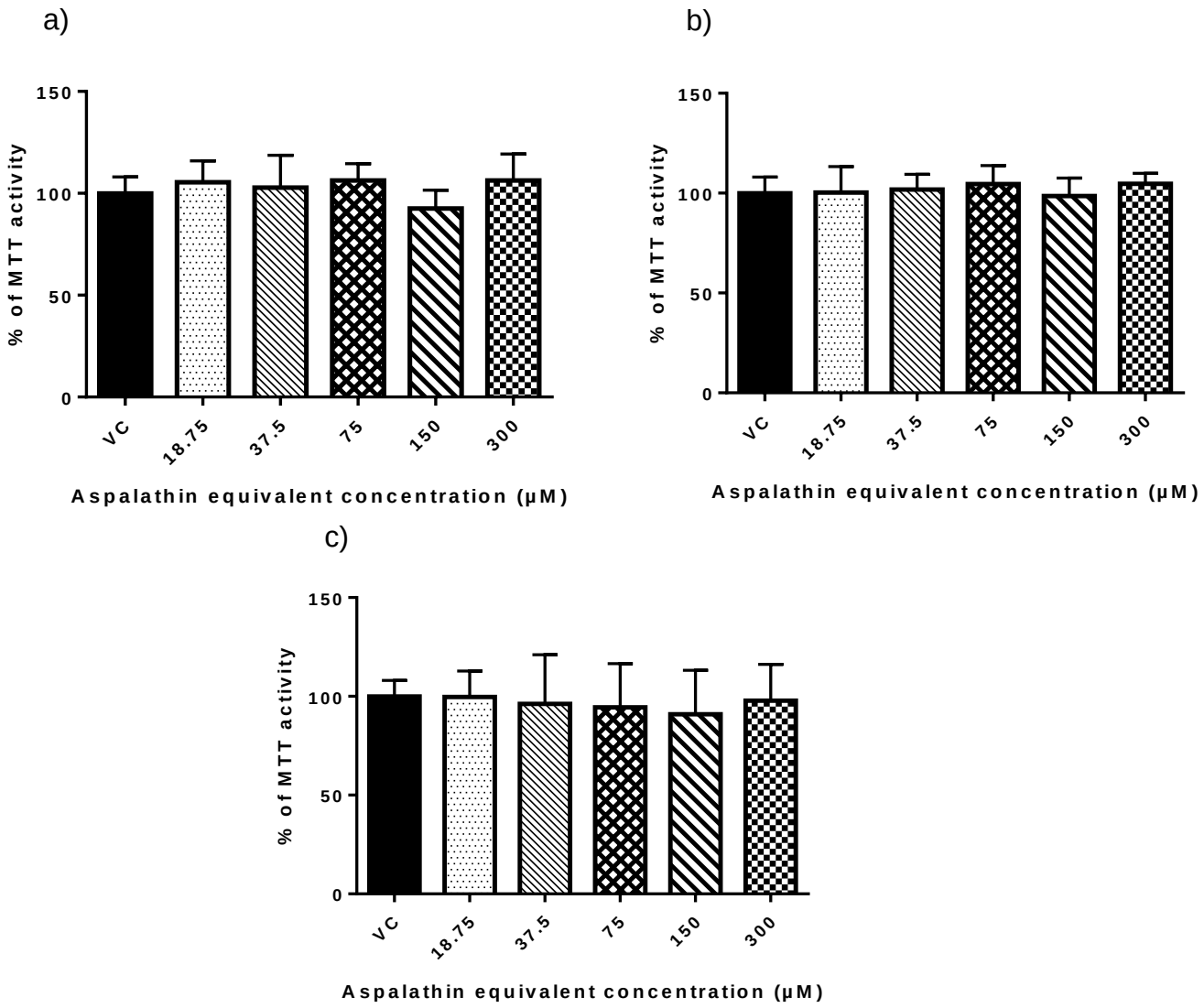


Figure 15: The MTT activity of Caco-2 cells exposed to a) ARC 1022 (60% ethanol extract batch 5) b) ARC 1022 (60% ethanol extract batch 9) and c) ARC 1023 (80% ethanol extract batch 5). The concentration of the extracts was standardized to aspalathin equivalent concentrations (Table 3). Caco-2 cells, cultured for 12 days were exposed to serial dilutions of the GREs for 2 h and the MTT was assessed using absorbance at 570 nm. Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean ± SD and are reflected as a percentage of the vehicle control set at 100%.

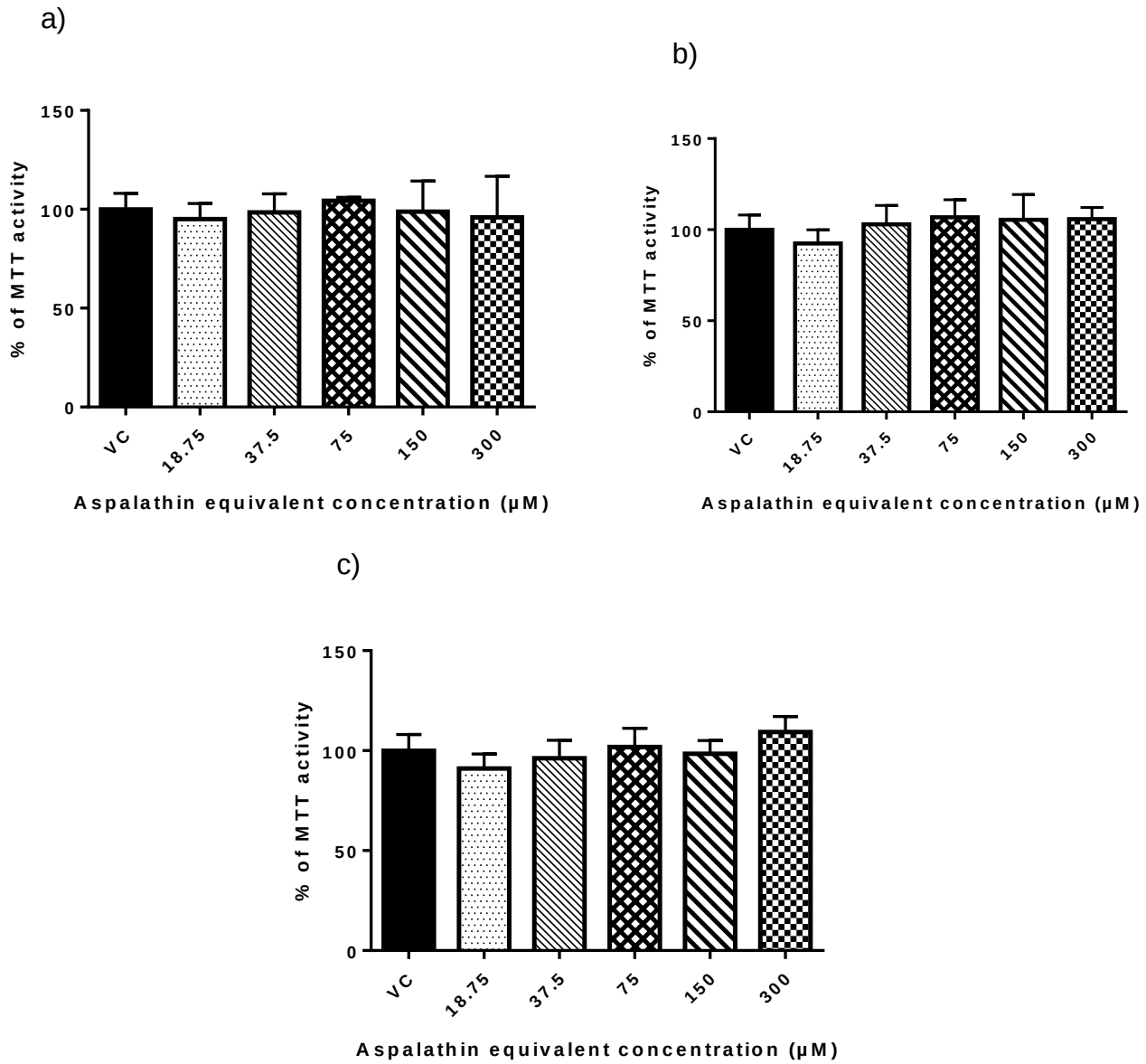


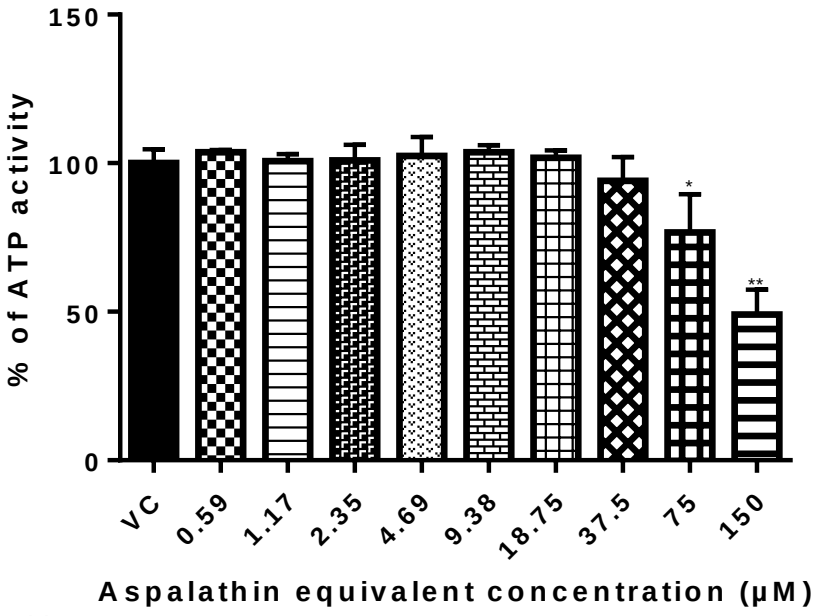
Figure 16: The MTT activity of Caco-2 cells exposed to a) ARC 1023 (80% ethanol extracts batch 9), b) ARC 1026 (aqueous extract batch 5) and c) ARC 1026 (aqueous extract batch 9). The concentration of the extracts was standardized to aspalathin equivalent concentrations (Table 3). Caco-2 cells, cultured for 12 days were exposed to serial dilutions of the GREs for 2 h and the MTT was assessed using absorbance at 570 nm. Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean ± SD and are reflected as a percentage of the vehicle control set at 100%.

4.3.2. Rooibos nanosome formulation (NovaSOL®) and Tween 20 Cytotoxicity

The Rooibos nanosome formulation (NovaSOL®) (containing 2% aspalathin) was assessed for cytotoxicity at an aspalathin equivalent concentration of 150 µM on Caco-2 cells for 2 h. The vehicle control used for the experiment was 0.125 % DMSO which was used to calculate the relative cell viability. The Rooibos nanosome formulation (NovaSOL®) was cytotoxic to Caco-2 cells at concentrations ranging between 75 µM and 150 µM, significantly reducing cell viability by ca. 30% to 50%, respectively (Figure 17).

