

In vitro* assessment of cytochrome P450 and drug transporters modulation by polyphenolic constituents of *Cyclopia genistoides



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Declaration

I, Nonkululeko Purity Nokuphila Mchunu, affirm that I have carefully read and understood the University`s policies and rules applicable to postgraduate research and as such I surely confirm that this dissertation is a product of my own hard work and effort, all the original authors have been dully acknowledged where applicable. More so, I believe that it is acknowledged as being in line with normal academic standard.

Signature..... Signed on this day _____ of _____ 2019

Abstract

Historically, medicines were administered in the form of herbal concoctions, and this is still true for many traditional medicines. These ancient remedies are often consumed straight from the bush/bark or leaf, ground up as powders or brewed as teas. In these forms, the active ingredient, as part of the mixture, is often found at low concentrations and have a low bioavailability. The primordial use of natural medicine is growing in popularity and as science has advanced, chemists have shown it possible to extract active ingredients from natural sources to make more potent medications under the term “nutraceutical”. As the health-conscious increase “superfoods” into their daily dietary intake, it has also been shown that a large portion of the South African populations will use natural products as their primary source of medication and more frequently as adjunctive therapeutics.

Herb-drug interactions (HDI) are acknowledged as a growing public health problem that can lead to life-threatening adverse drug reactions (ADR). When boosting the efficacy of herbal products, methods are employed to produce products enriched in bioactive phytochemicals. However, the increased exposure to these bioactive phytochemicals poses the risk of HDI should the user be simultaneously prescribed conventional drugs. The potential for ADR originating from HDI should require assessment before herbal products are made available on the market, but no current legislature makes this testing mandatory.

Cyclopia genistoides has shown potential to be developed into an antidiabetic nutraceutical because of its α -glucosidase inhibitory activity and high phenolic content. In this study, we assessed the effect of a *Cyclopia genistoides* extract for its interaction with cytochrome P450 (CYP) enzymes and drug transporters to investigate whether the use of *C. genistoides* extract or its bioactive phenolic constituents has the potential for herb-drug interactions. The potential for hepatotoxicity of a methanol *C. genistoides* extract, xanthone and benzophenone enriched fractionations and associated pure compounds (mangiferin, isomangiferin, 3- β -D-glucopyranosyl-4- β -D-glucopyranosyloxyiriflophenone (IDG) and 3- β -D-glucopyranosyliriflophenone (IMG)) was assessed using C3A liver cells. The inhibitory effects of these test entities on major CYP isoforms were assessed using

Vivid® blue screening kits in both time and concentration-dependent assays. qRT-PCR was performed using Taqman probes in order to investigate the modulatory effect of *C. genistoides* constituents on the expression of drug transporters (SLCO1B3, ABCB1 and ABCG2). Translation of these drug transporter genes was confirmed through Western blot analysis.

C. genistoides was deemed non-toxic to C3A hepatocytes at the concentrations tested. ARC188 showed inhibitory activities on all CYP isoforms tested (50.27%, 72.47%, 70.68%, 50.48% and 78.49% inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively). ARC188x inhibited CYP1A2, CYP2D6 and CYP3A4 by 57.15%, 56.56% and 50.61%, respectively. Mangiferin inhibited CYP 3A4 (60.83 % inhibition), CYP2D6 (52.12% inhibition) and CYP2C9 (82.08% inhibition). While ARC188_B only inhibited CYP3A4 (53.28%) and the benzophenone pure compounds (IMG and IDG), and isomangiferin had no inhibitory effects on CYP activity. All tested *C. genistoides* constituents up-regulated the efflux drug transporter ABCB1.

Xanthone-enriched extracts, especially those with a high mangiferin content, show potential towards HDI especially with antidiabetic drugs such as sulfonylureas and warrant further investigation into the possibility of ADR.

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“Fear not, for I am with you; be not dismayed, for I am your God. I will strengthen you, yes, I will help you, I will uphold you with my righteous right hand.” Isaiah 41:10

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For my father in heaven, I hope you are proud of me, Macingwane.

List of abbreviations

ABC	Adenosine-5 triphosphate binding cassette
ABCB1	Adenosine-5 triphosphate binding cassette B1
ABCG2	Adenosine-5 triphosphate binding cassette G2
ADR	Adverse drug reactions
ANOVA	Analysis of variance
ATCC	American Type Culture collection
ATP	Adenosine-5 triphosphate
BCRP	Breast cancer resistant protein
BOMCC	7-benzyloxymethyloxy-3-cyanocoumarin
BSA	Bovine serum albumin
CAM	Complementary or alternative medicine
CDI	Concentration-dependent inhibition
cDNA	Complementary deoxyribonucleic acid
CO ₂	Carbon dioxide
Ct	Cycle threshold
CYP	Cytochrome P450
CYP1A2	Cytochrome P450 1A2
CYP2C19	Cytochrome P450 2C19
CYP2C9	Cytochrome P450 2C9
CYP2D6	Cytochrome P450 2D6
CYP3A4	Cytochrome P450 3A4
DAD	Diode-array detector
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
EX	Excision
EM	Emmission
EMEM	Eagles minimum essential medium
EOMCC	7-ethyloxymethyloxy-3-cyanocoumarin

FBS	Fetal bovine serum
HDI	Herb-drug interactions
FDA	Food and drug administration
HPLC	High performance liquid chromatography
IC ₅₀	half maximal inhibitory concentration
IDG	3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone
IMG	3-β-D-glucopyranosyliriflophenone
mRNA	Messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium
NaCl	Sodium chloride
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NEAA	Non-essential amino acids
OATP	Organic anion transporter polypeptide
OATP1B1	Organic anion transporter polypeptide 1B1
OATP1B3	Organic anion transporter polypeptide 1B3
OH	Hydroxyl
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PXR	pregnane X receptor
qRT-PCR	Quantitative real time Polymerase chain reaction
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SAMRC	South African Medical Research Council
SLC	Solute carrier
SLCO1B1	Solute Carrier O 1B1
SLCO1B3	Solute Carrier O 1B3
T2D	Type-2 diabetes
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing Tween-20
TBST	Tris-buffered saline with Tween-20

TC	Tissue culture
TDI	Time-dependent inhibition
Tris	Trizma
WHO	World Health Organization

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Chapter 1

1. Introduction

African traditional medicine is one of the oldest forms of medicine and incorporates the use of herbal medicines. In South Africa, the use of herbal medicines is still widely popular (Van Wyk, 2011). Approximately 70% of the South African population in rural areas primarily use herbal medicines for therapy (Street *et al.*, 2008). Globally, it has been reported that about 80% of the world's populations in developing countries use herbal medicines (WHO, 2003). The use of herbal medicines as complementary or alternative medicine (CAM) has recently become popular in develop countries as well, as people try to deviate from using synthetic chemical products in hopes of a healthy lifestyle (Alostad *et al.*, 2018).

In South Africa there is a rich biodiversity, there are about 30 000 species of higher plants with about 8 000 of which are endemic to the country and about 3 000 plant species are used in the production of herbal medicines (Van Wyk, 2011). With the rising interest in herbal medicines, research on medicinal plants has increased and many more plants with medicinal properties have emerged. Amongst such plants is *Cyclopia genistoides*, a short shrub that has historically been used in the production of a South African herbal tea known for its sweet taste, aroma and lack of caffeine and tannins, honeybush tea (Joubert *et al.*, 2011; Schulze *et al.*, 2015). The plant is endemic to the Cape Flora fynbos biome of South Africa and belongs to the *Fabaceae* family and *Podalrieae* tribe (North *et al.*, 2017). Of the approximate 23 *Cyclopia* species, *C. genistoides* together with 2 other species, *C. intermedia* and *C. subternata* are the only species used in the commercial production of the tea with *C. genistoides* containing the highest phenolic content when compared to the other plant species (Joubert *et al.*, 2003, 2014). *C. genistoides* has been found to be rich in xanthenes and benzophenones which have been associated with many health benefits including antidiabetic, anti-inflammatory, anti-oxidative and immune modulating properties (Joubert *et al.*, 2003; Verhoog *et al.*, 2007; Le Roux *et al.*, 2008; Kokotkiewicz and Luczkiewicz, 2009; Malherbe *et al.*, 2014). Recently, *C. genistoides* xanthenes and benzophenones have been shown to inhibit the activity of α -glucosidase, an enzyme that aides in post prandial carbohydrate metabolism, *in vitro* (Beelders *et al.*, 2014; Bosman *et al.*, 2017). These recent findings together with the plant's high phenolic content increases the possibility of *C. genistoides* to be used as an anti-diabetic

adjunctive that aides in decreasing the metabolic rate of postprandial carbohydrates thus lowering postprandial glucose levels in the gut. Such an adjuvant will most-likely be taken concurrently with other oral antidiabetic drugs used to treat type-2 diabetes (T2D) which may lead to herb-drug interactions, thus it is important to investigate the safety of *C. genistoides* in that regard.

Communities perceive herbal medicines as “safe” since they are merely plant products, as a result herbal medicines are generally unregulated (Corns, 2003; Folashade *et al.*, 2012). In South Africa herbal medicines are not included in regulation laws for medical products, thus there are no regulations regarding the safety, quality and/or therapeutic efficacy of such products (Richter, 2003; Mkunga *et al.*, 2008). There are also no license requirements for distributing and selling herbal products in the country. This causes a public health risk as anyone can just sell a product with unknown properties to the public (Thomford *et al.*, 2016). Another health risk associated to herbal medicines is the simultaneous use of these products with conventional drugs. Most people do not disclose their use of herbal medicine to healthcare professionals before taking prescriptions of conventional drugs leading to herb-drug interactions with unwanted side effects (Chavez *et al.*, 2006). Apart from potential hepatotoxicity, likely dose and time dependent toxicity may occur due to contamination of the plant harvest or interactions of the herbal product with conventional drugs leading to adverse side effects (Jordan *et al.*, 2010). Herbal medicines are generally made from plant extracts containing high concentrations of bioactive plant polyphenols (Pan *et al.*, 2013; Schulze *et al.*, 2015). Plant polyphenols have been associated with several herb-drug interactions with unwanted side effects (Valli and Giardina, 2002; Cordier and Steenkamp, 2011). They are able to change the activity of major drug metabolizing enzymes and drug transporters, causing major changes on the pharmacokinetics of conventional drugs co-administered with them (Kimura *et al.*, 2010; Hussain *et al.*, 2016).

Safety concerns about Rooibos tea, a popular South African herbal tea closely related to *Cyclopia* which has been used as a nutraceutical as an extract have recently come to surface (Engels *et al.*, 2013). Unfermented green (unoxideised) rooibos extracts were found to potentially cause hepatotoxicity and inhibit major drug metabolizing enzymes which has led to scientific assessment of the safety of Rooibos extracts in

general (Patel *et al.*, 2016a; Reddy *et al.*, 2016). To the same end, there is little knowledge of the cytotoxic potential of the phenolic compounds present in *C. genistoides*. Due to the potential use of honeybush as adjunctive therapies, food supplements and/or pharmaceutical/nutraceutical products, it is essential to assess possible adverse biological effects.

This study explored whether the use of *C. genistoides* or its phenolic constituents, with α -glucosidase inhibitory activity, will have implications for herb-drug interactions.

1.1. Aim and Objectives

The aim of this study was to identify possible modulation of drug transporters and Cytochrome P450 enzymes by polyphenolic constituents of *C. genistoides* using a C3A liver cell model.

In order to address the specific aim our objectives were:

- To assess cell viability and contribute to subsequent dose optimisation and safety of *C. genistoides* consumption using a C3A liver cell model.
- To identify potential risk of herb-interactions induction by monitoring induction of CYP enzymes herb-drug (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4).
- To investigate modulation of expression of drug transporters (OATP1B1, OATP1B3, PgP and BCRP1) in C3A liver cells treated with polyphenolic constituents of *C. genistoides*, using cDNA synthesis.

1.2. Intended contribution to the existing body of Knowledge

Basic understanding of the pharmacodynamics and pharmacokinetics of herbal products are important in order to avoid toxicity and herb-drug interference in drug metabolism. The current study will reveal the potential effects of *C. genistoides* on CYP enzyme activity and expression of drug transporters, yielding important information regarding dose optimization during subsequent progression into animal and human nutraceutical development.

Chapter 2

2. Literature review

2.1. Herbal medicines

Herbal medicines derived from plants and plant material, are used for therapeutic purposes, as described by the World Health Organization (WHO, 1999). Plant polyphenols such as tannins, flavonoids and other phenolic compounds have been shown to possess health-promoting properties (Edeoga *et al.*, 2005). Over the last decade the use of herbal medicines has increased (Corns, 2003). In 1992 WHO estimated that approximately 80% of the world population, especially in developing countries rely on medicinal plants as their primary source of healthcare (WHO, 2003). South African statistics have shown that about 70% of the general public make use of medicinal plants (Stree *et al.*, 2008). In South Africa, traditional healers play a vital role in the distribution of herbal medicines to people in rural areas who often prefer their holistic services to those of clinic doctors (Awortwe *et al.*, 2018). In addition to this, a large majority of South Africans make use of herbal medicines either alone or together with prescribed medication (Rafieian-Kopaei and Sewell, 2014).

In recent years, the popularity of living a more healthy lifestyle has driven the increased use of herbal medicines as the public shies away from exposure to the perceived “harmful” chemicals found in conventional synthetic drugs (Barnes *et al.*, 2007).

These medicines are highly unregulated and currently, in South Africa, there is no legislation that governs the production and distribution of herbal medicines (Alostad, Steinke and Schafheutle, 2018). In the year 2000, the African Traditional Medicines Experts Committee was appointed to advise the minister of health in trying to establish regulatory rules for herbal medicines (Department of health, 2010). However, today these rules are not strictly monitored or formally legislated.

Most herbal medicines are registered as herbal supplements by the Food and drug administration (FDA) (Sahoo *et al.*, 2010). Nevertheless, manufacturers of the dietary supplements do not necessarily have to get approval from the FDA before production and distribution and no scientific testing is required prior to distribution (Huang, 2006). This lack of regulation in terms of safety and efficacy of herbal medicines has the potential to pose a risk to society given the wide usage of these products.

2.2. Nutraceuticals

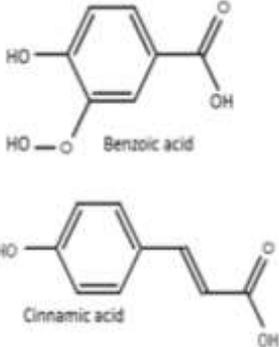
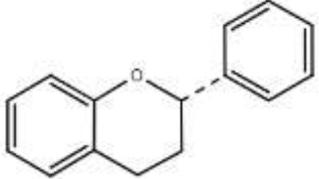
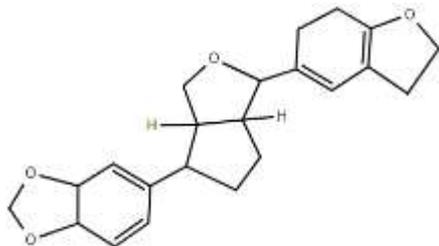
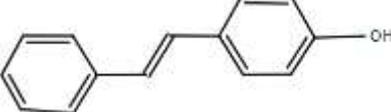
The term “nutraceutical” was derived from a combination of nutrition and pharmaceutical and combined by De Felice in 1979 (Lin *et al.*, 2016). Nutraceuticals are food items or food parts that provide health benefits such as disease prevention or treatment (Galanakis, 2013). Over the last few years nutraceuticals have been shown to play an important role in a number of biochemical processes in the body such as cell proliferation, gene expression, and safeguarding of mitochondrial integrity, amongst others (Tapas *et al.*, 2008). They are generally used to manage or treat chronic diseases such as obesity, diabetes, cancer and cardiovascular diseases either in monotherapy or as adjuvants, co-administered with conventional drugs (Vandeweerd *et al.*, 2012). Examples of nutraceuticals include; the immune booster Echinacea, Ginseng for colds and flu, cod liver oil capsules and folic acid supplements (Rajasekaran *et al.*, 2008). The nutraceuticals that are used in combinational therapy, are generally claimed to lower the effective dose of the conventional drug (Sarris *et al.*, 2016). The ideal nutraceutical candidate should be active enough to elicit an efficient therapeutic effect without interfering with the pharmacokinetics and pharmacodynamics of the conventional drug. Some such candidates exist in the form of plant polyphenols, often considered as nutraceuticals (Lin *et al.*, 2016).

2.3. Plant polyphenols

Plant polyphenolic compounds are secondary metabolites of plants, and are said to contribute to the many health-promoting effects associated with herbal medicines (Cardona *et al.*, 2013). They are abundantly found in fruits, vegetables, cereals and beverages. They contribute to the bitterness, flavour, odour and oxidative status of many plant based foods and beverages (Pandey and Rizvi, 2009). Plant polyphenols play important roles in plant function, and flower, fruit and seed pigmentation. These compounds also promote plant fertility and germination of pollen (Edirisinghe and Burton-Freeman, 2016). Some polyphenols protect the plant against ultra-violet penetration and act as signal molecules to protect plants from bacterial infections (Petti and Scully, 2009). Of the many different polyphenols in nature, more than 8000 have been identified, and divided into different classes including hydroxybenzoic acids, anthocyanins, benzophenones, flavanols, flavonoids, lignans, tannins and xanthenes (Manach, 2004). It is very difficult to estimate the concentration of polyphenols in food

because of their wide structural differences (Scalbert and Williamson, 2000). Though structurally diverse, plant polyphenols have a common phenylalanine backbone. The different types of polyphenols are shown in table 1. Polyphenols are not equally distributed in plants, e.g: soluble polyphenols are found in the vacuoles, while insoluble polyphenols are found on the cell wall of plant cells (Pandey and Rizvi, 2009), and are classified by their source of origin, biological function, and chemical structure. They often exist as glycosides with both sugar units and acylated sugars positioned at different stations of the polyphenol skeletons.

Table 1: The different classes of polyphenols

Polyphenols	Description	Structure	Reference
Phenolic acids	Two main types - benzoic acid and cinnamic acid derivatives.	 <p>Benzoic acid</p> <p>Cinnamic acid</p>	Heleno <i>et al.</i> , 2015; Scalbert <i>et al.</i> , 2015
Flavanoids	Have a common basic structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle.		Williamson, 2017
Lignans	Di-phenolic compounds that contain a 2,3-dibenzylbutane structure that is formed by the dimerization of two cinnamic acid residues.		Tsao, 2010
Stilbenes	Contain two phenyl moieties connected by a two-carbon methylene bridge.		Pandey and Rizvi, 2009

2.4. Food sources of polyphenols

Hundreds of different polyphenolic molecules have been identified in food sources, and these range from simple polyphenols with low molecular weights to very complex structures (Scalbert *et al.*, 2015). On average, most people consume 1 gram of polyphenols per day, which is said to increase due to the consumption of herbal medicines (Gharras, 2009). Fruits, vegetables and beverages such as tea, coffee and fruit juices contain the most polyphenols. A cup of coffee or tea is said to contain about 100 mg polyphenols and fruits contain approximately 200-300 mg polyphenols per 100 g fresh weight, but it is difficult to estimate the exact polyphenolic content in foods due to the structural diversity of polyphenols (Bahadoran, Mirmiran and Azizi, 2013; Ganesan and Xu, 2017; Guasch-Ferré *et al.*, 2017). The most widely distributed polyphenols are the flavonoids and phenolic acids, flavonoids are found in 50% of all foods containing polyphenols while phenolic acids account for 25% of dietary polyphenols (González *et al.*, 2011). A specific type of polyphenol may be more prominent in certain foods, or certain parts of food, for example certain isoflavones are prominent in soya, phlorizin is prominent in apples, yet apple peels contain more polyphenols than the flesh, and some benzophenones are found specifically in honeybush tea (*Cyclopia* spp.) (Kokotkiewicz *et al.*, 2013).

2.5. The role of polyphenols in promoting health

Studies have shown that chronic consumption of a diet rich in polyphenols can help prevent certain cancers, cardiovascular diseases, T2D, osteoporosis, pancreatitis, gastrointestinal problems and lung damage (Mandal and Jaganathan, 2009; González *et al.*, 2011; Edirisinghe and Burton-Freeman, 2016; Guasch-Ferré *et al.*, 2017). Polyphenols have the ability to scavenge free radicals, which reduces oxidative stress and eradicates many disease symptoms (Khan and Mukhtar, 2007). Polyphenols inhibit pro-inflammatory transcription factors by interacting with proteins involved in gene expression and cell signalling, leading to protective effects against many inflammation-mediated chronic diseases (Howell *et al.*, 2018).

2.6. Absorption of polyphenols

Polyphenol structure has a major impact on intestinal absorption (Silberberg *et al.*, 2006). Polyphenols are poorly absorbed in the small intestines because of their

glycoside linkages, thus most polyphenols are not absorbed in the small intestines but proceed to the gut where they are cleaved by bacteria belonging to gut microflora (Bravo, 1998). The most widely discussed structural parameters are molecular weight, glycosylation and esterification (Igel *et al.*, 2007).

2.7. Bioavailability of polyphenols

Bioavailability refers to the degree and rate at which an administered drug or xenobiotic reaches the systemic circulatory system. Understanding a drug's bioavailability often aids dose optimization for non-intravenous administration of the drug (Hu *et al.*, 2017). Bioavailability of polyphenols are variable; however polyphenols are thought to have similar pharmacokinetic patterns i.e. they are first cleaved to the aglycone and then undergo phase II metabolism (Lambert *et al.*, 2007). The structure of specific polyphenols play an important role in determining their bioavailability (Marín *et al.*, 2015). Most polyphenols are esters, glycosides or polymers that are conjugated and cannot be absorbed by the body (Scalbert *et al.*, 2002). These conjugates need to be hydrolysed to unconjugated aglycones prior to absorption (Brat *et al.*, 2006).

2.8. Metabolism of polyphenols

The absorbed glycosides and aglycone polyphenols are usually metabolized in the same way as xenobiotics in the body, whereby they are first oxidized and/or hydroxylated through phase I metabolism (Lambert *et al.*, 2007). The oxidized compounds are further conjugated into methylated, glucuronidated or sulphated derivatives through phase II metabolism in order to be easily excreted (Marín *et al.*, 2015). These are generally excreted through the urinary tract or in the bile (Liu and Hu, 2007). Absorption of polyphenols can be influenced by solubility, interaction with other dietary ingredients, molecular transformations, different cellular transporters, metabolism and the interaction with the gut microbiota, resulting in changes to their bioavailability (Sadeghi Ekbatan *et al.*, 2018). Metabolism of polyphenols is further complicated by their poor absorption. Polyphenolic entities that are absorbed by the small intestines are metabolized as xenobiotics (Sadeghi *et al.*, 2018), while the un-absorbed entities are usually cleaved by the enzymes produced by gut microflora (Dall'Asta *et al.*, 2012). Polyphenols usually occur as C- or O-glycosides in plants and foods, O-glycosides are highly insoluble and cannot be absorbed by the human body (Silberberg *et al.*, 2006). Bacteria in the gut are able to cleave the O-glucosides in

order to release aglycosides and hepatic O-glucuronides which are better absorbed (Scalbert and Williamson, 2000; Stevens and Maier, 2016). Positioning of the hydroxyl groups may influence their degradation, as recent studies indicate that flavonoids without hydroxyl groups at C5, C7, and C4 positions are degraded slower (Williamson, 2017). Some gut microbiota species that have been involved in this hydrolysis are *Bacteroides distasonis*, *Bacteroides uniformis*, *Bacteroides ovatus*, *Enterococcus casseliflavus* and *Eubacterium ramulus*. The type of glycosidic bond (C- or O-glycosides) has an influence on their degradation rates (Cardona *et al.*, 2013; Stevens and Maier, 2016; Espín *et al.*, 2017). Metabolism of a C-glycosidic bond is much slower than the hydrolysis of an O-glycosidic bond (Aura, 2008). The process of the metabolism of most polyphenols is summarised in Figure 1.

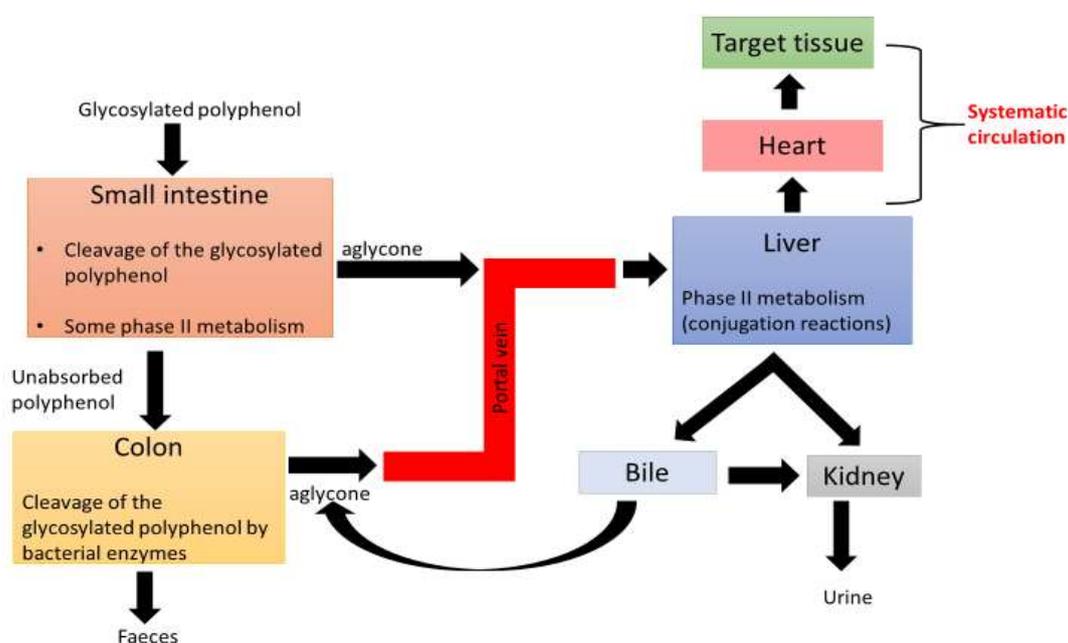


Figure 1: Schematic representation of the metabolism of polyphenols.

After oral ingestion, the glycosylated polyphenol is cleaved by enzymes in the small intestines and some phase II metabolism also occurs. The aglycone is absorbed and transported to the liver (phase II metabolism). After metabolism, polyphenol metabolites are excreted from the liver to the systematic circulation to reach target tissues. Waste products are excreted through the kidney, and the bile where they may be recycled to form aglycones, re-absorbed by the liver and re-metabolized. Some glycosylated proteins cannot be cleaved by enzymes in the small intestines and travel to the colon where they are cleaved by enzymes from the microbial flora of the colon and thereafter undergo absorption. Un-cleaved and unabsorbed glycosylated polyphenols in the colon are excreted in the form of faeces (adapted from Marín *et al.*, 2015).

2.9. *Cyclopia genistoides*

Cyclopia genistoides belongs to the *Fabaceae* family and *Podalrieae* tribe (North *et al.*, 2017). There are approximately 20 to 24 species in the *Cyclopia* genus, but the most popular species are *C. genistoides*, *C. intermedia* and *C. subternata* (Joubert *et al.*, 2014). Hot water extracts of *C. genistoides* have been shown to possess more of the bioactive phenolic compounds when compared to those of *C. intermedia*, *C. maculata* and *C. subternata*. The plant is also said to produce more biomass when compared to the other three well known *Cyclopia* species (Joubert *et al.*, 2003). *Cyclopia* species are generally short woody shrubs with a low leaf-to-stem ratio (Schutte, 1995). *Cyclopia* species have trifoliate leaves, however, leaf shape is species specific (De Nysschen *et al.*, 1995). *C. genistoides* has ribbed branches that are nude at the base but leafy at the top, small linear leaves which are 14 to 20 mm long and 1 to 2 mm wide, and large golden yellow flowers that have small red markings at the base (Figure 2) (Roza *et al.*, 2017). *C. genistoides* has hard shelled, kidney shaped seeds which usually germinate between summer and autumn (Joubert *et al.*, 2008a).

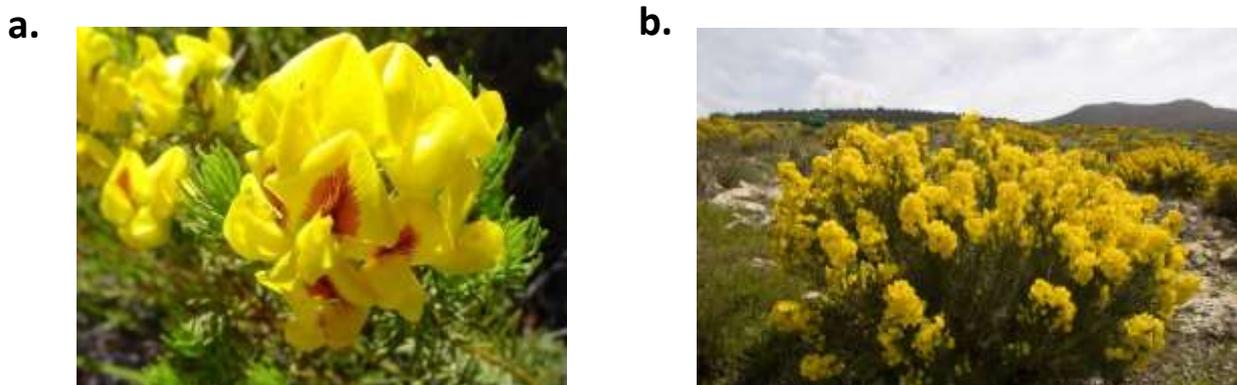


Figure 2: Image of *C. genistoides* (South African Honeybush Tea Association, no date).

Cyclopia species are endemic to the fynbos biome in parts of the Eastern and Western Cape region, South Africa (Joubert, 2008b). They can be found naturally all the way from the Cederberg Mountains, to the Cape peninsula in the South and Port Elizabeth in the Eastern Cape (Figure 3) (Joubert *et al.*, 2011). Species are usually restricted to very small areas and very specific habitats (North *et al.*, 2017). *C. genistoides* grows naturally in the coastal sandy areas from the west coast to Mossel Bay (Mbangcolo,

2008). The plant can also be found on the mountains near the Cape peninsula (Joubert *et al.*, 2014).

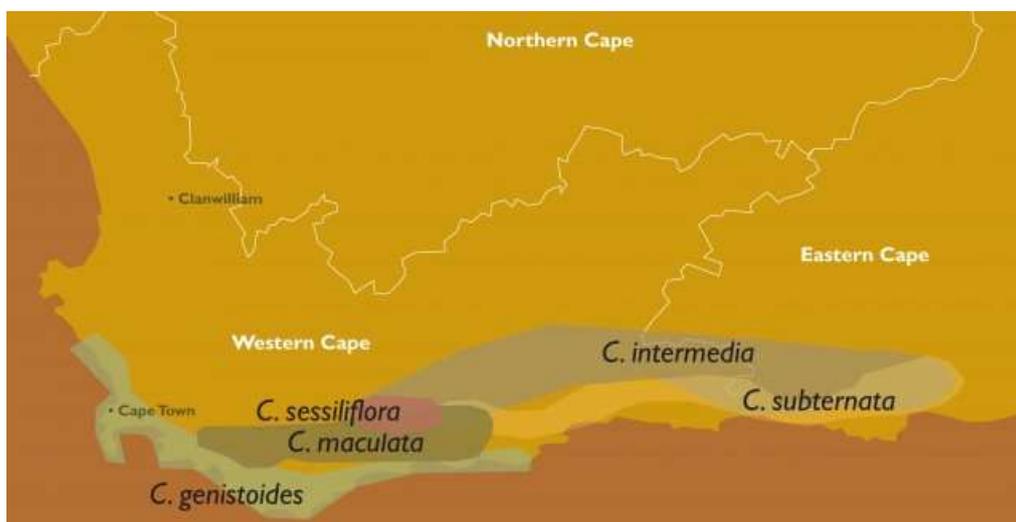


Figure 3: Geographic distribution of *C. genistoides*.

C. genistoides is generally found between the sandy coastal regions of the western cape and the Mountainous regions of the Cape peninsula (from South African Honeybush Tea Association, no date).

2.10. Historical use of *C. genistoides*

The earliest botanic information about *Cyclopiopsis* was published in the European taxonomic script of 1705, where the plant was referred to as *Genista rosmarinifolia triphyllos* Promont, Bonae Spei, and later changed to *C. genistoides* in 1825 (Joubert *et al.*, 2008b). It is not known when the medicinal utilization of *C. genistoides* commenced, however it is believed that people in the Western Cape regions of South Africa had been using infusions from *Cyclopiopsis* species to treat diarrhoea, colds, coughs and skin conditions, and to stimulate appetite and to promote milk production to breast feeding mothers for centuries (Joubert *et al.*, 2003). The medical use of *Cyclopiopsis* species gradually became less important, superseded by the use as “honeybush tea” (Joubert *et al.*, 2011).

2.11. General uses of *C. genistoides*

C. genistoides is popularly used to produce a South African herbal tea known as “honeybush tea”, a name given because of its naturally sweet flavour (Joubert *et al.*, 2006). Honeybush tea was originally produced from *C. genistoides* and, as the tea grew in popularity, the use of other species was included to meet the rising demand

(Le Roux *et al.*, 2008). The tea became a commercial commodity in the mid 1990s and is increasingly popular for its flavour, lack of caffeine and low tannin content, as well as its anecdotal health properties (Marnewick *et al.*, 2009). The first branded product, 'Caspa *Cyclopia* Tea', was presented commercially in South Africa in the 1960s (Joubert *et al.*, 2011) and honeybush is slowly becoming popular in the worldwide market (Le Roux *et al.*, 2008). Honeybush tea is made from the shoots (leaves, stems and flowers) of several *Cyclopia* species, including *C. genistoides*, by fermentation of the leaves, stems and sometimes flowers of the plants (Joubert *et al.*, 2017). It is estimated that about 125 tons of honeybush tea is produced in South Africa per year and more than 80% of the fermented tea is exported to countries like Germany, United Kingdom and USA (Kokotkiewicz and Luczkiewicz, 2009).

In recent years, *C. genistoides* has generated major scientific interest because of its health benefits. *In vitro* studies have shown that extracts from this plant have α -glucosidase inhibitory activity (Beelders *et al.*, 2014; Raaths, 2016; Bosman *et al.*, 2017), anti-inflammatory activity (Kokotkiewicz and Luczkiewicz, 2009), anti-cancer activity (Van der Merwe *et al.*, 2006), phyto-oestrogenic activity (Louw, Joubert and Visser, 2013), anti-oxidative activity (Joubert *et al.*, 2008b), anti-bacterial and anti-fungal activity (Coetzee *et al.*, 2008). These findings have generated an interest in the development of herbal nutraceuticals using *C. genistoides*. Research is still underway to determine the safety and efficacy of such potential products.

2.12. Phenolic composition of *C. genistoides*

Cyclopia species are rich in xanthenes; mangiferin and isomangiferin and in benzophenones; 3- β -D-glucopyranosyl-4- β -D-glucopyranosyloxyiriflophenone (IDG), 3- β -D-glucopyranosyliriflophenone (IMG) and 3- β -D-glucopyranosylmaclurin (MMG). It is also rich in the flavanones; hesperidin (the *O*-rutinoside of hesperitin), narirutin, eriocitrin, naringenin and their glycosides, and contain the isoflavones; orobol, calycosin, formononetin and afrormosin, the flavones; luteolin, diosmetin and luteolin, and the catechin epigallocatechin-3-*O*-gallate (De Nysschen, 2006; Kokotkiewicz *et al.*, 2012; Beelders, *et al.*, 2014b; Schulze *et al.*, 2015). The relative quantities of the phenolic compounds differ between the different *Cyclopia* species and are specific to the geographical area in which they are grown (Joubert *et al.*, 2014). Harvesting time and fermentation also affect the levels of polyphenols in the plants, plants harvested

in the summer were shown to have high levels of mangiferin, isomangiferin and IMG (Joubert *et al.*, 2003). The levels of polyphenols decrease greatly during or after fermentation due to heat exposure, which degrades most of the polyphenols, however *C. genistoides* was shown to retain 77% of its bioactive polyphenols when compared to other species (Beelders *et al.*, 2014b, 2017).

C. genistoides contains the highest levels of polyphenols when compared to other *Cyclopia* species used in the commercial production of honeybush tea. Hot water extracts of the plant were found to contain up to 3.61g/100 g of mangiferin, 0.54g/100g isomangiferin and 1.74g/100g hesperidin in a comparative study by (Joubert *et al.*, 2008a). There are two types of *C. genistoides* which are defined by the geographical area in which they grow, the “Overberg type” and the “West Coast type” (Raaths, 2016). The Overberg type of *C. genistoides* is known to have a very high content of mangiferin and a lower content of isomangiferin while the West coast type is known to have a very high content of hesperidin and similar contents of mangiferin and isomangiferin (Roza *et al.*, 2017).

2.12.1. Mangiferin

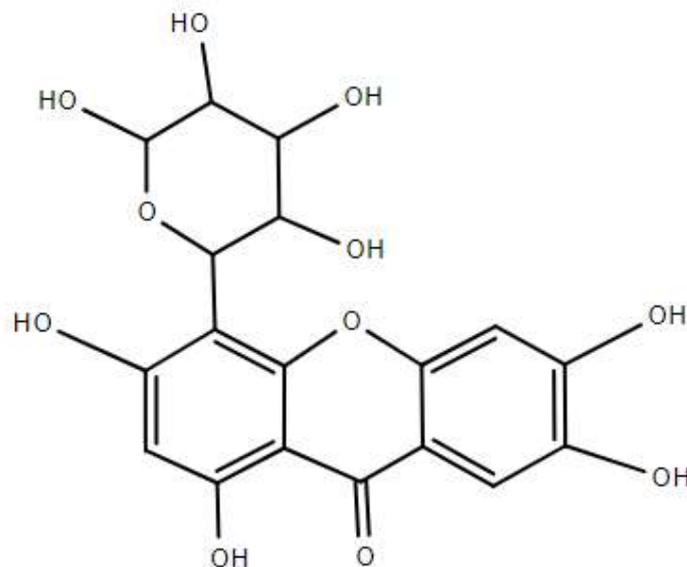


Figure 4: Chemical structure of mangiferin, a C-glucoside found in *C. genistoides* (Chemspider)

Mangiferin, a C-glucosyl xanthone, is a major biologically active compound in plant extracts (Van der Merwe *et al.*, 2012). It is widely distributed in plants and has been reportedly found in six families of ferns (Pardo Andreu *et al.*, 2005), as well as several

flowering plants such as *Mangifera indica* L. and the spice *Curcuma amda* (Van der Merwe *et al.*, 2012; Matkowski *et al.*, 2013). Mangiferin is found abundantly in the leaves of *Cyclopia* species and in especially high quantities in *C. genistoides* (Beelders *et al.*, 2014b). Seasonal changes may influence its production, with high concentrations of mangiferin observed in the leaves of *C. genistoides* when the plant is harvested in the summer (Joubert *et al.*, 2014). Mangiferin levels were shown to decline significantly in plants harvested in winter (Joubert *et al.*, 2003). Mangiferin has a wide range of medicinal properties which include antioxidant, anti-inflammatory, antidiabetic, anticancer and immunomodulatory effects (Bhatia *et al.*, 2008; de Beer *et al.*, 2009; Fomenko and Chi, 2016). This polyphenol is highly labile in alkaline conditions but can remain stable when heated at 80°C. However, it can undergo thermal degradation during high temperature (90°C) fermentation of *C. genistoides* plant material (Beelders *et al.*, 2017). Mangiferin is resistant to acid and enzyme hydrolysis because its glucose molecule is directly linked by a carbon-carbon bond to the aglycone (Figure 4) (Bock *et al.*, 2008).

To be able to determine the pharmacokinetics of mangiferin, the metabolic fate of mangiferin has been studied, and it has been shown that mangiferin is not always absorbed in its parent form as a C-glucoside. However it can be cleaved to norathyriol, the aglycone of mangiferin before absorption Bock, Waldmann and Ternes, 2008; Marnewick *et al.*, 2009). The human intestinal bacterium *Bacteroides* sp. releases an enzyme that is able to cleave the C-glucoside bond of mangiferin releasing norathyriol (Bock and Ternes, 2010). A study on the pharmacokinetics of mangiferin showed that absorption and volume of distribution of mangiferin increases when administered at a high dose and its elimination is accelerated (Hou *et al.*, 2011). The same study showed that uncleaved absorbed mangiferin mostly exists in the blood and extracellular fluid, with knowledge regarding specific tissue distribution lacking (Hou *et al.*, 2011). In a separate study, mangiferin could not be detected in its pure form in the faeces of pigs after oral administration of *C. genistoides* extracts (Bock and Ternes, 2010). Mangiferin is said to have a very low oral bioavailability due to first pass metabolism, which limits its use as a nutraceutical (Liu *et al.*, 2011). This polyphenol has also been implicated in potential herb-drug interactions, as it has been found to inhibit cytochrome P450 enzymes in human liver microsomes (Rodeiro *et al.*, 2013a).

Mangiferin has also been shown to inhibit the UDP-Glucuronosyltransferase in the same study (Rodeiro *et al.*, 2013).

