Intestinal absorption and metabolism of bush tea major phenolic compounds exhibiting anti-diabetic activity.

By:

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DECLARATION

By submitting this dissertation, I hereby declare that the entirety of the work contained in this document is my own, original work that has not been published for any degree or examination in any other institution. It is submitted for the Master's degree in Biochemistry in the department of Biochemistry and Microbiology at the University of Zululand. It has not been submitted before.

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ABSTRACT

Background: *Athrixia phylicoides* (*A. phylicoides*), popularly known as ‘bush tea’, is an indigenous aromatic shrub found in mountainous and grassland areas of the northern and eastern parts of Southern Africa. The plant is traditionally used for the treatment of several ailments, including coughing, treating infected wounds, boils, sore throat, hypertension and heart disease. An aqueous extract of *A. phylicoides,* has been shown to improve glucose uptake *in vitro* when tested in muscle, liver and fat cells. While an aqueous extract of *A. phylicoides* has been shown to have bioactivity, there is limited knowledge regarding absorption and bioavailability of phenolic compounds present in *A. phylicoides*.

Aim of the study: The present study aims to identify major phenolic compounds in an aqueous extract of *A. phylicoides*, and describe their transport characteristics across the Caco-2 monolayer.

Methods and materials: HPLC-DAD and LC/MS was used to identify major phenolic compounds within the extract as well as monitor the transport of these compounds across the intestinal barrier. Differentiated Caco-2 cells were used as a model to predict bioavailability and identify metabolite formation, respectively. Specific inhibitors were used to assess efflux characteristics of these compounds.

Results: Nine major phenolic compounds of the aqueous extract of *A. phylicoides* were identified with *para*-coumaric acid identified for the first time. Three other major phenolic compounds; protocatechuic acid (PCA), caffeic acid (CA) and *para*-coumaric acid (*p*-CA), were demonstrated to cross the Caco-2 cell monolayer in significant amounts, with Papp values 4.52, 4.35 and 23.80 (x 10^6) cm/s, respectively.

Conclusion: The present study reports, for the first time, the transport of phenolic compounds from an aqueous extract of *A. phylicoides* with PCA, CA and *p*-CA being the major transported compounds having relatively low bioavailability.

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This Thesis is dedicated to my Dad "Solomzi Ntamo", Thank you for all you have done for me. R.I.P Dad.

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List of Abbreviations

A. phylicoides	Aqueous extract of *Athrixia phylicoides*
ANOVA	Analysis of variance
AP	Apical
AP-BL	Apical to basolateral
ATCC	American type culture collection
ATP	Adenosine triphosphate
BL	Basolateral
BSA	Bovine serum albumins
CA	Caffeic acid
ca.	Circa (approximately)
Ca^{2+}	Calcium
Caco-2	Adenoma colorectal carcinoma epithelial cells
ChA	Chlorogenic acid
CL-channels	Chloride channels
CO_2	Carbon dioxide
dH2O	Deionized water
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco`s phosphate buffered saline
EDTA	Ethylene diamine tetra acetic acid
EMEM	Eagle’s minimum essential medium
ER	Efflux ratio

EtOH	Ethanol
FBS	Foetal bovine serum
GIT	Gastrointestinal tract
GLUT1	Glucose transporter (1,2,3,4,5)
H+/K+	Gastric hydrogen potassium
HBSS	Hanks balanced salt solution
HEPES	N-[-2-hydroxyethyl] piperazine-N'-[-2-ethanesulfonic acid]
HPLC	High performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography with diode-array detection
HPTLC	High-performance thin-layer chromatography
IDF	International diabetes federation
IR	Insulin resistance
IRS	Insulin receptor substrate
LC-MS	Liquid chromatography mass spectrometry
LY	Lucifer yellow
MCT	Monocarboxylic transporters
MDR-1	Multi-drug resistance-1
Na+/H+	Sodium-hydrogen
Na+/K+	Sodium-potassium pump
NEAA	Non-essential amino acids
PA	Pyrrolizidine alkaloids
PC	Polycarbonate membrane

p-CA	*Para*-coumaric acid
PCA	Protocatechuic acid
Pen-strep	Penicillin streptomycin
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate poly-acrylamide gel electrophoresis
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBST	TRIS-buffered saline containing Tween®20 detergent
TEER	Trans epithelial electrical resistance
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
WHO	World health organization

CHAPTER 1

Introduction

1. Introduction

There is a growing interest in the use of indigenous plants to treat various ailments either as adjunctive therapies or as substitutes for treatment (Gelderblom *et al*., 2016; Joubert and De Beer, 2011; Van Wyk and Gericke, 2000). The popularity in the use of these plants are mainly attributed to the belief that natural products have less side effects and are perceived to be as effective as current pharmaceutical therapeutics (Bahadoran *et al*., 2013). In South Africa, *Aspalathus linearis* (rooibos) and *Cyclopia spp*. (honeybush) have received a lot of attention for their health promoting effects. *Athrixia phylicoides* (DC), a lesser known endemic herbal specie, belongs to the family Asteraceae (daisy family) and is commonly known as bush tea or Zulu tea. It is an aromatic leafy shrub growing in mountainous and grassland areas of the northern and eastern parts of South Africa (Van Wyk and Gericke, 2000). In general, many health benefits of herbal plants are attributed to their phenolic constituents (Niwa and Miyachi, 1986). Several studies have demonstrated beneficial effects of *A. phylicoides in vitro,* including high anti-oxidative activity (similar to that of rooibos tea) and the ability to modulate glucose metabolism in muscle, liver and fat cells (Chellan *et al*., 2012; McGaw *et al*., 2007). This indicates that *A. phylicoides* may well have potential anti-diabetic benefits.

Surveys of rural and urban subjects in northern parts of South Africa revealed the use of the plant by traditional health practitioners as an active herb for: the treatment of boils, sore throat, as an anthelmintic, coughing, sore feet and hypertension. Its consumption as a tea by indigenous communities has also been reported (Rampedi and Olivier, 2005). It is envisioned that due to its health promoting effects, *A. phylicoides* may follow a similar path to rooibos and honeybush as an everyday healthy beverage known to be anti-oxidant-rich and caffeine-free (De Beer *et al*., 2011; Mavundza, 2010). However, for the development of a health product, the bioactive constituents need to be identified and their mode of action elucidated. Furthermore, scientific verification of the efficacy and safety are crucial. In addition, there is limited knowledge about the extent to which its active phenolic compounds are bioavailable thus limiting its use as a nutraceutical.

In an effort to better understand the extent and mechanisms whereby these phenolic compounds are absorbed, this study aimed to fully characterized the prominent phenolic compounds present in an aqueous extract of *A. phylicoides* and using a Caco-2 cell model, investigated the potential absorption of the major phenolic compounds present in an *A. phylicoides* aqueous extract using a fully differentiated Caco-2 cell model.

CHAPTER 2

Literature Review

2. Literature review

2.1. Diabetes Mellitus

Glucose is used by all cells in the body to produce energy in the form of adenosine triphosphate (ATP) (Van der Heiden *et al*., 2009) through a metabolic process called glycolysis which entails glucose breakdown into pyruvate and further into ATP by enzymatic reactions in the mitochondria (Van der Heiden *et al*., 2009). The inability of cells to respond adequately to insulin, type 2 diabetes (T2D), or the lack of insulin, type 1 diabetes (T1D), results in diabetes mellitus (DM), a metabolic disease that is clinically characterized by chronic hyperglycemia resulting from disturbances in carbohydrate, fat and protein metabolism due to defects in insulin signaling and secretion (American Diabetes Association, 2010; Noriega-Cisneros *et al*., 2012). Gestational diabetes is another type of diabetes that is diagnosed only during pregnancy (Bellamy *et al*., 2009). The 2040 global projection of T2D incidence is estimated at 439 million adults, with sub-Saharan Africa having 18.6 million adults with T2D by 2040 (IDF, 2015; Shaw *et al*., 2010; Wild *et al*., 2004). It has been reported that in South Africa T2D is the seventh most common cause of death (Bradshaw *et al*., 2007). Added to this, it has been shown that a total number of 1.97 million cases of people with T2D exist in South Africa (Bertram *et al*., 2013). In addition, it has also been estimated that 1 million people in South Africa are living with undiagnosed T2D (Bertram *et al*., 2013) and this is expected to have increased to 1.4 million by year 2040 (IDF, 2015; Shaw *et al*., 2010). Increasing cases of T2D places an enormous burden on South Africa's health resources and affects the socio-economic development of the country (IDF, 2015).

2.1.1. Insulin resistance

Insulin resistance (IR), is a defect through which insulin is unable to adequately achieve its physiological effects such as the stimulation of glucose uptake in the muscle and adipose tissues and inhibition of hepatic glucose output (Muoio and Newgard, 2008). IR is usually a underlying causal factor associated with impaired glucose tolerance as well as obesity and is known to be the major risk factor for the development T2D (Kahn *et al*., 2006; Kahn, 2003; Stumvoll *et al*., 2005). There are various factors that influence insulin sensitivity of tissues and these include: hereditary predisposition, metabolic defects, age,

obesity, sedentary lifestyle and diet (Roberts *et al*., 2013). The latter two factors are the leading modifiable contributing factors (Bahadoran *et al*., 2013; Kahn, 2003; Kahn *et al*., 1990; Stumvoll *et al*., 2005) .

2.1.2. Management and Treatment of Diabetes Mellitus

Worldwide, healthcare systems are struggling with the management and treatment of DM. According to the American Diabetes Association (ADA) (2010) and Boulton *et al*., (2005), reducing the over-consumption of energy rich foods, particularly those high in fats and sugars, as well as regular exercise are helpful in maintaining normal blood glucose levels. Interventions have been developed which aim at improving glycemic control and preventing complications resulting from diabetes, which include natural health products and functional foods (Bahadoran *et al*., 2013). As a treatment strategy, T1D patients depend upon lifelong insulin injection (Boulton *et al*., 2005). Individuals suffering from T2D generally require oral anti-diabetic therapies, diet and lifestyle interventions with regular monitoring of their blood glucose levels (Bahadoran *et al*., 2013).

Various drugs have been used for the treatment of T2D including biguanides, such as metformin (a commonly used first line drug), sulfonylureas, thiazolidinedione (TZDs), dipeptidyl peptidase IV inhibitors, glucagon-like peptidase-1 and alpha-glucosidase inhibitors (Table 1), as well as combinations of the various drugs. However, even though these drugs are effective they have side effects such as increased risk of lactic acidosis, hypoglycemia, weight gain, gastrointestinal effects, diarrhea and flatulence (Table 1) (Chiang *et al*., 2014; Chiasson *et al*., 2002; Irons and Minze, 2014; Lipska *et al*., 2011; Kunte *et al*., 2007; Zimmerman, 1999). The use of phytochemical formulations, particularly those containing bioactive phenolic compounds as supplements and as adjuncts to conventional anti-diabetic therapies is increasing (Bahadoran *et al*., 2013; Semaming *et al*., 2016).

A number of indigenous plants have been scientifically investigated and reported to possess anti-diabetic activity (Auddy *et al*., 2003; Bahadoran *et al*., 2013; Joubert *et al*., 2008; Hutchings *et al*., 1996) and of particular interest in this study is the South African indigenous plant *Athrixia phylicoides (A. phylicoides).* Phenolic compounds from indigenous plants have been documented to have various bioactivities, thus encouraging

their direct therapeutic use in allopathic medicine (Lehlohonolo *et al*., 2013). The medicinal applications of indigenous plants are attributed to specialized compounds derived through secondary plant metabolism, including flavonoids, terpenes, phenolic acids, alkaloids and amines (Mavundza, 2010; Nchabeleng *et al*., 2012).

Table 1: Anti-diabetic agents commonly used in the management of T2D

Class	Example	Mode of action	Target	Adverse effect (s)	Reference
Biguanides	Metformin	Decrease hepatic glucose outputs Promotes muscle glucose uptake Suppresses lipogenesis	AMP-kinase	GI upset Lactic acidosis	(Lipska *et al*., 2011; Zimmerman, 1999)
Sulfonylureas	Glipizide	Increase insulin secretion Promote fatty acid uptake	ATP-sensitive Potassium channels	Hypoglycemia Weight loss	(Amoroso *et al*., 1990; Bowker *et al*., 2006; Irons and Minze, 2014)
Dipeptidyl peptidase IV inhibitors	Sitagliptin	Inhibit glucagon release Increases insulin secretion Decreases blood glucose levels	DDP- IV inhibitor	Hypoglycemia Nasopharyngitis Headache Angioedema	(Deacon, 2016)
Glucagon-like peptidase-1	Albiglutide	Increases insulin secretion Inhibiting glucagon release	GLP-1 agonist	Headaches Nausea Increased sweating Constipation	(García-Compeán *et al*., 2015; Mor *et al*., 2015)
TZDs	Troglitazone	Increasing insulin sensitivity Increase glycemic control	PPAR-γ	Weight gain Edema Increased bladder cancer	(Chiasson *et al*., 2002; Irons and Minze, 2014)
Alpha-glucosidase inhibitors	Miglitol	Decrease carbohydrate absorption	Alpha-glucosidase	GI side effects	(Irons and Minze, 2014)

2.2. Overview of *Athrixia Phylicoides*

In Southern Africa, several plants have traditionally been used to manage and treat various ailments and diseases. Popular indigenous plants include the likes of *Aspalathus linearis* which is generally known as 'rooibos', various *Cyclopia* species known as 'honeybush' as well as *A. phylicoides* 'bush tea' (Gelderblom *et al*., 2016; Joubert *et al*., 2008). *A. phylicoides* forms the main focus of this study. The genus *Athrixia* contains 14 species found occurring in Southern Africa and Madagascar with 9 species including *A. phylicoides* endemic to South Africa (McGaw *et al*., 2007).

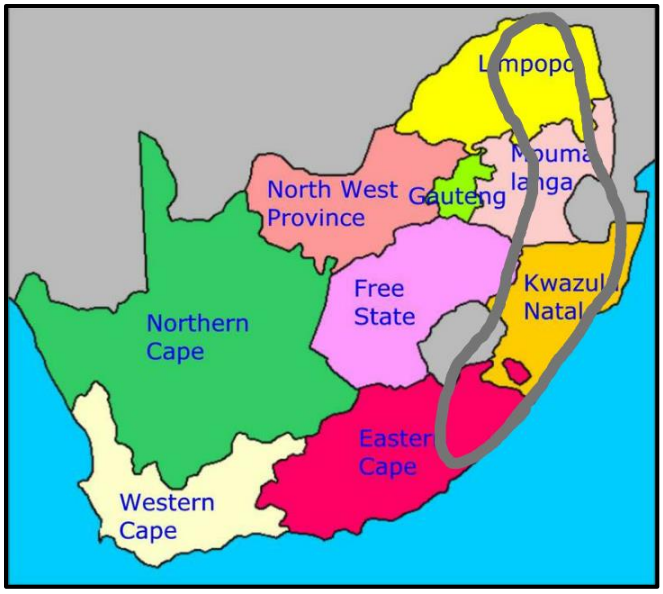


Figure 1: Geographical distribution of *A.phylicoides* Available at www.southafrica.to [Accessed 12 October 2015]. *A. phylicoides* is a South African plant that is widely distributed in the eastern part of Southern Africa running from northern parts of the Eastern Cape along the coast right up to sections of Limpopo (the areas circled in grey). The plant occurs commonly in grassland and forest margin scrub areas and has both medicinal and non-medicinal uses (McGaw *et al*., 2007).

2.2.1. Description, Distribution, Harvesting and Cultivation

A. phylicoides (DC), family *Asteraceae*, tribe Inucleae and sub-tribe *Athrixiinae* is an aromatic, leafy shrub with woolly stems that is perennial and usually reaches 1m in height (Fox *et al*., 1982; Nchabeleng *et al*., 2012). It is a South African plant with wide distribution in certain eastern parts of Mpumalanga, northern parts of the Eastern Cape, Limpopo and

KwaZulu-Natal province (Figure 1). The plant occurs frequently in grassland and forest margin scrub areas (Germishuizen, 2006).

Figure 2: Image of *Athrixia phylicoides* taken from (Pooley, 1988). *A. phylicoides* is indigenously referred as bush tea (English), bostee (Afrikaans) umtshanelo (Zulu), icholocholo (Xhosa), sephomolo (Sotho) and luphephetse (Swazi)(Mavundza, 2010; Padayachee, 2011). *A. phylicoides* flowers are daisy-like with pink–purple petals with bright yellow centres occurring throughout the year depending on the geographical area (Van Wyk and Gericke, 2000). The leaves are dark-green and appear to be shiny.

2.2.2. Indigenous uses

The aerial part and stems of the plant are used to produce a herbal tea by many indigenous groups. The Sotho and Xhosa people chew the plant in order to cure sore throats and coughs (Van Wyk and Gericke, 2000; Watt and Breyer-Brandwijk, 1932). It has been reported that the Venda people use a brew of *A. phylicoides* as an aphrodisiac (Hutchings *et al*., 1996; Van Wyk and Gericke, 2000), while the Zulu people consume herbal tea infusion of the plant as a blood purifier and for treatment of sores and boils (Hutchings *et al*., 1996; Rampedi and Olivier, 2005). Studies revealed that South African rural communities used *A. phylicoides* to treat hypertension, diabetes and heart disease (McGaw *et al*., 2013; Rampedi and Olivier, 2005) (Table 2). Scientific investigation is required to establish the proposed medicinal and therapeutic value of this plant. Categorization of health benefits of this tea, and commercialization thereof, holds developmental, economic and therapeutic potential for local communities.

Table 2: Traditional uses of *Athrixia phylicoides* by various ethnic groups (modified from Padayachee, 2011)

Traditional indication	Ethnic group(s)	Plant part used	Form of use	Source of Literature
Tea	Zulu, Xhosa Vhavenda	Leaves	Orally Ingested	(Hutchings *et al*., 1996; Mavundza, 2010)
Sore throat	Sotho Xhosa	Leaves	Gargle	(Hutchings *et al*., 1996; Mudau et *al*., 2007)
Anthelmintic	Vhavenda	Leaves Roots	Orally Ingested	(Lehlohonolo *et al*., 2013; Hutchings *et al*., 1996; McGaw *et al*., 2007)
Coughing	Sotho and Xhosa	Leaves	Orally Ingested, Chewed	(Hutchings *et al*., 1996; Nchabeleng et al., 2012)
Sore feet	Sotho	Leaves	Wash with, Soak with	(Hutchings *et al*., 1996; Lehlohonolo *et al*., 2013; Watt and Breyer-Brandwijk, 1932)
Treating boils	Zulu	Leaves	Apply on Boils	(Mudau *et al*., 2007)
Infected wounds	Zulu	Leaves and Roots	Apply on wounds	(Mudau *et al*., 2007; Nchabeleng et al., 2012)
Hypertension	Xhosa	Leaves and Roots	Orally ingested	(McGaw *et al*., 2007)
Skin problem rectifier	All communities	Leaves	Apply directly on affected area	(Hutchings *et al*., 1996; McGaw *et al*., 2007)
Diarrhea	Zulu	Leaves	Orally ingested	(McGaw *et al*., 2007)

2.2.3. Toxicity screening

Pyrrolizidine alkaloids (PA) are a well-recognized and relatively common group of plant toxins that occur in up to 3% of flowering plant species, including the Asteraceae family, and have numerous adverse health implications (Smith and Culvenor, 1981). In 2007, McGaw *et al*., used spectrophotometric and gas chromatography-mass spectrometry analysis to show that an aqueous extract of *A. phylicoides* does not contain any PA (McGaw *et al*., 2007). Cytotoxicity screening, using kidney cell lines and brine shrimp toxicity assays, showed that while the ethanol extract was relatively toxic**,** the aqueous extract was not (McGaw *et al*., 2007). The lack of toxic effects of the aqueous extract of *A. phylicoides* was confirmed by Chellan *et al*., both *in vitro* and *in vivo*, and the same extract was used in this study (Chellan *et al*., 2012; Chellan, 2011; Chellan *et al*., 2008).

2.2.4. Health Benefits of *Athrixia Phylicoides*

The Zulu and Khoisan people traditionally use dried twigs and leaves of *A. phylicoides* to produce both herbal tea and medicinal decoctions (Lehlohonolo *et al*., 2013;) (Table 2). The plant is taken either orally, for example for gargling when one has sore throat, or applied on infected wounds. Recent surveys conducted revealed that *A. phylicoides* used as a medicinal plant by African people, in rural and urban areas is often restricted to the consumption of *A. phylicoides* mainly as tea with no associated medicinal purposes (McGaw *et al*., 2007: Table 2). Scientific validation of the biological activities of *A. phylicoides* such as having anti-diabetic, anti-microbial and anti-inflammatory properties (Table 3) have been explored by various researchers in order to encourage the use of the plant as a health product (Lehlohonolo *et al*., 2013).

An aqueous extract of *A. phylicoides*, rich in phenolic acids, has previously displayed anti-diabetic potential *in vitro* by enhancing glucose uptake and metabolism in a matter mimetic to insulin in C2C12, Chang and 3T3-L1 cells (Chellan, 2011). The major phenolic acids, such as chlorogenic acid and caffeic acid present in an aqueous extract of *A. phylicoides* show similar metabolic benefits by preventing inflammation and bacterial infection when tested in *Staphylococcus aureus* (ATCC 25923) and chloroquine-resistant

Plasmodium falciparum (FCR-3) (Padayachee, 2011). The biological activities of *A. phylicoides* have been attributed to its phenolic composition (Auddy *et al*., 2003) and of particular interest are compounds such as *para*-coumaric acid (*p*-CA), caffeic acid (CA), protocatechuic acid (PCA) and chlorogenic acid (ChA) as they are thought to be the most bioactive compounds (Sato *et al*., 2011; Semaming *et al*., 2016; Torres, 2001).

Table 3: Biological activities of *A. phylicoides in vitro* and *in vivo*

Biological activity	Mechanism	Model used	Reference
Anti-diabetic activity	*In vitro* glucose uptake and metabolism	C2C12 (CRL-1772) Chang (CCL-13) 3T3-L-1 (CL-173)	(Chellan *et al*., 2012)
Anti-microbial activity	Bioautography Microdilution method	*Escherichia coli* (ATCC 25922) *Enterococcus faecalis* (ATCC 29212) *Staphylococcus aureus* (ATCC 25923)	(McGaw *et al*., 2007; Padayachee, 2011)
Anti-oxidant activity	DPPH$^{\cdot}$	*In vitro* spectrophotometrically *In vitro* by TLC	(Mavundza, 2010; McGaw *et al*., 2007)
Anti-malarial activity	Titrated hypoxanthine incorporated assay	Chloroquine-resistant *Plasmodium falciparum* (FCR-3)	(Padayachee, 2011)
Anti-inflammatory activity	5-Lipoxygenase assay	*In vitro* spectrophotometrically	(Padayachee, 2011)

2.3. Phytochemical Composition and biological activities

2.3.1. Phenolic composition

Phenolic composition of an aqueous extract of *A. phylicoides* has been well characterized by De Beer and co-workers using HPLC-DAD and LC-Mass spectrometry (De Beer *et al*., 2011). The composition of the aqueous extract of *A. phylicoides* includes several phenolic acids, flavonoids and flavanols such as chlorogenic acid, 1,3-dicaffeoylquinic acid, 5-hydroxy-6,7,8,3`,4`,5`-hexamethoxyflavon-3-ol and several hydroxycinnamic acid derivatives (Figure 3) (De Beer *et al*., 2011). Counter current chromatography in conjunction with liquid-liquid partitioning and semi-preparative reversed phase HPLC-DAD also detected 6-hydroxyluteolin-7-*O*-β-glucoside and quercetagetin-7-*O*-β-glucosidase (De Beer *et al*., 2011). In addition, Chellan and co-workers used high performance thin layer chromatography mass spectrometry (HPTLC/LC-MS) to identify phenolic compounds in a hot water extract of *A. phylicoides* (Chellan *et al*., 2012). There were quantitative differences when compared to the data from (De Beer *et al*., 2011). More so, Mashimbye and co-workers identified 3-*O*-dimethydigicitrin, quercetin and 5-hydroxy-6,7,8,3`,4`,5`-hexamethoxyflavon-3-ol, which was also identified by (Chellan *et al*., 2012; Mashimbye *et al*., 2015). Figure 3 shows chemical structures of some of the chemical compounds identified in *A. phylicoides* from the earlier-mentioned studies.

In order to better understand how phenolic compounds elicit their biological activities, it is important to have a deeper knowledge of the degree to which these phenolic compounds are absorbed and delivered to their target tissues (Joubert and Schultz, 2012).

2.3.2. *Para*-coumaric acid (*p*-CA)

p-CA, a hydroxycinnamic acid is found in abundance in pineapples and has wide distribution in other fruits and vegetables such as grapes, tomatoes and spinach (Alamed *et al*., 2009; Scalbert and Williamson, 2000). It shares structural similarity to caffeic acid (CA) and protocatechuic acid (PCA) (Choi *et al*., 2010; Wojdylo *et al*., 2007), and has

hypoglycemic effects and anti-diabetic activity (Torres, 2001). Studies have indicated that *p*-CA attenuates the oxidative stress associated with diabetes (Kusirisin *et al*., 2009). It has been reported that administration of *p*-CA to diabetic rats helped to improve their glycemic status by significantly increasing plasma insulin levels (Jung *et al*., 2007; Jung *et al*., 2006; Kusirisin *et al*., 2009). In addition, Shairibha *et al*. demonstrated that *p*-CA also significantly lowered the plasma glucose levels in streptozotocin (STZ) induced diabetic rats (Shairibha *et al*., 2014).

2.3.3. Caffeic acid (CA)

CA, frequently found in fruits and grains with known anti-diabetic properties, is another major phenolic compound present in an aqueous extract of *A. phylicoides*. Administration of CA in *db/db* mice resulted in significantly decreased fasting blood glucose levels (Hsu and Chung, 2000; Jung *et al*., 2006). In addition, Sato *et al*. showed CA exhibited protective effects against ischemia reperfusion injury in the rat small intestine (Sato *et al*., 2011). Amelioration of ischemic reperfusion injury as well as anti-oxidant, anti-thrombosis, anti-hypertension, anti-fibrosis, anti-virus and anti-tumor properties of CA have also been investigated (Chao *et al*., 2009; Jiang *et al*., 2005; Touaibia *et al*., 2011). CA has been reported to efficiently improve hypercholesterolemia and T2D (Choi *et al*., 2010; Kim *et al*., 1993).

