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HEPATOPROTECTIVE PROPERTIES OF GENTIANA SPP. AGAINST NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

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A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of Doctor of Philosophy

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a metabolic disease characterised by the accumulation of fat in the liver. It is estimated that 33 % of the UK population have NAFLD with 2-5 % progressing to non-alcoholic steatohepatitis (NASH). Due to a lack of an outright therapy for NAFLD, treatment has been mainly focussed on managing the conditions associated with the disease such as obesity, diabetes mellitus and hyperlipidaemia.

This study aimed to investigate the means by which hepatocyte protection is conferred by Gentiana plants (*Gentiana lutea*, *Gentiana macrophylla*, *Gentiana scabra* and *Gentiana rigescens*) used in herbal medicine for the management of non-alcoholic fatty liver diseases (NAFLD). The role played by some of the inherent Gentiana phytochemicals including: gentiopicroside, sweroside and swertiamarin in promoting hepatocyte protection against the cytotoxic effects of fatty acids were also investigated. Gentiana species: *lutea*, *macrophylla, rigescens,* and *scabra* are known to protect and enhance hepatocyte viability via their antioxidant, anti-inflammatory and bitter components including: amarogentin gentianine, iso-orientin, swertiamarin, gentiopicroside, and sweroside. This study was necessitated due to a lack of adequate research on the hepatoprotective effects of the above-named Gentiana species and phytochemicals with special emphasis on their effect on mitochondrial respiration in the presence of fatty acids.

At the time of submission, this was the first study to utilise the seahorse mitochondria stress assay to investigate the Gentiana species as well as phytochemicals: gentiopicroside, sweroside and swertiamarin. It was also found that the most abundant phytochemical in all four Gentiana species was gentiopicroside (up to 4.6% g/g), followed by swertiamarin (0.21–0.45% g/g), and sweroside (0.03- 0.4 % g/g). Furthermore, it was also observed that the methanolic extracts of all four Gentiana protected HepG2 and THLE-2 cells by inhibiting arachidonic acid from diminishing cell replication but showed a mitogenic effect mostly observed in gentiopicroside, *Gentiana lutea* and *Gentiana macrophylla*.

It was concluded that phytochemicals: gentiopicroside, sweroside and swertiamarin play key roles in the hepatocyte protection exerted by methanolic extracts of *Gentiana lutea*, *Gentiana macrophylla*, *Gentiana scabra* and *Gentiana rigescens* against the cytotoxic effects of fatty acids. This protection is conferred by enhancing mitochondrial function in terms of increasing maximal respiratory capacity in response to high influx of fatty acids, promoting ATP production as well as scavenging ROS produced as a result of high fatty acid influx and increased mitochondrial respiration. However, the mitogenic effect observed in gentiopicroside and Gentiana macrophylla requires further studies using unmodified primary hepatocytes to gain better understanding.

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Dedications

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Author's Declaration

I declare that all the material contained in this thesis is my own work.

Abbreviations

ACC - Acetyl-coA carboxylase

ALT – Alanine transaminase

AMPK - Adenosine monophosphate-activated protein kinase

AST – Aspartate transaminase

ATP – Adenosine triphosphate

COX - Cyclooxygenase

CTGF – Connective tissue growth factor

CYP2E1 - Cytochrom P450 2E1

DCF - Dichlorofluorescein

DMEM - Dulbecco's Modified Eagle Medium

FAS – Fatty acid synthase

FCCP – Carbonyl cynide 4-(trifluoromethoxy) phenylhadrazone

FFA – Free fatty acid

FOXO-1- Forkhead box protein O1

GC-MS – Gas chromatography

GL – *Gentiana lutea*

GM- *Gentiana macrophylla*

GPS – Gentiopicroside

GR – *Gentiana rigescens*

GS- *Gentiana scabra*

HNE - 4-Hydroxynonena

HPLC-ESI-Q/TOF-MS - High-performance liquid chromatography with electrospray ionization, quadruple time-of-flight mass spectrometry

IL-6 – Interleukin 6

LC-ESI-MS - Liquid chromatography– electrospray ionization-mass spectrometry

LDH- Lactate dehydrogenase

LDL – Low density lipoprotein

LKB1 – Liver kinase B1

LPG – Lipoprotein G

NAFLD – Non- alcoholic fatty liver disease

NASH – Non-alcoholic steatohepatitis

OCR – Oxygen consumption rate

PAI-1 - Plasminogen activator inhibitor-1

PPAR – α – Peroxisome proliferator activated receptor alpha

R123 - Rhodamine 123

ROS – Reactive oxygen species

SAA3 - Serum amyloid A

SPP - Species

SWE – Sweroside

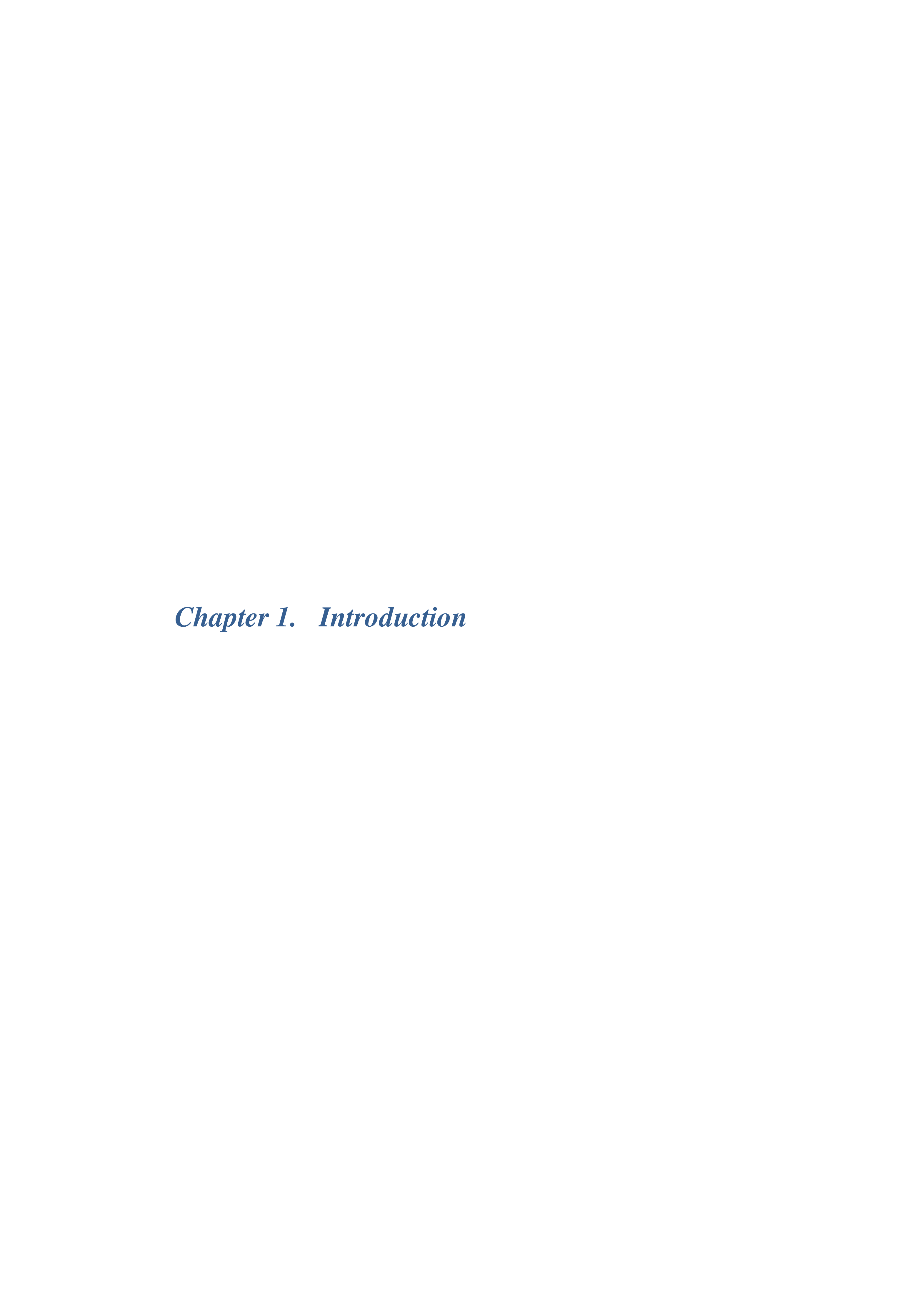
SWT- Swertiamarin

TG- Triglyceride

TMRE - Tetramethylrhodamine

TNF-α – Tumour necrosis factor alpha

VLDL - Very low-density lipoprotein



Chapter 1. Introduction

1.0 Overview of Gentiana Species Profile, Phytochemicals and Utilisation

The Gentiana genus which originates from the Gentianaceae family is composed of up to 300 different species of plants, some of which are: *G. lutea, G. macrophylla*, *G. rigescens, G. crassicaulis G. dahurica, G. asclepiadea, G. manshurica, G. straminea, G. olivieri* and *G. scabra* (Yang *et al.*, 2010, Tang and Eisenbrand, 2011). The species of plants found in the Gentiana genus have been found to possess several pharmacological activities including being: hepatoprotective, anti-inflammatory, antioxidant and antihypertensive. These actions may be attributed to inherent phytochemicals such as iridoids, flavonoids, xanthones, triterpenoids and secoiridoid (Jensen and Schripsema, 2002, Chong, 2008). Over 90 iridoid compounds, up to 34 flavonoids and 100 xanthones have been isolated from species of plants belonging to the Gentiana genus (Wang *et al.*, 2009a).

A typical example of pharmacological effects of plants in the Gentiana genus was observed in aerial parts of *Gentiana olivieri* which were administered subcutaneously to rats in assessing its effect on carbon tetrachloride induced hepatic damage. It was observed that *Gentiana olivieri* exerted anti-hepatotoxic effects via its phytochemical isoorientin (Orhan *et al.*, 2003). Methanolic extracts of Gentiana asclepiadea administered to Wistar rats exhibited hepatoprotective effects by significantly reducing the level of serum transaminases, alkaline phosphatase and total bilirubin in the presence of carbon tetrachloride. The extent of hepatoprotection conferred was comparable to silymarin which was used as a reference compound (Mihailovic *et al.*, 2013). Furthermore, anti-viral and anti-tumour effects of *Gentiana asclepiadea* have also been reported (Devic *et al.*, 2006). Hepatoprotective effects of *Gentiana manshurica* were shown by suppressing the elevation of malondialdehyde, promoting superoxide dismutase and glutathione production after being administered to mice intoxicated with alcohol (Lian *et al.*, 2010). According to the Chinese Materia Medica 2-10 g decoction of the rhizomes of *Gentiana manshurica* or *Gentiana scabra* or *Gentiana rigescens* can be administered in the treatment of jaundice and also for the improvement of liver and gallbladder functions (Enquin, 1990). Table 1.1 provides a summary of pharmacological effects of Gentiana plants, parts used and phytochemicals present.

Table 1.1 Summarised pharmacological effects of Some *Gentiana* plants.

Plant	Part Used	Phytochemicals Identified	Pharmacological Effect(s)	References
G. olivieri	Aerial parts	Isoorietin, vitexin, orientin	Anti-hepatotoxic	(Orhan *et al.*, 2003)
G. asclepiadea	Aerial parts Roots	Sweroside Swertiamarin Gentiopicroside	Hepatoprotective Anti-viral Anti-tumour	(Mihailovic *et al.*, 2013) (Devic *et al.*, 2006)
G. manshurica	Aerial parts Roots	Gentiopicroside Sweroside Swertiamarin	Anti-oxidant Reverses alcohol-induced steatosis Hepatoprotective	(Lian *et al.*, 2010) (Wang *et al.*, 2004) (Zhao *et al.*, 2004) (Enquin, 1990)
G. *lutea*	Aerial parts Roots	Loganic acid Gentiopicroside Sweroside Swertiamarin Amarogentin Gentisin Isogentisin Gentioside	Anti-inflammatory Hepatoprotective Anti-pyretic	(Mathew *et al.*, 2004) (Aberham *et al.*, 2007)
G. *macrophylla*	Aerial parts Roots	Gentiopicroside Sweroside Swertiamarin Loganic acid	Anti-rheumatoid arthritis Hepatoprotective	(Cao and Wang, 2010) (Zhao *et al.*, 2004) (Yu *et al.*, 2004)
G. *rigescens*	Aerial parts Roots	Gentiopicroside Sweroside Swertiamarin Loganic acid	Hepatoprotective Anti-fungal	(Xu *et al.*, 2009) (Zhao *et al.*, 2004) (Enquin, 1990) (Xu *et al.*, 2005)
G. *scabra*	Aerial parts Roots	Gentiopicroside Sweroside Swertiamarin	Hepatoprotective Anti-oxidant	(Liu *et al.*, 2013) (Zhao *et al.*, 2004) (Enquin, 1990) (Wang *et al.*, 2014)

The major secoiridoid glycoside found in the root and rhizome of Gentiana species is gentiopicroside whereas the minor entails amarogentin, sweroside amaroswerin and swertiamarin. It has been reported that in the Gentianaceae family which entails the Gentiana genus; sweroside, swertiamarin and gentiopicroside are the most commonly found phytochemicals with gentiopicroside and swertiamarin exclusively found in the Gentianaceae family (Jensen and Schripsema, 2002). It is estimated that the gentiopicroside content of Gentiana species after quantitation should not be less than 1.0 % g/g (Tang and Eisenbrand, 2011). Gentiopicroside, which can be obtained from *Gentiana macrophylla, Gentiana lutea, Gentiana rigescens as well as Gentiana scabra* has been indicated as an anti-viral, hepatoprotective and anti-inflammatory agent (Wu *et al.*, 2017, Tang *et al.*, 2016). Gentiopicroside has been used to treat a number of inflammatory conditions such as liver disease (hepatitis), rheumatoid arthritis, fever, digestive and intestinal disorders (Kondo *et al.*, 1994). Amarogentin which is a secoiridoid glycoside and the bitterest substance is used as an anticarcinogenic and antileishmanial agent. It inhibited the hyperproliferation of cancerous cells by downregulating cyclooxygenase (COX II) and upregulating apoptosis in a dermal carcinogenic model in mice. Amarogentin can be found in *Gentiana lutea* and *Swertia chirata* (Vanhaelen and Vanhaelen-Fastre, 1983, Saha *et al.*, 2006). Norswertianolin which is a xanthone found in *Gentiana campestris* as well as *Swertia davidi* has been indicated as an acetylcholinesterase inhibitor (Zeng *et al.*, 2004, Urbain *et al.*, 2004). Table 1.2 provides a summary of pharmacological effects of some Gentiana phytochemicals, their classification and plants from which they can be sourced.

Table 1.2 Summarised pharmacological effects of some Gentiana phytochemicals.

Phytochemical	Pharmacological Effect(s)/Toxicity	Class	Plant Source(s)	References
Norswertianolin	Acetylcholinesterase inhibitor	Xanthone	*Gentiana campestris* *Swertia davidi,*	(Zeng *et al.*, 2004) (Urbain *et al.*, 2004)
Amarogentin	Anticarcinogenic Antileishmanial	Secoiridoid glycoside	*Gentiana lutea* *Swertia chirata*	(Saha *et al.*, 2006) (Vanhaelen and Vanhaelen-Fastre, 1983)
Gentiopicroside	Antiviral Hepatoprotective Anti-inflammatory Gastro-protective	Secoiridoid glycoside	*Gentiana manshurica* *Gentiana lutea* *Gentiana macrophylla* *Gentiana scabra*	(Tang *et al.*, 2016) (Wu *et al.*, 2017) (Öztürk *et al.*, 2006) (Wang *et al.*, 2010a) (Niiho *et al.*, 2006)
Sweroside	Hepatoprotective Antifungal	Secoiridoid glycoside	*Gentiana lutea* *Gentiana tibetica* *Gentiana macrophylla* *Gentiana Scabra* *Fructus Corni*	(Tan *et al.*, 1998a) (Sun *et al.*, 2013a) (Tan *et al.*, 1996) (Ikeshiro and Tomita, 1983)
Swertiamarin	Analgesic Hepatoprotective Antioxidant Gastroprotective	Secoiridoid glycoside	*Gentiana macrophylla* *Gentiana manshurica* *Gentiana lutea* *Gentiana scabra*	(Jaishree and Badami, 2010) (Liu *et al.*, 1994) (Öztürk *et al.*, 2006)

				(Niiho *et al*., 2006)
Loganic Acid	Anti-inflammatory	Secoiridoid glycoside	*Gentiana manshurica* *Gentiana lutea* *Gentiana linearis*	(Wang *et al*., 2010b) (Lin *et al*., 2004) (Aberham *et al*., 2007) (Bergeron *et al*., 1997) (Ikeshiro and Tomita, 1983)
Amaroswerin	Gastro-protective	Secoiridoid glycoside	*Swertia japonica* *Gentiana Manshurica* *Gentiana Purpurea* *Gentiana punctate* *Gentiana lutea*	(Ishimaru *et al*., 1990) (Zhang *et al*., 1990) (Quercia *et al*., 1980) (Niiho *et al*., 2006)

Sweroside which can be obtained from *Gentiana lutea, Gentiana tibetica, Fructus corni, Gentiana macrophylla and Gentiana scabra* showed hepatoprotective effects against lipopolysaccharide-induced liver injury in mice and has also been indicated as an antifungal agent (Tan *et al*., 1998a, Ikeshiro and Tomita, 1983, Sun *et al*., 2013a). The administration of swertiamarin (100-200 mg/kg) body weight of rats significantly reduced liver injury and lipid peroxidation induced with d-galactosamine (Jaishree and Badami, 2010) Swertiamarin can be found in *Gentiana macrophylla*, *Gentiana manshurica*, *Gentiana lutea*, *Gentiana scabra* and *Gentiana lutea* (Liu *et al*., 1994, Öztürk *et al*., 2006, Wang *et al*., 2010b). Loganic acid presented an anti-inflammatory effect by inhibiting rat paw oedema induced with carrageenam by up to 44.4 % (del Carmen Recio *et al*., 1994). It can be isolated from *Gentiana manshurica, Gentiana lutea* (Aberham *et al*., 2007, Lin *et al*., 2004). Research by (Niiho *et al*., 2006) indicated that amaroswerin derived from *Gentiana lutea* prevented ethanol-induced gastritis in rats. Other sources of amaroswerin include *Swertia japonica, Gentiana manshurica, Gentiana purpurea and Gentiana punctata* (Quercia *et al*., 1980, Ishimaru *et al*., 1990, Zhang *et al*., 1990)

From the above, it could be deduced that Gentiana plants and their phytochemicals have variable pharmacological effects and applications. In this study however, the main point of focus was their hepatoprotective effects in terms of non-alcoholic fatty liver disease (NAFLD).

1.1. Non-alcoholic fatty liver disease (NAFLD)

The liver serves as a key determinant of the health status of an individual and hence the accumulation of increased amounts of fat in the liver produced detrimental effects on health and well-being. Non-alcoholic fatty liver disease (NAFLD) is a metabolic disorder which may include simple steatosis characterised by the accumulation of fat in the liver which does not originate from an inherent usage of alcohol. NAFLD. Non-alcoholic steatohepatitis also entails fat accumulation which occurs concomitantly with severe inflammation of the liver (Li *et al.*, 2013).

It is widely believed that NAFLD may be linked to obesity and a sedentary lifestyle. As a result, it is sometimes tagged as a disease which is more common among the affluent. Statistically NAFLD has been found to be prevalent in the general population in North America (34%) and other developed countries such as China (15%) (Dong *et al.*, 2012). According to the British Society of Gastroenterology (BSG), 33 % of the UK population have NAFLD with 2-5 % progressing to non-alcoholic steatohepatitis (NASH) (BSG, 2017).

Various clinical cases attest to NAFLD progressing to fibrosis, cirrhosis and hepatocellular carcinoma (Kristin *et al.*, 2009). NAFLD is sometimes described as a metabolic syndrome, and also denoted as the most common form liver disease with a high prevalence in the general population of Western countries (Bedogni *et al.*, 2005). Other diseases for which NAFLD has been reported to be an independent risk factor include: hypertension, hypertriglyceridemia and mixed hyperlipidaemia (Targher *et al.*, 2010). Furthermore, the pathogenesis of steatosis and cellular injury in NAFLD results in insulin resistance hepatic fat accumulation and oxidative stress (Soon Jr *et al.*, 2010). Due to the lack of an outright therapy for NAFLD, treatment has been mainly focussed on managing the conditions associated with the disease such as obesity diabetes mellitus and hyperlipidaemia. Reduction in weight helps to improve

the insulin sensitivity and prevent the progression to non-alcoholic steatohepatitis (NASH) (Trappoliere *et al.*, 2005).

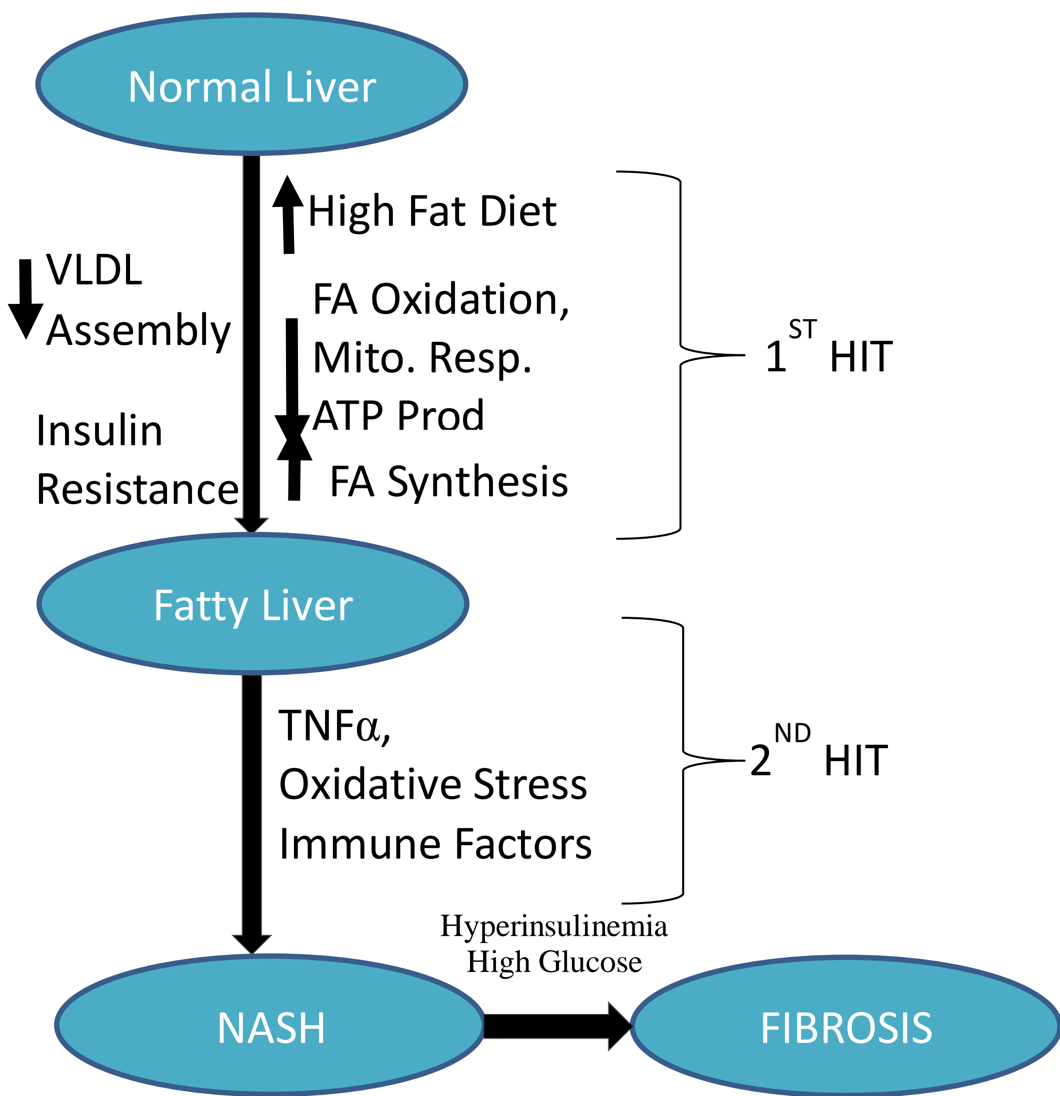


Fig 1.1 An illustration of causative factors of NAFLD and its complications. *Schematic depicting the first and second hits in NAFLD with their intermittent events eventually leading to liver fibrosis. The first hit comprises of a high fat diet associated with decreased fatty acid oxidation, decreased mitochondrial respiration as well as ATP production with an increased fatty acid synthesis. The second hit comprises increased inflammation markers notably TNF-α coupled with increased oxidative stress leading to NASH. High glucose coupled hyperinsulinemia leads to fibrosis via the activation of connective tissue growth factor (CTGF) (Paradis et al., 2001, Day and James, 1998)*

Fatty liver disease is the first stage (i.e. first-hit) in the two-hit model used to estimate NASH progression. This stage is propelled by factors such as decreased mitochondrial respiration, decreased fatty acid oxidation, decreased ATP production coupled with increased fatty acid intake and increased fatty acid synthesis. The second hit causes hepatic injury causing an increase in inflammation markers such as TNF- α alongside increased oxidative stress. These lead to NASH and then eventually liver fibrosis caused by the triggering of connective tissue growth factor (CTGF) by high glucose coupled with hyperinsulinemia as shown in Fig 1.1 (Day and James, 1998). The proceeding section evaluates the pathogenesis of NAFLD proposed NAFLD therapies and the use of Gentiana plants in managing NAFLD.