2.12.2. Isomangiferin

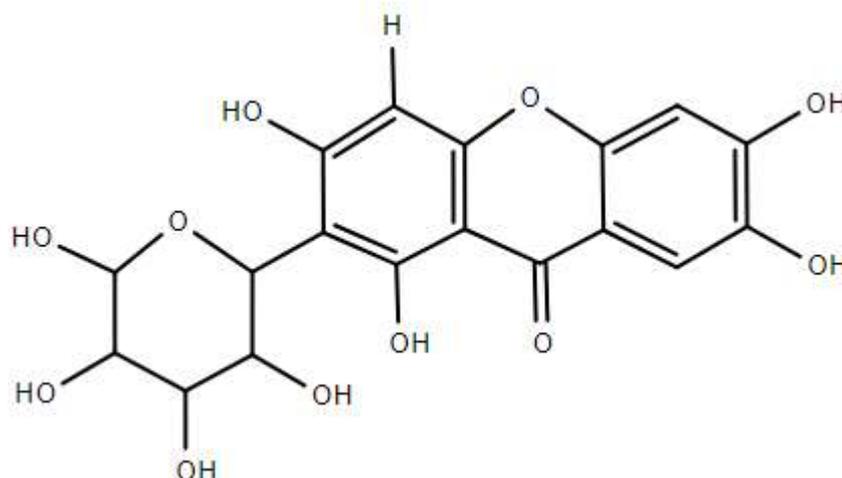


Figure 5: Chemical structure of isomangiferin, a regioisomer of mangiferin (chemspider).

Isomangiferin is a regio-isomer of mangiferin, differing only from mangiferin structurally in the position of the sugar moiety on the dibenzo- γ -pyrone structure (Bosman *et al.*, 2017). This polyphenol has been shown to have similar health promoting effects as mangiferin, including α -glucosidase inhibition (Beelders *et al.*, 2014a). Though slightly different in structure, the position of the sugar moiety on the dibenzo- c -pyrone structure does not affect the α -glucosidase inhibitory activity of the two xanthenes (Bosman *et al.*, 2017). Isomangiferin can also be converted to norathyriol before metabolism (Liu *et al.*, 2011). Similar to mangiferin, traces of isomangiferin were not detected in the faeces of pigs treated orally with *C. genistoides* extracts indicating that isomangiferin is also poorly absorbed and has a similar metabolic fate as mangiferin (Bock and Ternes, 2010). Isomangiferin is also found in the leaves of plants, especially those that contain mangiferin as well, and is also affected by seasonal variation (North *et al.*, 2017). High levels of isomangiferin can be observed in *Cyclopia* species, harvested around February (late summer) while low levels of this polyphenols can be observed in plants harvested in autumn (Joubert *et al.*, 2003). Thus far, isomangiferin has not been isolated from plants that lack mangiferin, indicating that it is likely that the two polyphenols are synthesised through the same process (Joubert *et al.*, 2014).

2.12.3. 3- β -D-glucopyranosyliriflophenone (IMG) and 3- β -D-glucopyranosyl-4- β -D-glucopyranosyloxyiriflophenone (IDG)

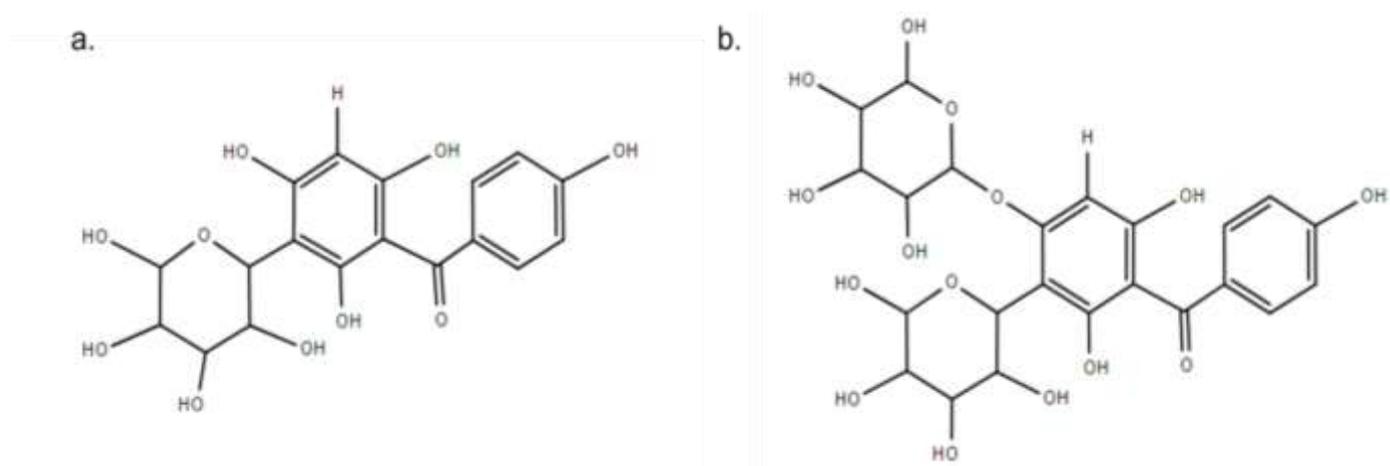


Figure 6: Chemical structures of IMG (a.) and IDG (b.), C-glucoside benzophenones found in *C. genistoides* (chemspider).

The benzophenone 3- β -D-glucopyranosyliriflophenone (IMG) is a C-glucoside benzophenone that is found mainly in the leaves of *C. genistoides* (Beelders *et al.*, 2014a). This polyphenol, together with isomangiferin, is said to contribute 5% of the dry leaf weight (Joubert *et al.*, 2003). IMG, also isolated from mango leaves, has been shown to regulate lipid and glucose homeostasis through the AMPK and PI3K/AKT signalling pathways (Zhang *et al.*, 2013b) and has also shown anti-oxidative and antidiabetic qualities (Malherbe *et al.*, 2014; Bosman *et al.*, 2017). It is an inhibitor of α -glucosidase (North *et al.*, 2017), and affected by seasonal variation, with high levels of IMG seen in summer and low IMG levels seen in spring (Joubert *et al.*, 2014).

Recently, the O-glucoside benzophenone 3- β -D-glucopyranosyl-4- β -D-glucopyranosyloxyiriflophenone (IDG) was isolated from the leaves of *C. genistoides* (Kokotkiewicz *et al.*, 2012). IDG has not been isolated in any other plant, although a compound with similar traits has been isolated in *C. subternata* (Malherbe *et al.*, 2014). Information regarding IDG is still relatively unknown, but evidence suggests that IDG inhibits α -glucosidase in a similar manner to IMG (Beelders *et al.*, 2014). Apart from the α -glucosidase inhibitory activity of IDG, the compound's biological effects have not been explored (Joubert *et al.*, 2008a).

2.13. Therapeutic effects of *C. genistoides*

Aside from those mentioned previously, *C. genistoides* has been associated with several health promoting effects both *in vitro* and *in vivo*. A study showed that *C. genistoides* compounds, especially isomangiferin and IMG had strong pro-apoptotic activity on human synovial cells isolated from patients with active rheumatoid arthritis, indicating the potential of the plant extracts to be developed into a nutraceutical to treat early arthritis (Kokotkiewicz *et al.*, 2013). *C. genistoides* extracts were also found to have phyto-estrogenic effects *in vitro*, whereby the extracts stimulated oestrogen production by inducing cell proliferation of the oestrogen sensitive MCF-7-BUS cells (Verhoog *et al.*, 2007). The xanthenes and benzophenones present in *C. genistoides* were found to inhibit α -glucosidase *in vitro* as well, with mangiferin and isomangiferin displaying similar α -glucosidase inhibitory activity patterns (Beelders *et al.*, 2014a; Bosman *et al.*, 2017). The mechanism by which *C. genistoides* inhibits α -glucosidase is not elucidated. *C. genistoides* has displayed many other health benefits including antimutagenic (Marnewick *et al.*, 2005) and anti-oxidant effects (Van de Merwe *et al.*, 2009). In the Cape, the plant has been used to treat diarrhoea for centuries (Joubert *et al.*, 2008b).

2.14. Inhibition of α -glucosidase

Alpha-glucosidase, located at the brush borders of the small intestines (Bischoff, 1995) is an enzyme that facilitates the metabolism of carbohydrates. The enzyme cleaves the 1,4- α bonds in starch or disaccharides such as maltose and sucrose to produce glucose which is absorbed in the upper jejunum (Lee *et al.*, 2001). In patients suffering from T2D, inhibition of α -glucosidase is a method used to regulate blood glucose levels, especially after meals high in carbohydrates (Subramanian, Asmawi and Sadikun, 2008). The use of α -glucosidase inhibitors began in the 1970s, however, the first pharmaceutical α -glucosidase inhibitor, acarbose, was approved by the FDA in the 1990 (Bedekar, Shah and Koffas, 2010). Inhibition of this enzyme results in a decline in the metabolism of carbohydrates by restricting the hydrolysis of oligosaccharides or disaccharides to produce glucose, thus reducing glucose absorption into the blood stream (Ghadyale *et al.*). Currently there are 3 commercially available pharmaceutical drugs that act as α -glucosidase inhibitors, namely; acarbose, miglitol and voglibose (Arvindkar *et al.*, 2012). α -Glucosidase inhibitors suppress the

activity of α -glucosidase in a competitive and reversible manner; these compounds are maltose-like and have a high affinity to α -glucosidase (DiNicolantonio, Bhutani and O'Keefe, 2015). Post-prandially, the action of α -glucosidase inhibitors may affect insulin secretion, due to decreased blood glucose levels. This type of treatment is not as effective for regulating fasting plasma glucose levels (Arvindekar *et al.*, 2012). α -Glucosidase inhibitors are said to act additively or synergistically with other anti-diabetic drugs such as sulfonylureas and/or biguanides, and a combination of these drugs has not been shown to have adverse effects because they have different mechanisms of actions (Bedekar *et al.*, 2010).

Herbal medicines have been used to alleviate symptoms related to hyperglycemia for many years (Jordan *et al.*, 2010). Phenolic compounds, which are sugar-like, have been shown to possess α -glucosidase inhibitory activity (Subramania *et al.*, 2008). The xanthone; mangiferin, and the benzophenone; IMG, have also displayed α -glucosidase inhibitory activities in a recent study (Beelders *et al.*, 2014a). Plant polyphenols exert their α -glucosidase inhibitory activity in a similar manner to the commercially available α -glucosidase inhibitors (Coman *et al.*, 2012). The sugar moieties have a high affinity for the enzyme and bind to the active site of α -glucosidase thus blocking the cleavage of carbohydrates by the enzyme (Kim *et al.*, 2008).

2.15. Potential toxicity of *C. genistoides*

There are no recorded incidences of *Cyclopia* extracts being toxic or exerting any undesired side-effects (Raaths, 2016). *Cyclopia* extracts are generally considered safe and have been used to alleviate gastrointestinal problems in toddlers for centuries without any undesired side effects (Joubert *et al.*, 2011). This indicates that extracts prepared from *Cyclopia* species may be safe and readily used without much consideration of dose. However, these extracts, especially *C. genistoides* extracts, are not completely free from risk. *C. genistoides* is rich in biologically active polyphenols such as the xanthone, mangiferin and the flavonoid, hesperidin which have been implicated in the inhibition of certain CYP enzymes (Rodeiro *et al.*, 2008, 2013). Therefore potential *C. genistoides* nutraceuticals, especially those targeting α -glucosidase inhibition, show probability of co-administration with conventional antidiabetic drugs and other drugs with an increased risk of potential herb-drug

interactions. This highlights the importance of investigating any potential CYP enzyme interactions that these nutraceuticals may have.

2.16. The liver as a detoxifying agent

The liver is the largest internal organ in the body and is found in the upper right quadrant of the abdomen (Grisham, 2009). The liver detoxifies many endogenous compounds such as products of carbohydrate, lipid and protein metabolism as well as exogenous substances including drugs, hormone enhancers, food additives, preservatives, food colourings, sweeteners, flavour enhancers, chemicals used in agriculture, alcohols, volatile organic compounds and inhaled gases (De Simone and Cortese, 1992; Hsu *et al.*, 2012; Mitra *et al.*, 2009). The liver is a source of stored bile, produced in the gall bladder, which breaks down fats during digestion and is also an energy source, storing glucose in the form of glycogen (Anil and Forbes, 1987; De Simone and Cortese, 1992; Marnewick *et al.*, 2003; Zani *et al.*, 2013). During detoxification, the liver filters the blood to remove large toxins, synthesizes bile which traps many fat-soluble toxins, and also enzymatically eliminates drugs and xenobiotics (Wu *et al.*, 2004). Many drugs enter the body as lipophilic substances where they are converted into hydrophilic substances that can be more easily excreted from the body (De Simone and Cortese, 1992).

2.17. Drug metabolism

About 70% of all drug metabolism occurs in the liver, though drug metabolism can also occur in other tissues such as the lungs, kidneys, placenta, epithelial cells of gastrointestinal tract, adrenals and skin (Blumenthal and Tavenner, 2010). The metabolism of drugs and xenobiotics is known as the “biotransformation process” of pharmaceutical drugs and other chemical substances that do not occur naturally in the body (xenobiotics) so that they can be more easily eliminated from the body (Arvindekar *et al.*, 2012). During drug metabolism, lipophilic compounds are converted into hydrophilic derivatives that are more easily excreted from the body, thus drug metabolism may also be defined as a “detoxification process” (Anil and Forbes, 1987).

Drug metabolism occurs primarily through two phases, phase I metabolism whereby compounds are oxidized, and phase II metabolism whereby the oxidized compounds

are conjugated with highly polar functional groups such as glutathione conjugates (Jancova, 2012).

Phase I reactions are non-synthetic reactions and involve formation of a new or modified functional group or cleavage whereby the OH group is formed or revealed through oxidation, reduction or hydrolysis (Bernhardt, 2006). Oxidation reactions are the most common phase I reactions and one of the major enzymes responsible for these reactions are cytochrome P450 (CYP) enzymes (Pelkonen *et al.*, 2008).

Phase II drug metabolic reactions are synthetic reactions that involve the conjugation of the drug metabolites with an ionized functional group, thus making the drug more polar, and also inactivating the drug and its metabolites (Josephy *et al.*, 2005). Ionic groups attached to the drug metabolites include the glutathione, methyl or acetyl groups (Xu *et al.*, 2005). Phase II reactions are catalysed by several enzymes including the UDP–glucuronosyltransferases, sulfotransferases and glutathione-S-transferases (Jin *et al.*, 2005). Phase II reactions are generally divided into two types: type 1 conjugation; in which the activated drug/metabolite is combined with a substrate to yield a conjugated product and type 2 conjugation; in which the substrate is activated and then combined with an amino acid to yield a conjugated product (Jančová and Šiller, 2012). After conjugation, the drug can then easily be excreted from the liver. The process of drug metabolism is summarised in Figure 7.

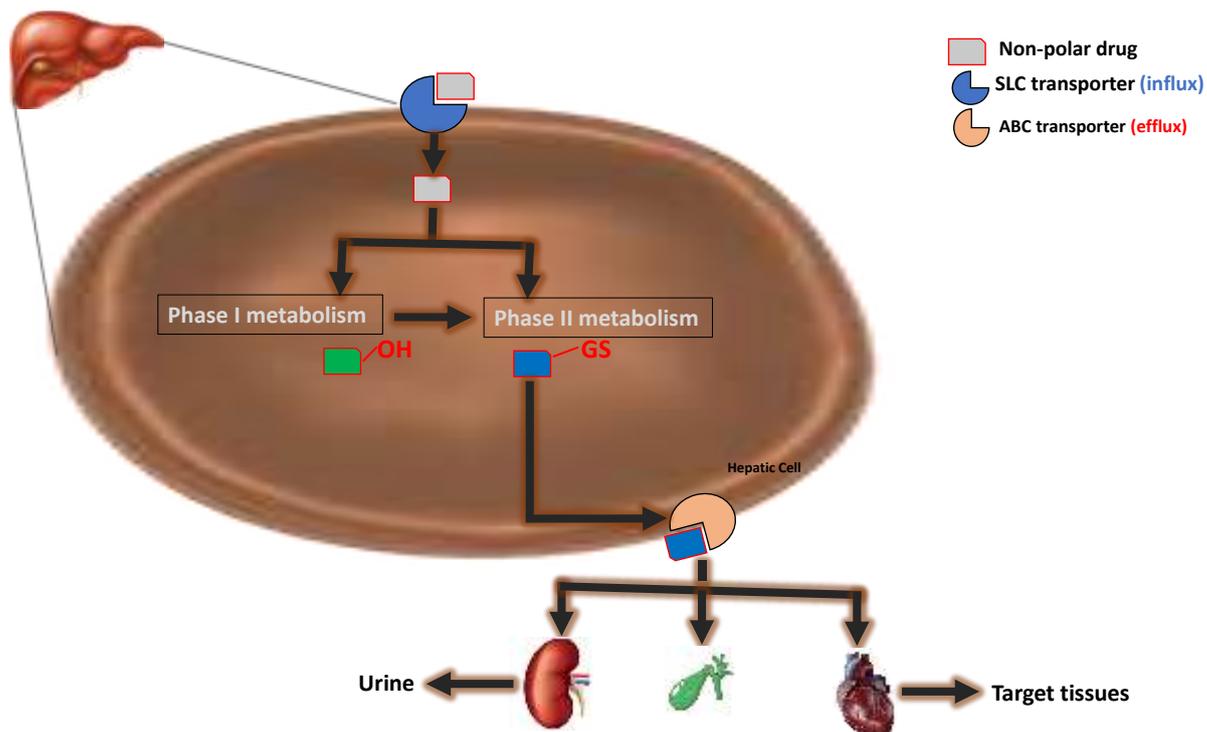


Figure 7: Schematic representation of the metabolism of drugs, which mainly occurs in the liver. After absorption of the non-polar drug by SLC transporters into the hepatocyte, it undergoes phase I metabolism. Phase I metabolism is mainly oxidation of the drug by CYP enzymes making the drug polar and active. The polar drug then undergoes phase II metabolism, which involves conjugation of the drug to make it more stable, this step sometimes de-activates drugs. Phase II metabolism may also occur simultaneously with phase I metabolism. After phase II metabolism, the conjugated drug can be excreted from the liver to target tissues or as waste to the kidney and bile for further excretion (adapted from drug metabolism for dummies, no date).

2.18. Cytochrome P450 (CYP) enzymes

Cytochrome P450 (CYP) is a large superfamily of haem-containing enzymes which catalyse the oxidative biotransformation of most drugs and other xenobiotics (Hasler *et al.*, 1999; Zanger and Schwab, 2013). CYPs are not only responsible for the biotransformation of drugs and xenobiotics, they also have a number of important functions such as the biosynthesis of steroid hormones, prostaglandins and bile acids, amongst others (Kirchheiner and Seeringer, 2007). CYP enzymes are so named because they contain a haem group that can bind with high affinity to carbon monoxide in a reduced state, and these bound complexes absorb UV light at a wavelength of 450 nm, hence the name cytochrome P450 (Bernhardt, 2006). Within the family, members sharing 85% sequence similarity, are grouped together to form a subfamily which is named by the alphabetic letter, and individual members of the family are named with a second Arabic number (Srinivas, 2017). CYP enzymes are widely

distributed in nature (Thomas *et al.*, 2013) and in humans, approximately 58 CYP enzymes, belonging to 18 families and 44 sub families have been identified (Smith and Wilson, 2010). CYP enzymes catalyse the oxidation of drugs using molecular oxygen (O₂). They transfer 1 atom of oxygen to a substrate and reduce the second atom to water using 2 electrons that are provided by NADPH, producing an oxidised substrate along with a molecule of water (Rendic *et al.*, 1999). The reaction of CYP mediated metabolism can be summarised in the following equation (Figure 8). Where R represents a CYP substrate (Lv *et al.*, 2016).

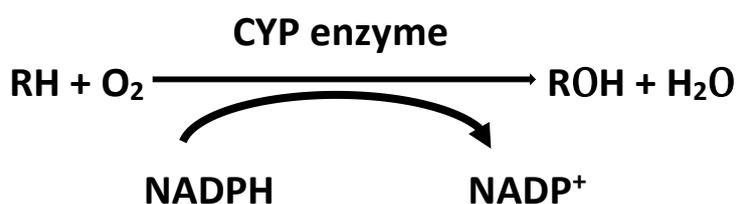


Figure 8: Schematic reaction of CYP mediated metabolism.

CYP enzymes donate O₂ to a non-polar compound thus revealing or introducing the OH- group to the compound and making it polar. NADPH is used as a co-factor (adapted from Bernhardt, 2006).

Binding of the substrate to the CYP enzyme lowers the redox potential of the enzyme, making it favourable for the co-factor, NADPH, to transfer an electron (Lamb *et al.*, 1999). This is followed by the first reduction, whereby the ferric (Fe³⁺) ion of the haem is reduced to a ferrous (Fe²⁺) ion by an electron transferred from NADPH via an electron transfer chain (Lewis and Pratt, 1998). The oxygen molecule (O₂) then binds to the reduced Fe²⁺ ion forming the Fe²⁺ + ·O₂ complex which undergoes a slow conversion to form Fe³⁺ + ·O₂⁻, a more stable complex (Isin and Guengerich, 2007). The binding of oxygen is followed by a second reduction, which is the rate limiting step of CYP mediated metabolism (Yun *et al.*, 2000; Taxak and Bharatam, 2014). During this step, the Fe³⁺ + ·O₂⁻ is reduced to Fe³⁺ + ·O₂²⁻ (Zhou *et al.*, 2010). Following the second reduction, the O-bond is cleaved to form water when O₂²⁻ reacts with 2 protons from the nearby solvent (usually water in the hepatocytes), leaving a Ferric oxide (FeO₂⁺) complex (De Monthellano, 2005). This leads to the formation of the product, whereby the Fe-ligated O-atom is transferred to the substrate to form a hydroxylated form of the substrate and then is released from the active site of the enzyme. After the

product is released, the enzyme active site returns to its initial state (Isin and Guengerich, 2007). The catalytic cycle of CYP enzymes is illustrated (Figure 9).

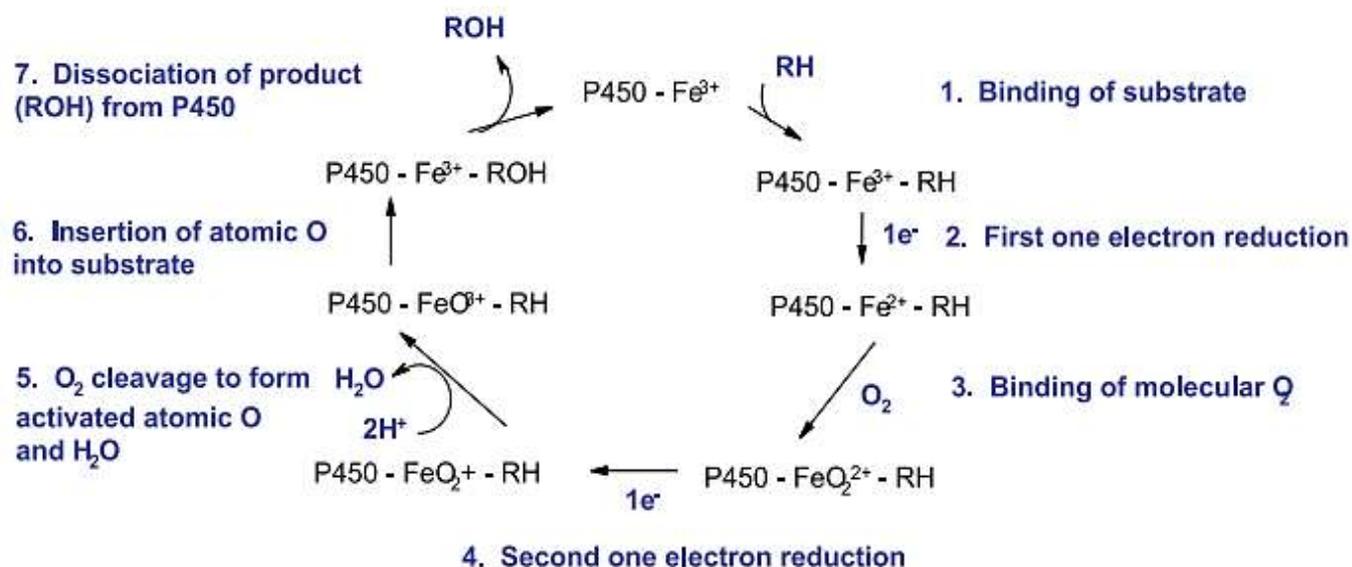


Figure 9: Schematic representation of the catalytic cycle of CYP reactions (Adapted from Stiborova *et al.*, 2017).

Unlike other enzymes, CYPs do not have tight substrate specifications, and can use substrates ranging in size from the relative molecular weight (Mr) of 28 g/mol to 1201 g/mol (Bernhardt, 2006; Isin and Guengerich, 2007); therefore substrate overlap is very common for these enzymes. Major drug metabolising enzymes belong to the CYP1, CYP2 and CYP3 families (Ogu and Maxa, 2000) and their approximate proportion in drug metabolism is shown in Figure 10. As a result, current FDA guidelines for the development of pharmaceutical products recommend that high throughput screening processes be performed to identify any potential drug interactions involving CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 for any new therapeutic product (FDA, 2012).

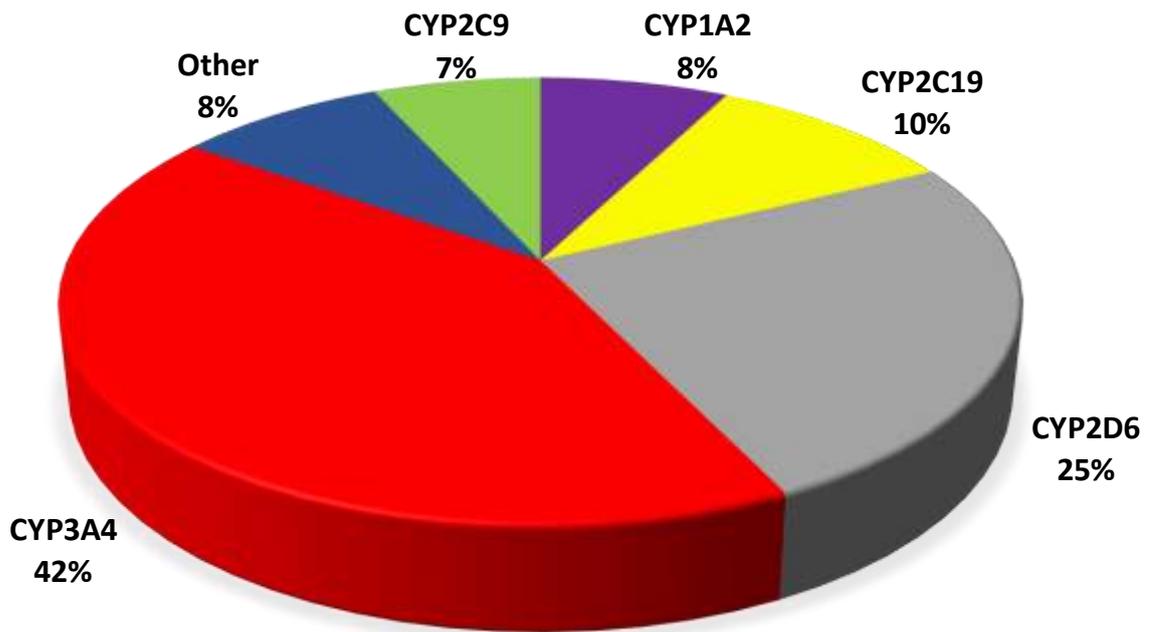


Figure 10: Proportion of drugs metabolised by CYP enzymes (adapted from Guengerich, 2005).

2.18.1. Cytochrome P450 1A2 (CYP1A2)

CYP1A2 belongs to the CYP1 family, as well as two other isozymes - CYP1A1 and CYP1B1 (Faber *et al.*, 2005). However, the expression of both CYP1A1 and CYP1B1 is very low and these enzymes contribute very little to the metabolism of drugs and xenobiotics (Sulem *et al.*, 2011). CYP1A2 contributes 13-15% of all CYP enzymes in the liver and is responsible for the metabolism of approximately 9% of commercially available pharmaceutical drugs (Nebert and Russell, 2002). CYP1A2 has been shown to have protective properties in animal studies. It is involved in the detoxification of PAHs, N-heterocyclic compounds and aromatic amines (Gong *et al.*, 2017). Common substrates of CYP1A2 include propranolol, melatonin, theophylline, clozapine and verapamil. Inhibitors of CYP1A2 include artemisinin, atazanavir, cimetidine, fluvoxamine and ciprofloxacin (Horn, 2005). Fluoroquinolone antibiotics, enoxacin and ciprofloxacin, also significantly inhibit CYP1A2. Potent inducers of CYP1A2 include carbamazepine, phenobarbital, rifampicin and tobacco (Mills, 2004).

2.18.2. Cytochrome P450 2C9 (CYP2C9)

Cytochrome P450 2C9 (CYP2C9), together with the other known isozymes in the CYP 2C family (CYP2C8, 2C19) contribute about 20% of all the CYP enzymes in the liver and 15% of the total intestinal CYP content (Shamsi *et al.*, 2017). The enzyme is

responsible for the metabolism of approximately 12% of pharmaceuticals (Lasker *et al.*, 1998). Common substrates of CYP2C9 include carvedilol, celecoxib, glipizide, ibuprofen, Irbesartan and losartan (FDA, no date). Potent inhibitors of CYP2C9 include amiodarone, fluconazole, fluoxetine, metronidazole, ritonavir and sulfamethoxazole (Lasker *et al.*, 1998). Carbamazepine, phenobarbital, phenytoin and rifampicin are some of the known potent inducers of this CYP enzyme (Lee *et al.*, 2002; Tessaneeyakul *et al.*, 2016) .

2.18.3. Cytochrome P450 2C19 (CYP2C19)

Cytochrome P450 2C19 contributes about 2% of the total intestinal CYP content and 16% of the total hepatic CYP content (Zanger and Schwab, 2013). The enzyme plays a significant role in the metabolism of protein pump inhibitors and the anti-epileptic drugs diazepam and mephenytoin (Lasker *et al.*, 1998). CYP2C19 is also one of the principal enzymes responsible for attacking various long-chain polyunsaturated fatty acids at their alkene bonds to form epoxide products that act as signaling agents (Poór *et al.*, 2017). Common substrates of CYP2C19 include warfarin, carisoprodol, phenytoin and tofacitinib (Thomford *et al.*, 2016). Potent inhibitors of CYP2C19 include amitriptyline, clomipramine, fluconazole, fluvoxamine, imipramine and ticlopidine. Common inducers of CYP2C19 include enzalutamide and rifampicin (Brandt *et al.*, 2007).

2.18.4. Cytochrome P450 2D6 (CYP2D6)

CYP2D6 is expressed in the liver and the brain. However, its physiological and pharmaceutical functions in the human brain are unknown (Lv *et al.*, 2016). There is variation in the efficiency and amount of CYP2D6 enzyme produced between individuals (Niwa *et al.*, 2018). Hence, for drugs that are metabolised by CYP2D6, certain individuals will eliminate these drugs quickly while others will not. The enzyme is responsible for the metabolism of approximately 30% of all therapeutic agents (Langhammer and Nilsen, 2013). CYP2D6 is also responsible for the metabolism of various endogenous substances such as hydroxytryptamines and neurosteroids, as well as m-tyramine and p-tyramine which then metabolizes into dopamine in the brain and liver (Hart *et al.*, 2008). This enzyme also activates prodrugs into pharmaceutically active agents. Common substrates of CYP2D6 include codeine, tramadol, bufuralol,

metoprolol, amitriptyline, paroxetine, venlafaxine, tamoxifen and flecainide (Kivistö and Kroemer, 1997). Strong inhibitors of CYP2D6 include bupropion, fluoxetine, metoclopramide, paroxetine and quinidine (Sanchez-Spitman *et al.*, 2018).

2.18.5. Cytochrome P450 3A4 (CYP3A4)

CYP3A4 is the most abundant CYP isozyme in the human liver and intestines. It is also the most versatile CYP enzyme. This enzyme contributes about 30% of adult hepatic CYP content and about 80% of intestinal content. CYP3A4 is responsible for the metabolism of approximately 50% of clinically prescribed medicines (Pal and Mitra, 2006). CYP3A4 is responsible for the biotransformation of many structurally different substances, common substrates of this enzyme include alprazolam, atorvastatin, cyclosporine, diazepam and simvastatin (Lynch *et al.*, 2007). Erythromycin, grapefruit juice, itraconazole, ketoconazole and ritonavir are some of the best known potent inhibitors of this enzyme (FDA, no date). Potent inducers of CYP3A4 include rifampicin, St. John's wort and carbamazepine (Williamson *et al.*, 2013).

2.19. Inhibition of CYP enzymes

Inhibition of CYP enzymes occur when a compound opposes the metabolic activity of one or more CYP enzymes. Some substrates may covalently bind to the CYP-active site and change the conformation of the active sites of the enzymes, thus blocking their activity (Pelkonen *et al.*, 1998). CYP enzyme inhibition occurs rapidly (Smith *et al.*, 2012) and there are many different ways by which CYP inhibition occurs, the 2 major mechanisms are reversible and/or non-reversible inhibition (Rendic, 2002). Reversible inhibition may be classified as competitive, non-competitive and mixed inhibition (White, 2016). During competitive inhibition, an inhibitor competes with the substrate by binding to the active site of the enzyme, thus preventing the formation of the enzyme-substrate complex which delays subsequent metabolism of the substrate (Costantino, Brown and Kelly, 1990). The binding of the inhibitor to the enzyme is reversible, and the overall structure of the enzyme is not affected during this binding, so the enzyme can still metabolize its prospective substrate after the inhibitor detaches from it (White, 2016). Non-competitive inhibition occurs when an inhibitor changes the structure of the enzyme by binding to parts of the enzyme, other than the active site, thus affecting the enzyme's ability to bind to the active site (Haque *et al.*, 2017). During mixed inhibition an inhibitor can bind to the active site of the enzyme before the

substrate is bound or it can bind to the enzyme-substrate complex, after the substrate is bound to the enzyme, thus breaking the complex before the reaction is complete (Thomas *et al.*, 2013). During non-reversible inhibition, also known as time-dependent inhibition, the inhibitor becomes more effective over time (Rendic, Nolteernsting and Schänzer, 1999).

2.20. Drug transporters

Drug transporters are large membrane-bound proteins that are expressed in tissues all over the body (Giacomini *et al.*, 2010). They can affect drug pharmacokinetics, and explain mechanisms underlying drug–drug interactions (DeGorter *et al.*, 2012). Drug transporters may be subdivided into two major families: (1) solute carrier transporters which facilitate the uptake of substances from the extracellular space into cells, and (2) ATP-binding cassette transporters which actively discharge substances out of cells (Liang *et al.*, 2015). They account for 1.7% of the human genome and are found in many tissues including the liver, kidneys, brain and small intestines (Huang *et al.*, 2008). Drug transporters localized in the basolateral membranes play an important role in the disposition of drugs and they, together with drug metabolism, determine the role of the liver as a detoxifying organ (Badolo *et al.*, 2014). Drug transporters have a significant impact on the efficacy and toxicity of drugs, changes in the activity of drug transporters may result in drug interactions with adverse side effects (Williamson *et al.*, 2013). There are 2 major ways in which the activity of drug transporters may be altered, i.e. genetic polymorphism and modulation (induction or inhibition) of transporter activity by an external substance (Stieger and Hagenbuch, 2014).

Drug transporters play an important role in the absorption, distribution and excretion of drugs, they also heavily influence drug metabolism by regulating access of the drug to the drug metabolizing enzyme (Fromm and Kim, 2011). In the last decade drug transporters have been implicated in drug interactions (Manzi and Shannon, 2005). Drug transporters of clinical significance include P-gp/ABCB1, BCRP/ABCG2, OATP1B1/SLCO1B1 and OATP1B3/SLCO1B3, amongst other transporters (Pang *et al.*, 2010). Similar to CYP enzyme modulations drug interactions involving drug transporters involve induction or inhibition of the transporter proteins resulting in drastic changes in drug pharmacokinetics and efficacy (Thompson, 2011). Therefore it is important to investigate possible modulations of these proteins for any new

therapeutics to determine the safety of the new product (Giacomini *et al.*, 2010). The FDA recommends that in vitro through put screenings for potential drug interactions involving drug transporters be conducted (FDA, 2012). Investigation of mRNA expression of drug transporters is a major technique of monitoring induction of drug transporters in vitro (Pang *et al.*, 2010). Another method is to conduct transport experiments.

2.20.1. The solute carrier superfamily

The solute carrier (SLC) is a large superfamily of membrane-bound transporters that move substrates across membranes through many different mechanisms, including facilitated diffusion, ion coupling, and ion exchange, which may be driven by an ion gradient that is maintained by the active efflux pumps (Elmeliegy *et al.*, 2011). Members of this family share 20-25% sequence homology, and include: the organic anion transporters (OATs), encoded by the SLC22A gene; the organic anion transporting polypeptides (OATPs), encoded by the SLCO gene; organic cation transporters (OCTs), encoded by the SLC22A gene and the recently discovered multidrug and toxin extrusion transporters (MATE transporters), encoded by the SLC47A gene (Gao *et al.*, 2000; Englund *et al.*, 2006; Karlgren *et al.*, 2012; Williamson *et al.*, 2013). The expression of drug transporters is very important for the pharmacokinetics of the drugs (Badolo *et al.*, 2014).

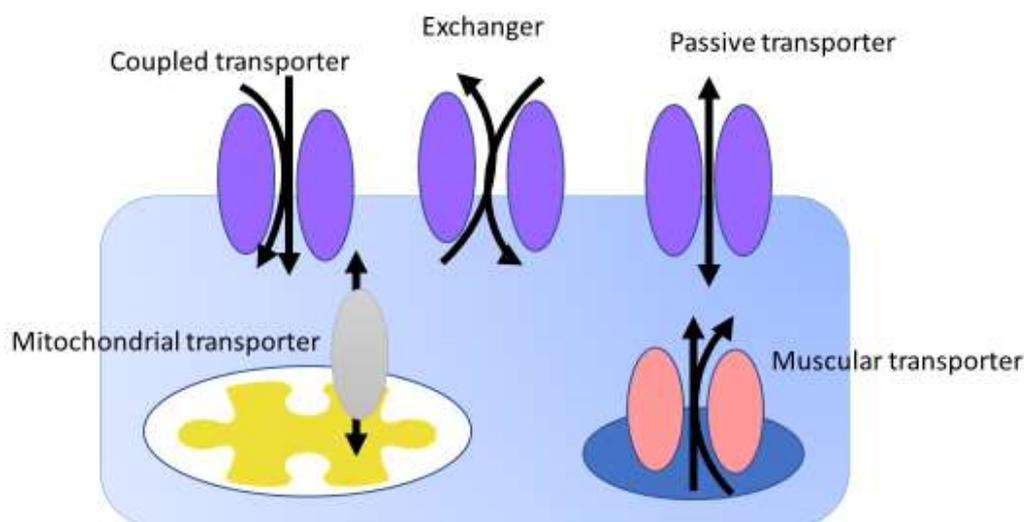


Figure 11: Different types of SLC transporters (adapted from Tyagi, 2005).

2.20.2. Organic anion transporting polypeptides (OATPs/SLCOs)

Organic anion transporting polypeptides (OATPs) are membrane proteins that facilitate the sodium-independent transport of substances into cells and tissues (Zaïr *et al.*, 2008). These transporters are found in humans and rats/mice in nature with human organic anion transporting polypeptides represented by OATPs and rat/mice organic anion transporting polypeptides represented as OATPs (Huang *et al.*, 2008). The first OATP/Oatp was identified in 1994 through expression cloning by Jacquenin and colleagues, the rat Oatp1a1 (Gao *et al.*, 2000). To date, over 40 transporters belonging to the OATP/Oatp subfamily have been identified and in humans there are 12 known OATP transporters (Liang *et al.*, 2015). They are encoded by the SLCO genes in humans which are generally found in a gene locus on chromosome 12 (Pasanen *et al.*, 2008). Structurally, OATPs contain 12 transmembrane domain helices and have a large extracellular domain between the 9th and 10th transmembrane domains. They also have the amino acid sequence D-X-RW-(I,V)-GAWW-X-G-(F,L)-L which is common for all SLC members (Englund *et al.*, 2006). The tertiary structure of OATPs is relatively unknown (Van De Steeg *et al.*, 2012). OATPs generally transport negatively charged solutes that are relatively large, ranging from 334 Da to 1143 Da and with diverse characteristics (Thompson, 2011). They have wide substrate specificity for amphipathic molecules such as the drugs, rifampicin, methotrexate and various antidiabetic drugs and statins (Hagenbuch and Meier, 2004). Endogenous substrates of OATPs include bile salts, organic dyes, steroid conjugates, thyroid hormones and anionic oligopeptides, while exogenous substrates include several drugs and xenobiotics (Zaïr *et al.*, 2008). These transporters are found in many different tissues especially the brain, liver, and kidney (Shitara, 2011). OATPs are involved in many drug interactions and are highly influential in determining the clinical efficacy of many drugs (Dolton *et al.*, 2012).