To further investigate if the toxicity observed for Rooibos nanosome formulation (NovaSOL®) was due to the presence of Tween 20 as disclosed by the manufacturer, cytotoxicity experiments were conducted. The cytotoxicity results for Tween 20 showed toxicity at 0.0625% in Caco-2 cells treated for 2 h. Tween 20 was cytotoxic to Caco-2 cells at concentrations ranging between 0.0625% and 1%, significantly reducing cell viability by ca. 60% to 99%, respectively.

a)



b)

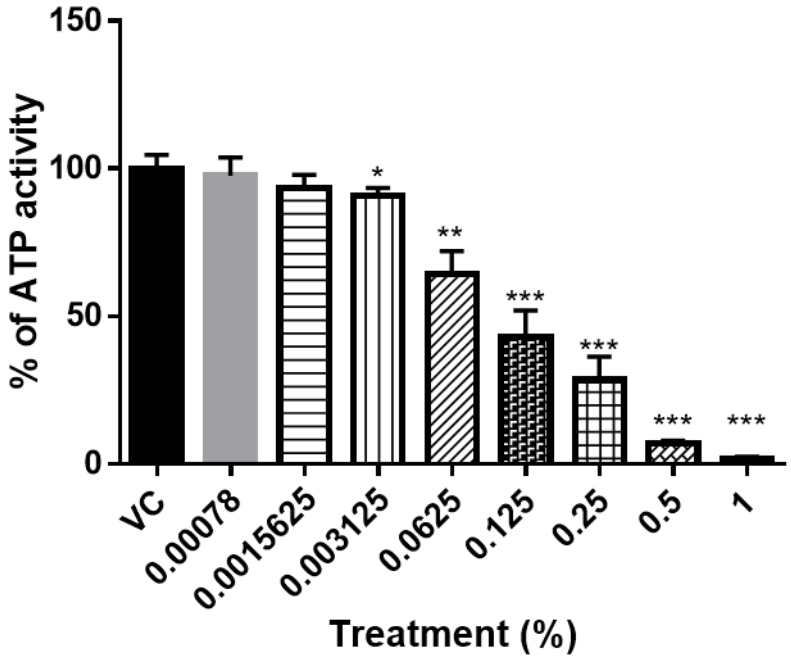


Figure 17: The ATP activity of Caco-2 cells exposed to a) the Rooibos nanosome formulation (NovaSOL®) formulated using Tween 20 and b) Tween 20. The concentration of the Rooibos nanosome formulation was standardized to an aspalathin equivalent concentration of 150 µM. Caco-2 cells, cultured for 12 days, were exposed to serial dilutions of the Rooibos nanosome formulation for 2 h and the cellular ATP content assessed using chemoluminescence. Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean ± SD and are reflected as the percentage of the vehicle control set at 100%. *p<0.05, **p<0.01, ***p<0.001 student's paired two tailed t-test.

4.4. Chromatographic characterization of GREs

Representative HPLC chromatograms of samples taken from the apical side at time 0 are shown in Figures 18-21, showing more or less the same fingerprint, irrespective of solvent used. HPLC chromatograms of samples taken at the same time point to the standardized 150 µM aspalathin equivalent concentration.

Initially the GREs were tested at 1 mg/mL, prior to standardization of the extracts to a 150 µM aspalathin equivalent concentration. At 1 mg/mL aspalathin and the major flavonoids present in the extract (nothofagin, orientin and isoorientin) could be detected on the basolateral side (Figure 22). At 150 µM aspalathin equivalent only traces of these other phenolic compounds could be detect on the basolateral side.

Table 4: Summary of aspalathin, nothofagin, isoorientin and orientin content of the respective GREs (g /100 g soluble solids).

Extracts	Batch no.	Extract type	Asp%	Not%	Ori%	Isoo%
ARC 2	n/a	80% ethanol	18.44	1.29	1.05	2.05
ARC 1022	5	60% ethanol	15.12	1.78	1.05	1.60
ARC 1022	9	60% ethanol	12.86	1.01	0.87	1.31
ARC 1023	5	80% ethanol	19.75	2.35	1.22	1.76
ARC 1023	9	80% ethanol	20.54	1.66	1.19	1.71
ARC 1026	5	aqueous	10.78	1.19	0.86	1.10
ARC 1026	9	aqueous	10.40	0.79	0.83	1.06

Summary of flavonoid *C*-glucosides in GREs showing phenolic compound dissimilarity. Asp-aspalathin, Not-nothofagin, Ori-orientin and Isoo-isoorientin.

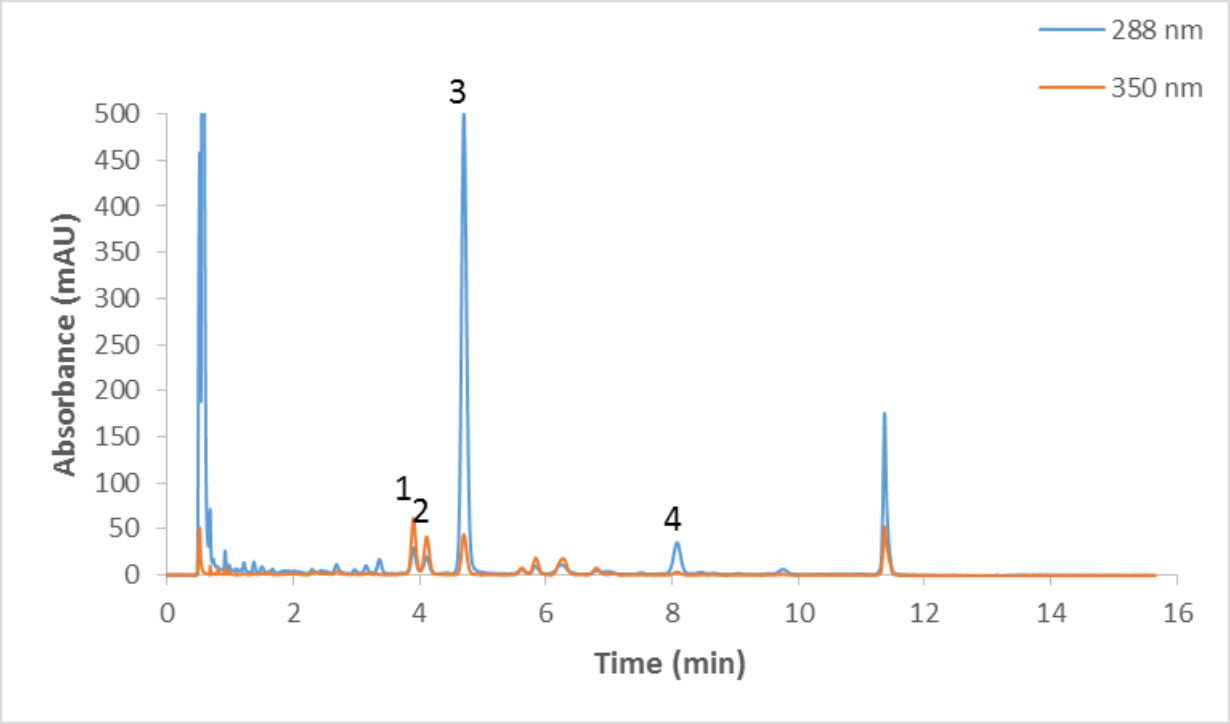
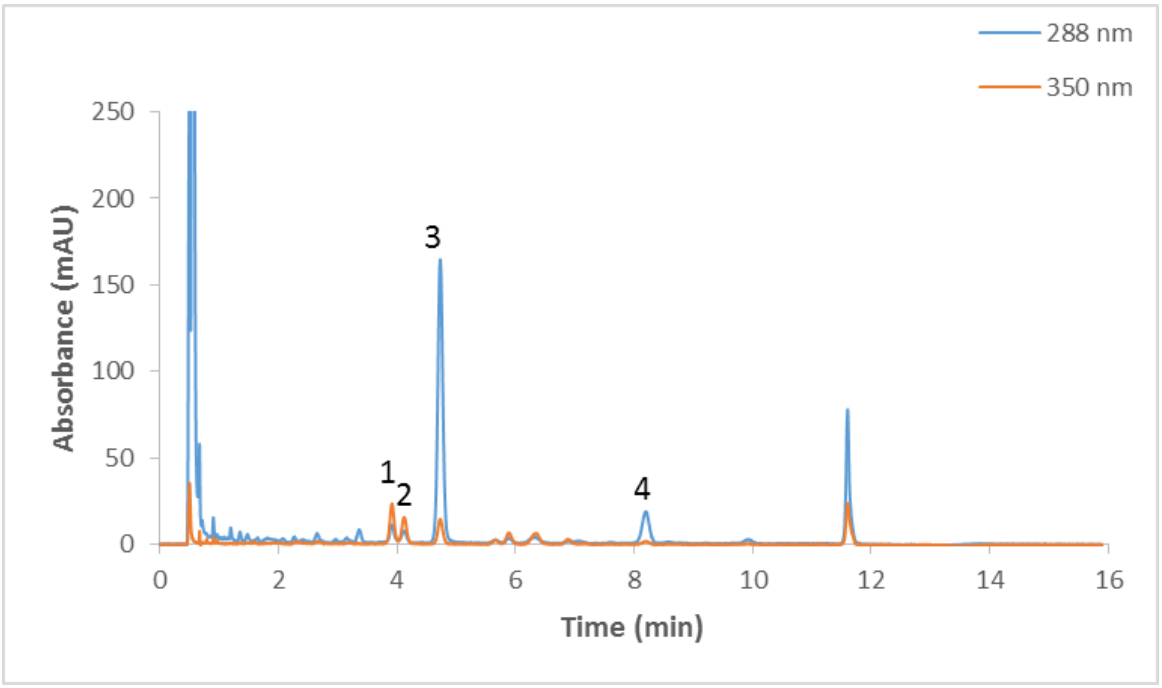


Figure 18: Apical HPLC-DAD fingerprint of the reference GRE ARC 2 at 1 mg/mL. HPLC analysis was conducted using a SB-C18 column (50 x 4.6 nm Dynama C18), with the flow rate of 1.0 mL/min and detection at 288 nm and, 350. 1 - Isoorientin, 2 - orientin, 3 - aspalathin, 4-nothofagin.

a)



b)

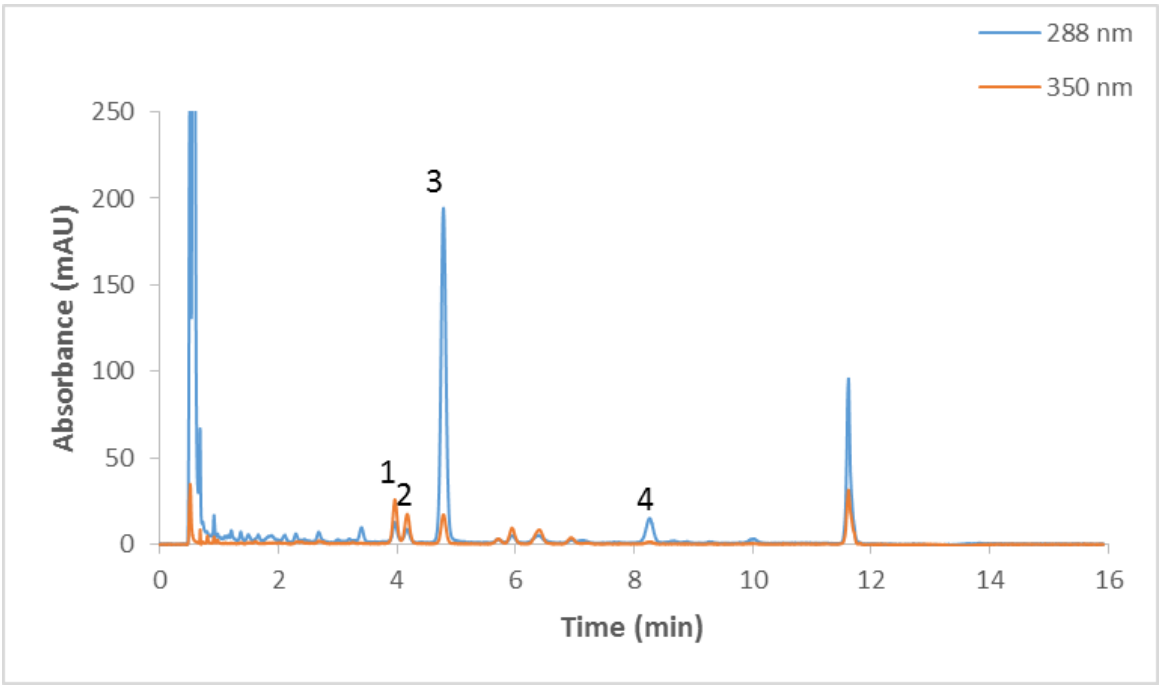
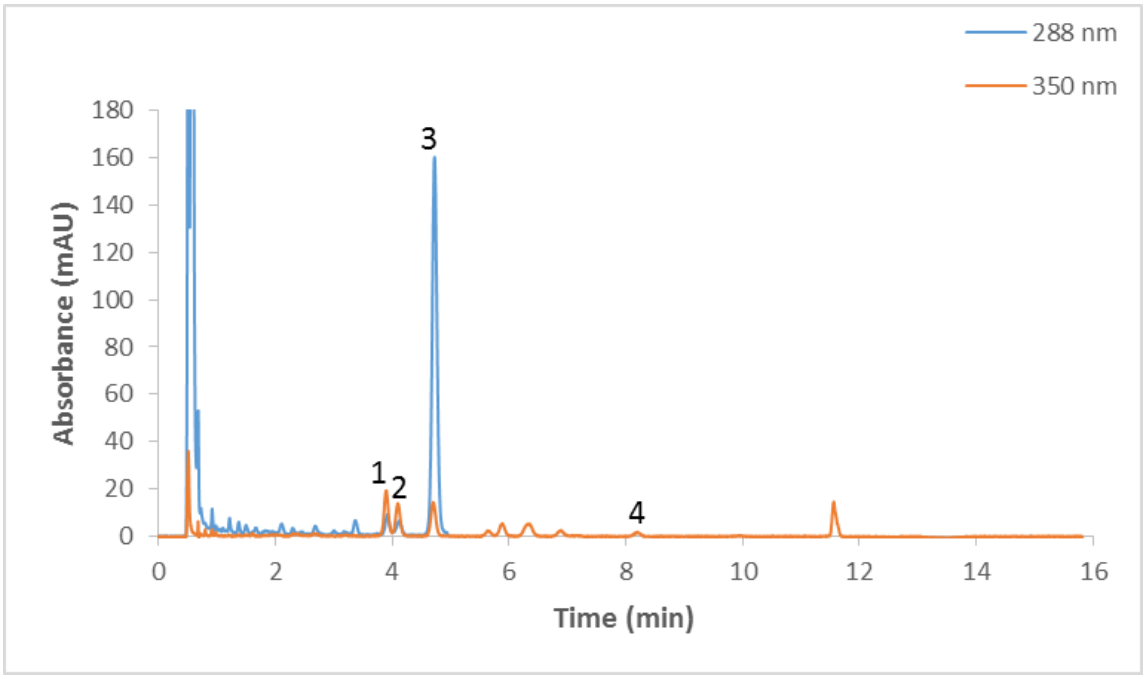