1.2 Pathogenesis and Therapeutics of non-alcoholic fatty liver disease

The continuous consumption of a high fat and or carbohydrate diet coupled with a sedentary lifestyle promotes the steatosis stage of NAFLD (Raszeja-Wyszomirska *et al.*, 2008). As a consequence of this, there is insulin resistance, due to increased levels of glucose, free fatty acids (FFAs) and insulin. Increased levels of free fatty acids cause a decrease in PPAR-α activity in the liver, resulting in significant reduction in β-oxidation. High carbohydrate intake increases expression of PPAR-γ, carbohydrate response element-binding protein-1 and sterol regulatory element-binding protein-1 with a resultant increase in fatty acid synthesis in the liver (Anderson and Borlak, 2008, Raszeja-Wyszomirska *et al.*, 2008).

A high fat diet causes Kupffer cells to release pro-inflammatory cytokines TNF-α leading to apoptosis and necrosis (Gyamfi and Patel, 2009) as shown in Fig 1.1. Silymarin is an active extract from the milk thistle plant (*Silybum marianum*) which has been mostly used in the therapeutic management of liver diseases (Comelli *et al.*, 2016). Silymarin was also found to protect hepG2 cells against palmitate-induced necrosis. Pre-treatment of HepG2 cells with silymarin prevented palmitate-induced inhibition of Akt kinase and eventual cell death. Furthermore, other studies suggested that silymarin could be an effective phytochemical against saturated fatty acid induced cell death in hepatocytes and useful in managing NASH (Song *et al.*, 2007). Hence silymarin was used as standard to which the Gentiana plants and phytochemicals were compared while investigating their hepatoprotective effects in this research. Fig 1.2 also shows the assayed possible points of intervention by Gentiana plants after the ingestion of a high fat diet. FFAs from high fat diet intake also increased lipid peroxidation, tissue inflammation and reactive oxygen species (ROS) such as: peroxides, superoxides and hydroxyl radicals leading to elevation of transaminases. The ability of gentian to stabilise the levels of transaminase enzymes; the levels of which are key determinants of liver diseases was a key feature in studies conducted by (Handoussa *et al.*, 2009). This action is mirrored by ethanol-induced LPS activation which also compels Kupffer cells to release pro-inflammatory cytokines TNF-α, consequently resulting in apoptosis as seen in the case of a high fat diet. On the other hand, decreased PPAR-α level leads to steatosis and a resultant increase in serum cholesterol, LDL - C and TG. These avenues serve as points of intervention by ostole an O-methylated coumarin which can be isolated from *Angelica pubescens*

which exerts hepatoprotective effects by decreasing TNF-α levels, increasing mRNA expression of PPAR-α and decreasing the expression of diacylglycerol acetyltransferase, 3-hydroxy-3-methylglutaryl-CoA reductase and cholesterol 7 alpha-hydroxylase (Sun *et al.*, 2009). Tectoridin which is an isoflavone isolated from *Pueraria thunbergiana* acts by modulating the peroxisome-proliferator activated receptor (PPAR) and preventing mitochondrial injury (Liu *et al.*, 2012).

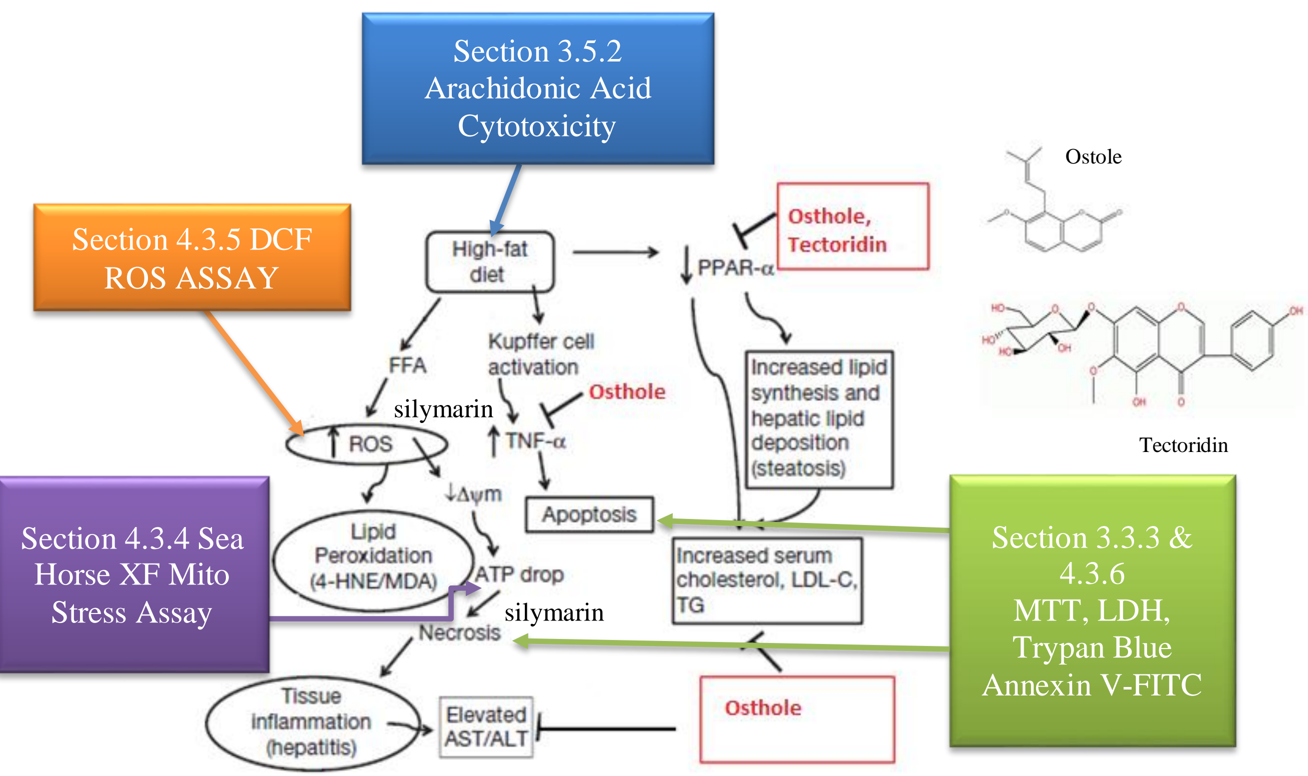


Fig 1.2 Metabolic pathways of a high fat diet leading to NAFLD. *This diagram depicts the metabolic pathways of a high fat diet and the sections of this thesis investigating possible points of intervention by Gentiana spp and phytochemicals in the fat metabolism pathway. Therapeutic intervention can be produced by silymarin (a mixture of flavonolignans extracted from milk thistle (Silybum marianum), osthole: an O-methylated coumarin which can be isolated from Angelica pubescens and tectoridin: an isoflavone which can be isolated from Pueraria thunbergiana. (Gyamfi, et al, 2009)(Song et al., 2007).*

There are studies which suggest that the administration of ghrelin hormonal therapy may have a preventive or therapeutic effect on rat NAFLD models. It was reported that ghrelin hormonal therapy caused a significant improvement in NAFLD-induced liver injury, oxidative stress, inflammation, and apoptosis by restoring the LKB1/AMPK and PI3 K/Akt pathways (Yan *et al*., 2013). There is no well-established therapy for NAFLD, however various therapies used in managing the disease are targeted at the risk factors involved in the pathogenesis aimed at reducing or fully eliminating any chance of reaching end-stage liver disease. Well-known therapeutic measures are centred on lifestyle changes, reducing sedentarism through increased physical activity; all with an aim of promoting insulin sensitivity, as well as using medications such as metformin and glibenclamide in the therapeutic process (Raszeja-Wyszomirska *et al*., 2008). Other researchers believe that a combination of N-acetyl-D,L-homocysteine-thiolactone, L-cysteine, and D-fructose can confer a certain degree of hepatoprotective effect (Stosiek *et al*., 2013).

1.3 Gentiana Plants, Silymarin and Phytochemicals Used in Treating NAFLD

The roots of *Gentiana lutea* were used in 180 BC as a tonic and *Gentian Macrophylla* used as the principal plant species in a Chinese folkloric proprietary blend called Longdan Xiegan Tang, mainly prepared as a decoction which comprises of other plants such as *Scutellariae radix, Gardeniae fructus, Alismatis rhizoma, Angelicae sinensis, Rehmanniae radix, Glycyrrhizae radix* and *Plantaginis semen* (Wang, 2007).

The Chinese Materia Medica reports that Gentian causes a reduction in jaundice while promoting gall-bladder function (Bensky *et al*., 2004). Gentian may be prepared as a tincture, alkaline mixture or acid Gentian mixture (BP, 2012). There have been reported pharmacological properties of Gentiana genus plants attributed to the presence of bitter glycosides. Notable phytochemicals found in plants belonging to the Gentianaceae family include: getianine, gentisin, amarogentin, gentiopicroside, sweroside, swertiamarin, amaroswerin, bellidifolin, swerchirin, norswertianolin and gentianadine (Singh, 2008). *Gentiana manshurica* reduced the serum levels of aspartate transaminase (AST) alanine transaminase (ALT) in rats with acute ethanol-induced hepatitis. It protects hepatocytes from ethanol-induced acute liver steatosis by potentially blocking CYP2E1-mediated free radical production and SREBP-1-regulated fatty acid synthesis (Lian *et al*., 2010).

There is an increasing interest in discovering and investigating drugs which exhibit hepatoprotective actions due to a surge in liver diseases. Statistics showing the spread of non-alcoholic fatty liver disease (NAFLD) quotes a figure of between 20–42% in the Western hemisphere and up to 24% in China (Amarapurkar *et al*., 2007).

There is an evident characterisation of liver damage portrayed through increased lipid peroxidation and depletion of glutathione levels (Rao and Raju, 2010). Although the precise mechanisms responsible for NAFLD are poorly understood, reports have shown perturbed mitochondrial function is central to the pathology, as fatty acids cause reduced mitochondrial respiration, increased free radical production and cell death (Gyamfi and Patel, 2009, Patel *et al*., 2007).

Silymarin is a mixture of flavonolignans extracted from milk thistle consisting of: silybin A, silybin B, isosilybin A, isosilybin B, silydianin and silychristin (Lee and Liu, 2003). It has also been found to significantly reduce the levels of elevated hepatic enzymes: aspartate transaminase (AST) and alanine transaminase (ALT) (Solhi *et al.*, 2014). Silybin A and B reduces the effect of NAFLD by scavenging reactive oxygen species and inhibiting the membrane absorption of phalloidine (an F-actin) and α-amanitine (a toxin). This is achieved by preventing phalloidin from binding to the cell surface whilst diminishing the membrane transporting system. The silybins have a cell membrane as well as cell nuclei effect whereby they increase the ribosomal synthesis of proteins by simulating the polymerases and RNA transcription. The reinvigoration of protein synthesis is a key step in repairing liver tissue damaged as a result of inflammation stemming from NAFLD (Hajaghamohammadi *et al.*, 2008). Supporting this point, invitro studies by (Fuchs *et al.*, 1997) suggest that silybin, which is the main component of the flavonoid silymarin scavenged free radicals and stimulated hepatocyte RNA synthesis while suppressing the growth of hepatic stellate cells and the accumulation of collagen. After inducing fibrosis in rats, silybin was found to decrease the deposition of collagen and lipid peroxidation (Trappoliere *et al.*, 2005). The antioxidant properties of silymarin have been demonstrated in both *in vitro* and *in vivo studies* (Wellington and Jarvis, 2001)

Plants such as: *Andrographis herba, Glycyrrhizae radix et rhizoma*, *Ginseng radix*, *Lycii fructus*, *Coptidis rhizoma* have all been categorized as hepatoprotective plants with anti-inflammatory and free-radical scavenging abilities. Berberine and resveratrol have been studied as bioactive compounds used in the treatment of NAFLD. Resveratrol's mechanism of action entails cell signalling, anti-apoptosis, gene expression and prevention of oxidative injury (Kovacic and Somanathan, 2010). As shown in Table 1.3 resveratrol which has a polyphenolic structure can be obtained from red grapes and other plants including *Rhizoma Polygoni Cuspidati* and *Veratrum Nigrum* whereas berberine which is an alkaloid, can be found in *Coptis chinensis.*

Apart from initiating hypoglycaemic effects, berberine is also believed to activate adenosine monophosphate-activated protein kinase (AMPK) which is a serine/protein kinase actively involved in the regulation of cellular metabolism. Resveratrol exhibits an anti-lipogenic action by up-regulating the FOXO-1 signalling pathway leading to a

reduced expression of SREBP-1, acetyl-coA carboxylase (ACC) and fatty acid synthase (FAS) with a combined effect of reduced lipogenesis and eventually a marked reduction in hepatic storage. Hepatic inflammation is also reduced through the decreased expression of TNF-α (Wang *et al.*, 2009b). This is further depicted by the schematic in Fig 1.2. Table 1.3 presents a summary of hepatoprotective phytochemicals and their bioactivities.

Table 1.3 Summary of hepatoprotective phytochemicals and their bioactivities

Phytochemical	Chemical Structure	Bioactivity	Reference
Silymarin		-ROS Scavenger -Hepatoprotective -Anti-inflammatory -↑Oxygen Consumption -↓LDH leakage -Enhanced mitochondrial fn. -↑ATP Production -↑Respiratory control ratio (RCR)	(Farghali *et al.*, 2000) (Karim, 2014) (Ligeret *et al.*, 2008)
Berberine		-Hepatoprotective -↓Lipid peroxidation -Anti-inflammatory (↓TNF-α and ↓COX-2)	(Domitrović *et al.*, 2011) (Dong *et al.*, 2012)
Resveratrol		-Antioxidant conferring protection form oxidative injury -Hepatoprotective	(Kovacic and Somanathan, 2010) (Dong *et al.*, 2012)
Osthole		-Reduction of liver injury and stabilization of liver enzymes (AST, ALT) -Hepatoprotective	(Okamoto and Kobayashi, 2007)
Tectoridin		-↓PPAR-α - Enhanced mitochondrial function	(Xiong *et al.*, 2010)

This study focused mainly on the secoiridoid glycoside phytochemicals found in Gentiana plants i.e.: gentiopicroside, swertiamarin and sweroside. This is because, these phytochemicals have been shown to possess hepatoprotective effects but have not been extensively researched (Chen *et al.*, 1993). At a dose of 25 -50 mg/kg mice body weight, gentiopicroside and sweroside showed hepatoprotective effects against d-galactosamine/lipopolysaccharide-induced liver injury (Lian *et al.*, 2010). Furthermore, the administration of swertiamarin at a dose of 100-200 mg/kg mice body weight prior to exposure to d-galactosamine exerted hepatoprotective effects by prevented the alteration of several hepatic parameters and reduced lipid peroxidation as well as oxidative stress significantly (Jaishree and Badami, 2010)

Fig. 1.3 Gentiopicroside, GPS

Gentiana lutea was found to possess hepatoprotective, anti-inflammatory and hypoglycaemic effects (Balijagić *et al.*, 2012). GPS (Fig. 1.3), present in most *Gentian spp* has shown hepatoprotective activity in mice intoxicated with carbon tetrachloride CCl_4 (Wang *et al*, 2010). Substantial amounts of gentiopicroside can be found in *Gentiana lutea*, *Gentiana macrophylla, Gentiana rigescens* and *Gentiana scabra* (Rahman, 2006). Despite the widespread use of *Gentian spp* there have been few studies on how the root extracts of the herb can be used as hepatoprotective agents. Hence this study investigates extracts of the four-above-named species of Gentiana and their phytochemicals to determine whether or not they possess any hepatoprotective characteristics and also compare them to determine the most viable species among them in this regard. Table 1.4 reviews some investigations already carried out on Gentiana plants, their aims and objectives, methodology employed, outcomes and comments on areas not covered in that spectrum which this research aims to help build up on.

Table 1.4 Summary of Research Conducted on Gentiana Plants

<table>
<tr><th>Title/Ref.</th><th>Aims and Objectives</th><th>Methodology</th><th>Results</th><th>Comments</th></tr>
<tr>
<td>Preliminary results on study of the hepatoprotective and antimicrobial effects of Gentiana asclepiadea ethanolic extract (Suciu et al., 2012)</td>
<td>To demonstrate the hepatoprotective and antimicrobial effects of gentian.</td>
<td>The active principles were extracted in 80% ethanol for 24hrs and analyses using a GC-MS. The extracts were administered to mice; and a liver transaminase analysis, histology and ultrastructural analyses of the liver conducted along with GC-MS analysis of the extracts, and microbiology tests against a number of pathological strains</td>
<td>The ethanolic extract of Gentiana asclepiadea had a hepatoprotective effect, as shown by the enzyme analysis where it reduced the ALT and AST levels in comparison to the control group, and the histology and ultrastructure analyses, both of which showed a decrease in cellular degradation as compared to the positive and negative control groups.
<table>
<tr><th></th><th>CTRL</th><th>INTOX</th><th>TREAT</th></tr>
<tr><td>AST (U/L)</td><td>278</td><td>1012</td><td>463</td></tr>
<tr><td>ALT (U/L)</td><td>137</td><td>219</td><td>70</td></tr>
</table>
Histologically, the gentian treated group showed less amounts of lipids compared to the intoxicated group.</td>
<td>This study was useful in depicting gentian as having hepatoprotective properties. However, the study did not identify and quantitate phytochemicals in the bioactive fractions. It also focused mainly on the transaminases and did not explore mitochondrial function (oxygen consumption and membrane potential), reduction of oxidative stress (free radical levels, antioxidant status, cytochrome c release). Finally, the study did not provide a clear understanding of the mechanism of action of phytochemicals in Gentiana asclepiadea. Hence the need for a</td>
</tr>
</table>

				study to investigate these phytochemicals.
Chemical profile, radical scavenging and cytotoxic activity of yellow gentian leaves (*Gentiana lutea*) grown in northern regions of Montenegro (Balijagić *et al.*, 2012)	To investigate the chemical profile cytotoxicity and radical scavenging activity of yellow gentian leaves	LC-ESI-MS and HPLC were used for the identification of the constituents from *Gentiana lutea* leaves collected at different localities, as well as for quantification of the main compounds. Concentrations of five constituents (swertiamarin, gentiopicrin, isovitexin, mangiferin and isogentisin) were determined. The relationship between concentrations of y-pyrones and altitude was observed with statistically significant correlation (r = 0.94). The extracts were also evaluated for their content of total phenolics, and	The leaf extract exhibited moderate cytotoxic effects toward HeLa cells with an IC50 value of 41.1 microg/mL, while gentiopicrin, mangiferin and isogentisin exerted strong activity against HeLa cells, with IC50 values ranging from 5.7 to 8.8 microg/mL. The results confirm the traditional usage of *Gentiana lutea* leaves and suggest their possible utilisation as hepatoprotective, hypoglycemic and anti-inflammatory agents.	This investigation placed more focus on the chemical profile of *Gentiana lutea* and its potential cytotoxic properties but was not fully focused on aligning the active phytochemicals to gentian's hepatoprotective property prompting the need for further study to determine this. The study also did not examine the mechanism of action of the isolated phytochemicals.

		antiradical and cytotoxic activities.		
Spicatic acid: A 4-carboxygentisic acid from *Gentiana spicata* extract with potential hepatoprotective activity (Handoussa *et al.*, 2009)	To investigate the hepatoprotective activity of the aqueous alcoholic extract of *Gentiana spicata* (Gentianaceae) on carbon tetrachloride treated rats was investigated.	A concentration of 1 mL/kg CCl_4 used and results derived by comparing the effects of pre-treatment with plant extracts.	The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased upon treatment with CCl_4. However, pre-treatment with gentian and its individual components significantly prevented the increase in these enzymes, which are the major indicators of liver injury.	This study also focused more on the transaminases and not mitochondrial function and membrane potential. Furthermore, quantitation and collation of bioactive phytochemicals were not carried out.
Hepatoprotective effects of *Gentiana scabra* on the acute liver injuries in mice (Jiang and Xue, 2005)	To study the hepatoprotective effect of the aerial parts and the roots of *Gentiana scabra* on acute liver injury models.	Acute liver injury models were induced by CCl_4, TAA (thioacetimidic acid) and D-GlanN in mice, and the levels of serum enzyme ALT, AST and ALP on acute liver injury mice with extracts of the aerial parts and the roots of *Gentiana scabra* determined.	Different dosages of the aerial part extract could significantly reduce the levels of serum enzyme ALT, AST and ALP ($P < 0.05$) on CCl_4 and TAA model mice, but the serum enzymes reduction of D-GlanN model mice was not significant.	This study also focussed mainly on the transaminases and on a single bioactive compound: succedaneum and did not seek to identify and quantify other bioactive phytochemicals. It also failed to elucidate a clear mechanism of action.

Gastroprotective effects of bitter principles isolated from gentian root and swertia herb on experimentally-induced gastric lesions in rats (Niiho *et al.*, 2013)	To study gastroprotective effects of the methanol extract of gentian root using different gastric lesion models	Gentian extracts were orally and duodenally administered in rats with acute gastric ulcer induced by aspirin plus pylorus ligation, water immersion restraint stress-induced ulcers, and gastric mucosal injury induced by ethanol to determine protection conferred. Amarogentin, gentiopicroside, amaroswerin, and swertiamarin, obtained from gentian root or swertia herb, were studied for their protective effects against stress-induced ulcers and ethanol-induced gastric mucosal injury	In pylorus-ligated rats, administration of gentian in the duodenum suppressed gastric juice secretion and total acid output in a dose-dependent manner. Oral or duodenum administration of gentian showed significant protection against acute gastric ulcer induced by aspirin plus pylorus ligation, water immersion restraint stress-induced ulcers, and gastric mucosal injury induced by ethanol. 500 mg/kg completely suppressed gastric juice secretion, but had no effect on ethanol-induced gastric mucosa damage at 1,000 mg/kg. Gentiopicroside obtained from n-BuOH soluble fraction of gentian root also had no effect. In contrast, 125 mg/kg ethyl acetate soluble fraction of gentian root had no effect on gastric juice secretion, but significantly protected against ethanol induced mucosal damage	This study focused more on the gastroprotective phase of gentian's broad range of effects but raised the possibility of gentian being a hepatoprotective. Building up on this information, gentiopicroside was examined for effects from a hepatoprotective point of view in this research rather than a gastroprotective point of view as covered already.

1.4 Hypothesis

This study will examine the hypothesis that the methanolic extracts and selected phytochemicals of the four Gentiana species: *lutea*, *macrophylla*, *rigescens* and *scabra* exhibit hepatoprotective effects in non-alcoholic fatty liver disease (NAFLD).

1.5 Aim

To investigate the means by which hepatocyte protection is conferred by Gentiana plants used in herbal medicine for the treatment of non-alcoholic fatty liver diseases (NAFLD).

1.6 Objectives

1. To assess Gentiana spp. extracts in order to:
   i. Identify some known phytochemicals in the extracts by HPLC and HPTLC.
   ii. Quantify selected phytochemicals in the Gentiana spp extracts by HPLC prior to screening on hepatocytes to determine their bioactivity.
2. To screen *in vitro,* the resistance of hepG2 and THLE-2 cells to fatty acid (arachidonic acid) induced cytotoxicity in the presence of Gentiana spp. as follows:
   i. Pre-treatment of hepG2 cells with Gentiana spp followed by fatty acids treatment.
   ii. Co-administration of Gentiana spp and fatty acids to hepG2 cells.
   iii. Post-treatment of hepG2 cells with Gentiana spp after they have been exposed to fatty acids
3. To investigate the effects of bioactive Gentiana spp. extracts and phytochemicals on mitochondrial function, apoptosis and reduction of oxidative stress on HepG2 cells in the presence of fatty acids in order to:
   i. Understand and evaluate the mode of hepatocyte protection conferred by bioactive extracts and phytochemicals in acting at cellular and molecular levels in the treatment of NAFLD.
   ii. Propose synergistic combinations of Gentiana spp. phytochemicals in enhancing hepatocyte protection.