2.3.4. Protocatechuic acid (PCA)

PCA has structural similarities to CA, *p*-CA, vanillic acid and ferulic acid (Robbins, 2003). This phenolic acid has been detected in various fruits such as plums, gooseberries, grapes and nuts. In addition to its abundance in nuts and fruits, PCA has been detected in *A. phylicoides* (De Beer *et al*., 2011). PCA has been noted to have many biological effects including anti-oxidant, anti-bacterial, hyperlipidemic as well as anti-diabetic activities, amongst others (Choi *et al*., 2010; Liu *et al*., 2002; Scalbert *et al*., 2005; Varì *et al*., 2011). In terms of its anti-diabetic activity, PCA has been proposed to have insulin-like properties through activation of PPAR-γ. The relationship between PPAR-γ activation, adiponection and the upregulation of the GLUT-4 transporter supports the

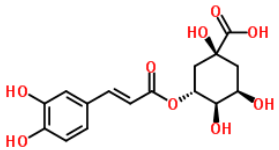
claims about anti-diabetic activity of PCA (Harini and Pugalendi, 2010; Semaming *et al*., 2016; Scazzocchio *et al*., 2011).

2.3.5. Chlorogenic acid (ChA)

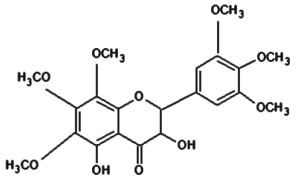
This hydroxycinnamic acid, is one of the major bioactive compounds present in *A. phylicoides* (Chellan *et al*., 2012). It has been reported to be a potent anti-oxidant due to its free radical scavenging activity (De Beer *et al*., 2011; Sato *et al*., 2011). Intestinal microflora hydrolyze ChA into its aromatic acid metabolites CA and quinic acid (Gonthier *et al*., 2003). ChA together with its derivative CA has been reported to efficiently improve hypercholesterolemia and T2D (Choi *et al*., 2010; Kim *et al*., 1993).

2.3.6. Quercetin

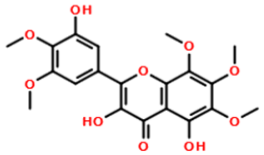
Quercetin is a flavonol that forms part of a subclass of flavonoids. This flavanol is abundantly found in fruits and vegetables and has been isolated previously from the aerial parts of *Hypericum hyssopifolium* (Cakir *et al*., 2003), the leaves of *Castanea crenata* (Haung *et al*., 1999) and the flowers of *Campsis radicans* (Cai *et al*., 2004). In addition to this, quercetin forms part of the compounds that have been found present in an aqueous extract of *A. phylicoides* (Chellan *et al*., 2012; De Beer *et al*., 2011; Mashimbye *et al*., 2015). Quercetin has been documented to possess biological activities such as protecting against several degenerative diseases such as cancer, atherosclerosis and chronic inflammation through preventing lipid peroxidation (Hollman *et al*., 1997; Murota and Terao, 2003).



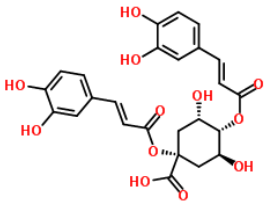
chlorogenic acid



5-hydroxy-6,7,8,3`,4`,5`-hexamethoxyflavon-3-ol



3-*O*-dimethyldigicitrin



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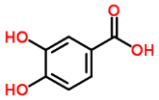
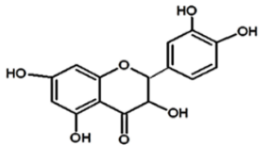
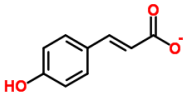


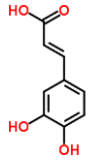
protocatechuic acid



quercetin



para-coumaric acid



caffeic acid

Figure 3: Chemical Structures of some of the phenolic compounds found in *A. phylicoides*
Structures retrieved from http://www.chemspider.com/chemical structure.23327294.html [Accessed 3 August 2016].

2.4. Absorption, Distribution, Metabolism and Excretion (ADME)

Intestinal absorption is the movement of molecules across the gastrointestinal tract (GIT) into the hepatic system. During the process of absorption, nutrients undergo various steps that enable passage through channels in the intestinal wall and into the bloodstream (Wardlaw and Kessel, 2002). Fundamental to the efficacy of any compound *in vivo*, is the degree to which compounds are absorbed and become bioavailable in target responsive tissues in the body (Ting *et al*., 2014).

Absorption of compounds is known to primarily occur in the small intestinal lumen by either one of four absorption mechanisms i.e. active transport, facilitated diffusion, passive diffusion as well as endocytosis (Mohn and Johnson, 2015). Rapid absorption into the plasma has been reported for some polyphenolic compounds such as quercetin and various other flavonols, but the absorption of phenolic compounds is dependent on factors such as molecular size, configuration, lipophilicity and solubility. These factors are determinants of the ability of the compound to cross the lining of the gut wall (Bahadoran *et al*., 2013, Hollman, 2004; Manach *et al*., 2004). After reaching their target tissues, polyphenols need to be reabsorbed from the blood in order to elicit their biological properties (Nijveldt *et al*., 2001; Niwa and Miyachi, 1986; Semaming *et al*., 2016). GI transporters can be grouped into two major classes. Solute carriers (SLC) and ATP-binding cassette (ABC) transporters are known to play a major role in the disposition and removal of drugs/compounds (Rao and Sankar, 2009). The ABC transporters, P-gp, MDR-1 and BCRP, are all expressed in the brush border membrane of intestinal cells where they have an important role as gatekeeper in the gut, limiting the oral bioavailability of many drug substrates (Xia *et al*., 2007). The expression of MDR1/P-gp varies over the total length of the GIT, gradually increasing from the stomach and duodenum with the highest levels in the colon (Murakami and Takano, 2008). 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 (Dobson and Kell, 2008). In particular, P-gp is an important member of the ATP-dependent membrane transporter which is expressed in various tissues such as brain, liver, kidney and intestinal epithelial cells (Rao and Sankar, 2009). P-gp is a well-

recognized efflux transporter, which has been reported to have influential activity on the absorption of several drugs due to its widely reactive spectrum (Dobson and Kell, 2008). In addition to this, P-gp is known to mediate the export of drugs from cells located in the small intestine, blood-brain barrier, liver cells and kidney proximal tubule, thus it serves as protective tool for the body against foreign substances (Wessler *et al*., 2013). Absorption is not only influenced by P-gp as the BCRP is also known to be capable of extrusion of glucuronides and sulfate conjugates formed during phase II metabolism in the enterocytes.

Estimations of the absorbability of polyphenolic compounds is not readily predictable due to various factors that affect the absorption, such as pH, regional permeability and mucosal enzymes (Charman *et al*., 1997, Lipinski *et al*., 2012, Martinez and Amidon, 2002). Changes in pH drastically affect the solubility as well as the dissolution of the compounds (Friedman and Jürgens, 2000). In addition, the rate of gastric emptying time is yet another important factor that stands as a limiting factor of the rate of absorption (Deshpande *et al*., 1996). To elucidate the role dietary phenolic compounds have in human health, it is vital to know the concentrations and the forms that are present in plasma after digestion (Hollman, 2004). The degree of hydroxylation of phenolic compounds is a determinant of their degradation tendency in the colon (Karakaya, 2004; Karakaya, 1999).

Compounds efficiently elicit their bioactivities after being successfully absorbed, and distributed into their respective compartments via the bloodstream (Illum, 2010). This distribution entails transferring the compound from one effector compartment to another (Pallasch, 1988). Thereafter, the compound may be dispensed into surrounding organs, usually to differing extents. The process of distribution is known to mostly result in lowering the plasma concentration of the compounds which in turn may hinder the effectiveness of the compound (Pallasch, 1988). There are many factors that affect compound distribution such as, molecular size of the absorbed compound, polarity as well as binding ability to serum proteins (Van De Waterbeemd, 2003).

After distribution, the compounds undergo a series of catabolic steps into precursor molecules (Scalbert and Williamson, 2000). The metabolism of phenolic compounds

occurs extensively both in intestinal tissues, through colonic microbiota, as well as in the liver (Landete, 2012; Scalbert and Williamson, 2000). Upon absorption into the GIT, dietary polyphenols become substrates for several hydrolyzing and conjugating enzymes in the small intestine, colon and liver (Rechner *et al*. 2004). Lactase phloridzin hydrolase, found in the brush border of the small intestines, plays an important role during absorption of some phenolic compounds such as quercetin glucosides as it has been shown to facilitate deglycosylation of quercetin-3-*O*-glucoside (Schulze, 2014). Manach *et al*. reported that many, but not all, polyphenols become conjugated to form *O*-glucuronides, sulphate esters and *O*-methyl ethers prior to their metabolism (Manach *et al*., 1998). The first conjugation occurs in the gut barrier and then progresses to the liver where polyphenols are further metabolized (Donovan *et al*., 2001; Landete, 2012). Absorbed ingested substances are subjected to both phase I and phase II metabolism. Primarily acting as defensive barriers against foreign substances. Phase I reactions mainly consists of oxidation, reduction and hydrolysis whereas phase II consists of methylation, sulfonation and gluconidation (Dobson and Kell, 2008). The focus of these reactions is to change the physico-chemical structure of the compound thus making it more hydrophilic to facilitate phase II reactions. Phase II reactions are carried out by a group of closely related isozymes known as cytochrome P450-depedent mixed function oxidases (Scheepens *et al*., 2010).

Metabolism of bulk xenobiotics entering the body orally is carried out by CYP1A, CYP2C, CYP2D and CYP3A (Jancova *et al*., 2010) which are the cytochrome (CYP) P450 enzymes playing a major role during various metabolic and biosynthetic processes. CYP3A4 has a broad spectrum and specificity and is important in xenobiotic metabolism. The liver and intestines respectively have 30-70% CYP3A4/5 enzymes (Jancova *et al*., 2010). The metabolites produced from phase I and phase II metabolism tend to be more polar and soluble derivatives of the xenobiotic (Jancova *et al*., 2010). The metabolites formed during phase II metabolism are subsequently distributed out of the liver into the bloodstream where they are carried to the kidneys, and further excreted into the urine. Alternatively, metabolites may be distributed back into the bile and released into the gut lumen (Stalmach *et al*., 2009). Phase II metabolites have two fates; i.e. excretion in the feces or being subject to reabsorption resulting to further metabolism. The liver has a

significant capacity to efficiently remove drugs with high protein binding from the blood circulation. (Zhou, 2008). The uptake of drugs by the liver is frequently followed by Phase I and Phase II biotransformation and efflux of the metabolite(s) into bile (Masereeuw and Russel, 2001; Zhou, 2008). The efflux transporters expressed at the basolateral and apical membrane of hepatocytes have been well renowned to be critical determinants of drug/ compound elimination or excretion (Giacomini *et al*., 2010). Efflux transporters expressed in the apical membrane signify the final step in the vectorial transport of drugs and drug metabolites from blood into bile (Masereeuw and Russel, 2001; Zhou, 2008). Excretion of type I and II cationic drugs across the apical membrane is mediated mostly by MDR1 and P-gp, respectively (Wessler *et al*., 2013).

The bioactivity of compounds found in *A. phylicoides* has been reported to be dependent on the processes involved during the preparation of the tea, GI digestion, absorption into the human body as well as its metabolism (Bahadoran *et al*., 2013). Determination of the bioavailability of the major phenolic compounds found in an aqueous extract of *A. phylicoides* is of great importance to their biological significance in order to gain clear insight into their therapeutic potential (Chellan *et al*., 2012). A number of studies have examined the bioavailability and metabolism of the herbal tea based flavonoids, however, neither the bioavailability nor the metabolism of *A. phylicoides* has yet been investigated.

2.4.1. Absorption of the major phenolic compounds

2.4.1.1. *Para*-coumaric acid

In 2003, Konishi *et al*., showed that the trans-epithelial transport of *p*-CA was pH dependent in Caco-2 cell monolayers (Konishi *et al*., 2003). Lowering the pH in the apical compartment led to increased permeation of *p*-CA over time while permeation of the compound was relatively low in the absence of the proton gradient experiment. Furthermore, *p*-CA is known to be transported via the monocarboxylic transporters (MCT) and this was confirmed when the compound permeability ceased in the presence of the metabolic inhibitor sodium azide (NaN_3) (Konishi *et al*., 2003). MCT 1-4 substrates such as benzoic acid and acetic acid subsequently reduced the permeation of *p*-CA while lactic acid, another substrate had no effect suggesting that the compound`s absorption is mediated by a MCT other than MCT 1-4 (Konishi *et al*., 2004; Konishi *et al*., 2003).

2.4.1.2. Caffeic acid

CA is a 3,4-dihydroxycinnammic acid. The transport of CA has been shown to be mediated by MCT paracellularly (Konishi *et al*., 2004). CA has been shown to be rapidly absorbed by Caco-2 cells in comparison with its esterified precursor, ChA. The net absorption of perfused CA in rats accounted for 19.5% in perfusion effluent, bile and plasma (Lafay *et al*., 2006b). Furthermore, only a small fraction of the CA (ca. 0.5%) that was perfused became metabolized in the intestinal wall and thereafter secreted back into the gut lumen in the form of ferulic acid (Olthof *et al*., 2001). The absorption of CA was determined to be 95 ± 4% in the small intestines of the subjects in the study by Olthof *et al*., 2001.

2.4.1.3. Protocatechuic acid

PCA, chemically known as 3,4-dihydroxybenzoic acid, is a phenolic acid belonging to the group dihydroxybenzoic acids (Kakkar and Bais, 2014). It is a major metabolite found in green tea with anti-oxidant properties (Archivio *et al*., 2007). However, there is sparse information available on its absorption.

2.4.1.4. Chlorogenic acid

Bioavailability of ChA has been reported to be poor in humans. In humans who have ingested coffee, known to contain high levels of caffeoylquinic acid and pure ChA, it was discovered that intact ChA recovery was rather low when urine samples were analysed (Lafay *et al*., 2006a; Olthof *et al*., 2001). Upon reaching the colon, ChA becomes de-esterified by microbial enzymes into its metabolites, CA and quinic acid (Plumb *et al*., 1999). The release of both CA and quinic acid resulting in their absorption in the colon prior to their circulation in the blood and metabolism in the liver and kidney and subsequent excretion via urine (Olthof *et al*., 2003). A study by Olthof *et al*. demonstrated that only one third of the ChA in hot water consumed was absorbed in the small intestine of ileostomy subjects (Olthof *et al*., 2001). Another study, in subjects with an intact colon, demonstrated that from the total ingested ChA only about 1.7% was recovered in the urine, mainly as ChA metabolites (Gonthier *et al*., 2003; Olthof *et al*., 2003). Metabolism of CA mainly by the liver includes not only methylation but also sulphation and glucuronidation (Nardini *et al*., 2002). The bioavailability of ChA has been demonstrated to be affected greatly by microbial degradation and absorption in the colon (Olthof *et al*., 2003).

2.4.1.5. Quercetin

Rapid absorption into plasma has been reported for quercetin and various other flavonols (Bahadoran *et al.*, 2013; Hollman, 2004; Manach *et al*., 2004). In 1997, Hollman reported that the absorption of quercetin aglycones in human was relatively poor (~1%). Orally administered quercetin aglycone in rats was however reported to be 20% (Hollman, 1997; Gugler *et al.,* 1995; Karakaya, 2004). Hollman *et al*. investigated the pharmacokinetics as well as the bioavailability of various forms of quercetin in 9 volunteering healthy individuals. Results from their investigation revealed that quercetin originating from onions had a rapid absorption reaching its peak maximum at about 0.9 hours after ingestion (Hollman *et al*., 1997). In contrast, quercetin 3-rutinoside (found abundant in tea) and quercetin-β-glucoside and β-xyloside (found in apples) had delayed and intermediate absorption respectively, reaching peak maximum at 9 hours and 2.5 hours respectively.

2.4.2. Metabolism of some of the phenolic compounds found in *A. phylicoides*

Using Caco-2 cells, de-esterification, glucuronidation, sulphation and methylation of ChA was observed indicating that the human small intestinal epithelium may contribute to the metabolism of ChA (Gonthier *et al*., 2003). ChA epithelial transport occurs paracellularly whereas CA transport occurs at least partially by the MCT (Konishi and Kobayashi, 2004).

An investigation into the metabolism of ChA and CA, using rat's small intestines, (Lafay *et al*., 2006a) showed that perfusion of ChA into the upper intestinal tract directed the recovery of both ChA and CA in the emission. However, when CA was perfused into the upper intestinal tract, CA and ferulic acids were recovered in the emission (Lafay *et al*., 2006a). CA is a phenolic acid produced during ChA de-esterification. These phenolic acids have a rapid absorption and a portion of them are rapidly methylated into ferulic acid and isoferulic acid via a reaction carried out by catechol-*O*-methyl transferase (Lafay *et al*., 2006a; Lafay *et al*., 2006b). Thus, part of ChA could be absorbed from the stomach without modification while another part could be absorbed from the small intestine, hydrolysed by the enterocytes prior to reaching the circulation.

PCA mainly undergoes phase II metabolism such as methylation, glucuronidation, and sulfonation (Tarko *et al*., 2013). The enzymatic degradation of PCA is known to be carried out by protocatechuate decarboxylase which metabolizes the 3, 4-dihydroxybenzoate to produce catechol and carbon dioxide (Pietta et al., 1998a; Pietta et al., 1998b).

p-CA is biosynthesized from cinnamic acid through an action of the P450-dependent enzyme 4-cinnamic acid hydroxylase (Oksana *et al*., 2012). The metabolism of *p*-CA has been reported by Zhang *et al*. (2007). *p*-CA was detected in plasma but not in GIT extracts or feces of rats. The consistent absorption pharmacokinetic curve at different time points showed a prolonged elimination phase with detection of *p*-CA in the kidneys as well as excretion in its unmodified form at 12 and 24 hours, respectively (Zhang *et al*., 2007).

2.5. Caco-2 cell model

Intestinal absorption of drugs/compounds for oral dosing has been well studied using the Caco-2 cell model (Artursson, 1990). The Caco-2 cell line is derived from adenoma colorectal carcinoma cells (Table 3). These cells are known to function similarly to the human intestinal epithelium (Hochman *et al*., 2000; Prime-Chapman *et al*., 2004).

In culture, Caco-2 cells readily form a differentiated monolayer with a distinct brush border on the apical surface and intercellular tight-junctions (Hochman *et al*., 2000) (Figure 4). Traditionally, when fully differentiated, Caco-2 monolayers are viewed as a single barrier rather than a polarized cell monolayer which consist of various metabolic enzymes with various transporters (Sun *et al*., 2008). Differentiated Caco-2 cells exhibit several functions of normal enterocytes and express various transport and efflux proteins, some P450 cytochrome (CYP) isoenzymes as well as phase II metabolizing enzymes (Hochman *et al*., 2000). Thus, these cells present themselves as good candidates to predict human intestinal permeability and to investigate the potential of drug efflux. Most laboratories use the Caco-2 cell line when screening for new chemical`s over prediction of the chemical`s bioavailability as well as the possibility of drug-drug interactions in the gut lumen (Awortwe *et al.*, 2014). The Caco-2 model is further employed in identification of substrates and inhibitors of drug transporters. Caco-2 cells are advantageous over other models employed in absorption studies because they have a transepithelial resistance that is four times that of HT 29 cell monolayers, and they spontaneously differentiate expressing morphological and functional characteristics resembling mature small intestinal enterocytes with various drug metabolizing enzymes such as aminopeptidase, esterase and sulfatase (Sambuy *et al*., 2005; Sarmento *et al*., 2012; Van Breemen and Li, 2005).

However, compared to the other models, Caco-2 cells are known to present an array of disadvantages. They do not contain goblet cells and as such do not produce mucus that is observed *in vivo*, their maintenance is time consuming, as they reach a fully differentiated stage only after 21 days and lastly, for quantitation of transported

compounds, LC-MS and HPLC identification are necessary (Sarmento *et al*., 2012; Van Breemen and Li, 2005). Even though the cells are an excellent model for studying the mechanism of trans-epithelial transport, Caco-2 cells often poorly express phase I and II enzymes (Liu and Hu, 2002). Apparent permeability is monitored through the rate of transport of the compounds from the apical to basolateral direction across a fully differentiated Caco-2 cell monolayer. Assessing bi-directional transport, in the apical to basolateral (AP-BL) and basolateral to apical (BL-AP) directions, across the cell monolayer allows for the determination of an efflux ratio, which subsequently provides an indicator as to whether a compound undergoes efflux or not. An efflux ratio above 2 signifies that the compound is subjected to an active efflux mechanism which in turn results in low bioavailability (Dahan and Amidon, 2008).

Table 4: The summary of the characteristics of Caco-2 cell model

Caco-2 cell characteristics	
Origin	Human colorectal adenocarcinoma
Growth in culture	Monolayer epithelial cells
Differentiated	21 days post seeding
Morphological appearance	Polarized cells containing tight junctions, distinct brush border and desmosomes
Active transport	Amino acids, sugars, vitamins and hormones
Membrane ionic transport	Na^+/K^+ ATPase, H^+/K^+ ATPase, Na^+/H^+ exchange, Apical CL^-channels
Electrical parameter	High electrical resistance
Receptors	Vitamin B12, vitamin D, epidermal growth factors and sugar transporters (GLUT1, GLUT2, GLUT3, GLUT5 and SGLT-1)

Na^+/K^+: Sodium-Potassium Pump; H^+/K^+: Gastric hydrogen potassium, Na+/H+: sodium–hydrogen; CL-channels: Chloride channels; GLUT1: Glucose transporter (1,2,3,5) and SGLT-1: Sodium-dependent glucose transporter-1.

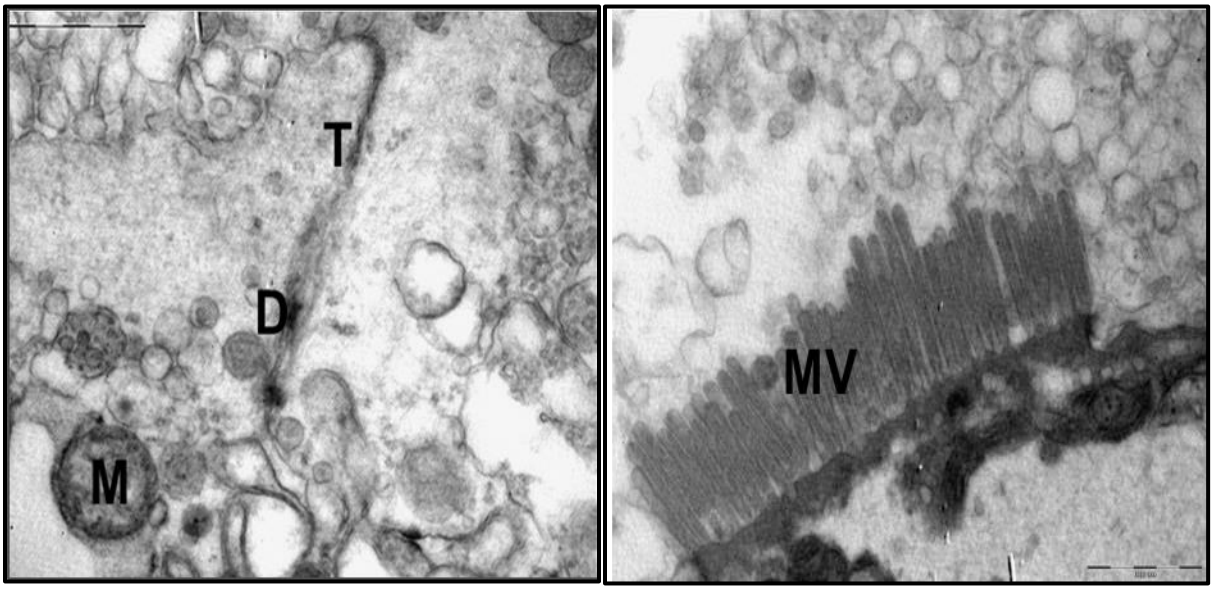


Figure 4: Image representing the microscopic images of fully differentiated Caco-2 cells grown for 25 days at standard tissue culture conditions. T: tight junctions, D: desmosomes, M: mitochondrion, MV: micro villi.

CHAPTER 3

Materials and Methods

3.1. Hypothesis

Bioactive polyphenolic compounds present in an aqueous extract of *A. phylicoides* are bioavailable and are absorbed in the digestive tract.

3.2. Aim of the study

The proposed aim of the study is to evaluate the absorption of phenolic compounds from an aqueous extract of *A. phylicoides* using Caco-2 cells as a model representing human enterocytes.

3.3. Objectives

In order to address the specific aim of the study, we sought to:

- Establish a safe non-cytotoxic concentration of use for an aqueous extract *A. phylicoides* in Caco-2 cells.
- HPLC analysis of apical and basolateral samples from transport experiments to determine concentration, stability, production of metabolites and passage mechanisms of the compounds through the monolayer to better understand what type of transport system is employed as well as relative stability of the compounds.
- Obtain P_{app} values representing the rate of absorption of an aqueous extract of *A. phylicoides* across the intestinal barrier.

3.4. Materials

3.4.1. Source of extract, plant preparation, cell line and chemicals and reagents

All cell culture supplies were purchased from Corning (New York, USA) unless otherwise specified. Caco-2 cells were obtained from the European Collection of Cell Cultures: cat # 86010202 (Salisbury, England). Transwell inserts and 6 well plates were from SPL Life Sciences (Gyeonggi-do, Korea). Verapamil, caffeine and HPLC grade acetonitrile were purchased from Sigma-Aldrich (St Louis, MO, USA). Eagle's Minimum Essential Medium (EMEM), Hank's balanced salt solution (HBSS), pyruvate, non-essential amino acids, L-Glutamine, trypsin-EDTA and Penicillin/Streptomycin was purchased from Lonza (Basel, Switzerland). Hyclone fetal bovine serum (FBS) was purchased from Thermo Scientific (Waltham, MA, USA). RIPA buffer was purchased from Cell Signaling Technology (Danvers, MA, USA); MDR-1(D-11) primary antibody, and anti-mouse IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, INC, USA). All other consumables and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), unless otherwise specified. All other materials, suppliers and product numbers are listed in Appendix A.