Figure 19: Apical HPLC-DAD fingerprint of green Rooibos extract ARC 1022 a) batch 5 and b) batch 9 at 150 µM aspalathin equivalent concentration. . HPLC analysis was conducted using a SB-C18 column (50 x 4.6 nm Dynama C18), with the flow rate of 1.0 mL/min and detection at 288 nm and, 350. 1 - Isoorientin, 2 - orientin, 3 - aspalathin, 4-nothofagin.

a)



b)

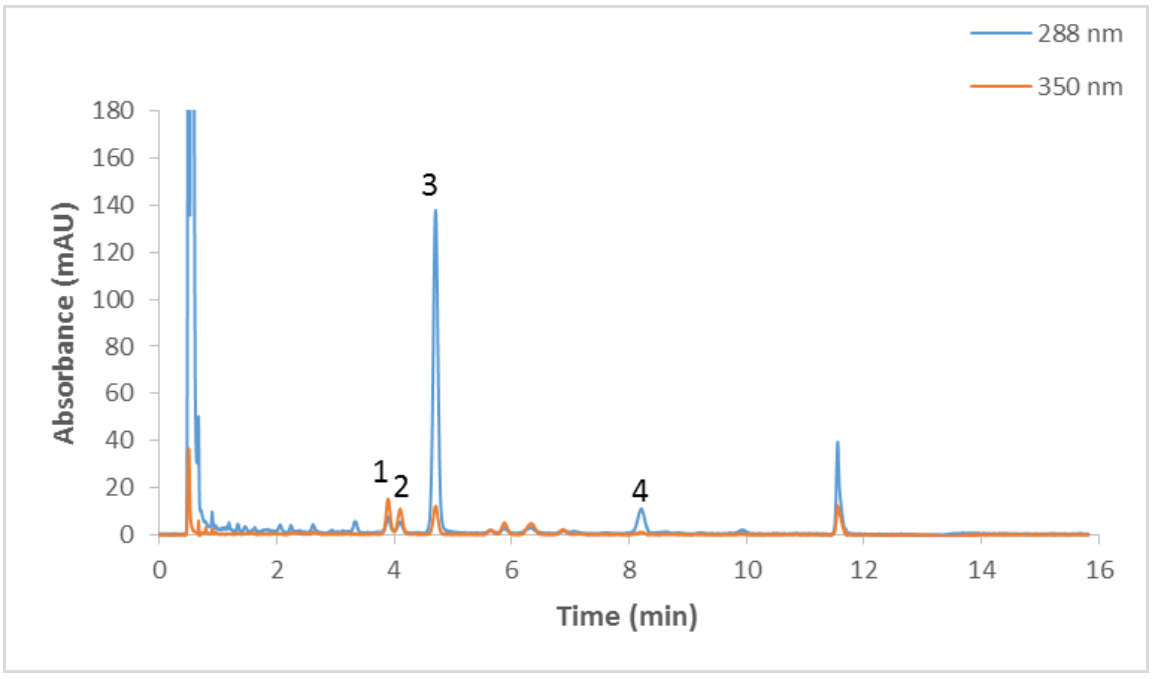
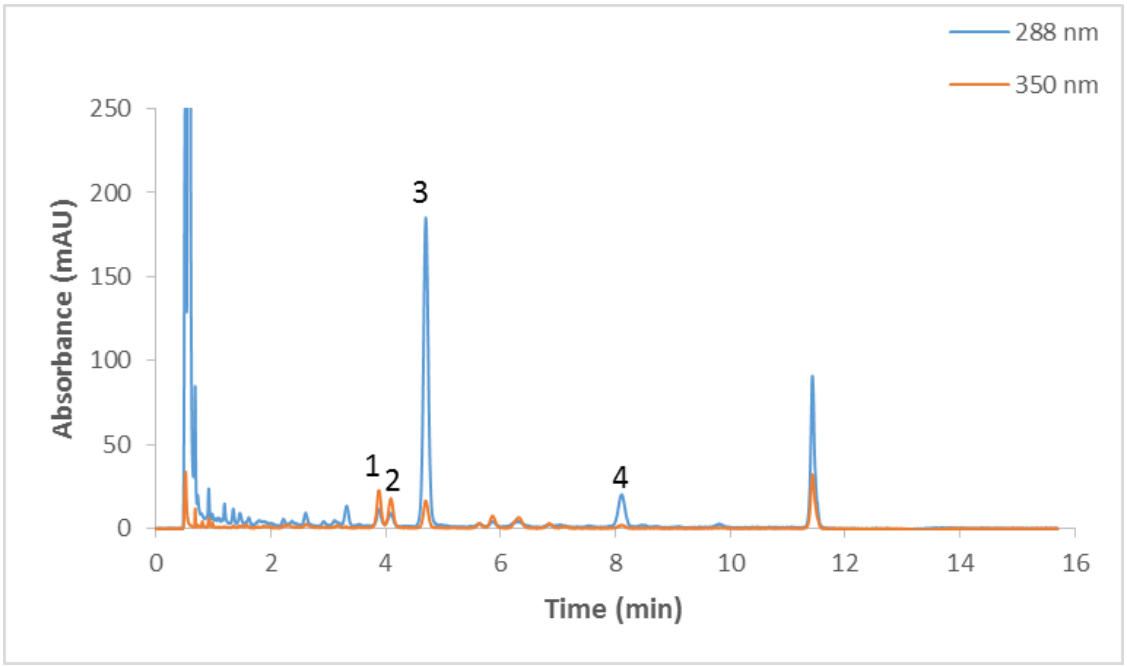


Figure 20: Apical HPLC-DAD fingerprint of green Rooibos extract ARC 1023 a) batch 5 and b) batch 9 at 150 µM aspalathin equivalent concentration. HPLC analysis was conducted using a SB-C18 column (50 x 4.6 nm Dynama C18), with the flow rate of 1.0 mL/min and detection at 288 nm and, 350. 1 - Isoorientin, 2 - orientin, 3 - aspalathin, 4-nothofagin.

a)



b)

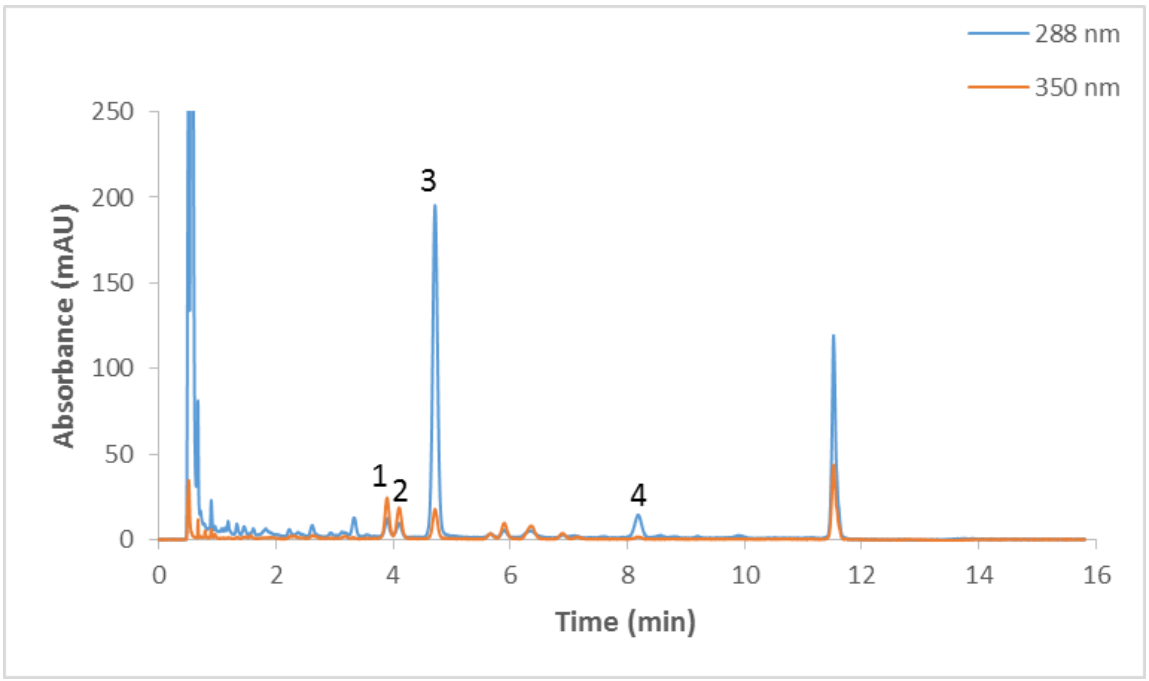
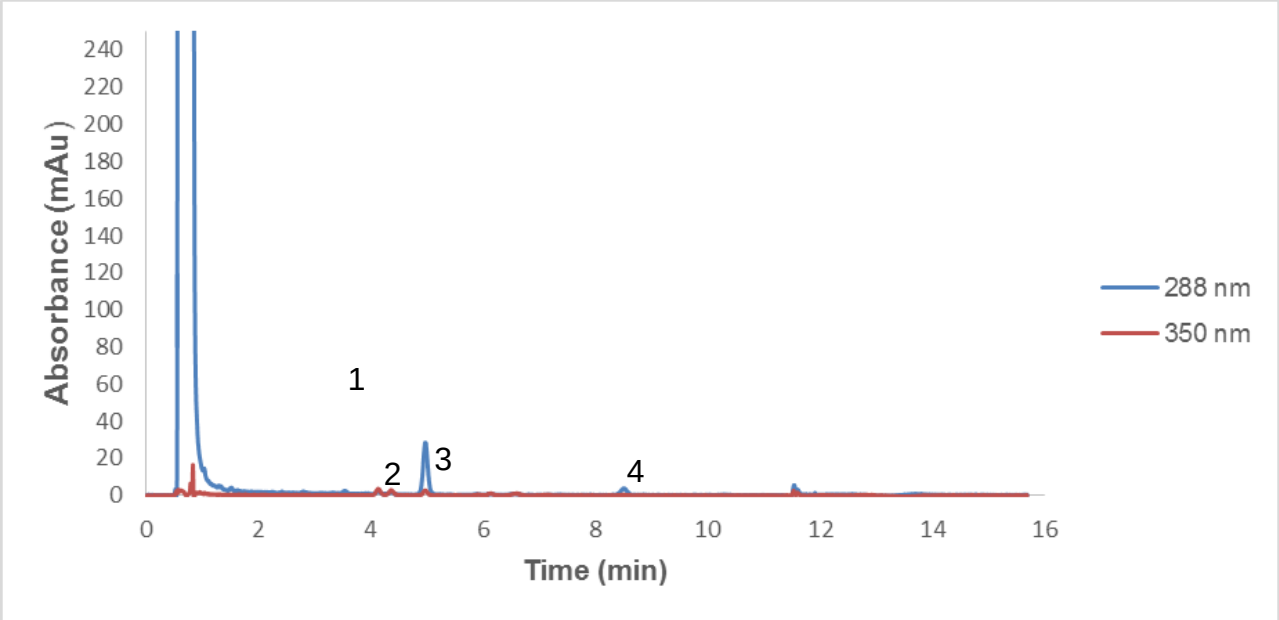