Chapter 2. Qualitative and Quantitative Analysis of Gentiana: Lutea, Macrophylla, Rigescens and Scabra

2.1 Introduction

Gentiana lutea, *Gentiana macrophylla*, *Gentiana scabra* and *Gentiana rigescens* shown in Fig 2.1 are four species plants found in the Gentianaceae family of flowering plants which is composed of approximately 900-1200 species (Daniel and Sabnis, 1978).

Morphologically, *Gentiana lutea* possesses yellow flowers with spaces of 5 to 10 cm in-between, arising from four to ten pairs of pseudo-umbels (Kery *et al*., 2000). *Gentiana macrophylla* has ovato-elliptic and narrowly elliptic late basal leaves and dark-blue corolla (Zhao *et al*., 2010). In terms of *Gentiana scabra,* the flowers and leaves are sessile and opposite. The calyx is conical, membranous and has a measure of 1 cm. The leaves of *Gentiana rigescens* are simple, sessile and opposite. It has flowers which are also sessile with a 1 cm long calyx as well as a violet corolla which is bell-shaped and 2.5 cm long. Furthermore, it has a cuneate blade with nerves tapering at the base (Wiart, 2012).

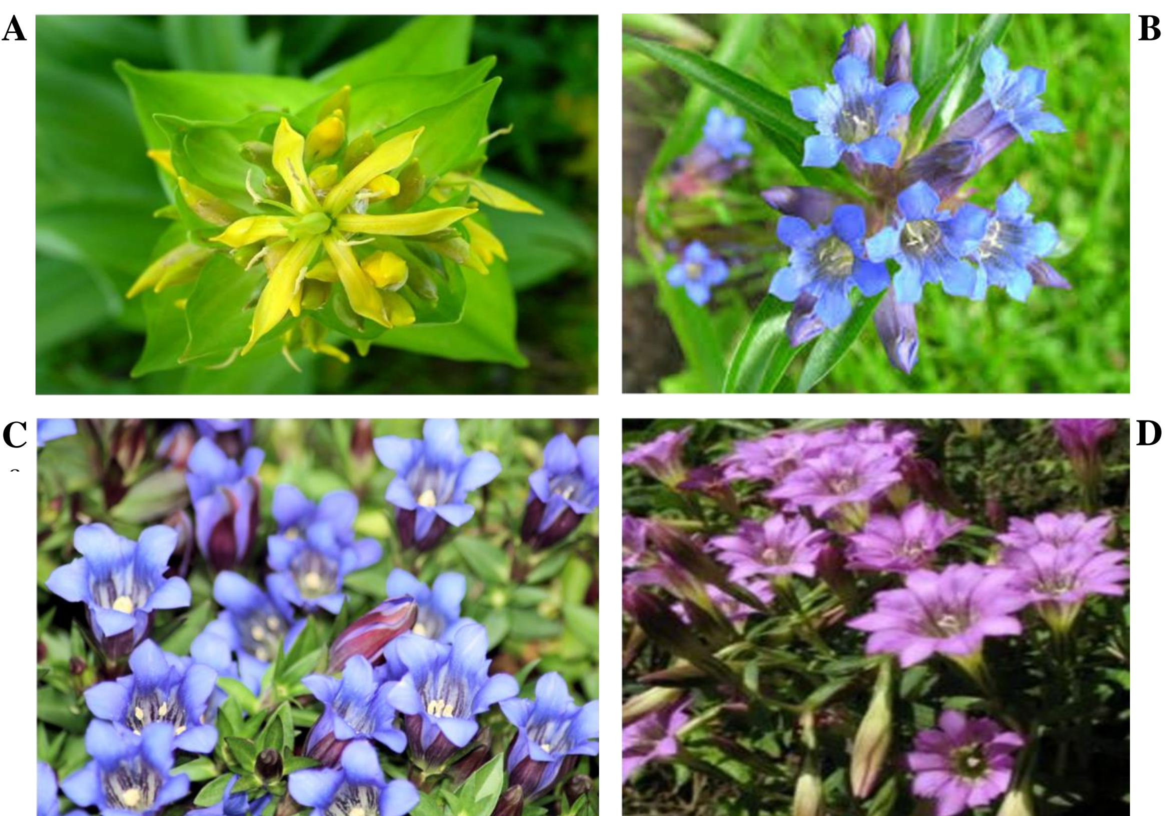


Fig 2.1. Flowering parts of Gentiana spp. *Flowering parts of: (A) Gentiana lutea, (B) Gentiana macrophylla, (C) Gentiana scabra and (D) Gentiana rigescens.*

There are variable methods used to extract dried and powdered roots of Gentiana species; most notable among them being methanolic extraction via sonication and methanolic extraction via refluxing in the presence of a heat source. According to the Chinese pharmacopoeia, 5g of Gentiana species root extract can be extracted with 20 mL of methanol under reflux for 30 min. The extract obtained is then evaporated under reduced pressure to dryness (Zhonghua Renmin, 1997). In another instance, 1 g of Gentiana species root powder was extracted using 10 mL of ethanol, refluxed for 30 mins and evaporated to dryness (Wagner *et al.*, 2016).

It has been reported that the powdered root of *Gentiana macrophylla* (10 mg) was extracted with methanol (10 mL) via sonication for 45 min at room temperature, yielding a drug/extract ratio of 35.2% (w/w) (Mustafa *et al.*, 2015). Sonication extraction method was also used by Hayata *et al.*, (2011) to extract *Gentiana cruciata* (100 mg) in 2 mL of methanol at room temperature. After HPLC, the dominant phytochemical elucidated was gentiopicroside 2.86% (w/w). Furthermore, *Gentiana macrophylla*, *Gentiana. straminea*, *Gentiana crassicaulis*, *Gentiana dahurica*, *Gentiana officinalis* and *Gentiana siphonantha* were extracted via sonication with methanol 20 mL at room temperature for 40 min (Cao and Wang, 2010).

As far as refluxing is concerned, methanolic extraction via refluxing was used in the extraction of dried root powder of *Gentiana lutea* (15 g) by refluxing the powder for 40 min in 180 mL of methanol. Quantitative HPLC assay of the extract yielded gentiopicroside, loganic acid and swertiamarin (46.3, 10.8 and 4.1 g/kg). Using this method, 1 g of dried *Gentiana lutea* root was refluxed with methanol 10 mL, for 10 min followed by filtration (Camelia *et al.*, 2008). *Gentiana rodentha* was successfully extracted by refluxing thrice with methanol leading to the identification, quantitation and isolation of rodenthoside via NMR and HPLC (Ma *et al.*, 1994). Table 2.1 presents a compilation of Gentiana species extraction methods and findings.

Table 1.1 Compilation of Gentiana spp extraction methods and findings

Plant & Quantity	Extraction Method	Phytochemicals Extracted & Quantities	References
Gentiana lutea (1 g)	Sonication	GPS (3.53 % g/g) SWE (0.15% g/g)	(Mustafa *et al.*, 2015)
Gentiana macrophylla (10 mg)	Sonication	GPS (9.7±2.0 %)	(Zheng *et al.*, 2011)
Gentiana macrophylla (0.5 g)	Sonication	GPS (65.45±1.02 mg/mL) SWE 0.18±0.002 mg/mL)	(Cao and Wang, 2010)
Gentiana scabra (0.1 g)	Sonication	GPS (2.27 mg/g) SWE 0.0162 mg/g)	(Yang *et al.*, 2009)
Gentiana rigescens (0.25g)	Refluxing	GPS (1110.6-1846.3 µg/mL) SWE (7.8-12.9 µg/mL) SWT (63-106 µg/mL	(Pan *et al.*, 2015)
Gentiana lutea (15 g)	Refluxing	GPS (28.2-62.6 g/kg) SWT (4.8 – 15.5 g/kg)	(Carnat *et al.*, 2005)
Gentiana macrophylla (500 g)	Refluxing	GPS (N/A)	(Yu *et al.*, 2004)
Gentiana rigescens (2 g)	Refluxing	Extract: material ratio (1:4-1:12 g/mL)	(Chu *et al.*, 2015)

Key: GPS- Gentiopicroside; SWE- Sweroside; SWT- Swertiamarin

Apart from the above-mentioned researchers who quantitated phytochemicals in Gentiana spp. using HPLC, other researchers have also used HPLC, although with adapted variations to suit their intended outcomes. After extracting 0.5 g powdered roots of *Gentiana manshurica*, *Gentiana scabra*, *Gentiana triflora* and *Gentiana rigescens* in methanol (10 mL) under ultrasound both gradient and isocratic HPLC conditions were used to quantify phytochemicals present in the plant species. The mobile phase used consisted of H_2O and CH_3CN as follows: isocratic - H_2O (80%): acetonitrile CH_3CN (20%); whereas for gradient, 0-22.5min - H_2O (90%): CH_3CN (10%) and then 22.5 – 25min H_2O (80%): CH_3CN (20%). UV spectra were measured with a diode-array detector from 200 to 400 nm (Jiang *et al.*, 2005). A gradient condition entailing aqueous phosphoric acid (0.4 %) was used linearly with methanol

(10-40 %) between 0 – 40 min with 5 µL of samples injected at a flow rate of 1 mL/min and detection wavelength 242 nm. The study resulted in the quantitation of loganic acid, swertiamarin, sweroside and gentiopicroside (6.4, 7.8, 65.4 and 0.1 mg/g) (Cao and Wang, 2010). Table 2.2 presents Gentiana species HPLC methods and conditions

Table 2.2 Gentiana spp HPLC methods and conditions

Plant (S)	HPLC Method	Detection (nm)	Column	Mobile Phase	Phytochemicals	Ref.
GS, GR	Gradient & Isocratic	200-400	2.5x3cm	H_2O (80%): acetonitrile CH_3CN (20%)	SWT	(Jiang and Xue, 2005)
GM	Gradient	242	C18 150x4.6mm, 5µm	Phosp. acid MeOH	GPS, SWT SWE	(Cao and Wang, 2010)
GL	Gradient	254&280	C18 5 µm	H_2O, MeOH	GPS, SWE, SWT	(Szucs *et al.*)
GL	Gradient	232	C18 150x4.6mm, 5µm	H_2O, Acetonitrile	GPS, SWT	(Aberham *et al.*, 2007)
GR	Isocratic		XR-ODS (72x1.6)	MeOH: 0.1% forminc acid in H_2O (95:5 v/v)	Gentiside (A-K)	(Pan *et al.*, 2014)
GR	Gradient		XR-ODS III (150x2.0 mm, 2.2µm)	Acetonitrile Formic acid	GPS, SWE, SWT	(Pan *et al.*, 2015)

Besides the use of HPLC, other researchers validated HPLC outcomes via high performance thin layer chromatography (HPTLC) which uses very high-resolution silica plates in a fully automated system which minimises the influence of human error experienced in conventional TLC. HPTLC has been used for the quantification of gentiopicroside in the root extracts of *Gentiana lutea* as well as for qualitative purposes (Bodart *et al.*, 1996). Swertiamarin and amarogentin have been quantified from Swertia species by HPTLC using ethanol, methanol and water. The recovery of amarogentin and swertiamarin was 94.5 % and 96.5 % respectively (Bhandari *et al.*, 2006). Gentiana rigescens extracts were analysed with HPTLC using a solvent mixture: toluene and ethyl acetate (15:1). The data obtained were analysed using three

multivariate analysis namely principal component analysis, partial least squares discrimination analysis (PLS-DA) and orthogonal PLS-DA. HPTLC model score plot showed excellent spatial distribution in all three multivariate analysis stated above. This outcome, coupled with the reproducibility and predictivity of results confirmed HPTLC as a robust method for qualitative and quantitative analysis of Gentiana plants (Ogegbo *et al.*, 2012).

Profiling Gentiana plants extracted through sonication or refluxing is a key step in obtaining a clear overview of phytochemicals present in the plants. The application of HPLC and HPTLC qualitative and quantitative methods provides further verification of the authenticity of the plants while serving as a reference point for understanding and tracing bioactive fractions of the plant extracts.

2.2 Aim

The investigations carried out in this chapter aimed at employing sonication and refluxing extraction techniques to extract *Gentiana lutea* (GL), *Gentiana macrophylla* (GM), *Gentiana scabra* (GS), and *Gentiana rigescens* (GR), after which high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) were used to qualitatively and quantitatively assess inherent phytochemicals. Gaining a clear understanding and estimations of three phytochemicals (gentiopicroside, sweroside and swertiamarin) in the Gentiana species: *lutea, macrophylla, scabra* and *rigescens* helped to portray an overview of the chemical nature of the plant extracts and outlined the basis for invitro tests carried out in chapter 3 on the hepatocytes.

2.3 Materials and Methods

2.3.1 Extraction of Gentiana spp. via Refluxing Extraction Method

Gentiana spp. extracted were *Gentiana lutea* (GL), *Gentiana macrophylla* (GM), *Gentiana scabra* (GS), and *Gentiana rigescens* (GR). Powdered roots were procured from (Beijing Tong Ren Tang, UK) and verified by Botanist Prof. Peter Li Hong Wu (Shanghai University of Traditional Chinese Medicine, China). Gentiana spp. root powder (5 g) was extracted in methanol/distilled water (75:25) by refluxing for 30

min, and the extracts were filtered via Buchner filtration and then rotary evaporated to dryness. After rotary evaporation, the extract was freeze-dried for 72 h.

2.3.2 Gentiana spp. Extraction via Sonication

The four Gentiana species mentioned in section 2.3.1, 0.2 g each was weighed and extracted with methanol (5 mL) and ultrasonicated for 30 min at room temperature. The extracts were centrifuged at 5000 rpm for 10 min and supernatants were filtered with a 0.22 μm pore membrane (Merck, Ireland) into vials for use in HPLC and HPTLC.

2.3.3 Preparation of Standard Phytochemicals: Gentiopicroside, Sweroside and Swertiamarin

For HPTLC analysis, gentiopicroside (Abcam, UK) 200 μg/mL, sweroside (Sigma-Aldrich, UK), 200 μg/mL and swertiamarin (Sigma-Aldrich, UK) 200 μg/mL were prepared in methanol. An initial stock solution was made for each phytochemical and then diluted to the desired concentration. Standards of the four phytochemicals for HPLC were prepared as follows: 0.5, 1, 5, 10, 15, 20 and 50 μg/mL in methanol.

2.3.4 HTPLC Analysis of Gentiana spp.

HPTLC was performed using the CAMAG ADC2 (CAMAG, Switzerland). Stationery phase used was 10x10 cm HPTLC plates silica gel 60 F 254 (Merck, UK), whereas a mobile phase comprising of ethyl acetate: methanol: water (10:2:1) was utilised at a solvent front position of 70 mm. For every specie of Gentian, methanolic extract (3 μL) was injected per HPTLC run, whereas gentiopicroside (3 μL) of 200 μg/mL standard solution was applied per run. After initial visualization, plates were immersed into H_2SO_4 (10%) for two seconds, dried on TLC plate heater at 105°C for 3 min before second visualization at 366 nm.

2.3.5 HPLC Analysis of Gentiana spp.

2.3.5.1 Isocratic HPLC Method for Qualitative Assessment of Gentiana spp. Extracted by sonication.

As part of qualitative experiments an isocratic RP-HPLC of the Gentian spp. extracted via sonication and gentiopicroside (standard) was performed using DIONEX AS50 (DIONEX, USA). Stationary phase used was Kinetex C18 150x4.6 mm (Phenomex, USA). A mobile phase consisting of methanol/water (18:82) was utilised in an isocratic manner at a flow rate of 1 mL/min and injection volume of 10 µL for each specie of Gentiana. Each specie was run for 45 min and detected at 233, 254 and 270 nm. Retention times and peak areas were noted and compared to that of the standard (gentiopicroside) prepared in section 2.33.

2.3.5.2 Gradient HPLC Method for Qualitative and Quantitative Assessment of Gentiana spp.

Qualitative and quantitative gradient HPLC was performed on Gentiana spp. and standards: gentiopicroside, sweroside and swertiamarin with Ultimate 3000 (Thermo Fisher Scientific, UK) using a stationary phase Kinetex C18 150x4.6 mm (Phenomex, USA). A mobile phase comprising of methanol/water with methanol (5-70 %) between 0 – 25 min gradient was utilised and 10 µL of samples injected at a flow rate of 1 mL/min. Peaks were detected at wavelengths 233, 254 and 270 nm after which peak areas were collated and used in quantifying phytochemicals presents via calibration curves. This assay was carried out for the four Gentiana species extracted by both refluxing and sonication after which quantities of phytochemicals were compared. The R square values and linear equations of the calibration curves were also noted and presented in Table 2.5.

2.3.6 Method Validation and Statistics

The reference standard solutions of gentiopicroside, sweroside and swertiamarin were prepared for a seven-point calibration curve by accurately weighing, dissolving in methanol and diluting as follows: 0.5, 1, 5, 10, 15, 20 and 50 µg/mL. Triplicate injections were made at each of the seven different concentrations. The linearity of each standard curve was made by plotting the peak area against concentration. The

resultant calibration curves were used in calculating the quantities of phytochemicals present in the four Gentiana species tested. The limit of detection (LOD) and quantitation (LOQ) under the chromatographic conditions were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. R^2 values of calibration curves ranged from 0.9958 – 0.9983. Relative standard deviation (RSD) of retention times for isocratic qualitative assessment given. All results given as ± standard deviation and are average values from three to five runs per sample in each experiment; which were also repeated at least thrice.

2.4 Results

2.4.1 HPTLC Profile of Gentiana: lutea, macrophylla, scabra and rigescens

A preliminary study to determine the phytochemical components of Gentian spp. was conducted via HPTLC analysis which showed the presence of gentiopicroside in all four species of Gentian (Fig 2.2.). For gentiopicroside, an Rf value of 0.51 was recorded. The chromatogram for *Gentiana lutea* appeared to have a vast array of bands which were also higher in intensity when compared to the three remaining Gentiana species. This was followed by *Gentiana scabra, Gentiana macrophylla* and *Gentiana rigescens* in order of decreasing band intensity.

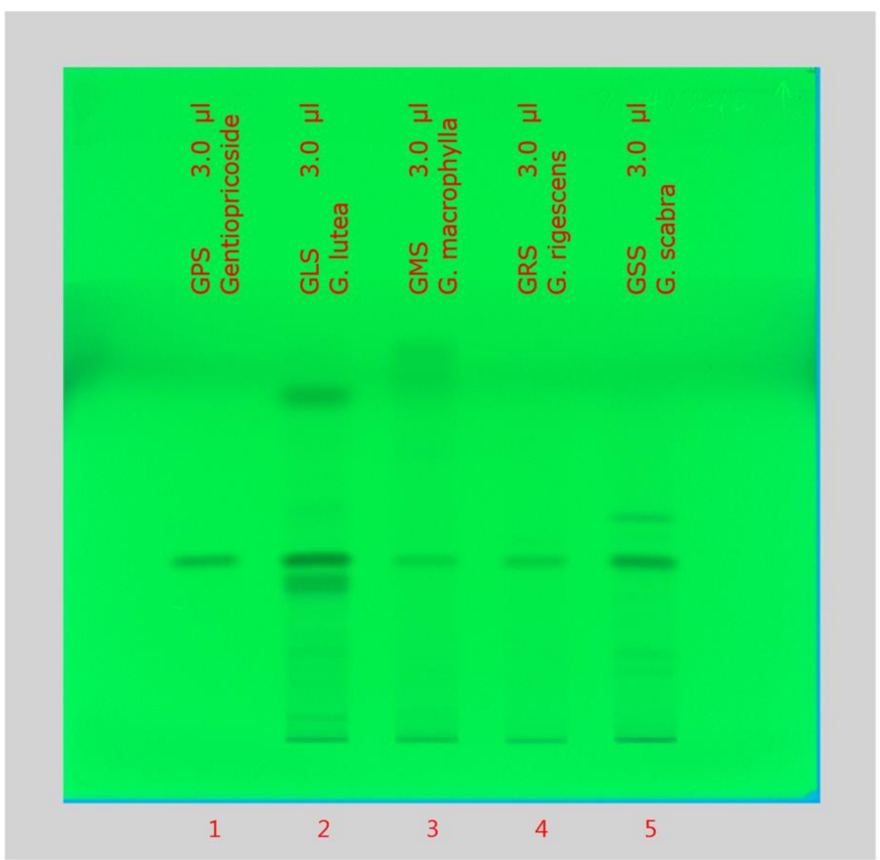


Fig 2.2 HPTLC of Sonicated Gentiana Spp. *Preliminary Priming HPTLC run of 200 µg/mL sonicated Gentiana lutea (2), Gentiana macrophylla (3), Gentiana scabra (4) and Gentiana rigescens (5) alongside standard (gentiopicroside) (1) with a band depicting the presence of gentiopicroside with RF value 0.51 in all four Gentiana spp. under 254 nm developed remissions*

In the follow-up HPTLC assays, bands representing other phytochemicals (sweroside and swertiamarin) which were also identified alongside gentiopicroside as shown in Fig 2.3-2.4. Swertiamarin presents an Rf value of 0.46 whereas sweroside presented 0.55.

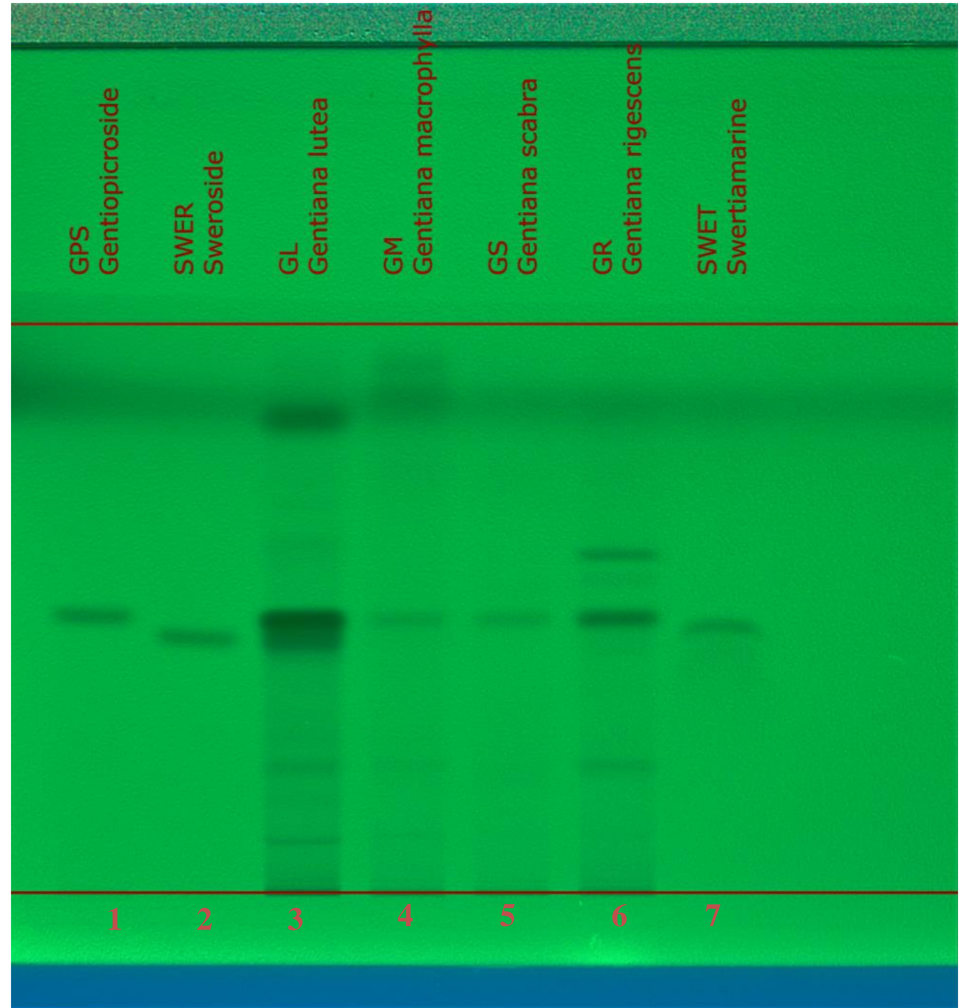


Fig 2.3 HPTLC of sonicated Gentiana spp. compared with three reference standards. *HPTLC run of refluxed 200 µg/mL Gentiana lutea (3), Gentiana macrophylla (4), Gentiana scabra (5) and Gentiana rigescens (6) alongside standards: gentiopicroside (1), sweroside (2) and swertiamarin (7) with bands depicting the presence of gentiopicroside (RF= 0.51), sweroside (RF=0.55) and swertiamarin (RF=0.46) in all four Gentiana spp. under 366 nm developed remissions*

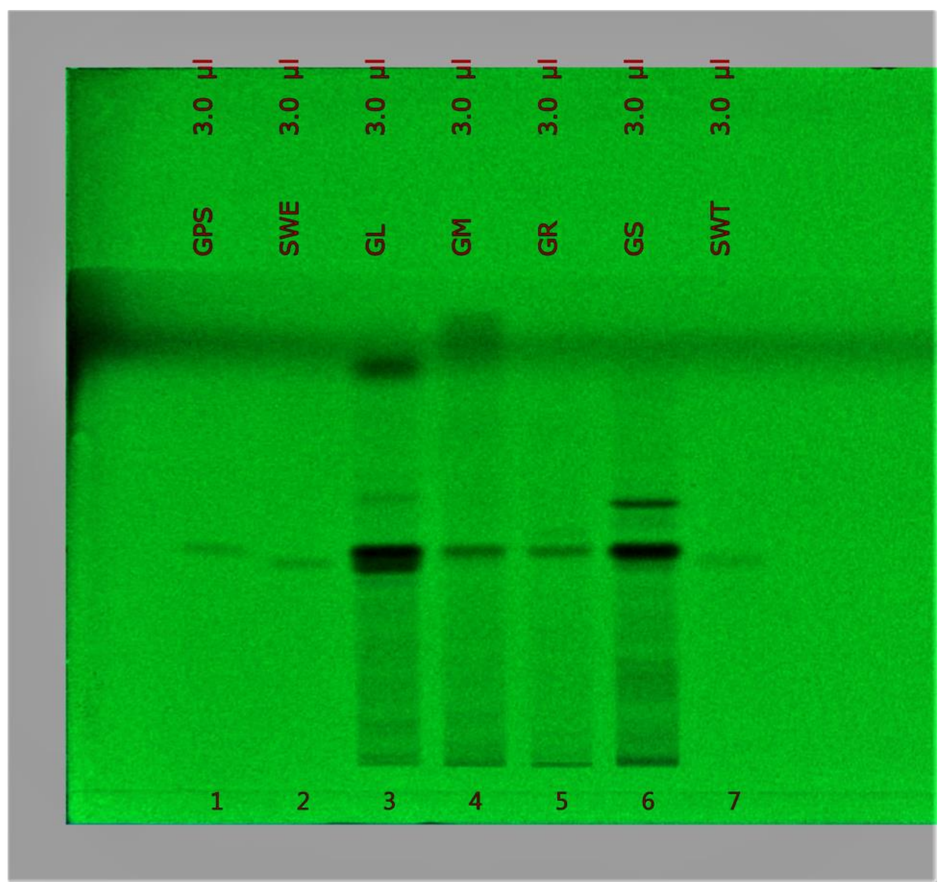


Fig 2.4 HPTLC of Refluxed Gentiana spp. compared with three reference standards. *HPTLC run of refluxed 200 µg/mL Gentiana lutea (3), Gentiana macrophylla (4), Gentiana scabra (5) and Gentiana rigescens (6) alongside standards: gentiopicroside, sweroside and swertiamarin with bands depicting the presence of gentiopicroside (RF= 0.51), sweroside (RF=0.55) and swertiamarin (RF=0.46) in all four Gentiana spp. under 366 nm developed remissions*

It was generally observed that bands generated for the refluxed Gentiana species were slightly more intense compared to bands from the sonicated Gentiana species. However there remained similarities between inter-species comparison of bands derived from Gentiana species extracted via both refluxing and sonication. Two distinctively intense green bands were observed for Gentiana lutea extracted by both refluxing and sonication. Table 2.3 presents a summary of Rf values for gentiopicroside, sweroside and swertiamarin.