Members of the SLCO1B1 and SLCO1B3 subfamilies are located on the basolateral membrane of hepatocytes and facilitate the uptake of substances into the liver via the portal vein (Niemi, 2007). SLCO1B1 and SLCO1B3 are of clinical significance since they are involved in many drug interactions due to polymorphisms and wide substrate specificity, thus the FDA recommends that screenings for potential drug interactions

involving these two transporters be performed for any new therapeutics (Huang, 2006).

2.20.3. Organic anion transporting polypeptide 1B1 (OATP1B1)

OATP1B1 which is encoded by the gene *SLCO1B1*, is the most studied human OATP and is believed to contain the most polymorphism (Shitara, 2011). This transporter is mainly expressed on the basolateral membrane of the liver and is mainly responsible for the hepatic uptake of antidiabetic drugs and statins that use the liver as a target organ (X. Zhang *et al.*, 2013). Apart from drug transport, OATP1B1 also transports endogenous substances like bile acids, bilirubin, steroid hormones and thyroid hormones (Treiber *et al.*, 2007). The OATP1B1 protein is made up of 691 amino acids and is encoded by the *SLCO1B1* gene (Louisa *et al.*, 2016). Expression of this transporter is known to be regulated by the nuclear hormone receptors farnesoid X receptor (FXR), hepatocyte nuclear factor-1- α (HNF1 α), hepatocyte nuclear factor-3- β (HNF3 β) and hepatocyte nuclear factor-4- α (HNF4 α) (zu Schwabedissen *et al.*, 2010; Svoboda *et al.*, 2011). *SLCO1B1* mRNA has been shown to be expressed in other tissues such as the intestines and kidneys, however the translation of this gene to protein was not confirmed in these tissues (Shitara, 2011).

2.20.4. Organic anion transporting polypeptide 1B3 (OATP1B3)

OATP1B3 is very similar to OATP1B1 with 80% sequence similarity (Schwarz *et al.*, 2011). OATP1B3 is also expressed in the basolateral membrane of the liver and facilitates the uptake of antidiabetic drugs and statins (Keppler, 2014; Vildhede *et al.*, 2014). It also transports taxanes and many small peptides. However, the role of OATP1B3 in taxane transport is not fully elucidated (Nieuweboer *et al.*, 2014). Since they are so similar, OATP1B1 and OATP1B3 have the same endogenous and exogenous substrates leading to substrate overlap (Hirano *et al.*, 2006). However, OATP1B3 has been shown to exclusively transport amanitin, a toxin from *Amanita* mushrooms (Letschert *et al.*, 2006).

2.20.5. Adenine triphosphate (ATP)-binding cassette transporters

Adenine triphosphate (ATP)-binding cassette (ABC) transporters are a large family of drug transporters that secrete substances out of cells against a concentration gradient

using energy generated from the hydrolysis of ATP (Schinkel and Jonker, 2012). It is estimated that hydrolysis of two ATP molecules results in the transport of one molecule of substrate (Mohammad *et al.*, 2018). Examples include the P-glycoprotein (P-gp), multidrug resistance proteins (MRPs) and the breast cancer resistance proteins (BCRP) (Yamaura *et al.*, 2016). The ABC family is made up of 48 members that are grouped into seven subfamilies (ABCA to ABCG) based on sequence similarity of the ATP-binding domain(s) (Dean and Annilo, 2005). Members of the ABC family have two transmembrane domains and two nucleotide-binding domains (Elmeliegy *et al.*, 2011). They all have a structural fold containing a core of six transmembrane α -helices per transmembrane domain (Jasinski *et al.*, 2015). ABC transporters protect internal organs from toxins, control drug distribution in the body, mediate inflammatory responses and transport lipids (Crowe, 2011). The mechanism by which ABC transporters operate is not uniform for all members of the superfamily, slight differences in the general mechanism have been observed for different members (Mohammad *et al.*, 2018). Generally, membrane domains of ABC transporters act as gates that are tightly bound to nucleotide binding domains (Wilkins, 2015). The transmembrane domains interchange between open and closed states. When open, the substrate can bind to the transmembrane domains thus causing conformation changes on whole protein and increasing the affinity of ATP to bind to the ATP binding domains (Liang *et al.*, 2015). Two molecules of ATP then bind to the ATP binding domains causing the transmembrane domains to close and change conformation, thus, forcing the substrate to be released out of the transmembrane domains and out of the cells (Mohamma *et al.*, 2018). Both ATP molecules are cleaved when the transmembrane domains are still closed releasing ADP, water and energy. This energy release forces the outward movement of the substrate and also re-sets the ABC transporter (Dohse *et al.*, 2010). The general action of the ABC transporters is illustrated in Figure 12.

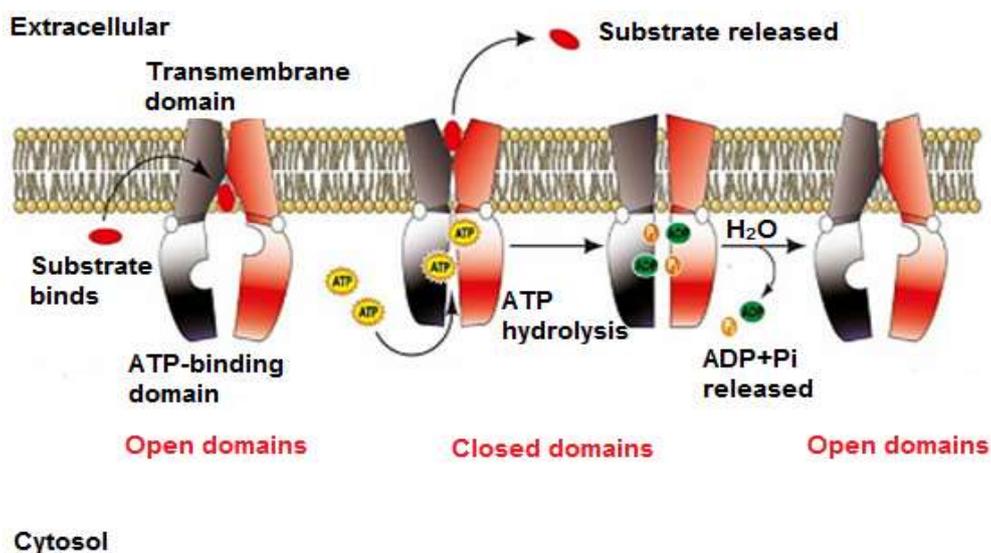


Figure 12: Simple diagram of ABC transporter activity.

A substrate binds to the open transmembrane domains increasing affinity for ATP to bind to the ATP binding domains. ATP then binds to the ATP binding domain and cleaved thus releasing energy. The energy release causes conformation changes of the transmembrane domain of the protein, allowing the drug transporter to efflux drugs to the extracellular space, against a concentration gradient (adapted from Mohammad, He and Yin, 2018).

2.20.6. ABCB1 (P-glycoprotein)

ABCB1/Pg-p is an ATP-dependent efflux pump with broad substrate specificity that is found in animals, fungi and bacteria (Leschziner *et al.*, 2007). In humans, the drug transporter is encoded by the ABCB1 gene (Hong *et al.*, 2006). It is extensively expressed in many tissues such as the intestines, liver, brain and kidneys (Noguchi *et al.*, 2014). ABCB1, first discovered by Juliano and Ling in 1976 and is the most studied efflux transporter to date (Dufort and Chimini, 2008). It was found to be over-expressed in drug resistant tumour cell lines, indicating that it promotes drug resistance to certain anti-cancer drugs (Igel *et al.*, 2007). Structurally, ABCB1 is a large protein weighing about 170 kDa and with 1276-1280 amino acids. It is a complete ABC transporter with 2 ATP binding cassette domains, with each domain containing 6 transmembrane domains (Li *et al.*, 2010). Substrates of ABCB1 are usually amphipatic or hydrophobic in nature and contain aromatic groups, however, non-aromatic linear and circular molecules can also be transported by this protein (Wang *et al.*, 2003). Common substrates of ABCB1 include the drugs diagon, rosuvastatin, gluburide, quinidine, ritonavir, nitrorantion, and dipyridamole, dietary compounds porphyrins and the endogenous substances such as bile acids, steroids and bilirubin (Chan *et al.*, 2004;

Hong *et al.*, 2006; Leschziner *et al.*, 2007; Li *et al.*, 2010; M.J. *et al.*, 2013) ABCB1 eliminates drugs into the lumen, thus reducing the absorption and bioavailability of the drug (Lin and Yamazaki, 2003). The expression of ABCB1 can be influenced by environmental factors and genetic factors (Greiner *et al.*, 1999). Common inducers of ABCB1 expression include: the drug rifampicin; the nutraceutical St. John's wort and dietary compounds such as green tea and grapefruit juice (Fromm, 2004). Polymorphisms of the ABCB1 genome results in inter-individual variation of the functioning of ABCB1 (Tabe *et al.*, 2006; Wolking *et al.*, 2015). There is substrate overlap between ABCB1 and the CYP enzyme CYP3A4, and these two proteins are co-expressed and have almost the same substrates (Elkayam *et al.*, 2006). Induction of P-gp/ABCB1 is speculated to be through the activation of pregnane X receptor (PXR) pathway (Awortwe *et al.*, 2015; Geick *et al.*, 2001). PXR is known to also regulate the induction of CYP3A4 expression. Exposure to an inducer activates the receptor and the PXR then moves to the nucleus where it forms heterodimer complexes with the retinoid X receptor (RXR). These complexes activate the response elements present in the regulatory region of the ABCB1 and CYP3A4 genes resulting in the up-regulation of their expression (Geick *et al.*, 2001).

2.20.7. Breast cancer resistant protein (ABCG2)

ABCG2, the breast cancer resistant protein (also known BCRP) was the last ABC transporter to be identified so far (Schinkel and Jonker, 2012). It was first cloned in MCF7 cells highly resistant to doxorubicin, MCF-7/AdrVp hence its name (Abbott, 2003). In humans, the protein is encoded by the ABCG2 gene (Schinkel and Jonker, 2012). ABCG2 is a 72 kDa protein encoding 655 amino acids. It appears as a half type ABC transporter with a single N-terminal ATP binding cassette and 6 transmembrane segments (Abbott, 2003). The drug transporter is usually co-expressed with ABCB1 and shares most of its substrates, inducers and inhibitors (Noguchi *et al.*, 2014). Since the two drug transporters (ABCG2 and ABCB1) are co-expressed, they are said to act synergistically, resulting in enhanced excretion of their substrates which drastically decreases the bioavailability of such substrates (Giacomini *et al.*, 2012). Expression of ABCG2 can also result in multidrug resistance of cancer cells to anticancer drugs and effects the pharmacokinetics of drugs in normal cells (Zhou *et al.*, 2001).

2.21. Herb-drug interactions

The continued global use of herbal medicine leans to an overwhelming increase in co-administration with conventional drugs, raising concern for public health. The concomitant use of herbal medicines and conventional drugs has been found to cause pharmacokinetic and pharmacodynamics changes in conventional drugs, often coupled to adverse side effects (Posadzki *et al.*, 2013). Most herbal medicines and conventional drugs are therapeutic at high specific dose windows and can often become toxic or ineffective when the dose changes, with even minor changes held responsible for severe side effects (Fugh-Berman and Ernst, 2001; Poppenga, 2002; Kar *et al.*, 2015).

The side effects associated with conventional drugs are well publicised, due to legislation requirements. Somewhat controversially, herbal remedies are alleged to be mild and safe because they are derived from natural plant materials (Jordan *et al.*, 2010). However, the actual chemical composition of many herbal medicines and plant extracts is largely unknown which may contain some toxicity risk (Mahomoodally, 2013). Plant material may also be contaminated with toxic heavy metals and microorganisms which may lead to toxicity when consumed by humans (States *et al.*, 2000). Improper preparation of the herbal medicine such as accidental removal or misidentification of the bioactive compounds within the herbal medicine may also lead to adverse reactions and potential toxicity (Corns, 2003). Studies have shown that the simultaneous use of herbal medicines with some antidiabetic drugs may lead to several adverse effects (Purohit and Mishra, 2017).

Pharmacokinetic herb-drug interactions occur when the herbal medicine interferes with the absorption, distribution, metabolism and/or elimination of the conventional drug (Fasinu *et al.*, 2016). Modulations in the activities of drug metabolizing enzymes such as cytochrome P450s, and/or drug transporters is said to be a major mechanism of pharmacokinetic herb-drug interactions (Hu *et al.*, 2005). Conversely, herbal medicines may decrease the effect of conventional drugs leading to reduced drug efficacy and drug failure (Mahomoodally, 2013). It is therefore important to screen for any potential interactions involving these proteins for any new therapeutic herbal products in order to avoid such interactions.

Many herbal medicines have been implicated in herb-drug interactions in the past (Posadzki *et al.*, 2013). One of the most noteworthy examples is that of St. John's wort. St. John's wort is a herbal product developed in the early 20th century, that has been shown to induce the activity of CYP3A4 and ABCB1 via the PXR, resulting in decreased plasma concentrations of many drugs including cyclosporine, digoxin, indinavir, irinotecan, warfarin and simvastatin (Madabushi *et al.*, 2006).

Green rooibos extracts have recently been found to inhibit several CYP enzymes (CYP2C8, CYP2C9 and CYP3A4), potentially slowing down the metabolism of pioglitazone and atorvastatin, which may also cause life threatening side effects such as extreme hypoglycemia (Posadzki *et al.*, 2013; Patel *et al.*, 2016).

Plant phenolic compounds have the ability to interfere with the normal activity of enzymes that catalyse the biotransformation of drugs as well as the normal activity of the drug transporting proteins (Gurib-fakim, 2006). A confounding factor of many adverse drug reactions originating from herb-drug interaction is that many individuals take herbal medicines together with pharmaceutical drugs without disclosing this concomitant use to prescribing doctors (Hu *et al.*, 2005).

2.21.1. High-throughput screening techniques

The use of high-throughput screening technologies has significantly assisted drug development (Trubetskoy *et al.*, 2005). Performing high-throughput screenings for CYP enzyme modulation earlier in the process of drug development helps to eliminate molecules with unwanted metabolic properties (Shamsi *et al.*, 2017). Most high-throughput screening assays include long and complicated post-reaction steps that limit their usefulness (Trubetskoy *et al.*, 2005). In recent years, fluorescent and/or luminescent assays have been more successful in high-throughput screenings because they require no postreaction separation steps (Marks *et al.*, 2002). The principle behind fluorescent assays is that fluorogenic CYP substrates are metabolized by CYP enzymes to yield highly fluorogenic products (White, 2016). The Vivid[®] CYP450 screening kits are designed to monitor CYP enzyme inhibition through fluorogenic reactions (Life technologies, 2012). Vivid substrates form highly fluorescent products when metabolized by CYP enzymes and inhibition of the CYP enzymes is observed through the lack of fluorescence (Shamsi *et al.*, 2017). The use

of Vivid® CYP450 screening kits is a relatively new technique of investigating possible CYP-mediated drug interactions. They predict CYP inhibition using relatively low reagent volumes and are very rapid making them an effective technique in drug discovery platforms (White, 2016).

2.22. Study model

2.22.1. The C3A liver cell line

Hepatocytes are the most abundant cell type and the primary functional unit of the liver (Hewitt et al., 2007). They perform many tasks, including the metabolism of xenobiotics (Iyer et al., 2010). HepG2 cells are hepatocellular carcinoma cells and are easily maintained and expanded in culture, and have been shown to express a wide range of liver-specific functions (Abdel-Lateef et al., 2016). Since HepG2 cells are cancer cells of the liver, they exhibit both hepatocyte and tumour cell characteristics (Filippi et al., 2004). They can be used for basic studies of hepatocyte cellular physiology such as response to inflammatory stimuli and as metabolically relevant models for in vitro toxicology studies (Elkayam et al., 2006). C3A cells are a clonal derivative of the HepG2 cell line which display an improved differentiated hepatocyte phenotype (Iyer et al., 2010). C3A cells are easily maintained in culture and have been shown to express a wide range of liver-specific functions. These cells retain biological characteristics of the adult liver (Nelson et al., 2017). For example, they switch from a foetal phenotype during the sub-confluent log growth phase to a mature liver phenotype upon reaching confluence and express several functional cytochrome P450 enzymes (Iyer et al., 2010).

2.23. Rational for this study

In order to develop a new chemical entity for use as a conventional drug, the drug safety pharmacology will involve the mechanisms of many drug-drug interactions in order to establish the risk of metabolism-based drug-drug interaction associated with its use (Zhou *et al.*, 2007). The inhibition or induction of major drug metabolizing enzymes such, as CYP enzymes, drug transporters and efflux proteins are part of the mandatory assessment using guidelines set out by the FDA (FDA, 2012). Many CYP enzymes and drug transporters are responsible for drug metabolism and are consequently involved in both drug-drug and herb-drug interactions (Posadzki *et al.*,

2013). However, most studies currently conduct the first screen using the 5 major CYP enzymes (CYP1A2, 2C9, 2C19, 2D6 and 3A4) and the drug transporter ABCB1, responsible for metabolism of over 90% of conventional drugs (Ogu and Maxa, 2000; Thompson, 2011). In this study, we use the Vivid® screening kits to assess the effect of *C. genistoides* constituents on these major CYP enzymes and drug transporters (ABCB1, ABCG2, SLCO1B1 and SCLO1B3) as a measure to evaluate the potential for herb-drug interactions.

Chapter 3

3. Materials and methods

3.1. C3A liver cells

C3A liver cells are human hepatocytes derived from hepatoblastoma based HepG2 cells and were purchased from the American Type Culture Collection (ATCC HB-8065, American Type Culture Collection, Manassas, VA, USA) and supplied at passage number 5.

3.2. Thawing of C3A liver cells

C3A liver cells were preserved in Eagles Minimum Essential Medium (EMEM) (cat no: 12-662F, Lonza, Walkersville, MD, USA) containing 100 mM sodium pyruvate, 1% non-essential amino acids (NEAA) (M7145, Sigma-Aldrich, St. Lucia, MA, USA), 10% fetal bovine serum (FBS), 2 mM L-glutamine (G7513, Sigma-Aldrich, St. Lucia, MA, USA) and 7% (v/v) dimethyl sulfoxide (DMSO)(1.02952, Sigma-Aldrich, St. Lucia, MA, USA). The cryopreservation tube containing frozen cells was placed in a water bath at 37°C in order to thaw the cells. Once the C3A liver cells were partially thawed, they were gently transferred into a 75 cm² flask (708003, Nest, Wuxi, Jiangsu, China) with 17 mL of warm complete EMEM containing 100 mM sodium pyruvate (TMS-005, Sigma-Aldrich, St. Louis, MO, USA), 1% non-essential amino acids (NEAA) (M7145, Sigma-Aldrich, St. Lucia, MA, USA), 10% fetal bovine serum (FBS), 2 mM L-glutamine (G7513, Sigma-Aldrich, St. Lucia, MA, USA). The cell suspension was mixed by gentle pipetting several times up and down with a serological pipette to allow for the equal distribution of C3A liver cells in the media. The flask was then incubated at standard tissue culture conditions (37°C, 5% CO₂ in humidified air). Media was changed in two to three day intervals to provide the C3A liver cells with fresh nutrients. C3A liver cells were used for twenty passages; thereafter a new vial of C3A liver cells was thawed to prevent genetic and phenotypic changes. Cells used for mRNA and protein expression experiments were used at the same passage number for repeated experiments.

3.3. Counting of C3A liver cells

The cell suspension was stained with 0.4% (w/v) trypan blue (T93595; Sigma-Aldrich, St. Lucia, MA, USA) in Dulbecco's phosphate buffered saline (DPBS) (17-513, Lonza, MD, USA) at a 1:1 ratio. Ten microliters of the cell suspension were diluted with 10 µL of trypan blue. The solution was mixed thoroughly by gently pipetting the contents up

and down several times. Ten microliters of the dilution were transferred onto the counting chamber of the Neubauer haemocytometer. C3A liver cells were counted using the 100X magnification lens of an inverted, phase contrast light microscope (Olympus ck x31, Tokyo, Japan). Cell concentration was calculated using the formula below:

$$\text{Cells/mL} = \text{average cells counted per mm}^2 \times 2 \times 10\,000$$

(1)

3.4. Sub culturing of C3A liver cells

C3A liver cells were added into a 75 cm³ flask, containing 18 mL of pre-warmed complete EMEM, at a density of 110 000 cells/mL. They were incubated under standard tissue culture conditions until they reached 70 - 80% confluence. The media was refreshed in two to three-day intervals.

The C3A liver cells were trypsinised when the recommended confluence was reached and subsequently seeded at assay specific densities. Briefly, media was aspirated and the C3A liver cells were gently washed with 10mL of pre-warmed DPBS. Thereafter, 2 mL of trypsin (17-161F Lonza, MD, USA) was added to the cells and incubated at 37°C for 8 minutes. After incubation, the flask was gently tapped with the palm of the hand to ensure that the cells were detached and confirmed by microscopic observation. Trypsin was inactivated by adding 8 mL of EMEM media. The cell suspension was then transferred into a 50 mL centrifuge tube (602002, Nest, Wuxi, Jiangsu, China) and centrifuged for 5 minutes at 800 xg. The cell pellet was re-suspended in 6 mL of complete EMEM. An aliquot of the cell suspension (about 500 µL) was collected into a 1.5 mL Eppendorf tube (0030 123.328, Eppendorf, Hamburg, Germany) and cells were counted as described previously.

3.4.1. Seeding into a 96-well plate

C3A liver cells were trypsinised and counted as described (3.3). After counting, cells were seeded into a Corning clear 96 well plate (CLS9102, Sigma-Aldrich, St. Lucia, MA, USA) at a density of 11 000 /well and were grown under standard tissue culture

conditions for 5 days. Media was refreshed every two to three days to ensure optimum growth.

3.4.2. Seeding into a 6-well plate

To seed C3A liver cells into a 6 well plate (CLS3516, Sigma-Aldrich, St. Lucia, MA, USA), C3A liver cells were trypsinised and resuspended as described above. The cell suspension was seeded at a density of 300 000 cells/well and the cells were cultured under standard culture conditions for 5 days. Media was refreshed every two to three days to ensure optimum growth.

3.5. Preparation of extracts and pure compounds

For the purpose of this study, the freeze-dried *C. genistoides* methanol extract (ARC188), and the benzophenone and xanthone enriched fractions (ARC188_B and ARC188_x) were obtained from the Agricultural Research Council (ARC), in a powder form. The compounds were dissolved in 10% dimethyl sulfoxide (DMSO) to make stock solutions of 5 mg/mL (ARC188) and 20 mg/mL (ARC188_B and ARC188_x), based on the highest soluble concentrations of each treatment sample. Single use 100 µL aliquots of the stock solutions were made and stored at -20°C until subsequent use. Isomangiferin, IMG and IDG were also obtained from the ARC, already dissolved in 10% DMSO as stock solutions of 1.021 mg/mL, 1.0446 mg/mL and 0.973 mg/mL respectively. One hundred microliters of the stock solutions were also stored at -20°C until subsequent use. Mangiferin from *Mangiferin indica* (cat no.: M3547), was purchased from Sigma-Aldrich. Mangiferin was dissolved in 100% DMSO to make a stock solution of 10 mg/mL and was also stored at -20°C, as single use 100 µL stocks.

A more detailed description of the preparation of reagents as well as experimental methods can be found in Appendix B. Dr. CJ Malherbe at the Agricultural Research Council, Infruitec-Nietvoorbij in Stellenbosch supplied the chromatograms, performed the extraction of chemical compounds from honeybush plant and fractionations of the extracts as well as the ultra-high-performance liquid chromatography with diode array detection (UHPLC-DAD) and high-performance liquid chromatography with diode array detection (HPLC-DAD).

3.6. Determination of cell viability

To evaluate the cytotoxic effect of the methanol extracts and fractions of *Cyclopia genistoides* in an *in vitro* model using C3A liver cells, cytotoxicity assays were performed. Time and concentration dependant experiments were performed to determine potential cytotoxicity of the test samples, using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (M5655; Sigma-Aldrich, St. Lucia, MA, USA) assay and confirmed using the ATP assay. These assays were performed in order to ensure that *C. genistoides* test samples (ARC188, ARC188_B and ARC188_x) were used at non-toxic concentrations as well as for future dose optimization.

3.6.1. MTT assay

The MTT assay is an NAD(P)H-dependent colorimetric assay where cellular metabolic activity can be measured through the reduction of the water-soluble tetrazolium MTT dye to the insoluble formazan crystals by mitochondrial dehydrogenases (Wang *et al.*, 2010). The activity is observed by the colour change of the solution from yellow to purple, the purple formazan crystals are then solubilized with DMSO and the activity is quantified using a spectrophotometer at 570 nm.

On day 5 of growth (80 – 90% confluence), the C3A liver cells were exposed to ARC188, ARC188_B and ARC188_x at concentrations ranging from 0.1µg/ml to 250 µg/ml (ARC188), and 1µg/ml to 1000µg/ml (ARC188_B and ARC188_x), as well as a 0.5% DMSO concentration (vehicle control), for 3, 6, 24 and 48 hours. Thereafter, cell viability was evaluated using an MTT assay following the manufacturer's instructions. Briefly, C3A liver cells were washed with 100 µL of pre-warmed DPBS per well to remove traces of the treatments and medium. Fifty microliters of 1 mg/mL MTT solution was added to each well. Thereafter, the plate was incubated for 30 minutes at 37°C. After incubation, the MTT was aspirated, the formation of formazan crystals was observed and 200 µL of 100% DMSO and 25 µL of Sorenson's Glycine buffer were added into each well. The plate was gently shaken at 500 x g using an orbital plate shaker (20197, Stovall life Science Inc., Bloomberg, USA) to dissolve the crystals. The absorbance was measured using the BioTeK ELX 800 plate reader

(BioTek Instruments Inc., Winooski, VT, USA) at 550 nm. Cell viability was calculated as a percentage of the vehicle control using the formula:

$$\text{Cell viability (\%)} = \left(\frac{\text{average test OD} - \text{blank OD}}{\text{average vehicle control OD} - \text{blank OD}} \right) \times 100$$

(2)

3.6.2. ATP assay

To confirm the results obtained in the MTT assay, an ATP assay was performed using 100 µg/mL of all the previously tested compounds. The ViaLight™ plus kit rapidly determines cell proliferation and cytotoxicity of mammalian cells and cell lines based on their ATP levels. The kit uses the bioluminescent measurement of ATP that is present in all metabolically active cells. The luminescent method utilizes the enzyme luciferase which catalyses the formation of light from ATP and luciferin (Lonza, 2018).

C3A liver cells were cultured under standard tissue culture conditions. At 70-80% confluence, cells were seeded into a white-walled clear bottom Corning 96 well plate (CLS3610, Sigma-Aldrich, St. Lucia, MO, USA) at a density of 11 000 cells/mL. After 5 days of culture, when the C3A liver cells were about 80 – 90% confluent, they were treated with 100 µg/mL of ARC188, ARC188_B and ARC188_X for 24 hours. Thereafter, cell viability was evaluated using the ViaLight™ plus kit (LT07-221, Lonza, Basel, Switzerland) following the manufacturer's instructions. Briefly, 50 µL of cell lysis reagent was added into each well and the plate was incubated for 10 minutes at 37°C. Thereafter, 100 µL of ATP monitoring reagent (AMR) was added into each well and the plate was incubated for 2 minutes at room temperature. Cell number was then determined by a luminometric measurement using the BioTeK FLX 800 plate reader (BioTek Instruments Inc., Winooski, VT, USA).

3.6.3. Bradford test for protein concentration determination

The Bradford test was performed using Bradford reagent (Cat no.: B6916 Sigma-Aldrich, St Louis, MO) to normalize data to protein. After lysing the C3A liver cells, 5 µL of the sample from each well was inoculated into a new 96 well plate. Following this, 200 µL of Bradford reagent was added into each well and the plate was incubated in a dark place for 10 minutes. After the 10-minute incubation period, absorbance was

measured using a BioTeK ELX 800 plate reader (BioTek Instruments Inc., Winooski, VT, USA). Cell viability was calculated as a percentage of the vehicle control, as normalised to the protein content using the following formulae:

$$\text{Normalised data} = \frac{\text{luminescence of test compound}}{\text{optical density (protein) of test compound}} \quad (3)$$

$$\text{Cell viability (\%)} = \left(\frac{\text{Normalised test compound}}{\text{Normalised vehicle control}} \right) \times 100 \quad (4)$$

3.7. Potential inhibitory effect of *C. genistoides* on CYP enzymes

Potential CYP enzyme inhibition by *C. genistoides* was investigated using the Vivid[®] CYP450 screening kits (Life Technologies[™], Carlsbad, CA, USA) to predict potential herb-drug interactions. Initially, the inhibitory effect of the organic solvent used as a vehicle control (DMSO), on the activity of the major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) was established using a kinetic screening assay. Following this, an initial one-point screening was performed for CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 using *C. genistoides* constituents (mangiferin, isomangiferin and IMG) to determine possible inhibitory effects. Thereafter, inhibitory screenings were performed to detect the test compounds that inhibited the 5 major CYP enzymes. *C. genistoides* constituents, that exhibited an inhibition of 50% or more, were selected for further analysis, and respective IC₅₀ values were calculated.

The SpectraMax i3x (Molecular Devices, LLC, Sunnyvale, CA, USA) was used to measure fluorescence at 415 nm and 460 nm excitation (EX) and emission (EM) wavelengths, respectively. For each Vivid[®] experiment two assay specific mixes, i.e master mix I and master mix II, were prepared following the manufacturer's instructions. Master mix I contained the Vivid[®] buffer I (200mM potassium phosphate) or the Vivid[®] buffer II (100 mM potassium phosphate), the Vivid[®] regeneration system (333 mM glucose-6-phosphate and 30 U/mL glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate, pH 8.0) and the Vivid[®] BACULOSOME[®] Plus reagent coding each CYP isoform. Master mix II contained Vivid[®] buffer I or the Vivid[®] buffer

II, the Vivid[®] blue substrate 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC) or 7-ethyloxymethyloxy-3-cyanocoumarin (EOMCC) and NADP⁺. Different positive controls were used for each CYP enzyme screening procedure as recommended by the manufacturer (table 2). The concentrations of pure compounds were selected based on the IC₅₀ values obtained for *C. genistoides* alpha glucosidase inhibitory activity as well as relative content (%) in the extract and fractions (Raaths, 2017). Concentrations of the extract and fraction were selected based on the results of C3A cytotoxicity assays in previously experiments. Compounds were either dissolved in 0.1% DMSO, 0.2% DMSO, 0.5% DMSO or 0.1% methanol (ketoconazole), based on their solubility. Substrates and positive controls used of each CYP isozyme are shown in table 3.1. below.

Table 2: Reaction set-up for Vivid[®] blue screening assays

Vivid[®] BACULOSOME	Vivid[®] Substrate	Positive control	Concentration of positive control
CYP1A2	EOMCC	α-naphthoflavone	10 μM
CYP2C9	BOMCC	sulfaphenazole	30 μM
CYP2C19	EOMCC	miconazole	30 μM
CYP2D6	EOMCC	quinidine	10 μM
CYP3A4	BOMCC	ketoconazole	10 μM

*BOMCC = 7-benzyloxymethyloxy-3-cyanocoumarin

*EOMCC = 7-ethyloxymethyloxy-3-cyanocoumarin

3.7.1. The inhibitory effect of DMSO on CYP activity

The potential inhibitory effect of DMSO on the activity of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 was investigated to ensure that the DMSO used as a solvent was not interfering with the CYP inhibitory reactions. In Corning black, clear bottom 96 well plates, 40 μL of either 0.1%, 0.2% or 0.5% DMSO, a positive control and Vivid[®] buffer I or Vivid[®] buffer II were added into each well. Thereafter, 50 μL of master mix I was added into each well. The mix was incubated at 37°C for 10 minutes. After incubation fluorescence was measured at of EX: 415 nm and EM: 460 nm wavelengths using the SpectraMax i3x to monitor any background fluorescence emitted from the samples. Ten microliters of master mix II was added in each well to

initiate the reaction. Fluorescence was monitored at EX: 415 nm and EM: 460 nm wavelengths using the SpectraMax i3x, kinetically, at 5 minute intervals for 30 minutes.

3.7.2. Initial 30-minute CYP inhibitory screening

Initial CYP inhibitory screening was performed using the Vivid® CYP450 screening kits (Life Technologies™, Carlsbad, CA, USA), following the manufacturer's instructions. Firstly, 40 µL of test samples (ARC188, ARC188_B, ARC188_X, IMG, IDG, mangiferin and isomangiferin) were added into each well of a black Corning 96-well plate (CLS3904, Sigma-Aldrich, St. Lucia, MO, USA). Concentrations of the test samples used are shown in table 3.3. Thereafter, 50 µL of master mix I was added to the test compounds. The plate was pre-warmed by incubating at 37°C for 10 minutes. After incubation, fluorescence was measured at EX: 415 nm and EM: 460 nm excitation and emission wavelengths using the SpectraMax i3x (Molecular Devices, LLC, Sunnyvale, CA, USA) to monitor any background fluorescence emitted from the samples. Ten microliters of master mix II was then added into each well to initiate the reaction. Fluorescence was then monitored at EX: 415 nm and EM: 460 nm wavelength using the SpectraMax i3x (Molecular Devices, LLC, Sunnyvale, CA, USA), kinetically, at 5 minute intervals for 30 minutes. Then finally the % inhibition was calculated as described in section 3.7.4.

Table 3: Concentrations of test samples used in initial Vivid® blue screening assays

Treatment	Concentration
Mangiferin	^a 5.6 µg/ml ^b 16 µg/ml
Isomangiferin	^a 3 µg/ml ^b 15 µg/ml
IMG	^a 5 µg/ml ^c 25 µg/ml
IDG	^a 2 µg/ml ^c 20 µg/ml
ARC188	100 µg/ml
ARC188 _B	100 µg/ml
ARC188 _X	100 µg/ml

^a = Representative of compound in ARC188

^b = Representative of compound in ARC188_X

^c = Representative of compound in ARC188_B

*IMG= 3-β-*D*-glucopyranosyliriflophenone

*IDG= 3-β-*D*-glucopyranosyl-4-β-*D*-glucopyranosyloxyiriflophenone

3.7.3. Concentration dependent screening

Following the initial time dependant screening, the test samples that exhibited high inhibitory effects (more than 50% inhibition) on the CYP enzymes were selected for further testing to determine the IC₅₀ values. Test samples were screened over a wide range of concentrations. The concentrations of the test samples are shown in table 3.4. Test samples and mastermixes were added into the black corning 96-well plate and pre-warmed as described above. Again, the initial fluorescence was measured as previously described to monitor background fluorescence. Ten microliters of master mix II was added in each well to initiate the reaction. The plate was incubated for 30 minutes at 37°C. Thereafter, the reaction was stopped by adding 20% Tris base ice-cold acetonitrile. Fluorescence was measured at EX: 415 nm and EM: 460 nm wavelengths. The enzyme activity and half maximal inhibitory concentration (IC₅₀) for each compound were determined as described in section 3.7.5.

Table 4: Concentrations of test samples

Test compound	Concentrations
ARC188	1 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 125 µg/mL and 250 µg/mL
ARC188 _B	1 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 500 µg/mL and 1000 µg/mL
ARCxf188 _x	1 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 500 µg/mL and 1000 µg/mL
Mangiferin	0.1 µg/mL, 1 µg/mL, 10 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL

3.7.4. Determination of percentage inhibition and remaining enzyme activity

For kinetic assays, the rate of reaction was calculated by measuring the change in fluorescence over time. The percentage inhibition due to the presence of test sample or positive inhibition control was calculated using the formula:

$$\% \text{ inhibition} = \left(1 - \frac{\text{rate of fluorescence observed for test sample}}{\text{rate of fluorescence observed for vehicle control}} \right) \times 100$$

(5)

For the end-point reaction, percentage inhibition due to the presence of test compound or positive inhibition control was calculated using the formula:

$$\% \text{ inhibition} = \left(1 - \frac{\text{fluorescence observed for test sample}}{\text{fluorescence observed for vehicle control}} \right) \times 100$$

(6)

The remaining enzyme activity was calculated using the formula:

$$\text{Remaining activity} = \left(\frac{\text{fluorescence observed for test sample}}{\text{fluorescence observed for vehicle control}} \right) \times 100$$

(7)

3.7.5. IC₅₀ determination

IC₅₀ values were determined using Graphpad prism® version 6 (GraphPad Software Inc., La Jolla, USA) software. Briefly, the remaining enzyme activity was plotted against the log concentration of the compounds in a line graph. The plot was analysed using Non-linear regression (curve fit). Dose response-inhibition was then selected and the variable slope (four parameters) was used to analyse the plot. Since the background fluorescence was initially subtracted from the final fluorescence results and it is impossible to obtain negative fluorescent results, the bottom variable was constrained to be equal to zero. The IC₅₀ values were calculated using the formula:

$$Y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + 10^{(\log \text{IC}_{50} - X) * \text{Hillslope}}} \quad (8)$$

Where: The top and bottom are plateaus in the units of the Y axis, the Hillslope describes the steepness of the slope and IC₅₀ is the concentration of the test sample that gives a response halfway between top and bottom (Graphpad, no date)

3.8. Modulatory effect of *C. genistoides* constituents on drug transporting

The effect of *C. genistoides* constituents on the expression of major drug transporters (SLCO1B3, ABCB1 and ABCG2) was investigated using C3A liver cells and TaqMan probes; then qRT-PCR was performed. Briefly, RNA was extracted from the cells, then the RNA was cleaned and quantified. Following RNA quantification, RNA was then treated with DNase to get rid of all genomic DNA, then cDNA was synthesized, and qRT-PCR was performed.

3.8.1. Treatment of C3A liver cells

C3A liver cells were cultured as described previously (section 3.4). Upon confluence, the cells were seeded into 6 well plates and cultured for 5 days, with media being refreshed every 2-3 days. On the 5th day of culture, C3A liver cells were treated with 250 µg/mL of ARC188, ARC188_B and ARC188_X, 34 µg/mL and 100 µg/mL mangiferin and 20 µM rifampicin used as a positive control for 12, 24 and 48 hours, respectively. The concentration (250 µg/mL) for ARC188, ARC188_B and ARC188_X was selected based on the viability assays performed on C3A

liver cells previously. After each time point, media was aspirated from the cells and washed with DPBS. Thereafter, 333 μ L of QIAzol lysis reagent (79306, Qiagen, Hilden, Germany) was added into each well. The plate was incubated at room temperature for 5 minutes and the cells were scraped from the plate using Nest cell scrapers (710011, Nest, Wuxi, Jiangsu, China). The cell lysates were immediately transferred into 2 mL Eppendorf tubes and put in ice. The cell lysates were then stored at -80°C until further use.

3.8.2. RNA extraction

RNA was extracted from C3A liver cells using a method developed at the South African Medical research Council (SAMRC), Biomedical Research and Innovations Platform (BRIP). Harvested C3A liver cells were thawed and then homogenised using the Qiagen tissuelyser (Qiagen, Hilden, Germany). C3A liver cells were homogenized by for 1 minute in the tissuelyser and put on ice for another minute, this was repeated twice. The homogenate was then centrifuged at $15000 \times g$ and 4°C for 10 minutes, using a microcentrifuge (Eppendorf, Hamburg, Germany). Thereafter, the *supernatant* was collected into 1.5 mL Eppendorf microfuge tube (0030123.328, Eppendorf, Hamburg, Germany) and 0.2 mL of chloroform (C2432, Sigma-Aldrich, St Louis, MO, USA) was added. The mixture was shaken vigorously for 15 seconds and then occasionally shaken for 3 minutes until the suspension was a pink milky colour. The mixture was centrifuged at $15000 \times g$ at 4°C for 15 minutes and the upper aqueous phase was transferred into 1.5 mL Eppendorf microfuge tube. Thereafter, 0.5 mL isopropanol (I9516, Sigma-Aldrich, St. Louis, Missouri, USA) was added to the aqueous phase, mixed thoroughly and finally incubated at -20°C overnight. The next morning the samples were removed from -20°C and centrifuged for 30 minutes at $15000 \times g$ and 4°C . The supernatant was discarded and 1 mL of 70% ethanol (96 – 100% ethanol molecular grade, E7023, Sigma-Aldrich, St Louis, MO, USA) was added to wash the pellet. Thereafter, the solution was centrifuged at $15000 \times g$ at 4°C for 10 minutes. The wash step with 70% ethanol was repeated twice. The supernatant was then removed and the pellet was air dried in the PCR hood for 30 minutes, or until dry. One hundred microlitres of RNase free water (AM9937, Ambion, Austin, TX, USA) was added to the pellet to redissolve the pellet. The RNA was incubated for 10 minutes at 55°C on a D1200-230V heating block (Labnet, Edison, NJ, USA) to redissolve the RNA.