Figure 21: Apical HPLC-DAD fingerprint of green Rooibos extract ARC 1026 a) batch 5 and b) batch 9 at 150 µM aspalathin equivalent concentration. HPLC analysis was conducted using a SB-C18 column (50 x 4.6 nm Dynama C18), with the flow rate of 1.0 mL/min and detection at 288 nm and, 350. 1 - Isoorientin, 2 - orientin, 3 - aspalathin, 4-nothofagin.

a)



b)

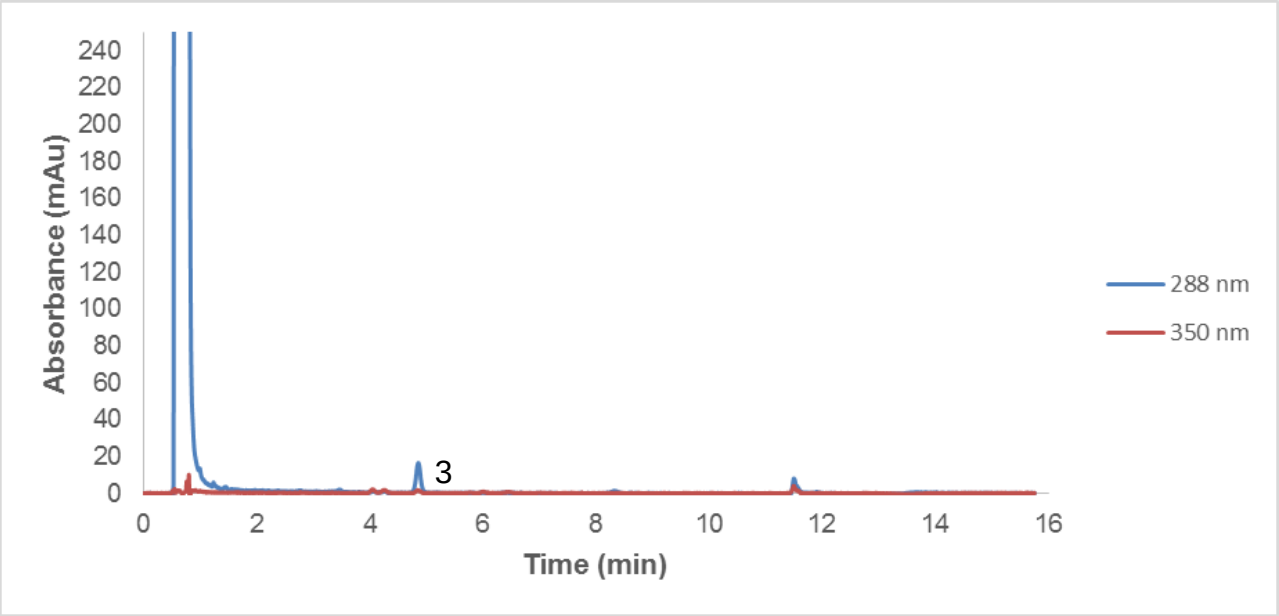


Figure 22: Basolateral HPLC-DAD fingerprint of green Rooibos extract ARC 1022 a) Batch no. 5 at 1 mg/mL and b) batch 5 at 150 µM aspalathin equivalent concentration. HPLC analysis was conducted using SB-C18 column (50X x 4.6 nm Dynama C18), with the flow rate of 1.0 mL/min and detection at 288 nm and, 350. 1-isoorienitn, 2-orientin, 3-aspalathin, 4-nothofagin.

4.5. Transport rate of flavonoid *C*-glucosides from different GREs

The transport of the compounds from the apical to basolateral side of the monolayer was assessed by quantifying the respective concentrations by HPLC-DAD. To achieve this, aliquots were removed from the basolateral side and replaced with buffer to maintain sink conditions throughout the experiment. The accumulative transport rate of aspalathin, nothofagin, orientin and isoorientin is represented in Figure 23 and 24. HPLC-DAD analysis confirmed that aspalathin passes through the monolayer, albeit at a low rate that was confirmed by calculating the P_{app} values.

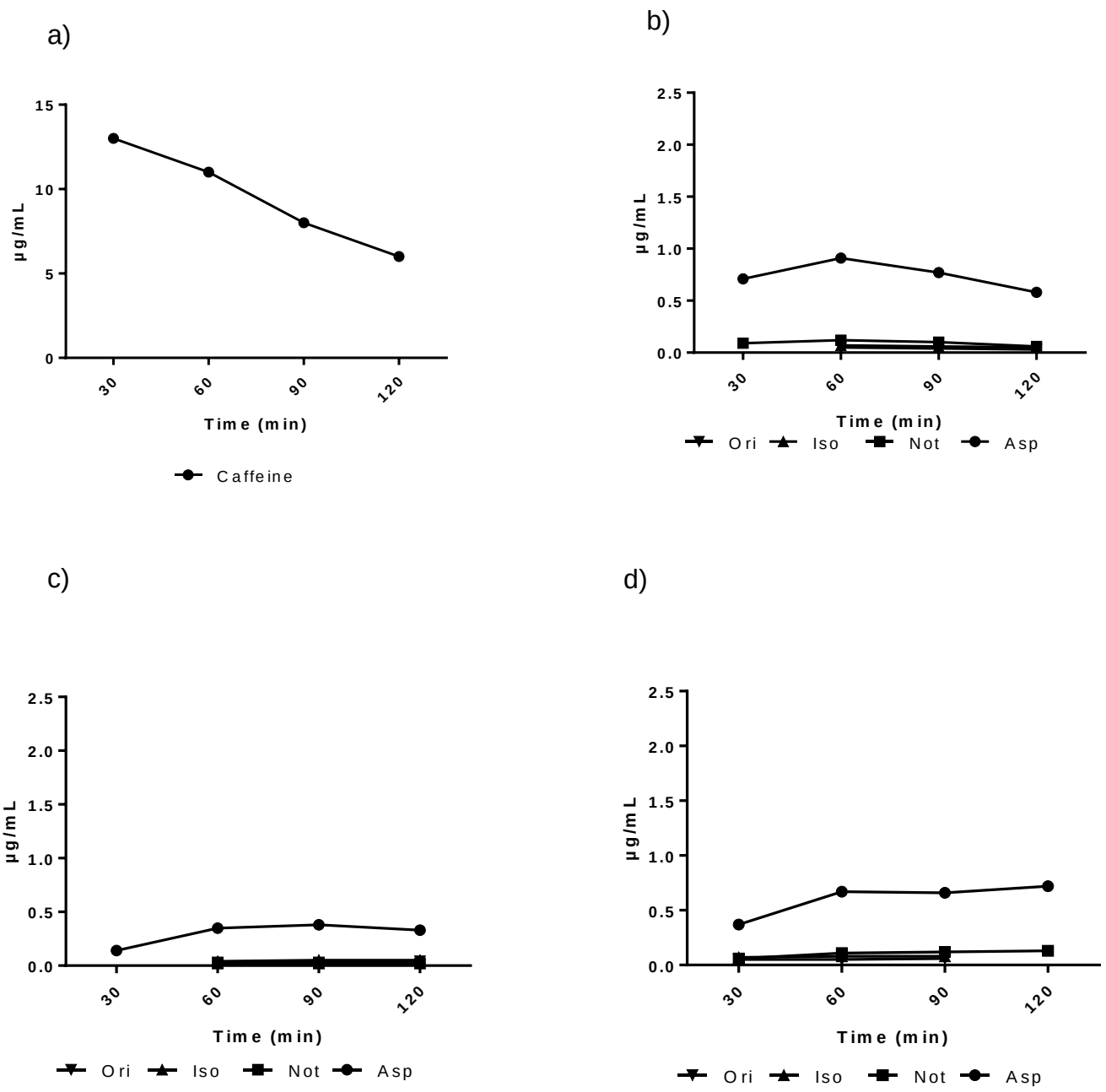


Figure 23: Accumulative flow rate of a) caffeine, aspalathin and its flavone derivatives in b) ARC 1022, c) ARC 1023 and d) ARC 1026 at 1 mg/mL. The accumulative flow rate of aspalathin and its flavone derivatives illustrates the compounds low absorption. Results are from a single experiment.

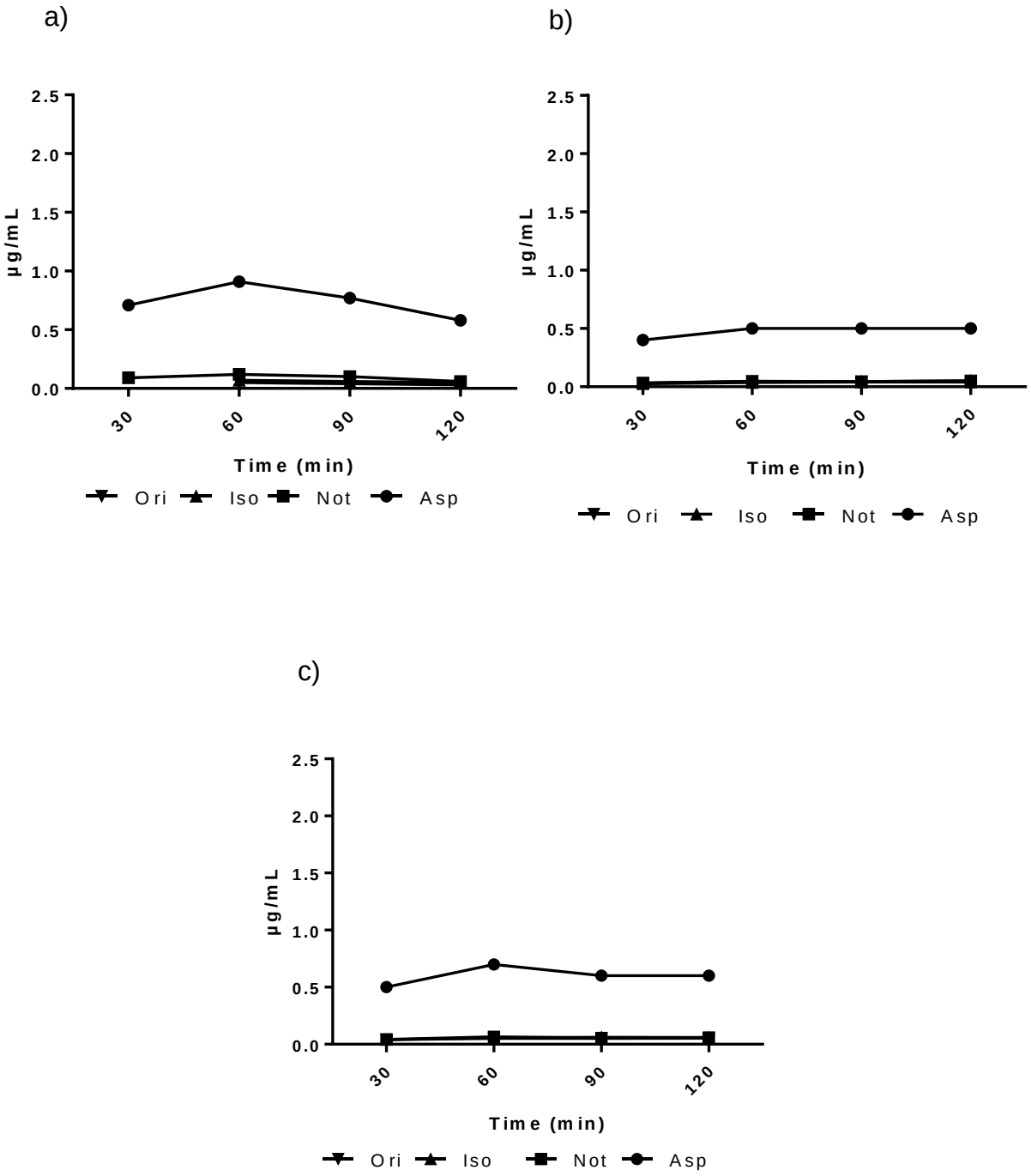


Figure 24: Accumulative flow rate a) ARC 2, b) ARC 2 in the presence of beta-cyclodextrin and c) ARC 2 in the presence of inulin at 1 mg/mL. The accumulative flow rate of aspalathin and its flavone derivatives illustrates the compounds low absorption. Results are from a single experiment.