Table 2.3 RF Values of Reference Standards

Reference Standard	**RF Value**
Gentiopicroside	0.51
Sweroside	0.55
Swertiamarin	0.46

2.4.3 HPLC Profile of Gentiana: lutea, macrophylla, scabra and rigescens

Further preliminary qualitative testing of Gentiana spp via HPLC (isocratic run) produced similar chromatograms for all four Gentian species with peak areas and retention as shown in (Fig.2.2). Drawing a comparison between these chromatograms and that of the standard (gentiopicroside), there was an indication of the presence of gentiopicroside in each of the Gentian species tested. The average retention time for gentiopicroside was 14.25 min (RSD 0.45 %) with the highest peak area of 12.8 mAU observed for *Gentiana lutea* (Table 2.5). This was followed by *Gentiana scabra*, *Gentiana macrophylla* and *Gentiana rigescens* in order of decreasing peak area.

Table 2.4 Comparison of Gentiopicroside Retention Times and Peak Areas Derived by Isocratic HPLC

SAMPLE NAME	RET. TIME (Min)	AREA MAU*min
Gentiopicroside	14.250	0.560
Gentiana lutea	14.267	12.797
Gentiana macrophylla	14.300	5.163
Gentiana scabra	14.267	11.665
Gentiana rigescens	14.233	1.567

%RSD of retention time =0.45

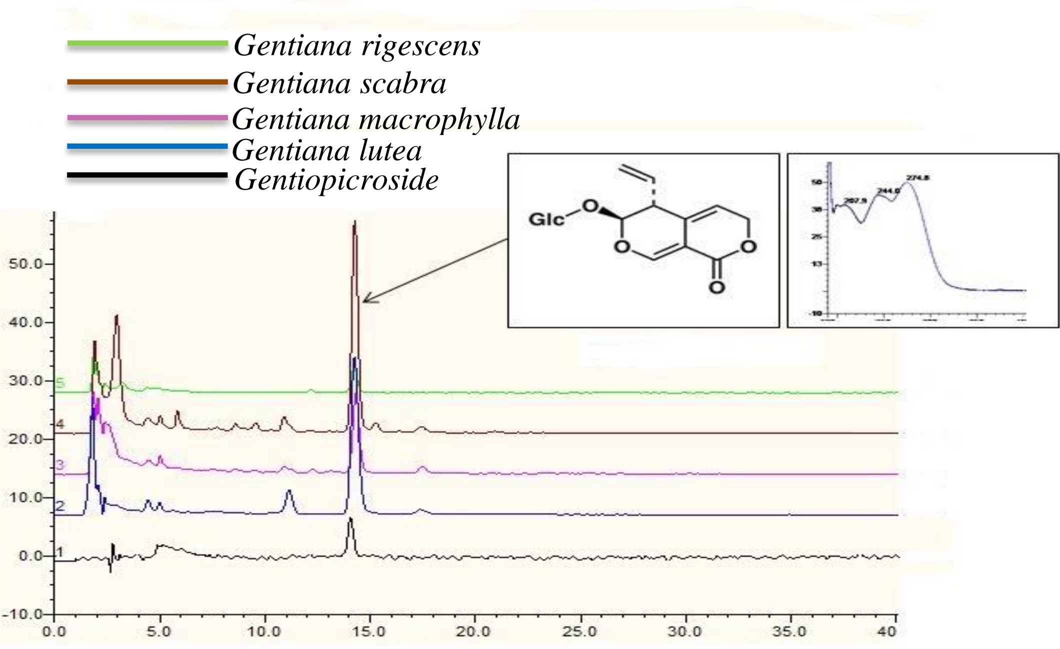


Fig. 2.5. Qualitative isocratic RP-HPLC assay of Gentian spp*. The chromatograms portray gentiopicroside bands in all four Gentiana species. RP-HPLC chromatogram: Gentiana lutea (1), Gentiana macrophylla, Gentiana scabra and Gentiana rigescens. Each species of Gentian contained gentiopicroside (arrowed) at 233 nm. Arrowed is the chemical structure of gentiopicroside.*

In order to obtain a full spectrum of phytochemicals present in the four Gentiana species, gradient HPLC was run which showed peaks representing gentiopicroside, sweroside and swertiamarin in each of the four Gentiana species (Fig 2.6). The average retention times were as follows: gentiopicroside (12.4 min), sweroside (12.9 min) and swertiamarin (11.7 min). Apart for the three afore-mentioned phytochemicals, other peaks also observed in the chromatograms obtained for each of the four Gentiana species. Notably in *Gentiana lutea,* the highest array of different peaks were observed which seemed to shed more light on the multiple bands observed in the HPTLC ass of sonicated *Gentiana lutea.*

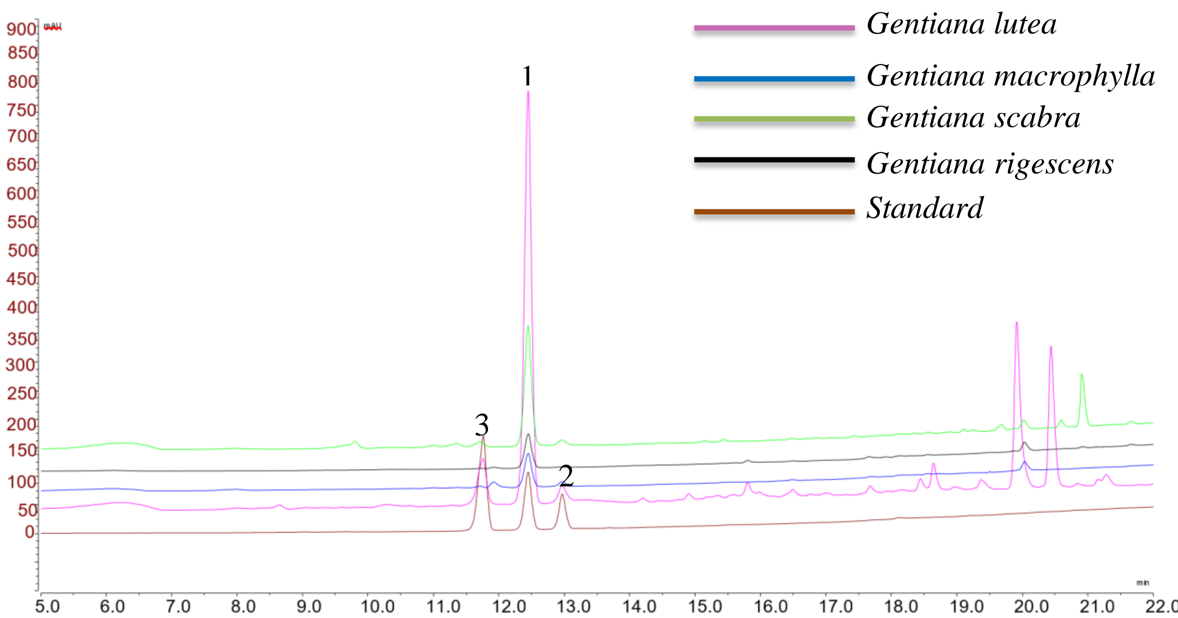


Fig 2.6 RP-HPLC-DAD Chromatograms of Gentiana spp extracted by sonication. *HPLC chromatograms at 233 nm showing the phytochemical profile of Gentiana lutea, Gentiana macrophylla, Gentiana scabra and Gentiana rigescens extracted by sonication with identified phytochemicals: (**1**) gentiopicroside (ret time 12.413), (**2**) sweroside (ret time 12.94) and (**3**) swertiamarin (11.717). Lutea species presents the highest amounts of each identified phytochemicals*

HPLC analysis of Gentiana species extracted by refluxing produced chromatograms in all four Gentiana species which were similar to chromatograms observed for Gentiana species extracted by sonication. The presence of gentiopicroside, sweroside and swertiamarin was also confirmed in each of the four species as shown in Fig 2.7. with retention times similar to those stated for the sonicated extracts. In this instance *Gentiana lutea* presented the most dominant array of peaks followed by *Gentiana scabra*, *Gentiana macrophylla* and *Gentiana rigescens*.

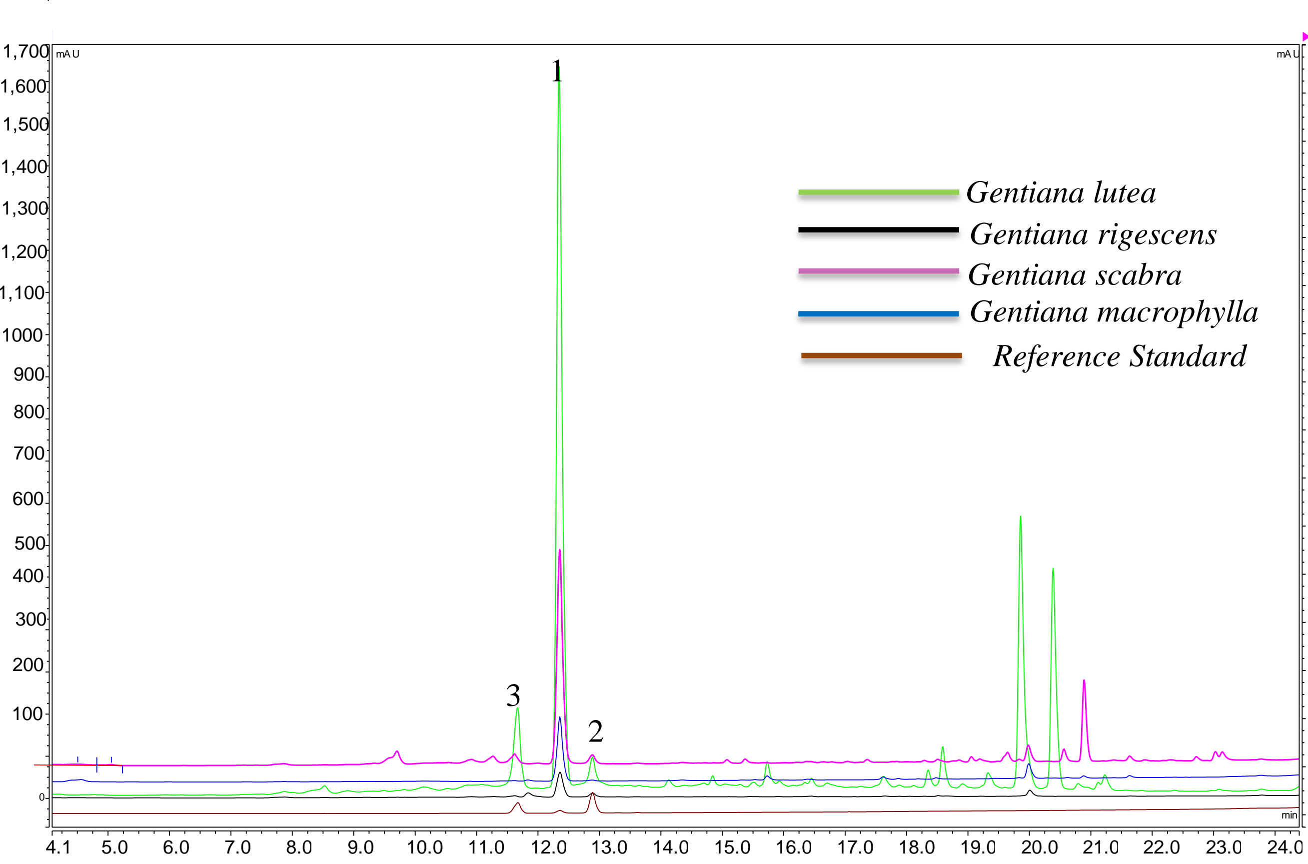


Fig 2.7 RP-HPLC-DAD Chromatogram Overlay for Gentiana spp extracted by refluxing*.* *HPLC Chromatograms overlay showing the phytochemical profile of Gentiana lutea, Gentiana macrophylla, Gentiana scabra and Gentiana rigescens extracted by refluxing aligned with standards phytochemicals: (**1**) gentiopicroside (ret time 12.413), (**2**) sweroside (ret time 12.93) and (**3**) swertiamarin (ret time 11.717) at 233nm.*

Quantitation of gentiopicroside, sweroside and swertiamarin was initiated by calibration of the standards at seven concentration points (0.5, 1, 5, 10, 15, 20, and 50 μg/mL). Details of intra-day gentiopicroside calibration tables can be found in Appendix A. As shown in fig 2.8 a mixture of the reference standards produced three peaks at 233 nm representing gentiopicroside, sweroside and swertiamarin. A liner equation of y=0.1371x + 0.0592 and R square value of 0.9982 was obtained for gentiopicroside as seen in Fig 2.9. There was a linear correspondence of gentiopicroside increment with peak area as observed in Fig 2.9

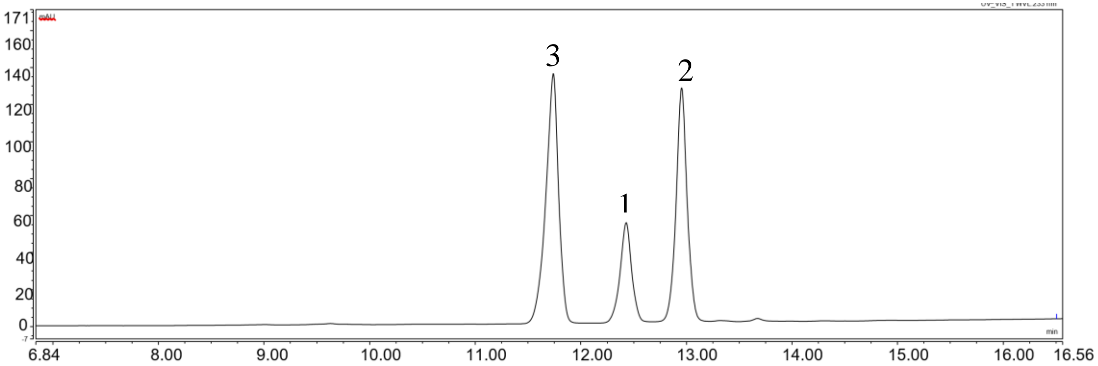


Fig 2.8 RP-HPLC-DAD Chromatograms of combined reference standards. *HPLC chromatograms at 233 nm showing standard phytochemicals: (**1**) gentiopicroside (ret time 12.413), (**2**) sweroside (ret time 12.94) and (**3**) swertiamarin (11.717).*

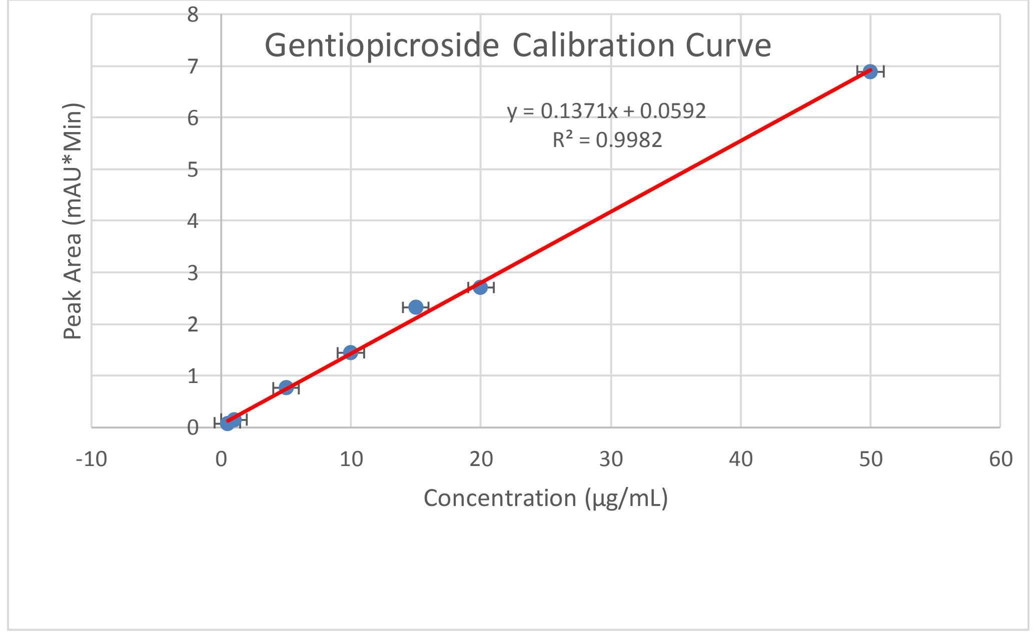


Fig 2.9 A graph of gentiopicroside peak area against concentration*. Calibration curve of gentiopicroside at concentrations 0.5, 1, 5, 10, 15, 20 and 50 µg/mL with line equation y=0.137x+0.0592 and R^2 value of 0.9982*

For sweroside, calibration was conducted at concentration points (0.5, 1, 5, 10, 15, 20, and 50 µg/mL). A rise in sweroside concentration corresponded with an increment in peak area represented by line equation y = 0.3043x + 0.0163 and R square value of 0.9998 as seen in Fig 2.10. Intra-day calibration tables for each of the seven concentrations can be found in Appendix B.

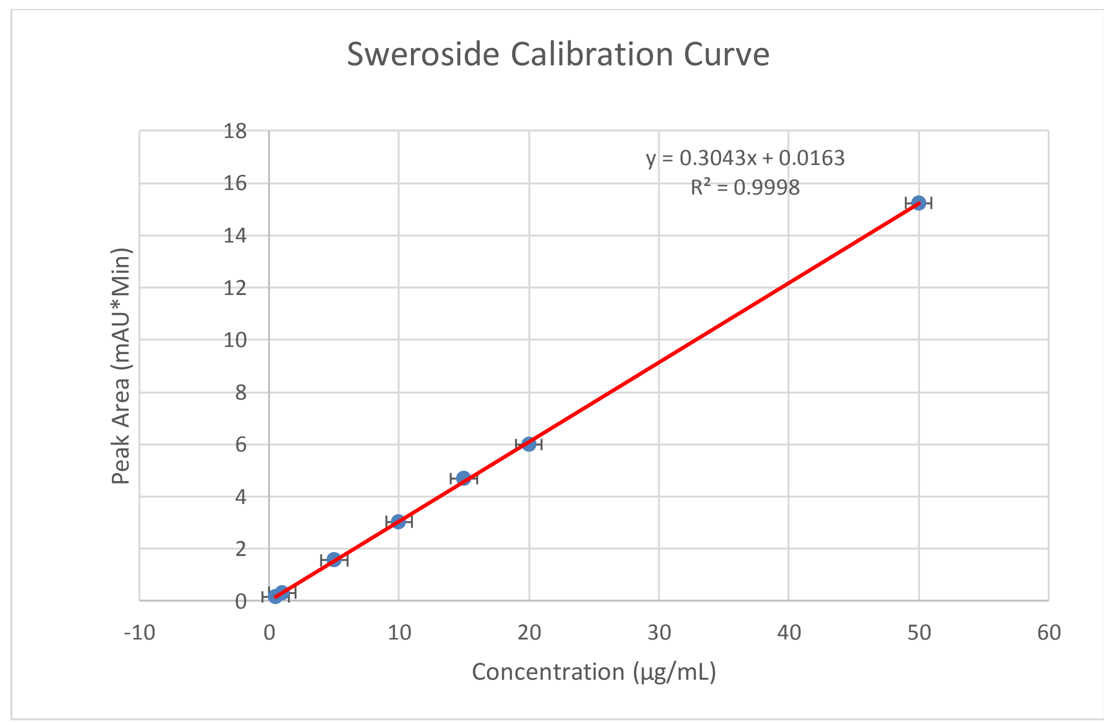


Fig 2.10 A graph of sweroside peak area against concentration. *Calibration curve of sweroside at concentrations 0.5, 1, 5, 10, 15, 20 and 50 µg/mL with line equation y=0.3043x+0.0163 and* R^2 *value of 0.9998*

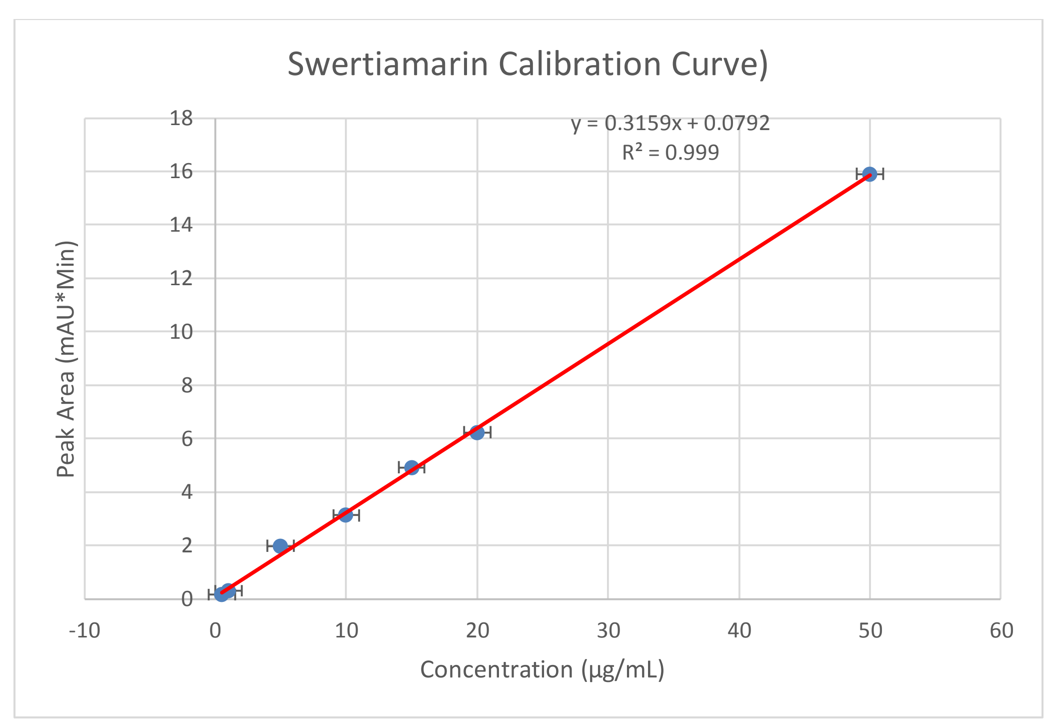


Fig 2.11 A graph of swertiamarin peak area against concentration. *Calibration curve of swertiamarin at concentrations 0.5, 1, 5, 10, 15, 20 and 50 µg/mL with line equation y=0.3159x+0.0802 and R^2 value of 0.999*

Calibration of swertiamarin at concentration points (0.5, 1, 5, 10, 15, 20, and 50 µg/mL) corresponded linearly to rise in peak areas with a line equation y = 0.3159x + 0.0792 and R square value of 0.999 as shown in Fig 2.10. Detailed calibration tables for swertiamarin can be found in Appendix C.