3.8.3. RNA quantification

Total RNA concentration and purity was quantified using the Nanodrop ND – 1000 (Nanodrop Technologies, Wilmington, DE, USA). After the machine had initialized, 1 μ L nuclease free water was used on the pedestal as a blank measurement. Both the pedestals of the machine were wiped clean then the total RNA concentration and purity was quantified by adding 1 μ L of each sample onto the pedestal of the machine and measuring absorbance. Two readings were made for each sample and the pedestals were cleaned after every reading. The machine was blanked after 20 readings. Samples were stored at -80°C until further use.

3.8.4. DNase treatment

Genomic DNA contamination was removed from the RNA samples using the Turbo DNA free kit (AM1907, Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions. Five microliters of DNase buffer and 1 μ L DNase were added to 20 ng of RNA and nuclease free water was added for a final volume of 50 μ L. The solution was mixed and incubated at 37°C for 30 minutes. After the incubation another 1 μ L of DNase was added into each tube and the solution was mixed and incubated at 37°C for 30 minutes. Then 10 μ L of the DNase inactivation reagent was added and the samples were incubated at room temperature for 5 minutes on an orbital shaker (Labnet, Edison, NJ, USA). The samples were centrifuged at 10 000 x g at 4°C for 90 seconds. The RNA was then transferred into a new tube, put on ice and quantified as described in section 3.8.3. After quantification RNA samples were stored at -80°C until further use.

3.8.5. cDNA synthesis

cDNA was synthesized using the High Capacity Reverse Transcription Kit (Cat no.: 4368813, Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions. Briefly, for each RNA sample, two solutions were prepared, one negative and one positive transcript. One microgram of RNA was added into a 0.2 mL Eppendorf tube. The final volume was adjusted to 10 μ L with nuclease free water, to dilute highly concentrated RNA samples. Two master mixes, a positive transcript master mix and a negative transcript mastermix, were prepared as described in Table 17 and Table 18, Appendix B. Ten microliters of the master mix was added to each

tube and the RNA solutions were mixed and pulse spinned. The tubes were put into the thermocycler for 4 heating cycles programmed as follows: step 1 at 25°C for 10 minutes, step 2 at 37°C for 2 hours, step 3 at 85°C for 5 seconds and lastly step 4 at 4°C with no time limit. The reaction of the thermocycler was set to 20 µL then the cycling programme was started. After the run was completed the cDNA was stored at 4°C until further use.

3.8.6. Testing of cDNA

In order to check if the synthesized cDNA had any genomic DNA contamination, quantitative Real time PCR (qRT-PCR) was performed using the Power SYBR green kit, and forward and reverse β – actin human primers. The SYBR green master mix was prepared as described in Table 19, Appendix B. One microliter of the cDNA was added into each well of a MicroAmp EnduraPlate Optical 96-well Fast Clear Reaction Plate (4483494, Applied Biosystems, Foster City, California, USA), and thereafter 24 µL of the master mix was added into each well as well. The plate was then covered with an adhesive PCR plate seal (AB0558, Thermofisher, Waltham, MA, USA) and pulse spun briefly, shaken at 500 rpm for 10 minutes and pulse spun again. Thereafter, the plate was placed in the thermal cycler and run using the following heating cycles: a pcr initiation activation step at 95°C for 15 minutes, a denaturation step at 94°C for 15 seconds, an annealing step at 50 - 60°C for 30 seconds, an extension step at 72°C and a cooling step at 4°C continuously. The reaction volume was set at 25 µL and a dissociation curve was added. Data was analysed using the default Ct and baseline settings, by subtracting the Ct obtained with the positive transcripts from the Ct obtained with the negative transcripts. The difference between the 2 Ct values indicated the degree of genomic DNA contamination, where a difference of more than 10 cycles was considered to be insignificant.

3.8.7. qRT-PCR

The effect of *C. genistoides* constituents on the expression of genes that code drug transporting proteins (SLCO1B1, SLCO1B3 ABCB1 and ABCG2) in C3A liver cells was investigated using qRT-PCR and the TaqMan probes SLCO1B1 (Assay ID: Hs00272374_m1, Thermofisher, Waltham, MA, USA) SLCO1B3 (Assay ID: Hs00251986_m1, Thermofisher, Waltham, MA, USA), ABCB1 (Assay ID: Hs00184500_m1, Thermofisher, Waltham, MA, USA) and ABCG2 (Assay ID:

Hs00184979_m1, ThermoFisher, Waltham, MA, USA) for human. Two other TaqMan genes were used as house-keeping genes to normalize the qRT-PCR data, human hypoxanthine phosphoribosyltransferase 1 (HPRT11) (Assay ID: Hs02800695_m1, ThermoFisher, Waltham, MA, USA) and β -actin (Assay ID: Hs99999903_m1, ThermoFisher, Waltham, MA, USA). The house-keeping genes β -actin and HPRT11 were previously found to be the most stable and unregulated genes in our labs at BRIP (Shabalala, 2016; Jack, 2016), hence they were selected, based on availability. In preparation for qRT-PCR, the standard curve was prepared by pooling all cDNA samples into one and making serial dilutions of that mixture. Seven 0.5 mL Eppendorf tubes were labelled S1 to S7 and 90 μ L of nuclease free water was added into tubes S2 to S7. Then 5 μ L of cDNA was collected from every sample and pooled into tube S1, then a serial dilution of 1:10 was performed for S2 to S7 with 10 μ L of sample diluted into 90 μ L nuclease free water. The standard curve dilutions (S1 to S7) were then stored at -20°C until further use. Standard curve dilutions were kept for 6 months, after which they were discarded and new standard curve dilutions were made.

The qRT-PCR master mix was prepared as described in Table 20, Appendix B using the TaqMan™ Protein Assays Fast Master Mix (cat no.: 4448616, Applied biosystems) and kept on ice. Then, 1 μ L of the cDNA samples and standard solutions were put into each well of the MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plate (cat no.: 4483354, Applied biosystems), and 9 μ L of the qRT-PCR master mix was added to each well. Samples and standard solutions were added in duplicates. The plate was then sealed with a MicroAmp™ Optical Adhesive Film (cat no.: 4311971, Applied biosystems) and covered with foil. Then it was pulse spun on a plate centrifuge and shaken with a plate shaker for 10 minutes. After shaking, the plate was pulse spun again and placed on the Applied biosystems 7500 qRT-PCR system (cat no.: 4351106, Applied biosystems) where a qRT-PCR run was performed at the following heating cycles: stage 1 at 50°C for 2 minutes, stage 2 at 95°C for 10 minutes, stage 3 at 95°C for 15 seconds, stage 3 was repeated for 40 cycles and stage 4 at 60°C for 1 minute. After the run had completed, data was analysed. Firstly, the standard curve results were viewed, the desired standard curve slope was set to 3, outliers from the standard curve were removed and data was re – analysed to yield new quantity yields. Data was then exported to Microsoft excel (Microsoft, Redmond, Washington, USA)

to determine the relative expression of the drug transporter genes using the formula below.

$$\text{Relative expression} = \frac{\text{quantity mean of transporter gene}}{\text{average quantity mean of house-keeping genes (HPRT1 and } \beta\text{-actin)}} \quad (9)$$

3.9. Western blot analysis

The effect of *C. genistoides* constituents on the translation of drug transporter genes into proteins was investigated using western blot analysis through the following sequence.

3.9.1. Isolation of protein from C3A liver cells

C3A liver cells were cultured as described (section 3.4.). Upon confluence, C3A liver cells were seeded into 6 well plates as described in Section 3.4. The cells were cultured for 5 days, with media refreshed every 2-3 days. On the 5th day of culture, C3A liver cells were treated as described in section 3.8.1 above. For protein analysis, cells were treated for 12 and 24 hours, respectively. After each time-point cells were harvested. The treatment media was aspirated from the cells, then the cells were washed with 500 μ L DPBS. Then 120 μ L of tissue lysis reagent (Cat no.: FNN0071, Invitrogen, Carlsbad, CA, USA) was added into each well and was incubated at room temperature for 5 minutes. The cells were scraped from the plate using Nest cell scrapers (Cat no.: 710011, Nest, Wuxi, Jiangsu, China). The cell lysates were immediately transferred into 2 mL Eppendorf tubes and put in ice. A stainless-steel ball was added into each tube and transferred to ice cold tissue lyser blocks. Samples were homogenised as described in section 3.8.1. This cycle was repeated 5 times in order to get a nice protein homogenate, then the homogenate was centrifuged at 4°C, 13500 x g for 15 minutes. The supernatant containing protein was transferred into new 1.5 mL tubes. Samples were then stored at -20°C until further use.

3.9.2. Determination of protein concentration from samples

The concentration of proteins was determined using Bio-Rad, Hercules, CA, USA BSA standards (0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1 mg/mL, 1.5 mg and

2 mg/mL), and Bio-Rad, Hercules, CA, USA Dc Reagents. Firstly, reagent A' was prepared by adding 20 μ L of Bio-Rad Dc Reagent S (500-0115, Bio-Rad, Hercules, CA, USA) into 1 mL of DC Reagent A (500-0113, Bio-Rad, Hercules, CA, USA). After which, 5 μ L of the Bio-Rad BSA standard (Cat no.: 500-007, Bio-Rad, Hercules, CA, USA) and 5 μ L of the samples were put into each well of a clear flat bottom 96 well plate in duplicates. This was followed by the addition of 25 μ L of Reagent A' and 200 μ L of Reagent B into each well. The plate was then shaken at 500 rpm in a plate shaker for 10 seconds to mix all the reagents and samples. After shaking, the plate was incubated at room temperature for 15 minutes and the optical density (OD) was measured at 695 nm using the BioTek ELX 800 plate reader (BioTek Instruments Inc., Winooski, VT, USA). After the optical density reading, data was exported into excel (Microsoft Excel, Microsoft, St. Lucia, California, USA) and protein concentration was determined. A standard curve was constructed with the formula:

$$y = mx + c \tag{10}$$

Then protein concentration was calculated using the formula:

$$\text{Protein concentration} = \frac{\text{OD}-C}{m} \tag{11}$$

3.9.3. Running acrylamide gels

Protein samples were first thawed and diluted in 4X sample buffer using 1-part sample buffer and 3 parts sample. The samples were denatured by heating at 95°C on the heating block for 5 minutes and then pulse spun and instantly placed on ice. A 10% Protean® TGX™ gel (161-0993, Bio-Rad, Hercules, CA, USA) was placed into the mini protein tetra cell tank (Bio-Rad, Hercules, CA, USA) and the tank was filled with 1X running buffer (161-0772, Bio-Rad, Hercules, CA, USA). Thereafter, 10 μ L of the Bio-Rad Precision Plus Western C marker (1610376, Bio-Rad, Hercules, CA, USA) and 60 μ g of each sample was loaded onto each lane of the gel. Gel electrophoresis was performed using the Bio-Rad power tank, the gel was run at 150V (90 minutes) until the dye front reached the bottom of the tank.

3.9.4. Transfer of proteins to a polyvinylidene fluoride (PVDF) membrane

After the gel had run, it was transferred into a polyvinylidene fluoride (PVDF) membrane (1704156, Bio-Rad, Hercules, CA, USA). The PVDF membrane was activated by placing in 99.9% methanol (1070182511, Merck, Whitehouse Station, NJ, USA) for 2 minutes, after which the membrane was washed in distilled water for 5 minutes. Thereafter, the membrane and Transblot stacks were incubated while shaking in Transblot transfer buffer (1704156, Bio-Rad, Hercules, CA, USA) using the orbital shaker for 3 minutes to equilibrate them. A transfer sandwich, with Transblot stack at the bottom, followed by the gel with the protein side up and then the PVDF membrane and the Transblot stack at the top, was assembled gently (as shown in Figure 4), air bubbles were gently rolled out using a roller. The sandwich was placed in the cassette of the Transblot transfer system which was placed between electrodes and gel transfer was commenced for 10 minutes.

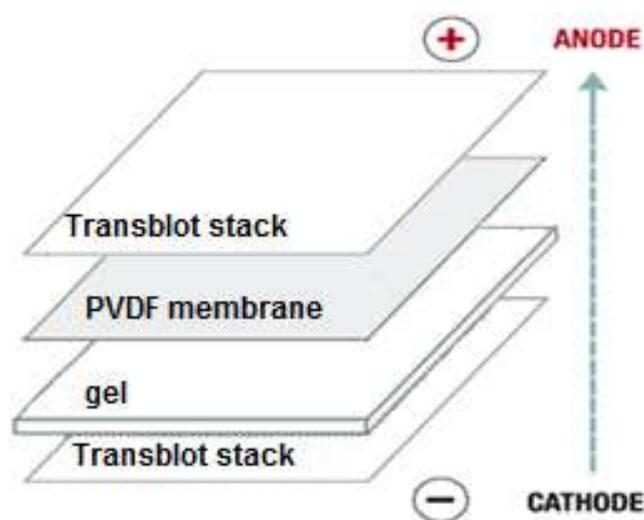


Figure 13: Schematic representation of the transfer sandwich

3.9.5. Ponceau S stain procedure

Once the transfer was complete the membrane was submerged in Ponceau S stain (P7170, Sigma-Aldrich, St. Lucia, MA, USA) and incubated for 5 minutes with shaking on an orbital shaker. The membrane was rinsed with double distilled water until the background was white. An image of the membrane was captured on a Chemi Doc MP system (Bio-Rad, Hercules, CA, USA) to see if the gel was successfully transferred.

3.9.6. Protein detection

After successful transfer, the membrane was blocked in 5% skim milk powder (700166, Sigma-Aldrich, Sigma-Aldrich, St Louis, MO, USA) prepared in 1X Tris base saline-tween 20 (TBST) for 2 hours. Thereafter, the membrane was incubated with the primary antibody overnight at 4°C. The following day, the membrane was washed with 1X TBST 3 times, for 10 minutes each, and incubated with the secondary antibody for 1.5 hours. Dilutions of antibodies are shown in Table 5 below. After the second incubation step, the membrane was washed with TBST for 10 minutes. This step was repeated, three times.

Table 5: Dilution concentrations of antibodies

Antibody	Catalogue number	supplier	Dilution ratio
β -tubulin	2146	Cell signalling	1:1500
ABCB1	Sc-55510	Santa Cruz	1:250
ABCG2	42078	Cell signalling	1:250
SLCO1B3	HPA004943	Sigma-Aldrich	1:250
Donkey anti-mouse IgG-HRP*	Sc-23181	Santa Cruz	1:1000
Donkey anti-mouse IgG-HRP*	Sc-2012	Santa Cruz	1:1000

*=secondary antibody

3.9.7. Imaging of the blots

The membrane was put onto a plastic tray and submerged into 1.5 mL of the Clarity Western EC substrates (1705060, Bio-Rad, Hercules, CA, USA) for 5 minutes. An image of the membrane was captured using the Bio-Rad Chemi Doc MP system.

3.9.8. Stripping and re-staining the membrane

If it was required to use more than one antibody to detect proteins of the same samples, the membrane was stripped using the restore plus stripping buffer (46430, Life Technologies Corporation, Carlsbad, CA, USA) in order to remove the previous antibody. The membrane was submerged in the stripping buffer and incubated on a shaker for 13 minutes at room temperature. After incubation the membrane was washed with DPBS for 5 minutes and washed with 1X TBST for a further 5 minutes. After stripping the membrane was blocked with 5% skim milk for 2 hours. The membrane was stripped and re-used 3 times for analysis.

3.9.9. Analysis of western blot images

The western blot images were analysed using the Bio-Rad image lab software version 5.2.1 to determine band intensity. Data was exported to Microsoft excel and protein levels were calculated using the following formula:

$$\text{Protein levels} = \frac{\text{band intensity of protein of interest}}{\text{band intensity of } \beta\text{-tubulin}}$$

(12)

3.10. Statistical analysis

For all experiments, unless otherwise specified, results were generated from 3 independent experiments and data was expressed in triplicate (n = 9). For mRNA expression analysis data was expressed in duplicates (n = 6). Data was analysed using Microsoft excel and statistical analysis was performed using one-way ANOVA, and unpaired student T-tests on GraphPad prism version 6.1. Statistical analysis was performed at a 5% interval with p < 0.05 regarded as statistically significant.

Chapter 4

4. Results

Extracts, fractions as well as isomangiferin, IMG and IDG pure compounds were prepared and characterised by the Agricultural Research Council (ARC, Stellenbosch). As detailed by Raaths (2016), the unfermented plant material of the ARC188 crude extract was sourced from the *Cyclopia* gene bank of the ARC. *Cyclopia genistoides* crude extracts and benzophenone or xanthone enriched fractions were prepared and evaluated based on preliminary mini-scale methanol-based extractions. All extracts and fractions were chemically characterised in terms of benzophenones and xanthenes content. These selected constituents were quantified in each of the extracts and fractions using high performance liquid chromatography with diode-array detection (HPLC-DAD) (Appendix C).

4.1. Cytotoxicity assays

To ensure that the *C. genistoides* extract and the benzophenone and xanthone enriched fractions (ARC188, ARC188_B and ARC188_X) were used at non-toxic concentrations, cytotoxicity experiments were performed over a wide range of concentrations, and time points, using both MTT and ATP assays. The potential cytotoxic effects of the pure compounds mangiferin, isomangiferin, IMG and IDG were not performed; however, these were assessed in the enriched fractions for the relative compounds.

The MTT assay was used to confirm cell viability based on mitochondrial dehydrogenase activity in time and concentration dependent experiments. The results obtained from the MTT are shown in Figure 14. Results from the MTT assay showed that there were no significant changes in cell viability when C3A liver cells were treated with ARC188, ARC188_B and ARC188_X for 3, 6 and 24 hours at all the tested concentrations (ARC188: 0 - 250 µg/mL, ARC188_B 0 - 1000 µg/mL and ARC_X: 0 - 1000 µg/mL). After 48 hours incubation, cell viability significantly decreased when cells were treated with the two highest concentrations of ARC188 (100 µg/mL = 86.3% $p < 0.0001$ and 250 µg/mL = 76.26% $p < 0.0001$) (Figure 14A). Treatment with the two highest concentrations of ARC188_B (500 µg/mL and 1000 µg/mL) also resulted in a significant decline in cell viability to 82.6% $p < 0.0001$ and 77.26% $p < 0.0001$, respectively after 48 hours (Figure 14B). Treatment with 10, 50, 100, 500 and 1000 µg/mL of ARC188_X

significantly reduced cell viability to 65.86% ($p < 0.01$), 64.32% ($p < 0.01$) and 67.88% ($p < 0.01$), respectively (Figure 14C)

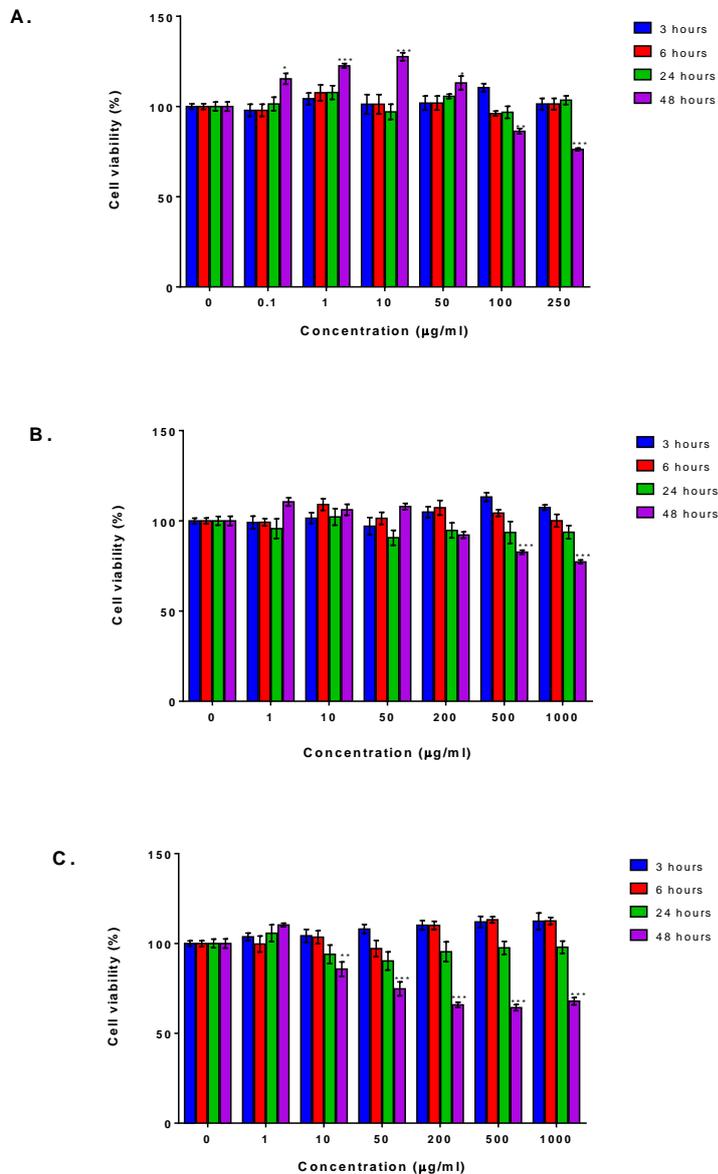


Figure 14: MTT activity of C3A cells treated with *C. genistoides* ARC188 (A), ARC188_B (B), and ARC188_x (C).

C3A cells were cultured for 5 days and were exposed to various concentrations of ARC188 (0 µg/ml – 250µg/ml) ARC188_B (0 µg/ml – 1000 µg/ml) and ARC188_x (0 µg/ml – 1000 µg/ml), respectively for 3, 6, 24 and 48 hours and absorbance was read at 570 nm. Results are from three independent experiments each done in triplicate ($n = 9$), expressed as the mean \pm SEM and are reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnet's post-hoc test if $p < 0.05$. * = $p < 0.05$, ** = $p < 0.02$ and *** = $p < 0.01$ against the vehicle control.

An ATP assay was used to validate the MTT cell viability results. The ATP assay determines ATP levels within cells to indicate cell viability. Being a luminescent quantification assay for ATP, it negates the possibility of polyphenols interfering with the activity of major enzymes responsible for the rate limiting steps of the reaction as in the MTT assay. In addition, the ATP assay is a more sensitive technique in determining cell viability. Since the ATP assay was only used for validation of the MTT assay, cells were treated with 100 $\mu\text{g}/\text{mL}$ of ARC188, ARC188_B and ARC188_x for 24 hours only. The assay was performed following the manufacturer's instructions and cell viability was expressed as a percentage of the vehicle control set at 100% and normalised to protein content using a Bradford protein analysis reagent (BioRad).

ATP results showed that 100 $\mu\text{g}/\text{mL}$ of ARC188 reduced cell viability to 80.2% ($p < 0.05$). There were no significant changes in cell viability after treatment with ARC188_B. ARC188_x reduced cell viability to 76.33% ($p < 0.02$) and 83% ($p < 0.05$) at 10 and 100 $\mu\text{g}/\text{mL}$, respectively. The results from the ATP assay are shown in Figure 15.

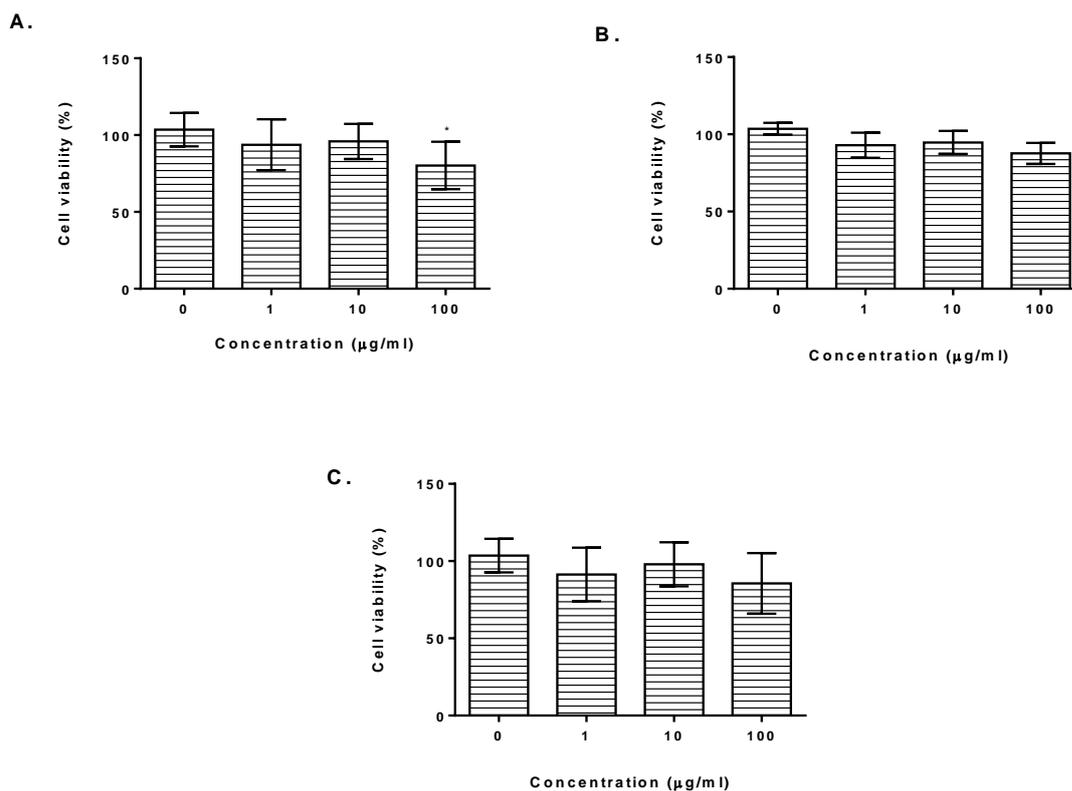


Figure 15: Cellular ATP content of C3A cells exposed to the extract of *C. genistoides* ARC188 (A), ARC188_B (B), ARC188_x (C).

The ATP assay was used to validate results from MTT assay in order to show more sensitivity and to enable correction for protein concentration using a RC\DC (BioRad, USA) assay. C3A cells were cultured for 5 days and subsequently exposed to ARC188 extract, ARC188_B and ARC188_x, at concentrations ranging from 0 µg/ml –100 µg/ml for 24 hours and luminescence was read at a temperature of 22°C. Results are from three independent experiments done in triplicate (n = 9), expressed as the mean ± SD and are reflected as a percentage of the vehicle control set at 100% and corrected for protein content using a Bradford reagent (BioRad). Statistical analysis was performed using one-way ANOVA and a Dunnet's post-hoc test if $p < 0.05$. * = $p < 0.05$, ** = $p < 0.02$ and *** = $p < 0.01$ against the vehicle control.

4.2. The effect of DMSO on CYP activity

DMSO was used to solubilize the *C. genistoides* constituents in this study. The effect of DMSO on the activity of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 was initially investigated, by incubating the Vivid® baculosomes with 0.1%, 0.2%, and 0.5% DMSO (to represent the various concentrations of vehicle control, used for respective solubilisation of compounds) and recording the change in fluorescent intensity for 30 minutes in a kinetic reaction where measurements were recorded every 5 minutes.

DMSO is known to interfere with the Vivid® screening kits at high concentrations (Table 24, Appendix C). It is recommended that it is used at low concentrations, hence the use in our experiments, at: 0.1%, 0.2% and 0.5%. A control (no inhibitor) was also included for this investigation for comparison. When the Vivid® substrates were incubated with the CYP baculosomes, highly fluorescent products formed at 415 nm EX and 460 nm EM wavelengths were signified by an increase in fluorescence. Fluorescence intensity was measured over time to show trends in enzyme activity with a decline in fluorescence indicating CYP enzyme inhibition.

DMSO did not significantly affect the activity of CYP1A2, CYP2C19, CYP2D6 and CYP3A4. Though there was slight decline in the formation of fluorescent intensity with treatment of 0.5% DMSO, the decline was not statistically significant. For CYP2C9 0.5% DMSO caused a decline in the formation of fluorescent products. Results obtained for the effect of DMSO on CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) over time are shown in Figure 16. To compensate for in batch variation the formation of fluorescence products in all tested CYP enzymes, were expressed relative to the background fluorescence at corresponding time points.

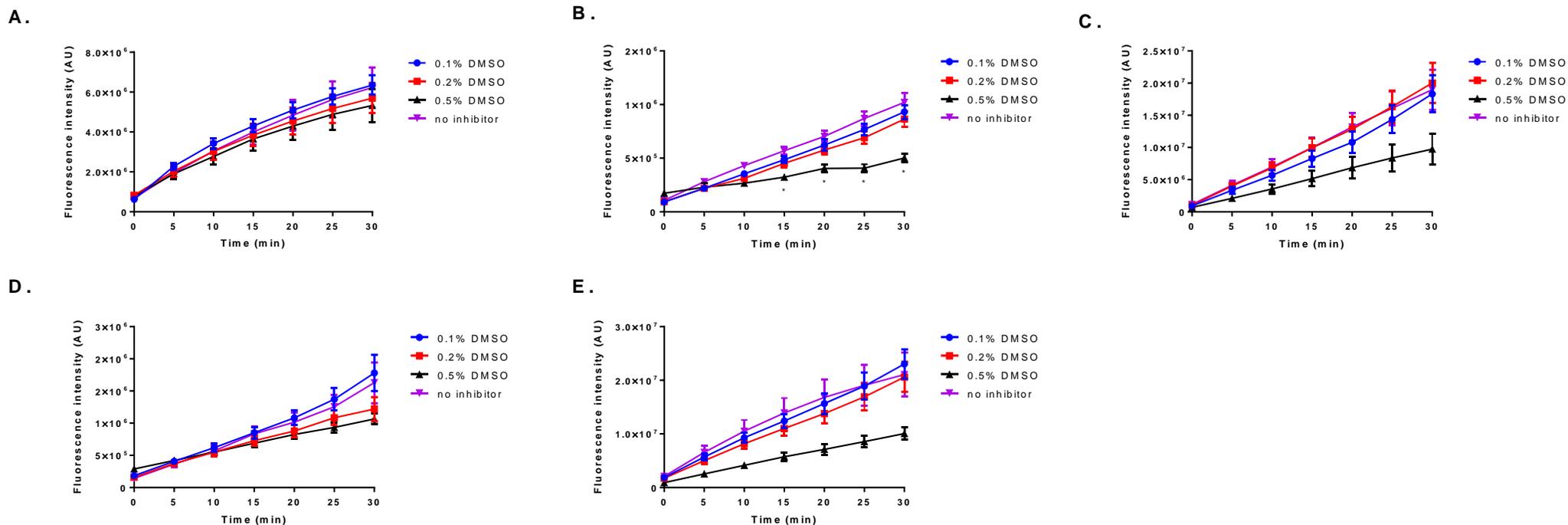


Figure 16: The effect of DMSO on CYP activity.

Formation of fluorescent metabolites observed when Vivid® baculosomes (**A.** CYP1A2, **B.** CYP2C9, **C.** CYP2C19, **D.** CYP2D6 and **E.** CYP3A4) were exposed to the organic solvent DMSO in kinetic reactions for 30 minutes. Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA and a Dunnet's post-hoc test if p < 0.05. Where * = p < 0.05, ** = p < 0.01 and *** = p < 0.001 against the negative control.

4.2. Screening of *C. genistoides* constituents on the activity of major CYP enzymes

Preliminary screening of the xanthone constituents of *C. genistoides* showed that ARC188, ARC188_x, mangiferin and isomangiferin inhibited CYP1A2 activity, shown by a decrease in the formation of fluorescent metabolite when compared to the control. The rate of inhibition observed over the 30-minute incubation was quantified and the degree of inhibition for ARC188, ARC188_x and mangiferin was found to be 50.27% ($p < 0.001$), 57.15% ($p < 0.001$) and 69.76% ($p < 0.001$), respectively. Isomangiferin showed 29.95% ($p < 0.05$) inhibition (Figure 17).

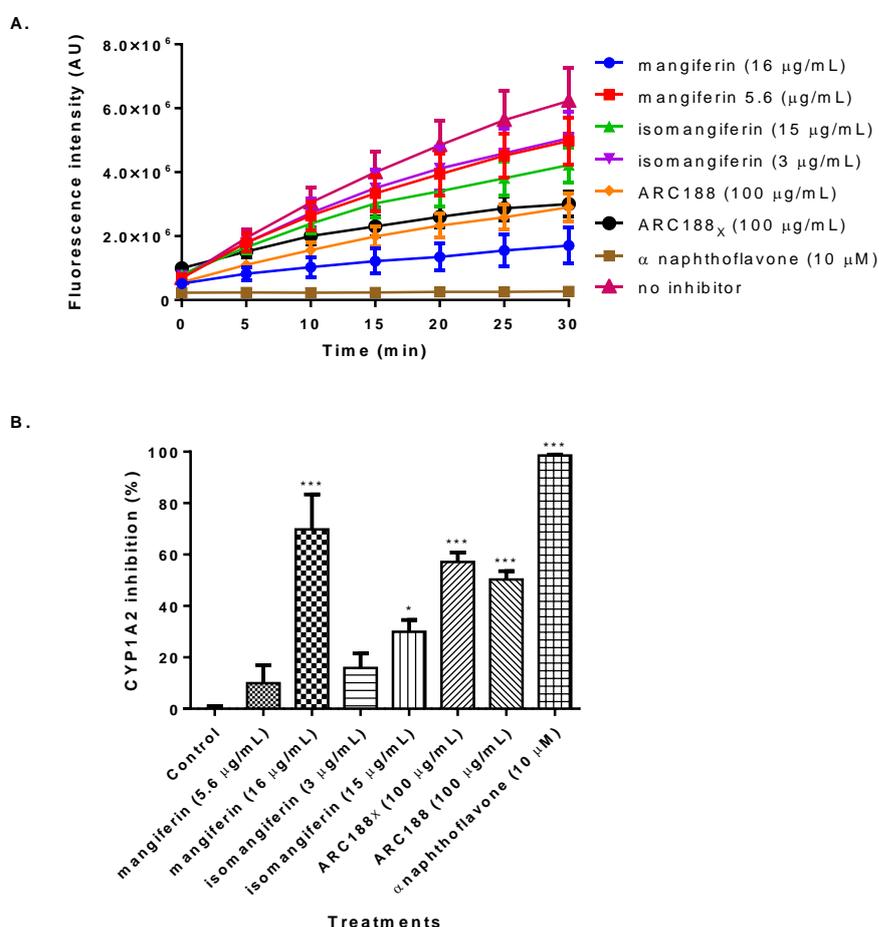


Figure 17: Effect of *C. genistoides* xanthenes on CYP1A2 baculosome activity.

CYP1A2 inhibitory assay incubated with *C. genistoides* xanthone constituents (A), and the rate of CYP1A2 inhibition over 30 minutes incubation (B). Results are derived from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnet's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Screening of the benzophenone constituents of *C. genistoides* showed that ARC188_B had a slight inhibitory effect on CYP1A2 activity shown by decreased fluorescent metabolite formation when compared to no inhibitor. The rate of inhibition observed over the 30-minute on CYP1A2 activity for ARC188_B was 32% ($p < 0.01$). The benzophenone pure compounds (IMG and IDG) had no significant effect on CYP1A2 activity (Figure 18).

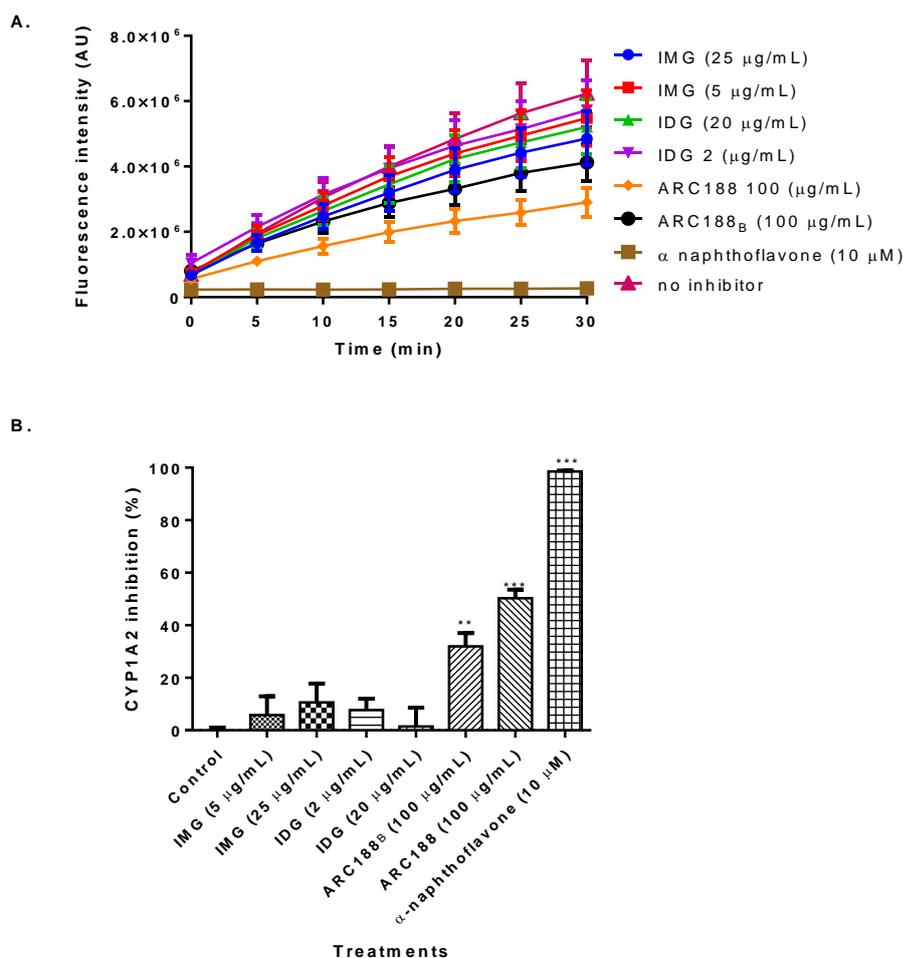


Figure 18: Effect of *C. genistoides* benzophenones on CYP1A2 baculosome activity.

Inhibitory activity of the benzophenone constituents of *C. genistoides* (A), and the rate of CYP1A2 inhibition over 30 minutes incubation (B). Results are derived from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnet's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Xanthone constituents of *C. genistoides* showed that ARC188, ARC188x and mangiferin inhibited CYP2C9 activity, shown by a decrease in the formation of fluorescent metabolite when compared to the control. These three constituents inhibited CYP2C9 activity by 72.47% ($p < 0.001$), 34.60% ($p < 0.05$) and 67.23% ($p < 0.001$), respectively (Figure 19) when the rate of inhibition observed over the 30-minute incubation was quantified.

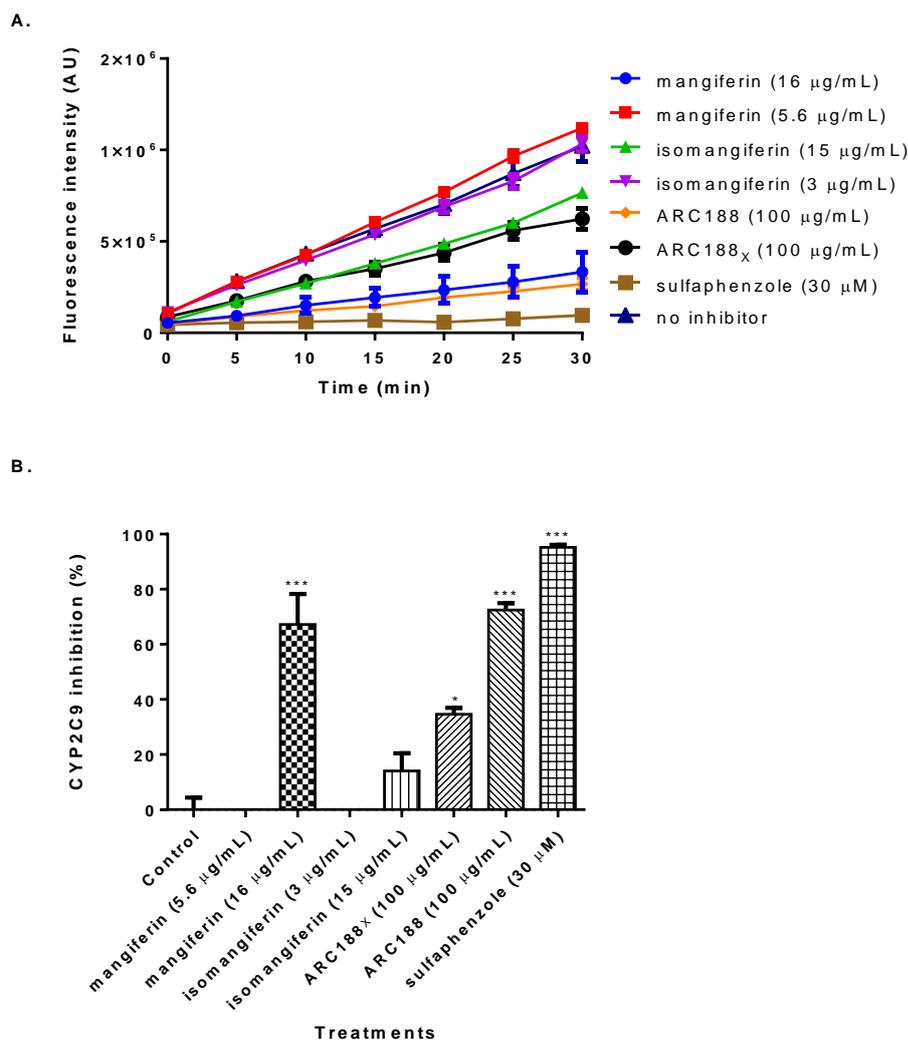


Figure 19: Effect of *C. genistoides* xanthenes on CYP2C9 baculosome activity.