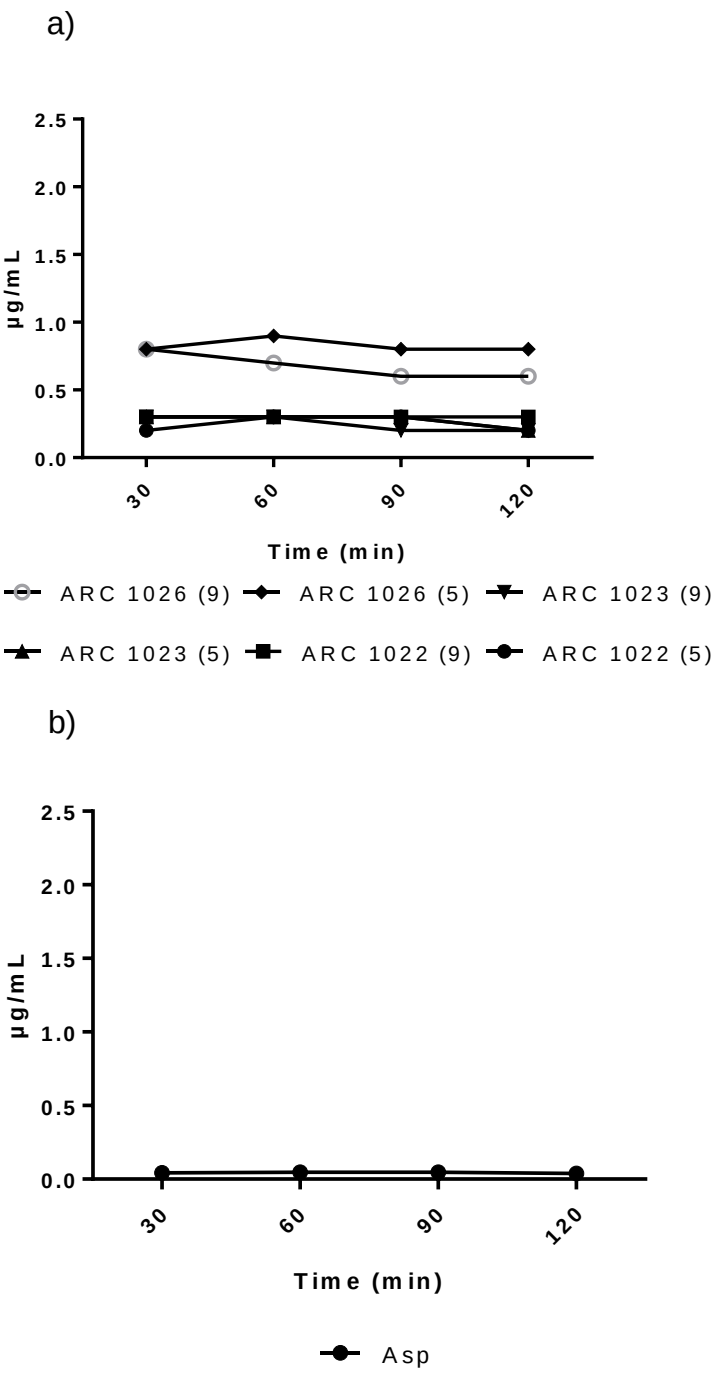


Figure 25: Accumulative flow rate of a) aspalathin in the different GREs and b) NovaSOL® at aspalathin equivalent concentration of 150 µM. The accumulative flow rate of aspalathin and its flavone derivatives illustrates the compounds low absorption in comparison to caffeine which was used as a positive control known to be rapidly absorbed.

4.6. Apparent permeability of flavonoid *C*-glucosides from different GREs

Apparent permeability (P_{app}), is defined as the rate at which a drug or compound is transported across the intestinal epithelium. P_{app} is determined by following the passage of compounds in apical-to-basolateral (A-B) transport experiments using the Caco-2 cell model. P_{app} values are used to predict bioavailability of compounds.

Table 5: P_{app} values for flavonoid *C*-glucosides from the different GREs at 1 mg/mL on the apical side.

Extract	Batch no.	Concentration in (mg/mL)	Compounds	P_{app} Values (cm/s) X 10^{-06}
ARC 1022	5	1	Aspalathin	1.21
			Isoorientin	1.26
			Orientin	1.13
			Nothofagin	1.26
ARC 1023	9	1	Aspalathin	0.88
			Isoorientin	0.84
			Orientin	0.60
			Nothofagin	0.92
ARC 1026	4	1	Aspalathin	1.23
			Isoorientin	1.24
			Orientin	0.91
			Nothofagin	0.75

P_{app} values for the transport in the A-B direction across the Caco-2 cell monolayers. At a concentration of 1 mg/mL, the dihydrochalchone *C*-glucosides aspalathin as well as isoorientin, orientin and nothofagin were detectable in the apical and basolateral samples. All the compounds had similar P_{app} values.

Table 6: P_{app} values for aspalathin in the different GREs at 150 µM aspalathin equivalent concentration on the apical side.

P_{app} Values (cm/s) of aspalathin from different GREs	
Compound	**P_{app} Values (cm/s)**
Caffeine	$6.65 \times 10^{-05} \pm 4.76 \times 10^{-06}$
ARC 2	$1.27 \times 10^{-06} \pm 6.36 \times 10^{-07}$
ARC 1022 batch 5 60% ethanol extract	$8.44 \times 10^{-07} \pm 5.73 \times 10^{-07}$
ARC 1022 batch 9 60% ethanol extract	$7.43 \times 10^{-07} \pm 5.13 \times 10^{-07}$
ARC 1023 batch 5 80% ethanol extract	$6.47 \times 10^{-07} \pm 4.27 \times 10^{-07}$
ARC 1023 batch 9 80% ethanol extract	$6.44 \times 10^{-07} \pm 4.36 \times 10^{-07}$
ARC 1026 batch 5 aqueous extract	$8.48 \times 10^{-07} \pm 2.93 \times 10^{-07}$
ARC 1026 batch 9 aqueous extract	$1.07 \times 10^{-06} \pm 4.83 \times 10^{-07}$

In comparison to the 1 mg/mL extract experiments, standardizing the extracts to an aspalathin content of 150 µM (Table 6), the major flavonoid *C*-glucosides present in the extracts (isoorientin, orientin and nothofagin) were not detectable by HPLC-DAD analysis. Therefore only P_{app} values for aspalathin could be determined.

Table 7: P_{app} values for the transport of aspalathin in the presence and absence of excipients and present in the nanosome.

P_{app} Values (cm/s) of aspalathin from different GREs	
Compound	**P_{app} Values (cm/s)**
ARC 2+ Beta-cyclodextrin	6.99×10^{-07}
ARC 2+ Inulin	1.22×10^{-06}
NovaSOL®	3.02×10^{-07}

Table 7 shows the respective P_{app} values of transport in the A-B direction in Caco-2 cell monolayers of aspalathin from ARC 2 in the presence of excipients and when encapsulated in nanosomes. The addition of the excipients and encapsulation in nanosomes did not improve the transport rate of aspalathin. Beta-cyclodextrin appeared to decrease the transport rate aspalathin. These results represent a single experiment.

Table 8: Aspalathin concentration on the apical side at different time points of the experiment in the presence of excipients.

	T = 0 min (µg/mL)	**T = 120 min (µg/mL)**
ARC 2	114.5	108.8
ARC 2 + Inulin	89.5	88.2
ARC 2 + Beta-cyclodextrin	118.2	95.8

Table 8 demonstrates the initial and final concentration of ARC 2 in the presence and absence of the excipients. The results show that ARC 2 in the presence of inulin had a significant decrease in the difference between the concentration of aspalathin on the apical side at the beginning and end of the experiment when compared to ARC 2 and ARC 2 in the presence of beta-cyclodextrin

4.7. Bile salts and linoleic acid TEER results

Caco-2 cells were exposed to bile salts (20 mM) and linoleic acid (1 mM) for 10 min prior to transport experiment. TEER readings were examined periodically for 24 h and a decreased resistance was observed after a 10 min exposure. This represented the increase in the spaces between the tight junctions. The cells have shown the ability to recover after 24 h of exposure, (TEER >300 Ω).

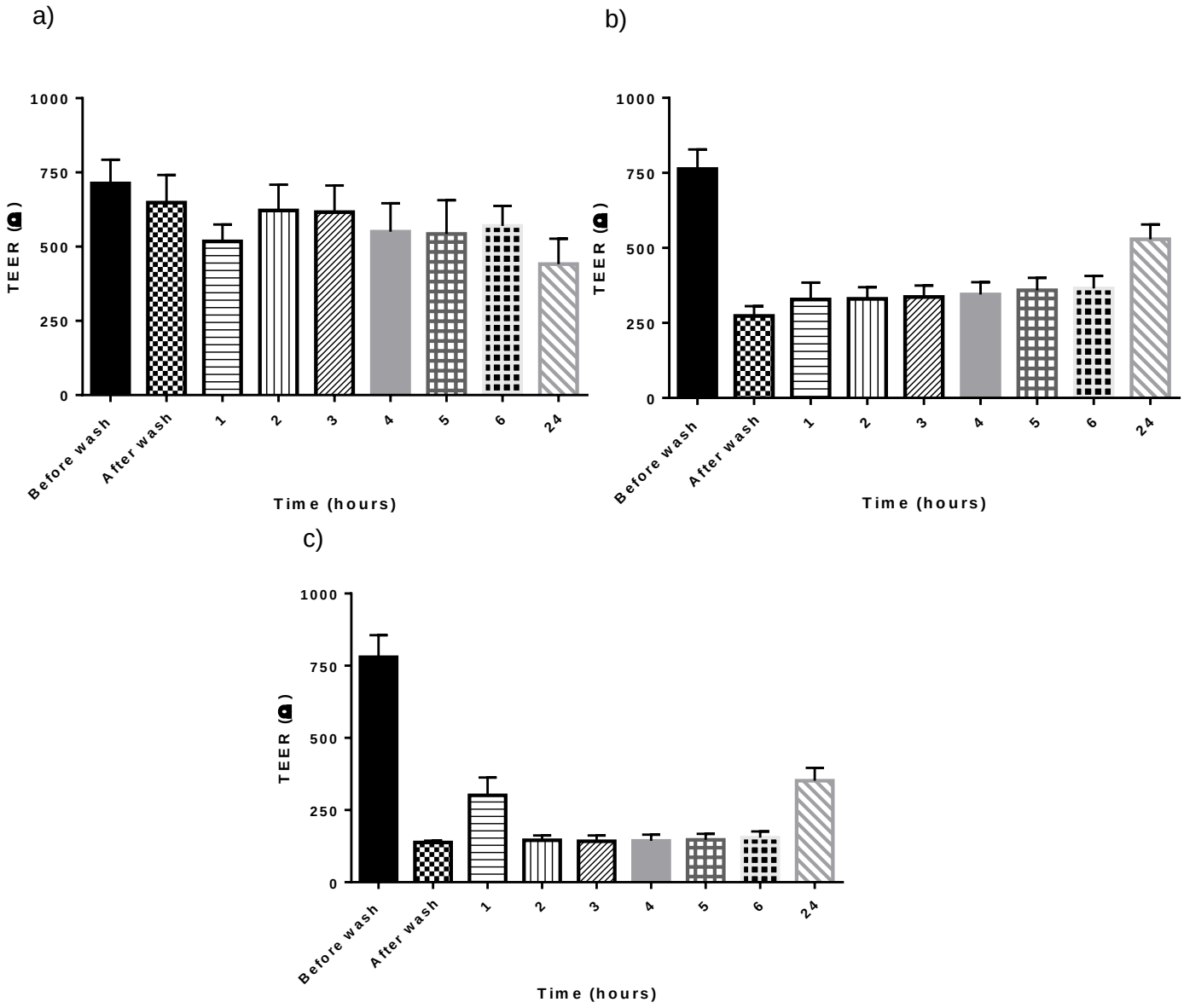


Figure 26: Transepithelial electric resistance readings of Caco-2 cells at day 25. Readings were taken prior to treatment with bile salts and linoleic acid, after the wash step and at 1, 2, 3, 4, 5, 6 and 24 h. a) TEER readings for cells treated with complete medium, b) TEER readings for cells treated with transport buffer only (experimental control) and c) TEER readings for cells treated with bile salts (20 mM) and linoleic acid (1 mM). A decrease in resistance correlates with the opening of the tight junctions. The improvement of TEER readings after 24 h (>300 Ω) indicated the recovery of the Caco-2 cell monolayer. Results are from three independent experiments.