An aqueous extract was prepared from fine twigs and leaves of *A. phylicoides* DC that was harvested in June 2005 in the Bushbuckridge area (Limpopo, South Africa) from a natural population. *A. phylicoides* was identified by the South African National Botanical Institute (SANBI) and supplied by Prof Jana Olivier, University of South Africa. The extract was prepared on a pilot-scale and freeze-dried by Dr Dalene De Beer, Nietvoorbij, Agricultural Research Council of South Africa. The extract was prepared by boiling dried fine twigs and leaves as described by Chellan *et al*. (Chellan *et al*., 2012).

3.5. Methods

Tissue culture

3.5.1. Source and storage of cell line

Cryo-vials containing Caco-2, originating from the European Collection of Cultures were cryo-preserved in freshly prepared freezing medium containing cryo-protectant (7% DMSO) in liquid nitrogen.

3.5.2. Thawing of cells

Vials of cryopreserved Caco-2 cells were removed from liquid nitrogen storage and thawed by placing cells in a 37 °C circulating water bath (Memmert; Heilbronn, Germany) until thawed. Immediately after thawing, the cryo-vial was sprayed with 70% ethanol, wiped and placed inside the biological safety cabinet where the cell suspension was slowly transferred from the cryo-vial into 16 mL of pre-warmed complete growth medium, (EMEM containing 100 mM sodium pyruvate, 1% non-essential amino acids (NEAA), 10% fetal bovine serum (FBS), 2 mM L-glutamine) and thereafter gently mixed by pipetting up and down. Cells were incubated under standard conditions (37 °C in 5% carbon dioxide and humidified air). Sub-culturing of cells was kept within 10 passages (between passages 48 and 58), in order to prevent phenotypic drift.

3.5.3. Counting cells

A 20 µL sample of the cell suspension was stained 1:1 with 0.4% Trypan Blue (Cat No: T93595; Sigma, Stanheim, Germany) in phosphate buffered saline (PBS) and cells counted using a hemocytometer (Figure 5). Briefly, 10 µL of the Trypan Blue/cell suspension was pipetted under the coverslip of the cell counting chamber slide of the hemocytometer and viewed under an inverted light microscope (Olympus; Melville, NY, USA) where the number of cells was counted in the four 1 mm^2 grid chambers (Figure 5). The average of the four counts (N) was used to determine the number of cells per mL in the diluted sample (D). The following formula was used:

$$\text{Cells/mL} = \frac{\text{Average count per mm}^2\text{ (1.1 cell suspension and trypan blue solution)}}{\text{Volume counted (1x1x0.1mm= 0.1µL)}}$$

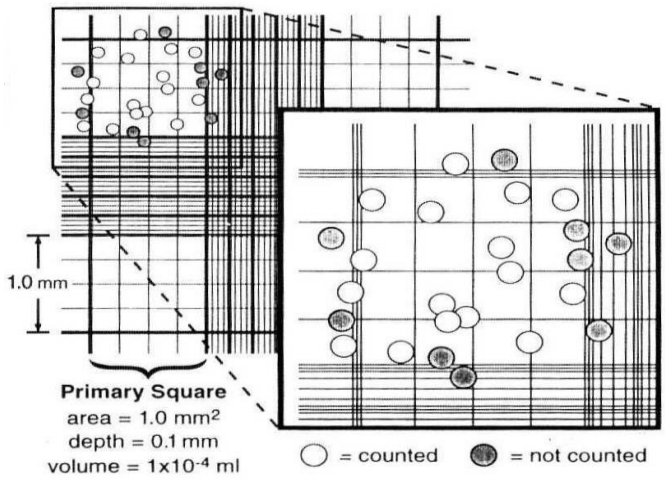


Figure 5. Schematic illustration of a haemocytometer chamber and a 1 mm² grid within the chamber.

3.6.1. Caco-2 cells

3.6.1.1. Sub-culturing of cells

Caco-2 cells were seeded in 75 cm^2 culture flasks at a density of 4×10^4 cells/cm^2 to a total volume of 18 mL in pre-warmed growth media. Cells were then incubated at 37 °C in 5% CO_2 and humidified air for three days until they were approximately 70-80% confluent. Media was refreshed every 2-3 days.

3.6.1.2. Seeding cells

When cells were approximately 70-80% confluent, media was removed by aspiration and cells were rinsed with 5 mL of pre-warmed (37°C) Dulbecco's phosphate buffered saline (DPBS) (Cat No: 17-513, Lonza, MD, USA). Cells were trypsinized by adding 2 mL trypsin (Cat No.: 17-161F Lonza, MD, USA) to the cell culture flask and incubated at 37 °C in 5% CO_2 and humidified air for 4 minutes to allow cells to detach from the flask. Cells were gently agitated by tapping the flask half way through incubation. An inverted light microscope was used to confirm detachment. Thereafter, the trypsinization reaction was stopped by adding 8 mL of complete growth media (containing 10% FBS). The cell suspension was mixed by pipetting up and down

slowly and gently against the growth surface of the flask in order to ensure a single cell suspension and to minimize clumping. The single cell suspension was then transferred into a 50 mL centrifuge tube and the tube was centrifuged at 800 × g (Benchtop centrifuge (SL16R), Thermo Fisher Scientific, Waltham, MA, USA) for 5 minutes. After centrifugation, the supernatant was discarded by aspiration and the pellet was re-suspended with complete growth media. The cells were counted (section 3.5.3) and the density recorded. Cells were routinely seeded at 1:3 – 1:5 split ratio.

3.6.2. Cytotoxicity

3.6.2.1. Seeding into a 96-well plate for cytotoxicity

After trypsinization, a single cell suspension was transferred into a 50 mL centrifuge tube and the tube was centrifuged at 800 × g (Benchtop centrifuge (SL16R), Thermo Fisher Scientific, Waltham, MA, USA) for 5 minutes. After centrifugation, the supernatant was discarded and the pellet was re-suspended with ~3 mL of complete growth medium. The cells were counted as described in section 3.5.3. Cells were seeded at 50 000 cells/mL in a volume of 200 µL per well. The plate was incubated under standard conditions. Media was refreshed every 2-3 days and cells were grown for 12 days.

3.6.2.2. ATP Assay for cell viability determination

The cytotoxic effect of *A. phylicoides* on Caco-2 cells was assessed by the ViaLight™ plus ATP kit (Cat No.: LT27-008 ViaLight, Whitehead Scientific, JHB, SA). Caco-2 cells were seeded at a density of 4×10^4 cells/cm^2 into white clear bottomed 96-well plates, and the medium refreshed every 2-3 days. After 12 days post-seeding, the medium was replaced with transport medium (HBSS with 10mM HEPES, pH 7.4) and incubated for 30 minutes at 37 °C under agitation (75 rpm), in order to wash the cells. The extract was reconstituted in 100% DMSO as a concentrated stock solution. The stock solution was diluted and added to the transport medium at varying concentrations (0.3125, 0.625, 1.25, 2.5 and 5 mg/mL) and corrected to an overall DMSO concentration of 0.125% in each treatment concentration, to establish the highest non-toxic dose. Herbal extract treatments were added, and incubated with the cells for 2 hours at 37 °C, 75 rpm agitation. HBSS without test samples, with the addition of DMSO to a final concentration of 0.125%, was used as a negative control.

After incubation, 50 µL of lysis buffer was added to each well and incubated at room temperature for 10 minutes, cell supernatant was diluted 1:1 with 50 µL DPBS (Cat No.: 17-513, Lonza, MD, USA) before addition to a new 96-well plate. Dilution of ATP in the supernatant was necessary in order for the result to be extrapolated in the format of a standard curve. A total volume of 100 µL of the ATP reaction mixture was added to each well and the plate incubated for further 2 minutes at room temperature. The ATP levels were quantified by luminometric measurement using BioTek ELX 800 plate reader (BioTek Instruments Inc., Winooski, VT, USA) and directly compared to that of the vehicle control to obtain percentage viability of cells. From this, the highest non-toxic dose of extract (>75% cell viability) was obtained and thus used for future experiments. See figure 6 for a representation of a typical plate layout as used in the ATP cytotoxicity assay.

		1	**2**	**3**	**4**	**5**	**6**	**7**
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	N.C	5mg/mL	2.5mg/mL	1.25mg/mL	0.625mg/mL	0.313mg/mL	Blank
C	Blank	N.C	5mg/mL	2.5mg/mL	1.25mg/mL	0.625mg/mL	0.313mg/mL	Blank
D	Blank	N.C	5mg/mL	2.5mg/mL	1.25mg/mL	0.625mg/mL	0.313mg/mL	Blank
E	Blank	N.C	5mg/mL	2.5mg/mL	1.25mg/mL	0.625mg/mL	0.313mg/mL	Blank
F	Blank	N.C	5mg/mL	2.5mg/mL	1.25mg/mL	0.625mg/mL	0.313mg/mL	Blank
G	Blank	N.C	5mg/mL	2.5mg/mL	1.25mg/mL	0.625mg/mL	0.313mg/mL	Blank
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Figure 6: ATP cytotoxicity 96 well- plate layout. N.C is the negative control which was HBSS without the test samples with added DMSO to a final concentration of 0.125%.

3.6.2.3. MTT Assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for cell viability determination of the pure compounds

Twelve days after seeding, cells were treated with increasing concentrations of protocatechuic acid (80 µM), caffeic acid (300 µM) and *para*-coumaric acid (280 µM) for 2 hours. After 2 hours of incubation, treatments were aspirated and cells were washed with pre-warmed DPBS (50 µL/well). Thereafter, MTT solution (Cat No.: M5655; Sigma, Stanheim, Germany) (2 mg/mL) was added at 50 µL per well and cells

incubated for 30 minutes at 37 °C and 5% CO_2. All media were aspirated, followed by the addition of 200 µL 4% phosphate buffered formaldehyde for 12 hours.

3.6.3. Transport assay experiments

3.6.3.1. Seeding cells in 6-well plates for transport assays

For cells seeded into 6-well plates for use in transport experiments, the complete medium was supplemented with 1% penicillin streptomycin (100 mM). The plate was prepared by incubation (37 °C in 5% CO2 and humidified air) with complete medium for 1 hour prior to seeding. After trypsinization, cells were centrifuged 800 × g (Benchtop centrifuge (SL16R), Thermo Fisher Scientific, Waltham, MA, USA), supernatant discarded and the pellet was re-suspended with complete growth media containing penicillin-streptomycin. The cells were counted as described in section 3.5.3. Cells were seeded at a density of 120 000 cells/mL (4000 cell/cm^2) in a volume of 1.5 mL per membrane on the apical side followed by the addition of 2.5 mL of complete media on the basolateral side. The plate with seeded cells was incubated at standard conditions for 5 hours, during this it was important not to disrupt the attaching cells for at least an hour after seeding. After incubation, the media was replaced and any cells that had not yet attached, discarded. Medium was refreshed every 2-3 days. The cell monolayer was grown for 25 days before transport assays were conducted. During this time, the monolayer was monitored for any detachment of cells, change in colour of media or turbidity which may indicate contamination.

3.6.3.2. Electron microscopy (method modified after Kay *et al*. 1967)

Cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at 4 °C for 4 – 6 hours 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 with 1.5% osmium tetroxide in Palade's buffer pH 7.4 for one hour and then rinsed with two changes distilled water to remove excess unbound osmium. Tissue was processed on further Leica EM (Leica Microsystems, Wetzlar, Germany) tissue processor in 2% uranyl acetate in 70% ethyl alcohol and further dehydrated in ascending concentrations of ethyl alcohol (70% for 5 minutes, 2x 96% for 5 minutes each, 3× 100% for 10, 15 and 20 minutes respectively). Following dehydration the tissue was impregnated initially with a 50/50 mixture of Spurr's resin (NSA× 13 mL, ERL 4206× 5 ml, DER × 3 mL and DMAE× 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 polymerize overnight at 60 °C. The tips of the capsules were trimmed with 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 with a glass knife fitted with a water trough. Sections were removed from the trough and placed on a drop of water and the sections are allowed to dry on a hot plate. Once the drop of water had evaporated and the sections adhered to the slide, the resin was then removed from the sections with 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 stained with warm 1% toluidine blue in 1% sodium tetraborate for 1 minute. 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 retrimmed into a small (max 2 ×1 mm) trapezium shape containing the area of interest by 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 a water trough. A ribbon of sections was then picked up, from the water bath, onto a G200 copper grid and allowed to dry on a filter paper. The sections were then stained for 5 minutes 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 minutes. 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.

3.6.3.3. Measuring Transepithelial electrical resistance (TEER)

The transepithelial electrical resistance (TEER) in ohms, was measured using Millicell-ERS volt ohmmeter (Merck KGaA, Darmstadt, Germany) by immersing the electrical

probe into the medium without disturbing the cell monolayer. Thereafter, cell culture inserts were briefly rinsed with calcium treated Hanks buffered saline solution (Ca^{2+}-HBSS) pH 6 which is representative of the pH in the jejunum (HBSS from Lonza # BE10-527F, MES from Sigma #M2933, Calcium chloride, pH adjusted with 1M KOH) and Ca^{2+}-HBSS pH 7.5 which is the serosal pH (HBSS from Lonza # BE10-527F, HEPES from Sigma #4034, Calcium chloride ($CaCl_2$) #C5670, pH set with 1M KOH) at the apical side and basolateral side respectively to remove traces of the culture media. Readings were taken before and after the wash, as well as after the 2-hour incubation with treatment in order to confirm the integrity of the monolayers throughout the transport experiments.

3.6.3.4. Lucifer yellow measurement

Lucifer yellow (LY) is a fluorescent indicator molecule. It was added at the same time as the treatment and monitored fluoremetrically by way of passage from apical to basolateral side of the monolayer. Samples collected at different time intervals during transport experiments were used to quantify LY in order to confirm the integrity of the Caco-2 monolayers during the experiments. Donor solutions (including respective treatments with LY) were prepared. Aliquots of 50 µL were transferred to a solid black plate and the percentage of LY passed through was measured fluoremetrically using Bio-TEK ELX 800 plate reader (BioTek Instruments Inc., Winooski, VT, USA). LY rejection values were calculated as a percentage value.

3.6.3.5. Preparation of treatments

Treatments containing *A. phylicoides* at 5 mg/mL (0.0161 g *A. phylicoides* dissolved in 80 µL of 10% DMSO) and 2.5 mg/mL (0.032 g *A. phylicoides* dissolved in 80 µL of 10% DMSO), negative control (80 µL 10% DMSO) and caffeine (0.0164 g caffeine dissolved in 4 mL 10% DMSO) were prepared and filter sterilized.

3.6.3.6. Transport experiment

Cell monolayers seeded as described in section 3.2.6.1, were grown for 25 days in order to reach a fully differentiated state. Prior to the transport experiment, TEER readings were recorded as described in section 3.2.6.2. The cell monolayers were incubated for 15 minutes at 37 °C under agitation (75 rpm) with transport buffer, pH 6.0 (HBSS with MES) on the apical side and pH 7.4 (HBSS with HEPES) on the basolateral side. If the experiment required the addition of an inhibitor (P-gp inhibitor,

verapamil hydrochloride), it was added during this wash step as well as during the subsequent incubation step with treatment. After the removal of transport buffers, for apical to basolateral compartment studies, 1.5 mL containing the respective treatment in HBSS (pH 6.0) was added to the apical compartment, followed by 2.4 mL addition of HBSS buffer (pH 7.4) at the basolateral compartment. For studies from the basolateral to apical compartment, 2.4 mL of treatment in HBSS (pH 7.4) was added to the basolateral compartment followed by 1.5 mL HBSS (pH 6) addition at the apical compartment. Experiments were performed in the presence and absence of efflux inhibitor, Verapamil hydrochloride (100 µM). To measure the concentration of transported and efflux of treatments, samples (1.2 mL) were withdrawn from the basolateral or the apical compartment respectively for determination of the rate of transport at (0, 0.5, 1, 1.5 and 2 hours) and replaced with an equal volume of relative transport buffer, thereby maintaining sink conditions. Samples were frozen after the addition of ascorbic acid to a final concentration of 1%, in liquid nitrogen and stored thereafter at -65°C until HPLC analysis was performed.

The apparent permeability coefficient, P_{app} (cm/s), was calculated using:

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

Where dQ/dt is the cumulative transport rate (nmol/s) defined as the slope obtained by linear regression of cumulative transport amount (Q) as a function of time (s); A is the surface area of inserts in the transwells; C_0 is the initial concentration of treatment on the donor side (µM).

The concentration of the transport of treatment compounds from the P_{app} (AP→BL) (apical-to-basolateral) and P_{app} (BL→AP) (basolateral-to-apical) respectively was measured using HPLC as described in the bioanalysis section (1.4) and the efflux ratio (ER) calculated based on the equation:

$$\text{Efflux ratio (ER)} = \frac{(P_{app}B/A)}{(P_{app}A/B)}$$

P_{app} (B/A) and P_{app} (AB) represent the apparent permeability of test compound from BL to AP side and AP to BL side of the cell monolayer respectively.

Results were corrected for dilution and expressed by means of the cumulative transport (% of initial dose) as a function of time. The experiments were performed in triplicate and the data expressed as mean ± standard deviation (SD). Differences between mean values were analyzed using a students paired two tailed t-test and were considered to be significant if the value $p < 0.05$.

3.7. Bioanalysis

3.7.1. Determination of individual compounds in *Athrixia phylicoides* extracts by HPLC-DAD

3.7.1.1. Sample preparation

Water was de-ionised and purified using a Millipore Advantage Water Purification System. Water for HPLC was further de-ionised using a Milli-Q Reference A+ water purification system. Thereafter, 10% Ascorbic acid was prepared by weighing ± 2 g of ascorbic acid into a 20 mL volumetric flask and de-ionised water added to dissolve and filled up to the mark, this was further stored in the fridge for 1 week. Thereafter, 1% *formic acid* was prepared by adding 20 mL of formic acid (using a 20 mL glass pipette) to a 2 L volumetric flask. The reagent was completed by filling up HPLC water (a fume hood was used for the preparation of this reagent). The reagent was mixed well and filtered using all-glass filter apparatus and a Millipore HV 0.45 µm membrane filter (Microsep; HVLP 047 00) Mr (HCOOH) = 46.03 $g.mole^{-1}$. Acetonitrile [Mr (CH_3CN) = 41.05 $g.mole^{-1}$] was used without filtering.

3.7.1.2. High-performance liquid chromatography with diode-array detection (HPLC-DAD)

A new method was developed, based in part on the existing method by De Beer *et al*. (2011), and validated for the analysis of extracts of *Athrixia phylicoides* with special emphasis to improve quantification of the nine (9) prominent phenolic compounds; i.e. protocatechuic acid (**1**), neochlorogenic acid (**2**), caffeic acid (**3**), chlorogenic acid (**4**), cryptochlorogenic acid (**5**), *para*-coumaric acid (**6**), 3,4-dicaffeoylquinic acid (**7**), 3,5-dicaffeoylquinic acid (**8**) and 4,5-dicaffeoylquinic acid (**9**). Analysis was performed using an Agilent 1200 HPLC system consisting of a quaternary pump with incorporated in-line degasser, autosampler, column thermostat and diode-array detector (Agilent

Technologies, Waldbronn, Germany). OpenLab CDS Chemstation software (Agilent Technologies) was used for instrument control and data analysis during HPLC-DAD analysis. Separation was achieved on a Kinetex C18 column (150 mm × 4.6 mm, 2.6 µm particle size, 100 Å) (Phenomenex, Santa Clara, CA, USA) protected by a guard column with the same stationary phase and a KrudKatcher Ultra inline filter (Phenomenex). The solvent system for separation consisted of 1% formic acid (A) and acetonitrile (B) at 1 mL/minute, with column temperature maintained at 20 °C, in the following multilinear gradient: 0–5 minutes, 5% B; 5–13.5 minutes, 5-7.3% B; 13.5–25 minutes, 7.3–13.1% B; 25–35 minutes, 13.1–19% B; 35–40 minutes, 19–21% B; 40–42 minutes, 21–25% B; 42–46 minutes, 25-80% B; 46–50 minutes, 50-5% B; 50–60 minutes, 5% B. UV–vis spectra were recorded for all samples from 190 to 400 nm with selective wavelength monitoring at 255, 288, 320 and 350 nm. Peaks were tentatively identified by comparing retention times and UV–vis spectra from HPLC-DAD analysis with those of authentic standards. Stock solutions of all nine standard compounds were prepared in DMSO and aliquots frozen at −20 °C until required. Dried extracts were dissolved in 10% DMSO: deionized water (v/v) prior to analysis by adding DMSO to the equivalent volume of 10% of the total volume, mixing and sonication, before deionized water was added to the equivalent volume of 90% of the total volume. Samples from the transport study was defrosted and mixed before filtration and analysis. The standard calibration mixtures and sample solutions were filtered using 0.22 µm pore-size Millex-GV hydrophilic PVDF syringe-driven filter devices (Millipore) with 4 and 33 mm diameter, respectively, prior to HPLC analysis.

3.7.1.3. High-performance liquid chromatography with electrospray ionisation mass spectrometry (HPLC-ESI-MS)

HPLC-ESI-MS analyses were performed on a Waters Acquity UPLC system equipped with a binary solvent manager, sample manager, diode-array detector and Synapt G2 Q-TOF mass spectrometer using electrospray ionization in both the negative and positive modes (Waters, Milford, MA, USA). HPLC-ESI-MS and –MS^E analyses were performed using the same column and gradient conditions as for the HPLC-DAD method, but with a slight modification by performing the separation at 32 °C. This modification affected retention times without affecting the elution order. The eluant was split 1:1 prior to introduction into the ionization chamber. Analyses were performed in MS^E mode (collision energy ramp from 20 to 50 V). The MS parameters

were as follows: desolvation temperature, 275 °C; nitrogen flow rate, 650 L/h; source temperature, 120 °C; capillary voltage, 3000 V; cone voltage, 15 V. Sodium formate was used as calibration standard and leucine enkephalin as reference standard for the lock mass. Data acquired were processed using MassLynx v.4.1 software (Waters). Peaks were identified by comparison of retention times, UV-Vis spectra, LC-ESI-MS and MS^E spectra with those of authentic standards as a measure of the specificity of the HPLC method.

3.8. Protein analysis

3.8.1. Protein extraction of concentration determination (method modified from RC DC)

Protein determinations were performed using the RC DC protein assay method according to the manufacturer's instructions (Bradford Bio-Rad Assay, Bio-Rad Laboratories). Briefly, cells were lysed by adding 400 µL Ripa buffer to the wells from the plates used for transport assays and cells were gently scrapped from the wells using a cell scrapper. 5 µL sample of cell homogenate or bovine serum albumin (BSA) standards (0.125, 0.25, 0.5, 0.150, 0.325) were added to wells of a 96-well plate in duplicate, followed by addition of 25 µL of reagent A (mixture of 1000 µL DC reagent A and 20 µL DC reagent C) and 250 µL of RC reagent I to each standard or sample. Plates were incubated in the dark while covered with aluminium foil for 10 minutes and absorbance was recorded thereafter at 630 nm on a BioTek® ELX 800 plate reader (BioTek Instruments Inc., Winooski, VT, USA). Protein concentration was calculated by extrapolating the absorbance measurement of the sample from the BSA standard curve. Protein concentration was expressed as mg/mL.

3.8.2. Western Blotting Analysis

3.8.2.1. Transfer of gel to PVDF membrane

Following gel electrophoresis (Trans-Blot Cell 170-4070, Bio-Rad), proteins were transferred to a 7 cm x 9 cm polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was briefly pre-wet with 100% methanol prior to submersion in transfer buffer for 5 minutes. The electro-blotting (Tank transfer) method was used to transfer proteins from the SDS-PAGE GEL to PVDF membrane. Gel and PVDF

were equilibrated in transfer buffer for 20 minutes. After equilibration, a ‘transfer sandwich' was prepared in the following order (pad-filter paper-gel-membrane-filter paper-pad) in a support grid as shown in Figure 7. The support gel sandwich was then place in the Tank filled with transfer buffer and an ice pack was placed on top to mitigate heat production during transfer.

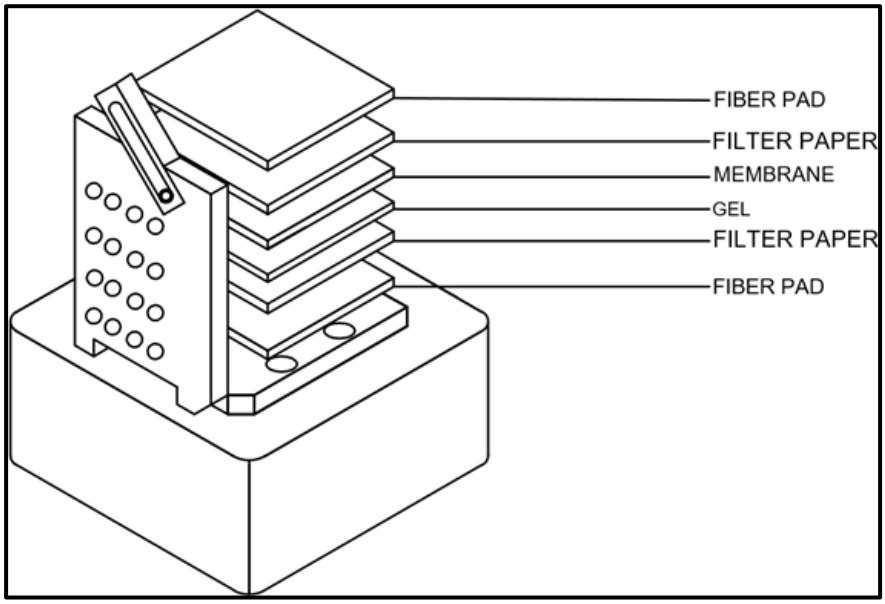


Figure 7: Image representing the transfer membrane sandwich

3.8.2.2. Ponceau S stain

After transfer, the blotted membrane was immersed in a sufficient amount of Ponceau S Staining Solution for 5 minutes. The proteins bands were clearly visible and the staining was reversed by washing the stained membrane with distilled water for another 5 minutes**.**

3.8.2.3. Labelling of membrane with MDR-1 antibody

After removal of Ponceau S stain, the PVDF membrane containing the proteins of interest were blocked with 5% (w/v) non-fat milk powder dissolved in 1× Tris-buffered saline containing Tween-20 (TBST-20) at room temperature for 2 hours on an orbital shaker 20197 (2.5 rpm) (Stovall life Science). Subsequently, the membrane was labelled with the relevant primary antibodies (1:1000) (Santa Cruz MDR-1, Cat#sc5510, Lot#G3013) overnight at 4 °C in 1× TBST. The following day the membranes were washed 3 times for 10 minutes each in 1× TBST-20 at room

temperature on an orbital shaker. Thereafter, membranes were labelled with the relevant horseradish peroxidase (HRP) (1:4000) conjugated secondary antibody (donkey anti-mouse HRP (Cat #sc2318, Santa Cruz) in 2.5% non-fat milk powder dissolved in 1× TBST-20 at room temperature for 90-minutes. After this period, membranes were washed for 10 minutes in 3 instances with 1× TBST-20.