Time-dependent CYP2C9 inhibitory assay incubated with *C. genistoides* xanthone constituents (A.), and the rate of CYP2C9 inhibition over 30 minutes incubation (B.). Results are from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnett's post-hoc test if $p < 0.05$. Where * = $p < 0.05$ and *** = $p < 0.001$.

Benzophenone constituents of *C. genistoides* showed that ARC188_B had a slight inhibitory effect on CYP2C9 activity shown by decreased fluorescent metabolite formation when compared to no inhibitor. The rate of inhibition of CYP2C9 observed over the 30-minute incubation for ARC188_B was 25.03% ($p < 0.05$). The benzophenone pure compounds (IMG and IDG) had no significant effect on CYP1A2 activity (Figure 20).

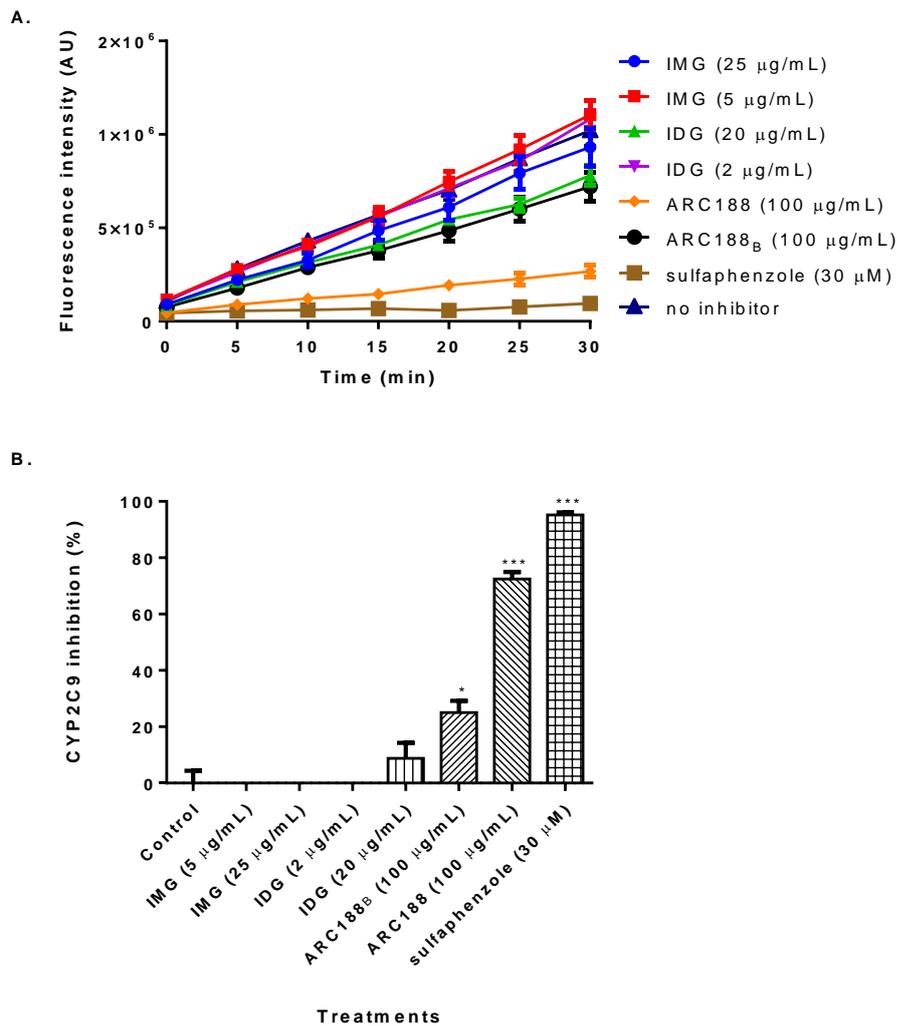


Figure 20: Effect of *C. genistoides* benzophenones on CYP2C9 baculosome activity.

Time-dependent CYP2C9 inhibitory assay incubated with *C. genistoides* benzophenone constituents (A.), and the rate of CYP2C9 inhibition over 30 minutes incubation (B.). Results are from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnett's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Screening of the xanthone constituents of *C. genistoides* showed that only ARC188 majorly inhibited CYP2C19 activity, shown by a decrease in the formation of fluorescent metabolite when compared to the control (Figure 21). The rate of inhibition of CYP2C19 activity observed over the 30-minute incubation for ARC188, ARC188_x and mangiferin was 70.68% ($p < 0.001$), 34.13% ($p < 0.001$) and 34.13% ($p < 0.001$), respectively (Figure 4.8 B.).

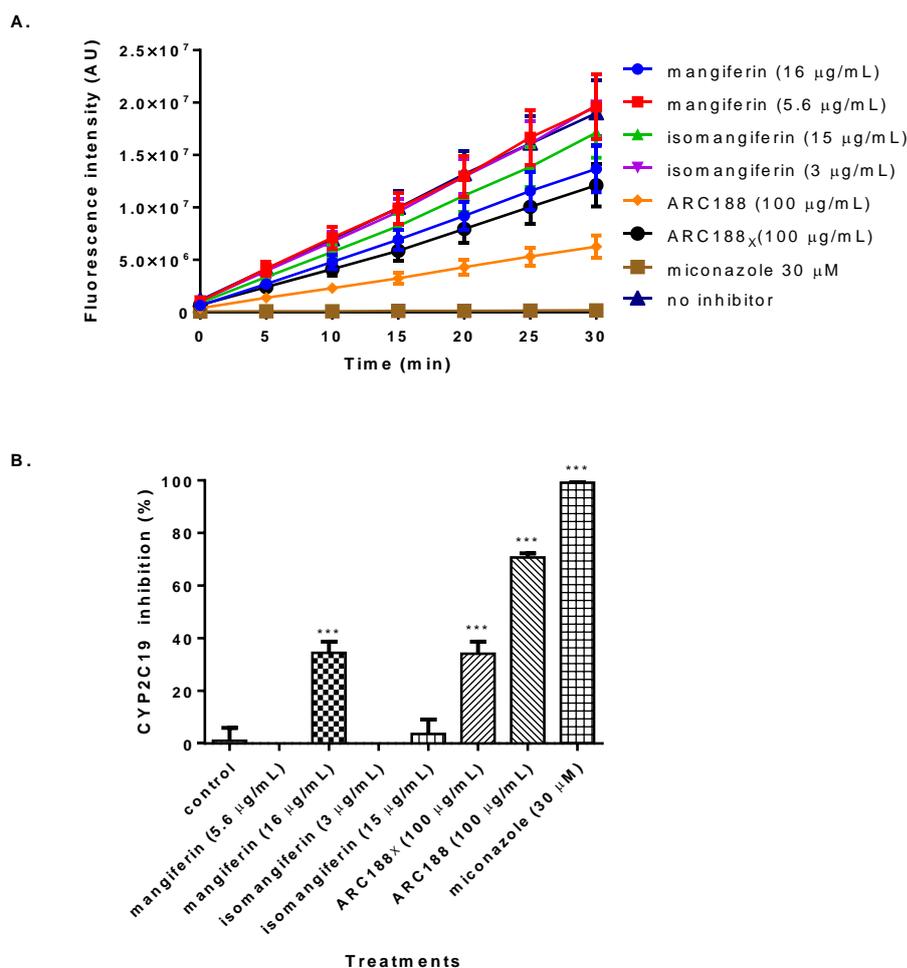
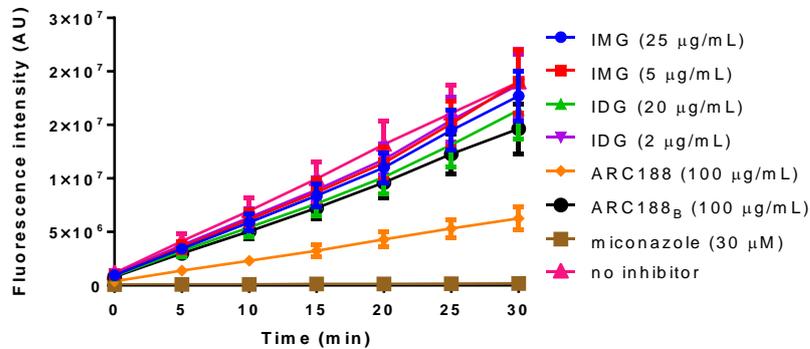


Figure 21: Effect of *C. genistoides* xanthenes on CYP2C19 baculosome activity.

Time-dependent CYP2C19 inhibitory assay incubated with *C. genistoides* xanthone constituents (A), and the rate of CYP2C19 inhibition over 30 minutes incubation (B). Results are from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnett's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

The benzophenone constituents of *C. genistoides* showed that the benzophenone constituents had no significant effects on CYP2C19 activity (Figure 22).

A.



B.

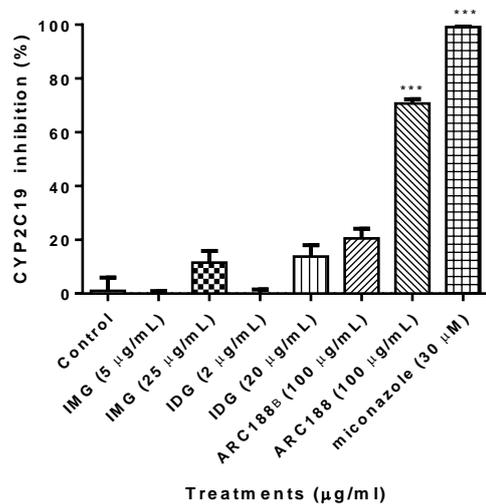


Figure 22: Effect of *C. genistoides* benzophenones on CYP2C19 baculosome activity.

Time-dependent CYP2C19 inhibitory assay incubated with *C. genistoides* benzophenone constituents (A.), and the rate of CYP2C19 inhibition over 30 minutes incubation (B.). Results are from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnett's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

The xanthone constituents of *C. genistoides* showed that ARC188, ARC188x and mangiferin inhibited CYP2D6 activity, shown by a decrease in the formation of fluorescent metabolite when compared to the control. These 3 constituents inhibited CYP2D6 activity by 50.48% ($p < 0.001$), 56.56% ($p < 0.001$) and 75.12% ($p < 0.001$), respectively (Figure 23).

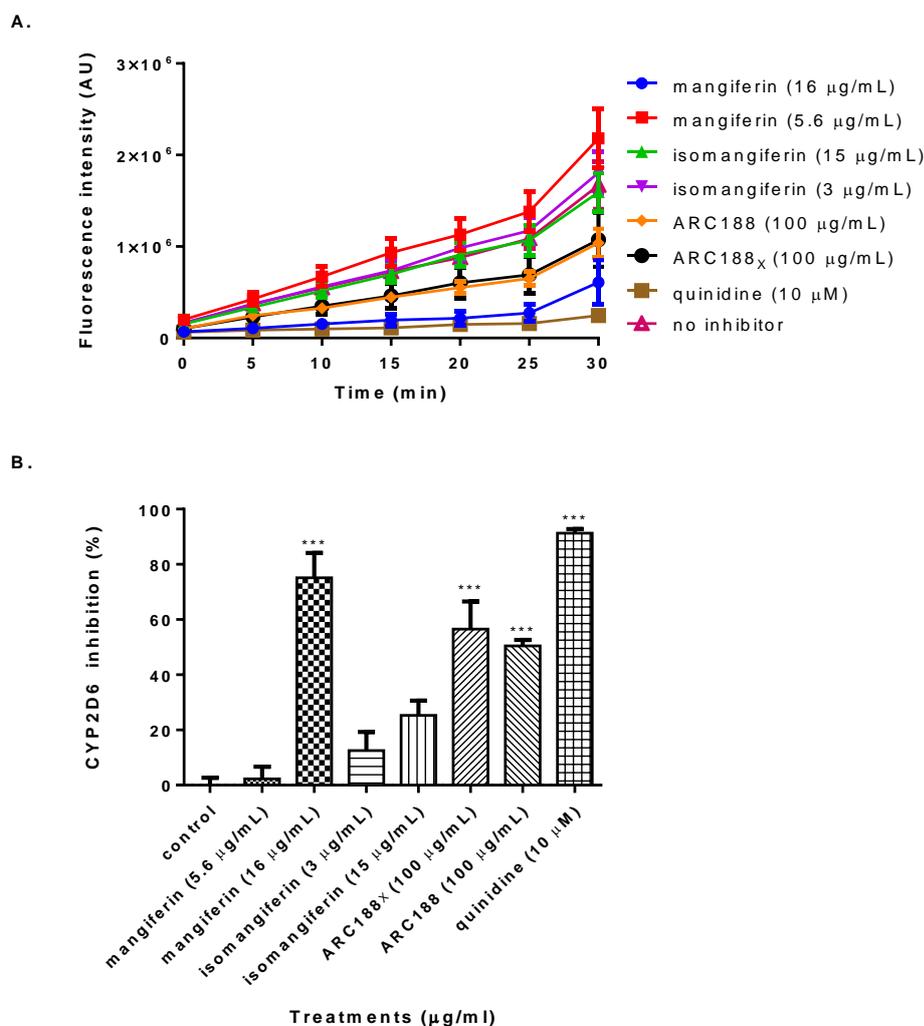


Figure 23: Effect of *C. genistoides* xanthenes on CYP2D6 baculosome activity.

Time-dependent CYP2D6 inhibitory assay incubated with *C. genistoides* xanthone constituents (A), and the rate of CYP2D6 inhibition over 30 minutes incubation (B). Results are from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnett's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

The benzophenone constituents of *C. genistoides* showed that ARC188_B inhibited CYP2D6 by 37.91% ($p < 0.05$) while IMG and IDG had no significant effects on CYP2D6 activity (Figure 24).

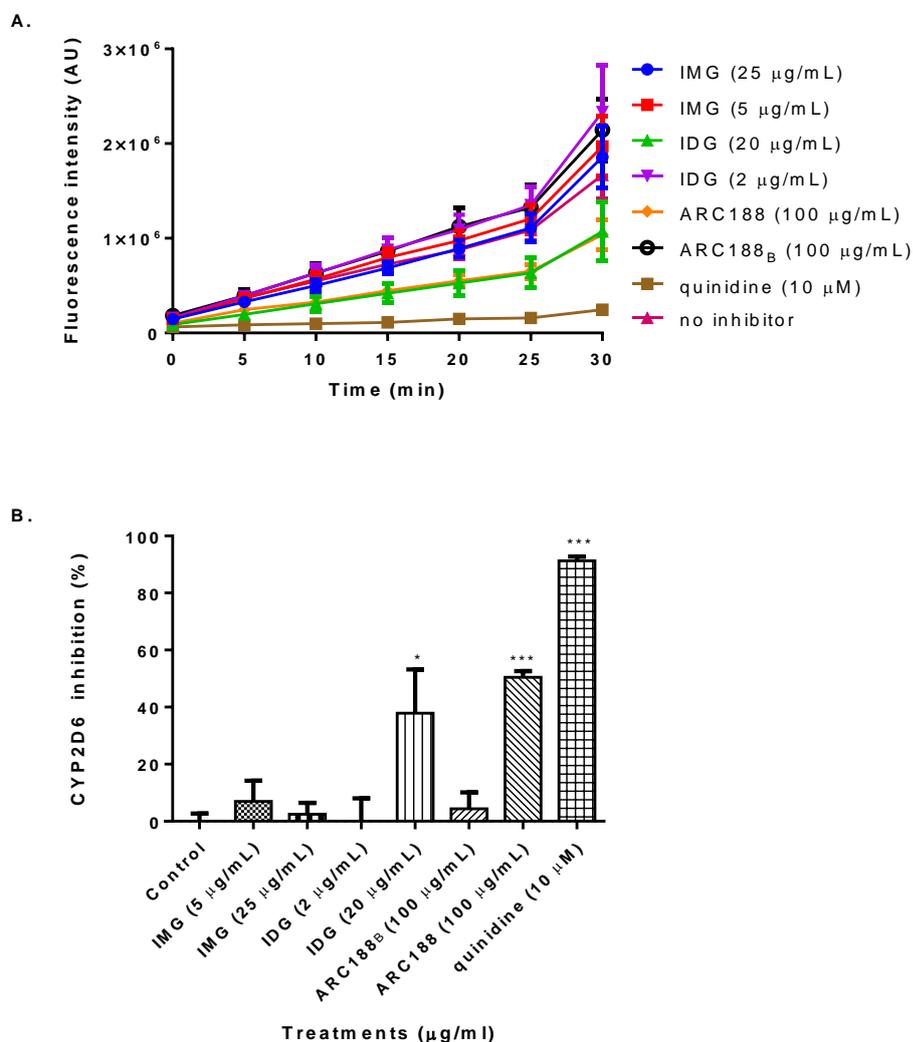


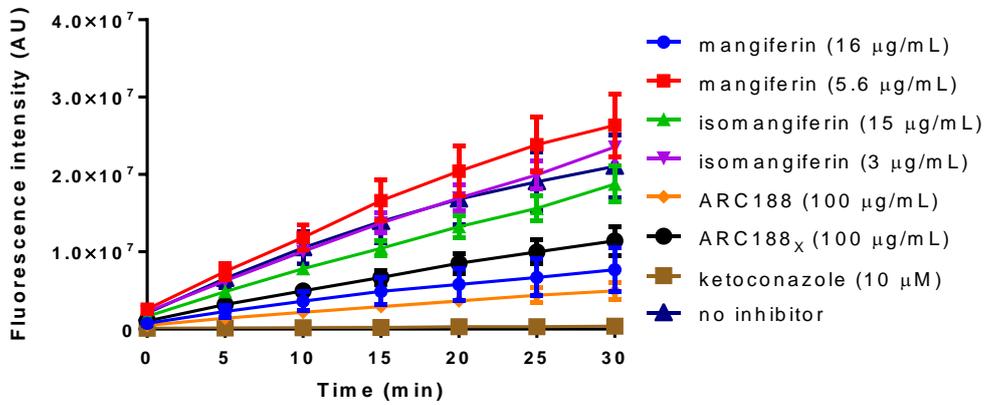
Figure 24: Effect of *C. genistoides* benzophenones on CYP2D6 baculosome activity.

Time-dependent CYP2D6 inhibitory assay incubated with *C. genistoides* benzophenone constituents (A.), and the rate of CYP2D6 inhibition over 30 minutes incubation (B.). Results are from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnett's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, and *** = $p < 0.001$.

The xanthone constituents of *C. genistoides* showed that ARC188, ARC188_B, ARC188_X and mangiferin inhibited CYP3A4 activity, shown by a decrease in the formation of fluorescent metabolite when compared to the control. These test samples

inhibited CYP3A4 activity by 78.49% ($p < 0.001$), 53.28% ($p < 0.001$), 50.61% ($p < 0.001$) and 83.10% ($p < 0.001$), respectively (Figure 25).

A.



B.

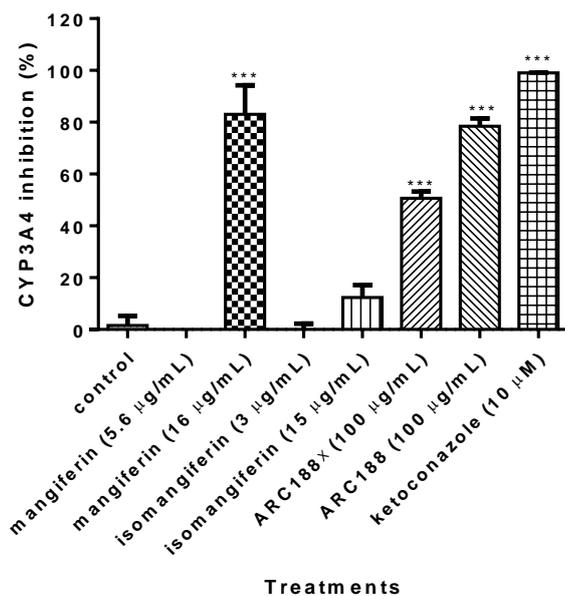
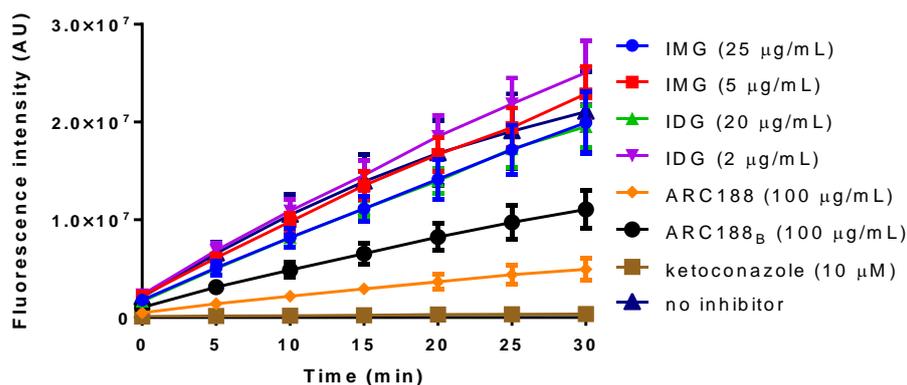


Figure 25: Effect of *C. genistoides* xanthenes on CYP3A4 baculosome activity.

Time-dependent CYP3A4 inhibitory assay incubated with *C. genistoides* xanthone constituents (A) and the rate of CYP3A4 inhibition over 30 minutes incubation (B). Results are from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnett's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Screening of the benzophenone constituents of *C. genistoides* showed that ARC188_B had an inhibitory effect on CYP3A4 activity shown by decreased fluorescent metabolite formation when compared to no inhibitor. ARC188_B inhibited CYP3A4 activity by 53.28% ($p < 0.001$). The benzophenone pure compounds (IMG and IDG) had no significant effect on CYP1A2 activity (Figure 26).

A.



B.

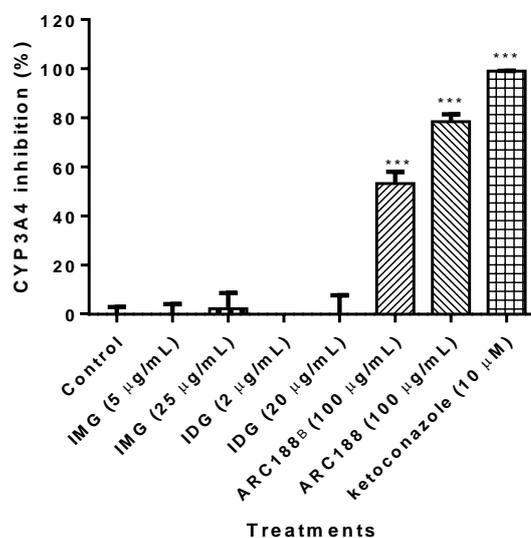


Figure 26: Effect of *C. genistoides* constituents on CYP3A4 baculosome activity.

Time-dependent CYP3A4 inhibitory assay incubated with *C. genistoides* benzophenone constituents (A.), and the rate of CYP3A4 inhibition over 30 minutes incubation (B.). Results are from three independent experiments done in triplicate ($n = 9$), expressed as the mean \pm SEM and CYP inhibition is reflected as a percentage of the vehicle control set at 100%. Statistical analysis was performed using one-way ANOVA and a Dunnett's post-hoc test if $p < 0.05$. Where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

4.3. IC₅₀ determination of *C. genistoides* constituents on major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4)

The IC₅₀ concentration can be used as a guide to classify the potency of a compound or extract as an inhibitor (Zou *et al.*, 2002 and Kong *et al.*, 2011). Extracts with an IC₅₀ of less than or equal to 20 µg/mL can be regarded as strong inhibitors. Moderate inhibition is shown when the IC₅₀ value is more than 20 µg/mL, but less than 100 µg/mL and weak inhibition if the IC₅₀ that is greater than 100 µg/mL (Kong *et al.*, 2011).

For CYP1A2, ARC188 extract, ARC188_x and mangiferin, the only constituents that displayed time-dependent CYP inhibition ≥50%, were analysed further for concentration-dependent activity. Mangiferin was shown to be the most potent entity against CYP1A2 activity with an IC₅₀ value of 35.06 ± 3.23 µg/mL. While ARC188_x was the least potent inhibitor of CYP1A2 with 102.0 ± 3.12 µg/mL IC₅₀. ARC188 had an IC₅₀ of 98.61 ± 2.54 µg/mL. The concentration-dependent results of CYP1A2 are shown in Figure 27.

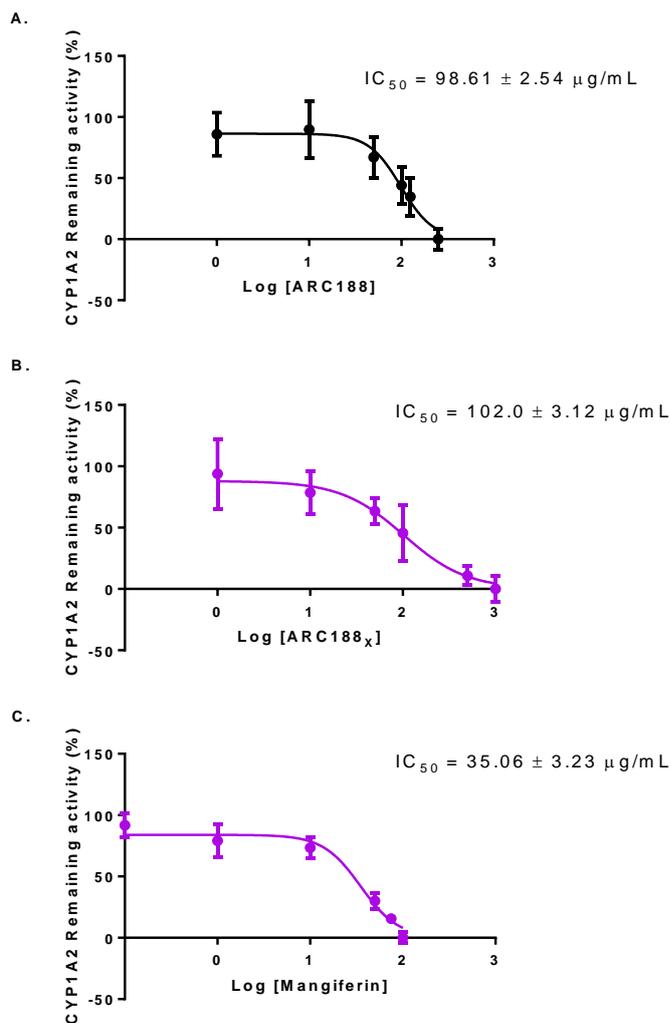


Figure 27: IC_{50} plots show *C. genistoides* constituents inhibition of CYP1A2.

ARC188 extract (A.), ARC188X (B.) and mangiferin (C.). Results are from three independent experiments done in triplicate ($n = 9$) and are expressed as the mean $IC_{50}(\mu\text{g/mL}) \pm \text{SEM}$.

For CYP2C9, only ARC188 extract and mangiferin displayed time-dependent CYP inhibition $\geq 50\%$ and were analysed further in concentration-dependent assays. ARC188 was less potent than mangiferin with IC_{50} values of 90.09 ± 4.66 and $57.86 \pm 5.50 \mu\text{g/mL}$, respectively. The results obtained from concentration-dependent inhibition of CYP2C9 are shown in Figure 28.

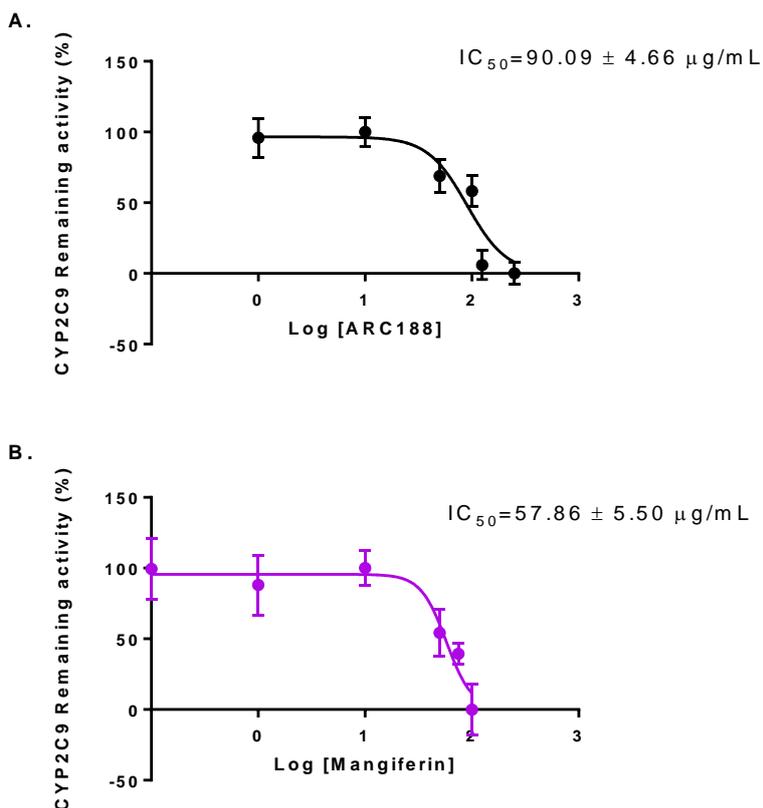


Figure 28: IC_{50} plots show *C. genistoides* constituents inhibition of CYP2C9.

ARC188 extract (A.) and mangiferin (B.). Results are from three independent experiments done in triplicate ($n = 9$) and are expressed as the mean IC_{50} ($\mu\text{g/mL}$) \pm SEM.

For CYP2C19, only ARC188 extract displayed time-dependent CYP inhibition $\geq 50\%$. The results obtained from concentration-dependent assays of ARC188 extract on CYP2C19 are shown in Figure 29 below. The IC_{50} value of ARC188 was $64.39 \pm 3.01 \mu\text{g/mL}$.

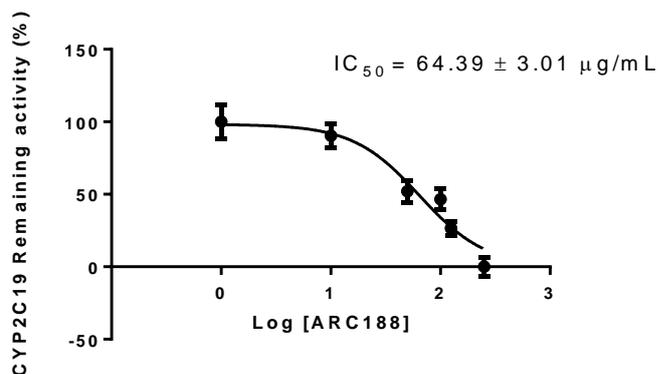


Figure 29: IC₅₀ plot show ARC188 extract inhibition of CYP2C19.

Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean IC₅₀ (µg/mL) ± SEM.

For CYP2D6, ARC188, ARC188_x and mangiferin, constituents that displayed CYP inhibition ≥ 50%, were analysed further in concentration-dependent assays. The results obtained from concentration-dependent inhibition assays of CYP2D6 are shown in Figure 30 below. Again, mangiferin was the most potent CYP2D6 inhibitor with an IC₅₀ of 7.19 ± 1.75 µg/mL, while ARC188 was the least potent inhibitor with 57.83 ± 2.07 µg/mL IC₅₀. ARC188_x had an IC₅₀ of 42.62 ± 3.79 µg/mL.

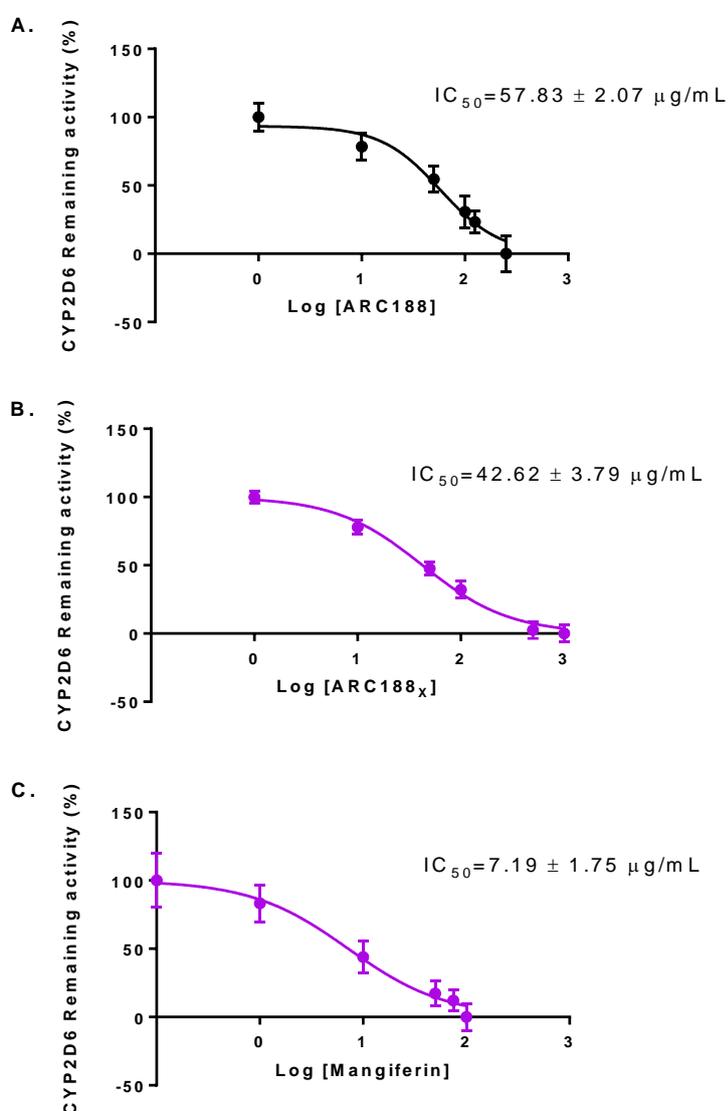


Figure 30: IC₅₀ plots showing the potential of *C. genistoides* constituents to inhibit CYP2D6.

ARC188 (A.), ARC188_x (B.) and mangiferin (C.). Results are from three independent experiments done in triplicate (n = 9) and are expressed as the mean IC₅₀ (µg/mL) ± SEM.

For CYP3A4, ARC188, ARC188_B and ARC188_x and mangiferin, all displayed time-dependent CYP inhibition ≥50%. ARC188_B was the least active entity against the activity of CYP3A4 with an IC₅₀ of 213.4 ± 2.94 µg/mL while mangiferin was the most active entity with an IC₅₀ of 23.12 ± 0.02 µg/mL. ARC188 and ARC188_x had IC₅₀ values of 57.91 ± 5.74 and 121.4 ± 4.31 µg/mL respectively (Figure 31).

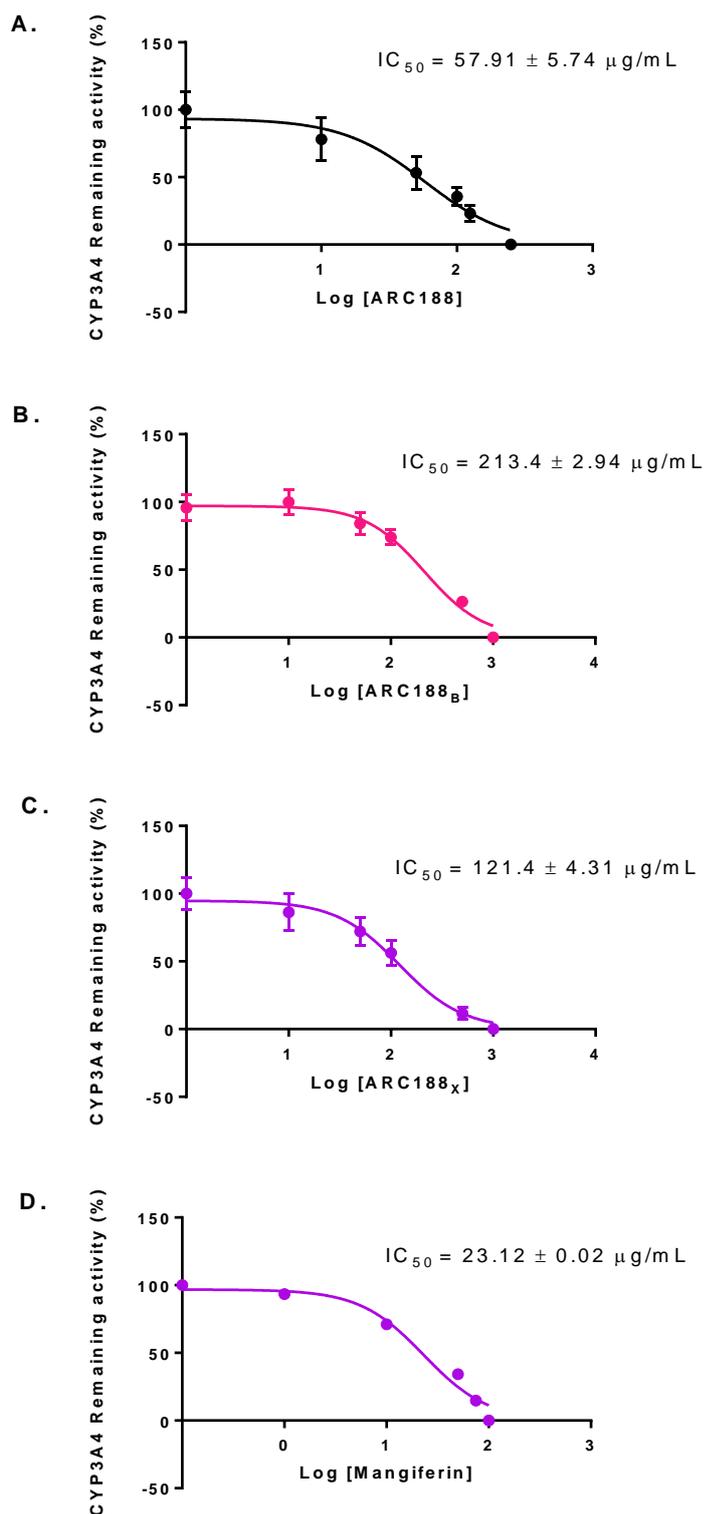


Figure 31: IC_{50} plots show *C. genistoides* constituents inhibition of CYP3A4.

ARC 188 extract (A.), benzophenone rich fractionation (B.), xanthone rich fractionation (C.) and mangiferin (D.). Results are from three independent experiments done in triplicate ($n = 9$) and are expressed as the mean IC_{50} ($\mu\text{g/mL}$) \pm SEM.

4.4. Summary of CYP inhibition

Table 6: Summary of the inhibitory effect of *C. genistoides* constituents on CYP activity

	IC ₅₀ (µg/mL±SEM)						
	ARC188	ARC188 _B	IMG	IDG	ARC188 _x	Mangiferin	Isomangiferin
CYP1A2	98.61 ± 2.543	No inhibition	No inhibition	No inhibition	102.0 ± 3.123	35.06 ± 3.228	No inhibition
CYP2C9	90.09 ± 4.664	No inhibition	No inhibition	No inhibition	No inhibition	57.86 ± 5.504	No inhibition
CYP2C19	64.39 ± 3.007	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
CYP2D6	57.83 ± 2.067	No inhibition	No inhibition	No inhibition	42.62 ± 3.789	7.187 ± 3.034	No inhibition
CYP3A4	57.91 ± 5.737	213.4 ± 2.944	No inhibition	No inhibition	121.40 ± 4.306	23.12 ± 8.262	No inhibition

Results are from 3 independent experiments in triplicates (n=9), expressed as mean± SEM

4.5. Solvent effect on mRNA expression of drug transporters (ABCB1, ABCG2 and SLCO1B3)

The effect of the vehicle control (0.5% DMSO) on the mRNA expression of drug transporters was investigated. There were no significant changes in the mRNA expression of ABCB1 and SLCO1B3 for the vehicle control when compared to the control at all time points. For ABCG2 there was a significant up-regulation of mRNA expression for 0.5% DMSO (5.05 ± 0.44 AU) compared to the control (2.66 ± 1.03 AU), $p < 0.01$. No significant changes were observed at 12 and 24 hours. Results for the effect of 0.5% DMSO on mRNA expression of drug transporters are shown in Figure 32.