4.8. Statistical Analysis

Statistical analysis was conducted using the unpaired student t-test and one-way ANOVA to compare their P_{app} values as a representation of the transport rate across the Caco-2 monolayer. The unpaired student t-test compared all extracts (ARC 1022, ARC 1023 and ARC 1026 and their batches 5 and 9) to ARC 2, which was the reference extract. There was no significant difference between the reference extract ARC 2 and ARC 1022 (5 or 9), ARC 1023 (9) nor ARC 1026 (5 or 9). ARC 1023 (5) had a slower rate of aspalathin transport compared to ARC 2, $p=0.04$. However, using one-way ANOVA to compare each extract to one other showed that there was no significant difference between extracts or batches.

Chapter 5

5. Discussion

5.1. Rooibos

The popularity of Rooibos is increasing amongst the health conscious for its numerous benefits relevant to metabolic diseases such as obesity, diabetes and cardiovascular disease. These health-benefits have been associated with the phenolic constituents of Rooibos. The variations in polyphenolic concentrations in Rooibos tea limit its potential use as an nutraceutical (Joubert and de Beer, 2011). Factors that determine the polyphenolic concentrations of Rooibos extracts include the variation in plant materials due to use of open-pollinated seeds for production (de Beer *et al*., 2016). Other factors are fermented versus green Rooibos start up material (Joubert, 1996) and the extraction methods and solvents used to enrich the phenolic content. Using these technologies to produce a nutraceutical of acceptable potency and efficacy is also dictated by financial considerations as the yields versus input costs of solvent extraction need to be considered. The use of standardized extract preparations from Rooibos, have been shown to deliver predictable biological effects amongst different laboratories (Kawano *et al*., 2009, Muller *et al*., 2012, van der Merwe *et al*., 2010). Identifying a strategy to optimize the quality of the raw green Rooibos material in terms of aspalathin content would allow consistent yield of this compound, limiting batch to batch variation and adding positively to Rooibos as a nutraceutical.

Flavonoid-enriched extracts prepared from unfermented Rooibos, with increased concentrations of flavonoid *C*-glucosides, in particular the dihydrochalcones C-glucoside aspalathin as well as its flavone derivatives, orientin and isoorientin, have been shown to increase bioactivity (Joubert and de Beer, 2011, Kamakura *et al*., 2015, Muller *et al*., 2012, Son *et al*., 2013). However, these compounds have been shown to have low bioavailability likely due to their physiochemical properties such as molecular size and hydrophilicity (Joubert *et al*., 2009). Resulting in almost undetectable concentrations of aspalathin in the blood plasma of humans following oral ingestion of green Rooibos infusions and enriched extract (Breiter *et al*., 2011, Courts and Williamson, 2009, Stalmach *et al*., 2009) in pigs (Kreuz *et al*., 2008). The low bioavailability of aspalathin and other related bioactive compounds restricts their therapeutic effects on target tissues and limits their potential as a nutraceutical. To address the issue of bioavailability, extracts using hot water and ethanol extraction,

produced from different plant batches to introduce further variation in terms of the extract matrix, were compared for their effect on the intestinal transport using the Caco-2 cell model. The Caco-2 *in vitro* model has been established and validated in our laboratory for assessing phenolic compounds derived from extracts produced from Rooibos and Bush tea (*Athrixia phylicoides*). To assess the rate of transport across fully differentiated monolayers, the passage of the major flavonoid *C*-glucosides in these extracts were monitored in the apical to the basolateral direction. The concentration of the compounds on the apical and basolateral sides was quantified, using HPLC analysis and the rate of transport was calculated. Excipients and the use of nanosomes, as a medium to encapsulate the Rooibos compounds, were investigated to improve bioavailability of aspalathin. Encapsulation of GRE in the nanosomes improve the stability of aspalathin in solution and aid solubility of the extract (Joubert *et al*., 2010).

The initial experiments in this study focused on validation of the Caco-2 cells as a suitable and functional enterocytic model in their fully differentiated form at day 25. Light microscopy confirmed the presence of a single intact layer of Caco-2 cells grown on a polycarbonate support (Figure 8). Transmission electron microscopy illustrated that the differentiated cells expressed several of the membrane specializations for absorption, including microvilli and indigitations of the basolateral basal membranes (Figure 9). Other ultrastructural features include junctional complexes, tight junctions, desmosomes and the presence of transport vesicles (Figure 9). In addition, the presence of various effector transport proteins, including GLUT2, SGLT1 and MDR1 abundantly found in the gastrointestinal tract (Hayashi *et al*., 2003), were confirmed using Western blot (Figure 10). To further confirm the integrity of the monolayer, TEER readings (Figure 11) were monitored consistently throughout the development of the monolayer, as well as during the transport experiments where there is evidence of increased TEER with maturity of the monolayer. In addition, Lucifer Yellow (LY) was added as an indicator molecule at the same time as the treatment and the regulation of its passage across the monolayer was monitored. For all experiments, TEER values obtained were above 300 Ω and Lucifer yellow passage was below 3%, both indicative criteria that the monolayer is suitable for intestinal transport studies.

It is essential to ensure that compounds remain in solution throughout the experiment and thus the assessment of solubility is a necessity. As such, the concentration of the

phenolic constituents should also be high enough to be readily detectable by HPLC, as well as at a concentration that is non-toxic to the Caco-2 cells. In this study, enriched GREs were used, selected based on their polyphenolic content and bioactivity. Due to the organic nature of the ethanol extracts, all extracts were dissolved in 10% DMSO and further diluted using HBSS transport buffer to reach a final DMSO concentration of not more than 0.125%. A concentration of 0.125% of DMSO has been shown to have minimal effects on cell viability (Da Violante *et al*., 2002). Under these conditions it was established that the extracts remained fully dissolved up to a concentration of 2 mg/mL. Cytotoxicity of the GREs (ARC 1022 batch 2, ARC 1023 batch 9 and ARC 1026 batch 4) on the Caco-2 cells was first conducted at the highest soluble concentration. Cytotoxicity was observed for ARC 1023 at a concentration of 2 mg/mL yielding <75% cell viability, while other extracts were non-cytotoxic at a concentration of 2 mg/mL. Therefore, the effect of concentration on the rate of transport of aspalathin, nothofagin, orientin and isoorientin was investigated using 1 mg/mL of each extract.

To efficiently compare the rate of transport, extracts containing various concentrations of aspalathin (10.40% to 20.54%) were standardized to contain a 150 µM aspalathin equivalent concentration. This concentration was derived from the Caco-2 cytotoxicity studies using pure aspalathin where 300 µM was shown to be slightly cytotoxic while 150 µM was shown to be non-toxic, and the transported aspalathin across the monolayer was detectable by HPLC (Bowles *et al*., 2016 in Press). Conversely, the Rooibos nanosome (NovaSOL®) proved to be cytotoxic at 150 µM aspalathin equivalent concentration. The lowest non-toxic concentration of NovaSOL® was found to be 37.5 µM aspalathin equivalent concentration. Although a detailed formulation of the NovaSOL® was not available (confidential information), the presence of Tween 20 and ascorbic acid was disclosed. Based on this information, further cytotoxicity using Tween 20 was investigated and found to be toxic to the Caco-2 cells at concentrations above 0.0032%, which suggest that either Tween 20 or other non-disclosed constituents present in the nanosome may be responsible for the observed cytotoxicity.

The recovery and isolation of phytochemicals from plant material is governed by the extraction step. Extraction yield is affected by physiochemical properties of the solvent such as the polarity and pH as well as extrinsic factors such as extraction temperature

and time, plant material, solvent ratio and particle size. These properties together with the extraction method were used determine the extraction efficiency (Stalikas, 2007).

In this study, Green Rooibos, i.e. the unfermented product, was used as a source material. Hot water was used to prepare extracts according to simulated industrial extraction conditions for preparation of a Rooibos food ingredient extract (De Beer *et al*., 2016). Two ethanol-water mixtures (60% and 80% ethanol) were used to produce GREs enriched in flavonoids, in particular aspalathin. The different extraction methods yielded various percentages of the major Rooibos flavonoid *C*-glucosides (Table 2). For the extracts prepared from the same batch of plant material, the highest flavonoid content was obtained for the 80% ethanol extracts, followed by the 60% ethanol extract and the aqueous extract. Extract yield gave the opposite trend, due to more selective extraction of phytochemicals (E. Joubert, ARC Infruitec-Nietvoorbij, personal communication). It is noteworthy that water and organic solvent extraction may facilitate the extraction of phytochemicals that are soluble in water and/or organic solvent, respectively. Ultimately, increasing the ethanol: water ratio will increase the total polyphenolic yield of the extracts.

ARC 2, also an 80% ethanol extract, was used as a reference extract due to its known bioactivity. When compared to the other extracts (ARC 2, ARC 1022 and ARC 1026), ARC 1023 had the highest concentration of known bioactive compounds (aspalathin, orientin, nothofagin, PPAG, isoquercitrin and hyperoside). However, ARC 2 displayed higher concentrations of isoorientin and rutin. The two plant batches (5 and 9) differed in flavonoid content, confirming the challenge to produce a standardised extract.

To compensate for the various factors related to the variety of extracts with different phenolic content due to extraction methods, extract concentrations were standardized by adjusting the aspalathin content to equimolar concentration of 150 µM. Using this approach, theoretically it would be possible to make direct comparison between the extracts and the reference extract ARC 2. In terms of plant batches and extract type. the standardization to aspalathin was comparable to nothofagin, however, for the flavone derivatives, orientin and isoorientin, the concentration was more variable. However, for ARC 2 produced commercially, the standardization was not as effective as the variation between ARC 2 and the other extracts (ARC 1022, ARC 1023 and ARC 1026) was more pronounced. This illustrates the difficulty in adjusting the

concentration of complex mixtures using only one chemical entity. The standardization of the extracts for aspalathin was successful to an extent, between plant extracts produced under the same conditions albeit from different plant batches.

Apical and basolateral samples collected from transport experiments with the different GREs at 1 mg/mL were analysed using HPLC-DAD. No metabolites of the compounds of interest were detected, indicating that these compounds were transported in their unmetabolised form. However, when the extracts were standardized to 150 µM aspalathin equivalent concentration, only aspalathin was detected on the basolateral side of the monolayer. This is visible in the HPLC chromatograms illustrating differences in the peak sizes of (1) isoorientin, (2) orientin, (3) aspalathin and (4) nothofagin when the 1 mg/mL concentration of the extracts is compared to the 150 µM aspalathin equivalent concentration (Figure 22).

Caffeine, used as a positive control (Figure 23 a), is absorbed rapidly, with most of the caffeine added, absorbed in the first 30 min. The rate of transport of aspalathin was consistent amongst the different GREs and not dependent on concentration. Accumulative flow rate graphs (Figure 23 b-g) show an increase in the concentration of the flavonoid *C*-glucosides on the basolateral side with time.

When the GREs were tested at 1 mg/mL and aspalathin equivalent concentration of 150 µM, low P_{app} values were observed (Table 5 and 6), indicating a low rate of transport for the flavonoid *C*-glucosides when compared to caffeine (5.79×10^{-05} cm/s) which is known to be rapidly absorbed. Most of the extract added to the apical side remained after monitoring the transport for 2 h. However, aspalathin was consistently detected in the basolateral samples intact and unmodified.