3.8.2.4. ChemiDoc detection

Detection of the MDR-1 protein was quantified using a ChemiDoc-XRS imager (Bio-Rad) and Quantity One software. The blot was marked with Clarity™ Western ECL Substrate Lumino/enhancer and Clarity™ Western ECL peroxide solution (Cat # 170-5061, Bio-Rad) by preparing the two solutions in a 1:1 ratio and 2 mL of the mixture was pipetted onto the membrane. An image of the membrane was captured with the Bio-Rad ChemiDoc with 1 second exposure time.

3.9. Statistical analysis

Specific attention was given to correct sample size, appropriate controls and the avoidance of type-II errors during the experimental design phase. *In vitro* results were expressed as the means of three independent experiments, with intra-experimental repeats per experiment. Error bars reflect the standard error of the mean. All data were entered into Microsoft Excel spreadsheets to generate graphs and GraphPad Prism (version 5.0) (GraphPad Software Inc., La Jolla, USA) was used for statistical analysis. Significant differences between groups (i.e. $p < 0.05$) were determined by student T-test.

CHAPTER 4

Results

4.1. Establishing and Validation of the Caco-2 model

Caco-2 cell model integrity to ensure that the grown Caco-2 cells truly represented the human intestinal epithelial cells was validated using Light microscopy (Figure 8 A&B) together with Transmission electron microscopy (Figure 8 C&D) in order to visually identify an intact single cell monolayer as well as ultrastructural features of Caco-2 cells such as tight junctions, distinct brush border and desmosomes.

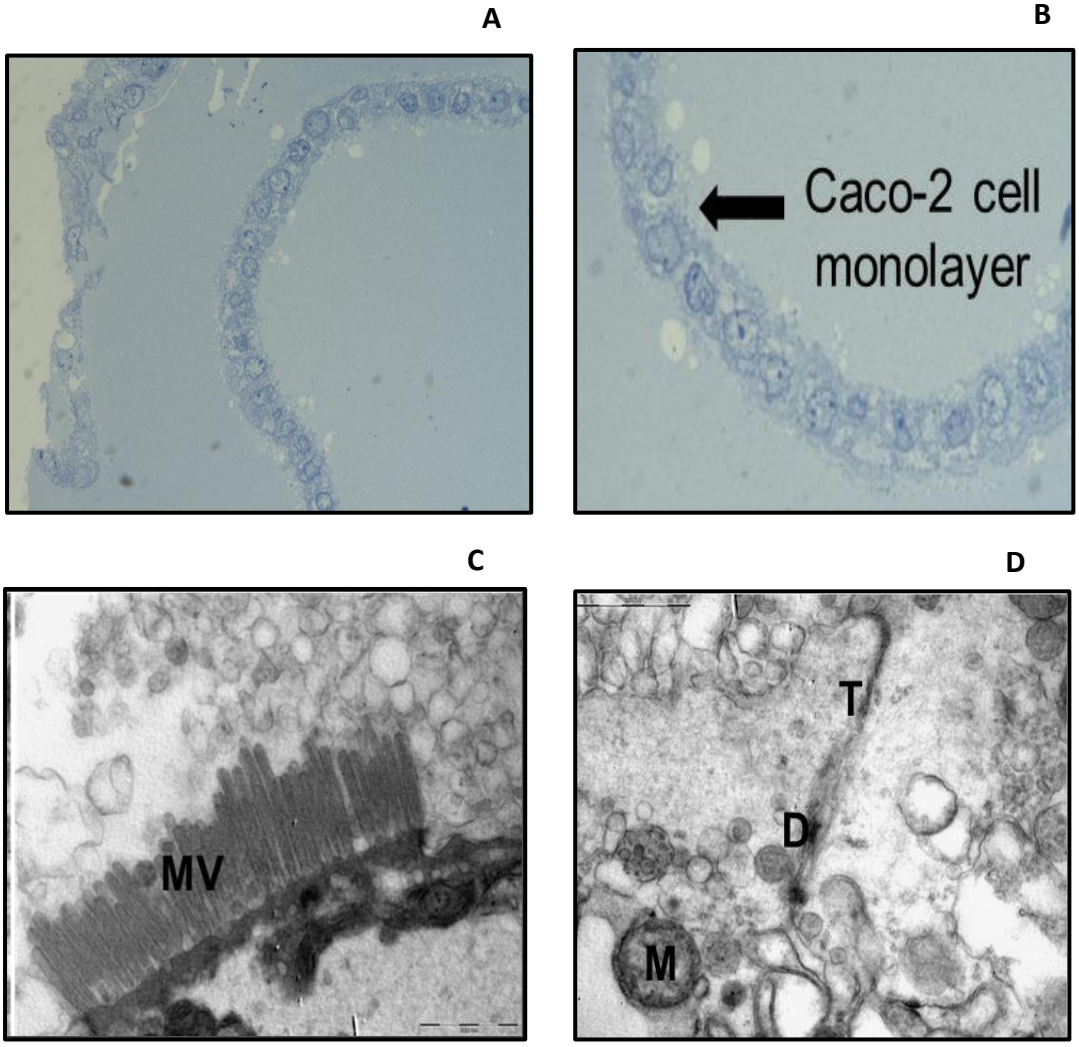


Figure 8: Validation and confirmation of the Caco-2 cell model. Transmission electron microscopy images of the fully differentiated Caco-2 cell monolayers grown for 23 days showing (A&B) an intact single cell monolayer (C) microvilli (MV), (D) tight junctions (T), Desmosomes (D) and mitochondria (M), which are ultrastructural features of differentiated Caco-2 cells representing the human intestinal epithelium.

4.2. Validation of the presence of MDR-1 expression in Caco-2 cells

Multi drug resistant protein-1 (MDR-1) is one of the major efflux proteins affecting the absorption of bioactive compounds within the small intestine. We confirmed the presence of MDR1 in Caco-2 cells by Western blot analysis (Figure 9).

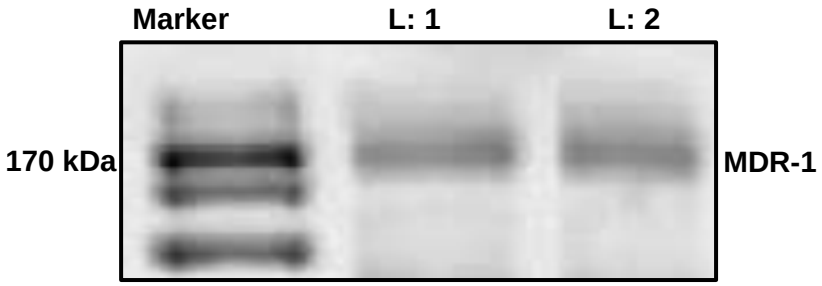


Figure 9: Protein quantification of MDR-1 in Caco-2 cells to illustrate the presence of MDR-1 in differentiated Caco-2 cells. Detection of MDR-1 at 170 kDa as shown by L: 1 (Lane 1; 20 µg/mL), and L: 2 (Lane 2; 40µg/mL). The blot is a representative of three independent experiments.

4.3. *In vitro* integrity assays of the cell monolayers

Two measures of monolayer integrity were monitored before, during and after all transport experiments. Transepithelial electrical resistance (TEER) was measured using a Millicell-ERS epithelial voltmeter (Millipore, Massachusetts, USA) where TEER readings above 300 Ω were regarded as a good indication of an intact Caco-2 monolayer (Gerbella *et al*., 2014). Lucifer yellow (LY) (Figure 11), is a fluorescent indicator molecule that was added at the same time as the treatment and monitored fluoremetrically by measuring the % of LY that was of passed from apical to basolateral side of the monolayer. Readings indicating passage of the molecule below 3%, were regarded as good indication of an intact monolayer.

For all experiments, cells were only used when TEER readings were above 300 Ω (Table 5) and only those samples taken from cells with LY readings below 3% were sent for HPLC-DAD and LC-MS analysis.

Table 5: Confirming the integrity of the Caco-2 monolayers by measuring TEER readings (an example of acceptable monolayer integrity conditions).

Plate layout	TEER (Ω) before	TEER (Ω) @ 15 minutes	TEER (Ω) after transport experiment
Control	866 ± 5.03	455 ± 0.00	498 ± 2.04
A.phylicoides	769 ± 4.04	316 ± 2.04	345 ± 1.30
***A.phylicoides* + Ver**	901 ± 4.04	401 ± 2.90	434 ± 2.90
PCA	856 ± 5.03	409 ± 4.04	418 ± 1.30
CA	876 ± 5.77	343 ± 2.20	399 ± 2.04
***p*-CA**	788 ± 5.03	316 ± 1.30	376 ± 0.00

Transepithelial resistance measurements used as confirmation of the integrity of the Caco-2 cell monolayers before, 15 minutes after wash and after the transport experiments. Results are represented as the mean ± SD of three independent experiments done in triplicates (n=9). *A. phylicoides* (an aqueous extract of *A. phylicoides*); *A. phylicoides* +Ver (*A. phylicoides* + verapamil hydrochloride); PCA (protocatechuic acid); CA (caffeic acid); *p*-CA (*para*-coumaric acid).

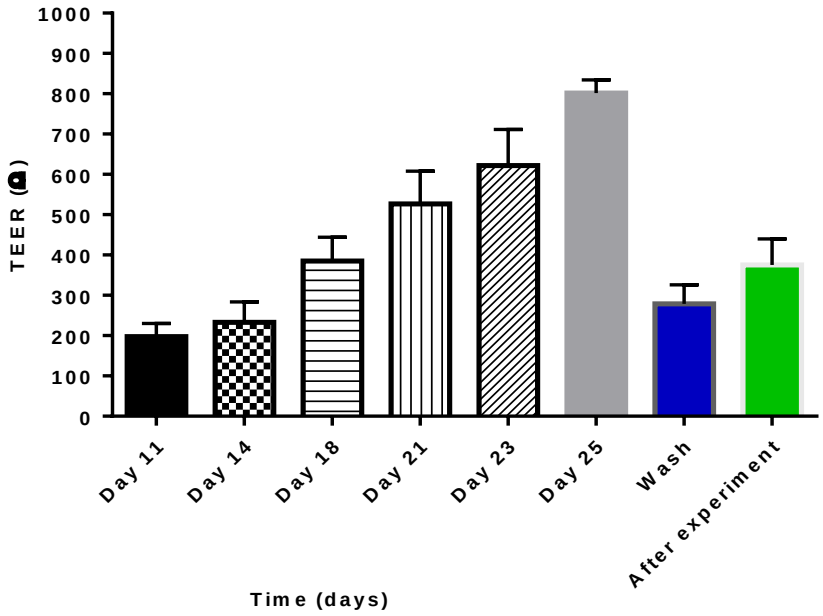


Figure 10: Confirming the integrity of the grown Caco-2 monolayer by measuring the transepithelial resistance (TEER) from day 11 post seeding to day 25 and during the wash and after the transport experiments. Results are represented as the mean ± SD of the three independent experiments done in triplicates (n=9).

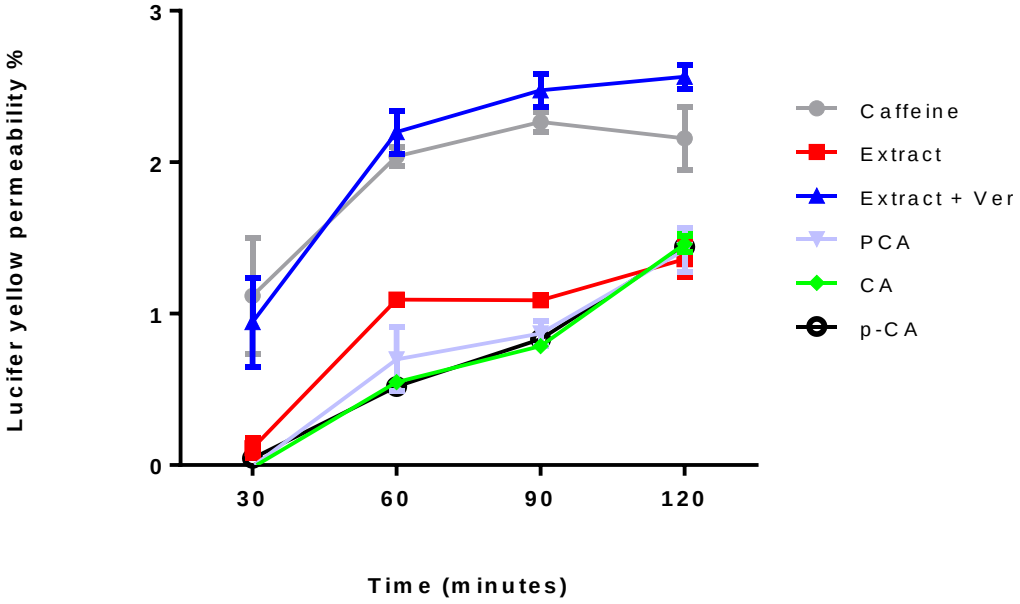


Figure 11: Confirming the integrity of the Caco-2 monolayer using Lucifer yellow. LY readings below 3% were regarded as a good indication of an intact monolayer that closely resemble the human intestinal epithelium (Saitoh *et al*.,2004). Results are represented as the mean ± SD of the three independent experiments done in triplicates (n=9).

4.4. Effect of *A. phylicoides* on cell viability

Caco-2 cells were exposed to various concentrations of an aqueous extract of *A. phylicoides* (0.3125, 0.625, 1.25, 2.5 and 5 mg/mL) with 5 mg/mL the highest soluble concentration of *A. phylicoides.* At this concentration, the cell ATP content of the Caco-2 cells was 74.6 ± 2.6 % relative to the vehicle control (0.0125% DMSO) (Figure 12).

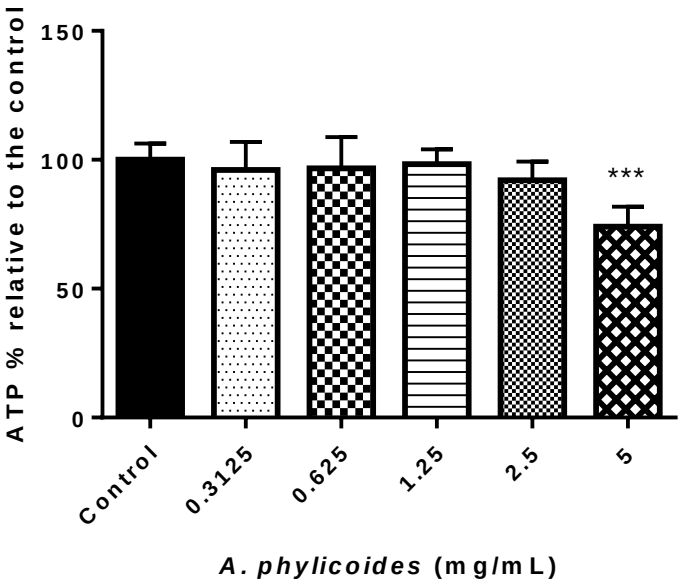


Figure 12. Caco-2 cell ATP content following a 2-hour exposure with an increasing range of concentrations of an aqueous extract of *A. phylicoides*. Results are expressed as mean ± SD of three independent experiments done in triplicates (n=9) relative to the vehicle control (0.125% DMSO) set at 100 %. *** = p<0.001 vs. vehicle control.

4.5. Effects of the major bioavailable compounds of *A. phylicoides* in their pure form on cell viability

PCA (1), CA(3) and *p*-CA (6) were identified as the most likely major bioavailable phenolic compounds from an aqueous extract of *A. phylicoides* (Figure 14 B). These pure compounds were individually tested for cytotoxicity to Caco-2 cells at their highest soluble concentrations (80 µM, 280 µM and 300 µM respectively) in 0.01% ethanol (Figure 13). Using the MTT-assay, the compounds were deemed non-toxic to Caco-2 cells at these concentrations over a 2-hour period.

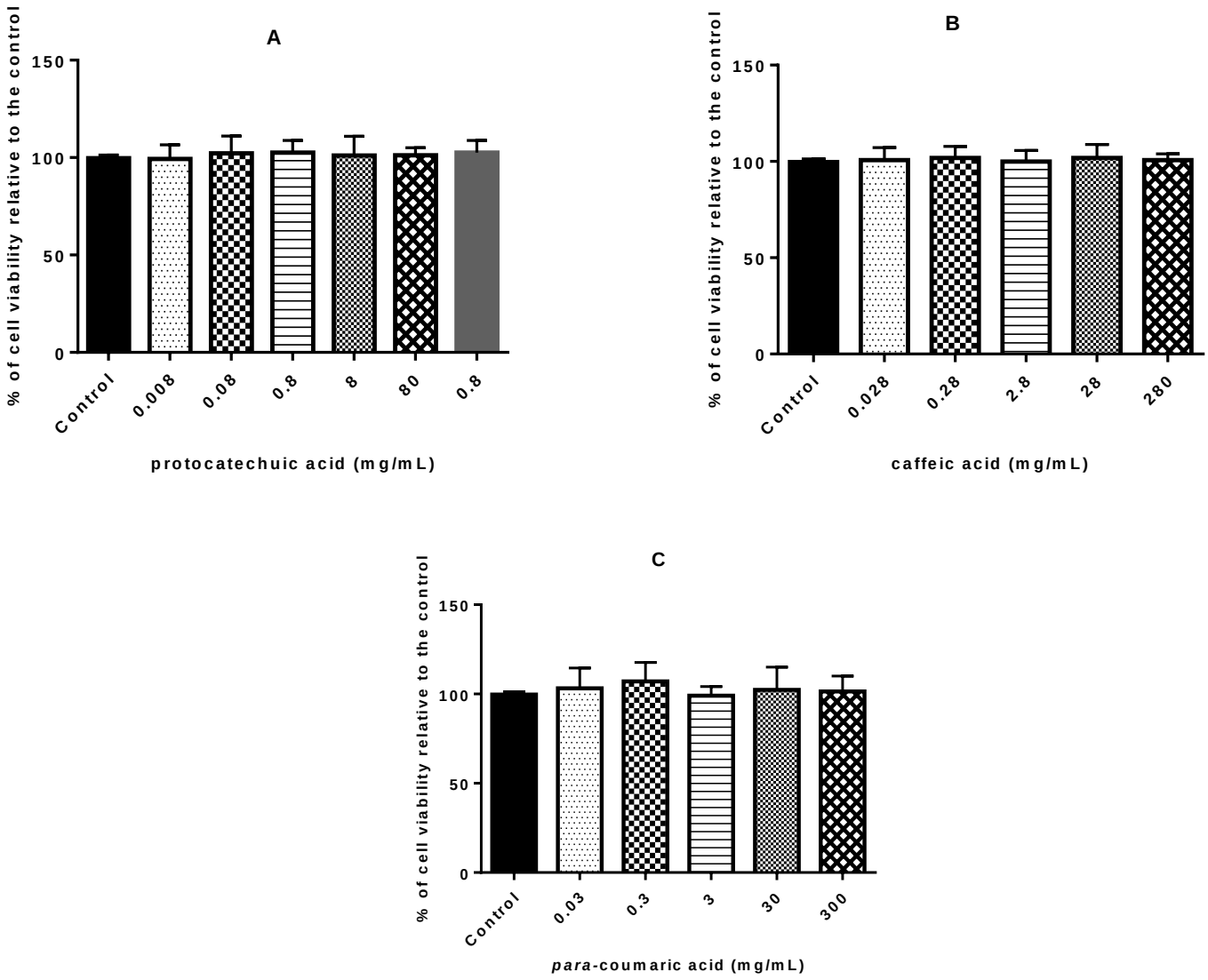


Figure 13: Effect of major phenolic compounds identified in an aqueous extract of *A. phylicoides* on MTT activity following a 2-hour incubation with an increasing range of concentrations of (**A)** protocatechuic acid, (**B)** caffeic acid and (**C)** *para*-coumaric acid. Results reflect the mean ± SD of three independent experiments done in triplicates (n=9) and are expressed as a percentage of the vehicle control (0.01% ethanol).

4.6. Validation of the HPLC-DAD methodology for characterization of an aqueous extract of *A. phylicoides*

The HPLC-DAD method was validated by evaluating the specificity, linearity, stability over 24 hours and intra- and inter-day repeatability (Table 6). The specificity of the adapted method was confirmed by examining the UV–Vis and MS spectra across the target analyte peaks and comparing them to those of the authentic reference standards.

The concentrations of the 9 major compounds are summarised in Table 7. Transport experiments across Caco-2 monolayers, identified three compounds (1, 3 and 6) that passed through the monolayer at significant levels (Figure 14 B).

Table 6: Validation parameters for HPLC-DAD method developed to analyse *A. phylicoides* extracts and samples from Caco-2 cell experiments

Parameter	1	2	3	4	5	6	7	8	9
Linearity (R^2) of calibration curve	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Stability 24 hours [a] (cal. mix)	4.02	0.38	0.39	0.56	0.59	0.33	0.34	0.35	0.30
Stability 24 hours [a] (AP)	0.81	2.31	0.55	0.82	1.59	0.94	1.18	0.81	0.97
Intra-day repeatability [b] (cal. mixture)	0.20; 0.20; 0.20	0.20; 0.20; 0.40	0.10; 0.20; 0.20	0.20; 0.20; 0.20	0.30; 0.40; 0.30	0.10; 0.20; 0.30	0.10; 0.20; 0.20	0.70; 0.20; 0.70	0.20; 0.30; 0.10
Intra-day repeatability [b] (AP)	0.30; 0.60; 0.30	0.70; 0.90; 0.80	0.30; 0.40; 0.40	0.50; 0.70; 0.50	0.90; 1.30; 1.20	0.60; 0.40; 0.90	0.90; 0.50; 0.50	0.80; 0.60; 0.70	0.40; 0.50; 0.70
Inter-day repeatability [c] (cal. mix)	0.20	0.30	0.20	0.30	0.40	0.20	0.20	0.60	0.30
Inter-day repeatability [c] (AP)	0.40	0.80	0.40	0.60	1.60	0.70	0.90	1.30	1.30

[a] %RSD for 13 injections over 24-hour period, [b]% RSD for 10 injections on each of 3 separate days, .[c]% RSD for the averages of 10 injections on each of 3 separate days. protocatechuic acid (1), neochlorogenic acid (2), caffeic acid (3), chlorogenic acid (4), cryptochlorogenic acid (5), para-coumaric acid (6), 3,4-dicaffeoylquinic acid (7), 3,5-dicaffeoylquinic acid (8) and 4,5-dicaffeoylquinic acid (9).

Abbreviations: *AP*, *Athrixia phylicoides* extract; cal mix, calibration mixture; %RSD, percentage relative standard deviation.

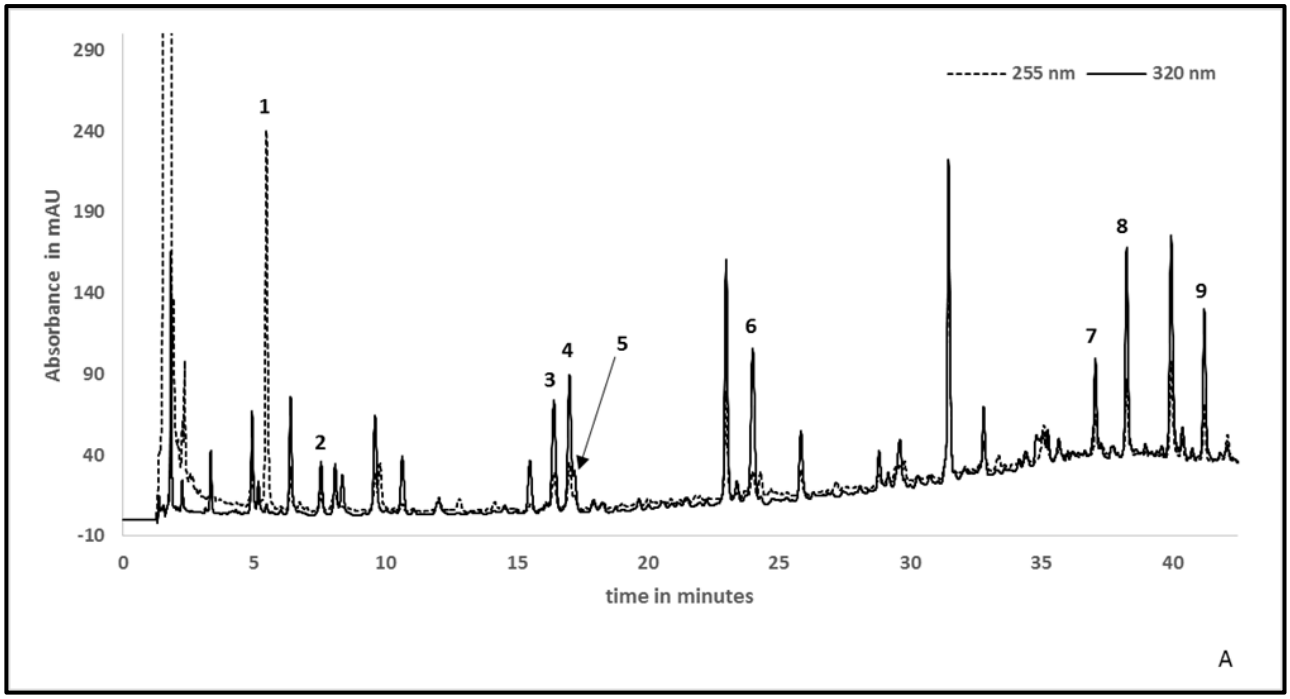
Table 7: Phenolic composition (g/100 g) of the aqueous extract of *A. phylicoides*

	1	2	3	4	5	6	7	8	9
***A. phylicoides* extract**	0.19	0.04	0.06	0.13	0.04	0.07	0.08	0.13	0.09

The concentrations of the 9 major phenolic compounds: protocatechuic acid (1), neochlorogenic acid (2), caffeic acid (3), chlorogenic acid (4), cryptochlorogenic acid (5), *para*-coumaric acid (6), 3,4-dicaffeoylquinic acid (7), 3,5-dicaffeoylquinic acid (8) and 4,5-dicaffeoylquinic acid (9).