As shown in Table 2.5 limit of detection (LOD) values of gentiopicroside were calculated 0.00153 with 0.00160 for sweroside and 0.00146 for swertiamarin. Limit of quantitation (LOQ) values calculated for gentiopicroside, sweroside and swertiamarin were (0.0153, 0.0160 and 0.0146) respectively.

Table 2.5 Summary Calibration Table for Gentiopicroside, Sweroside and Swertiamarin

Compound	**Regression Equation**	R^2	**LOD**	**LOQ**
Gentiopicroside	y=0.137x+0.0592	0.9982	0.00153	0.0153
Sweroside	y=0.3043x+0.0163	0.9998	0.00160	0.0160
Swertiamarin	y=0.3159x+0.0802	0.9991	0.00146	0.0146

As shown in Table 2.6 inter-day HPLC precision of gentiopicroside found in 200 µg/mL *Gentiana lutea* extracted by refluxing produced peak areas comparable to intra-day figures for the same amount of *Gentiana lutea* refluxed extracts. Gentiopicroside inter-day peak areas ranged from 2.8199-2.8921 mAU*Min whereas an average of 2.7547 mAU*Min was recorded intra-day.

Sweroside inter-day peak areas ranged from 0.1251-1.1424 mAU*Min with averaged 0.1184 mAU*Min intra-day.

Swertiamarin yielded inter-day peak areas ranging from 0.4083-0.4329 mAU*Min which was similar to the averaged intra-day peak area of 0.4437 mAU*Min. RSD values of gentiopicroside, sweroside and swertiamarin for both inter-day and intra-day precision studies were below 1 % as seen in Table 2.6.

Further intra-day precision data for peak areas of gentiopicroside, sweroside and swertiamarin obtained from 100, 500 and 1000 µg/mL *Gentiana lutea* extracted by refluxing can be seen in Appendices H, J and L

Table 2.6 Intra-day and Inter-Day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed *Gentiana lutea* Based on Peak Areas with RSD

	Inter-day (n=3)			**Intra-day (n=3)** **(mAU*Min)**
Compound	**Day 1** **(mAU*Min)**	**Day 2** **(mAU*Min)**	**Day 3** **(mAU*Min)**	
Gentiopicroside	2.8199 SD=0.0050 RSD =0.18 %	2.8921 SD=0.0040 RSD=0.13 %	2.8257 SD=0.0013 RSD=0.05 %	2.7547 SD=0.0012 RSD=0.44 %
Sweroside	0.1251 SD=0.006 RSD =0.18 %	0.1424 SD=0.001 RSD=0.99%	0.1284 SD=0.0005 RSD=0.36 %	0.1184 SD=0.0005 RSD=0.39 %
Swertiamarin	0.4329 SD=0.002 RSD =0.53 %	0.4414 SD=0.0013 RSD=0.29 %	0.4083 SD=0.002 RSD=0.49 %	0.4437 SD=0.0018 RSD=0.41 %

In the case of 200 µg/mL *Gentiana lutea* extracted by sonication, inter-day peak areas obtained for the three phytochemicals were comparable to the intra-day peak area average. Gentiopicroside yielded 0.8427-0.8548 mAU*Min comparable to an intra-day average of 0.8359 mAU*Min as seen in Table 2.7.

The inter-day values obtained for sweroside ranged from 0.0382-0.04217 mAU*Min compared to 0.04031 mAU*Min intra-day. Swertiamarin also had inter-day values ranging from 0.1147-0.1191 mAU*Min with 0.1149 mAU*Min as intra-day.

Further intra-day precision data for peak areas of gentiopicroside, sweroside and swertiamarin obtained from 100, 500 and 1000 µg/mL *Gentiana lutea* extracted by sonication can be seen in Appendices I, K and M.

Table 2.7 Intra-day and Inter-Day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated *Gentiana lutea* Based on Peak Areas with RSD

	Inter-day (n=3)			**Intra-day (n=3) (mAU*Min)**
Compound	**Day 1 (mAU*Min)**	**Day 2 (mAU*Min)**	**Day 3 (mAU*Min)**	
Gentiopicroside	0.8427 SD=0.003 RSD =0.30 %	0.8583 SD=0.002 RSD= 0.30 %	0.8584 SD=0.003 RSD=0.31 %	0.8359 SD=0.004 RSD= 0.48 %
Sweroside	0.0382 SD=0.0004 RSD = 1.14 %	0.04217 SD=0.001 RSD=1.35 %	0.0422 SD=0.0006 RSD=1.41 %	0.04031 SD=0.0002 RSD=0.50 %
Swertiamarin	0.1147 SD=0.003 RSD =2.49 %	0.1168 SD=0.001 RSD= 0.82%	0.1191 SD=0.002 RSD= 1.71%	0.1149 SD=0.001 RSD= 0.47 %

As shown in Table 2.8 inter-day HPLC precision of gentiopicroside found in 200 µg/mL *Gentiana macrophylla* extracted by refluxing produced peak areas comparable to intra-day figures for the same amount of *Gentiana macrophylla* refluxed extracts. Gentiopicroside inter-day peak areas ranged from 0.9917-1.0209 mAU*Min whereas an average of 0.9792 mAU*Min was recorded intra-day.

Sweroside inter-day peak areas ranged from 0.0875-0.0912 mAU*Min with averaged 0.0872 mAU*Min intra-day.

Swertiamarin yielded inter-day peak areas ranging from 0.1136-0.1234 mAU*Min which was similar to the averaged intra-day peak area of 0.1151 mAU*Min. RSD values of gentiopicroside, sweroside and swertiamarin for both inter-day and intra-day precision studies as seen in Table 2.8 were low indicating that the data is tightly clustered around the mean.

Further intra-day precision data for peak areas of gentiopicroside, sweroside and swertiamarin obtained from 100, 500 and 1000 µg/mL *Gentiana macrophylla* extracted by refluxing can be seen in Appendices N and P.

Table 2.8 Intra-day and Inter-Day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed *Gentiana macrophylla* Based on Peak Areas with RSD

	Inter-day (n=3)			**Intra-day (n=3)**
Compound	**Day 1 (mAU*Min)**	**Day 2 (mAU*Min)**	**Day 3 (mAU*Min)**	**(mAU*Min)**
Gentiopicroside	0.9917 SD=0.001 RSD =0.14 %	1.0209 SD=0.002 RSD= 0.23 %	0.9934 SD=0.002 RSD= 0.22 %	0.9792 SD=0.0013 RSD= 0.13%
Sweroside	0.0875 SD=0.0001 RSD = 2.12 %	0.0912 SD=0.003 RSD=2.82 %	0.0835 SD=0.001 RSD=1.21%	0.0872 SD=0.002 RSD=2.35 %
Swertiamarin	0.1234 SD=0.003 RSD = 2.43 %	0.1136 SD=0.001 RSD= 0.54 %	0.1140 SD=0.001 RSD= 4.5 %	0.1151 SD=0.004 RSD= 3.48%

The results for 200 µg/mL *Gentiana macrophylla*_extracted by sonication presented inter-day peak areas for the three phytochemicals comparable to the intra-day peak

area average. Gentiopicroside produced 0.0619-0.0.0671 mAU*Min comparable to an intra-day average of 0.06010 mAU*Min as seen in Table 2.9.

The inter-day values obtained for sweroside ranged from 0.0070-0.00757 mAU*Min compared to 0.0083 mAU*Min intra-day. Swertiamarin also had inter-day values ranging from 0.0080-0.0084 mAU*Min with 0.0089 mAU*Min as intra-day. Low RSD values obtained for inter and intra-day results denoted data clustering around the mean.

Further intra-day precision data for peak areas of gentiopicroside, sweroside and swertiamarin obtained from 100, 500 and 1000 µg/mL *Gentiana macrophylla* extracted by sonication can be seen in Appendices O and Q.

Table 2.9 Intra-day and Inter-Day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated *Gentiana macrophylla* Based on Peak Areas with RSD (in parenthesis)

	Inter-day (n=3)			**Intra-day**
Compound	**Day 1** (mAU*Min)	**Day 2** (mAU*Min)	**Day 3** (mAU*Min)	**(n=3)** (mAU*Min)
Gentiopicroside	0.0619 SD=0.0003 RSD =0.43 %	0.0647 SD=0.0006 RSD= 0.94 %	0.0671 SD=0.0004 RSD= 0.59%	0.06010 SD=0.001 RSD= 1.64%
Sweroside	0.00745 SD=0.0002 RSD =2.87 %	0.00757 SD=0.004 RSD=5.52 %	0.0070 SD=0.001 RSD=1.42 %	0.0083 SD=0.003 RSD=0.36 %
Swertiamarin	0.0082 SD=0.0001 RSD = 1.22 %	0.0084 SD=0.0003 RSD=3.6 %	0.0080 SD=0.0001 RSD= 1.25%	0.0089 SD=0.0001 RSD= 1.12 %

A similar trend in results obtained from precision studies was observed for *Gentiana scabra* and *Gentiana rigescens* extracted by refluxing and sonication. Tables representing these results can be found in Appendices D-G.

Quantitation results showed the most dominant phytochemical in all the four Gentiana species notwithstanding the method of extraction was gentiopicroside. The highest amount of gentiopicroside (4.7 % g/g) was found in the root powder of *Gentiana lutea* extracted by refluxing. This was followed by (1.9 % g/g) found in *Gentiana scabra* also extracted by refluxing. It appeared that the refluxed extracts contained higher quantities of phytochemicals than sonicated extracts. A slightly higher amount of sweroside (0.0022% g/g) more was found in refluxed *Gentiana macrophylla* root powder when compared to *Gentiana lutea* extracted by the same method. Finally, the highest quantity of swertiamarin (0.8% g/g) was contained in refluxed *Gentiana lutea* root powder as shown in Table 2.10. More details about the quantity of phytochemicals in extracts administered to hepatocytes during this study can be found in chapter 3 which deals with cell work and hepatocyte treatments.

Table 2.10 Summary Quantitation of Gentiana Spp. Extracted Via Refluxing and Sonication (RSD Values in Parenthesis)

	SONICATED G.SPP	**REFLUXED G.SPP**	
G. LUTEA	**Root Powder (%g/g)**	**Crude Extract (%g/g)**	**Root Powder (%g/g)**
Gentiopicroside	3.7460 (0.52)	10.1185 (0.24)	4.6545 (0.17)
Sweroside	0.1728 (1.90)	0.8016 (2.0)	0.4050 (1.4)
Swertiamarin	0.3079 (2.31)	1.3204 (1.0)	0.7580 (0.7)
G. MACROPHYLLA	**SONICATED G.SPP**	**REFLUXED G.SPP**	
	Root Powder (% g/g)	**Crude Extract (%g/g)**	**Root Powder (%g/g)**
Gentiopicroside	0.2804 (0.11)	3.3520 (0.35)	1.5928 (0.2)
Sweroside	0.0267 (0.17)	0.9080 (1.3)	0.4072 (0.83)
Swertiamarin	0.0934 (1.5)	0.6001(0.6)	0.2715 (1.7)
G. RIGESCENS	**SONICATED G.SPP**	**REFLUXED G.SPP**	
	Root Powder (% g/g)	**Crude Extract (%g/g)**	**Root Powder (%g/g)**
Gentiopicroside	0.2816 (0.8)	0.9001 (0.2)	0.4010 (1.5)
Sweroside	0.0140 (2.5)	0.0841 (3.8)	0.0331 (2.1)
Swertiamarin	0.0170 (1.4)	0.0968 (2.01)	0.0427 (0.3)
G. SCABRA	**SONICATED G.SPP**	**REFLUXED G.SPP**	
	Root Powder (% g/g)	**Crude Extract (%g/g)**	**Root Powder (%g/g)**
Gentiopicroside	0.9312 (0.1)	3.6011 (0.27)	1.850 (0.18)
Sweroside	0.0276 (1.4)	0.7134 (2.3)	0.3270 (1.5)
Swertiamarin	0.1076 (3.3)	0.9083 (1.8)	0.5030 (3.5)

2.5 Discussion

Performing the HPTLC and RP-HPLC analysis of all Gentian species was a very important stage in further validating their authenticity and usefulness in carrying out the remaining experiments on cells. The confirmation of substantial amounts of gentiopicroside, sweroside and swertiamarin in all four Gentiana species tested first by HPTLC and then further substantiated by RP-HPLC provided a key point of reference and foundation for understanding their varied effects on liver cells as shown in Chapters 3 and 4. The quantitation also served as a basis for aligning phytochemicals to the hepatocyte protective effects which were observed.

Methanolic extracts of *Gentiana lutea,* which were extracted under vacuum and tested qualitatively by RP-HPLC-DAD contained gentiopicroside, amarogentin, sweroside, swertiamarin, gentisin and gentioside isomers (Szucs, 2002). The quantities of gentiopicroside (3.7 %g/g), sweroside (0.2 %g/g) and swertiamarin (0.3 %g/g) obtained in sonicated *Gentiana lutea* were similar to the quantitation range obtained by (Mustafa *et al*., 2015) who also extracted the lutea species via sonication to obtain gentiopicroside (1.85–3.97 %g/g), sweroside (0.05–0.35 %g/g) and swertiamarin (0.08–0.3 %g/g), making lutea the species with the highest amounts of all three phytochemicals. Furthermore, investigations by (Hayta *et al*., 2011) on the underground parts of wild growing *Gentiana curcurita* resulted in the identification of the presence of three main secoiridoid-glycosides : gentiopicroside which was in higher quantities as well as, swertiamarin and sweroside both of which were always in lower quantities. In a study to determine the amounts of gentiopicroside and swertiamarin in *Gentiana macrophylla*, *Gentiana rigescens* and *Gentiana scabra* all extracted via sonication, (Zhao *et al*., 2004) found swertiamarin (0.17% g/g) in Gentiana macrophylla which was close to the 0.1% g/g swertiamarin found for sonicated macrophylla species in this study. However, there were slight variations in the quantities of gentiopicroside, sweroside and swertiamarin found in rigescens and scabra. For instance, the study found a nil (or too low to quantitate) amount of swertiamarin in rigescens whereas this investigation found 0.02% g/g swertiamarin in the rigescens species.

These differences could be attributed to the different climate, soil, species and growth periods of the plants. An amount of 0.5g of fourteen different *Gentiana macrophylla*

samples grown in different climatic conditions were extracted in 20 ml of methanol for 30 min by (Qi *et al*., 2012) which upon comparison to the aforementioned research quantitated gentiopicroside in a wide range of between 0.04% g/g to 0.78% g/g showing the relevance of climate and growth conditions when quantifying Gentiana spp. The quantity of gentiopicroside obtained for this study (0.30% g/g) however fell within this stipulated range. Dried roots of *Gentiana scabra* which were extracted by sonication contained gentiopicroside (1.1% g/g) and sweroside (0.05% g/g) (Jiang *et al*., 2005). This was comparable with 0.9% g/g and 0.03% g/g for gentiopicroside and sweroside respectively obtained in this study. In considering Gentiana plants extracted by refluxing, (Carnat *et al*., 2005), quantified gentiopicroside (2.8% g/g to 6.2% g/g) in naturally dried *Gentiana lutea* species which corresponded to 4.6% g/g obtained for this study. It is noteworthy that the study by Carnat *et al*., (2005) also highlighted that differences in quantities of phytochemicals were caused by different drying methods used. The similarities between the earlier stated results and that of this study may be due to the use of refluxing extraction in both cases and the fact that *Gentiana lutea* roots which were commercially obtained had been dried naturally as well.

It was generally observed that extracts obtained by refluxing in this study contained higher levels of phytochemicals gentiopicroside, sweroside and swertiamarin compared to sonicated extracts. This was also evidenced in the quantities of the afore-mentioned phytochemicals derived from the earlier stated investigations which utilised sonication compared to the quantities derived from investigations which applied the refluxing method. Hence, the refluxed extracts were chosen for cell work. These were freeze-dried and used for all the tests on liver cells discussed in the follow-up chapters. Considering both refluxed and sonicated Gentiana species, *lutea* emerged with the highest amounts of gentiopicroside, sweroside and swertiamarin followed by scabra, *macrophylla* and *rigescens* in descending order. Between (0.21–0.45% g/g) of swertiamarin, and up to 9.53% g/g of the most dominant compound gentiopicroside was found in different samples of *Gentiana lutea* plants tested at the same time.

Other compounds such as amarogentin found in Gentiana are in trace amounts (Aberham *et al*., 2007). It has been reported that methanol, water, ethanol and chloroform are ideal solvents for separating iridoid glycosides such as gentiopicroside (Giddings, 1983). In this study however, utilising ethyl acetate: methanol: water

(10:2:1) in HPTLC of Gentian spp. elucidated gentiopicroside, sweroside and swertiamarin. Gentiopicroside bands obtained in all four Gentiana species had an RF value of 0.51. This was similar to an Rf value range of 0.55-0.56 obtained for gentiopicroside identified in *Gentiana lutea* via HPTLC (Camelia *et al.*, 2008). Furthermore, a mobile phase of methanol/water 82:18 used under HPLC isocratic conditions, for the quantitative study yielded a dominant peak representing gentiopicroside, however there were inconspicuous peaks seen for sweroside, swertiamarin and xanthone glycosides as seen in typical chromatograms of the Gentiana species extracted via sonication but analysed in a gradient HPLC. Using methanol/water under gradient conditions described in the methodology a wider spectrum of peaks was observed and hence that method was implemented in proceeding quantitation experiments. A broad spectrum of peaks were observed for the extraction of *Gentiana lutea* under gradient conditions with mobile phase composed of 0.085% (v/v) of phosphoric acid in water and acetonitrile (Aberham *et al.*, 2011).

After verifying the presence of gentiopicroside, sweroside, and swertiamarin in the four Gentiana species tested and gaining a preliminary profile of the quantities of gentiopicroside, sweroside and swertiamarin phytochemicals in them, the next step entailed testing the extracts to determine their effects on liver cells, factoring in the influence of fatty acids.

2.6 Conclusion

This study achieved the aim of employing sonication and refluxing extraction techniques to extract the four Gentiana species, after which high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) were used to qualitatively and quantitatively assess three of the inherent phytochemicals. The identified and quantified phytochemicals were gentiopicroside, sweroside and swertiamarin. After satisfying the aim of this chapter, the next step was to test, first the whole plant extracts followed by the individual phytochemicals identified on liver cells exposed to fatty acids to determine whether or not they possessed any hepatocyte protective effects. These aspects are covered in Chapter 3.

Chapter 3. Influence of Gentiana Spp. Extracts on Cell Viability of Hepatocytes Treated with Lipid (arachidonic acid)

3.1 Introduction

An understanding of the effects of exposing hepatocytes to fatty acids such as decreased ATP production, lipid peroxidation and deceased cell viability are key to deciphering any possible interventions caused by Gentiana plant extract treatment. Fatty acids (FA) play a pivotal role in intracellular signaling and form an important component of ligands which bind onto nuclear receptors making them crucial for cell viability (Chawla *et al.*, 2001). This chapter examines the outcomes of pre-treating hepatocytes with Gentiana spp. extracts before fatty acid exposure, co-administering fatty acids and Gentiana spp. extracts to hepatocytes and finally, pre-treating hepatocytes with fatty acids before the administration of Gentiana spp. extracts. These outcomes were assessed via trypan blue assay, LDH assay, MTT assays and analysed via statistical methods setting a precedent for detailed mitochondrial stress, ATP production, apoptosis and ROS studies carried out in chapter 4.

Studying fatty acid uptake is crucial in understanding steatosis, which is a prominent feature of non-alcoholic fatty liver disease (NAFLD). The increase in serum-free fatty acids causes a rise in hepatocyte fatty acid uptake in excess of metabolic requirements. This leads to excessive storage of triglycerides resulting in steatosis and provides a substrate for lipid peroxidation (Bradbury, 2006).

Fatty acids such as arachidonic acid, palmitic acid and oleic acid decrease mitochondrial function by uncoupling oxidative phosphorylation (Schönfeld and Wojtczak, 2008). Arachidonic acid and palmitic acid have effectively disrupted mitochondrial membrane potential after 24 h exposure to hepatocytes (VA-13 cells) with arachidonic acid causing a greater degree of mitochondrial membrane potential disruption (Gyamfi, 2012). Rat hepatoma cells exposed to oleic, palmitic and arachidonic acid caused reduced cellular mitochondrial function with the highest damage being recorded in the presence of arachidonic acid (López-Gómez *et al.*, 1993). Arachidonic acid caused more disruption in bovine heart mitochondrial function compared to palmitic acid (Cocco *et al.*, 1999). Ethanol and arachidonic acid are toxic to HepG2 cells which express CYP2E1 (Chen *et al.*, 1998). Hence arachidonic acid was found to be most instrumental in eliciting not only cytotoxicity in hepatocytes but also increasing ROS production which is a key factor in NAFLD assessed in subsequent chapters.

As shown in Fig 3.1, after crossing the hepatocyte membrane, lipoproteins are converted by the liver to VLDL and LDL which transports triglycerides back into the blood and adipose tissue whereas other lipids undergo β-oxidation to produce energy. Triglycerides stored in adipose tissue are hydrolysed to free fatty acids (FFAs) and glycerol via a hormone sensitive lipase and transported back into the liver. Some of the FFAs from the adipose tissue are re-esterified to triglyceride in the adipose tissue whereas others are converted to triglycerides in the liver. Increase in mitochondrial β and ω-oxidation as well as peroxisomal β-oxidation in a normal liver leads to energy production whereas a decrease in oxidation in a fatty liver resulting from mitochondrial dysfunction may lead to an increase in unoxidized fatty acids (Reshef *et al.*, 2003), (Zechner *et al.*, 2005). Carnitine palmitoyl transferase I (CPT1) catalyses the entry of activated fatty acids into the mitochondria of hepatocytes by attaching carnitine to fatty acids to enable them to cross the mitochondrial membrane. Once inside the mitochondria, fatty acids are detached into the β-oxidation cycle leading to the generation of acetyl coenzyme A molecules and hence ATP generation (Dunning *et al.*, 2010).

The accumulation of lipid in the liver can also be the end result of high fat intake culminated with reduced energy combustion which is mediated by the mitochondria via peroxisome proliferator-activated receptor (PPAR)-α and peroxisomal fatty acid β-oxidation Fig 3.1. A dysfunctional or under-functioning cellular mitochondria may have a bearing on the level of fatty acids accumulated in liver cells by affecting PPAR-α, which functions as a lipid sensor, resulting in diminished fatty acid metabolism, hepatic steatosis and steatohepatitis (Reddy and Rao, 2006). Furthermore, the accumulation of fatty acids in the mitochondria beyond cellular metabolic capacity leads to the production high amounts of reactive oxygen species eventually causing lipid peroxidation (Schrauwen and Hesselink, 2004). All these factors have a bearing on the cell viability of hepatocytes. The studies in this chapter investigated the capacity of Gentiana spp. extracts to preserve the viability of hepatocytes in the presence of increased amounts of fatty acids (i.e. arachidonic acid).