The apparent permeability (P_{app}) for aspalathin when present in the different GREs was comparable from extract to extract, indicating that difference in the matrix had no effect. There was no significant difference in the transport rate (P_{app}) between the reference extract ARC 2 and ARC 1022 (5 or 9), ARC 1023 (9) or ARC 1026 (5 or 9). The unpaired student t-test compared the transport rate of aspalathin present in ARC 2 to that of ARC 1023 (5) demonstrated a significant difference ($p = 0.04$). The reference extracts, ARC 2 and ARC 1023 batch 5, are both 80% ethanol extracts. However, the one-way ANOVA Tukey post-hoc compared each extract to the other and showed that there was no significant difference between extracts or batches. The

variation of these statistical comparisons shows that further investigation should be conducted to evaluate whether the difference observed was due to experimental limitations or matrix effects. The rate of transport of aspalathin, regardless of the plant batch origin or extraction method, consistently indicated a low predicted absorption.

The naturally occurring polysaccharide and oligosaccharide excipients were investigated for their influence on the rate of transport of the flavonoid *C*-glucosides. For this study, two excipients were used i.e. inulin and beta-cyclodextrin. The physiochemical properties displayed by the excipients made them ideal candidates (dose, stability and release of active pharmaceutical ingredient (API) from formulation) to investigate their ability to improve the absorption of aspalathin, nothofagin, orientin and isoorientin. However, the experiments in this study were not able to show an improvement to the transport rate of these compounds. The size of the aspalathin excipient-complex could have been the underlying limitation to inhibiting the effective transport of the flavonoid *C*-glucosides (Fincher *et al*., 1973). This is supported by a study by Hoffman *et al* (1993), who demonstrated that complexation of cyclodextrin with the non-steroidal anti-inflammatory drug, naproxen and tolbutamide increased their dissolution rate but inhibited the diffusion of the complexes across the epithelium. In addition to this, it was difficult to prove the resultant encapsulation of compounds by the excipients. When comparing the concentration of aspalathin added to the monolayer, which was performed by removing samples before and after the transport experiments from the apical side of the monolayer, a large amount of aspalathin was not accounted for (neither remaining in the apical sample, nor transferred to the basolateral compartment). This indicates a possibility that the aspalathin-excipient complex was bound to either the apical or the basolateral membrane, or removed when the sample was filtered prior to HPLC analysis.

The effect of the Rooibos nanosome formulation (NovaSOL®) was also investigated for its effect on the rate of transport of the major flavonoid *C*-glucosides. Briefly, NovaSOL® is a highly sophisticated technology that enables the encapsulation of single compounds or plant extracts for use as functional ingredients in a nanostructure. Experiments done with green tea extract encapsulated in such nanosomes showed increased absorption of these compounds (Kim *et al*., 2012). The experiments conducted with NovaSOL® in this study, however, showed no improvement in absorption of aspalathin. Based on the NovaSOL® cytotoxicity experiments, the

aspalathin concentration was limited to 37.5 µM aspalathin equivalents. At a higher aspalathin concentration the nanosomes were toxic to the Caco-2 cells. However, disruption of the integrity of the monolayer was observed (cells lifted off the membrane) at an aspalathin concentration of 37.5 µM. To avoid this disruption of membrane integrity, further experimentation established that NovaSOL® could only be used at an aspalathin equivalent concentration of 1.17 µM. At this concentration, only aspalathin, and none of the other major flavonoid *C*-glucosides could be detected in basolateral samples by HPLC-DAD. This scenario illustrates the limitations of the Caco-2 cell model as compared to the *in vivo* mouse model. The Caco-2 cell model demonstrates a single barrier and the absence of mucins secreted by the goblet cells, amongst other protective features essential in shielding the intestinal epithelium (Blaser, 2007). Further *in vivo* pharmacokinetics studies on NovaSOL® will be of great interest.

Our laboratory has shown that aspalathin is very likely to be transported across the Caco-2 monolayer via the paracellular route (Bowles *et al*., 2016 in Press). This entices the investigation of the use of regulatory compounds, such as bile salts, able to modify the dynamic and structural changes in tight junctional complexes. These compounds can modulate the space between epithelial cells without causing irreversible changes to the monolayer, allowing characteristic features closer to that of in vivo conditions. Preliminary work entailed optimising the combination and concentration of bile salts (20 mM) and linoleic acid (1 mM), as well as the exposure time (10 min exposure of the Caco-2 monolayer prior to transport experiment) to an extent where TEER readings decreased, representing an increase in the space between the tight junctions. These cells have shown the ability to recover with 6 h of exposure (TEER > 300 Ω). These experiments have not been conducted in the presence of extracts. Future work will include experiments with bile salts and linoleic acid to demonstrate whether these compounds effectively and reversibly modulate the tight junctional paracellular space in the presence of GREs. Using TEM analysis the effect of these regulatory compounds will be used to illustrate the opening and recovery of the Caco-2 cell monolayer, and how this mechanism effects the transport, absorption and predicted bioavailability of flavonoid *C*-glucosides present in GREs (Figure 27).

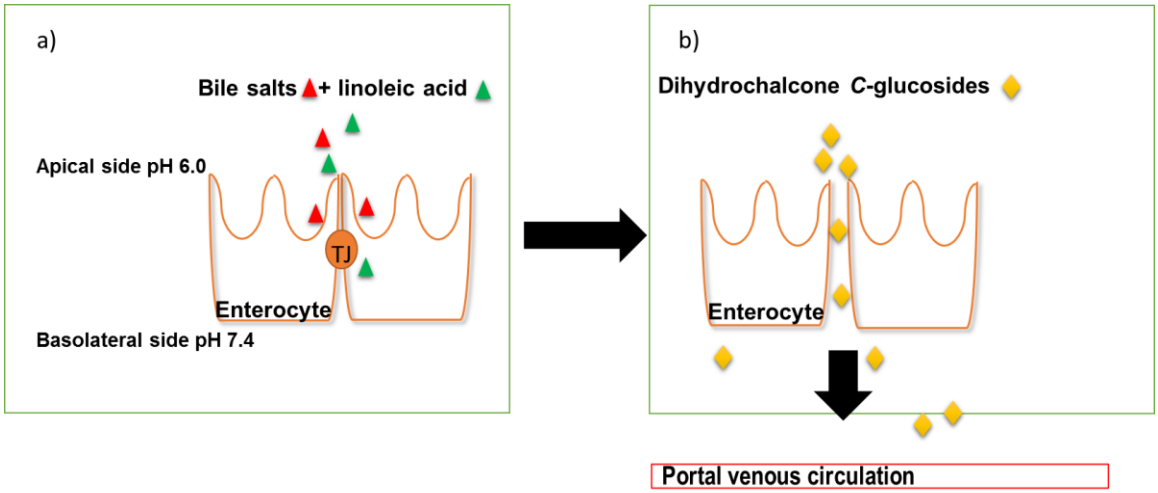


Figure 27: Predicted modulation of tight junctions of fully differentiated Caco-2 monolayers using bile salts and linoleic acid. Schematic b represents pre-treatment of cells using bile salts (red triangles) and linoleic acid (green triangles). Schematic b follows with addition of flavonoid *C*-glucosides (yellow diamonds) within GREs, which pass through the monolayer between the enterocytes (paracellular transport).

5.2. Conclusion

In general, herbal products have a poor reputation because of inconsistent efficacy due largely to variation in the phytochemical content of the raw material and dissimilarity introduced by agroprocessing. Variation in the phytochemical content of the plant material is due to a multitude of factors e.g. soil quality, seasonal fluctuation, environmental variability and genetics. The use of standardized extracts as therapeutics have several advantages including predictable potency and safety. This is also true for Rooibos extracts which due to their health properties are growing in popularity and available for application by the cosmeceutical and nutraceutical industries. The bioactivity of Rooibos is attributed to its polyphenolic content. To increase the potency and consistency of Rooibos extract as a nutraceutical several approaches can be considered. The polyphenolic content of the extract can be increased by the selection of plant material producing high levels of selected bioactive compounds, such as aspalathin, and the application of extraction methods to enrich the extracts in aspalathin. Alternatively, the bioavailability of these bioactive constituents can be enhanced by the addition of excipients improving their overall bioavailability.

In this study, it was shown that the different extraction methods were effective in producing dissimilar GREs in terms of enrichment of flavonoid *C*-glucosides. Their transport rates were not altered by the extraction method used or the variation of plant batches, nor was the transport rate found to be concentration dependent. In the investigation of using plant-based excipients to enhance intestinal absorption, the use of excipients tested was demonstrated not to improve the transport rate of these compounds. The formulation of the nanosomes (with Tween 20 as emulsifier) interfered with the intactness of the monolayer during transport experiments at an aspalathin concentration required for detection on the basolateral side on the monolayer. Aspalathin is transported at a consistent rate.

This study confirms that aspalathin is consistently transported across the monolayer at a constant rate, irrespective of concentration, type of extract (and thus extract matrix) or the addition of tested excipients at a rate that confirms low bioavailability. The implications of this study is that the low bioavailability of aspalathin remains a likely pharmacokinetic limitation for its therapeutic use. Increasing bioavailability of

aspalathin will require structural modifications to the molecule (analogues) or modulation in the permeability of the intestinal barrier.

5.3. Shortcomings of the study

- The transport rate of the major Rooibos flavonoid *C*-glucosides when green Rooibos extract is encapsulated in nanosome could not be determined due to sensitivity of the Caco-2 cell model for Tween 20, the emulsifying agent used to prepare the nanosomes.
- Excipients other than inulin and beta-cyclodextrin could not be investigated for their effect to enhance the intestinal absorption of the flavonoid *C*-glucosides due to resource and time limitations.
- Transport experiments with bile salts and linoleic acid and pure aspalathin could not be investigated due to time limitations.
- Experiments confirming the successful encapsulation of the flavonoid *C*-glucosides also could not be performed due resource limitations.

5.4. Future work

Our labs have been able to show that aspalathin is very likely to be transported via the paracellular route (Bowles *et al* 2016 in Press). This led us to investigate compounds to modify the tight junctional structures enhancing the space in between the epithelial cells allowing enhanced transport of the flavonoid *C*-glucosides. We successfully optimized concentration and exposure time for the combination bile salts 20 mM and linoleic acid 1 mM where TEER reading improved after treatment (>300 Ω). Therefore, transport experiments with bile salts, linoleic acid and pure aspalathin need to be conducted. This should be followed by transmission electron microscopy analysis to show the opening and recovery of the Caco-2 cell monolayer.

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Appendix 1

List of reagents

Product name	Catalogue number	Supplier and country
ATP assay kit	LT27-008	Whitehead Scientific, JHB, SA
3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) (MTT)	M2003	Sigma, St Louise, MO, USA
96 well plates	3300	Corning, MA, USA
Ascorbic acid	A7506	Sigma, St Louise, MO, USA
Beta-cyclodextrin	C4767	Sigma, St Louise, MO, USA
Benchtop centrifuge	SL16R	Thermo Fisher Scientific, Waltham, MA, USA
Bio Rad Bradford Protein assay kit	500-0201	Bio-Rad, Hercules, CA, USA
Bio Rad Gel Doc	170-8650	Bio-Rad, Hercules, CA, USA
Caco-2 cell line	812010202 Sigma	European Collection of Cell Cultures, Salisbury in UK
Caffeine	C0750	Sigma, St Louise, MO, USA
Carbon dioxide	K239C	Air Products, Centurion, SA
Cell counting chamber slides	C10228	Life Technologies Corporation, Carlsbad, CA, USA
Cell extraction lysis Buffer	FNN0011	Life Technologies Corporation, Carlsbad, CA, USA
Centrifuge (Temperature control) Tubes 2.0 mL	K82967 3906	Sigma-Aldrich, St Louis, MO, USA
Dimethyl sulfoxide (DMSO)	276855	Sigma, St Louise, MO, USA
Dulbecco's phosphate buffered saline (DPBS)	17513-F	Lonza, Walkersville, MD, USA
Eagles Minimum Essential Medium	BE12-126F	Lonza, Walkersville, MD, USA
Eppendorf tubes	0030123.301	Sigma, St Louise, MO, USA
Ethanol absolute, 200 proof for molecular	E7023-500	Sigma, St Louise, MO, USA
Fetal Bovine Serum (Hyclone)	10499-044	Gibco
Fine balance scale	AR2140	United Scientific, Cape town, SA
Glucose transporter2 antibody	ab54460	abcam, Cambridgeshire, United Kingdom
Hanks Balanced Salt Solution	BE20-527F	Lonza, Walkersville, MD, USA
Heating block	S62927099	Labinet, USA
Inulin	12255	Sigma, St Louise, MO, USA