4.7. Characterization of the prominent and major transported phenolic compounds of *A. phylicoides*

The prominent phenolic compounds present in an aqueous extract of *A. phylicoides* were determined by HPLC-DAD and LC-MS (Figure 14 A). Compound 1 was the only phenolic compound quantified at 255 nm (Figure 14 A & B), while the other phenolic compounds were quantified at 320 nm (Figure 14 A &B).



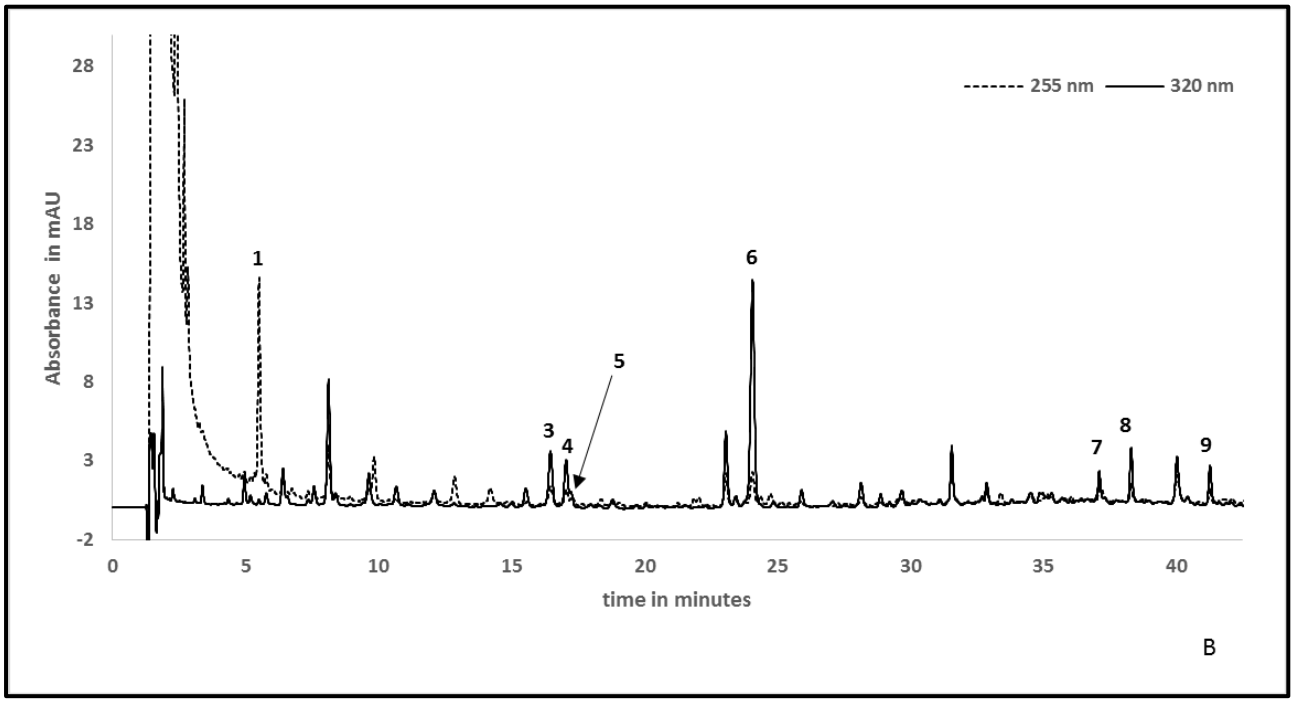


Figure 14: HPLC-DAD chromatograms identifying (A) the prominent phenolic compounds present in an aqueous extract of *A. phylicoides* in the apical compartment at time 0 during AP-BL transport studies, protocatechuic acid (1), neochlorogenic acid (2), caffeic acid (3), chlorogenic acid (4), cryptochlorogenic acid (5), *para*-coumaric acid (6), 3,4-dicaffeoylquinic acid (7), 3,5-dicaffeoylquinic acid (8) and 4,5-dicaffeoylquinic acid (9), and (B) phenolic compounds in the basolateral compartment at time 120 minutes during AP-BL transport studies, transported across the fully differentiated Caco-2 monolayer, protocatechuic acid (1), neochlorogenic acid (2), caffeic acid (3), chlorogenic acid (4), cryptochlorogenic acid (5), *para*-coumaric acid (6), 3,4-dicaffeoylquinic acid (7), 3,5-dicaffeoylquinic acid (8) and 4,5-dicaffeoylquinic acid (9).

4.8. Rate of transport of prominent phenolic compounds of an aqueous extract of *A. phylicoides*

The apparent rate of transport (P_{app}) of phenolic compounds of an *A. phylicoides* aqueous extract across Caco-2 cell monolayers were calculated from concentrations obtained from HPLC-DAD analysis (Table 7). Caffeine was used as a positive control as it has been well documented as a compound that is passively transported at a high rate (Joo *et al*., 2016; Kamimori *et al*., 2002).

4.9. Percentage of phenolic compounds remaining in the apical compartment

Using the change in the initial concentration in the apical compartment, the percentage cross over of each of the nine prominent phenolic compounds was calculated. The percentage cross over of the major phenolic compounds (1, 3 and 6) was 4.61%, 6.21% and 32.61% respectively (Table 7), which is complementary to their particular P_{app} (rate of transport) values.

Table 8: Apparent permeability and % cross over for the prominent phenolic compounds of an aqueous extract of *A. phylicoides* in Caco-2 cells.

Compound	P_{app} (a/b) $\times 10^6$ cm/s	% cross over
Caffeine	69.04 ± 0.35	79.60 ± 0.00
1	4.52 ± 0.66	4.61 ± 3.70
2	0.09 ± 0.01	3.70 ± 3.10
3	4.35 ± 0.88	6.71 ± 2.90
4	0.27 ± 0.02	1.00 ± 2.50
5	0.23 ± 0.11	-0.33 ± 2.50*
6	23.76 ± 0.51	32.36 ± 2.80
7	0.35 ± 0.27	-3.34 ±1.70*
8	0.31 ± 0.09	-2.66 ±4.50*
9	0.43 ± 0.11	-0.55 ±2.30*

P_{app} values defined as apparent permeability coefficient Apical to Basolateral (a/b) represent the rate of absorption of the prominent phenolic compounds from an aqueous extract of *A. phylicoides* across a fully differentiated Caco-2 cell monolayer together with their respective % cross over. Caffeine was used as a positive control. Percentage cross over of the phenolic compounds present (as % remaining in the apical compartment) during apical to basolateral transport experiments with an aqueous extract of *A. phylicoides*. The results are represented as mean ± SD of three independent experiments (n=9). The * values indicate negative % readings, which represent efflux of the compounds. Protocatechuic acid (1), neochlorogenic acid (2), caffeic acid (3), chlorogenic acid (4), cryptochlorogenic acid (5), *para*-coumaric acid (6), 3,4-dicaffeoylquinic acid (7), 3,5-dicaffeoylquinic acid (8) and 4,5-dicaffeoylquinic acid (9). The compounds written in red are the major transported phenolic compounds.

4.10. Effects of transporter inhibitors on the transport of the prominent phenolic compounds of *A. phylicoides*

Efflux was validated by use of verapamil hydrochloride (an inhibitor for the efflux protein P-gp). In the presence of verapamil, there was a significant decrease (*p <0.05; **p <0.01; (***p <0.001) in the rate of transport of effluxed compounds (4, 5, 7, 8 and 9) in the basolateral to apical direction (Table 9). P_{app} values from the apical to basolateral direction remained relatively unchanged in the presence or absence of the inhibitor.

Table 9: Apparent permeability for the phenolic compounds of an aqueous extract of *A. phylicoides* in Caco-2 cells in the presence of a P-gp inhibitor verapamil hydrochloride.

	(-) Verapamil hydrochloride			(+) Verapamil hydrochloride			
Compound	**P_{app} (a/b) $\times 10^{-6}$ cm/s**	**P_{app} (b/a) $\times 10^{6}$ cm/s**	**Efflux ratio**	**P_{app} (a/b) $\times 10^{6}$ cm/s**	**P_{app} (b/a) $\times 10^{6}$ cm/s**	**Efflux ratio**	**P value**
Caffeine	69.04 ± 0.35						
1	4.52 ± 0.66	6.90 ± 0.50	1.53	1.07 ± 0.66	1.23 ± 0.62	0.27	0.90
2	0.09 ± 0.01	3.50 ± 0.14	36.45	0.76 ± 0.01	0.09 ± 0.01	0.95	0.01*
3	4.35 ± 0.88	7.74 ± 0.06	1.78	1.82 ± 0.88	2.83 ± 1.00	0.65	0.0063**
4	0.27 ± 0.02	4.27 ± 0.06	16.02	0.39 ± 0.02	6.28 ± 0.38	2.36	<0.001***
5	0.23 ± 0.11	5.52 ± 0.06	24.32	0.61 ± 0.11	0.01 ± 0.01	0.01	0.10
6	23.76 ± 0.51	2.29 ± 0.58	0.96	15.81 ± 0.51	12.36 ± 3.50	0.52	0.15
7	0.35 ± 0.27	2.62 ± 0.08	7.33	0.60 ± 0.27	0.14 ± 0.04	0.40	0.42
8	0.31 ± 0.09	3.36 ± 0.09	10.90	0.29 ± 0.09	0.40 ± 0.05	1.28	<0.001***
9	0.43 ± 0.11	3.18 ± 0.10	7.42	0.22 ± 0.11	0.25 ± 0.03	0.58**	< 0.001***

P_{app} values defined as apparent permeability coefficient [apical to basolateral (a/b) and basolateral to apical (b/a)] I the presence and absence of verapamil hydrochloride, representing the rate of transport of compounds. The results are presented as the mean of ± SD of three independent experiments done in triplicate (n=9). In the presence of verapamil, efflux ratio of the effluxed phenolic compounds was significantly decreased (*p <0.05; **p <0.01; (***p <0.001). Efflux mechanisms are known to be involved when the efflux ratio (b/a)/(a/b) is >2. protocatechuic acid (1), neochlorogenic acid (2), caffeic acid (3), chlorogenic acid (4), cryptochlorogenic acid (5), *para*-coumaric acid (6), 3,4-dicaffeoylquinic acid (7), 3,5-dicaffeoylquinic acid (8) and 4,5-dicaffeoylquinic acid (9). The compounds written in red are the major transported phenolic compound

4.11. Rate of transport of the major phenolic compounds

The apparent rate of transport of major phenolic compounds (1, 3 and 6) in their pure forms across fully differentiated Caco-2 monolayers were calculated using concentrations obtained by HPLC-DAD analysis (Table 10).

Table 10: Apparent permeability for the phenolic compounds across fully differentiated Caco-2 cell monolayers.

Compound	P_{app} (a/b) x 10^6 (cm/s)	P_{app} (b/a) x 10^6 (cm/s)	Efflux ratio
Caffeine	69.04 ± 0.35		
1	70.20 ± 0.66	30.33 ± 0.62	0.04
3	22.70 ± 0.88	0.03 ± 1.00	0.00
6	46.61 ± 0.51	29.83 ± 3.50	0.64

P_{app} values defined as apparent permeability coefficient (apical to basolateral and basolateral to apical), representing the rate of absorption of 3 major phenolic compounds in their pure forms across a fully differentiated Caco-2 monolayer. The results are represented as the mean of ± SD of three independent experiments done in triplicate (n=9).

CHAPTER 5

Discussion

5.1. Potential of an aqueous extract of *A. phylicoides* as a nutraceutical

The consumption of beverages prepared from indigenous plants such as *Aspalathus linearis* (rooibos) and *Cyclopia spp*. (honeybush) has been successfully commercialized. The rationale behind the popularity in these plants is attributed to their numerous health promoting benefits (Auddy *et al*., 2003; Bahadoran *et al*., 2013; Joubert *et al*., 2008). *A. phylicoides*, commonly known as bush tea, is another South African indigenous tea that has notable interesting history of traditional medicinal uses (Joubert *et al*., 2008; Lehlohonolo *et al*., 2013; Lerotholi *et al*., 2016; Oksana *et al*., 2012; Van Wyk and Gericke, 2000) (summarized in Table 2) and thus has the potential to follow suite from a commercialization aspect. The medicinal properties attributed to the extract purified from *A. phylicoides* have been scientifically validated in terms of its anti-oxidant potential in an aqueous extract (De Beer *et al*., 2011; Padayachee, 2011), anti-bacterial activity in an ethanol extract (Mavundza, 2010) and anti-inflammatory, anti-microbial and anti-malarial activities in a methanol extract (Padayachee, 2011). In addition to this, Chellan *et al*. has shown the aqueous extract stimulates glucose uptake and metabolism in muscle, liver and adipose tissues *in vitro*, suggesting that consumption of *A. phylicoides* tea could potentially ameliorate metabolic disorders related to T2D (Chellan *et al*., 2012). Despite the potential pharmacological effects elicited by *A. phylicoides*, there is very limited evidence relating to its efficacy as an anti-diabetic nutraceutical and the absorption and bioavailability of its major bioactive phenolic compounds. Hence, this study investigates the potential absorption of compounds present in an aqueous extract of *A. phylicoides* using a Caco-2 cell model in an effort to substantiate this information. Caco-2 cells in their fully differentiated form, are structurally and functionally representative of human intestinal epithelial cells and are an important model to study the transport rate of compounds across the GIT (Farrell *et al*., 2012; Hubatsch *et al*., 2007; Takenaka *et al*., 2016; Van Breemen and Li, 2005). Understanding the absorption mechanisms of the major phenolic compounds in *A. phylicoides* will highlight the potential bioactive compounds as well as reveal prospects of additional chemical manipulation of the compounds improving the efficacy of *A. phylicoides* as a nutraceutical.

Initially, the experiments in this study concentrated on validation of the Caco-2 cell model to ensure an accurate structural and functional mimicry of the human intestinal epithelium. Light microscopy was used to confirm the integrity of the Caco-2 monolayer grown on a polycarbonate membrane support, where an intact single cell polarized epithelial monolayer was observed (Figure 8 A&B). Subsequently, transmission electron microscopy verified that the fully differentiated Caco-2 monolayers expressed several membrane specialties for absorption such as microvilli (MV) and indigitations of the basolateral basal membranes (Figure 8 C). Furthermore, well developed junctional complexes including apical tight junctions (T) and desmosomes (D) were visualized between adjacent cells (Figure 8 D). In addition to this, the presence of other transport proteins including the functional efflux protein (MDR-1) was confirmed using Western blot (Figure 9). In support of this, the presence of MDR-1 protein in Caco-2 and Caco-2 subclones has been confirmed previously (Da Silva *et al*., 2015).

Functionally, confirmation of the cell monolayer integrity was attained using two accomplished methods: transepithelial resistance measurement (TEER) and Lucifer yellow permeability (LY). In this study, TEER readings were continuously monitored from the establishment of a monolayer (day 11) until full differentiation (day 25). A good indication of an intact Caco-2 monolayer (Table 4) (Figure 10) was TEER readings above 300 Ω (Doguer *et al*., 2016). Lucifer yellow passage across the Caco-2 monolayer into the basolateral compartment, was monitored consistently throughout the transport experiments. The percentage passage of LY was calculated and remained below 3%, which is also a good indication of an intact monolayer (Figure 11). These two indications of monolayer integrity were in agreement with other publications (Price *et al*., 2014; Srinivasan *et al*., 2015) and confirmation of the model was fundamental in accurately monitoring the rate of transport of test compounds.

The solubility of the extract was assessed by conducting solubility experiments, as it was of utmost importance to keep the solvent concentration (DMSO) at a level that was not adverse towards the cell monolayer. The extract was soluble at 5 mg/mL in 0.25% DMSO and no signs of precipitation were evident upon dilution with the HBSS transport buffers. Likewise, the pure compounds tested were dissolved in ethanol (0.01%) which showed no signs of precipitation in the HBSS buffers once diluted.

Consequently, extract and pure compounds were used at their highest soluble concentrations.

For transport experiments, it was important to determine a concentration of the extract as well as the pure compounds that was non-toxic to the cells. Cytotoxicity was assessed by monitoring the cellular activity as a representation of cell viability. The ATP assay was used to assess cytotoxicity of the *A. phylicoides* extract (Figure 12). Exposing the cells to increasing concentrations of *A. phylicoides* for 2 hours did not have a significant effect ($p > 0.05$) on cell viability. At 2.5 mg/mL (100% cell viability) the aqueous extract was not cytotoxic. For the purpose of this study, it was important to also take into consideration the concentration of the extract allowing for optimal HPLC-DAD detection. More phenolic compounds were identified using HPLC-DAD in the basolateral compartment by using 5 mg/mL in comparison to 2.5 mg/mL extract after transport experiments. At 5 mg/mL, the viability of the cells were reduced to 75%. TEER readings in Table 5 together with LY readings in Figure 11 confirmed no significant effect on the monolayer integrity during transport experiments. As such, although 5 mg/mL of the extract decreased cell viability as determined by the ATP content without affecting TEER and LY results, it was deemed fit for use for further experiments as this allowed for the maximum detection of the nine prominent phenolic compounds (Figure 13 A).

To compare the transport rates of the individual major phenolic compounds to that of the complex mixture of phenolic compounds present in the extract, pure compounds, at highest soluble concentrations protocatechuic acid (80 µM), caffeic acid (280 µM) and *para*-coumaric acid (300 µM) in 0.01% ethanol were found not to be toxic (MTT assay, 100% cell viability) to the cells after a 2-hour exposure (Figure 13). During transport experiments, caffeine was used as a positive reference compound, as it is reported to be passively transported at a relatively high rate of transport (Joo *et al*., 2016; Kamimori *et al*., 2002). The rate of transport of caffeine was similar in the extract and pure compound experiments, thus confirming consistency between the two experimental conditions.

In this study, a new method was developed to characterize *A. phylicoides*, by modification of the method by De Beer *et al*. (2011) and validated for the analysis of

extracts of *A. phylicoides* with special emphasis on improving quantification of prominent phenolic compounds.

An aqueous extract of *A. phylicoides* has been previously characterized using countercurrent chromatography coupled with semi-preparative HPLC to identify a number of phenolic compounds such as 6-hydroxyluteolin-7-*O*-glucoside, chlorogenic acid, protocatechuic acid, 1,3-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and a methoxy-flavonol derivative (Chellan *et al*., 2012; de Beer *et al*., 2011). Characterization of this extract resulted in the identification of nine prominent phenolic compounds as shown in Figure 14 A identifying similar compounds to previous findings from (Chellan *et al*., 2012; De Beer *et al*., 2011). The nine identified phenolic compounds in this study were protocatechuic acid (1), neochlorogenic acid (2), caffeic acid (3), chlorogenic acid (4), cryptochlorogenic acid (5), *para*-coumaric acid (6), 3,4-dicaffeoylquinic acid (7), 3,5-dicaffeoylquinic acid (8) and 4,5-dicaffeoylquinic acid (9). Eight out of the nine phenolic compounds identified have been previously identified in an aqueous extract of *A. phylicoides*, while *para*-coumaric acid (compound 6) was identified for the first time.

HPLC-DAD was used to monitor the transport of phenolic compounds in an apical to basolateral compartment direction and to quantify the concentration of these compounds and thus, determine their respective rates of transport. Bi-directional transport experiments (including the basolateral to apical compartment direction) allowed for the identification of potential efflux of any of the compounds within the extract.

All nine identified phenolic compounds of *A. phylicoides* were shown to be transported across the Caco-2 model. However, differences were observed in the concentration levels in which the compounds were found present in the basolateral compartment, as protocatechuic acid (1), caffeic acid (3) and *para*-coumaric acid (6) were found at noticeably higher levels in comparison to the other six compounds present at lesser concentrations (Figure 14 B).

It is known that phenolic compounds are subject to enzymatic metabolism in the enterocytes, which may lead to structural changes and the formation of secondary metabolites (Vetrani *et al*., 2016). The formation of secondary metabolites was assessed using LC-MS on concentrated basolateral samples, however, no evidence

of the formation of any secondary metabolites were found in this study, suggesting that the phenolic compounds found within an aqueous extract of *A. phylicoides* are transported without being modified by enterocytic enzymes present in the Caco-2 cells.

The apparent permeability coefficient (P_{app}), indicative of the rate of transport of compounds across the monolayer, was calculated for each of the identified phenolic compounds of *A. phylicoides*. Compared to caffeine, the phenolic compounds 1, 2, 3, 4, 5, 7, 8 and 9 had relatively slow rates of transport. An exception was para-coumaric acid (6) that had a fast transport rate (Table 8). Although the other two major phenolic compounds (1 and 3) had a rate slower than *para-coumaric* acid (6), they had a relatively faster rate of transport (4.52×10^6 and 4.35×10^6 cm/s) when compared to the less bioavailable compounds (Table 8).

Following this, in the basolateral to apical compartment direction, the transport rate of the compounds 1, 3 and 6 remained relatively unchanged (6.90×10^6, 7.74×10^6 and 22.85×10^6 cm/s respectively), in comparison to the apical to basolateral compartment direction. The efflux ratio of the major transported phenolic compounds (1, 3 and 6) were calculated to be 1.53, 1.78 and 0.96, respectively; all these values <2 (Table 9), signifying no efflux mechanism present but rather indicative of passive diffusion (Farrell, *et al*., 2012).

On the contrary, the six less bioavailable compounds had a relatively higher rate of transport in the basolateral to apical compartment direction (Table 8), which is a classic indication of efflux from the basolateral to apical compartment. The efflux ratio of the six minor compounds were >2, a clear indication of the presence of efflux of these compounds (Farrell *et al*., 2012). In support of this, it has been documented that dicaffeoylquinic acids and chlorogenic acids are well-known to be effluxed (Farrell *et al*., 2012).

Efflux of the minor phenolic compounds was confirmed by conducting transport experiments in the presence of a P-gp inhibitor, verapamil hydrochloride. The P_{app} values in the presence of the verapamil hydrochloride were calculated using compound concentrations obtained from HPLC-DAD analysis (Table 9). Compared to bi-directional transport experiments, in the absence of the inhibitor, the rate of transport of the compounds in the basolateral to apical compartment direction was significantly increased, indicating effective inhibition of efflux. The inhibitor did not

decrease the transport rate of the compounds from apical to basolateral compartment direction, thus confirming P-gp as a major transporter of these effluxed phenolic compounds.

Determination of P_{app} values and efflux ratios allowed for the prediction of the absorption rate of prominent phenolic compounds. In order to support predicted absorption, the percentage cross-over of the nine prominent phenolic compounds from apical to basolateral compartment, was calculated as a percentage of the initial concentration added (Table 8). The percentage of the major phenolic compounds (protocatechuic acid, caffeic acid and *para*-coumaric acid) were 4.61%, 6.21% and 32.61%, respectively. These percentages confirm the P_{app} values and are thus an indication of predicted absorption rate of each individual compound. *Para*-coumaric acid was shown to have a relatively faster rate of transport (P_{app}), high % cross over, with no evidence of efflux. *Para*-coumaric acid is known to be absorbed by the monocarboxylic acid transporter (MCT) in Caco-2 cells (Konishi *et al*., 2004) with rapid absorption in the GIT in an intact form (Konishi *et al*., 2004). Apart from *para*-coumaric acid showing a comparatively high absorption rate, the two other major transported phenolic compounds (1 and 3) showed a moderate rate of absorption. The other less bioavailable phenolic compounds are shown to have a low absorption rate and underwent efflux back into the apical compartment.

In comparison to the compounds in the extract, the P_{app} values of the pure compounds (protocatechuic acid, caffeic acid and *para*-coumaric acid) showed relatively fast transport rates 70.20 $\times 10^6$, 22.72 $\times 10^6$ and 46.60 $\times 10^6$ cm/s respectively (Table 10). Their respective efflux ratio values were <2, similar to those present in the extract (Table 9). These results indicate that compared to the P_{app} of the same compounds in the extract, the compounds in pure forms pass through the Caco-2 monolayer at relatively faster rates (Table 10). This faster rate of transport of the major compounds in the pure form may suggest that there is some competitive transport between the compounds in the extract. However, this cannot be concluded as the concentration of the pure compounds was not standardized to that found in the extract.

In summary, not only was *p*-CA identified for the first time in an aqueous extract of *A. phylicoides* as one of the major transported phenolic compounds, it also had the

highest predicted absorption of the compounds identified in the extract. ChA and quinic acids have been shown to be well absorbed in humans and rats (Meng *et al*., 2013; Zhao *et al*., 2012) and *in vivo* experiments in the rat have shown that *p*-CA can be absorbed in all sections of the GIT (stomach, jejunum, ileum and colon) with the highest absorption in the jejunum (Putschögl *et al*., 2008). In previous *in vitro* Caco-2 transport experiments, CA was shown to be rapidly absorbed compared with its esterified precursor, ChA (Konishi and Kobayashi, 2004). *p*-CA is known to have a much higher bioavailability than its conjugates ChA and CA (Konishi *et al*., 2004; Konishi and Kobayashi. 2004), which is consistent with our studies.