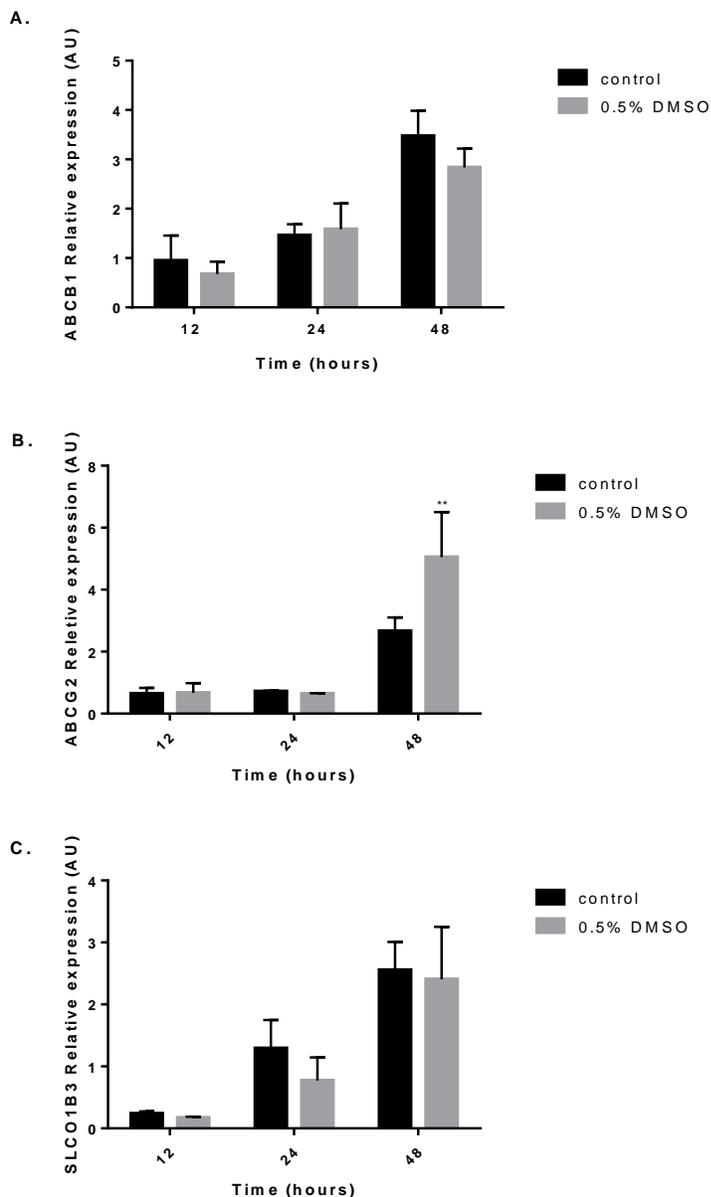


Figure 32: Effect of 0.5% DMSO on mRNA expression of drug transporter genes (A.) ABCB1, (B.) ABCG2 and (C.) SLCO1B3 after 12, 24 and 48 hours.

Cells were cultured for 5 days and treated with 0.5% DMSO for 12, 24 and 48 hours. mRNA was extracted, and cDNA was synthesized thereafter, and qRT-PCR was performed. ABCG2 mRNA expression was expressed relative to the housekeeping genes HPRT1 and β -actin. Results are from three independent experiments done in duplicates ($n = 6$) and are expressed as the mean \pm SEM. Statistical analysis was performed using student T-tests with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ against the control.

4.6. The effect of *C. genistoides* constituents on the expression of drug transporter genes (ABCB1, ABCG2 and SLCO1B3)

The modulatory effect of *C. genistoides* on the gene expression of the drug transporters; ABCB1, ABCG2 and SLCO1B3 was investigated. ABCB1 and ABCG2 are major drug efflux pumps and are involved in many herb-drug interactions with the herbal medicines having an ability to influence their expression thus causing a decrease in the efficacy of drugs. SLCO1B3 is a major uptake drug transporter which transports many drugs, including anti-diabetic drugs. Investigation of how the *C. genistoides* constituents influence these drug transporters may give an indication of any potential herb-drug interactions that may involve compounds from *C. genistoides*. To evaluate the effect of *C. genistoides* on the expression of ABCB1, ABCG2 and SLCO1B3 cells were treated with 250 µg/mL of ARC188 extract, 250 of µg/mL ARC188_B and ARC188_X fractionations and 34 and 100 µg/mL of mangiferin for 12, 24 and 48 hours. The pure compounds, isomangiferin, IMG and IDG were not investigated for their effect on mRNA expression due to the lack of CYP inhibition, the scarcity of compounds and cost.

Cells were also treated with 20 µM of rifampicin (positive control), known to induce the expression of drug transporters and CYP enzymes. RNA was then extracted, and cDNA was synthesized. mRNA expression of the drug transporter genes (ABCB1, ABCG2, SLCO1B1 and SLCO1B3), as well as 2 house-keeping genes (β-actin and HPRT11), was quantified using qRT-PCR for each time point. Results were expressed as relative expression of the drug transporter genes, normalized to the 2 house-keeping genes and expressed relative to the vehicle control at each respective time-point.

ABCB1 relative expression was increased from 1.29 ± 0.42 arbitrary units (AU) (control) to 5.34 ± 0.26 , 7.09 ± 0.70 , 9.09 ± 0.07 , 3.51 ± 0.24 , 3.30 ± 0.13 and 3.96 ± 0.37 AU by mangiferin (34 µg/mL), mangiferin (100 µg/mL), ARC188, ARC188_B, ARC188_X and rifampicin, respectively, after 12 hours. Mangiferin (34 µg/mL), mangiferin (100 µg/mL), ARC188, ARC188_B, ARC188_X and rifampicin up-regulated ABCB1 expression to 2.31 ± 0.51 , 3.26 ± 0.41 , 6.12 ± 0.01 , 2.92 ± 1.50 , 4.14 ± 0.17 and 5.19 ± 0.51 AU, respectively, versus the control (1.01 ± 0.14) after 24 hours. There were no significant changes in

ABCB1 expression after 48 hours. The effect of *C. genistoides* constituents on the expression of ABCB1 is illustrated in Figure 33 below.

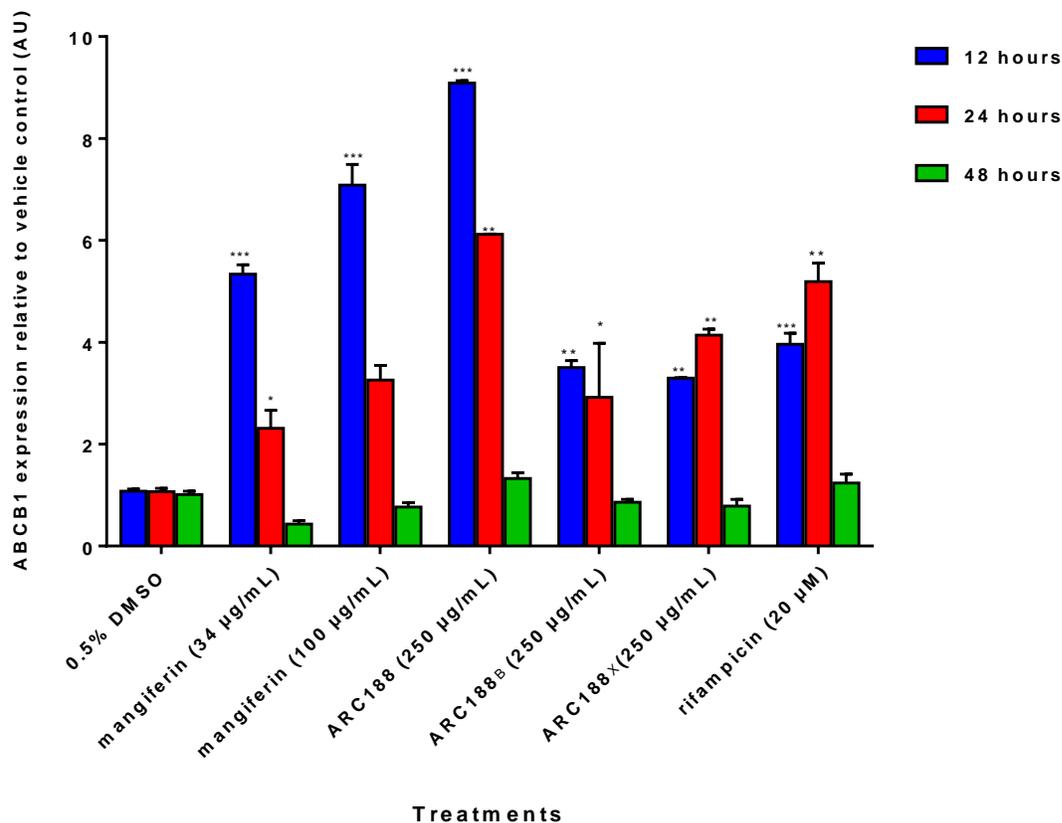


Figure 33: The effect of *C. genistoides* constituents on ABCB1 mRNA expression.

C3A cells were cultured for 5 days and subsequently exposed to 250 µg/mL of ARC188, 250 µg/mL of ARC188_B, 250 µg/mL of ARC188_X, 34 and 100 µg/mL of mangiferin and 20 µM of rifampicin for 12, 24 and 48 hours. mRNA was extracted, and cDNA was synthesized thereafter, and qRT-PCR was performed. ABCB1 mRNA expression was expressed relative to the housekeeping genes HPRT1 and β-actin and normalised to the vehicle control. Results are from three independent experiments done in duplicate (n = 6) and are expressed as the mean ± SEM. Statistical analysis was performed using unpaired student T-tests with * = p < 0.05, ** = p < 0.01 and *** = p < 0.001 against the vehicle control.

For ABCG2, mangiferin (34 $\mu\text{g/mL}$) (3.23 ± 0.53 AU), mangiferin (100 $\mu\text{g/mL}$) (3.88 ± 0.70 AU), ARC188 (7.41 ± 0.56 AU), ARC188_x (4.38 ± 0.15 AU), and rifampicin (4.81 ± 0.68 AU) up-regulated gene expression when compared to the control (0.96 ± 0.18 AU) after 12 hours. After 24 hours, mangiferin (100 $\mu\text{g/mL}$) (2.64 ± 0.91 AU), ARC188 (2.85 ± 0.52 AU), ARC188_x (6.25 ± 2.97 AU) and rifampicin (4.83 ± 5.50 AU) up-regulated compared to the control (1.01 ± 0.14 AU). No significant changes on ABCG2 expression were observed for ARC188_B at all time-points tested. Also, no significant changes in ABCG2 expression after 48 hours. Results showing the effect of *C. genistoides* constituents on ABCG2 expression are illustrated in Figure 34.

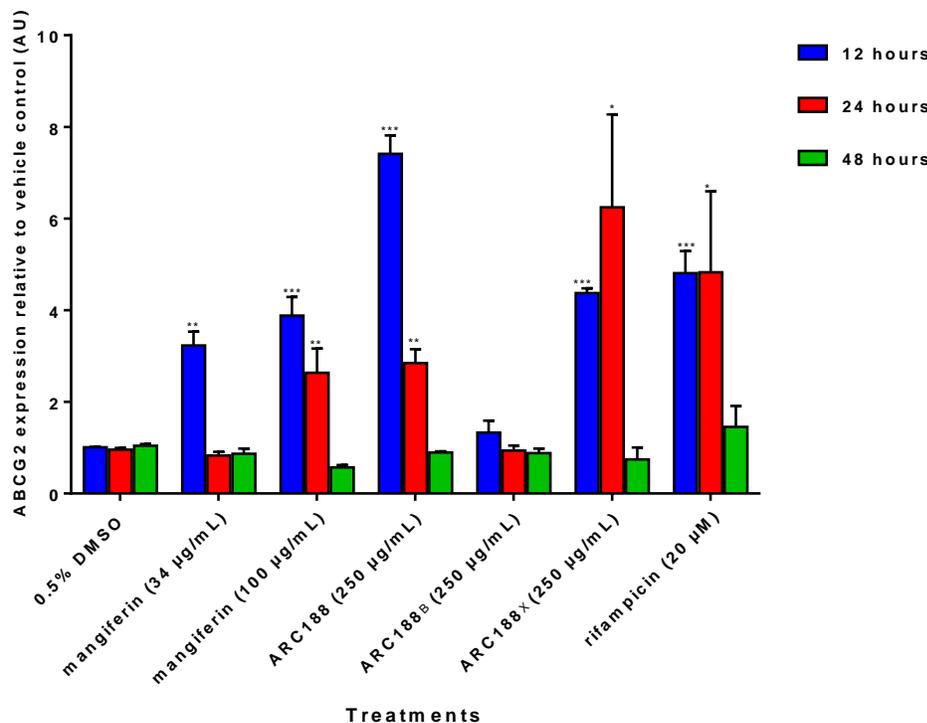


Figure 34: The effect of *C. genistoides* constituents on ABCG2 mRNA expression.

C3A cells were cultured for 5 days and subsequently exposed to 250 $\mu\text{g/mL}$ of ARC188 extract, 250 $\mu\text{g/mL}$ of ARC188_B and ARC188_x fractionations, 34 and 100 $\mu\text{g/mL}$ of mangiferin and 20 μM of rifampicin for 12, 24 and 48 hours. mRNA was extracted, and cDNA was synthesized thereafter, and qRT-PCR was performed. ABCG2 mRNA expression was expressed relative to the housekeeping genes HPRT1 and β -actin and normalised to the vehicle control. Results are from three independent experiments done in duplicate ($n = 6$) and are expressed as the mean \pm SEM. Statistical analysis was performed using unpaired student T-tests with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ against the vehicle control.

ARC188 significantly down regulated SLCO1B3 after 12, 24 and 48 hours treatment (0.925 ± 0.308 vs 1.367 ± 0.100 relative expression), (0.504 ± 0.035 vs 1.293 ± 0.350 relative expression) and (0.529 ± 0.082 vs 1.556 ± 0.082 AU), respectively compared to the negative control. Mangiferin at a high concentration significantly down-regulated SLCO1B3 expression after 12, 24 and 48 hours treatment. After 12 hours of treatment with mangiferin ($100 \mu\text{g/mL}$), SLCO1B3 mRNA expression was 0.388 ± 0.170 vs 1.367 ± 0.100 . after 24 hours of treatment with mangiferin ($100 \mu\text{g/mL}$) SLCO1B3 mRNA expression was 0.065 ± 0.037 vs 1.293 ± 0.350 against the negative control and after 48 hours SLCO1B3 mRNA expression was 0.041 ± 0.019 . No significant changes were observed in SLCO1B3 expression after treatment with ARC188_B and ARC188_X at all time-points. Results of the effects of *C. genistoides* constituents on the expression of SLCO1B3 are shown in Figure 35.

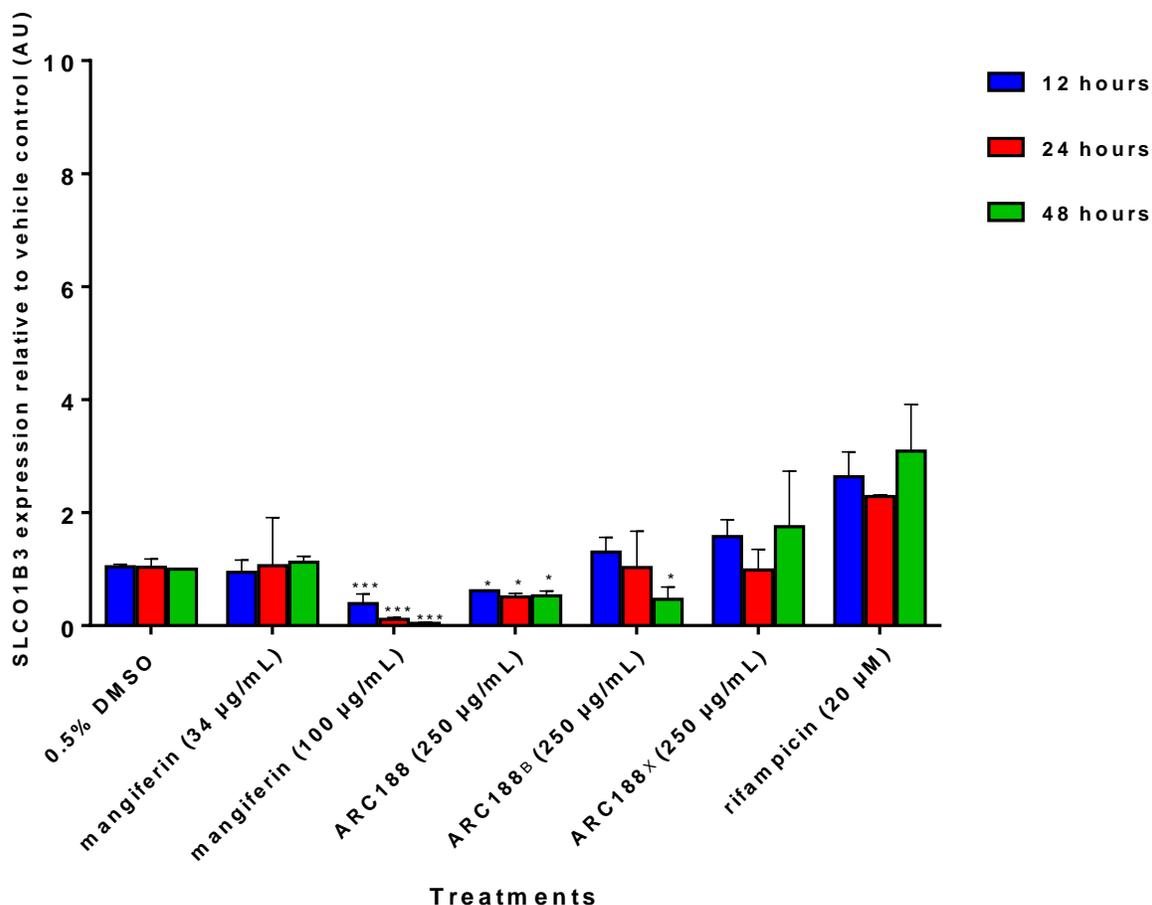


Figure 35: The effect of *C. genistoides* constituents on SLCO1B3 mRNA expression.

C3A cells were cultured for 5 days and subsequently exposed to 250 µg/mL of ARC188 extract, 250 µg/mL of ARC188_B and ARC188_X fractionations, 34 and 100 µg/mL of mangiferin and 20 µM of rifampicin for 12, 24 and 48 hours. mRNA was extracted, and cDNA was synthesized thereafter, and qRT-PCR was performed. ABCG2 mRNA expression was expressed relative to the housekeeping genes HPRT1 and β-actin and normalised to the vehicle control. Results are from three independent experiments done in duplicates (n = 6) and are expressed as the mean ± SEM. Statistical analysis was performed using unpaired student T-tests with * = p < 0.05, ** = p < 0.01 and *** = p < 0.001 against the control.

4.7. Drug transporter protein expression

Protein levels for drug transporter proteins; ABCB1, ABCG2 and SLCO1B3 were measured in C3A cells. C3A cells were treated with *C. genistoides* constituents at the same concentrations used for mRNA expression (250 µg/mL of ARC188 extract, 250

$\mu\text{g/mL}$ of ARC188_B and ARC188_x and 34 and 100 $\mu\text{g/mL}$ of mangiferin) for 12 and 24 hours, respectively. Protein expression results were normalised to β -tubulin. The pure compounds, isomangiferin, IMG and IDG were not investigated for their effect on protein expression due to the scarcity of these compounds and cost. Rifampicin was also used as a positive control. Proteins were then extracted, and Western blot analysis was performed to illustrate the expression of specific drug transporter proteins (ABCB1, ABCG2 and SLCO1B3). β -tubulin was used as a house-keeping protein. Images of the Western blots were captured on the BioRad Chemidoc-imager. Protein expression levels were quantified using Image Lab software to quantify band intensity of the proteins relative to the intensity of β -tubulin. Results were expressed as protein levels (AU) relative to β -tubulin expression and normalized to the vehicle control (0,5% DMSO).

ARC188_B, ARC188_x and rifampicin (positive control) significantly up-regulated ABCB1 expression relative to the vehicle control (0.5% DMSO) after 12 hours of treatment (0.67 ± 0.15 , 0.70 ± 0.09 and 0.61 ± 0.08). ARC188 and mangiferin (100 $\mu\text{g/mL}$) also showed an increase in expression of ABCB1 after 12 hours, but this was not shown to be significant. Protein levels of ABCB1 after 12 hours are shown in Figure 36.

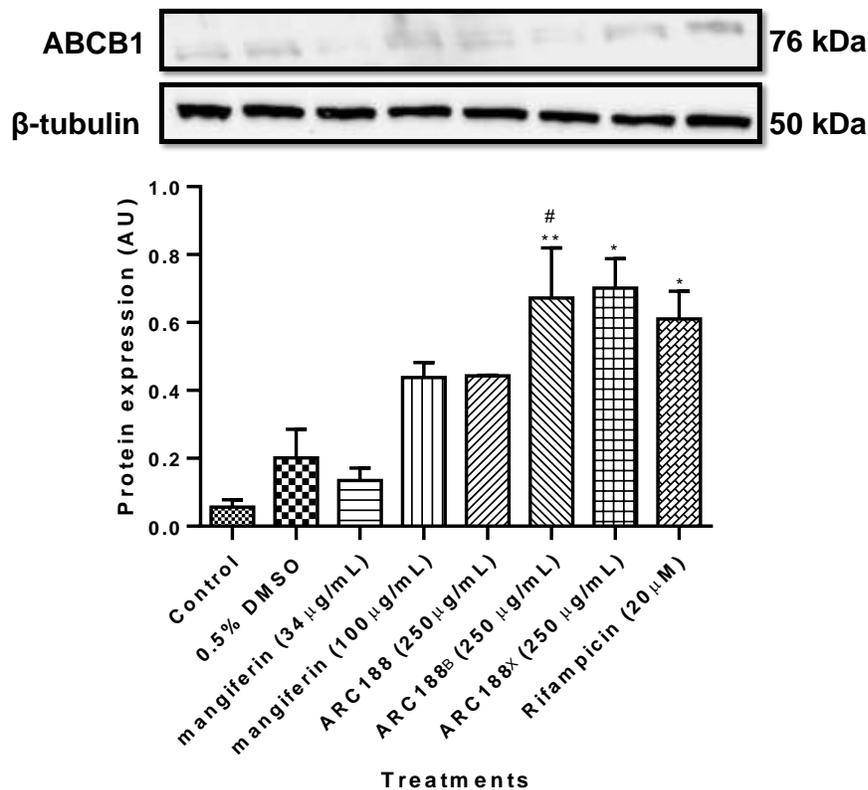


Figure 36: Protein levels of ABCB1 after 12 hours of treatment with *C. genistoides* constituents.

C3A cells were cultured for 5 days and subsequently exposed to 250 µg/mL of ARC188, 250 µg/mL of ARC188_B and ARC188_X, 34 and 100 µg/mL of mangiferin and 20 µM of rifampicin for 12. Proteins were isolated and Western blot analysis was performed, then protein levels were quantified on image lab and expressed relative to β-tubulin and normalized to the vehicle control (0.5% DMSO). Results are from three independent experiments done in duplicates (n = 6) and are expressed as the mean ± SEM. Statistical analysis was performed using unpaired student T-tests with * = p < 0.05, ** = p < 0.01 and *** = p < 0.001 against the vehicle control.

After 24 hours, mangiferin (100 µg/mL) significantly up-regulated ABCB1 expression (0.11 ± 0.05 AU) relative to the control while the other test samples did not show any significant changes in ABCB1 protein expression. The protein levels of ABCB1 after 24 hours are shown in Figure 37.

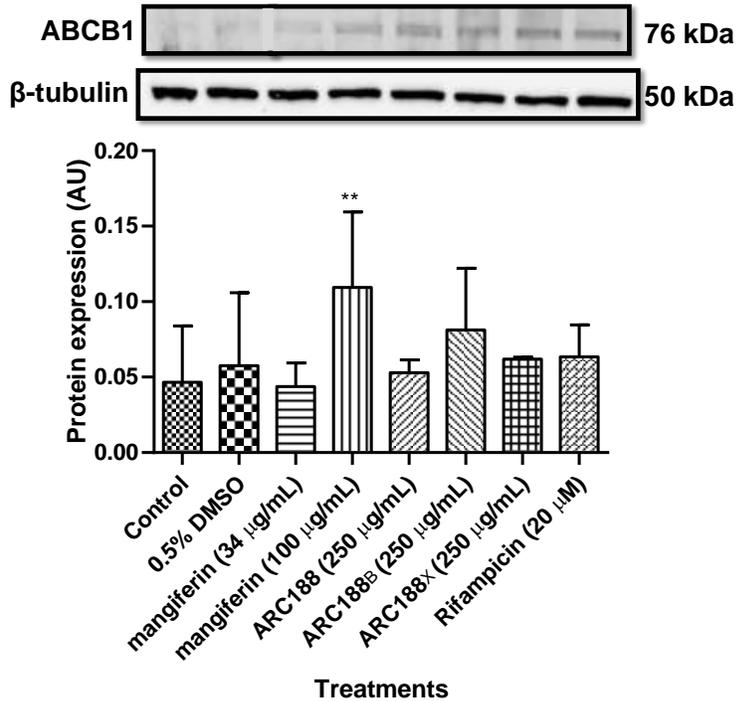


Figure 37: Protein levels of ABCB1 after 24 hours of treatment with *C. genistoides* constituents.

C3A cells were cultured for 5 days and subsequently exposed to 250 µg/mL of ARC188, 250 µg/mL of ARC188_B and ARC188_X, 34 and 100 µg/mL of mangiferin and 20 µM of rifampicin for 24. Proteins were isolated and Western blot analysis was performed, then protein levels were quantified on image lab and expressed relative to β-tubulin. Statistical analysis was performed using unpaired student T-tests with * = p < 0.05, ** = p < 0.01 and *** = p < 0.001 against the vehicle control.

For ABCG2 both mangiferin concentrations (34 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$) down-regulated protein formation to 0.09 ± 0.01 and 0.13 ± 0.01 AU, respectively, compared to the control (0.21 ± 0.09 AU) after 12 hours. However, the change was statistically insignificant ($p > 0.05$). No significant changes were observed on ABCG2 protein formation with the other test samples at 12 hours. Results for ABCG2 protein levels are shown in Figure 38.

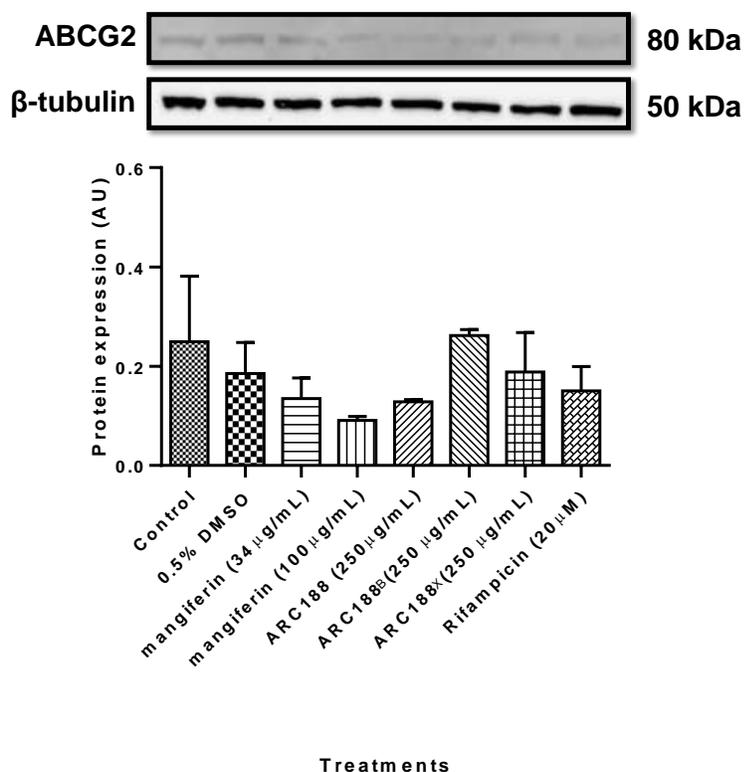


Figure 38: Protein levels of ABCG2 12 hours of treatment with *C. genistoides* constituents.

C3A cells were cultured for 5 days and subsequently exposed to 250 $\mu\text{g}/\text{mL}$ of ARC188 extract, 250 $\mu\text{g}/\text{mL}$ of ARC188_B and ARC188_X fractionations, 34 and 100 $\mu\text{g}/\text{mL}$ of mangiferin and 20 μM of rifampicin for 12 hours. Proteins were isolated and Western blot analysis was performed, then protein levels were quantified on image lab and expressed relative to β -tubulin. Results are from 3 independent experiments in duplicates ($n = 6$) and are expressed as mean \pm SEM. Statistical significance was performed using unpaired student T-tests with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ against the vehicle control.

After 24 hours there were no significant changes in the protein levels for all test samples. Mangiferin (100 $\mu\text{g}/\text{mL}$) slightly up-regulated ABCG2 protein expression (0.22 ± 0.17 AU) compared to the control 0.21 ± 0.20 AU, however the change was not significant ($p > 0.05$). Results for protein levels of ABCG2 after 24 hours are shown in Figure 39.

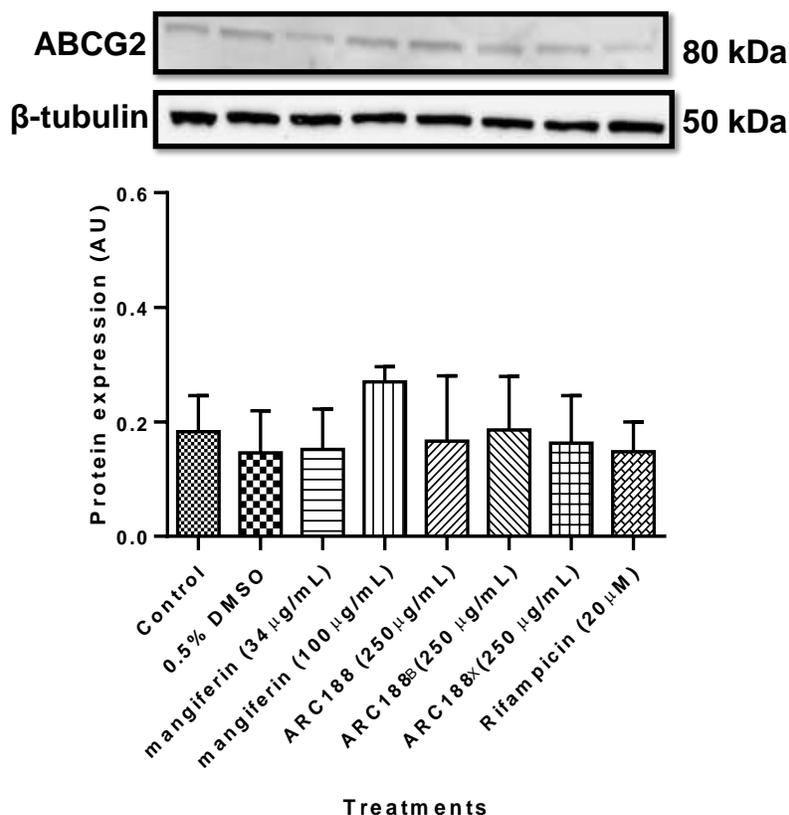


Figure 39: Protein levels of ABCG2 24 hours of treatment with *C. genistoides* constituents.

C3A cells were cultured for 5 days and subsequently exposed to 250 $\mu\text{g}/\text{mL}$ of ARC188 extract, 250 $\mu\text{g}/\text{mL}$ of ARC188_B and ARC188_X fractionations, 34 and 100 $\mu\text{g}/\text{mL}$ of mangiferin and 20 μM of rifampicin for 24 hours. Proteins were isolated and Western blot analysis was performed, then protein levels were quantified on image lab and expressed relative to β -tubulin. Results are from 3 independent experiments in duplicates ($n = 6$) and are expressed as mean \pm SEM. Statistical significance was performed using unpaired student T-tests with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ against the vehicle control.

There were no significant changes in the protein expression levels of SLCO1B3 after 12 and 24 hours treatment with *C. genistoides* constituents. The vehicle control (0.5% DMSO), mangiferin 34 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$, ARC188_x and rifampicin (the positive control) down-regulated SLCO1B3 protein expression, though the effects were insignificant ($p > 0.05$), after 12 hours and 24 hours (Figure 40 and 41, respectively).

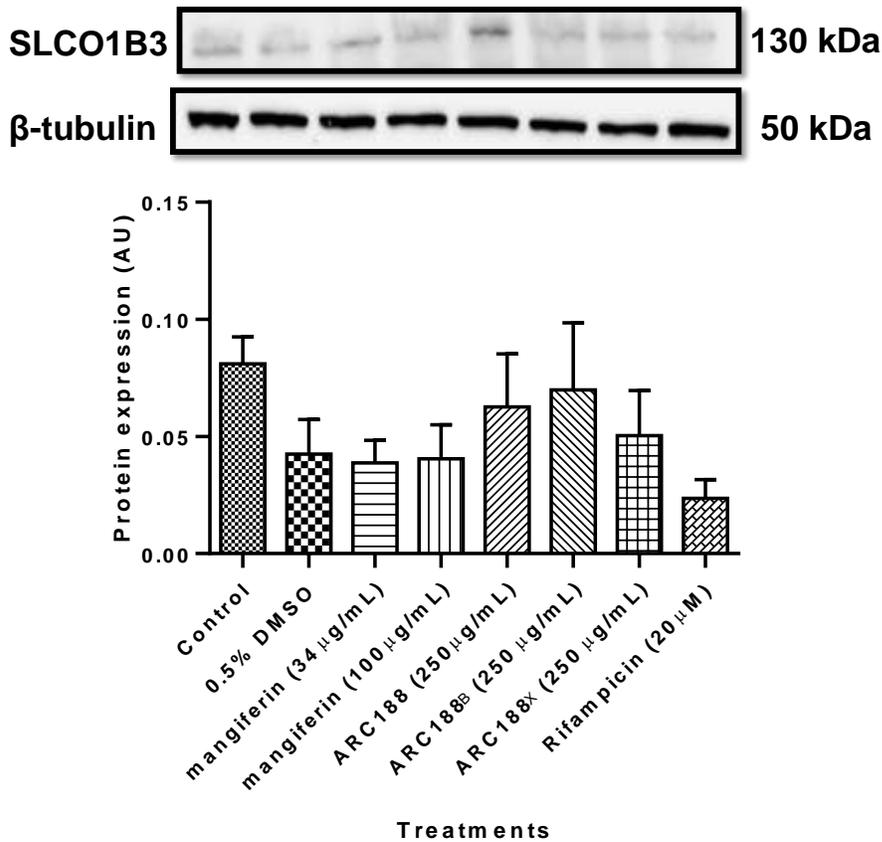


Figure 40: Protein levels of SLCO1B3 12 hours of treatment with *C. genistoides* constituents.

C3A cells were cultured for 5 days and subsequently exposed to 250 $\mu\text{g}/\text{mL}$ of ARC188 extract, 250 $\mu\text{g}/\text{mL}$ of ARC188_B and ARC188_x fractionations, 34 and 100 $\mu\text{g}/\text{mL}$ of mangiferin and 20 μM of rifampicin for 12 hours. Proteins were isolated and Western blot analysis was performed, then protein levels were quantified on image lab and expressed relative to β -tubulin. Results are from 3 independent experiments in duplicates ($n = 6$) and are expressed as mean \pm SEM. Statistical significance was performed using unpaired student T-tests with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ against the vehicle control.

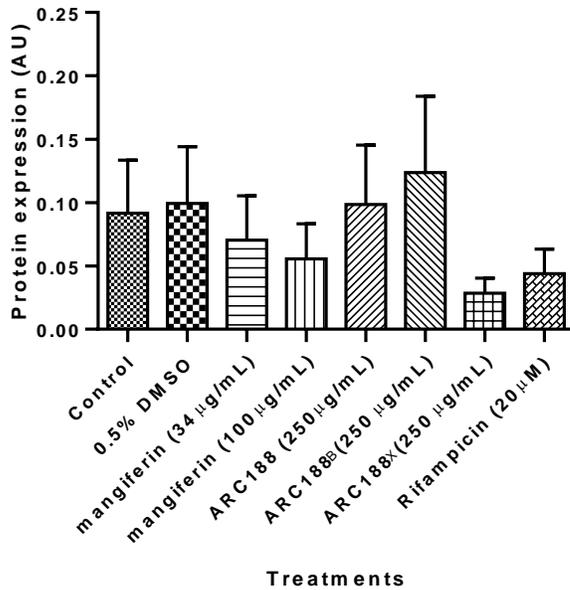
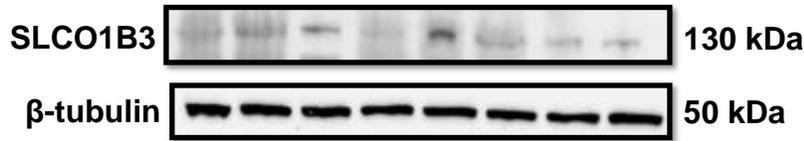


Figure 41: Protein levels of SLCO1B3 24 hours of treatment with *C. genistoides* constituents.

C3A cells were cultured for 5 days and subsequently exposed to 250 μ g/mL of ARC188 extract, 250 μ g/mL of ARC188_B and ARC188_X fractionations, 34 and 100 μ g/mL of mangiferin and 20 μ M of rifampicin for 24 hours. Proteins were isolated and Western blot analysis was performed, then protein levels were quantified on image lab and expressed relative to β -tubulin. Results are from 3 independent experiments in duplicates ($n = 6$) and are expressed as mean \pm SEM. Statistical significance was performed using unpaired student T-tests with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ against the control.

Chapter 5

5. Discussion

Herbal medicine and the use of medicinal plants, are the oldest existing form of medication where evidence suggests that herbal medicines have been used for 60 000 years (Rafieian-Kopaei and Sewell, 2014). The use of herbal medicines has been on the rise since the beginning of the 21st century (Folashade *et al.*, 2012). An attempt to live a healthy lifestyle, has contributed to this drastic increase in the use of herbal medicines (Wang *et al.*, 2015). Approximately 50% of all conventional drugs, either isolated or chemically modified for therapeutic use, originate from medicinal plants (Pan *et al.*, 2013). Herbal medicines are relatively easy to acquire in comparison to most conventional drugs. They can be purchased as over the counter (OTC) supplements at supermarkets, pharmacies and, health food stores, and can be obtained without prescription (Corns, 2003; Rafieian-Kopaei and Sewell, 2014). Well-known health promoting compounds, in herbal medicines are polyphenols, secondary metabolites of plants which have been reported to have numerous health benefits, such as anti-oxidant, anti-inflammatory, anti-microbial, anti-cancer, cholesterol lowering, cardio- and hepatoprotective properties (Ganesan and Xu, 2017; Mandal and Jaganathan, 2009; Odongo *et al.*, 2017). Herbal medicines are perceived to be safe and non-toxic, since they are derived from “natural” materials. So much so, that recommended dosages are often merely a suggestion rather than a scientific verified dose (Jordan *et al.*, 2010). Side effects and drug dependency are often associated with conventional drugs alone causing a subsequent increased interest in the use of herbal medicines as adjuncts to conventional medication (Tachjian *et al.*, 2010). However, herbal medicines also have their own risks, which often go ignored (Mahomoodally, 2013). Furthermore, supplementation of conventional medication often potentiates an increased likelihood for adverse drug reactions linked to herb-drug interactions (Calapai, 2008).

Clinically, α -glucosidase inhibitors such as acarbose and miglitol have been used in the management of T2D since 1996 and have been found to be effective in regulating postprandial glycaemic levels for diabetic patients (Proença *et al.*, 2017). However, these therapeutics have been associated with a number of unwanted side effects thus a search for alternative α -glucosidase inhibitors is on the rise (Dineshkumar *et al.*, 2010; Proença

et al., 2017). In T2D therapy α -glucosidase inhibitors are usually taken in conjunction with other antidiabetic drugs to yield a wholesome effect (DiNicolantonio *et al.*, 2015). Herbal medicines are amongst the favourite candidates in the development of effective α -glucosidase inhibitors with less side effects. As such many plant products have been shown to possess α -glucosidase inhibitory activity both *in vitro* and *in vivo*, such as *C. genistoides* (Dineshkumar *et al.*, 2010).

C. genistoides, a fynbos shrub rich in the bioactive xanthenes; mangiferin and isomangiferin as well as benzophenones; IMG and IDG, has been found to contain many health-promoting effects including glucose modulating effects due to α -glucosidase inhibition in the gastrointestinal tract (Bosman *et al.*, 2017). Alpha-glucosidase cleaves polysaccharides into simple sugars such as glucose after meals rich in carbohydrates and starch. Inhibition of this enzyme regulates post-prandial glycaemic levels (Benalla *et al.*, 2010). Current α -glucosidase inhibitors such as acarbose and miglitol reduce glycated haemoglobin (HbA1c) levels by roughly 0.5% but these drugs have been associated with unwanted side effects including diarrhoea, abdominal cramps and sometimes bloody stools (DiNicolantonio *et al.*, 2015). As such, alternative forms of α -glucosidase inhibitors such as those of herbal medicines are sought after (Imran *et al.*, 2015). Since α -glucosidase inhibitors only regulate glycaemic levels after meals, they are usually co-administered with other oral antidiabetic drugs used for effective regulation of glycemic levels in T2D patients (Toeller, 2010). Since *C. genistoides* has demonstrated α -glucosidase inhibitory activity *in vitro*, largely attributed to the xanthone and benzophenone compounds, (Beelders *et al.*, 2014; Bosman *et al.*, 2017), there is an interest in developing extracts rich in these compounds into α -glucosidase inhibiting nutraceuticals to be taken with other oral antidiabetic drugs (Raaths, 2016).