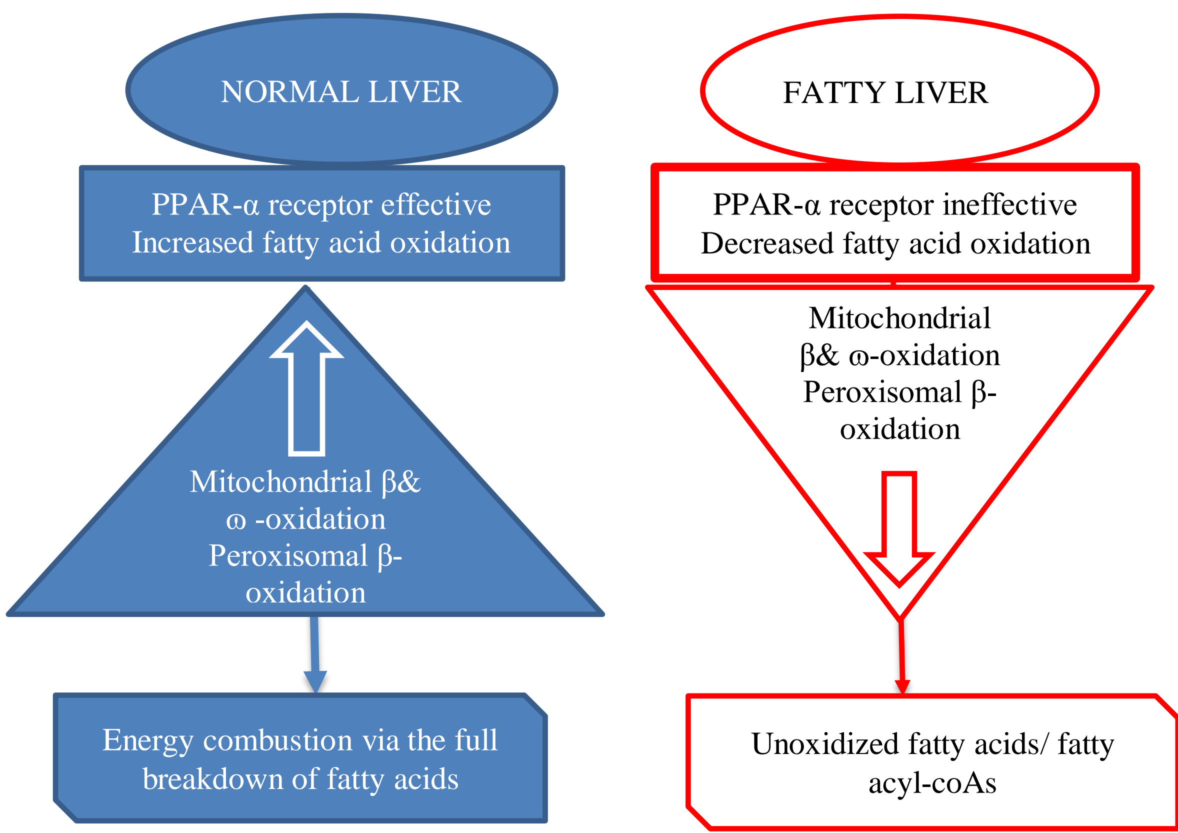


Fig 3.1. Fatty acid metabolism. *Schematic showing the metabolism of fatty acids prior to entering the liver. Triglycerides stored in adipose tissue are hydrolysed to free fatty acids (FFAs) and glycerol via a hormone sensitive lipase and transported into the liver. Increase in mitochondrial β and ώ-oxidation as well as peroxisomal β-oxidation in a normal liver leads to energy production whereas a decrease in oxidation in a fatty liver resulting from mitochondrial dysfunction may lead to an increase in unoxidized fatty acids eventually diminishing liver function (Reshef et al., 2003), (Zechner et al., 2005)*

The trypan blue technique for determining cell viability has been found to be more widely used and safer when compared to the use of eosin and acrylic which are toxic to cells when used to determine cell viability (Altman *et al.*, 1993). Both LDH assay and MTT assay are effective ways of assessing the viability of cells, however a comparison of the two methods showed MTT assay as being more accurate and reliable in determining the viability of cells (Fotakis and Timbrell, 2006). Notwithstanding the merits and demerits of each of the above-listed cell viability assay methods, all of them were assessed in this study to deepen understanding of the cytotoxicity of lipids on hepatocytes, optimise the experimental methods and aid in practical research skill development.

Mitochondrial dehydrogenase plays an active role in the β-oxidation of fatty acids by dehydrogenating long-chain fatty acids to produce a trans double bond between c2 and c3. A properly functioning mitochondrion contains active mitochondrial dehydrogenases which convert yellow (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) into purple coloured formazan (Berg *et al.*, 1990). This assay was used to validate the viability of hepatocytes in the presence of fatty acids and Gentiana spp. extracts. In subsequent chapters, cellular condition was further assessed to determine whether cells were necrotic, apoptotic or viable. Arachidonic acid was the fatty acid of choice for determining the level by which Gentiana spp. guard against fatty acid induced cytotoxicity because studies have shown that ROS production was significantly increased in hepatocytes (HepG2) with arachidonic acid exhibiting a greater effect than palmitic acid

As per reviewed literature on the interaction of Gentiana spp. extracts with hepatocytes exposed to fatty acids, gentiopicroside which can be found in Gentiana spp. significantly lowered liver lipid peroxidation in mice caused by tetrachloromethane (Yuan, 2015). *Gentiana scabra* root extracts exhibited anti-lipid peroxidation and superoxide radical scavenging activities with IC_{50} values of 45.8, 183.4, and 56.3 µg/mL, respectively (Ko *et al.*, 2011). *Gentiana macrophylla* root extracts showed strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radical scavenging activity (Yu *et al.*, 2004). Furthermore, methanolic extracts of *Gentiana lutea* roots have been found to enhance hepatocyte viability by scavenging superoxide anion, hydroxyl radical and hydrogen peroxide responsible for many cell disorders through

their action on lipids (Kusšar *et al*., 2006). Bearing these information in mind, the first step in this study was to determine the effects of the Gentiana spp. extracts alone on the hepatocytes followed by arachidonic acid alone and then assess how the extracts interact with cells in the presence of fatty acids by using LDH, trypan blue and MTT assays.

3.2 Aim

This work aimed at assessing the cytotoxicity of arachidonic acid (10, 30 and 80 µM) in VA-13, HepG2 and THLE-2 cell lines in the presence of Gentiana spp (*lutea, macrophylla, scabra* and *rigescens*) pre-treatment, co-administration and post-treatment. The extracts used were ones obtained by refluxing as described in Chapter 2.

3.3 Materials and Methods

3.3.1 Cell Line, Cell Culture and Passaging

The cell lines used for this study were VA-13 cells (Hep G2 cells that efficiently express alcohol dehydrogenase), human hepatocellular (HepG2) cells and THLE-2 cells. The THLE-2 cells were obtained from (ATTC, UK) whereas VA-13 and HepG2 cells were obtained from (Dan Clement, University of Nebraska). VA-13 and HepG2 were cultured in Dulbecco's modified eagle media (DMEM) with 4 g/L glucose (Lonza, Slough, UK) supplemented with foetal bovine serum (FBS) 10 % (Biosera, Sussex, UK), sodium pyruvate 1 % (*Sigma-Aldrich*, UK), L-glutamine 1 % (*Sigma-Aldrich*, UK), and penicillin-streptomycin 1% (BioWest, USA). THLE-2 cells were cultured in bronchial epithelial growth medium (BEGM) (Lonza, UK) supplemented with epidermal growth factor (EGF) 20 µg/mL (*Sigma-Aldrich*, UK), phosphoethanolamine 2.5mg/mL (*Sigma-Aldrich*, UK) and foetal bovine serum 10 % (FBS) (Biosera, Sussex, UK). When thawing cells from liquid nitrogen, vials were quickly defrosted at 37 °C in a water bath containing distilled water, washed in 5 mL of DMEM containing foetal bovine serum (FBS) 10 % and seeded in to suitable culture flask. Prior to seeding of THLE-2 cells flasks were coated for 24 h with a coat consisting of 0.1% FBS, collagen 5mg/mL (ATTC, UK) and fibronectin 1 mg/mL (*Sigma-Aldrich*, UK). All cells were maintained in a 37°C incubator (Binder APT Germany), and media changes made every three days or earlier if needed. DMEM with

1 g/L glucose (Lonza, Slough, UK) supplemented with 1% FBS was used during each assay. When the cells reach the required confluency (70-80%) they were passaged or frozen for storage. During passage, cells were washed once with Dulbecco's phosphate buffered saline (DPBS) free from calcium and magnesium (Sigma-Aldrich, UK), trypsinised with trypsin 0.25% (1X) solution, with 0.1% EDTA (Thermo Scientific, UK) and neutralised with DMEM containing FBS 10%. Cells were centrifuged at 500 rpm for 5 min, re-suspended in DMEM containing FBS 10 % and seeded into a new flask. When freezing cells, they were re-suspended in DMEM containing DMSO 10% and kept at -80°C for 24 h prior to storage in liquid nitrogen.

3.3.2 Method Optimization - Determination of Cell Viability and Cytotoxicity in the Presence of Arachidonic Acid

3.3.2.1 Trypan Blue Exclusion Assay

VA-13 cells were seeded onto 12-well plates at a concentration of $2.5x10^5$ mL DMEM per well for 24 h. The media was discarded, and cells treated with various concentrations of arachidonic acid (AA, 20, 40 and 80 µM) and Gentian spp (0.001, 0.01 and 0.1 µg/mL), i.e. co-administration. Cells were then incubated for 24 h at 37°C. After treatment, the media was removed from cells in the presence of FCS 1% into respective labelled tubes, washed once with PBS and trypsinized. Media and cells were centrifuged at 2000 rpm for 5 min, re-suspended in 1 mL of PBS and cells treated with 0.1 mL of 0.05% trypan blue for 5 min. Excluded or stained cells were counted in a haemocytometer under a light microscope and viability expressed as: [Staining $_{total}$/ (Staining $_{total}$ + Excluding $_{total}$)] x 100%

3.3.2.2 LDH Assay

VA-13 cells were seeded onto 96-well plates at a concentration of $2.5x10^4$ µL DMEM per well for 24 h. The media was discarded, and cells treated with 40 µM AA with 1% FBS DMEM. The cells were incubated for a period 24 h after which they were centrifuged at 250 x g for 4 min to pellet cells. Media was then removed into respective Eppendorf tubes. To the cells, LDH assay lysis solution 40 µL was added and incubated at 37°C for 45 min. The plates were centrifuged at 250 g for 4 min and supernatants (lysates) collected and diluted (1:10) i.e. 20 µL lysate + 180 µL PBS/DH_2O. A total of 50 µL of supernatants (media and lysates) was transferred to a new 96-well flat bottom plate and lactate dehydrogenase assay mixture prepared by

mixing equal amounts of LDH assay substrate, cofactor and dye solutions. Assay mixture (100 µL) was added to each sample and mixed by shaking for 10s. The plate was covered with aluminium foil to protect from light and incubated at room temperature for 20-30 min. Absorbance was spectrophotometrically measured at a wavelength of 490 nm whereas background absorbance of multi-well plates were measured at 650 nm and subtracted from the primary wavelength measurement. Percentage LDH released was measured as follows: [(LDH media (A))/ (LDH media (A)+LDH lysate (B))] where media (A) was the media removed from the cells prior to LDH assay and media (B) includes lysates.

3.3.3 MTT Assay for Measuring Cell Viability in the Presence of Arachidonic Acid and Gentian spp

HepG2 cells were trypsinized and seeded at a concentration of $25x10^3$ /200 µL DMEM per well for 24 h. The media was then removed, and three different types of treatment applied. MTT assay was performed after 24 h by removing treatments/media and replacing with 90 µL of media. Thiazole blue tetrazolium bromide (TBT) (*Sigma-Aldrich*, UK) 10 µL containing 5 mg/mL TBT in PBS was added per well and incubated at 37°C for 2 h. This was removed and then DMSO 50 µL added per well. The plates were read at 550 nm after being incubated at room temperature for 15 min. MTT assay was used extensively due to its accuracy and minimalization of human error. Cell viability/growth was presented as a percentage of control cells with DMSO.

3.3.3.1 Co-administration MTT Assay

Cells were treated with 0.01 mg/mL Gentiana species, alongside AA (10, 30 and 80 µM) and incubated at 37°C (Binder APT.line) for 24 h after which MTT assay was performed as previously described in section 3.3.3.

3.3.3.2 Pre-treatment MTT Assay

Cells were pre-treated with 0.01 mg/mL Gentiana species and incubated at 37°C (Binder APT) for 24 h and then treatment removed and replaced with (10, 30 and 80 µM) arachidonic acid and incubated again for 24 h at 37°C followed by MTT assay.

3.3.3.3 Post-treatment MTT Assay

Cells were pre-treated with arachidonic acid (10, 30 and 80 μM) and incubated at 37°C (Binder APT.line) for 24 h and then treatment removed and replaced with (GL, GM, GR, GS) 0.01 mg/mL. This was incubated again for 24 h at 37°C and then assayed by MTT.

3.3.3.4 Timeline Post-treatment MTT Assay

Cells were treated with 30 μM AA at 0 h, and then given subsequent treatment of GL and GM at different time intervals: 2, 4, 8 and 24 h. One set of control cells had AA replaced with media at the above stated hours. MTT assay was performed at the end of the timeline period.

3.3.3.5 Timeline Cell Viability Enhancement Experiment

Cells were treated with GL, GM, GR and GS, 0.01 mg/mL at 0 h, and treatments replaced with media at different time intervals: 2, 4, 8 and 24 h. After applying the above treatments for the designated hours, the treatments were removed and replaced with plain media. MTT was then carried out as stated in section 3.3.3.

3.3.4 Statistics

Results refer to mean ± standard deviation and are average values from three to seven values per experiment; which were also repeated at least thrice. In order to evaluate arachidonic acid toxicity or hepatocyte protection conferred by Gentiana spp. comparison among experimental groups was performed via the unpaired t test with Welch's correction, one-way ANOVA with Dunnett's multiple comparison test and finally two-way ANOVA respectively based on the experimental design. Differences at $p<0.05$ were considered significant.

3.4 Results

3.4.1 Cytotoxicity of Arachidonic Acid on Hepatocytes

In order to determine the level of AA (Arachidonic Acid) cytotoxicity on VA-13 and HepG2 cells, trypan blue assay, LDH and MTT assays were performed. VA-13 cells actively secrete alcohol dehydrogenase and are more adapted for the investigation of ALD. HepG2 cells do not secrete alcohol dehydrogenase and are more suitable for NAFLD studies. Due to this, HepG2 cells were more widely used for this study (Clemens, 1998). Furthermore, MTT assay was more widely used because it was economically viable and also minimised human error. LDH assay of VA-13 cells treated with 40 µM AA showed percentage LDH release 90-98% whereas control cells showed LDH release of 9-10% (Fig 3.2). Percentage viability of VA-13 cells treated with AA (20, 40, and 80 µM) decreased with increase in AA dosage after being assayed via trypan blue assay (Fig. 3.3). The lowest percentage viability of 18% was recorded for 80 µM AA whereas the highest percentage viability of 77.7% was observed for 20 µM AA. Following similar trend MTT assay of hepatocytes treated with AA (10, 30 and 80 µM) showed significant decrease in viability compared with control cells without any AA exposure (Fig 3.4). The lowest viability of 39.5% was recorded for hepatocytes treated with 80 µM AA whereas the highest viability of 63.3% was recorded for 10 µM AA in line with previous observations for both LDH and trypan blue assays.

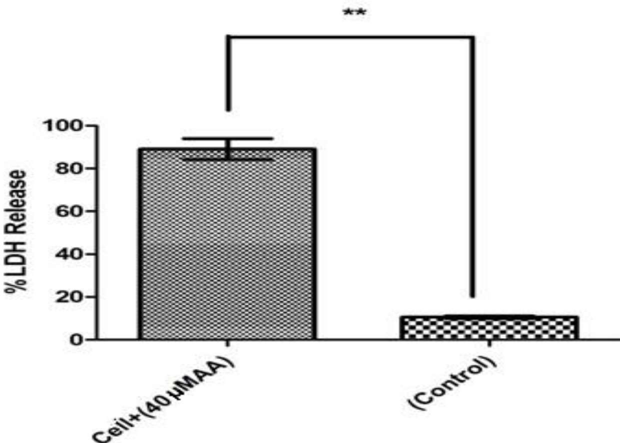


Fig. 3.2.Cytotoxicity effect of Arachidonic Acid (AA) on hepatocytes. *Percentage LDH released by VA-13 cells treated with AA 40 µM: 90-98% whereas control cells showed LDH release of 9-10%. Lower LDH release represented higher cell viability. Data analysed by unpaired t test with Welch's correction and data shown as mean ± SEM, n=3 (**p =0.0040)*

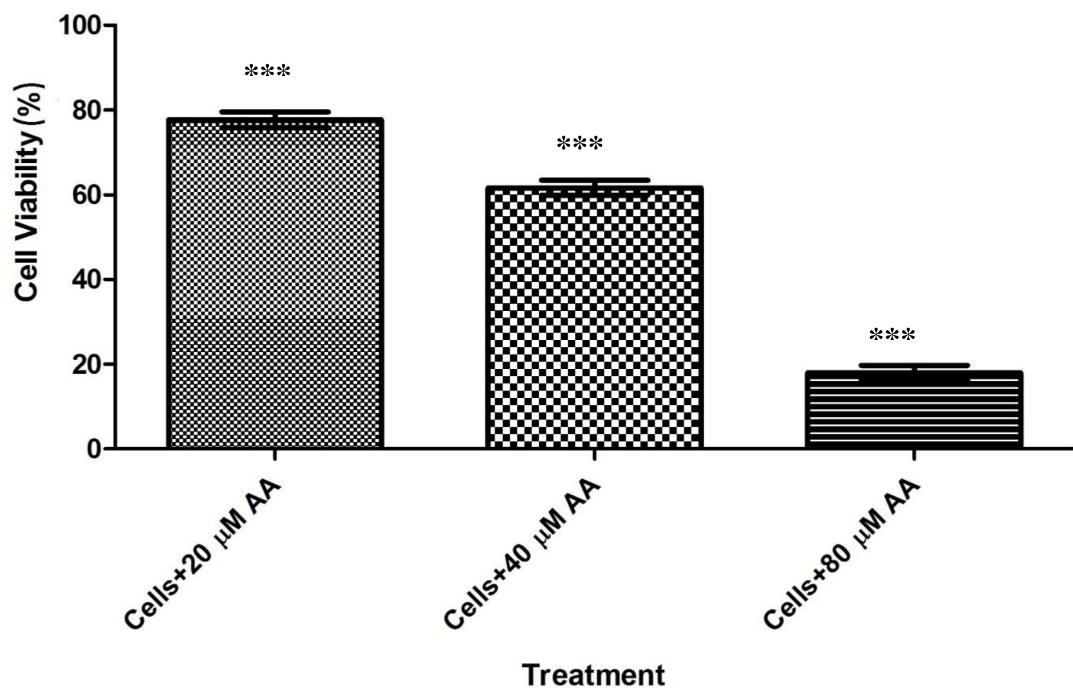


Fig. 3.3. Cytotoxicity of AA on hepatocytes. *Trypan blue assay showed cytotoxicity of AA increased with increasing concentration of AA. HepG2 cells treated with AA (20, 40 and 80 μM) produced viabilities with statistically significant mean (one-way anova, Dunette's multiple comparison test) differences compared to control ***$p<0.05$.*

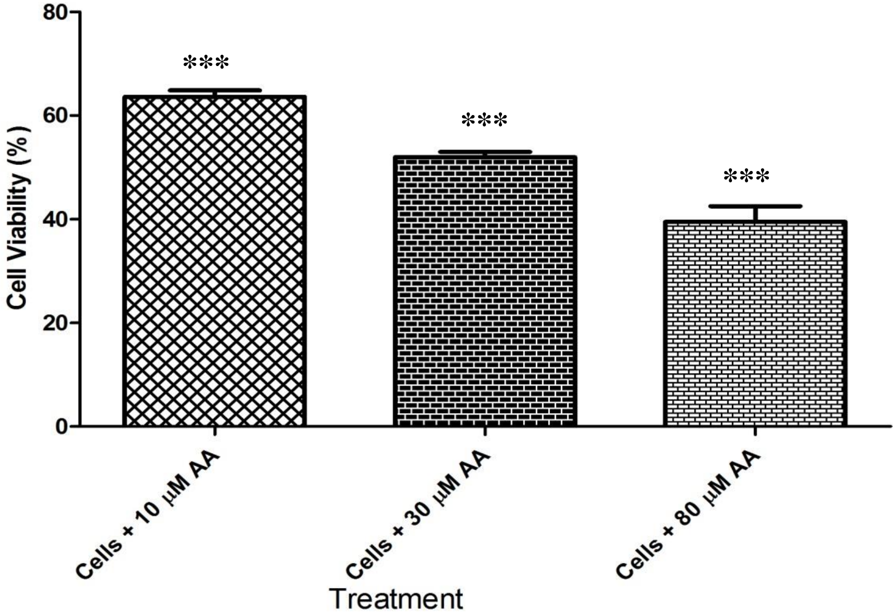


Fig. 3.4. Cytotoxicity of AA on hepatocytes. *MTT assay showed cytotoxicity of AA in HepG2 cells increasing with increase in dose of AA. HepG2 cells treated with AA (10, 30 and 80 μM) produced viabilities with statistically significant mean (one-way ANOVA, Dunette's multiple comparison test) differences compared to control *** $p<0.05$.*

3.4.2 Assessment of Gentian Spp Effect on Hepatocytes (HepG2)

The level by which Gentian spp enhanced the viability and growth of HepG2 cells in a dose-dependent manner was assessed by treating HepG2 cells seeded at $25x10^3$ /200 µL DMEM per well with GL, GM, GR and GS (0.01 and 0.001 mg/mL) for 24 h followed by an MTT assay. A timeline assessment of cell viability enhancement by the four Gentian was performed by treating HepG2 cells seeded at $25x10^3$ /200 µL DMEM per well with GL, GM, GR and GS and then replacing treatment with media at time intervals 2, 4, 8 and 24 h. It was observed that the cell viability increased from 2-24h in the presence of Gentiana treatments (Fig. 3.5). The highest percentage cell growth as well as mitogenic characteristic was observed in cells treated with GM with 146 % after 24 h. This was followed by GR with 142 % after 24 h. It was generally observed that decreasing treatment dose from 0.01-0.001 mg/mL reduced cell viability across all species of Gentiana. Other control cells treated with DMEM containing 0.01 and 0.001 % DMSO presented cell viabilities of 101 % and 103 % respectively. It was observed that hepatocytes treated with GM (0.01 mg/mL) showed the highest percentage viability of 141% (i.e. 41% increase compared to control cells with only media and no treatment); hence portraying a degree of mitogenicity. This was followed by GR (0.01 mg/mL) with 140% (Fig. 3.6).