This study has illuminated the potentially bioactive compounds present in an aqueous extract of *A. phylicoides* with a special focus on their respective intestinal absorption dynamics. However, none of the identified compounds are unique to *A. phylicoides*. Many of the phenolic compounds present in *A. phylicoides* are well known bioactives present in in various fruits, vegetables and coffee. As such, *p*-CA exists either in free or conjugated form (Pei *et al*., 2016) with structural similarities to CA and PCA (Alamed *et al*., 2009; Choi *et al*., 2010; Scalbert and Williamson, 2000). Others have described the biological activities of *p*-CA to include hypolipidemic, anti-diabetic and anti-oxidant effects (Kusirisin *et al*., 2009; Pei *et al*., 2016; Torres, 2001). *In vivo*, *p*-CA improved plasma insulin levels in diabetic rats and has been shown to attenuate the oxidative stress associated with their disease state (Amalan *et al*., 2016; Kusirisin *et al*., 2009). ChA, has received a great amount of attention by displaying convincing anti-diabetic effects through regulating GLUT-4 in L-6 myotubes as well as increasing PPAR-γ gene expression (Meng *et al*., 2013; Upadhyay and Mohan, 2013). CA, 3,4-dicaffeoylquinnic acid, PCA and other hydroxycinnamic acids, have also been previously reported to be responsible for modulating diabetes, anti-oxidant and anti-inflammatory effects (De Beer *et al*., 2011; Harini and Pugalendi, 2010; Joubert and Schultz, 2012).

CHAPTER 6

Conclusion

6.1. Conclusion

With the increasing changing socio-economic conditions, adaptations to unhealthy Western lifestyles and the undesirable side effects of the currently used anti-diabetic therapeutics, interest has been drawn to the use of indigenous plants as adjuncts to conventional therapies (Baharvand *et al*., 2016; Denver *et al*., 2016; Joubert and Schultz, 2012; Van Wyk and Gericke, 2000). Scientific verification of the potential health benefits of plant-based therapeutics has been documented. Knowledge about the efficacy, absorption and bioavailability of the plant is essential in order to gain some insight into its pharmaceutical relevance.

In this study, we have measured the transport of major phenolic compounds present within an aqueous extract of *A. phylicoides* across a fully differentiated Caco-2 cell monolayer. Of the nine identified compounds, *para*-coumaric acid was identified for the first time in this extract. Not only was this compound identified for the first time but it was also observed to have a relatively faster absorption rate, thus suggesting a high predicted bioavailability. Evidence from this study allows speculation that *p*-CA could be chiefly responsible for the biological activities elicited by *A. phylicoides*. However, the biological activities of the major identified compounds in their pure form was not studied and perhaps further studies will provide an appreciable insight to confirming this speculation.

Future work

Using the knowledge gained form this study, further experiments will include testing the biological activities of the compounds found in *A. phylicoides* in their pure forms as well as the combinations thereof to elucidate any possible synergistic effect. Monitoring compound transport in co-cultured cells (Caco-2 monolayers with human liver cells) will also be useful in order to fully investigate the absorption and metabolism of the major phenolic compounds.

References

Alamed, J., Chaiyasit, W., McClements, D.J., Decker, E.A., 2009. Relationships between free radical scavenging and antioxidant activity in foods. *Journal of agricultural and food chemistry* **57**(5), pp.2969-2976.

Amalan, V., Vijayakumar, N., Indumathi, D., Ramakrishnan, A., 2016. Antidiabetic and antihyperlipidemic activity of *p*-coumaric acid in diabetic rats, role of pancreatic GLUT 2: *In vivo* approach. *Biomedicine and Pharmacotherapy* **84**, pp.230-236.

Amoroso, S., Schmid-Antomarchi, H., Fosset, M., Lazdunski, M., 1990. Glucose, sulfonylureas, and neurotransmitter release: role of ATP-sensitive K+ channels. *Science* **247** (4944), pp.852-854.

Artursson, P., 1990. Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorbtive (Caco-2) cells. *Journal of Pharmaceutical Sciences* **79**(6), pp.476-482.

American Diabetes Association., 2010. Diagnosis and classification of diabetes mellitus. *Diabetes Care* **33**(1), pp.S62-S69.

Atlas, I.D., 2015. International Diabetes Federation, Brussels, 2015. Available from: [Last accessed: 5 March 2014], *www.diabetesatlas.org/resources/2015-atlas.html.*

Auddy, B., Ferreira, M., Blasina, F., Lafon, L., Arredondo, F., Dajas, F., Tripathi, P., Seal, T., Mukherjee, B., 2003. Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. *Journal of Ethnopharmacology* **84**(2), pp.131-138.

Awortwe, C., Fasinu, P., Rosenkranz, B., 2014. Application of Caco-2 cell line in herb-drug interaction studies: current approaches and challenges. *Journal of pharmacy and pharmaceutical sciences: a publication of the Canadian Society for Pharmaceutical Sciences* **17**(1),pp.1.

Bahadoran, Z., Mirmiran, P., Azizi, F., 2013. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. J*ournal of Diabetes and Metabolic Disorders* **12**(1), pp.1.

Baharvand-Ahmadi, B., Bahmani, M., Tajeddini, P., Naghdi, N., Rafieian-Kopaei, M., 2016. An ethno-medicinal study of medicinal plants used for the treatment of diabetes. *Journal of Nephropathology* **5**(1), pp.44.

Bellamy, L., Casas, J.P., Hingorani, A.D., Williams, D., 2009. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *The Lancet* **373** (9677), pp.1773-1779.

Boulton, A.J., Vinik, A.L., Arezzo, J.C., Bril, V., Feldman, E.L., Freeman, R., Malik, R.A., Maser, R.E., Sosenko, J.M., Ziegler, D., 2005. Diabetic neuropathies a statement by the American Diabetes Association. *Diabetes Care* **28**(4), pp.956-962.

Bowker, S.L., Majumdar, S.R., Veugelers, P., Johnson, J.A., 2006. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* **29**(2), pp.254-258.

Cakir, A., Mavi, A., Yıldırım, A., Duru, M.E., Harmandar, M., Kazaz, C., 2003. Isolation and characterization of antioxidant phenolic compounds from the aerial parts of Hypericum hyssopifolium L. by activity-guided fractionation. *Journal of Ethnopharmacology* **87**(1), pp.73-83.

Cai, Y., Luo, Q., Sun, M. and Corke, H., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences* **74**(14), pp.2157-2184.

Chao, P., Hsu, C., Yin, M., 2009. Anti-inflammatory and anti-coagulatory activities of caffeic acid and ellagic acid in cardiac tissue of diabetic mice. *Nutrition and Metabolism* **6**(1), pp.1.

Charman, W.N., Porter, C.J., Mithani, S., Dressman, J.B., 1997. Physicochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH. J*ournal of Pharmaceutical Sciences* **86**(3), pp.269-282.

Chellan, N., 2011. The antidiabetic and antioxidant properties of *Athrixia phylicoides* aqueous extract: an *in vitro* and *ex vivo* assessment. Masters dissertation, University of Stellenbosch.

Chellan, N., De Beer, D., Muller, C., Joubert, E., Louw, J., 2008. A toxicological assessment of *Athrixia phylicoides* aqueous extract following sub-chronic ingestion in a rat model. *Human and Experimental Toxicology* **27**(11), pp.819-825.

Chellan, N., Muller, C., De Beer, D., Joubert, E., Page, B., Louw, J., 2012. An *in vitro* assessment of the effect of *Athrixia phylicoides* DC. aqueous extract on glucose metabolism. *Phytomedicine* **19**(8), pp.730-736.

Chiang, J.L., Kirkman, M.S., Laffel, L.M., Peters, A.L., 2014. Type 1 diabetes through the life span: a position statement of the American Diabetes Association. *Diabetes Care* **37**(7), pp.2034-2054.

Chiasson, J.L., Josse, R.G., Gomis, R., Hanefeld, M., Karasik, A., Laakso, M., 2002. Acarbose for prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial. *The Lancet* **359**, pp.2072-2077.

Choi, U.K., Lee, O.H., Yim, J.H., Cho, C.W., Rhee, Y.K., Lim, S.I., Kim, Y.C., 2010. Hypolipidemic and antioxidant effects of dandelion (Taraxacum officinale) root and leaf on cholesterol-fed rabbits. *International Journal of Molecular Sciences* **11**(1), pp.67-78.

Da Silva, J.B, J., Marinho, D.T., Bersani, D.A., Dos Reis, S.C., 2015. Evaluating Potential P-gp Substrates: Main Aspects to Choose the Adequate Permeability Model for Assessing Gastrointestinal Drug Absorption. *Mini Reviews in Medicinal Chemistry* **15**(10), pp.858-871.

Dahan, A., Amidon, G.L., 2008. Segmental dependent transport of low permeability compounds along the small intestine due to P-glycoprotein: the role of efflux transport in the oral absorption of BCS class III drugs. *Molecular Pharmaceutics* **6**(1), pp.19-28.

De Beer, D., Joubert, E., Malherbe, C.J., Brand, D.J., 2011. Use of countercurrent chromatography during isolation of 6-hydroxyluteolin-7-*O*-β-glucoside, a major antioxidant of *Athrixia phylicoides*. J*ournal of Chromatography A* **1218**(36), pp.6179-6186.

Deacon, C.F., 2016. In : Handbook od incretin-based Therapies in Type 2 Diabetes. Oxford: Springer International Publishing.

Denver, D., Diana, G., Quinton, J., 2016. Ethnobotanical survey of medicinal plants used to manage High Blood Pressure and Type 2 Diabetes Mellitus in Bitterfontein, Western Cape Province, South Africa. *Journal of Ethnopharmacology*, **194**, pp.755-766.

Deshpande, A., Rhodes, C., Shah, N., Malick, A., 1996. Controlled-release drug delivery systems for prolonged gastric residence: an overview. *Drug development and Industrial Pharmacy* **22**(6), pp.531-539.

Dobson, P.D., Kell, D.B., 2008. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule. *Nature Reviews Drug Discovery* **7**(3), pp.205-220.

Doguer, C., Shay, N., Collins, J.F., 2016. Improved Bioavailability of Iron from Amino Acid Chelates in Fully Differentiated Human Intestinal Epithelial (Caco-2) Cells. *The Federation of European Biochemical Societies Journal* **30**(1), pp.918.

Donovan, J.L., Crespy, V., Manach, C., Morand, C., Besson, C., Scalbert, A., Rémésy, C., 2001. Catechin is metabolized by both the small intestine and liver of rats. The *Journal of Nutrition* **131**(6), pp.1753-1757.

Farrell, T.L., Poquet, L., Dew, T.P., Barber, S., Williamson, G., 2012. Predicting phenolic acid absorption in Caco-2 cells: a theoretical permeability model and mechanistic study. *Drug Metabolism and Disposition* **40**(2), pp.397-406.

Fox, B., James, K., Morgan, B., Seed, A., 1982. Distribution of fatty and fibrous plaques in young human coronary arteries. *Atherosclerosis* **41**(2), pp.337-347.

Friedman, M., Jürgens, H.S., 2000. Effect of pH on the stability of plant phenolic compounds. *Journal of Agricultural and Food Chemistry* **48**(6), pp.2101-2110.

García-Compeán, D., González-González, J.A., Lavalle-González, F.J., González-Moreno, E.I., Maldonado-Garza, H.J., Villarreal-Pérez, J.Z., 2015. The treatment of diabetes mellitus of patients with chronic liver disease. *Annals of Hepatology* **14**, pp.780-788.

Gelderblom, W., Joubert, E., Gamieldien, K., Sissing, L., Malherbe, C., Maritz, G., 2016. Rooibos (Aspalathus linearis), honeybush (*Cyclopia* intermedia) and cancer bush (Sutherlandia frutescens subsp. microphylla) protect against tobacco-specific mutagenesis *in vitro*. *South African Journal of Botany*.

Germishuizen, G., Meyer, N.L., Steenkamp, Y., Keith, M., 2006. A Checklist of South African Plants. *Southern African Botanical Diversity Network* Report No. 41*,* Pretoria.

Giacomini, K.M., Huang, S.M., Tweedie, D.J., Benet, L.Z., Brouwer, K.L., Chu, X., Dahlin, A., Evers, R., Fischer, V., Hillgren, K.M., Hoffmaster, K.A., 2010. Membrane transporters in drug development. *Nature Reviews Drug Discover*y, **9**(3), pp.215-236.

Gonthier, M.P., Verny, M.A., Besson, C., Rémésy, C., Scalbert, A., 2003. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. The *Journal of Nutrition* **133**(6), pp.1853-1859.

Harini, R., Pugalendi, K.V., ,2010. Antihyperglycemic effect of protocatechuic acid on streptozotocin-diabetic rats. *Journal of Basic and Clinical Physiology and Pharmacology* **21**(1), pp.79-92.

Hochman, J.H., Chiba, M., Nishime, J., Yamazaki, M., Lin, J.H., 2000. Influence of P-glycoprotein on the transport and metabolism of indinavir in Caco-2 cells expressing cytochrome P-450 3A4. *Journal of Pharmacology and Experimental Therapeutics* **292**(1), pp.310-318.

Hollman, P.C., 2004. Absorption, bioavailability, and metabolism of flavonoids. *Pharmaceutical Biology* **42**, pp.74-83.

Hollman, P.C., van Trijp, J.M., Buysman, M.N., Mengelers, M.J., de Vries, J.H. and Katan, M.B., 1997. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *Federation of European Biochemical Societies Letters*, **418**(1-2), pp.152-156.

Hsu, S., Chung, H.Y., 2000. Interactions of konjac, agar, curdlan gum, κ-carrageenan and reheating treatment in emulsified meatballs. *Journal of Food Engineering* **44**(4), pp.199-204.

Huang, Y.T., Hwang, J.J., Lee, P.P., Ke, F.C., Huang, J.H., Huang, C.J., Kandaswami, C., Middleton, E., Lee, M.T., 1999. Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on cell growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. *British Journal of Pharmacology*, **128**(5), pp.999-1010.

Hubatsch, I., Ragnarsson, E.G., Artursson, P., 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nature Protocols* **2**(9), pp.2111-2119.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A.B., 1996. Zulu medicinal plants: an inventory. KwaZulu-Natal: University of Kwazulu Natal Press.

Illum, L., 2000. Transport of drugs from the nasal cavity to the central nervous system. *European Journal of Pharmaceutical Sciences* **11**(1), pp.1-18

Irons, B.K., Minze, M.G., 2014. Drug treatment of type 2 diabetes mellitus in patients for whom metformin is contraindicated. Diabetes, metabolic syndrome and obesity: *Targets and Therapy* **7**, pp.15.

Jancova, P., Anzenbacher, P. and Anzenbacherova, E., 2010. Phase II drug metabolizing enzymes. *Biomedical Papers*, **154**(2), pp.103-116.

Jiang, R.W., Lau, K.M., Hon, P.M., Mak, T.C., Woo, K.S., Fung, K.P., 2005. Chemistry and biological activities of caffeic acid derivatives from Salvia miltiorrhiza. *Current Medicinal Chemistry* **12**(2), pp.237-246.

Joo, S., Kim, E., Kang, W., 2016. Effect of terpinyl acetate on the percutaneous absorption of caffeine in rats. Experimental Dermatology, Accepted Author Manuscript. doi:10.1111/exd.13219.

Joubert, E., De Beer, D., ,2011. Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany* **77**(4), 869-886.

Joubert, E., Gelderblom, W.C., Louw, A., De Beer, D., 2008. South African herbal teas: *Aspalathus linearis*, *Cyclopia spp*. and *Athrixia phylicoides*--a review. *Journal of Ethnopharmacology* **119**(3), pp.376-412.

Joubert, E., Schultz, H., 2012. Production and quality aspects of rooibos tea and related products. A review. *Journal of Applied Botany and Food Quality* **80**(2), pp.138-144.

Jung, E.H., Ran Kim, S., Hwang, I.K., Youl Ha, T., 2007. Hypoglycemic effects of a phenolic acid fraction of rice bran and ferulic acid in C57BL/KsJ-*db/db* mice. *Journal of Agricultural and Food Chemistry* **55**(24), pp.9800-9804.

Jung, U.J., Lee, M.K., Park, Y.B., Jeon, S.M., Choi, M.S., 2006. Antihyperglycemic and antioxidant properties of caffeic acid in *db/db* mice. *Journal of Pharmacology and Experimental Therapeutics* **318**(2), pp.476-483.

Kahn, S., 2003. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of type 2 diabetes. *Diabetologia* **46**(1), pp.3-19.

Kahn, S.E., D'Alessio, D.A., Schwartz, M.W., Fujimoto, W.Y., Ensinck, J.W., Taborsky, G.J., Porte, D., 1990. Evidence of cosecretion of islet amyloid polypeptide and insulin by β-cells. *Diabetes* **39**(5), pp.634-638.

Kahn, S.E., Hull, R.L., Utzschneider, K.M., 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444**(7121), pp.840-846.

Kakkar, S., Bais, S., 2014. A review on protocatechuic acid and its pharmacological potential. *International Scholarly Research Network pharmacology*, pp. 952943-952943.

Kamimori, G.H., Karyekar, C.S., Otterstetter, R., Cox, D.S., Balkin, T.J., Belenky, G.L., Eddington, N.D., 2002. The rate of absorption and relative bioavailability of caffeine administered in chewing gum versus capsules to normal healthy volunteers. *International Journal of Pharmaceutics* **234**(1), pp.159-167.

Karakaya, S., 2004. Bioavailability of phenolic compounds. *Critical Reviews in Food Science and Nutrition* **44**(6), pp.453-464.

Karakaya, S.S.N., 1999. Quercetin, luteolin, apigenin and kaempferol contents of some foods. *Food Chemistry* **66**(3), pp.289-292.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., Ferrara, N., 1993. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*, *Nature*, **362**(6423), pp. 841-844.

Konishi, Y., Hitomi, Y., Yoshioka, E., 2004. Intestinal absorption of *p*-coumaric and gallic acids in rats after oral administration. *Journal of Agricultural and Food Chemistry* **52**(9), pp.2527-2532.

Konishi, Y., Kobayashi, S., 2004. Microbial metabolites of ingested caffeic acid are absorbed by the monocarboxylic acid transporter (MCT) in intestinal Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry* **52**(21), pp.6418-6424.

Konishi, Y., Kobayashi, S., Shimizu, M., 2003. Tea polyphenols inhibit the transport of dietary phenolic acids mediated by the monocarboxylic acid transporter (MCT) in intestinal Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry* **51**(25), pp.7296-7302.

Kunte, H., Schmidt, S., Eliasziw, M., del Zoppo, G.J., Simard, J.M., Masuhr, F., Weih, M., Dirnagl, U., 2007. Sulfonylureas improve outcome in patients with type 2 diabetes and acute ischemic stroke. *Stroke* **38**(9), pp.2526-2530.

Kusirisin, W., Srichairatanakool, S., Lerttrakarnnon, P., Lailerd, N., Suttajit, M., Jaikang, C., Chaiyasut, C., 2009. Antioxidative activity, polyphenolic content and anti-glycation effect of some Thai medicinal plants traditionally used in diabetic patients. *Medicinal Chemistry* **5**(20), pp.139-147.

Lafay, S., Gil-Izquierdo, A., Manach, C., Morand, C., Besson, C., Scalbert, A., 2006a. Chlorogenic acid is absorbed in its intact form in the stomach of rats. *The Journal of Nutrition* **136**(5), pp.1192-1197.

Lafay, S., Morand, C., Manach, C., Besson, C., Scalbert, A., 2006b. Absorption and metabolism of caffeic acid and chlorogenic acid in the small intestine of rats. *British Journal of Nutrition* **96**(1), pp.39-46.

Landete, J., 2012. Updated knowledge about polyphenols: functions, bioavailability, metabolism, and health. *Critical Reviews in Food Science and Nutrition* **52**(10), pp.936-948.

Lehlohonolo, N., Mariga, I.K., Ngezimana, W., Mudau, F.N., 2013. bush tea (*Athrixia phylicoides* dc.) success stories in south africa a review. *Crop Production* **2**(2), pp.37-43.

Lerotholi, L., Chaudhary, S., Combrinck, S., Viljoen, A., 2016. Bush tea (*Athrixia phylicoides*): A review of the traditional uses, bioactivity and phytochemistry. *South African Journal of Botany*.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 2012. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Reviews* **64**, pp.4-17.

Lipska, K.J., Bailey, C.J., Inzucchi, S.E., 2011. Use of metformin in the setting of mild-to-moderate renal insufficiency. *Diabetes Care* **34**(6), pp.1431-1437.

Liu, C.L., Wang, J.M., Chu, C.Y., Cheng, M.T., Tseng, T.H., 2002. *In vivo* protective effect of protocatechuic acid on tert-butyl hydroperoxide-induced rat hepatotoxicity. *Food Chemical Toxicology* **40**(5), pp.635-641.

Manach, C., Morand, C., Crespy, V., Demigné, C., Texier, O., Régérat, F., Rémésy, C., 1998. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *The Federation of European Biochemical Societies letters* **426**(3), pp.331-336.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* **79**(5), pp.727-747.

Martinez, M.N., Amidon, G.L., ,2002. A mechanistic approach to understanding the factors affecting drug absorption: a review of fundamentals. *The Journal of Clinical Pharmacology* **42**(6), pp.620-643.

Masereeuw, R., Russel, F.G., 2001. Mechanisms and clinical implications of renal drug excretion. *Drug Metabolism Reviews* **33**(3-4), pp.299-351.

Mashimbye, M.J., Mudaua, F.N., Soundy, P., Van Ree, T., 2015. A new flavonol from *Athrixia phylicoides* (bush tea). *South African Journal of Chemistry* **59**(1), pp.1-2.

Mavundza, E.J., 2010. Antioxidant and antibacterial activities of ethanol extract and flavonoids isolated from *Athrixia phylicoides*. Doctoral dissertation, University of Pretoria.

McGaw, L.J., Bagla, V.P., Steenkamp, P.A., Fouche, G., Olivier, J., Eloff, J.N., Myer, M.S., 2013. Antifungal and antibacterial activity and chemical composition of polar and non-polar extracts of *Athrixia phylicoides* determined using bioautography and HPLC. *BMC Complementary and Alternative Medicine* **13**(1), pp.1.

McGaw, L.J., Steenkamp, V., Eloff, J.N., 2007. Evaluation of *Athrixia* bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. *Journal of Ethnopharmacology* **110**(1), pp.16-22.

Meng, S., Cao, J., Feng, Q., Peng, J., Hu, Y., 2013. Roles of chlorogenic acid on regulating glucose and lipids metabolism: a review. *Evidence-Based Complementary and Alternative Medicine*, pp.1-16.

Mohn, E.S., Johnson, E.J. 2015. Nanotechnology and Functional Foods: Effective Delivery of Bioactive Ingredients. West Sussex: John Wiley and Sons Ltd.

Mor, A., Berencsi, K., Svensson, E., Rungby, J., Nielsen, J., Friborg, S., Brandslund, I., Christiansen, J., Vaag, A., Beck-Nielsen, H., 2015. Prescribing practices and clinical predictors of glucose-lowering therapy within the first year in people with newly diagnosed Type 2 diabetes. *Diabetic Medicine* **32**(12), 1546-1554.

Mudau, F.N., Araya, H.T., Du Toit, E.S., Soundy, P., Olivier, J., 2007. Bush tea (*Athrixia phylicoides* DC.) as an alternative herbal and medicinal plant in Southern Africa: Opportunity for Commercialization.

Muoio, D.M., Newgard, C.B., 2008. Molecular and metabolic mechanisms of insulin resistance and β-cell failure in type 2 diabetes. *Nature Reviews Molecular Cell Biology* **9**(3), pp.193-205.

Murakami, T., Takano, M., 2008. Intestinal efflux transporters and drug absorption. *Expert Opinion on Drug Metabolism and Toxicology* **4**(7), pp.923-939.

Murota, K. and Terao, J., 2003. Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. *Archives of Biochemistry and Biophysics* **417**(1), pp.12-17

Nardini, M., Cirillo, E., Natella, F., Scaccini, C., 2002. Absorption of phenolic acids in humans after coffee consumption. *Journal of Agricultural and Food Chemistry* **50**(20), pp.5735-5741.

Nchabeleng, L., Mudau, F., Mariga, I., 2012. Effects of chemical composition of wild bush tea (*Athrixia phylicoides* DC.) growing at locations differing in altitude, climate and edaphic factors. *Journal of Medicinal Plants Research* **6**(9), pp.1662-1666.

Nijveldt, R.J., Van Nood, E., Van Hoorn, D.E., Boelens, P.G., Van Norren, K., Van Leeuwen, P.A., 2001. Flavonoids: a review of probable mechanisms of action and potential applications. *The American Journal of Clinical Nutrition* **74**(4), pp.418-425.

Niwa, Y., Miyachi, Y., 1986. Antioxidant action of natural health products and Chinese herbs. *Inflammation* **10**(1), pp.79-91.

Noriega-Cisneros, R., Ortiz-Ávila, O., Esquivel-Gutiérrez, E., Clemente-Guerrero, M., Manzo-Avalos, S., Salgado-Garciglia, R., Cortés-Rojo, C., Saavedra-Molina, A., 2012. Improvement in blood lipid levels by Eryngium carlinae in diabetic rats. *The Federation of American Societies for Experimental Biology Journal* **26**(1 Supplement), pp.759-5.

Oksana, S., Marian, B., Mahendra, R., Bo, S.H., 2012. Plant phenolic compounds for food, pharmaceutical and cosmetics production. J*ournal of Medicinal Plants Research* **6**(13), pp.2526-2539.

Olthof, M.R., Hollman, P.C., Buijsman, M.N., Van Amelsvoort, J.M., Katan, M.B., 2003. Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. *The Journal of Nutrition* **133**(6), pp.1806-1814.

Olthof, M.R., Hollman, P.C., Katan, M.B., 2001. Chlorogenic acid and caffeic acid are absorbed in humans. *The Journal of Nutrition* **131**(1), pp.66-71.

Padayachee, K., ,2011. The phytochemistry and biological activities of *Athrixia phylicoides*. Doctoral dissertation, University of the Witwatersrand.

Pallasch, T.J., 1988. Principles of pharmacotherapy: II. Pharmacokinetics. *Anesthesia Progress*, **35**(4), p.133.

Pei, K., Ou, J., Huang, J., Ou, S., 2016. *p*-Coumaric acid and its conjugates: dietary sources, pharmacokinetic properties and biological activities. *Journal of the Science of Food and Agriculture* **96**(1), pp.2952-2962.

Pietta, P., Simonetti, P., Gardana, C., Brusamolino, A., Morazzoni, P., Bombardelli, E., 1998a. Catechin metabolites after intake of green tea infusions. *Biofactors* **8**(1,2), pp.111-118.