C. genistoides thus has a potential to be developed into an α -glucosidase inhibiting nutraceutical. However, the potential for *C. genistoides* to elicit adverse side-effects due to herb-drug interactions has not been fully explored. The potential concomitant use of these *C. genistoides* extracts and oral anti-diabetic drugs or any other conventional drugs poses a potential for herb-drug interaction and warrants proper screening for such interactions.

Most herb-drug interactions occur due to modulations in the activity of drug metabolizing enzymes, particularly the CYP enzymes, and drug transporters (Fugh-berman, 2000). This study investigated the inhibitory effect of *C. genistoides* extracts rich in xanthenes and benzophenones on the activity of major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) and the extracts' effects on the mRNA expression of the drug transporters (ABCB1, ABCG2 and SLCO1B3).

5.1. Cytotoxicity assays

Cell viability assays are used to assess the effect of various treatments on cell survival and/or proliferation or as a measure of cytotoxicity (Petty *et al.*, 1995). There are various assays available that use standard plate readers to measure metabolic markers to evaluate the number of viable cells in culture (Boncler *et al.*, 2014). Of these assays, each has their own set of advantages and disadvantages. MTT and ATP assays are widely used to determine cell viability (Weyermann *et al.*, 2005). The MTT assay is one of the most commonly used cell viability assays (Murain *et al.*, 2009). The MTT assay, however is a colorimetric assay and sometimes the colour of the test samples may interfere with its reactivity thus compromising its effectiveness (Wang *et al.*, 2010). Polyphenols may also interfere with MTT assays, the plant polyphenols may either induce or inhibit mitochondrial dehydrogenase activity thus resulting in false results (Karakas *et al.*, 2017). As a result, MTT assays are often validated with a different cell viability assay, usually a fluorescent and/or a luminescent assay such as the ATP assay (Ulukaya *et al.*, 2008). The ATP detection assay is a more sensitive, luminescent assay and has a lesser degree of interference of the treatment compounds, however the ATP assay is more expensive and is often used simply for validation of results yielded from an MTT assay (Petty *et al.*, 1995). ATP assays were hence selected to validate the results obtained from MTT assays.

Previous studies have shown, both *in vitro* and *in vivo*, that extracts of *Cyclopia* species have not been found to be toxic (Joubert *et al.*, 2008b). A hot water *C. intermedia* extract, was not found to affect the functioning of both the liver and kidneys when consumed by rats using this as the only fluid intake for 10 weeks (Marnewick *et al.*, 2003). Another

study also showed that no negative side effects were observed on the liver and kidneys of rats given fermented and unfermented *Cyclopia intermedia* extracts over a 10-week period (Joubert *et al.*, 2008b). Both these studies suggest that extracts from *Cyclopia* species are not toxic and may be readily consumed without any significant negative side effects. It cannot however be assumed that one sub-species of *Cyclopia* will exert the same helpful and harmful effects as the next. Although the extracts tested in our study used a unique extraction protocol, based on previous studies (de Beer and Joubert, 2010; Beelders, De Beer, Stander and Joubert, 2014; Bosman *et al.*, 2017), the extracts and enriched fractions were not expected to be toxic *in vitro*.

Although calculation on effective dose, based just on IC₅₀ values is an oversimplification of determining an effective dose, for the purpose of this study, based on the α -glucosidase activity of acarbose, the clinically approved α -glucosidase inhibitor (IC₅₀ = 41.88 μ g/mL) (Dineshkumar *et al.*, 2010), given at a dose of 50 mg three times a day, it is reasonable, based on the IC₅₀ values of ARC188 (IC₅₀ = 150 μ g/mL), ARC188_x (IC₅₀ = 59 μ g/mL) and ARC188_B (IC₅₀ = 121 μ g/mL) (Raaths, 2016) as well as the pure compounds that we extrapolate what the effective dose could be. In terms of the extract (ARC188), the relative dose would be in the order of approximately 353 mg three times a day. In comparison, the relative dose for mangiferin as a pure compound, would be in the order of approximately 86 mg three times a day.

Based on these theoretical doses, these increased levels, for effective alpha-glucosidase inhibition justified investigation into potential toxicity and likely herb-drug interactions. This is of particular importance when the extract is designed to be incorporated into a gastric-retentive system, used to enhance its inhibitory activity by lengthening its passage through the gastro-intestinal tract. As this system will retain the extract in the gastro-intestinal tract for an extended period, the potential for toxicity is increased, and thus further investigation was required regarding the safety and potential for herb-drug interactions.

For these reasons, a broad range of each test sample was screened for potential cytotoxicity in C3A liver cells. The range was initially determined based on solubility in

0.5% DMSO as a vehicle control. The highest concentrations tested for each test sample, was the highest soluble concentration in 0.5% DMSO.

Results from the MTT assay show that at the concentrations tested; ARC188 (0-250 µg/mL), ARC188_B (0 – 1000 µg/mL) and ARC_x (0 – 1000 µg/mL), there was a significant concentration-dependent decrease in cell viability. For ARC188, the two highest concentration tested (100 and 200 µg/mL) showed a significant decrease in cell viability (86.3% $p < 0.01$ and 76.26% $p < 0.01$), respectively (Figure 14 A). For ARC188_B, the two highest concentrations tested (500 and 1000 µg/mL) showed a significant decrease in cell viability (82.6% $p < 0.01$ and 77.26% $p < 0.01$), respectively (Figure 14 B). For ARC188_x, the five highest concentration tested (10, 50, 250, 500 and 1000 µg/mL) showed a significant decrease in cell viability to 65.86% ($p < 0.01$), 64.32% ($p < 0.01$) and 67.88% ($p < 0.01$), respectively (Figure 14 C).

For ARC188, the four lowest concentrations tested (0.1, 1, 10 and 50 µg/mL) showed a significant increase in cell viability, which was increased to 115.35% ($p < 0.01$), 122.51% ($p < 0.01$), 127.62%, ($p < 0.01$) and 113.09% ($p < 0.01$) respectively, representative of likely cell proliferation or mitochondrial activity (Figure 14 A.). This effect was not shown at any of the concentrations of ARC188_B or ARC188_x tested.

The ATP assay has two major benefits over the MTT assay. First is its sensitivity in terms of fluorescence over the colorimetric MTT assay, thus negating any significant interference with colour absorption, and the ATP assay also avoids interference of polyphenols with mitochondrial dehydrogenases (Ulukaya *et al.*, 2008; Maioli *et al.*, 2009). The ATP assay also negates the interference of polyphenols on enzyme activity (Karakas *et al.*, 2017). Secondly, the ATP assay enabled the use of subsequent protein determination using a Bradford protein determination assay (Riss *et al.*, 2013; Kruger, 2009). This normalizes the results obtained from the ATP assay, where ATP activity represents cellular activity, to the total protein content and takes into account any cell proliferation caused by test samples (Muelle *et al.*, 2004), but excludes cell death.

The concentrations of the test samples used in the ATP assay were 1, 10 and 100 µg/mL for all test samples (ARC188, ARC188_B and ARC188_x). These concentrations were

chosen based on non-cytotoxic concentrations determined from the MTT assay (as detailed above), but also to incorporate reported α -glucosidase inhibitory activity IC_{50} values, for each test sample (Table 22, Appendix C). For the ATP activity assay, our results show that, ARC188 showed a significant decrease in cell viability at the highest concentration tested (100 $\mu\text{g}/\text{mL}$) where there was a decrease to 80.2% ($p < 0.05$) in comparison to the vehicle control and corrected for total protein content ARC188_B (0 $\mu\text{g}/\text{mL}$ -100 $\mu\text{g}/\text{mL}$) showed no significant change in cell viability at any of the concentrations tested. While the highest concentration of ARC188_X showed a significant decrease in cell viability ($p < 0.05$), the total viability of the cells was greater than 85% ($85.6\% \pm 6.931$) at the highest concentration tested (100 $\mu\text{g}/\text{mL}$) (Figure 15).

Based on the cytotoxicity experiments and published IC_{50} concentration for alpha-glucosidase inhibition (Raaths, 2016), subsequent CYP inhibition experiments used 100 $\mu\text{g}/\text{mL}$ as a test concentration for the extract and fractions (ARC188, ARC188_B, ARC188_X).

Subsequent experiments included pure compounds, at concentrations present in each extract or respective enriched fraction i.e. mangiferin (5.6 and 16 $\mu\text{g}/\text{mL}$), isomangiferin (5 and 15 $\mu\text{g}/\text{mL}$), IDG (2 and 20 $\mu\text{g}/\text{mL}$) and IMG (5 and 25 $\mu\text{g}/\text{mL}$). The higher concentrations were representative of the concentration in the relative enriched fractionation (ARC 188_B; IDG/IMG and ARC 188_X; mangiferin and isomangiferin), and the lower concentration, representative of the concentration of that specific compound in the extract (ARC 188). This allowed insight into whether there was a concentration-based inhibition effect for specific constituents i.e. xanthone and benzophenone enriched fractions.

Although the pure compounds within the two fractions were not singularly assessed for cytotoxicity, the xanthenes have been used in previous studies at the same or similar concentrations and have not been shown to be cytotoxic (Satish *et al.*, 2009; Kaivalya *et al.*, 2011). In terms of benzophenones, which are commercial unaffordable, cytotoxicity assessment was done using the enriched fractions, and the concentrations of all the pure compounds used never exceeded those of what were present in the extracts/fractions.

By including these pure compounds, the effects of the pure compounds alone, as well as their contribution in the presence of other compounds (in the extract and enriched fractions) could be determined, which would allow for the prediction of compounds responsible for causing any effect on CYP activity and drug transporter expression.

5.2. The effect of DMSO on CYP activity

DMSO was used to solubilize the various test samples at concentrations varied to ensure absolute solubility (Table 23, Appendix C). The concentration of DMSO never exceeded 0.5%, and this concentration is known to have no significant effect on C3A cell viability. DMSO has, however been shown to interfere with CYP activity, especially at high concentrations (Table 24, Appendix C). Owing to this, a range of DMSO concentrations (0.1, 0.2, 0.5%), representative of the concentrations used to solubilize test samples, was tested for the potential effect on each CYP enzyme tested respectively. While the lower concentrations (0.1 and 0.2%) showed no significant effect on any of the CYP enzymes tested, 0.5% DMSO showed significant inhibition of CYP2C9 (Figure 16). It was therefore necessary to include the various DMSO concentrations used as vehicle controls in each CYP activity experiment and subsequently normalize the results of each test sample to the relative DMSO vehicle control.

5.3. Vivid CYP activity assays

ARC188, ARC188_B and ARC188_X, as well as pure compounds, were screened to identify the effect of each entity on CYP activity. The IC₅₀ concentration determines the degree of enzyme inhibition and shows the potency of an enzyme inhibitor (Paris *et al.*, 2009; Chou, 2010). According to (Zou *et al.*, 2002; Kong *et al.*, 2011), an extract is a strong enzyme inhibitor if the resultant IC₅₀ is less than or equal to 20 µg/mL. Moderate inhibition is shown when the IC₅₀ value is more than 20 µg/mL, but less than 100 µg/mL and weak inhibition is exhibited by an IC₅₀ that is greater than 100 µg/mL (Kong *et al.*, 2011).

5.4. Inhibitory effect of *C. genistoides* on the activity of CYP1A2

CYP1A2 plays a role in the metabolism of caffeine and other thiazolino agents (Thelingwani *et al.*, 2009). Previously, interactions involving CYP1A2 were not of clinical

importance as inhibition of this enzyme was usually associated with increased caffeine in the body, which had no adverse health implications (Cornelis *et al.*, 2006). However, over the past decade, the number of identified CYP1A2 substrates has increased drastically which can be attributed to the increased probability of CYP1A2 related drug interference (Kim *et al.*, 2010). The increased knowledge of substrate specifically associated with the inhibition of this enzyme, may have some adverse reactions such as toxicity due to increased drug exposure or decreased therapeutic efficiency (Tracy *et al.*, 2005). For example, it has recently been shown that inhibition of CYP1A2 causes a decline in the absorption of glucose from the small intestines, facilitated by pioglitazone, and rosiglitazone, resulting in uncontrolled hypoglycaemia (reviewed by Purohit and Mishra, 2017).

In this study, 30 min time-dependent screenings of CYP1A2 showed that ARC188 and ARC188_x as well as mangiferin at a high concentration (16 µg/mL), displayed inhibitory effects greater than 50% on the activity of this CYP. ARC188 showed 50.27% ($p < 0.01$) inhibition and mangiferin at a high concentration exerted the highest inhibition against CYP1A2 activity with 69.75% ($p < 0.01$) inhibition. Interestingly, isomangiferin (regio-isomer of mangiferin, only differing in the position of the sugar moiety C2-C5) and ARC188_B exerted low inhibition against the activity of this CYP enzyme with 29.94% ($p < 0.05$) and 32% ($p < 0.02$) inhibition, respectively. While IMG and IDG exerted negligible inhibition against the activity of this CYP with the average inhibitory potentials of 8.2% and 4.58%, respectively (Figure 17 and Figure 18). ARC188, ARC188_x and mangiferin at a high concentration (16 µg/mL) displayed potential inhibition of CYP1A2 and the degree of these effects was further quantified in concentration dependent screenings. It is noteworthy that the inhibitory effects of *C. genistoides* constituents were much lower than the effect of the positive control α -naphthoflavone, which diminished the activity of CYP1A2 with 98.56% ($p < 0.01$) inhibition (Figure 17 and Figure 18).

Upon further investigation, in concentration dependent screenings, it was indicated that ARC188, and mangiferin exhibited moderate inhibition of the CYP enzyme as indicated by the IC₅₀ values obtained (98,61 µg/mL \pm 2.54 and 35,06 µg/mL \pm 3.23, respectively)

while ARC188_x showed weak inhibition with the IC₅₀ value of 102,00 µg/mL ± 3.12 obtained (Figure 27).

Based on these findings and taking into consideration the type of inhibition which has not been fully elucidated in this study, overweight and diabetic patients taking pioglitazone and statins may have to be cautious when consuming these compounds as co-administration of the xanthone enriched extracts may lead to uncontrolled hypoglycaemia since CYP1A2 inhibition may lead to a decline in the clearance of co-administered drugs (Langhammer and Nilsen, 2013; Mallhi *et al.*, 2015)

5.5. Inhibitory effect of *C. genistoides* on the activity of CYP2C9

Members of the CYP2C family are responsible for the metabolism of about 12% of all therapeutic drugs including most antidiabetic drugs (Johnson *et al.*, 2011). CYP2C9 has been associated with the metabolism of thiazolidinediones such as glibenclamide, glimepiride and glipizide, and many other non-steroidal anti-inflammatory drugs (Lynch *et al.*, 2007). Inhibition of this enzyme, through potential herb-drug interactions, may lead to hypoglycaemia due to reduced clearance of oral antidiabetic drugs and may cause many other adverse effects including haemorrhaging, especially when using the anti-coagulant warfarin. Clearance of glibenclamide was greatly reduced due to CYP2C9 inhibition in diabetic rats (Li *et al.*, 2012).

C. genistoides constituents were also investigated for their potential to inhibit CYP2C9 using Vivid CYP2C9 blue screening kits as mentioned previously. Upon analysis, it was determined that mangiferin (16 µg/mL) and ARC188 exhibited major inhibition against the activity of this enzyme, with inhibition of 67.22% ($p < 0.01$) and 72.47% ($p < 0.01$), respectively. ARC188_x and ARC188_B also showed enzyme inhibition of 34.60% ($p < 0.05$) and 25.02% ($p < 0.05$), respectively. However, their inhibitory potential did not exceed 50%, and thus an IC₅₀ determination was not warranted. IMG, IDG and isomangiferin had no significant effects on the activity of this CYP isozyme in comparison to the vehicle control. Sulfaphenazole, the positive control showed major CYP inhibition (95.18%, $p < 0.01$) (Figure 19 and Figure 20). Further concentration dependent screening of mangiferin

and ARC188 showed that both compounds were moderate inhibitors of the enzyme with IC_{50} values of $57,86 \mu\text{g/mL} \pm 5.50$ and $90,09 \mu\text{g/mL} \pm 4.66$, respectively (Figure 28).

Again, taking into consideration the type of inhibition which has not been fully elucidated in this study, patients taking sulphonylureas, meglitinides and thiazolidinediones, may have to be cautious as co-administration of the xanthone enriched extracts, particularly those rich in mangiferin, may lead to impaired substrate metabolism and decreased efficacy of the antidiabetic drugs.

5.6. Inhibitory effect of *C. genistoides* on the activity of CYP2C19

CYP2C19 is involved in the metabolism of various drugs including medications for HIV therapy, anti-cancer, protein pump inhibitors, anti-depressants, beta-adrenoceptor blockers and anti-epileptic drugs (Flockhart and Desta, 2009). A great degree of polymorphism is reported for CYP2C19 which causes inter-individual variations in the activity of this enzyme (Desta *et al.*, 2002). As a result, the degree of drug interactions involving CYP2C19 commonly differs for different individuals (Lynch and Price, 2015). One person may experience adverse side effects due to a particular drug interaction involving CYP2C19 and the other person may not, even though they may be taking the same medications (Ingelman-sundberg *et al.*, 2007).

ARC188 showed an inhibitory effect of 70.68% enzyme inhibition in comparison to the vehicle control. Mangiferin at $16 \mu\text{g/mL}$ and ARC188x had similar effects against the activity of this CYP enzyme with 34.47% and 34.13% inhibition, respectively. The positive control, miconazole, showed an inhibition of 99.14% (Figure 21 and Figure 22). Benzophenone constituents had no effect on the activity of CYP2C19. During concentration dependent screening, the inhibitory effect of ARC188 on CYP2C19 was moderate with an IC_{50} of $95,20 \mu\text{g/mL} \pm 3.01$ (Figure 29).

Besides the xanthenes and benzophenones, the crude *C. genistoides* extract contains other polyphenols and phenolic compounds such as flavanols which may contribute to the inhibitory effect of ARC188 on CYP2C19 activity. The flavanol hesperidin, found in citrus fruit such as grapefruit juice and also isolated in *Cyclopia* species has been proven

to inhibit CYP2C19 in other studies (Marchetti *et al.*, 2007; Ujita *et al.*, 2008). This polyphenol might be attributed to ARC188 activity against CYP2C19.

5.7. Inhibitory effect of *C. genistoides* on the activity of CYP2D6

CYP2D6 is the second most versatile CYP enzyme, after CYP3A4, and is responsible for the metabolism of approximately 20% of all therapeutic drugs (Ahmed *et al.*, 2016). The enzyme only accounts for 2 – 9% of hepatic CYPs and is involved in the synthesis of neurotransmitters and the activation of pro-drugs (Lynch *et al.*, 2007). Pro-drugs are inactive drugs that become active compounds upon metabolism. The active compounds exert the therapeutic effects of the drug while pro-drugs remain inactive (Rautio *et al.*, 2008). An example of such activation is the conversion of codeine to morphine through CYP2D6 mediated metabolism (Crews *et al.*, 2012).

In the current study, ARC188, ARC188_x and the high mangiferin concentration (16 µg/mL), exhibited inhibitory effects of 50.45% ($p < 0.01$), 56.56% ($p < 0.01$) and 75.12% ($p < 0.01$), respectively. ARC188_B and IMG had no inhibitory effects against the activity of CYP2D6 while high concentrations of isomangiferin (15 µg/ml) and IDG (25 µg/ml) exhibited slight inhibition of 25.29% and 37.91% ($p < 0.05$), respectively. The positive control, quinidine, showed an inhibition of 91.29% ($p < 0.01$) (Figure 23 and Figure 24).

Subsequent CDI screening showed that ARC188 and ARC188_x induced moderate inhibition with IC₅₀ values of 57.83 µg/mL ± 2.07 and 42,62 µg/mL ± 3.789, respectively. Mangiferin exhibited strong inhibition of the enzyme with an IC₅₀ value of 7,97 µg/mL ± 3.03 (Figure 30).

Inhibition of CYP2D6 may affect the pharmacokinetics of sulfonylureas and other antidiabetic drugs, delaying their metabolism which may lead to hypoglycemia (Purohit and Mishra, 2017). Mangiferin has been shown to be a strong inhibitor of CYP2D6 and CYP3A4 in previous studies (Gomez-Lechon *et al.*, 2008). Potential interactions may occur when consuming ARC188, ARC188_x or mangiferin with conventional drugs primarily metabolised by CYP2D6. A study showed that inhibition of CYP2D6 by *Aloe vera* juice co-administered with glibenclamide significantly reduced blood glucose levels

in rats (reviewd by Purohit and Mishra, 2017). The authors suggested that inhibition of this enzyme increased the efficacy of the drug, although the effect of *Aloe vera* on glycaemia should also be considered (Yimam *et al.*, 2014). This effect could be true for *C. genistoides* test samples as well.

5.8. Inhibitory effect of *C. genistoides* on the activity of CYP3A4

CYP3A4 is the most versatile CYP isoform and is thus susceptible to major modulations. Studies have shown that inhibition of this enzyme may lead to many adverse reactions, and the most aberrant of which is liver toxicity (Miltra and Pal, 2006). Many herbal products have been associated with herb-drug interactions involving CYP3A4, including St. John's wort, which has been shown to induce the activity of CYP3A4 *in vitro* (Markowitz *et al.*, 2003). A study showed that inhibition of CYP3A4 by *Andrographis paniculata* increased the efficacy of pioglitazone and led to an increased glucose transport through GLUT4 (Rani *et al.*, 2012). Inhibition of this enzyme by *Aloe vera* has also been shown to increase the efficacy of pioglitazone (Yimam *et al.*, 2014).

In this study, CYP3A4 inhibition was the most prominent of all the isozymes tested. ARC188, ARC188_x, ARC188_B and mangiferin (16 µg/mL) all exhibited inhibition against the activity of this CYP isozyme greater than 50%. As such, these entities show the potential to severely inhibit the activity of CYP3A4 with 78.49%, 50.66%, 53.28% and 83.10% inhibition, respectively. IMG and IDG had no effect on the activity of this CYP isozyme and once again isomangiferin at a high concentration (15 µg/mL) showed low inhibitory activity at 12.38%. The positive control, ketoconazole inhibited CYP3A4 activity by 99.08% (Figure 25 and Figure 26). Further assessment of the CDI effect of ARC188, ARC188_B, ARC188_x and mangiferin, showed that mangiferin ($IC_{50} = 23,12 \mu\text{g/mL} \pm 8.262$) and ARC188 ($IC_{50} = 57.91 \mu\text{g/mL} \pm 5.7$) exhibited moderate CYP3A4 inhibition, while ARC188_B and ARC188_x showed weak CYP3A4 inhibition ($IC_{50} = 213.4 \mu\text{g/mL} \pm 2.944$ and $121.40 \mu\text{g/mL} \pm 4.306$, respectively) (Figure 31).

5.9. Summary of CYP inhibition

In summary, it was evident that the benzophenones as pure compounds (both IMG and IDG) had no inhibitory effect on any of the CYP isozymes tested. Accordingly, ARC188_B

also showed no inhibitory activity towards any of the CYP isozymes tested, barring a weak inhibition towards CYP3A4.

Mangiferin, known for CYP inhibition, showed strong inhibitory effects towards CYP2D6 as well as CYP3A4, which has been shown in previous publications (Matkowski *et al.*, 2013; Rodeiro *et al.*, 2013a). It was also shown to be a moderate inhibitor of CYP1A2 as well as to CYP2C9, however no significant inhibition of CYP2C19 was found.

ARC188_x, consisting of approximately 54% mangiferin also showed inhibition of most of the CYP isozymes tested. There was no significant inhibition on CYP2C9 or CYP 2C19. However, it showed moderate inhibition of CYP2D6 and weak inhibition towards CYP1A2 and CYP3A4.

ARC188 showed moderate inhibition of all the CYP isozymes tested. The percentage of mangiferin in ARC188 extract is known to be approximately 14%, which suggests that mangiferin content is the major contributor to the activity observed for ARC188 and ARC188_x.

The presence of maclurin-3-*C*-glucoside, in ARC188 (1% ca) as well as in ARC188_B (11% ca) cannot be ignored, especially since ARC188_B was shown to be a weak CYP3A4 inhibitor. The other major constituents (IDG and IMG) of ARC188_B were not likely to be responsible for this inhibition as the pure compounds alone did not shown any significant inhibition on any of the CYP isozymes.

Most interestingly, isomangiferin, a raeo-isomer of mangiferin, differing only in the positioning of the sugar moiety, showed no effect on any of the CYP isozymes, whilst mangiferin, its isomeric counterpart proved to be a strong CYP inhibitor. Polyphenols that are raeoisomers have been found to show contradicting activities previously. The flavones isovitexin and vitexin, raeo-isomers differing only on the position of the sugar moiety (sugar moiety located on C6 and C4 of A-benzyl ring, respectively) inhibited α -glucosidase differently, with isovitexin remaining inactive in the inhibition of the enzyme (Shibano *et al.*, 2008). This shows that the position of the sugar moieties of phenolic compounds, may have an impact on CYP enzyme activity without affecting the efficacy

of the α -glucosidase activity. This highlights the usefulness of considering regio-isomers that retain effectiveness but avoid the potential for herb-drug interactions i.e mangiferin and isomangiferin.

5.10. Effect of DMSO on mRNA and protein expression of drug transporters

DMSO has been shown to interfere with the expression of some genes when used at high concentrations because of its complex and toxic nature (Sumida *et al.*, 2011). In this study, there were no significant changes in mRNA and protein expression of drug transporters with 0.5% DMSO. Thus 0.5% DMSO could be used as a vehicle control as it did not interfere with mRNA and protein expression of the drug transporters after 12 and 24 hours. After 48 hours, 0.5% DMSO up-regulated ABCG2 mRNA expression

5.11. Effect of *C. genistoides* on the mRNA and protein expression of drug transporters

The drug transporters, ABCB1, ABCG2 and SLCO1B3, are of clinical importance as the number of drug interactions involving them have increased over the last few years (Brouwer *et al.*, 2015). Regulation of the expression of drug transporters is governed by both environmental and genetic factors (Gerk and Vore, 2002). A number of drugs and plant products have been implicated in the induction and inhibition of these proteins (Giacomini *et al.*, 2010). Synthesis of drug transporters follows the same patterns of other proteins and occurs in two major steps. First a single strand of mRNA coding a particular sequence is transcribed from a DNA molecule (Selbach *et al.*, 2008), then following mRNA transcription, the mRNA is then translated into protein. Nucleic receptors are involved in the induction of drug transporter expression, and the efflux transporters ABCB1 and ABCG2 are induced through the activation of the PXR (Williamson, Kelly E. Dooley, *et al.*, 2013).

Drug transporters play a significant role in drug pharmacokinetics as they facilitate drug absorption, disposition and elimination (Chen *et al.*, 2016). These proteins are localised in all major tissues and are found in ample amounts on the apical and basolateral membranes of gut enterocytes, as well as the basolateral and canalicular membranes of

hepatocytes (Fromm and Kim, 2011). They act as barriers of many other tissues such as the brain, testes, kidneys and placenta (Mallhi *et al.*, 2015). Modulations in the activity, and expression of drug transporters affects the plasma concentrations of the drug, and hence the drug's efficacy (Konig *et al.*, 2013). Over expression of the efflux drug transporters (ABC transporters; ABCB1 and ABCG2) leads to decreased absorption and bioavailability of the drug while causing rapid clearance of the drug, decreasing the efficacy of the said drug (Zhou *et al.*, 2007). Over expression of uptake drug transporters (SLC transporters, SLCO1B3) has an opposite effect and promotes rapid absorption of the drug leading to increased drug bioavailability (Liang *et al.*, 2015). Evidence suggests that drug transporters such as SLCO1B1 are poorly expressed in HepG2 cells (Louisa *et al.*, 2016).

After investigation of possible CYP inhibition by *C. genistoides* constituents, the effect of *C. genistoides* on the mRNA and protein expression of the drug transporters in C3A liver cells was evaluated using quantitative RT-PCR and western blotting. mRNA expression of the drug transporters was expressed relative to the negative control at the relevant time point. Based on the cell viability assays, where the maximum soluble concentration of 250 µg/mL was shown not to be toxic, the mRNA and protein expression experiments were performed. ARC188, ARC188_B and ARC188_X were tested at 250 µg/mL, and mangiferin was tested at the relative concentrations present in the extract and fraction, respectively (34 µg/mL and 100 µg/mL).

5.12. Effect of *C. genistoides* on ABCB1 mRNA expression

The membrane-associated protein encoded by the gene ABCB1 is a member of the superfamily of ATP-binding cassette (ABC) transporters, responsible for transport of various molecules across extra- and intra-cellular membranes (Leschziner *et al.*, 2007). This protein is a member of the MDR/TAP subfamily, members of which are involved in multidrug resistance of various cancer cells (Schinkel and Jonker, 2012). The protein encoded by this gene is an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity and functions as a transporter in the blood-brain barrier (Albermann *et al.*, 2005). The ABCB1 drug transporter actively excretes drugs out of the cells. As such, an increased expression would yield an increased clearance of the drug,

causing a decline in drug efficacy (Vaidyanathan *et al.*, 2016). Many chemical substances such as conventional drugs, hormones and polyphenols are known to influence the expression and activity of ABCB1 (Vilaboa *et al.*, 2000).

In this study, the relative expression of ABCB1 showed no significant difference in the expression of the vehicle control and the negative control (media only) at any of the time points tested. It was observed that all *C. genistoides* constituents significantly up-regulated the expression of ABCB1 in C3A liver cells at both 12 hours as well as 24 hours. After 12 hours, the expression of ABCB1 was especially high in ARC188 in comparison to the negative control (9.09 ± 0.31 vs 1.29 ± 0.42 relative expression). At 24 hours, there was still a significant increase in ABCB1 expression, but less than that observed at 12 hours (6.12 ± 0.01 vs 1.01 ± 0.14 relative expression). While both mangiferin concentrations, 34 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$, also showed a large increase in ABCB1 expression (5.35 ± 0.26 vs 1.29 ± 0.42 relative expression) and (7.09 ± 0.70 vs 1.29 ± 0.42 relative expression) after 12 hours, respectively. The expression of ABCB1 was also shown to be diminished after 24 hours. The enriched fractions ARC188_B and ARC188_X had a smaller effect on ABCB1 expression (3.50 ± 0.23 vs 1.29 ± 0.42 relative expression) and (3.30 ± 0.01 vs 1.29 ± 0.42 relative expression) after 12 hours respectively. A similar effect was observed after 24 hours with 2.92 ± 1.50 vs 1.01 ± 0.14 relative expression and 4.14 ± 0.17 relative expression for ARC188_B and ARC188_X, respectively, but there was also a significantly large increase in upregulation at both time points ($p < 0.001$). Rifampicin was used as a positive control, which showed a large increase in ABCB1 gene expression in comparison to the vehicle control (3.96 ± 0.37 vs 1.29 ± 0.42 relative expression) for 12 hours and the effect continued (5.19 ± 0.51 vs 1.01 ± 0.14 relative expression) for 24 hours. No significant changes in the expression of ABCB1 mRNA were observed at 48 hours and this may have been due to the degradation of the extracts after incubation for such a long time as most polyphenolic compounds are not stable for long periods of time in cells (Fang and Bhandari, 2010). Rifampicin, a known inducer of ABCB1 expression (Burger *et al.*, 2005; Williamson, Kelly E. Dooley, *et al.*, 2013) is said to function for 24 hours at the most, in healthy patients without renal failure and the average half-life of the drug is 2-3 hours (Büdingen *et al.*, 2014).

Mangiferin has been previously found to modulate the expression of ABCB1 *in vitro*. A study showed that 200 μM (84 $\mu\text{g}/\text{mL}$ ca) of mangiferin significantly up-regulated ABCB1 expression in HK-2 cells after 24 hours, but drastically declined after 72 hours of incubation (Chieli *et al.*, 2010). This indicates that mangiferin regulated ABCB1 mRNA expression in a time and concentration-dependent manner. Interestingly, mangiferin was found to inhibit the activity of ABCB1 in breast cancer cells (MCF-7 cells) thus increasing sensitivity of such cells to anti-cancer drugs (Louisa *et al.*, 2014).

5.13. Effect of *C. genistoides* on mRNA expression of ABCG2

ABCG2 is an efflux protein and drug transporter that is responsible for the efflux of drugs out of the liver and/or other excretory organs (Zhou *et al.*, 2001). The rapid excretion of drugs by this transporter results in increased drug clearance and decreased drug efficacy (Woodward *et al.*, 2009).

With regards to the relative expression of ABCG2, there was no significant difference in the expression of the vehicle control and the negative control (media only) over any of the times points tested.

It was observed that all *C. genistoides* constituents significantly up-regulated the expression of ABCG2 in C3A liver cells at 12 hours barring ARC188_B, which showed no significant change in expression at any of the incubation times. After 12 hours, the expression of ABCG2 was especially high in ARC188 in comparison to the negative control (7.41 ± 0.56 vs 0.96 ± 0.18 relative expression). Expression of ABCG2 decreased after treatment with ARC188 after 24 hours (2.85 ± 0.52 vs 1.01 ± 0.14 relative expression), though ABCG2 was still up-regulated compared to the control. ARC188_X also showed a large increase in expression (4.38 ± 0.15 vs 0.96 ± 0.18 relative expression) and (6.25 ± 2.87 vs 1.01 ± 0.14 relative expression) after 12 and 24 hours respectively. Both mangiferin (34 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$) concentrations also showed a large increase in ABCG2 expression (3.23 ± 0.53 vs 0.96 ± 0.18 relative expression) and (3.88 ± 0.70 vs 0.96 ± 0.18 relative expression) after 12 hours respectively, which was decreased in the higher mangiferin concentration (100 $\mu\text{g}/\text{mL}$) after 24 hours (2.64 ± 0.91 vs 1.01 ± 0.14 relative expression) in comparison to the vehicle control, but still largely

significant ($p < 0.002$). At the lower concentration of mangiferin (34 $\mu\text{g/mL}$), after 24 and 48 hours there was no noticeable change in expression. Rifampicin was used as a positive control and has been found to induce the expression of ABCG2 through the PXR receptor (Albermann *et al.*, 2005). In this study, we showed a large increase in ABCG2 gene expression in comparison to the vehicle control (4.81 ± 0.68 vs 0.96 ± 0.18 relative expression) for 12 hours. ABCG2 mRNA expression remained steady after treatment with rifampicin for 24 hours. As with ABCB1, no significant changes in the expression of ABCG2 expression were observed at 48 hours, which again may have been due to the degradation of the extracts after incubation for such a long time.

Similar to ABCB1, mangiferin is known to down-regulate ABCG2 expression in cancer cells contributing to the anti-cancer effects of chemotherapies (Louisam *et al.*, 2014), and increase expression of this drug transporter in non-cancer cells (Abbott, 2003). Up-regulation of ABCG2 by ARC188, ARC188_x and mangiferin may cause herb-drug interactions when co-administered with conventional medication thus caution may be required. ABCG2 transports a number of drugs including glyburide, rosuvastatin and nitrofurantoin (Abbott, 2003). Over expression of ABCG2 may lead to clearance of such drugs rendering them ineffective.

5.14. Effect of *C. genistoides* on mRNA expression of SLCO1B3

SLCO1B3 is located in the basolateral membrane of the liver and moves drugs and other exogenous compounds into the liver cells down a concentration gradient (Karlgrén *et al.*, 2012). The drug transporter plays an important role in drug absorption (Shitara, 2011). Induction of this drug transporter increases hepatic absorption of the drug and may lead to toxicity when drug hepatic concentrations hike above the safe therapeutic range (Williamson *et al.*, 2013). Inhibition of SLCO1B3 causes a decline in the hepatic uptake of the drug, leading to increased drug plasma concentrations, which can alter the effective (Shitara, 2011).

With regards to the relative expression of SLCO1B3, ARC188 and mangiferin significantly down regulated gene expression after 12, 24 and 48 hours treatment compared to the negative control. No significant changes were observed in SLCO1B3 expression after

treatment with ARC188_B and ARC188_X at all time-points. Results of the effects of *C. genistoides* constituents on the expression of SLCO1B3 are shown in Figure 35.

SLCO1B3 expression is highly susceptible to disruptions because of the highly polymorphic nature of this gene (Nozawa *et al.*, 2005). Mangiferin has been found to down-regulate SLCO1B3 expression in primary human hepatocytes (Rodeiro *et al.*, 2013). Flavonoids are known inhibitors of SLCO1B3 expression and inhibition and the flavanol hesperidin, which is also present in ARC188, is a known SLCO1B3 inhibitor (Dolton *et al.*, 2012). Presence of hesperidin in ARC188 may have been a contributing factor to SLCO1B3 down-regulation by ARC188.

5.15. Effect of *C. genistoides* on the synthesis of ABCB1

Western blot analysis of protein expression of prominent drug transporters (ABCB1, ABCG2 and SCLO1B3) was attempted in C3A liver cells using the available antibodies. Results for ABCG2 and SCLO1B3 were inconclusive. The quality of the antibodies could account for the lack of significant findings involving these transporters. These Western blots had a high background due very high concentrations of the antibodies needed to detect the proteins as they were lowly expressed in C3A liver cells. It has been shown that using high concentrations of the antibody may cause the formation of a high background (Taylor and Posch, 2014). However, ABCB1 protein expression results were acceptable. Eighty percent of drugs are transported by ABCB1. As such, investigation of the effect of *C. genistoides* constituents on the protein expression of this drug transporter provides relevant information related to potential herb-drug interactions.

ARC188_B, ARC188_X and rifampicin (positive control) significantly up-regulated ABCB1 expression relative to the vehicle control (0.5% DMSO) after 12 hours of treatment (0.67 ± 0.15 , 0.70 ± 0.09 and 0.61 ± 0.08). Mangiferin (100 µg/mL) also showed an increase in expression of ABCB1 after 12 hours, but this was not shown to be significant. After 24 hours, mangiferin (100 µg/mL) significantly up-regulated ABCB1 expression (0.11 ± 0.05 relative expression).

C. subternata extracts have been shown to up-regulate ABCB1 expression in liver microsomes in a previous study (Abrahams, 2011). Mangiferin was also found to up-regulate the expression of ABCB1 in hepatocytes (Louisa *et al.*, 2014).

5.16. Summary of the effect of *C. genistoides* on the expression of major drug transporters

In summary, gene expression of ABCB1 was significantly increased by all test samples over both 12 and 24 hours, these results were supported by protein expression at 12 hours. Gene expression of ABCG2 was increased by ARC188, ARC188_x and the highest dose of mangiferin at both 12 and 24 hours, and also by the lower mangiferin concentrations at 12 hours. Gene expression of SLCO1B3 was significantly decreased by the DMSO vehicle control, confounding the effects of the other test samples on this transporter, and therefore making it difficult to make assumptions on the potential added effects of the *C. genistoides* constituents.

Chapter 6

6. Conclusion

In this study, the *C. genistoides* constituents were investigated for potential herb-drug interactions by assessing their effect on relevant CYP enzymes and drug transporter expression at a gene and protein level. The *C. genistoides* extract (ARC188), was shown to be a moderate inhibitor of all the major CYP enzymes tested (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4). Of the two fractions, the benzophenone enriched fraction ARC188_B, as well as its two major benzophenones IMG and IDG, did not induce significant inhibition on CYP activity. The xanthone enriched fractions ARC188_x demonstrated inhibition for CYP1A2, CYP2D6 and CYP3A4 and these activities could be attributed to mangiferin, the major xanthone component. Interestingly, isomangiferin, the regioisomer of mangiferin showed no inhibitory effects against any of the CYP enzymes tested.

To make a conclusive assumption on the effect of *C. genistoides* constituents and their involvement in herb-drug interaction, one cannot use only the CYP enzyme activity, but one also needs to consider the effect these constitutes may have on drug transporters. In terms of ABCB1, the most prominent drug transporter, there was increased expression at a gene level, by all the test entities at 12 and 24 hours. This was confirmed in protein expression at 12 hours. This suggest the potential for increased potential drug efflux by enterocytes into the gut as well as efflux by the liver into the bile enteric secretion. In terms of ABCG2 gene expression, ARC188_B had no effect, however ARC188, ARC188_x and mangiferin all upregulated the expression of this drug transporter, however this could not be confirmed at a protein level. Furthermore, mangiferin significantly down-regulated SLCO1B3 gene expression, although this could not be confirmed at a protein level. This suggests a potential for decreased drug absorption by the liver and enterocytes.

Taking all the information into consideration, ARC188_x and mangiferin are the major contributors of potential herb-drug interactions based on their inhibitory effect on the prominent CYP enzymes as well as their effect on ABCB1, ABCG2 and SLCO1B3, whilst ARC188_B can be considered as unlikely to cause herb-drug interactions.

In summary, the major potential for herb-drug interaction can be attributed to mangiferin, which has been previously shown. According to our knowledge, what is novel to this study, is the lack of interaction by isomangiferin. This shows, for mangiferin, stereochemistry plays a large role in the observed potential herb-drug interaction.