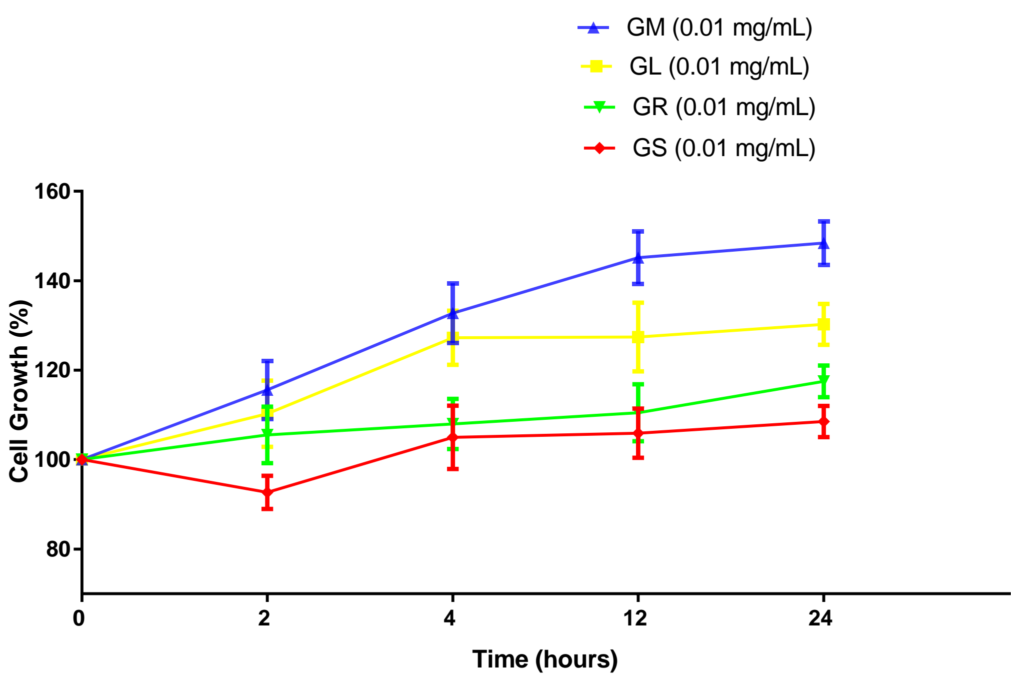


***Fig. 3.5. HepG2 cell growth enhancement by Gentian spp timeline**. HepG2 cells were incubated in media containing GL, GM, GR and GS (0.01 mg/mL) for varying periods of 2-24 h. After treatments, cell growth was assessed by MTT assay. Results presented as mean±SD (two-way ANOVA). Gentian spp treatment factor significant when compared viability and time of treatment p<0.05*

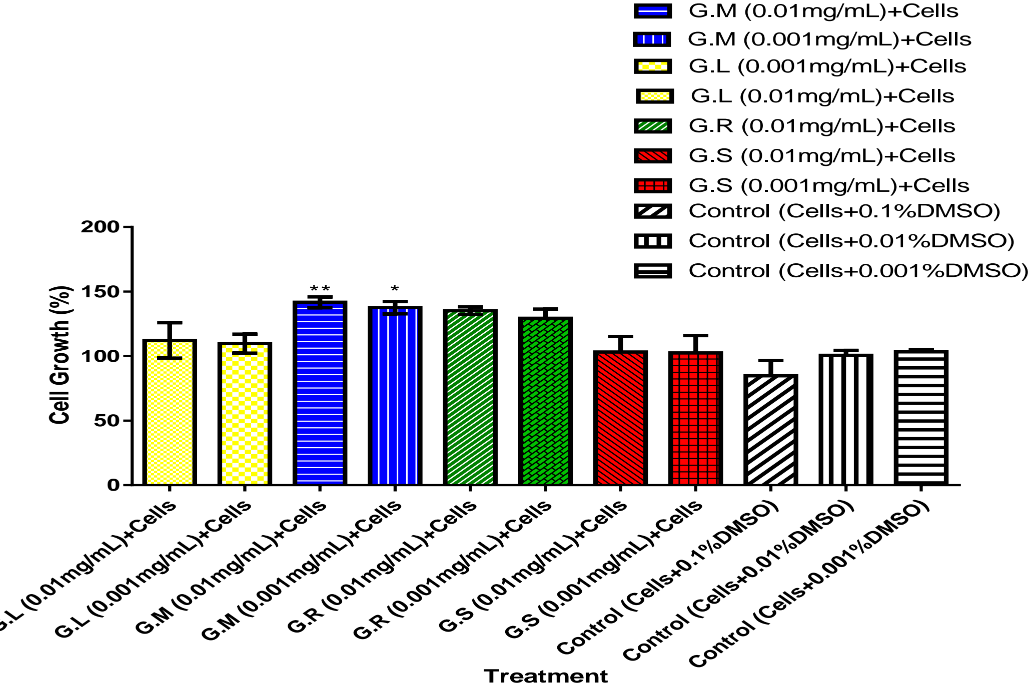


***Fig. 3.6. HepG2 cell growth enhancement by Gentiana spp**. MTT assay showed increase in cell growth alongside increase in dose of Gentian from 0.01-0.001 mg/mL. HepG2 cells treated with GL, GM, GR and GS (0.01 and 0.001 mg/mL) produced viabilities with statistically significant mean (one-way anova) differences compared to control *p<0.05 and **p=0.0029.* Percentage *viabilities ranged between (103-142 %) with the highest viability shown in Gentiana macrophylla*

3.4.3 Effects of Concurrent Exposure of Gentian spp and Fatty Acids to Hepatocytes

In order to investigate cell viability and also determine whether or not *Gentiana spp* inhibits AA cytotoxicity upon concurrent exposure of both to HepG2 cells; GL, GM, GR and GS (0.01 mg/mL) were administered to HepG2 cells in the presence of AA (10, 30 and 80 µM) for 24 h. Cytotoxicity as well as percentage cell viability were then assessed by MTT assay. Control cells administered with only AA (10, 30 and 80 µM) for 24 h were also assessed by MTT assay. In the presence of lower AA levels (i.e. 10 µM), hepatocytes treated with GM had the highest cell viability of 115 %. However, in the presence of higher AA levels (i.e. 30 and 80 µM), GL-treated hepatocytes presented with the highest viabilities of 80.5 and 50.9 % respectively. There was a general trend of AA cytotoxicity decreasing in the presence of Gentian spp particularly at 10 µM AA treatment (Fig 3.7).

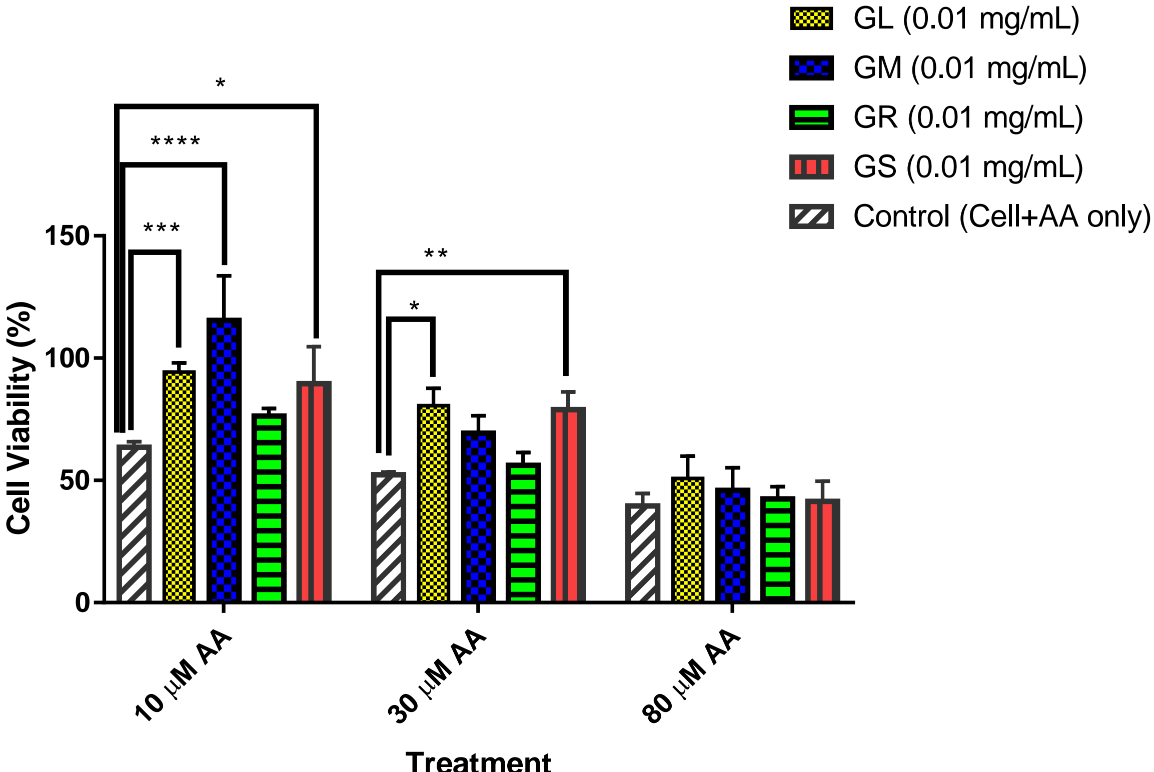


Fig. 3.7. Cytotoxicity of AA on HepG2 in the presence of Gentian spp. *AA cytotoxicity decreased in the presence of Gentian spp. GM and GL treated hepatocytes presented the highest viabilities (50.9-115.4%) in the presence of lower and higher levels of AA (10-80 µM) Data presented as mean±SD Two-way ANOVA with Tukey Multiple Comparison of Gentian spp treatment factor and control (*p<0.05) (**p=0.0025) (***p=0.0009) (****p=0.0001)*

3.4.4 Effects of Gentiana spp. on Fatty Acid Pre-treated Cells

The amount by which Gentiana spp. sustain growth or reverse AA cytotoxicity in hepatocytes previously exposed to AA for 24 h was investigated by treating hepG2 cells with AA (10, 30 and 80 µM) for 24 h. After that period, media containing AA treatment was removed and replaced with media containing GL, GM, GR and GS (0.01 mg/mL) for 24 h. MTT assay was undertaken after the 24 h incubation period to determine percentage cell viability after these two treatments. In this instance, GM treated hepG2 cells constantly presented the highest percentage cell viability of (60.7-96.8%) across all the three AA concentrations used. As observed in the previous Gentiana spp. concurrent and pre-treatment experiments, in this case also, cell viability in hepatocytes having Gentiana spp. treatment was better than hepatocytes without any treatment (Fig 3.8.). Furthermore, GL-treated hepatocytes had the second highest percentage viability of (41-76%) across all AA treatments. In the time course experiment, hepG2 cells were pre-treated with AA (30 µM) and treatment replaced with GL and GM (0.01 mg/mL) at 2, 4, 12 and 24 h respectively. Cell viability was then analysed by MTT assay. Cytotoxicity in Gentian-treated hepatocytes decreased for both GM and GL treated hepatocytes. GM-treated hepatocytes presented the highest viabilities (89-95%) (Fig 3.9.)

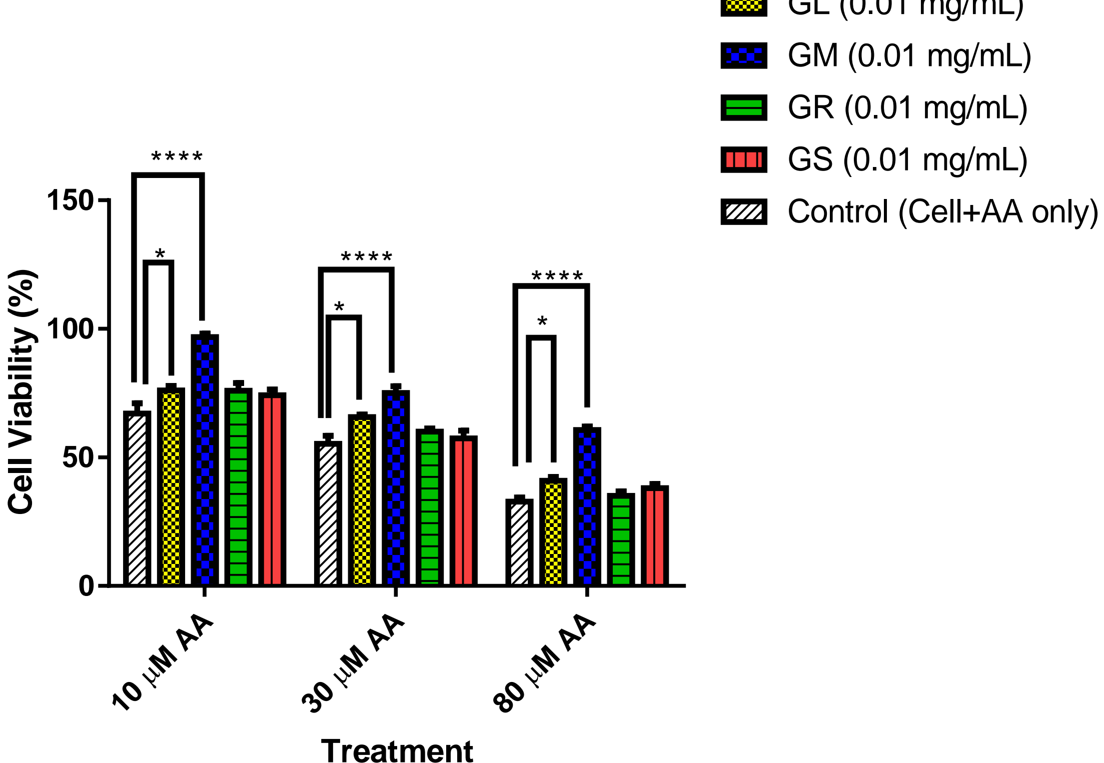


Fig. 3.8. Cell viability of fatty acid pre-treated cells followed by Gentiana spp treatment. *Cell viability of HepG2 cells exposed to AA (10, 30 and 80 µM) for 24 h before Gentian spp treatment. Cytotoxicity in Gentian-treated hepatocytes decreased for all concentrations of AA used. GM-treated hepatocytes presented the highest viabilities* (60.7-96.8%) *in the presence of lower and higher levels of AA (10-80 µM) Data presented as mean±SD (Two-way ANOVA with Tukey Multiple Comparison of Gentian spp treatment factor and control (*p<0.05) (****p=0.0001)*

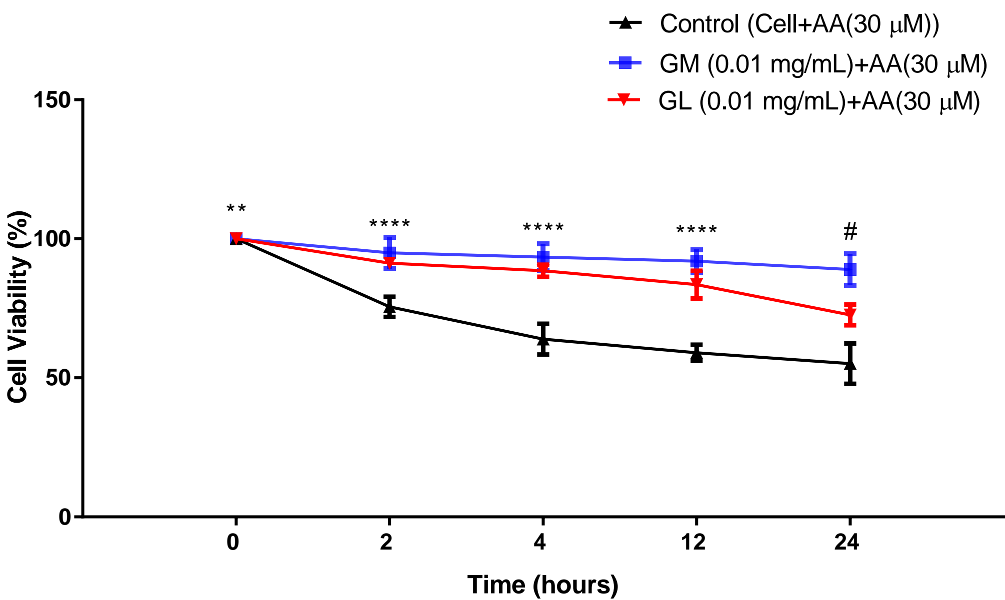


Fig. 3.9. Time course cell viability of HepG2 cells pre-treated with AA and then GL or GM. *Cytotoxicity in Gentian-treated hepatocytes from 2-24 h decreased for both GM and GL treated hepatocytes compared to control cells. GM-treated hepatocytes presented the highest viabilities* (89-95%) *in the presence of AA (30 µM) Data presented as mean±SD Two-way ANOVA with Tukey Multiple Comparison of Gentian spp treatment factor and control (#p=0.02) (**p=0.0048) (***p=0.0008) (****p=0.0001)*

3.4.5 Effects of Fatty Acids on Gentian Pre-treated Hepatocytes

This study aimed to establish whether pre-treating cells with Gentian prior to fatty acid treatment conferred a degree of hepatocyte protection to the cells. In order to establish this, HepG2 cells were treated with GL, GM, GR and GS (0.01 mg/mL) for 24 h after which treatment was replaced with media containing AA (10, 30 and 80 µM) for another 24 h. Cell viability was then studied via MTT assay. AA cytotoxicity was observed in GM pre-treated hepatocytes with percentage viabilities ranging from (81.2-118%). It appeared that hepatocytes pre-treated with Gentian spp fared better in viability than untreated hepatocytes which had the lowest cell viabilities of up to 46% at the highest AA dose of 80 µM (Fig. 3.10).

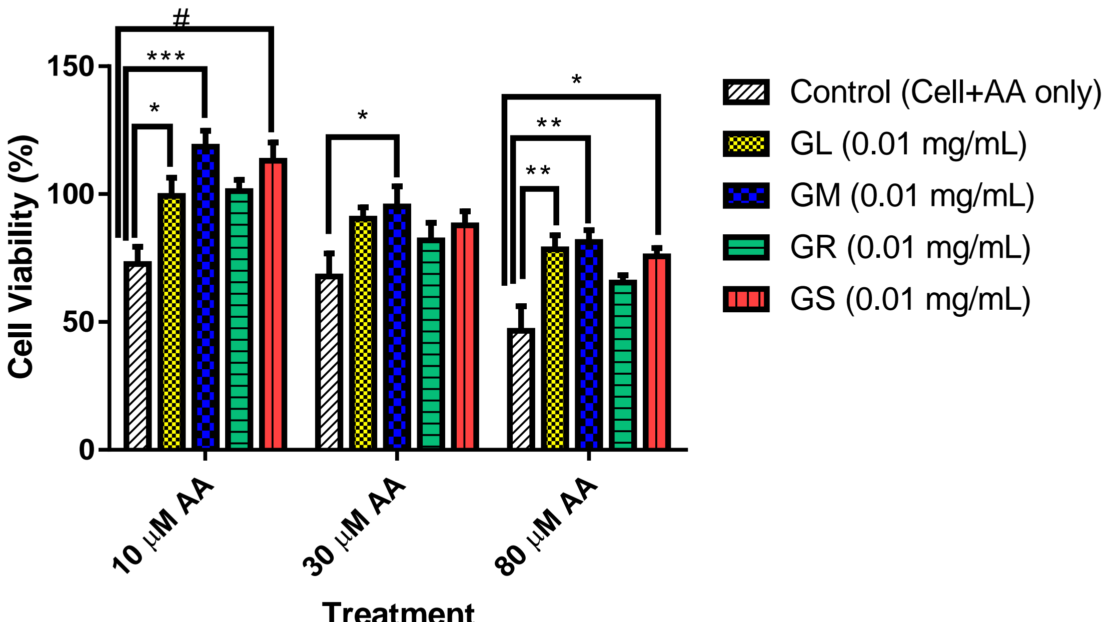


Fig. 3.10. HepG2 cell protection conferred by Gentian spp pre-treatment for 24 h. *For all Gentian pre-treated hepatocytes, AA cytotoxicity decreased compared with untreated cells. GM-treated hepatocytes presented the highest viabilities* (81.2-118%) *in the presence of AA (10-80 µM) Data presented as mean±SD Two-way ANOVA with Tukey Multiple Comparison of Gentian spp treatment factor and control (*p<0.05) (#p=0.0002) (**p=0.0046) (***p=0.0008)*

3.4.6 Effects of Fatty Acids on Gentian Pre-treated THLE-2 cells

The aim of this experiment was to determine the effects of fatty acids on Gentian pre-treated THLE-2 cells which are hepatocytes transformed with SV40 large T antigen. As shown in Fig 3.10, pre-treatment of THLE-2 cells with Gentiana spp generally provided hepatocyte protection against cytotoxic effects of arachidonic acid. Cell viabilities ranged from 70 to 103 % with the highest viability recorded in *Gentiana macrophylla,* followed by *Gentiana lutea*, *Gentiana scabra* and *Gentiana rigescens* in a decreasing order. Control THLE-2 cells which were not primed with Gentiana spp extracts had very low viabilities, markedly in the presence of 80 µM AA which decreased cell viability up to 38 %. Furthermore the priming of THLE-2 cells with Gentiana extracts on its own did not appear to diminish cellular viability with increased viabilities up to 105 % as shown in Fig 3.11.

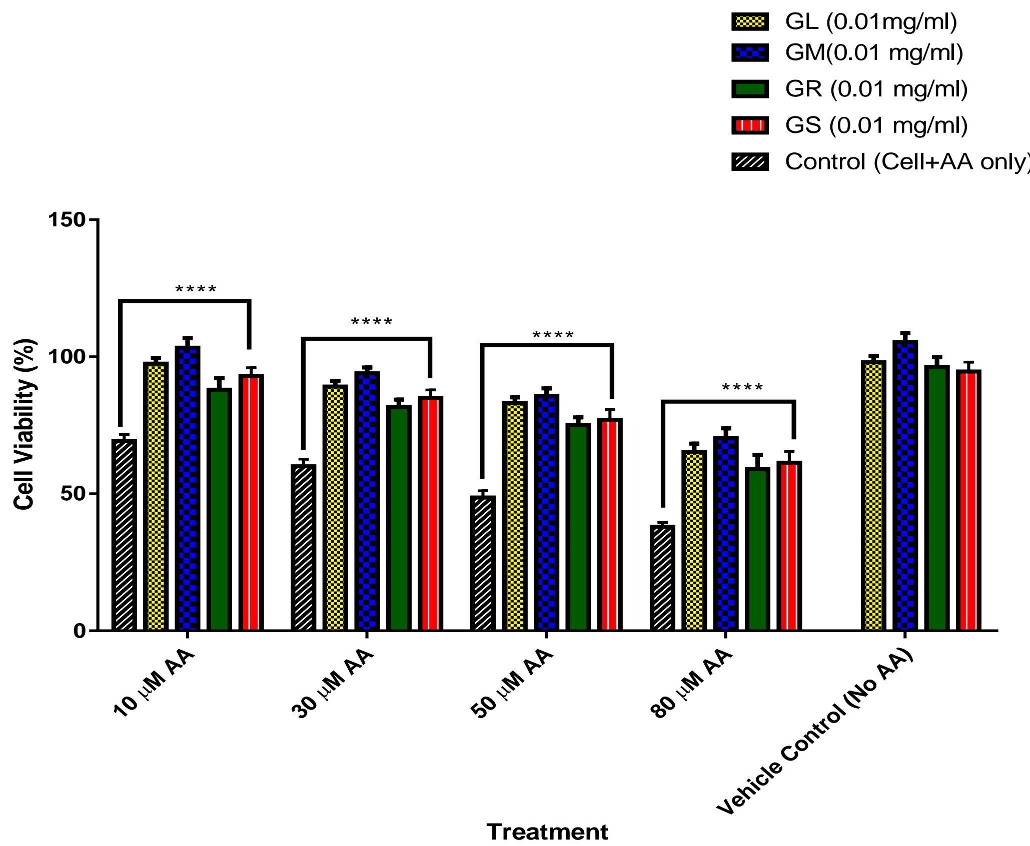


Fig. 3.11. Hepatocyte protection conferred on THLE-2 cells by Gentian spp pre-treatment for 24 h. *For all Gentian pre-treated hepatocytes, AA cytotoxicity decreased compared with untreated cells. GM-treated hepatocytes presented the highest viabilities* (70-103%) *in the presence of AA (10-80 µM) Data presented as mean±SD Two-way ANOVA with Tukey Multiple Comparison of Gentian spp treatment factor and control ****p=0.0001*

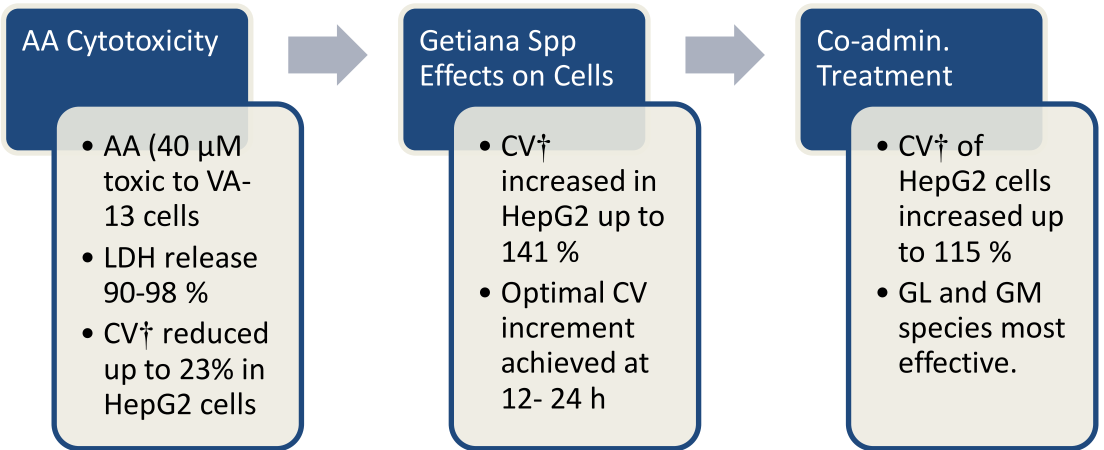
3.5 Discussion

3.5.1 Introduction

The study aimed to investigate hepatocyte protection conferred by four different species of Gentiana in a comparative manner to determine the best species in this regard. In the optimisation stage of the study trypan blue, LDH and MTT assays were performed to primarily to assess in a dose-dependent manner, the level of cytotoxicity caused by the treatment of hepatocytes with AA (10-80 μM). The use of trypan blue assay to assess cell viability after treatment with 60-80 μM arachidonic acid exposure to Jurkat cells indicated in cell viabilities of up to 28% within 24-48 h (Siddiqui *et al.*, 2001). In this study however, percentage viability of VA-13 cells treated with AA (20, 40, and 80 μM) decreased with increase in AA dosage after being assayed via trypan blue assay. The lowest percentage viability of 18% was recorded for 80 μM AA whereas the highest percentage viability of 77.7% was observed for 20 μM AA (Fig 3.3).

As shown below in Fig 3.12, the study began by assessing and confirming the cytotoxicity of AA on cell lines (VA-13 and HepG2), followed by a study of the effects of Gentiana spp. alone on hepatocytes in terms of cell viability. Co-administration of Gentiana spp. and AA studies were conducted on hepatocytes after determining that Gentiana species enhanced cell viability to a great degree whereas AA showed toxicity to hepatocytes.