Pietta, P., Simonetti, P., Mauri, P., 1998b. Antioxidant activity of selected medicinal plants. *Journal of Agricultural and Food Chemistry* **46**(11), pp.4487-4490.

Plumb, G.W., Garcia-Conesa, M.T., Kroon, P.A., Rhodes, M., Ridley, S., Williamson, G., 1999. Metabolism of chlorogenic acid by human plasma, liver, intestine and gut microflora. *Journal of the Science of Food and Agriculture* **79**(3), pp.390-392.

Price, D.B., Ackland, M.L., Burks, W., Knight, M.I., Suphioglu, C., 2014. Peanut Allergens Alter Intestinal Barrier Permeability and Tight Junction Localisation in Caco-2 Cell Cultures1. *Cellular Physiology and Biochemistry* **33**(6), pp.1758-1777.

Prime-Chapman, H.M., Fearn, R.A., Cooper, A.E., Moore, V., Hirst, B.H., 2004. Differential multidrug resistance-associated protein 1 through 6 isoform expression and function in human intestinal epithelial Caco-2 cells. *Journal of Pharmacology and Experimental Therapeutics* **311**(2), pp.476-484.

Putschögl, M., Zirak, P., Penzkofer, A., 2008. Absorption and emission behaviour of trans-*p*-coumaric acid in aqueous solutions and some organic solvents. *Chemical Physics* **343**(1), pp.107-120.

Rampedi, I., Olivier, J., 2005. The use and potential commercial development of *Athrixia phylicoides*. *Acta Academica-University of the Free State* **37**(3), pp.165.

Rechner, A.R., Smith, M.A., Kuhnle, G., Gibson, G.R., Debnam, E.S., Srai, S.K.S., Moore, K.P., Rice-Evans, C.A., 2004. Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radical Biology and Medicine* **36**(2), pp.212-225.

Robbins., R.J., 2003. Phenolic acids in foods: an overview of analytical methodology. *Journal of Agricultural and Food Chemistry* **51**(10), pp.2866-2887.

Roberts, C.K., Hevener, A.L., Barnard, R.J., 2013. Metabolic Syndrome and Insulin Resistance: Underlying Causes and Modification by Exercise Training. *Comprehensive Physiology* **3**, pp.1-58.

Russel, F.G., 2010. Enzyme-and transporter-based drug-drug interactions. New York: Springer.

Saitoh, R., Sugano, K., Takata, N., Tachibana, T., Higashida, A., Nabuchi, Y., Aso, Y., 2004. Correction of permeability with pore radius of tight junctions in Caco-2 monolayers improves the prediction of the dose fraction of hydrophilic drugs absorbed by humans. *Pharmaceutical Research* **21**(5), pp.749-755.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M., Stammati, A., Zucco, F., 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-

related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology* **21**(1), pp.1-26.

Sarmento, B., Andrade, F., Da Silva, S.B., Rodrigues, F., Das Neves, J., Ferreira, D., 2012. Cell-based *in vitro* models for predicting drug permeability. *Expert Opinion on Drug Metabolism and Toxicology* **8**(5), pp.607-621.

Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., Sugawara, M., Iseki, K., 2011. *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. *International Journal of Pharmaceutics* **403**(1), pp.136-138.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., Jiménez, L., 2005. Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition* **45**(4), pp.287-306.

Scalbert, A., Williamson, G., 2000. Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition* **130**(8), 2073S-2085S.

Scazzocchio, B., Varì, R., Filesi, C., D'Archivio, M., Santangelo, C., Giovannini, C., Iacovelli, A., Silecchia, G., Volti, G.L., Galvano, F., 2011. Cyanidin-3-O-β-glucoside and protocatechuic acid exert insulin-like effects by upregulating PPARγ activity in human omental adipocytes. *Diabetes* **60**(9), pp.2234-2244.

Scheepens, A., Tan, K. and Paxton, J.W., 2010. Improving the oral bioavailability of beneficial polyphenols through designed synergies. *Genes and Nutrition*, **5**(1), pp.75-87.

Schulze, C., 2014. From molecule to men: Inhibition of intestinal glucose absorption by polyphenols and plant extracts for reducing the glycemic response. Doctoral dissertation, Universitätsbibliothek der TU München.

Semaming, Y., Kukongviriyapan, U., Kongyingyoes, B., Thukhammee, W., Pannangpetch, P., 2016. Protocatechuic Acid Restores Vascular Responses in Rats With Chronic Diabetes Induced by *Streptozotocin*. *Phytotherapy Research* **30**(2), pp.227-233.

Shairibha, S.R., Rajadurai, M., Kumar, N.A., 2014. Effect of *p*-coumaric acid on biochemical parameters in streptozotocin-induced diabetic rats. *Journal of Academia and Industrial Research* **3**(5), pp.237.

Shaw, J.E., Sicree, R.A., Zimmet, P.Z., 2010. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice* **87**(1), pp.4-14.

Smith, L., Culvenor, C., 1981. Plant sources of hepatotoxic pyrrolizidine alkaloids. *Journal of Natural Products* **44**(2), pp.129-152.

Srinivasan, B., Kolli, A.R., Esch, M.B., Abaci, H.E., Shuler, M.L., Hickman, J.J., 2015. TEER measurement techniques for *in vitro* barrier model systems. *Journal of Laboratory Automation* **20**(2), pp.107-126.

Stalmach, A., Mullen, W., Barron, D., Uchida, K., Yokota, T., Cavin, C., Steiling, H., Williamson, G., Crozier, A., 2009. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug Metabolism and Disposition* **37**(8), pp.1749-1758.

Stumvoll, M., Goldstein, B.J., Van Haeften, T.W., 2005. Type 2 diabetes: principles of pathogenesis and therapy. *The Lancet* **365** (9467), pp.1333-1346.

Takenaka, T., Harada, N., Kuze, J., Chiba, M., Lwao, T., Matsunaga, T., 2016. Application of a Human Intestinal Epithelial Cell Monolayer to the Prediction of Oral Drug Absorption in Humans as a Superior Alternative to the Caco-2 Cell Monolayer. *Journal of Pharmaceutical Sciences* **105**(2), pp.915-924.

Tarko, T., Duda-Chodak, A., Zajac, N., 2013. Digestion and absorption of phenolic compounds assessed by *in vitro* simulation methods. *A review. Roczniki Państwowego Zakładu Higieny*, **64**(2), pp.79-84.

Ting, Y., Jiang, Y., Ho, C.T., Huang, Q., 2014. Common delivery systems for enhancing *in vivo* bioavailability and biological efficacy of nutraceuticals. *Journal of Functional Foods* **7**, pp.112-128.

Torres Y, R.J., John P.N., ,2001. Microbial Transformations of p-Coumaric Acid by Bacillus m egaterium and Curvularia l unata. *Journal of Natural Products* **64**(11), pp.1408-1414.

Touaibia, M., Jean-Francois, J., Doiron, J., 2011. Caffeic acid, a versatile pharmacophore: an overview. *Mini Reviews in Medicinal Chemistry* **11**(8), pp.695-713.

Upadhyay, R., Mohan, L.J., 2013. An outlook on chlorogenic acids-occurrence, chemistry, technology, and biological activities. *Critical Reviews in Food Science and Nutrition* **53**(9), pp.968-984.

Van Breemen, R.B., Li, Y., 2005. Caco-2 cell permeability assays to measure drug absorption. *Expert Opinion on Drug Metabolism and Toxicology* **1**(2), pp.175-185.

Van De Waterbeemd, H., Gifford, E., 2003. ADMET in silico modelling: towards prediction paradise. *Nature Reviews Drug Discovery* **2**(3), pp.192-204.

Van Wyk, B.E., Gericke, N., 2000. People's plants: A guide to useful plants of Southern Africa. Briza Publications, ISBN 1875093192.

Van der Heiden, M.G., Cantley, L.C., Thompson, C.B., 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**(5930), pp.1029-1033.

Varì, R., D'Archivio, M., Filesi, C., Carotenuto, S., Scazzocchio, B., Santangelo, C., Giovannini, C., Masella, R., 2011. Protocatechuic acid induces antioxidant/detoxifying enzyme expression through JNK-mediated Nrf2 activation in murine macrophages. *The Journal of Nutritional Biochemistry* **22**(5), pp.409-417.

Vetrani, C., Rivellese, A.A., Annuzzi, G., Adiels, M., Borén, J., Mattila, I., Orešič, M., Aura, A.M., 2016. Metabolic transformations of dietary polyphenols: comparison between *in vitro* colonic and hepatic models and *in vivo* urinary metabolites. The *Journal of Nutritional Biochemistry* **33**, pp.111-118.

Wardlaw, G., Kessel, M., 2002. Minerals: dietary needs, absorption, transport and excretion. Perspectives in Nutrition, 5th edition. New York: Mc Graw-Hill Companies Inc.

Watt, J.M., Breyer-Brandwijk, M.G., 1932. The Medicinal and Poisonous Plants of Southern Africa. The medicinal and poisonous plants of southern Africa.

Wessler, J.D., Grip, L.T., Mendell, J., Giugliano, R.P., 2013. The P-glycoprotein transport system and cardiovascular drugs. *Journal of the American College of Cardiology* **61**(25), pp.2495-2502.

Wild, S., Roglic, G., Green, A., Sicree, R., King, H., 2004. Global prevalence of diabetes estimates for the year 2000 and projections for 2030. *Diabetes Care* **27**(5), pp.1047-1053.

Wojdyło, A., Oszmiański, J., Czemerys, R., 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chemistry* **105**(3), pp.940-949.

Xia, C.Q., Milton, M.N., Gan, L.S., 2007. Evaluation of drug-transporter interactions using *in vitro* and *in vivo* models. *Current Drug Metabolism* **8**(4), pp.341-363.

Zhang, Y., Tie, X., Bao, B., Wu, X., Zhang, Y., 2007. Metabolism of flavone C-glucosides and p-coumaric acid from antioxidant of bamboo leaves (AOB) in rats. *British Journal of Nutrition* **97**(3), pp.484-494.

Zhao, Y., Wang, J., Ballevre, O., Luo, H., Zhang, W., 2012. Antihypertensive effects and mechanisms of chlorogenic acids. *Hypertension Research* **35**, pp.370-374.

Zhou, S.F., 2008. Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4. *Current Drug Metabolism*, **9**(4), pp.310-322.

Zimmerman, H.J., 1999. Hepatotoxicity: the adverse effects of drugs and other chemicals on the liver. 2nd edition. Philadelphia: Lippincott Williams and Wilkins.

Appendix A

List of materials

Description	Catalogue No.	Manufacturer
10mL Serological pipettes	760159	Greiner bio-one, Frickenhausen, Germany
10mL Syringes	R5MA55246	Merck Millipore
15mL Centrifuge tubes	188261	Greiner bio-one, Frickenhausen, Germany
25mL Serological pipettes	760160	Greiner bio-one, Frickenhausen, Germany
2mL Eppendorf Centrifuge tubes	30123301	Sigma-Aldrich, St Louis, MO, USA
3,4-dicaffeoylquinic acid	PubChem CID: 5281780	TransMIT GmbH ,Gieβen, Germany
3,5-dicaffeoylquinic acid	PubChem CID: 6474310	TransMIT GmbH ,Gieβen, Germany
4,5-dicaffeoylquinic acid	PubChem CID: 6474309	TransMIT GmbH ,Gieβen, Germany
4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT)	M2003	Sigma-Aldrich, St Louis, MO, USA
50mL Centrifuge tubes	227261	Greiner bio-one, Frickenhausen, Germany
50mL Serological pipettes	14120441	Greiner bio-one, Frickenhausen, Germany
75cm^2 flasks	658975	Greiner bio-one, Frickenhausen, Germany
ATP Vialight assay kit	LT27-008	Whitehead Scientific, JHB, SA
Bio-Rad Bradford protein assay kit	500-0201	Bio-Rad, Hercules, CA, USA
Bovine serum albumin (BSA)	100-10SB	Sigma-Aldrich, St Louis, MO, USA
Bradford assay kit	3#500-0203	Bio-Rad Laboratories, California, USA
Caco-2 cell line	812010202	Sigma originating from the European Collection of Cell Cultures, Salisbury in UK

Caffeic acid	PubChem CID: 689043	Sigma-Aldrich, St Louis, MO, USA
Caffeine	C0750	Sigma-Aldrich, St Louis, MO, USA
Carbon dioxide (CO_2)	K239C	Air Products, Centurion, SA
Cell bind -6 well plates	3335	Corning, MA, USA
Cell bind -96 well plates	3300	Corning, MA, USA
Cell counting chamber slides	C10228	Life Technologies Corporation, Carlsbad, CA, USA
Cell culture 96-well plates (non-cell bind)	655180	Greiner bio-one, Frickenhausen, Germany
Cell culture tested water	W3500	Sigma-Aldrich, St Louis, MO, USA
Cell extraction lysis Buffer	FNN0011	Life Technologies Corporation, Carlsbad, CA, USA
Cell scrapers	P0500	Sigma-Aldrich, St Louis, MO, USA
Centrifuge (Temperature control) Tubes; mL; 2.0 mL	K82967 3906	Sigma-Aldrich, St Louis, MO, USA
chlorogenic acid	PubChem CID: 1794427	Sigma-Aldrich, St Louis, MO, USA
Cryotubes	430659	Greiner Bio-One, Frickenhausen, Germany
Cryptochlorogenic acid	PubChem CID: 9798666	Vestenbergsgreuth, Nürnberg, Germany
Dimethyl sulfoxide (DMSO)	D4540	Sigma-Aldrich, St Louis, MO, USA
Donkey anti-mouse HRP	sc2318	Santa Cruz
Dulbecco's phosphate buffered saline (DPBS)	BE17-513F	Lonza, Walkersville, MD, USA
Eagles modified essential medium (EMEM)	12-662F	Lonza, Walkersville, MD, USA
Ethanol	2875	Sigma-Aldrich, St Louis, MO, USA
Ethanol absolute, 200 proof for molecular	E7023-500	Sigma, St Louise, MO,USA

Fetal bovine serum (FBS)	BC/S0615-HI	Thermo Fisher Scientific
Glycine	50046	Sigma-Aldrich, St Louis, MO, USA
Heat inactivated fetal Bovine serum (FBS)	1050-064	GIBCO, Invitrogen, Auckland
L-Glutamine	G8540	Sigma-Aldrich, St Louis, MO, USA
Lucifer yellow CH dipotassium salt	L0144	Sigma-Aldrich, St Louis, MO, USA
Lysis buffer	9803	Cell Signalling Technology, Danvers, MA, USA
Millex-GP syringe filter unit	SLGP033RS	Millipore PES membrane, Merck
NaOH	10252	AnalaR Laboratories, Poole, England
Neochlorogenic acid	PubChem CID: 5280633	PhytoLab GmbH & Co. KG
Non-fat milk powder clover	N/A	Pick and Pay
Para-coumaric acid	PubChem CID: 637542	Sigma-Aldrich, St Louis, MO, USA
Pasteur pipettes	6112 361	Greiner bio-one, Frickenhausen, Germany
Phenylmethanesulfonyl fluoride (PMSF)	11206893001	Roche, Basel, Switzerland
PolyvinylideneFluoridine Membrane (PVDF)	88585	Pierce, Rockford, IL, USA
Ponceau S Stain	p23295	Sigma-Aldrich , St Louis, MO, USA
Protocatechuic acid	PubChem CID: 72	Sigma-Aldrich, St Louis, MO, USA
Running buffer	161-0772	Bio-Rad, Hercules, CA, USA
SDS-PAGE gels	161-0993	Bio-Rad, Hercules, CA, USA
Serological pipettes-2ml	CLS4487	Corning, MA, USA
Serological pipettes-10ml	4101	Corning, MA, USA
Serological pipettes-25ml	4251	Corning, MA, USA
Serological pipettes-50ml	4490	Corning, MA, USA
Stainless steel beads 5mm	69989	Qiagen, Hilden, Germany

Sterile TC water	59900C	Lonza, Walkersville, MD, USA
Stripping buffer	46430	Thermo Fisher Scientific
Syringe Filters	SLGP033RS	Merck Millipore
Tris	93352	Sigma-Aldrich, St Louis, MO, USA
Trypan blue	T93595	Sigma-Aldrich , St Louis, MO, USA
Trypsin	17-161F	Lonza, Walkersville, MD, USA
Tween-20	58980C	Sigma-Aldrich , St Louis, MO, USA
ViaLight™ plus ATP kit	LT07-321	Lonza, Walkersville, MD, USA
Whatman 3 MMChr sheets	3030-931	Sigma-Aldrich , St Louis, MO, USA

List of reagents

1. 1 × Tris-buffered saline and Tween 20 (1 × TBST)

1 × 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.

2. Complete growth media (complete EMEM)

Complete media was made by adding 5 mL Pyruvate, 5 mL Sodium pyruvate, 50 mL FBS and 5 mL L-glutamine to 500 mL EMEM and carefully mixing. The media was kept at 4°C.

3. Transfer buffer for Western blot

25 mM Tris + 192 mM glycine + 200 mL made up to 1 L in distilled water. Buffer was kept at 4°C.

4. Sorensen's glycine buffer

Sorensen's glycine buffer was prepared by adding 0.751 g glycine (0.1M) +0.584g NaCl (0.1M) in 100 mL cell culture tested water.

5. Sodium hydroxide buffer

NaOH solution was made up by mixing NaOH (1M) in 100 mL cell culture tested water. The buffer pH was adjusted to pH 10.5.

Equipment

Agilent 1200 HPLC system	Agilent Technologies, Waldbronn, Germany
Benchtop centrifuge (SL16R)	Thermo Fisher Scientific, Waltham, MA, USA
BioTek ELX 800 plate reader	BioTek Instruments Inc, Winooski, VT, USA
Bio-Rad Gel Doc (170-8650)	Bio-Rad, Hercules, CA, USA
Biosafety cabinets	Airvolution
CO_2 incubator	RS Bio Tech
Equipment	Manufacturer/ Supplier
Fine balance scale (AR2140)	United scientific
FLX800 fluorescence microplate reader	BioTek 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
JEOL JEM-1011	JEOL Ltd, Tokyo, Japan
Kinetex C18 column (150 mm × 4.6 mm, 2.6 µm particle size, 100 Å)	Phenomenex, Santa Clara, CA, USA
Leica EM	Leica Microsystems, Wetzlar, Germany
Leica EM UC7	Leica Microsystems
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
Reichert TM60® block trimmer	Reichert, Vienna, Austria
Scale (ARC120)	United scientific
The Millicell-ERS volt ohmmeter	MERS00002 Merck KGaA, Darmstadt, Germany
Tissue lyser	Qiagen, Hilden, Germany
Trans-Blot Cell (170-4070)	Bio-Rad, Hercules, CA, USA
Waterbath	Memmert, Heilbronn, Germany

Appendix B

Research outputs

1. SEDMSA conference 14-17th April 2016

Poster: Abstract 20 page 16

Title: Intestinal absorption and metabolism of Bush tea major phenolic compounds Exhibiting Anti-diabetic activity

Ntamo Y[2], Muller C.J.F[1], Malherbe C.J[3], Kappo A.M.P[2], Louw J[1] and Bowles S[1]

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2. Early Career Young Scientist Convention 19-20th October 2016

Poster: page 116

Title: Intestinal absorption and metabolism of Bush tea major phenolic compounds Exhibiting Anti-diabetic activity

Ntamo Y[2], Muller C.J.F[1], Malherbe C.J[3], Kappo A.M.P[2], Louw J[1] and Bowles S[1]

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3. Manuscript to be submitted

Phenolic acids, major constituents of an aqueous extract of *Athrixia phylicoides*, are transported across a Caco-2 monolayer.

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Abstract

Ethnopharmacological relevance: Athrixia phylicoides (*A. phylicoides*), popularly known as `bush tea`, is an indigenous aromatic shrub found in mountainous and grassland areas of the northern and eastern parts of Southern Africa. The plant is traditionally used for the treatment of several ailments, including coughing, treating infected wounds, treating boils and sore throat, hypertension and heart disease. Potential ant-diabetic effects have also been demonstrated *in vitro*.

Aim of the study: To investigate the intestinal transport of phenolic constituents of a characterized aqueous extract of *A*. *phylicoides,* previously shown to have bioactivity, using a fully differentiated Caco-2 monolayer.

Materials and Methods: HPLC-DAD and LC/MS were used to identify and quantify phenolic compounds within the extract. Intestinal transport of the phenolic compounds was assessed using a differentiated Caco-2 monolayer model in order to predict bioavailability and identify metabolite formation. Rate of transport, efflux and percentage crossover were calculated for the respective phenolic compounds.

Results: Nine prominent compounds, present in the aqueous extract of *A. phylicoides,* were identified. Of these, three phenolic acids (protocatechuic acid, caffeic acid and *para*-coumaric acid), crossed the Caco-2 cell monolayer in significant amounts, with Papp values of 4.52, 4.35 (x 10^6 cm/s) and 2.38 (x 10^5 cm/s), respectively. *para*-Coumaric acid was shown to have the highest predicted bioavailability.

Conclusions: ***para*-Coumaric acid, identified for the first time in *A. phylicoides,* was shown to have the highest predicted bioavailability suggesting that it could play a major role in the bioactivity of *A. phylicoides*.**

Key words: *Athrixia phylicoides,* Caco-2 transport, protocatechuic acid, caffeic acid, *para*-coumaric acid.

Chemical compounds studied in this article: protocatechuic acid (PubChem CID: 72), caffeic acid (PubChem CID: 689043), *para*-coumaric acid (PubChem CID: 637542), chlorogenic acid (PubChem CID: 1794427), cryptochlorogenic acid (PubChem CID: 9798666), neochlorogenic acid (PubChem CID: 5280633), 3,4 di-*O*-caffeoylquinic acid (PubChem CID: 5281780), 3,5 di-*O*-caffeoylquinic acid (PubChem CID: 6474310), 4,5 di-*O*-caffeoylquinic acid (PubChem CID: 6474309)

1. Introduction

Athrixia phylicoides DC. (Family Asteraceae - daisy family, tribe Inucleae and subtribe Athrixiinae) is an aromatic, indigenous South African herbal shrub with medicinal properties inferred by ingestion of the plant (McGaw et al., 2007). It has been reported to have various traditional uses by a vast number of ethnic groups for several purposes including hypertension, diarrhea, coughing, wound healing, heart disease and diabetes (Mavundza, 2010; McGaw et al., 2007; Rampedi and Olivier, 2005). Scientific validation of its biological activity has focused on anti-oxidant, anti-bacterial, anti-inflammatory, anti-microbial and anti-malarial activities (de Beer et al., 2011; Mavundza, 2010; Padayachee, 2011). Of particular interest, are the potential metabolic benefits, including its anti-diabetic effects, attributed to the phenolic compounds of *A. phylicoides* (Chellan et al., 2012). These phenolic compounds include protocatechuic acid, caffeic acid and *para*-coumaric acid, which share structural similarities through their benzoic acid backbone. These compounds are differentiated by the addition of specific acrylic functional groups in different positions to the benzoic acid backbone (Robbins, 2003). Caffeic acid has displayed antidiabetic activity by decreasing fasting blood glucose levels in *db/db* mice (Jung et al., 2006; Meng et al., 2013). Protocatechuic acid is reported to possess anti-oxidant, anti-bacterial, hyperlipidemic as well as anti-diabetic activities (Lin et al., 2009; Zheng and Wang, 2001). *para*-Coumaric acid, has a high potency to attenuate oxidative stress associated with diabetes by activating PPARγ, adiponectin and the upregulation of the GLUT-4 transporter in liver cells (Jung et al., 2006; Kusirisin et al., 2009; Scazzocchio et al., 2011).

The use of natural products and their phenolic compounds as health supplements to assist in the management of chronic metabolic diseases is increasing (Semaming et al., 2016), however, there is limited knowledge concerning their bioavailability. Fundamental to the efficacy of any compound *in vivo*, is the degree to which it is absorbed into responsive target sites in the body (Ting et al., 2014). Caco-2 cells in their fully differentiated form, are structurally and functionally representative of human intestinal epithelial cells and are an important model to study the transport rate of compounds across the gastrointestinal tract (Farrell et al., 2012; Hubatsch et al., 2007). Establishing the absorptive dynamics of phenolic compounds from *A. phylicoides*, supported by pharmacokinetics, as well as dose optimization, is essential for launching its use as nutraceutical (Joubert et al., 2008). This study fully characterizes the prominent phenolic compounds present in an aqueous extract of *A. phylicoides* and, using a Caco-2 cell model, investigates their potential transport mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

All cell culture consumables were obtained from Corning (New York, USA) unless otherwise specified. Caco-2 cells were obtained from the European Collection of Cell Cultures (cat # 86010202) (Salisbury, England). Transwell inserts and 6 well plates were procured from SPL Life Sciences

(Gyeonggi-do, Korea). Verapamil hydrochloride, caffeine and HPLC gradient grade acetonitrile, solvents and other reagents of analytical grade were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Eagle's Minimum Essential Medium (EMEM), pyruvate, non-essential amino acids, L-Glutamine, trypsin-EDTA and Penicillin/Streptomycin were purchased from Lonza (Basel, Switzerland). Hyclone fetal bovine serum (FBS) was purchased from Thermo Scientific (Waltham, MA, USA). Laboratory grade deionized water was prepared using a Millipore Elix Advantage 5 water purification system (Merck Millipore, Darmstadt, Germany), which was further purified to HPLC grade water using a Milli-Q Reference A+ system (Merck Millipore). The Millicell-ERS volt ohmmeter was also obtained from Merck-Millipore.Calibration standards were purchased from the following suppliers; Sigma-Aldrich (as Fluka products: protocatechuic acid, caffeic acid and chlorogenic acid, and as Sigma products: *para*-coumaric acid and caffeine), PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Nürnberg, Germany) (neochlorogenic acid and cryptochlorogenic acid) and TransMIT GmbH (Gieβen, Germany) (3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid).

2.2. Plant material and extraction

Fine twigs and leaves from *A. phylicoides* DC. were harvested in June 2005 in the Bushbuckridge area (Limpopo, South Africa) from a natural population and identified by the South African National Biodiversity Institute (SANBI). The plant material was supplied by Prof. Jana Olivier, University of South Africa, and after air-drying at room temperature, a hot water extract of the plant material was prepared and stored. The same aqueous extract, as prepared by Chellan *et al.,* was used in the current study (Chellan et al., 2008).