6.1. Limitations and future work

The present study used a crude extract (ARC188), two fractions rich in xanthenes and benzophenones, respectively (ARC188_B and ARC188_X). All of these test entities were extracted with methanol. While methanol is used industrially as a solvent, pesticide, and alternative fuel source, it is a biohazardous alcohol and ingestion may lead to methanol poisoning. As such it is generally not accepted as a food grade extraction alcohol. It is therefore recommended that future studies make use of other solvents to prepare *C. genistoides* extracts and fractions, such as ethanol, which is known to be safer for human consumption, especially as their development would ultimately lead to the consumption of *C. genistoides* as a nutraceutical. Complications may arise, as the use of different solvents may produce test entities showing different results to those of this study, as the phenolic composition of the extract may change. However, Joubert *et al.* (2014) has shown that even hot water extracts of *C. genistoides* maintain a high xanthone and benzophenone content.

In the current study, IC₅₀ values could not be generated for all test entities as proposed, since the IMG, IDG and isomangiferin showed no significant inhibitory effects on CYP activity at the concentrations tested. Although, taking into account their relatively low concentrations in both ARC188 and enriched fractions (ARC188_X and ARC188_B) and their solubility in DMSO, we decided not to perform CDI experiments for these compounds. However, it may be beneficial to consider other solvents to increase the soluble concentrations, in order to enable IC₅₀ calculation across all test entities.

In terms of the gene and protein expression experiments, the data generated was inconsistent, and therefore only ABCB1 expression was acceptable to be considered for potential herb-drug interactions of the *C. genistoides* constituents. Although difficult to pinpoint the exact cause of irreproducibility, technical aspects such as mRNA and protein

extraction, as well as the available antibodies could be likely causes. From a quality control point of view, some of the Western blots could not be used for quantification, due to promiscuity of the antibody. It would be beneficial to trouble shoot and optimize these shortcomings and repeat these experiments.

Further, the experimental outcome of the mRNA expression levels in this study (12 – 48 hours) was not truly a representation of a time-dependent effect of the *C. genistoides* constituents on the mRNA expression of drug transporters as baseline expression levels were not taken into account. The concentrations used to assess the effect of the *C. genistoides* constituents on the mRNA and protein expression of drug transporters were quite high. Xanthenes and benzophenones have an extremely low bioavailability, so it is highly unlikely that they will be present in the liver at the concentrations tested in this study to invoke such an effect. For future purposes, baseline mRNA and protein expression evaluation should be performed in order to incorporate the time-dependent effect of test samples on mRNA and protein expression. It would also be beneficial to use much lower concentrations of test samples to accommodate the low bioavailability of phenolic compounds.

In order to further explore the type of inhibition involved in respective CYP450 enzymes by each test entity, it would be beneficial to perform a time-dependent inhibition (TDI) study and normalize the expression levels to those at baseline. Using other models such as S9 liver fractions and human liver microsomes would help elucidate the potential for herb-drug interactions. These studies would add value in determining the type of inhibition involved in the interactions. Whether the inhibition is reversible/non-reversible, for example, would determine greatly the degree of adversity perpetuated by the herb-drug interactions.

Based on the evidence this study has revealed, a low expression of drug transporters in C3A liver cells has been confirmed. The results obtained for the mRNA expression of SLCO1B1 were inconclusive. Only the neat and highly concentrated standards (S1 and S2) were amplified by qRT-PCR. Thus, a standard curve could not be constructed for these results making it difficult to determine if the amplification was accurate or correct.

Using human THLE-2 liver cells in place of C3A liver cells for a better profile of mRNA and protein expression of drug transporters and efflux proteins may be beneficial during future studies, as they have functional cytochrome P450 pathways. These immortalized human liver cells constitute an *in vitro* model for toxicological studies (Pfeifer *et al.*, 1993). Also, since western blotting proved to be troublesome, the effect of *C. genistoides* in the protein expression of drug transporters should be reinvestigated using other techniques such as immunoprecipitation and purified antibodies, as these techniques are highly sensitive and generally provide clearer results.

Since this study showed obvious potential herb-drug interactions of various test entities, it would be beneficial to determine the efficacy of these test entities as adjunctive therapies with chronic medication such as hypoglycaemic and hyperlipidaemic treatments. To understand the underlying pharmacokinetic interactions between these test entities (drug-compound interactions) and chronic medication would be useful to align any findings with dose adjustment/optimization. For an accurate insight into these pharmacological interactions, it would be necessary to explore *in vivo* experimental options.

Probably the most interesting finding was the difference in CYP inhibition and drug transporter expression elicited by mangiferin and its isomeric counterpart, isomangiferin. A chance to further expose the nature of these differences using *in silico* modelling, might be interesting in future work.

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Appendix A: Plant extracts, chemical reagents, consumables and equipment

Table 7: List of the plant extracts

Plant extract	Code	Company
<i>C. genistoides</i> methanol extract	ARC188	Agricultural Research Council, Stellenbosch, SA
<i>C. genistoides</i> benzophenone enriched fraction	ARC188 _B	Agricultural Research Council, Stellenbosch, SA
<i>C. genistoides</i> xanthone enriched fraction	ARC188 _X	Agricultural Research Council, Stellenbosch, SA
Isomangiferin		Agricultural Research Council, Stellenbosch, SA
IMG		Agricultural Research Council, Stellenbosch, SA
IDG		Agricultural Research Council, Stellenbosch, SA

Table 8: List of chemicals and reagents

Chemical/Reagent	Catalogue number	Company
10% Protean® TGX™ gels	161-0993	Bio-Rad, Hercules, CA, USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	M5655	Sigma-Aldrich, St Louis, MO, USA
Chloroform	136112-00-0	Sigma-Aldrich, St Louis, MO, USA
Clarity Western EC substrates	1705060	Bio-Rad, Hercules, CA, USA
Dimethyl sulfoxide (DMSO)	1.02952	Sigma-Aldrich, St Louis, MO, USA
Dulbecco's phosphate buffered saline (DPBS)	17-513	Lonza, Walkersville, MD, USA
Ethanol	2875	Sigma-Aldrich, St Louis, MO, USA
Fetal bovine serum	BC/S0615-HI	Lonza, Walkersville, MD, USA
Fetal Bovine Serum (FBS)	1634.00 SEK / 500mL	Thermofisher, Waltham, MA, United States
Glycine	G7126	Sigma-Aldrich, St Louis, MO, USA
Hybond-P PVDF Membrane	1704156RNP1416F	Bio-Rad, Hercules, CA, USA
Isopropornol	I9516	Sigma-Aldrich, St Louis, MO, USA
Ketoconazole	K1003	Sigma-Aldrich, St Louis, MO, USA
L-glutamate	G7513	Sigma-Aldrich, St Louis, MO, USA
Mangiferin	M3547-100MG	Sigma-Aldrich, St Louis, MO, USA

Methanol	1070182511	Merck, Whitehouse Station, NJ, USA
Miconazole	M3512	Sigma-Aldrich, St Louis, MO, USA
Minimum Essential Medium Eagle, MEM Eagle EBSS (EMEM)	12-662F	Lonza, Walkersville, MD, USA
Non-fat milk powder	700166	Sigma-Aldrich, St Louis, MO, USA
Phenylmethanesulfonyl fluoride (PMSF)	11206893001	Roche, Basel, Switzerland
Phenylmethanesulfonyl fluoride (PMSF)	P7626	Sigma-Aldrich, St Louis, MO, USA
Phos Stop	4906845001	Sigma-Aldrich, St Louis, MO, USA
Ponceau S Stain	P23295	Sigma-Aldrich, St Louis, MO, USA
Precision plus western C marker	1610376	Bio-Rad, Hercules, CA, USA
Precision protein streptactin-HRP conjugatew	1610380	Bio-Rad, Hercules, CA, USA
Protease Inhibitors	11206893001	Roche, Basel, Switzerland
Qiazol stainless steel beads (0.5 mm)	79306	Qiagen, Hilden, Germany
Quinidine	Q3625	Sigma-Aldrich, St Louis, MO, USA
RC/DC Reagent A	50013	Bio-Rad, Hercules, CA, USA
RC/DC Reagent B	50014	Bio-Rad, Hercules, CA, USA
RC/DC Reagent S	50015	Bio-Rad, Hercules, CA, USA
RNase free water	Am9937	Ambion, Austin, TX, USA

Running buffer SDS	161-0772	Bio-Rad, Hercules, CA, USA
Sulfaphenazole	S0758	Sigma-Aldrich, St Louis, MO, USA
Tissue lysis reagent	FNN0011	Life Technologies Corporation, Carlsbad, CA, USA
Trypsin	71-161F	Lonza, Walkersville, MD, USA
Turbo blot Transfare buffer	1704156	Bio-Rad, Hercules, CA, USA
Tween-20	P1379	Sigma-Aldrich, St Louis, MO, USA
α -naphthoflavone	N5757	Sigma-Aldrich, St Louis, MO, USA

Table 9: List of consumables

Consumables	Catalogue number	Company
0.2 PCR tubes, flat cap	AX/PCR-02-C/S	Axygen, Corning, NY, USA
0.5 mL Eppendorf safe-lock tubes	0030 123.301	Eppendorf, Hamburg, Germany
1.5 mL Eppendorf safe-lock tubes	0030 123.328	Eppendorf, Hamburg, Germany
2 mL Eppendorf safe-lock tubes	0030 123.344	Eppendorf, Hamburg, Germany
15 mL centrifuge tubes	602072	NEST Biotechnology, Jiangsu China Wuxi
50 mL centrifuge tubes	601001	NEST Biotechnology, Jiangsu China Wuxi
CELLBIND 6-well plates	3335	Corning, MA, USA
CELLBIND 96-well clear plates	3300	Corning, MA, USA
CELLBIND 96-well black clear bottom plates	3340	Corning, MA, USA
CELLBIND 96-well white clear bottom plates	3610	Corning, MA, USA
Filter Pads	23385	Sigma-Aldrich, St Louis, MO, USA
MicroAmp Optical PCR 96-well clear plates	N8010560	Applied Biosystems, Foster City, CA, USA
75 cm ³ flask	708003	Nest, Wuxi, Jiangsu, China

Table 10: List of gene probes

TaqMan probe	Assay ID	Company
Beta-Actin	Hs99999903_m1	Life Technologies Corporation, Carlsbad, CA, USA
ABCB1	Hs00184500_m1	Life Technologies Corporation, Carlsbad, CA, USA
ABCG2	Hs00184979_m1	Life Technologies Corporation, Carlsbad, CA, USA
HPRT11	Hs02800695_m1	Life Technologies Corporation, Carlsbad, CA, USA
SLCO1B1	Hs00272374_m1	Life Technologies Corporation, Carlsbad, CA, USA
SLCO1B3	Hs00251986_m1	Life Technologies Corporation, Carlsbad, CA, USA

Table 11: List of antibodies

Antibodies	Catalogue number	Company
β -tubulin	2146	Cell signaling technology, MA, USA
Donkey anti-mouse IgG-HRP	Sc-2318	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Donkey anti-rabbit IgG-HRP	Sc-2012	Santa Cruz Biotechnology, Santa Cruz, CA, USA
MDR1	Sc-55510	Santa Cruz Biotechnology, Santa Cruz, CA, USA
SLCO1B3	HPA004943-100UL	Sigma-Aldrich, St Louis, MO, USA
ABCG2	42078	Cell signaling technology, MA, USA

Table 12: List of assay kits

Experimental kits	Catalogue no	Company
ViaLight ATP assay kit	LT27-008	Lonza, Walkersville, MD, USA
Bio-Rad, Hercules, CA, USA RC DC protein kit	500-0201	Bio-Rad, Hercules, CA, USA
High Capacity cDNA kit	PN 4375575	Applied biosystem, Foster City, CA, USA
Turbo DNase kit	AM1907	Ambion, Austin, TX, USA
RNeasy mini kit	74106	Qiagen, Hilden, Germany
Clarity™ Western ECL substrate	1705060	Bio-Rad, Hercules, CA, USA
Vivid® CYP1A2 Blue Screening Kit	P2863	Life Technologies Corporation, Carlsbad, CA, USA
Vivid® CYP2C9 Blue Screening Kit	P2861	Life Technologies Corporation, Carlsbad, CA, USA
Vivid® CYP2D6 Blue Screening Kit	P2972	Life Technologies Corporation, Carlsbad, CA, USA
Vivid® CYP2C19 Blue Screening Kit	P2864	Life Technologies Corporation, Carlsbad, CA, USA
Vivid® CYP3A4 Blue Screening Kit	P2858	Life Technologies Corporation, Carlsbad, CA, USA

Table 13: List of equipment

Equipment	Product Number	Supplier
Agilent Bioanalyser	G2946-90004	Agilent Technologies, Santa Clara, California
D1200-230V Heating block	S62927099	Labnet, Adison, NJ, USA
IKA® MS 3 digital shakers	IKA 331900X	Sigma-Aldrich, St Louis, MO, USA
Benchtop Centrifuge	SL16R	Thermo Fisher Scientific, Waltham, MA, USA
Micro-centrifuge	001977	Eppendorf, Hamburg, Germany
Mini Protean casting frame	165-3304	Bio-Rad, Hercules, CA, USA
Mini Protean casting stand	165-3303	Bio-Rad, Hercules, CA, USA
Mini Protein tetra cell	165-8030	Bio-Rad, Hercules, CA, USA
Mini Trans-blot cell	170-4070	Bio-Rad, Hercules, CA, USA
NanoDrop™ 1000 Spectrophotometer	A984	Thermo Fisher Scientific, Waltham, MA, USA
Orbital shaker ITPSIC 10	20197	Torrey pines Scientific
Powerpack HC	165-8025	Bio-Rad, Hercules, CA, USA
Visualiser	85300	Qiagen, Hilden, Germany
ABI 7500 Real time PCR system	4351104	Applied Biosystem, Foster City, CA, USA
Biotek® FLX 800 plate reader	FLX 800	BioTek Instruments Inc., Winooski, VT, USA
BioTek® ELX800 plate reader	ELX800	BioTek Instruments Inc., Winooski, VT, USA
Orbital shacker	20197	Stovall life Science

Appendix B: Preparation of reagents

1.1. Preparation of complete EMEM

In 500 mL EMEM containing 1% non-essential amino acids and 100 mM sodium pyruvate, the following was added:

- 50 mL Fetal Bovine Serum
- 5 mL L-glutamine (20 mM)

Media was stored at 4°C.

1.2. Extraction and fractionation process of ARC188, ARC188_B and ARC188_x

Unfermented *C. genistoides* plant material (ARC188) was collected from the *Cyclopia* gene bank of the Agricultural Research Council (ARC). The leaves and fine shoots of the plant material were dried at 40°C for 16 hours in the drying tunnel to obtain a moisture content of less than 10%, while the thick shoots were discarded. The dried plant material was then milled to a fine powder using a Retsch rotary mill (1.0 mm sieve; Retsch, GmbH, Haan, Germany).

The crude *C. genistoides* methanol extract (ARC188) was extracted using the method adapted from Raaths, 2017. Briefly, a mini-scale extraction was first performed then the extraction was up scaled. For miniscale extraction, 40% methanol was prepared by diluting 200 mL 99.9% methanol (1070182511, Merck, Whitehouse Station, NJ, USA) with 300 mL double distilled water. Forty milligrams of the plant material were weighed in 5 mL Reactivials (Sigma-Aldrich, St. Lucia, MA, USA) and 4 mL of the 40% methanol solvent was added to each Reactivials and closed tightly. Reactivials were placed in a pre-heated Stuart SBH200D/3 block heater (Bibby Scientific, Staffordshire, UK) at 93°C for 1 hour with mixing every 5 minutes. Samples were then cooled and filtered using a 0.45-micron Millipore Miller PVDF syringe filters (Merck-Millipore, Darmstadt, Germany). Two millilitres from each sample was further diluted with 18 mL of double distilled water. One-point five millilitres was then taken from the diluted samples and stored at -20°C until further use. A preservative (1% vitamin C) was added to each sample aliquot to prevent oxidation, then the major phenolic compounds present in the extract were characterised

and quantified using high performance liquid chromatography (HPLC) containing a diode-array detector (DAD) (HPLC-DAD). After quantification and characterization of phenolic compounds, the extraction process was up scaled. The plant material that would provide the extract was chosen based on their phenolic content as determined by the mini-scale screening process. Five litres of 40% methanol (v/v) was prepared. One hundred and fifty grams of the plant material was obtained from each group and weighed into each clean 2 L glass bottle and 1.5 L of the 40% methanol was added to each bottle. Bottles were tightly closed, and samples were placed in a pre-heated water bath at 93°C for 30 minutes with mixing every 5 minutes. The extract was then poured through a 200-mesh stainless steel sieve and filtered under vacuum using Whatman papers (Whatman #4 filter, Whatman plc, Maidstone, UK). Methanol was then evaporated from the extract using a Büchi Rotavapor R-215 (BÜCHI Labortechnik, Flawil, Switzerland). The *C. genistoides* extract was then chemically characterised using HPLC-DAD (Chromatograms available in Figure C1-C3, Appendix C).

In order to ensure that *C. genistoides* constituents were separated adequately, HPLC-DAD was performed using a method described by Beelders *et al.*, 2014 with minor adjustments as adapted from Raaths, 2017. An Agilent 1200 series system consisting of an in-line degasser, quaternary pump, autosampler, column thermostat and diode-array detector was used to conduct HPLC-DAD experiments. The instrument was controlled, and data was analysed using Openlab Chemstation software (Agilent Technologies Inc., Santa Clara, CA, USA). UV-Vis spectra were recorded between 200–700 nm with selective wavelength monitoring at 288 and 320 nm. The benzophenones, IDG and IMG, were monitored at 288 nm, while the xanthenes, mangiferin and isomangiferin, and the benzophenone, maclurin, were monitored at 320 nm. The enriched fractions were collected by open column chromatography using XAD180 and a methanol-water solvent gradient.

In order to ensure that *C. genistoides* constituents were separated adequately, HPLC-DAD was performed using a method described by Beelders *et al.*, 2014 with minor adjustments as adapted from Raaths, 2017. An Agilent 1200 series system consisting of an in-line degasser, quaternary pump, autosampler, column thermostat and diode-array

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1.3. Preparation of MTT reagent

- 100 µg/mL MTT (3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide) (Cat No.: M5655; Sigma-Aldrich, St Louis, MO, USA)
- 50 mL Dulbecco's phosphate buffered saline (DPBS) (Cat No.: 17-513, Lonza, MD, USA)

The mixture was vortexed until it was a clear yellow colour, then covered in foil and refrigerated at 4°C until further use.

1.4. Preparation of Sorenson's buffer

- 100 mM glycine
- 100 mM sodium chloride (NaCl)
- 50 mL sterile TC water

The pH of the mixture was adjusted to 10.5 using 0.2 M Potassium hydroxide (KOH). The pH was measured using a pH meter. Stored at 4°C.

1.5. Preparation of Vivid® reagent

Vivid® buffer I and buffer II working solutions were prepared by diluting the 2X buffers into DPBS in a 1:1 ratio. Stored at room temperature.

Vivid® substrates were reconstituted as described in table 14 and stored at -20°C.

Table 14: Reconstitution of the Vivid® Substrate

CYP enzyme	Vivid® Substrate	Volume of acetonitrile
CYP1A2	Vivid® EOMCC	205 µL
CYP2C9	Vivid® BOMCC	160 µL
CYP2C19	Vivid® EOMCC	205 µL
CYP2D6	Vivid® EOMCC	205 µL
CYP3A4	Vivid® BOMCC	160 µL

Master mix I was prepared as described in table 15 and used immediately.

Table 15: Preparation of master mix I

CYP enzyme	Buffer used	Volume of buffer	Volume of regeneration system	Volume of baculosome
CYP1A2	Buffer I	2.425 mL	50 µL	25 µL
CYP2C9	Buffer II	2.400 mL	50 µL	50 µL
CYP2C19	Buffer II	2.425 mL	50 µL	25 µL
CYP2D6	Buffer I	2.400 mL	50 µL	50 µL
CYP3A4	Buffer I	2.425 mL	50 µL	25 µL

Master mix II was prepared as described in table 16 and used immediately.

Table 16: Preparation of master mix II

CYP enzyme	Vivid Substrate	Volume of buffer	Volume of Vivid® substrate	Volume of NADP⁺
CYP1A2	Vivid EOMCC	477.5 µL	7.5 µL	15 µL
CYP2C9	Vivid BOMCC	460 µL	25 µL	15 µL
CYP2C19	Vivid EOMCC	460 µL	25 µL	15 µL
CYP2D6	Vivid EOMCC	460 µL	25 µL	15 µL
CYP3A4	Vivid BOMCC	460 µL	25 µL	15 µL

1.6. Preparation of the cDNA transcripts

cDNA transcripts were prepared as described in table 17 and table 18 and used immediately.

Table 17: Preparation of the positive transcript for cDNA synthesis

Component	Volume (μL) per 1 sample
10 X RT buffer	2
25 X dNTP mix	0.8
10 X random primers	2
RNase inhibitor	1
Nuclease free water	3.2
Reverse transcriptase	1
Total master mix volume	10

Table 18: Preparation of the negative transcript master mix for cDNA synthesis

Component	Volume (μL) per 1 sample
10 X RT buffer	2
25 X dNTP mix	0.8
10 X random primers	2
RNase inhibitor	1
Nuclease free water	3.2
Total master mix volume	10

1.7. Preparation of SYBR green master mix

The SYBR green mastermix prepared as described in table 19 and used immediately

Table 19: Preparation of the SYBR green mastermix

Component	Volume (μL) per 1 sample
SYBR green PCR mix	12.5
Forward primer	1
Reverse primer	1
RNase free water	9.5
Total SYBR green master mix volume	24

1.8. Preparation of qRT-PCR master mix

The qRT-PCR mastermix was prepared as described in table 20 and used immediately.

Table 20: Preparation of the qRT – PCR master mix

Component	Volume (μL) per 1 sample
TaqMan™ Protein Assays Fast Master Mix	5
TaqMan™ probe	0.5
Nuclease free water	3.5
Total volume	10

1.9. Preparation of tissue lysis reagent

In 100 mL of Tissue lysis reagent the following was added

- 2X protease inhibiting tablets

- 10X Phos Stop tablets

Tissue lysis reagent was stored at -20°C. Just before use 10 µL of PMSF were added to 1 mL of the tissue lysis reagent

1.10. Preparation of 10X Tris buffered saline (TBS)

- 200 mM tris
- 1.37 M sodium chloride
- pH 7.6

Weigh out 24.22 g of Tris and 80.0 g of NaCl, dilute the two with double distilled water to make 1 L.

1.11. Preparation of 1X TBS

One-time TBS (1X TBS) was made by diluting 100 mL 10X TBS with 900 mL double distilled water.

1.12. Preparation of 1X TBST

One-time TBST (1X TBST) was made by diluting 100 mL of 10X TBS with 900 mL double distilled water and adding 1 mL Tween – 20.

Appendix C: Supplementary data

Unfermented *C. genistoides* shoots and leaves were collected and phenolic compounds were quantified and characterised using HPLC-DAD. The plant material was then extracted using HPLC-DAD and 40% methanol as a solvent. The xanthenes, mangiferin and isomangiferin, and the benzophenone maclurin were observed at 288 nm wavelength. While the benzophenones, IDG and IMG, were observed at 320 nm wavelength. Chromatograms illustrating the peaks of the pure compounds are shown in Figures 42-44.

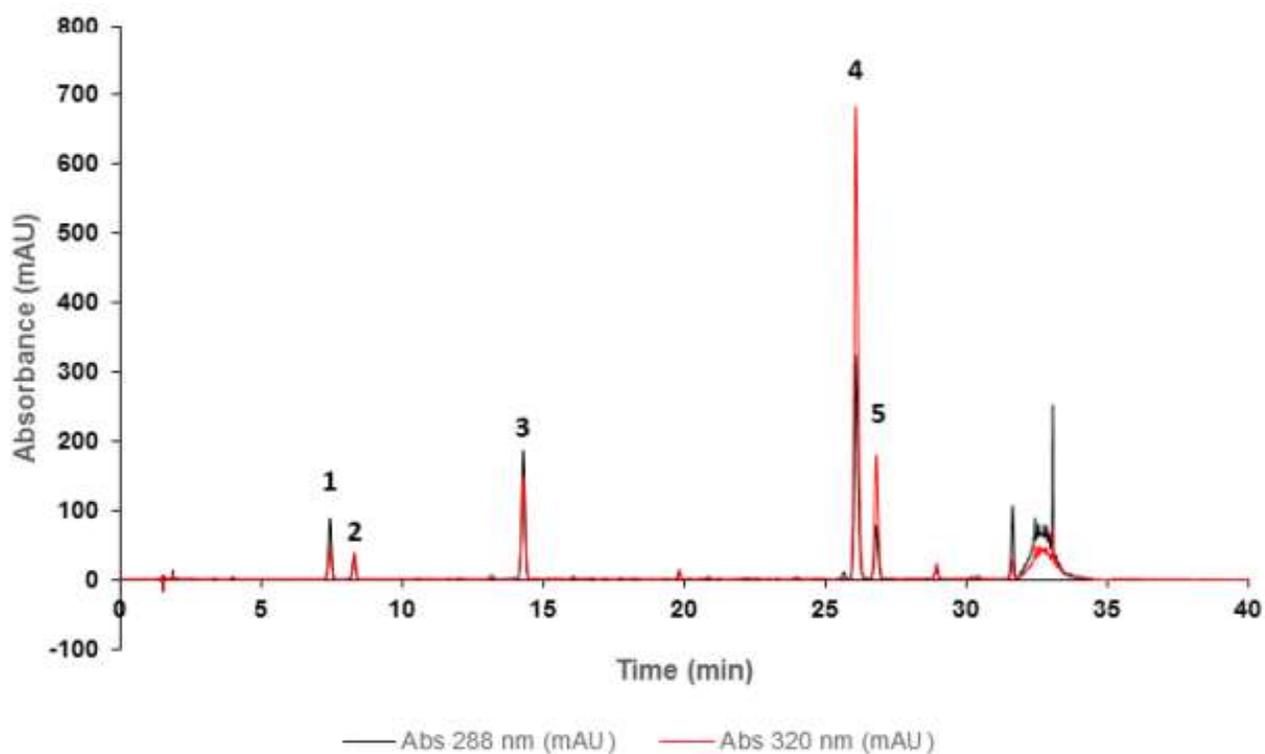


Figure 42: Chromatogram illustrating HPLC-DAD of (ARC188 1) IDG, 2) maclurin, 3) IMG, 4) mangiferin and 5) isomangiferin) (adapted from Raaths, 2016).

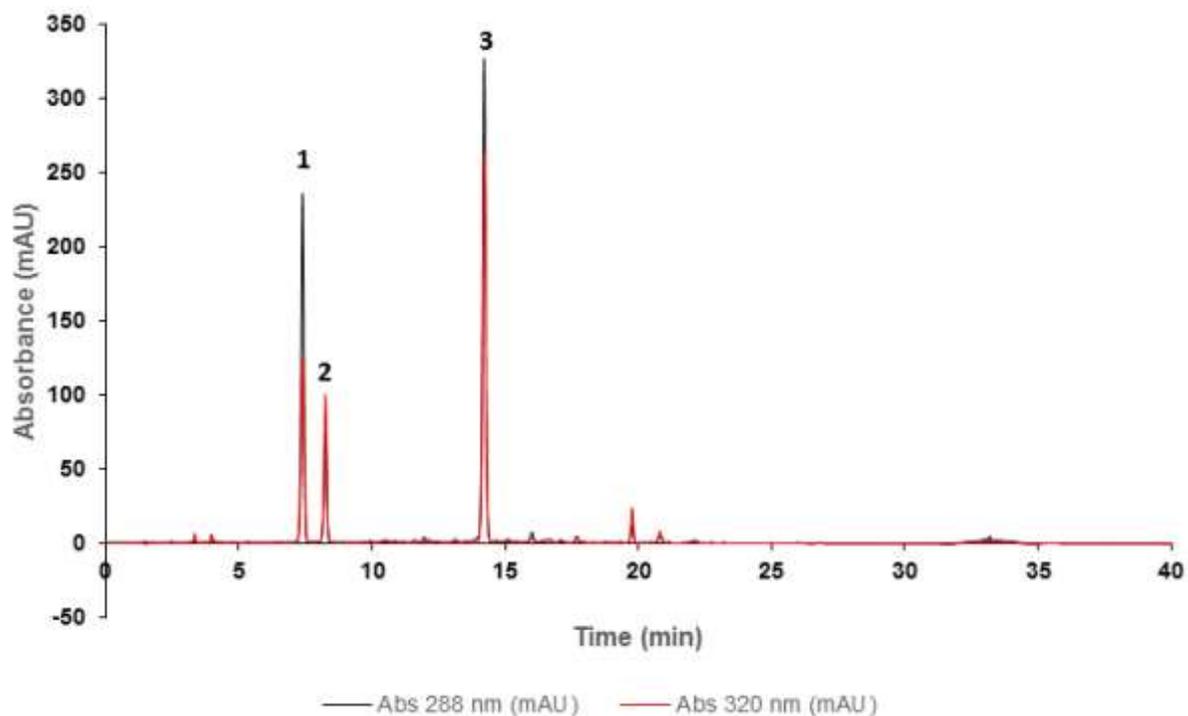


Figure 43: Chromatogram illustrating HPLC-DAD of ARC188_B (1) IDG, 2) maclurin and 3) IMG) (adapted from Raaths, 2016).

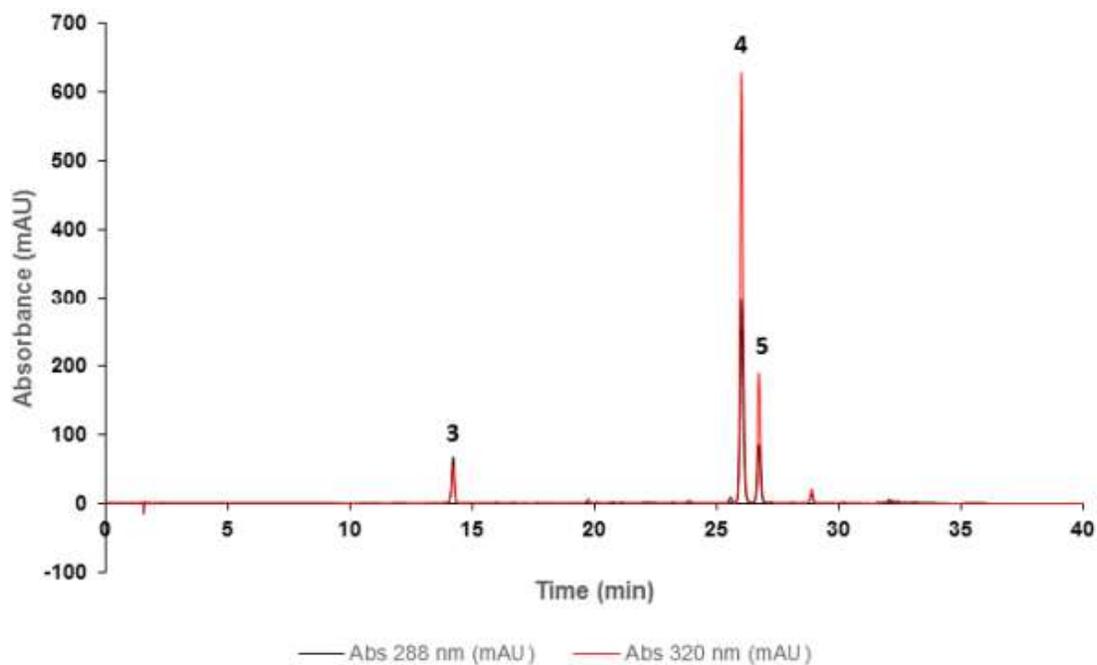


Figure 44: Chromatogram illustrating HPLC-DAD fingerprint of ARC188_x (3) IMG, 4) mangiferin and 5) isomangiferin) (adapted from Raaths, 2017).

Table 21: Summary of the composition of ARC188, ARC188_B and ARC188_x

<i>C. genistoides</i>	IDG (g/100g)	IMG (g/100g)	Mangiferin (g/100g)	Isomangiferin (g/100g)
ARC188	2,214	3,885	13,811	3,193
ARC188_B	23,267	28,272	0	0
ARCfX188_x	0	5,804	54,016	14,352

Table 22: Inhibitory effects of *C. genistoides* constituents on α -glucosidase activity

<i>C. geistoides</i> constituent	α-glucosidase inhibition: (μg/mL)	IC₅₀	Reference
ARC188	150		Raaths, 2017
ARC188 _B	121		Raaths, 2017
ARC188 _x	59		Raaths, 2017
Mangiferin	41.88		Dineshkumar <i>et al.</i> , 2010

Table 23: DMSO solubilization concentration for kinetic Vivid® screening assays

Test compound	Concentration of DMSO
ARC188	0.2% DMSO
ARC188 _B	0.1% DMSO
ARC188 _x	0.1% DMSO
Mangiferin (5.6 µg/mL)	0.1% DMSO
Mangiferin (16 µg/mL)	0.2% DMSO
Isomangiferin (3 µg/mL)	0.1% DMSO
Isomangiferin (15 µg/mL)	0.1% DMSO
IMG (5 µg/mL)	0.1% DMSO
IMG (25 µg/mL)	0.2% DMSO
IDG (2 µg/mL)	0.1% DMSO
IDG (20 µg/mL)	0.2% DMSO

Table 24: interference of solvents on Vivid® CYP enzyme activity. Values are given as percent inhibition at the indicated solvent concentration. Values preceded by a “+” indicate an increase in activity. Dashed lines indicate inhibition not detected (<https://www.lifetechnologies.com/vivid>).

Vivid Kit	[Solvent] (%)	DMSO (% inhibition)	Acetonitrile (% inhibition)	Methanol (% inhibition)	Ethanol (% inhibition)
1A2 Blue	1	13	18	--	27
	0.1	--	--	--	8
	0.01	--	--	--	--
2A6 Blue	1	68	75	34	72
	0.1	14	31	--	17
	0.01	--	--	--	--
2B6 Blue	1	35	19	29	58
	0.1	--	--	17	21
	0.01	--	--	--	9
2C8 Green	1	+10	10	--	--
	0.1	--	--	--	--
	0.01	--	--	--	--
2C9 Blue	1	69	--	58	79
	0.1	36	--	18	41
	0.01	15	--	--	--
2C9 Green	1	--	--	42	51
	0.1	--	--	15	21
	0.01	--	--	--	--
2C9 Red	1	14	8	50	59
	0.1	--	--	19	20
	0.01	--	--	--	--
2C19 Blue	1	37	16	30	62
	0.1	--	--	13	19
	0.01	--	--	--	--
2D6 Blue	1	45	--	47	58
	0.1	11	--	16	15
	0.01	--	--	11	10
2D6 Cyan	1	14	13	40	50
	0.1	--	--	14	13
	0.01	--	--	--	--
2E1 Blue	1	99	52	84	100
	0.1	81	18	46	96
	0.01	34	7	19	79
2J2 Cyan	1	46	--	28	67
	0.1	11	--	11	26
	0.01	--	--	--	6
3A4 Blue	1	70	9	19	26
	0.1	34	4	26	15
	0.01	21	--	11	12
3A4 Green	1	38	+5	--	5
	0.1	10	--	--	--
	0.01	--	--	--	--
3A4 Red	1	61	--	20	25
	0.1	25	--	22	11
	0.01	11	--	11	12
3A5 Blue	1	79	11	29	46
	0.1	45	8	33	21
	0.01	23	--	19	19
3A5 Green	1	25	--	--	--
	0.1	--	--	--	--
	0.01	--	--	--	--

Appendix D: Research outputs

1. University of Zululand, University of Limpopo, SAMRC joint research meeting: Stellenbosch, 25 -28 July 2018

In vitro assessment of cytochrome P450 inhibition by polyphenolic constituents of *Cyclopia genistoides*.

N.P.N. Mchunu, S.L. Bowles, A.M.P. Kappo, C. Awortwe, C.J. Malherbe, C.J.F. Muller

2. Joint scientific meeting of ARC, MRC and TUAT: SAMCR Cape town, 17 September 2018

In vitro assessment of cytochrome P450 and drug transporters modulation by polyphenolic constituents of *Cyclopia genistoides*.

N.P.N. Mchunu, S.L. Bowles, A.M.P. Kappo, C. Awortwe, C.J. Malherbe, C.J.F. Muller

3. BRIP annual symposium: SAMCR Cape town, 16 October 2018

In vitro assessment of cytochrome P450 and drug transporters modulation by polyphenolic constituents of *Cyclopia genistoides*.

N.P.N. Mchunu, S.L. Bowles, A.M.P. Kappo, C. Awortwe, C.J. Malherbe, C.J.F. Muller

Appendix E: Ethical clearance certificate

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



RESEARCH & INNOVATION

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Tel: 035 902 6731
Fax: 035 902 6222
Email: Msutshane@baubosbu.ac.za

ETHICAL CLEARANCE CERTIFICATE

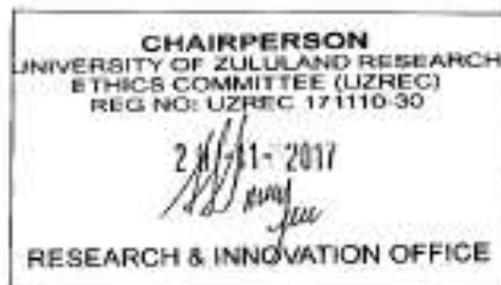
Certificate Number	UZREC 171110-030 PGM 2017/439		
Project Title	In vitro assessment of CYP modulation by polyphenolic constituents of <i>Cyrtia genistoides</i> in C3A liver cell.		
Principal Researcher/ Investigator	Mchunu N		
Supervisor and Co- supervisor	Dr S Bowles & Prof AP Kappo	Prof C Muller, Dr C Awoetwe and Dr C Malherbe	
Department	Biochemistry and Microbiology		
Faculty	Science & Agriculture		
Type of Risk	Low risk – Desktop research		
Nature of Project	Honours/4 th Year	Master's	<input checked="" type="checkbox"/> 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. The Researcher may therefore commence with data collection as from the date of this Certificate, using the certificate number indicated above.

- Special conditions:
- (1) This certificate is valid for 2 years from the date of issue.
 - (2) Principal researcher must provide an annual report to the UZREC in the prescribed format [due date-01 July 2018]
 - (3) Principal researcher must submit a report at the end of project in respect of ethical compliance.
 - (4) The UZREC must be informed immediately of any material change in the conditions or undertakings mentioned in the documents that were presented to the meeting.

The UZREC wishes the researcher well in conducting research.

Professor Gideon De Wet
Chairperson: University Research Ethics Committee
Deputy Vice-Chancellor: Research & Innovation
16 November 2017



Nonkululeko Mchunu MSc. Thesis

by Nonkululeko Mchunu

Submission date: 09-Dec-2018 11:09PM (UTC+0200)

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1. Introduction

African traditional medicine is one of the oldest forms of medicine and incorporates the use of herbal medicines. In South Africa, the use of herbal medicines is still widely popular. Approximately 70% of the South African population in rural areas primarily use herbal medicines for therapy. Globally, it has been reported that about 80% of the world's populations in developing countries use herbal medicines. The use of herbal medicines as complementary or alternative medicine (CAM) has recently become popular in developed countries as well, as people try to deviate from using synthetic chemical products in hopes of a healthy lifestyle.

The herbal medicine industry is dependent on the availability of plants. In South Africa there is a rich biodiversity, there are about 30 000 species of higher plants with about 8 000 of which are endemic to the country and about 3 000 plant species are used in the production of herbal medicines. With the rising interest in herbal medicines, research on medicinal plants has increased and many more plants with medicinal properties have emerged. Amongst such plants is *Cyclopia genistoides*, a short shrub that has historically been used in the production of a South African herbal tea known for its sweet taste, aroma and lack of caffeine and tannins, honeybush tea. The plant is endemic to the Cape Flora fynbos biome of South Africa which belongs to the *Fabaceae* family and *Podalrieae* tribe. Of the approximate 23 *Cyclopia* species, *C. genistoides* together with 2 other species, *C. intermedia* and *C. subternata* are the only species used in the commercial production of the tea with *C. genistoides* containing the highest phenolic content when compared to the others. *C. genistoides* has been found to be rich in xanthenes and benzophenones which have been associated with many health benefits including antidiabetic, anti-inflammatory, antioxidative and immune modulating properties. Recently, *C. genistoides* xanthenes and benzophenones have been shown to inhibit the activity of α -glucosidase, an enzyme that aids in post prandial carbohydrate metabolism, *in vitro*. These recent findings together with the plant's high phenolic content increases the possibility of *C. genistoides* to be used as an anti-diabetic adjunctive that aids in decreasing the metabolic rate of postprandial carbohydrates thus lowering postprandial glucose levels in the gut. Such an adjuvant will most-likely be taken concurrently with other oral antidiabetic drugs used to treat type-2 diabetes (T2D) which may lead to herb-drug

Nonkululeko Mchunu MSc. Thesis

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