Inserts and 6 well plates 0.4 µm pore	PBIO35006	Biosmart, United Kingdom
Inverted light microscope	CKX 41	Olympus, Melville, NY, USA
L-Glutamine	G8540	Sigma, Stanheim, Germany
Linoleic acid	L9530	Sigma, St Louise, MO, USA
Low fat milk powder	2082054	Clover, JBH, SA
Lysis buffer	9803	Cell Signalling Technology, Danvers, MA, USA
Lucifer yellow CH dipotassium salt	L0144SIGMA	Sigma, St Louise, MO, USA
Methanol	67-56-1	Sigma, St Louise, MO, USA
Millex-GP syringe filter unit	SLGP033RS	Millipore PES membrane, Merck
Multidrug Resistance Protein antibody	sc55510	Sansa Cruise
Orbital shaker	20197	Stovall life science, USA
Pasteur pipettes	6112 361	Greiner bio-one, Frickenhausen, Germany
Penicillin streptomycin	17-602E	Lonza, Walkersville, MD, USA
pH meter	702038	Lansec, Gauteng, SA
Ponceau S Stain	P23295	Sigma, St Louise, MO, USA
Running buffer SDS	161-0772	Bio-Rad, Hercules, CA, USA
SDS-PAGE gels	161-0993	Bio-Rad, Hercules, CA, USA
Serological pipettes -2mL	4501	Corning, MA, USA
Serological pipettes-10mL	4101	Corning, MA, USA
Serological pipettes-25mL	4251	Corning, MA, USA
Serological pipettes-50mL	4490	Corning, MA, USA
sodium-glucose linked transporter antibody	ab14686	abcam, Cambridgeshire, United Kingdom
Stainless steel beads 5mm	69989	Qiagen, Hilden, Germany
Sterile TC water	59900C	Lonza, Walkersville, MD, USA
Taurocholic acid sodium salt hydrate	T9034	Sigma, St Louise, MO, USA
Tissue lyser	85220	Qiagen, Hilden, Germany
Tris	93352	Sigma, St Louise, MO, USA
Trypan blue	T8154	Sigma, St Louise, MO, USA
Trypsin/versene	15050-065	Invitrogen, Carlsbad, CA USA
Tween 20	3030-931	Sigma, St Louise, MO, USA

Appendix 2

Preparation of reagents

HBSS/25 mM HEPES (pH 7.4) (250 mL)

The HBSS HEPES buffer was prepared by dissolving 1.498 g of HEPES in 250 mL of HBSS (Lonza, Cat # BE10-527). The buffer was allowed to reach room temperature and the pH was adjusted to 7.4 using 1M KOH.

HBSS/10 mM MES (pH 6) (250 ml)

The HBSS HEPES buffer was prepared by dissolving 0.488 g of HEPES in 250 mL of HBSS (Lonza, Cat# BE10-527). The buffer was allowed to reach room temperature and the pH was adjusted to 6 using 1M KOH.

Sorenson's buffer

The buffer was prepared by dissolving 0.1 M of glycine (0.7151 g in 100 ml distilled water) and 0.1 M NaCl (0.584 g in 100 mL). The pH of the buffer was then adjusted to 10.5 using NaOH.

Destaining buffer

The buffer was prepared by adding 150 mL of 15% methanol and 200 mL of 20% acetic acid which was topped up with 650 mL of distilled water to make up a total volume of 1L.

Transfer buffer for Western Blot

The buffer was prepared by dissolving 25 mM of Tris (3.03 g) and 192 mM glycine (14.4 g) topped up with 800 mL of distilled water, 200mL of methanol to make up a total volume of 1L.

10X Tris-buffered saline

The buffer was prepared by dissolving 200 mM of Tris (24.22 g) and 1.37 mM NaCl (80.06 g) topped up with 1000 mL of distilled water to make up a total volume of 1L.

1X Tris-buffered saline and Tween 20 (1X TBST)

1X TBST was prepared by diluting 100 mL of 10 x TBST with 900 mL of distilled water (v/v), thereafter 1 mL Tween 20 was added. The buffer was kept at 4°C.

Transport Assay treat preparation

Preparation of treatment for extracts

- 75 uL of extract
- 60 uL of Lucifer yellow
- 5865 uL of HBSS (pH 6)
  Total volume 6mL which was filter sterilize

Extract-excipient treatment

- 150 uL of extract-excipient solution
- 60 uL of Lucifer yellow
- 5790 uL of HBSS (pH 6)
  Total volume 6mL which was filter sterilize

Negative Control

- 75 uL of 10% DMSO
- 60 uL of Lucifer yellow
- 5865 uL of HBSS (pH 6)

Positive control

- 75 ul of caffeine
- 60 ul of Lucifer yellow
- 5865 of HBSS (pH 6)

Appendix 3

Agilent 1200 HPLC system	Agilent Technologies, Waldbronn, Germany
Benchtop centrifuge (SL16R)	Thermo Fisher Scientific, Waltham, MA, USA
Bio TEK ELX 800 plate reader	BioTek Instruments Inc., Winooski, VT, USA
Bio-Rad Gel Doc (170-8650)	Bio-Rad
Bio-Rad Gel Doc (170-8650)	Bio-Rad, Hercules, CA, USA
Biosafety cabinets	Airvolution
Carbon dioxide incubator	RS Bio Tech
ELX800 absorbance microplate reader	Bio Tek Instruments Inc; Friedrichshall, Germany
Equipment	Manufacturer
Fine balance scale (AR2140)	United scientific
FLX800 fluorescence microplate reader	Bio Tek Instruments Inc.; Friedrichshall, Germany
Gen5 software (version 1.05)	BioTek Instruments Inc., Winooski, VT, USA
GraphPad Prism® version 5.04	GraphPad Software Inc., La Jolla, USA
Heating block (S62927099)	Labinet
Inverted fluorescent microscope (Eclipse Ti)	Nikon; NY, USA
Inverted light microscope (CKX 41)	Olympus; Melville, NY, USA
Kinetex C18 column (150 mm × 4.6 mm, 2.6 µm particle size, 100 Å)	Phenomenex, Santa Clara, CA, USA
Mini Protean casting frame (165-3304)	Bio-Rad, Hercules, CA, USA
Mini Protean casting stand (165-3303)	Bio-Rad, Hercules, CA, USA
Mini Protean Tetra Cell (165-8025)	Bio-Rad, Hercules, CA, USA
OpenLab CDS Chemstation software	Agilent Technologies
Orbital shacker (20197)	Stoval life Science
pH meter (702038)	Lasec
Power pack (043BR16158)	Bio-Rad
Scale (ARC120)	United scientific
Tissue lyser	Qiagen, Hilden, Germany
Trans-Blot Cell (170-4070)	Bio-Rad, Hercules, CA, USA
The Millicell-ERS volt ohmmeter	MERS00002 Merck KGaA, Darmstadt, Germany
Water bath	Memmert; Heilbronn, Germany

Appendix 4

Research outputs

SEMDSA Conference

Poster title: Abstract 23 page 17

Evaluation of excipients for enhanced intestinal absorption of Rooibos flavonoids

Hlengwa N[2], Muller C.J.F[1], Basson A.K[2], Joubert E[3], Bowles S[1]

[1]Biomedical Research and Innovation Platform, South African Medical Research Council, Cape Town, South Africa. [2]Departmentment of Biochemistry and Microbiology, University of Zululand, Kwa-Dlangezwa, South Africa. [3]Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC), Infruitec-Nietvoorbij, Stellenbosch, South Africa.

Early Career Young Scientist Convention

Poster title: page 113

Evaluation of excipients for enhanced intestinal absorption of Rooibos flavonoids

Hlengwa N[2], Muller C.J. F[1], Basson A.K[2], Joubert E[3], Bowles S[1]

[1]Biomedical Research and Innovation Platform, South African Medical Research Council, Cape Town, South Africa. [2]Departmentment of Biochemistry and Microbiology, University of Zululand, Kwa-Dlangezwa, South Africa. [3]Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC), Infruitec-Nietvoorbij, Stellenbosch, South Africa.

Appendix 5

Supplementary information

Table SP: Statistical analysis of aspalathin P_{app} value from the different GREs

Extract	Batch no.	Unpaired student t-test p value	Statistical Significance	One-way ANOVA
ARC 1022	5	0.12	P<0.05	P<0.05
ARC 1022	9	0.08	P>0.05	P>0.05
ARC 1023	5	0.04	P<0.05	P>0.05
ARC 1023	9	0.07	P>0.05	P>0.05
ARC 1026	5	0.07	P>0.05	P>0.05
ARC 1026	9	0.19	P>0.05	P>0.05

Supplementary table showing values for statistical analysis of GREs using unpaired student t-test and One-way ANOVA.

Appendix 6

Ethical clearance certificate

UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-030)

RESEARCH & INNOVATION
Website: http://www.unizulu.ac.za
Private Bag X1001
KwaDlangezwa 3886
Tel: 035 902 6887
Fax: 035 902 6222
Email: ManqeleS@unizulu.ac.za

ETHICAL CLEARANCE CERTIFICATE

Certificate Number	**UZREC 171110-030 PGM 2015/208**							
Project Title	**Evaluation of excipients for enhanced intestinal absorption of Rooibos (Aspalathus linearis) flovonolds**							
Principal Researcher/ Investigator	N Hlengwa							
Supervisor and Co-supervisor	Prof AK Basson		Dr. CJF Muller, Dr S Bowles and Prof E Joubert					
Department	Biochemistry and Microbiology							
Nature of Project	Honours/4th Year		Master's	x	Doctoral		Departmental	

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate.

Special conditions: (1) The Principal Researcher must report to the UZREC in the prescribed format, where applicable, annually and at the end of the project, in respect of ethical compliance.
(2) Documents marked "To be submitted" (see page 2) must be presented for ethical clearance before any data collection can commence.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

Appendix 7

Turnitin report

Nokulunga

by Nokulunga Hlengwa

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1. Introduction

Advancement of technology is increasingly making life more convenient and consequentially people are becoming less and less physically active. The resultant sedentary lifestyle, coupled to an unhealthy diet typically high in sugar, refined carbohydrates and saturated fats are major risk factors in the development of chronic diseases such as diabetes, heart diseases, obesity and cancer (Who and Consultation, 2003). A change in lifestyle including regular exercise and a healthier diet with an increased consumption of fibre, fruit, vegetables are recommended to help combat the development of these diseases (Li et al., 2014). An increased intake of phenolic compounds commonly found in tea, fruit and vegetables has been shown to have various health benefits. Increased consumption of these compounds are particularly helpful in the management of metabolic disorders such as insulin resistance, a major causal factor in the development of metabolic diseases (Landete, 2012, Muller et al., 2012). Phenolic compounds are produced by plants as secondary metabolites, primarily as protectants against plant pathogens, herbivores, and are associated with sensory attributes such as colour, bitterness and astringency (Shi et al., 2003). These phenolic compounds, sequestered from the plant in the form of an enriched extract, can act as potent dietary supplements. The identification and characterization of specific bioactive phenolic compounds responsible for exhibiting health-promoting properties is essential for the development of products that are safe and effective.

Rooibos (*Aspalathus linearis)* is a plant that is indigenous to South Africa. Research into the health properties associated with Rooibos consumption is gathering momentum both locally and internationally. Most of the health benefits associated with Rooibos are linked to its phenolic composition (Joubert et al., 2008, Mikami et al., 2015, Lee and Bae, 2015, Ku et al., 2015, Kamakura et al., 2015, Muller et al., 2016b). Aspalathin, a dihydrochalcone *C*-glucoside, uniquely present in Rooibos is the most abundant flavonoid. Aspalathin has gathered a lot of interest due to its hypoglycaemic (Son et al., 2013), antidyslipidaemic (Beltran-Debon et al., 2011), anti-inflammatory and anticancer effects (Joubert et al., 2005). Aspalathin has also been found to reduce insulin resistance (Mazibuko et al., 2013) stimulate insulin secretion (Kawano et al., 2009) and lower cardiovascular disease risk (Marnewick et

Nokulunga

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PRIMARY SOURCES

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