2.3. Cell culture and treatment

Cells were cultured at standard culture conditions in EMEM containing sodium 100mM pyruvate supplemented with 2mM L-glutamine, 1% non-essential amino acids and 10% heat inactivated fetal bovine serum (FBS). Cell cultures were split when 70-80% confluent, using trypsin/EDTA. The culture medium was refreshed every 2-3 days with media pre-warmed to 37˚C. Cells were seeded at 1 x 10^4 cells per cm^2 and a sub-cultivation ratio of 1:3 was routinely used. For transport experiments cells were seeded with the addition of Penicillin/Streptomycin (10 000 units/ml Potassium Penicillin and 10 000 µg/ml Streptomycin sulfate) at 4 x 10^4 cells/cm^2 into 0.4 µm polycarbonate 6-well inserts with an insert area of 4.52 cm^2. To prevent phenotypic drift only cells from passage 50 – 60 were used.

2.4. Cytotoxicity of an aqueous extract of *A. phylicoides* against Caco-2 cells.

The cytotoxicity of the aqueous extract of *A. phylicoides* on Caco-2 cells was assessed using a ViaLight plus kit as per the manufacturer's instructions (Lonza, Walkersville, MD, USA). Briefly, Caco-2 cells were seeded at a density of 4 x 10^4 cells/cm^2 into a white clear-bottom 96-well plate and grown for 12 days. On the day of the experiment, the medium was replaced with transport buffer (HBSS with 10mM HEPES, pH 7.4) and incubated for 30 min at 37˚C under agitation (75 rpm), in order to wash the cells. The freeze-dried extract of *A. phylicoides* was dissolved in DMSO as a concentrated stock solution, and added to the transport medium at varying concentrations to establish the highest non-

toxic dose (final DMSO concentration was standardized to 0.25 %) and incubated for 2 hours. Cell viability was assessed as a measure of the ATP concentration/ levels as determined by using a Bio TEK ELX 800 plate reader (BioTek Instruments Inc., Winooski, VT, USA). Subsequently, the highest non-toxic dose of extract (~75% viability) was used for future experiments.

2.5. Transport experiments

The monolayer was cultured in complete EMEM for 23 days to ensure a fully differentiated intact Caco-2 monolayer. For the experiment, the media was removed, the cell monolayer washed with transport buffer, pH 6.0 (HBSS adjusted with MES) on the apical side and pH 7.4 (HBSS adjusted with HEPES) on the basolateral side to remove any traces of the culture media. Subsequently, the cultured monolayers were incubated with transport buffer for 30 min at standard cell culture conditions. After the removal of transport buffer, for apical to basolateral compartment studies, 1.5 mL treatment in HBSS (pH 6.0) was added to the apical compartment. For studies measuring transport in the opposite direction, 2.4 mL of treatment in HBSS (pH 7.4) was added to the basolateral compartment. Samples (1.2 mL) were withdrawn from the basolateral or the apical compartment respectively; at 0, 0.5, 1, 1.5 and 2 hours and replaced with equal volume of relative transport buffer, thereby maintaining sink conditions. Samples were stored at -65º C for further analysis by HPLC-DAD and LC-MS. To ensure stability of the samples, ascorbic acid, to a final concentration of 1 %, was added to each sample after which they were frozen in liquid nitrogen before storage at -65 ˚C. Monolayer integrity was maintained by ensuring TEER readings remained above 300 ohms throughout the experiment. In addition Lucifer yellow was added to the treatment, its passage was monitored spectrophotometrically and a measurement below 3 %, was deemed as indication of membrane integrity (Gerbella et al., 2014). The bidirectional transport of phenolic compounds was quantified by HPLC-DAD as described in section 2.6. To establish the rate of transport, the accumulated concentrations of the compounds were used to calculate the apparent permeability with 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 transwell inserts; C_0 is the initial concentration of treatment on the donor side (µM).

The bidirectional Papp values were used to calculate the efflux ratio where Papp_BA are the calculated permeability coefficient of basolateral to apical compartment and Papp_AB is permeability coefficient from apical to basolateral compartment the based on the equation:

$$\text{Efflux ratio} = \frac{Papp\_BA}{Papp\_AB}$$

To confirm efflux, transport experiments were performed in the presence and absence of the efflux (P-glycoprotein) inhibitor, verapamil hydrochloride (100 uM), that was added during the wash step as well as during the subsequent incubation step.

2.6. High-performance liquid chromatography with diode-array detection (HPLC-DAD)

A new method was developed, based in part on the existing method by (de Beer et al., 2011), and validated for the analysis of extracts of *Athrixia phylicoides* with special emphasis to improve quantification of the prominent phenolic compounds. Analysis was performed using an Agilent 1200 HPLC system consisting of a quaternary pump with incorporated in-line degasser, autosampler, column thermostat and diode-array detector (Agilent Technologies, Waldbronn, Germany). OpenLab CDS Chemstation software (Agilent Technologies) was used for instrument control and data analysis during HPLC-DAD analysis. Separation was achieved on a Kinetex C18 column (150 mm × 4.6 mm, 2.6 µm particle size, 100 Å) (Phenomenex, Santa Clara, CA, USA) protected by a guard column with the same stationary phase and a KrudKatcher Ultra inline filter (Phenomenex). The solvent system for separation consisted of 1% formic acid (A) and acetonitrile (B) at 1 mL/min, with column temperature maintained at 20 °C, in the following multilinear gradient: 0–5 min, 5% B; 5–13.5 min, 5-7.3% B; 13.5–25 min, 7.3–13.1% B; 25–35 min, 13.1–19% B; 35–40 min, 19–21% B; 40–42 min, 21–25% B; 42–46 min, 25-80% B; 46–50 min, 50-5% B; 50–60 min, 5% B. UV–vis spectra were recorded for all samples from 190 to 400 nm with selective wavelength monitoring at 255, 288, 320 and 350 nm. Peaks were tentatively identified by comparing retention times and UV–vis spectra from HPLC-DAD analysis with those of authentic standards. Stock solutions of all nine standard compounds were prepared in DMSO and aliquots frozen at −20 °C until required. Dried extracts were dissolved in 10% DMSO: deionized water (v/v) prior to analysis by adding DMSO to the equivalent volume of 10% of the total volume, mixing and sonication, before deionized water was added to the equivalent volume of 90% of the total volume. Samples from the transport study were defrosted and mixed before filtration and analysis. The standard calibration mixtures and sample solutions were filtered using 0.22 µm pore-size Millex-GV hydrophilic PVDF syringe-driven filter devices (Merck Millipore) with 4 and 33 mm diameter, respectively, prior to HPLC analysis.

2.7. High-performance liquid chromatography with electrospray ionization mass spectrometry (HPLC-ESI-MS)

HPLC-ESI-MS analyses were performed on a Waters Acquity UPLC system equipped with a binary solvent manager, sample manager, diode-array detector and Synapt G2 Q-TOF mass spectrometer using electrospray ionization in both the negative and positive modes (Waters, Milford, MA, USA). HPLC-ESI-MS and $-MS^E$ analyses were performed using the same column and gradient conditions as for the HPLC-DAD method, but with a slight modification by performing the separation at 32 °C. This modification affected retention times without affecting the elution order. The eluent was split 1:1 prior to introduction into the ionization chamber. Analyses were performed in MS^E mode (collision energy ramp from 20 to 50 V). The MS parameters were as follows: desolvation temperature, 275 °C; nitrogen flow rate, 650 L/h; source temperature, 120 °C; capillary voltage, 3000 V; cone voltage, 15 V. Sodium formate was used as calibration standard and leucine enkephalin as reference standard for

the lock mass. Data acquired were processed using MassLynx v.4.1 software (Waters). Peaks were identified by comparison of retention times, UV-Vis spectra, LC-ESI-MS and MS^E spectra with those of authentic standards.

3. Statistical analysis

All experiments were performed in triplicate and the data expressed as mean ± standard deviation (SD). Differences between samples were analyzed using a Students paired two-tailed t-test. Differences were considered significant if the value $p < 0.05$.

4. Results

4.1. Cytotoxicity of the aqueous extract of *A. phylicoides* against Caco-2 monolayers

An aqueous extract of *A. phylicoides* was not cytotoxic to Caco-2 cell monolayers up to 5 mg/mL (Supplementary material, Figure S1).

4.2. HPLC-DAD detection of absorbed phenolic acids present in an aqueous extract of *A. phylicoides*

The HPLC-DAD method was validated by evaluating the specificity, linearity, stability over 24 hours, intra- and inter-day repeatability (Supplementary material, Table S1). The specificity of the adapted method was confirmed by examining the UV–Vis and MS spectra across the target analyte peaks and comparing them to those of the authentic reference standards.

HPLC-DAD and LC-MS identified the presence of 9 prominent phenolic compounds in an aqueous extract of *A. phylicoides*.; i.e. protocatechuic acid (**1**), neochlorogenic acid (**2**), caffeic acid (**3**), chlorogenic acid (**4**), cryptochlorogenic acid (**5**), *para*-coumaric acid (**6**), 3,4-dicaffeoylquinic acid (**7**), 3,5-dicaffeoylquinic acid (**8**) and 4,5-dicaffeoylquinic acid (**9**) (Table 1 and Figure 1A). Compound **1** was the only phenolic compound quantified at 255 nm (Figure 1A and B), while the other phenolic compounds were quantified at 320 nm (Figure 1A and B).

4.3. Rate of transport of major phenolic compounds of an aqueous extract of *A. phylicoides.*

The apparent rate of transport of phenolic compounds in the presence and absence of P-gp inhibitor, verapamil hydrochloride, across differentiated Caco-2 monolayers, were calculated using concentrations obtained by HPLC-DAD analysis (Table 1).

4.4. The percentage of treatment transported to the basolateral compartment

The percentage cross over the nine phenolic compounds of an aqueous extract of *A. phylicoides* was calculated as a percentage of the initial concentration added (Table 1).

FIGURE 1 to be inserted here.

TABLE 1 to be inserted here.

5. **Discussion**

There is a growing interest in the use of dietary phenolic compounds, including those present in *A. phylicoides* (bush tea), to treat various metabolic diseases (Joubert et al., 2008; Joubert and de Beer, 2011). An aqueous extract of *A. phylicoides*, rich in phenolic compounds, has previously displayed anti-diabetic potential *in vitro* by enhancing glucose uptake in C2C12, Chang and 3T3-L1 cells (Chellan et al., 2012; Stratton et al., 2000). Of these phenolic compounds, identified in *A. phylicoides* have reputable biological benefits such as anti-inflammatory, anti-bacterial activity (Padayachee, 2011), anti-carcinogenic (Semaming et al., 2016) and anti-oxidant (de Beer et al., 2011; Mavundza, 2010).

In this study, we have developed a new HPLC method to chemically characterize *A. phylicoides,* based in part on the existing method by De Beer *et al*., (2011), and validated for the analysis of extracts of *A. phylicoides* with special emphasis on improving quantification of the most abundant phenolic compounds. The concentrations of 9 prominent phenolic compounds i.e.; protocatechuic acid (**1**), neochlorogenic acid (**2**), caffeic acid (**3**), chlorogenic acid (**4**), cryptochlorogenic acid (**5**), *para*-coumaric acid (**6**), 3,4-dicaffeoylquinic acid (**7**), 3,5-dicaffeoylquinic acid (**8**) and 4,5-dicaffeoylquinic acid (**9**) (summarized in Table 1) were quantified using this new method.

To scientifically validate the potential use of *A. phylicoides* as a nutraceutical, the intestinal absorption and bioavailability of the bioactive phenolic compounds needed to be estimated**.** Caco-2 cells are functionally and physically similar to the human intestinal epithelium, e.g. they express various transport and efflux proteins such as MDR-1 (Hochman et al., 2000; Prime-Chapman et al., 2004), which were confirmed in our Caco-2 model (Supplementary material, Figure S2).

Bi-directional transport assays (apical to basolateral and basolateral to apical) were performed to determine the rate of transport and efflux of the prominent phenolic compounds in an aqueous extract of *A. phylicoides* (Table 1). Caffeine was used as a positive control, as it is passively transported at a high rate (Kamimori et al., 2002; Ting et al., 2014), three compounds (**1**, **3** and **6**) were found to be transported at noticeably higher levels, and these were regarded as the phenolic compounds with the highest potential bioavailability (Figure 1B).

Of the 9 prominent phenolic compounds, compounds **1**, **3** and **6** had a relatively faster rate of transport (4.52×10^6 cm/s, 4.35×10^6 cm/s and 2.38×10^5 cm/s, respectively) in comparison to the other 6 phenolic compounds (Table 3). In the basolateral to apical direction, the transport of compounds **1**, **3** and **6** remained relatively unchanged (6.903×10^6 cm/s, 7.743×10^6 cm/s and 2.285×10^5 cm/s, respectively) in comparison to the apical to basolateral direction, however the 6 other compounds had a higher rate of transport in the basolateral to apical direction (Table 3). This is a classic indication of efflux as defined by an efflux ratio of greater than 2 (Farrell et al., 2012). In support of this, it has been documented that dicaffeoylquinic acids and chlorogenic acids, are renowned to be effluxed (Farrell et al., 2012). In contrast, the efflux ratio of compounds **1**, **3** and **6** were 1.53, 1.78 and 0.96 respectively (Table 3) signifying no efflux mechanism present but rather indicative of passive diffusion (Farrell et al., 2012). Furthermore, the rate of efflux of the 6 other

phenolic compounds was significantly decreased (Table 3) by verapamil hydrochloride. In addition to these results, the percentage crossover of the nine phenolic compounds were calculated as a percentage of the initial concentration added to the donor side (Table 3). The percentage crossover of compounds **1**, **3** and **6** were 4.61%, 6.21% and 32.61%, respectively, correlating with the Papp values (Table 3). These results, in combination, confirmed that compound **6** (*para*-coumaric acid) is likely to be the most bioavailable compound from the aqueous extract of *A. phylicoides*.

In summary, this study has documented the rate of transport of nine prominent phenolic compounds present in an aqueous extract of *A. phylicoides* across a fully differentiated Caco-2 cell monolayer, which is a good indication of relative bioavailability of each compound. Chellan *et al*., (2012), have previously identified eight out of the nine identified phenolic compounds in the extract. Optimisation of the HPLC method demonstrated the presence of *para*-coumaric acid for the first time in *A. phylicoides*. The kinetic bi-directional transport of phenolic compounds from *A. phylicoides* across this monolayer showed a consistent increase in concentration over time of compounds 1, 3 and 6 while compounds 2, 4, 5, 7, 8 and 9 had a comparatively slower rate of transport in the apical to basolateral direction.

6. Conclusions

The transport of major phenolic compounds, present in an aqueous extract of *A. phylicoides*, across a Caco-2 cell monolayer was investigated. Of these compounds, *para*-coumaric acid, identified for the first time in *A. phylicoides* was shown to have the highest predicted bioavailability suggesting that it could play a major role in the bioactivity of *A. phylicoides*.

7. Author contributions

S.B wrote the manuscript together with Y.N. and C.J.M. Both S.B and Y.N were responsible for all Caco-2 *in vitro* experiments and analysis of data from HPLC-DAD. C.J.F.M was PI of project, responsible for study conception and revision of draft paper. C.J.M. performed the HPLC-DAD and LC-MS/MS experiments, including the data analysis for Caco-2 transport studies and revision of draft paper. JL Director of Unit, reviewed the manuscript. A.K Student co-supervisor, Reviewed the manuscript. All authors approved the final version of the manuscript.

The Authors declare no conflict of interest.

8. Acknowledgements

Research reported in this publication was supported by the South African Medical Research Council through funding received from the South African National Treasury. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the South African Medical Research Council. We would like to acknowledge Agricultural research council, SA for performing HPLC-DAD and the Central Analytical facility of Stellenbosch University for LC-MS and MS^E analyses. Professor Jana Olivier is also acknowledged for harvesting and graciously providing the *A. phylicoides* sample. Ms. N Hlengwa, for technical assistance.**Figure Captions**

Figure 1: HPLC-DAD chromatograms identifying (A) the prominent phenolic compounds present in an aqueous extract of *A. phylicoides* in the apical compartment (**1** was quantified at 255nm and **2** to **9** was quantified at 320nm) and (B) phenolic compounds in the basolateral compartment, transported across the fully differentiated Caco-2 monolayer. [protocatechuic acid (**1**), neochlorogenic acid (**2**), caffeic acid (**3**), chlorogenic acid (**4**), cryptochlorogenic acid (**5**), *para*-coumaric acid (**6**), 3,4-dicaffeoylquinic acid (**7**), 3,5-dicaffeoylquinic acid (**8**) and 4,5-dicaffeoylquinic acid (**9**)]

References

Chellan, N., Beer, D.D., Muller, C., Joubert, E., Louw, J., 2008. A toxicological assessment of *Athrixia phylicoides* aqueous extract following sub-chronic ingestion in a rat model. Hum. Exp. Toxicol. 27, 819–825.

Chellan, N., Muller, C.J.F., de Beer, D., Joubert, E., Page, B.J., Louw, J., 2012. An *in vitro* assessment of the effect of *Athrixia phylicoides* DC. aqueous extract on glucose metabolism. Phytomedicine Int. J. Phytother. Phytopharm. 19, 730–736.

de Beer, D., Joubert, E., Malherbe, C.J., Jacobus Brand, D., 2011. Use of countercurrent chromatography during isolation of 6-hydroxyluteolin-7-*O*-β-glucoside, a major antioxidant of *Athrixia phylicoides*. J. Chromatogr. 1218, 6179–6186.

Farrell, T.L., Poquet, L., Dew, T.P., Barber, S., Williamson, G., 2012. Predicting phenolic acid absorption in Caco-2 cells: a theoretical permeability model and mechanistic study. Drug Metab. Dispos. 40, 397–406.

Gerbella, M., Baccarini, M., Borra, E., Rozzi, S., Luppino, G., 2014. Amygdalar connections of the macaque areas 45A and 45B. Brain Struct. Funct. 219, 831–842.

Hochman, J.H., Chiba, M., Nishime, J., Yamazaki, M., Lin, J.H., 2000. Influence of P-glycoprotein on the transport and metabolism of Indinavir in Caco-2 cells expressing cytochrome P-450 3A4. J. Pharmacol. Exp. Ther. 292, 310–318.

Hubatsch, I., Ragnarsson, E.G.E., Artursson, P., 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nat. Protoc. 2, 2111–2119.

Joubert, E., de Beer, D., 2011. Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. South Afr. J. Bot., Special issue on Economic Botany 77, 869–886.

Joubert, E., Gelderblom, W.C.A., Louw, A., de Beer, D., 2008. South African herbal teas: *Aspalathus linearis*, *Cyclopia spp*. and *Athrixia phylicoides*: a review. J. Ethnopharmacol. 119, 376–412.

Jung, U.J., Lee, M.-K., Park, Y.B., Jeon, S.-M., Choi, M.-S., 2006. Antihyperglycemic and antioxidant properties of caffeic acid in *db/db* mice. J. Pharmacol. Exp. Ther. 318, 476–483.

Kamimori, G.H., Karyekar, C.S., Otterstetter, R., Cox, D.S., Balkin, T.J., Belenky, G.L., Eddington, N.D., 2002. The rate of absorption and relative bioavailability of caffeine administered in chewing gum versus capsules to normal healthy volunteers. Int. J. Pharm. 234, 159–167.

Kusirisin, W., Srichairatanakool, S., Lerttrakarnnon, P., Lailerd, N., Suttajit, M., Jaikang, C., Chaiyasut, C., 2009. Antioxidative activity, polyphenolic content and anti-glycation effect of some Thai medicinal plants traditionally used in diabetic patients. Med. Chem. Shariqah United Arab Emir. 5, 139–147.

Lin, C.-Y., Huang, C.-S., Huang, C.-Y., Yin, M.-C., 2009. Anticoagulatory, antiinflammatory, and antioxidative effects of protocatechuic acid in diabetic mice. J. Agric. Food Chem. 57, 6661–6667.

Mavundza, E.J., 2010. Antioxidant and antibacterial activities of ethanol extract and flavonoids isolated from *Athrixia phylicoides,* MSc dissertations, University of Pretoria, Pretoria, viewed 20161130 <http://hdl.handle.net/2263/25977>.

McGaw, L.J., Steenkamp, V., Eloff, J.N., 2007. Evaluation of Athrixia bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. J. Ethnopharmacol. 110, 16–22.

Meng, S., Cao, J., Feng, Q., Peng, J., Hu, Y., 2013. Roles of chlorogenic acid on regulating glucose and lipids metabolism: a review. Evid.-Based Complement. Altern. Med. ECAM 2013, 801457.

Padayachee, K., 2011. The phytochemistry and biological activities of *Athrixia phylicoides.* PhD Thesis, University of Witwatersrand, Johannesburg, viewed 20161130 <https://core.ac.uk/download/pdf/39669529>

Prime-Chapman, H.M., Fearn, R.A., Cooper, A.E., Moore, V., Hirst, B.H., 2004. Differential multidrug resistance-associated protein 1 through 6 isoform expression and function in human intestinal epithelial Caco-2 cells. J. Pharmacol. Exp. Ther. 311, 476–484.

Rampedi, I., Olivier, J., 2005. The use and potential commercial development of *Athrixia phylicoides*. Acta Acad. 37, 165–183.

Robbins, R.J., 2003. Phenolic acids in foods: an overview of analytical methodology. J. Agric. Food Chem. 51, 2866–2887.

Scazzocchio, B., Varì, R., Filesi, C., D'Archivio, M., Santangelo, C., Giovannini, C., Iacovelli, A., Silecchia, G., Li Volti, G., Galvano, F., Masella, R., 2011. Cyanidin-3-*O*-β-glucoside and protocatechuic acid exert insulin-like effects by upregulating PPARγ activity in human omental adipocytes. Diabetes 60, 2234–2244.

Semaming, Y., Kukongviriyapan, U., Kongyingyoes, B., Thukhammee, W., Pannangpetch, P., 2016. Protocatechuic acid restores vascular responses in rats with chronic diabetes induced by streptozotocin. Phytother. Res. PTR 30, 227–233.
Stratton, I.M., Adler, A.I., Neil, H.A., Matthews, D.R., Manley, S.E., Cull, C.A., Hadden, D., Turner, R.C., Holman, R.R., 2000. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. BMJ 321, 405–412.
Ting, Y., Jiang, Y., Ho, C.-T., Huang, Q., 2014. Common delivery systems for enhancing *in vivo* bioavailability and biological efficacy of nutraceuticals. J. Funct. Foods 7, 112–128.
Zheng, W., Wang, S.Y., 2001. Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem. 49, 5165–5170.

Ethical Clearance

UNIVERSITY OF ZULULAND
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ETHICAL CLEARANCE CERTIFICATE

Certificate Number	**UZREC 171110-030 PGM 2015/206**						
Project Title	**Intestinal absorption and metabolism of bush tea major phenolic compounds exhibiting anti-diabetic activity**						
Principal Researcher/ Investigator	Y Ntamo						
Supervisor and Co-supervisor	Dr. AP Kappo		Dr. CFJ Muller & Dr S Bowles & Dr CJ Malherbe				
Department	Biochemistry & 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

Classification:

Data collection	Animals	Human Health	Children	Vulnerable pp.	Other
					X

Low Risk	Medium Risk	High Risk
X		

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these would require approval.)

Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation			X
Health Research Ethics Committee recommendation			X
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants			X
Informed consent from parent/guardian			X
Permission for access to sites/information/participants			X
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire			X
Data collection instrument in appropriate language		Only if necessary	
Other data collection instruments		Only if used	

The UZREC retains the right to

- Withdraw or amend this Certificate if
  - Any unethical principles or practices are revealed or suspected
  - Relevant information has been withheld or misrepresented
  - Regulatory changes of whatsoever nature so require
  - The conditions contained in this Certificate have not been adhered to

- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research.

Professor Nokuthula Kunene
Chairperson: University Research Ethics Committee
12 November 2015

CHAIRPERSON
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Y Ntamo - PGM 2015/206

Plagiarism report

MSc Biochemistry Thesis

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to University of Stellenbosch, South Africa Student Paper	%1
2	de Beer, Dalene, Christiaan J. Malherbe, Theresa Beelders, Elize L. Willenburg, D. Jacobus Brand, and Elizabeth Joubert. "Isolation of aspalathin and nothofagin from rooibos (Aspalathus linearis) using high-performance countercurrent chromatography: sample loading and compound stability considerations$", Journal of Chromatography A, 2015. Publication	%1
3	www.fstarchina.com.cn Internet Source	%1
4	Chellan, N.. "An in vitro assessment of the effect of Athrixia phylicoides DC. aqueous extract on glucose metabolism", Phytomedicine, 20120615 Publication	%1
5	www.springer.com Internet Source	%1

with Caco-2 cell model: Cremophor EL as the surfactant", European Journal of Pharmaceutical Sciences, 2016.
Publication

---

123 de Beer, Dalene, Alexandra Schulze, Elizabeth Joubert, André de Villiers, Christiaan Malherbe, and Maria Stander. "Food Ingredient Extracts of Cyclopia subternata (Honeybush): Variation in Phenolic Composition and Antioxidant Capacity", Molecules, 2012.
Publication

<%1

---

124 Kirsten Mease. "Differential selectivity of efflux transporter inhibitors in Caco-2 and MDCK-MDR1 monolayers: A strategy to assess the interaction of a new chemical entity with P-gp, BCRP, and MRP2", Journal of Pharmaceutical Sciences, 05/2012
Publication

<%1

---

125 Ramos, Alice A., Filipe Marques, Manuel Fernandes-Ferreira, and Cristina Pereira-Wilson. "Water extracts of tree Hypericum sps. protect DNA from oxidative and alkylating damage and enhance DNA repair in colon cells", Food and Chemical Toxicology, 2013.
Publication

<%1

---

126 "ABSTRACTS", Drug Metabolism Reviews, 3/1/2006
Publication

<%1

127	Reichelt, K.V., P. Hoffmann-LÃ¼cke, B. Hartmann, B. Weber, J.P. Ley, G.E. Krammer, K.M. Swanepoel, and K.-H. Engel. "Phytochemical characterization of South African bush tea (Athrixia phylicoides DC.)", South African Journal of Botany, 2012. Publication	<%1

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