In order to determine the most effective means to administer Gentiana spp. extracts to cells further studies were conducted entailing AA pre-treatment of cells followed by Gentiana spp. extracts and then Gentiana spp. pre-treatment followed by AA treatment. The study was concluded by using the best treatment regimen i.e. Gentiana spp. extract pre-treatment method on THLE-2 cells to determine if cell growth enhancement was only limited to HepG2 cells or could be seen in other cell types such as THLE-2 cells which are hepatocytes transformed with SV40 large T antigen.



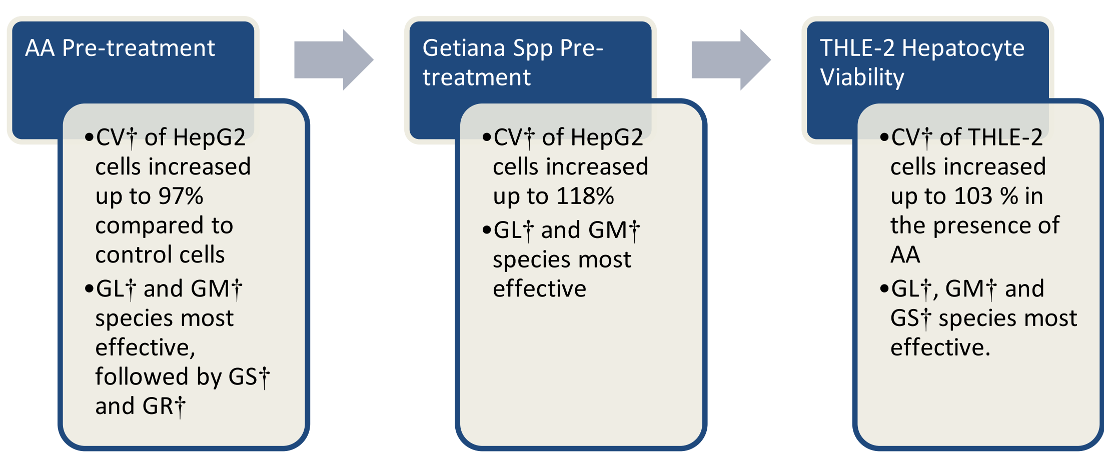


Fig 3.12. Chronological summary of studies on hepatocytes and outcomes. *The first point of study was AA cytotoxicity studies aimed at confirming the toxicity of AA to hepatocytes followed by studies to investigate the effect of Gentiana spp. on hepatocyte cell viability. A comparison was drawn between co-administration of AA and Gentiana spp. extracts, AA pre-treatment prior to Gentiana spp. exposure and Gentiana spp. pre-treatment prior to AA exposure to determine the most effective treatment sequence in terms of hepatocyte viability enhancement. Gentiana spp. pre-treatment which was the most effective treatment sequence was used to test the effects of AA on THLE-2 cells primed with Gentiana spp.*

†CV-Cell viability, GL-*Gentiana lutea* GM-*Gentiana macrophylla*, GR-*Gentiana rigescens* GS- *Gentiana scabra*

3.5.2 Assay of Cytotoxicity of Arachidonic Acid (AA)

Cytotoxicity in the presence of AA may be attributed to hepatocyte plasma membrane rupture and enzyme leakage which allows trypan blue staining to occur hence plasma membrane integrity can be assessed via cellular enzyme leakage and its interaction with vital dye staining. Exclusion of the vital dye trypan blue by hepatocytes at the time of isolation has become a widely accepted method of determining cell viability with major laboratories reporting 85-99% absorption of the dye by hepatocytes (Jauregui, 1981). Following a similar trend, LDH assay of VA-13 cells treated with 40 μM AA showed percentage LDH release 90-98 % whereas control cells showed LDH release of 9-10 % in Fig 3.1. This signified substantial increase of LDH release into the media in the presence of AA 40 μM. The LDH assay indirectly measures the number of viable cells either via the total cytoplasmic LDH or the amount of cytoplasmic LDH released into the media serving as an index for determining the percentage of cell viability (Yang *et al*., 2008).

MTT assay of hepatocytes treated with AA (10, 30 and 80 μM) showed significant decrease in viability of up to 39.5% compared with control cells without any AA treatment. After a 24 h incubation of HepG2 cells with AA, lactate dehydrogenase (LDH) release was induced, as well as cytotoxicity and alterations in cell proliferation. MTT assay of cells showed a significant decrease in viability up to 37%, $p<0.01$ (Holownia *et al*., 2014). MTT assay was more widely used in this experiment because it was economically viable in comparison to the LDH assay and also minimised human error more than the trypan blue assay.

3.5.3 Effects of Gentiana spp. on the Viability of HepG2 Cells

After establishing cytotoxicity of AA via the above methods in a dose-dependent manner, the next investigation was aimed at determining the effect of Gentian spp. on hepatocytes in a dose-dependent manner.

Apart from enhancing hepatocyte cell viability in a dose-dependent manner, length of treatment time also played a factor in determining the extent of viability conferred. Hepatocytes treated with Gentian spp. for the maximum time of 24 h showcased the highest percentage viability whereas low figures were recorded for the shortest

treatment time of 2 h (Fig. 3.6). This factor contributed to the choice of 24 h in investigating the level of arachidonic acid cytotoxicity reduction in the presence of Gentian pre-treatment, co-administration and post-treatment. It was observed that cell growth was more enhanced in Gentian treated hepatocytes than control hepatocytes lacking Gentian treatment.

A mitogenic effect was observed for *Gentiana lutea* as well as *Gentiana macrophylla.* Furthermore, percentage cell viability increased with an increase in dose of Gentian spp. from 0.001 to 0.01 mg/mL. However, the species which enhanced hepatocyte growth the most was GM with an increase of up to 142 (i.e. 42 % more than control cells without Gentiana treatment) compared to control cells followed by GR and GL with 12-39% increase in cell viability (Fig. 3.5). This observation was in line with studies which suggest that Gentiana species: lutea, *macrophylla*, *rigescens*, *scabra manshurica* and *olivieri* protect and enhance hepatocyte viability via their antioxidant, anti-inflammatory and bitter components including: amarogentin gentianine, iso-orientin, swertiamarin, gentiopicroside, and sweroside (Wang *et al*., 2010b).

3.5.4 Pre-treatment, Co-administration and Post-treatment Effects of Gentiana spp on Hepatocyte Viability in the Presence of Arachidonic Acid

Co-administration of Gentian with AA helped to decipher whether or not there was any interaction between the plant extracts and the fatty acid, and also whether or not that interaction was detrimental to hepatocyte viability. The results obtained appeared to show a lack of Gentian-AA interaction, detrimental to hepatocyte cell viability. (Fig 3.7)

Having established a lack of detrimental interaction, hepatocytes were then exposed to AA prior to Gentian treatment in order to ascertain whether or not the plant extracts could contribute in any way to aiding cellular recovery after fatty acid induced cytotoxicity. In this instance, the results indicated a degree of enhanced cellular recovery in Gentian-treated hepatocytes as compared to control cells which were treated with plain media after the AA exposure period.

Finally, a test was conducted to establish whether or not pre-treating or priming hepatocytes with Gentian before exposing them to AA conferred hepatocyte protection against fatty acid induced cytotoxicity. The results expressed Gentian pre-treatment provided protection to hepatocytes against fatty acid induced cytotoxicity. (Fig. 3.10) It was also noted that even though the lutea species contained the highest proportions of gentiopicroside and swertiamarin, among the four species studied, it only had a higher viability than macrophylla during co-administration, whereby the extract and the arachidonic acid were given at the same time for just 24 h. In all other instances during which cells were primed with extract before fatty acid exposure, GM had the highest viability. Research by (Balijagić *et al.*, 2012) states that *Gentiana lutea* extracts showed toxicity to HeLa cells at a dose of 41 µg/mL although it is a potent hepatoprotective and anti-inflammatory agent. This cytotoxicity was attributed to a mixture of secoiridoid glycosides, mangiferin, isogentisin and gentiopicrin. This may be one of the reasons why although lutea pre-treated cells had a lower cell viability than macrophylla pre-treated cells even though the (10 µg/mL) of lutea administered contained the highest gentiopicroside (1.0118 µg/mL) and swertiamarin (0.35 µg/mL). Macrophylla on the other hand contained a slightly more sweroside (0.24 µg/mL) than lutea but contained a lower amount of gentiopicroside than lutea (0.4330 µg/mL) based on quantitation results from Chapter 2. The HPLC chromatograms also showed peaks which were not seen in macrophylla for other secoiridoid glycosides which may be cytotoxic as mentioned above. Hence, with pre-treatment, cells were exposed to lutea and all the other possibly cytotoxic secoiridoid glycosides for up to 48 hours (i.e. during the 24 h for drug only treatment, and another 24 h when arachidonic acid is administered) before MTT assay hence lower viability compared to macrophylla. In the co-administration however, cells were exposed for only 24h (i.e. both extract and arachidonic given at the same time for 24 h) followed by MTT hence a lesser exposure time to both hepatoprotective and possibly cytotoxic secoiridoid glycosides making lutea perform better than macrophylla in that instance.

3.5.5 Viability of THLE-2 Hepatocytes Pre-treated with Gentiana spp Prior to Arachidonic Exposure

Having noted Gentiana spp. pre-treatment as the most effective means of securing hepatocyte protection based on the viability data obtained, this same method was

applied in testing hepatoprotective effects of Gentiana extracts on THLE-2 cells THLE-2 which are liver epithelial cells transformed with SV40 large T antigen (ATTC, 2017). This assay was necessary to draw a comparison between the effects of Gentiana pre-treatment on HepG2 cells which are replicating liver cells and THLE-2 cells which are uncancerous liver cells transformed with SV40 large T antigen. The results obtained for THLE-2 were consistent with results obtained for HepG2 cells with *Gentiana macrophylla* primed THLE-2 cells presenting the highest viability of up to 103 % in the presence of AA. This was not as high as the viability of 118 % recorded for *Gentiana macrophylla* in HepG2 cells. A study found HepG2 cells to possess higher sensitivity for basic compounds whereas THLE-2 cells possessed higher sensitivity for acidic and neutral compounds (Shah *et al*., 2014). As seen in Fig 3.11, the lower cell viability seen in THLE-2 in comparison with HepG2 cells could be attributed to their high sensitivity to the effects of arachidonic acid due to its acidity causing more damage in the THLE-2 cells than in the cancerous HepG2 cells.

The hepatoprotective effects of individual phytochemicals: gentiopicroside, sweroside and swertiamarin are investigated further in chapter 4. Other studies have shown that another plant: *Lippia noduflora* and silymarin also protected HepG2 cells by reducing reactive oxygen species in the presence of hepatotoxins. MTT assay of the HepG2 cells pre-treated with *Lippia noduflora and* silymarin showed a decrease in cell death by 16 % and 28 % respectively in the presence of hepatotoxins (Arumanayagam and Arunmani, 2015). In this study however, hepG2 cell death was decreased by as much as 31.8 % and 35.2 % respectively by pre-treating cells with the two best performing Gentian spp: *lutea* and *macrophylla* prior to AA exposure. Hence this study presents the Gentian spp. as potential hepatocyte protective.

3.6 Conclusion

This study found the four Gentian spp: *lutea*, *macrophylla*, *scabra* and *rigescens* as hepatocyte protectors and identified the presence of gentiopicroside in all four plants. Being the first study of its kind to compare hepatocyte-protective activity of the four-named species of Gentian, this study discovered *Gentiana lutea* and *Gentiana macrophylla* as the more dominant hepatocyte protectors among the plants investigated. Furthermore, the most effective means of conferring hepatocyte protection was by pre-treatment of hepatocytes with Gentiana plants prior to arachidonic acid exposure. The next step in this study entailed assays aimed at establishing the mode by which the Gentian species protect hepatocytes from fatty acid cytotoxicity and the role played individually by the single compounds: gentiopicroside, sweroside and swertiamarin in conferring hepatocyte protection. From a synergistic point of view, an understanding of the mode by which Gentiana phytochemicals protect liver cells and their mechanism of action will set a foundation for potential studies on the synergistic effect of using these phytochemicals with other well-known hepatocyte protectors such as silymarin or reducdyn (N-acetyl - D, L - homocysteine thiolactone). Finally, individual phytochemicals: gentiopicroside sweroside and swertiamarin will be studied in the follow-up chapters.

Chapter 4. Influence of Lipid (arachidonic acid) on Hepatocytes Pre-treated with Single Compounds: Gentiopicroside, Sweroside, Swertiamarin and Silymarin

4.1 Introduction

In the previous chapter, it was found that all the Gentiana species studied produced a degree of hepatocyte protection in terms of maintaining and improving cell viability of hepatocytes; most notable among them being *G. lutea* and *G. macrophylla* but this also raised many questions such as:

- How may Gentiana spp. extracts enhance cell viability of hepatocytes?
- Which phytochemicals in the Gentiana spp. extracts may be responsible for this cell viability enhancement?
- Do these phytochemicals work individually or synergistically to promote cell viability?
- Do Gentiana spp. promote hepatocyte protection and cell viability by preventing necrosis or apoptosis or both?
- Is cell viability preserved by the scavenging of reactive oxygen species (ROS) produced as a result of exposure to high concentrations of AA?
- The mitochondria plays an important role in fatty acid metabolism in terms of β-oxidation. Do Gentiana spp. extracts enhance mitochondrial function and capacity in any way? Especially in terms managing mitochondrial stress caused by high energy demand or high influx of arachidonic acid.
- Do Gentiana spp. extracts and phytochemicals affect the amount of ATP produced by hepatocytes, hence having a bearing on cellular metabolic rate
- Do Gentiana spp. extracts and phytochemicals affect cellular respiration in terms of oxygen consumption rate? And is that a mechanism for protecting liver cells and promoting cell viability observed in Chapter 3?

In order to answer these questions, this chapter further looks into the Gentiana species in terms of its phytochemicals gentiopicroside, sweroside (Fig. 4.1) and swertiamarin (Fig. 4.2) with the aim of determining if they play any role in providing protection to hepatocytes by first determining their individual effects on hepatocyte (HepG2 and THLE-2) cell viability via MTT. This was followed by a sequence of assays including the seahorse mitochondrial stress assay with focus on hepatocyte ATP production, non-mitochondrial respiration, proton leak, basal respiration, maximal respiration and spare respiratory capacity, in the presence of oligomycin, FCCP, antimycin and

rotenone. This was then followed by DCF ROS assay and annexin V-FITC apoptosis flow cytometric assay.

These phytochemicals were studied jointly with silymarin which is a well-known hepatoprotective phytochemical derived from milk thistle (*Silybum marianum*). Silymarin (Fig 4.1) has been shown to possess antioxidant and hepatoprotective protective properties (Saller *et al.*, 2001). Studies in rat modules have shown that silymarin promotes mitochondrial function by inhibiting lipid peroxide formation in the mitochondria of rat livers and microsomes (Bindoli *et al.*, 1977). Silymarin may act by supressing TNF-α activation of NF-κB dependent transcription as well as p50 and p65 nuclear translocation (Polyak *et al.*, 2010). Hepatocellular parameters such as mitochondrial ATP content, respiratory control ratio and glutathione were improved in the presence of silymarin (Ligeret *et al.*, 2008). Silymarin is well adopted for studies involving a wide range of toxic models and provides hepatoprotective effects via mechanisms such as: anti-lipid peroxidation, anti-fibrosis, anti-inflammation, providing membrane stability, immunomodulation and being an antioxidant (Pradhan and Girish, 2006). These studies projected silymarin as the best phytochemical for comparing gentiopicroside, swertiamarin and sweroside hepatoprotective effects via MTT assay, seahorse mitochondrial stress assay and DCF ROS assay to investigate reactive oxygen species scavenging effects of the Gentiana phytochemicals.

Studies have shown that gentiopicroside, sweroside and swertiamarin shown in Fig 4.1 were responsible for hepatoprotective effects exerted by *Gentiana manshurica* as well as *Gentiana turkestanerum* against carbon tetrachloride induced hepatic damage in mice (Zhu and Chen, 2007) (Yang *et al.*, 2017). Gentiopicroside, sweroside and swertiamarin found in Gentiana scabra exerted hepatoprotective effects on hepatocytes by diminishing oxidative stress (Ko *et al.*, 2011). In a rat liver damage model induced by α-naphthylisot hiocyanate, swertiamarin at a dose of 20 mg/kg portrayed hepatoprotective effects by significantly reducing alanine aminotransferase, aspartate aminotransferase and the total and direct bilirubin levels which had been increased in the presence of α-naphthylisot hiocyanate while conversely increasing bile flow (Tian *et al.*, 2014)

Fig 4.1 Structures of Gentiana phytochemicals. *Chemical structures of phytochemicals: (A) sweroside, (B) swertiamarin, (C)gentiopicroside and (D) silymarin*

The seahorse mito stress assay is a useful tool for assessing cellular mitochondrial stress resulting in a more detailed understanding and evaluation of mitochondrial dysfunction, signals, phenotypes and metabolic pathway. It performs these by measuring cellular oxygen consumption rate (OCR) via a probe in the presence of oligomycin which inhibits ATP synthase (complex V), cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) which uncouples OCR (i.e. affects the inner mitochondria by reducing the proton gradient; thereby affecting membrane potential) and then antimycin and rotenone A which block complex 1 and III. Fig 4.2 (Luz *et al.*, 2015). Exerting these effects on the electron transport chain enables the seahorse mito stress assay to measure parameters such as basal respiration (level of OCR required for ATP production), ATP production itself, proton leak (can deduce mitochondrial damage or ATP production regulation), maximal respiration (highest mitochondrial respiratory capacity with rapid oxidation of fatty acids), spare respiratory capacity (denotes cell

fitness and ability to respond quickly to energy requirements) and non-mitochondrial respiration (accounts for OCR of other cellular enzymes) (Agilant, 2017) (Lay *et al.*, 2016) These are pictorially illustrated by Fig 4.2 and summarized as follows:

- Basal Resp. = Basal OCR – Non-Mitochondrial Resp. (Rot & Ant A induced)
- ATP Prod. = Basal OCR – Oligomycin induced OCR
- Proton Leak = Oligomycin induced OCR - Non-Mitochondrial Resp.
- Maximal Resp. = FCCP induced OCR - Non-Mitochondrial Resp.
- Spare Resp. Capacity = FCCP induced OCR - Basal OCR
- Non-Mitochondrial Resp. = Rotenone & Antimycin A induced OCR

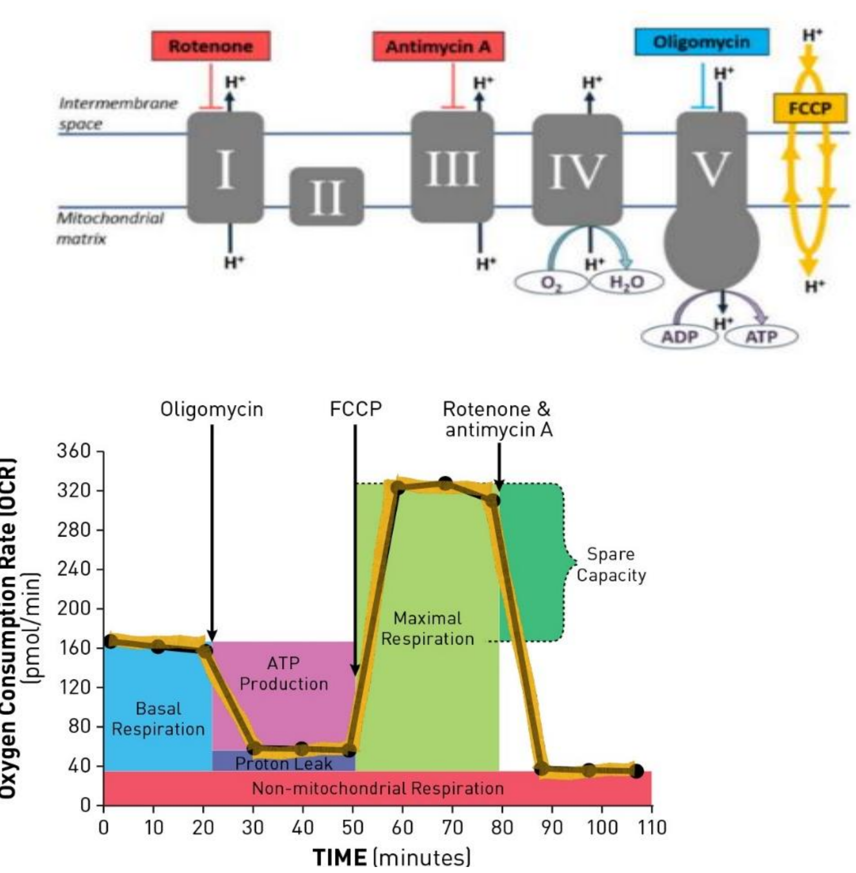


Fig. 4.2. Seahorse XF cell mitochondrial stress test profile. *Oligomycin inhibits complex V, FCCP is an uncoupler, rotenone and antimycin inhibits complex I & III respectively. Parameters such as basal respiration, ATP production, maximal respiration, spare respiratory capacity and non-mitochondrial respiration can be measured by the seahorse mito stress assay (Seahorse Bioscience, 2015)*

The seahorse mito stress assay was used in the presence of oligomycin, FCCP and rotenone which are sequentially injected to determine the oxygen consumption rates (OCR) of hepatocytes in a study on the effect of caspase-1 activity on hepatocyte protection after oxidative stress (Sun *et al.*, 2013b). Mitochondrial proton leakage and OCR of were measured by the seahorse mito stress assay in studies investigating the link between oxidative stress, mitochondrial dysfunction and obesity (Li *et al.*, 2010). In deciphering the effect of BNip3 (an apoptotic Bcl-2 protein) in regulating mitochondrial function and lipid metabolism in THLE-2 cells, the seahorse mito stress assay was used to detect increased hepatocellular respiration in the study which concluded that the role of BNip3 in diminishing mitochondrial mass while retaining mitochondrial integrity had key consequences for lipid metabolism in hepatocytes (Glick *et al.*, 2012). It has been confirmed independently that at the time of submitting this thesis, this was the first study which used the Seahorse mito stress assay to assess the four Gentiana species and phytochemicals.

ROS are generated continuously during hepatocellular anaerobic metabolism and plays a key protective and functional role in hepatocytes. An increase in ROS above the ROS scavenging threshold of hepatocytes can be detrimental to the viability of hepatocytes. The dichlorohydrofluorescein diacetate (DCF-DA) ROS assay is highly sensitive employs a cell permeability fluorescent chemiluminescent probe to measure hepatocellular redox (Eruslanov and Kusmartsev, 2010). This assay was used to determine whether or not phytochemicals in Gentiana spp extracts preserved cell viability by preventing the accumulation of reactive oxygen species. The DCF-DA ROS assay was chosen due to its high sensitivity, wide usage and ability to offer real-time monitoring of ROS changes in hepatocytes. By using the DCF-DA ROS assay (Huang *et al.*, 2008) demonstrated that oridonin stimulated hepatocyte (HepG2) mitochondrial transmembrane permeability in a ROS-dependent mechanism. Furthermore, mitochondrial mediated apoptosis triggered by ROS in hepatocytes (HepG2) cells was studied using DCF-DA ROS assay (Sharma *et al.*, 2012). ROS production triggered by tert-butyl hydroxyperoxide was found to be significantly reduced in the presence of 10 μM quercetin after conducting the DCF-DA ROS assay (Alía *et al.*, 2006).

Apoptosis is a programmed and controlled cell death which is pre-programmed as part of a cell's life cycle. Morphological transformations including nuclear condensation, cell surface changes, cell shrinkage and DNA transformation culminating in apoptosis (Andree *et al.*, 1990). Annexin V is a calcium-dependent, phospholipid-binding protein which selectively binds to phosphatidylserine. This assay is useful in determining apoptosis based on the assertion that mammalian cells relocate phosphatidylserine from the inner face of the plasma membrane to the cell surface as soon as apoptosis is triggered putting annexin V in a position selectively bind to the released phosphatidylserine (Zhang *et al.*, 1997). Using the annexin V-FITC assay to determine whether or not phytochemicals in Gentiana spp. prevented apoptosis was key to determining if the enhanced cell viability observed in chapter 3 was as a result of promoted cell longevity via a slowdown or prevention of the apoptotic process in hepatocytes. Studies have shown that arachidonic acid causes apoptosis by producing cytosolic phospholipase A_2 eventually causing mitochondrial permeability transition (Scorrano *et al.*, 2001). Annexin V-FITC was used to determine the anti-apoptotic properties of *Fumaria parviflora* against nimesulide induced apoptosis in hepatocytes (Tripathi *et al.*, 2010).

In summary, the workflow of this chapter entailed an initial determination of the ability of the phytochemicals: gentiopicroside, sweroside and swertiamarin to enhance hepatocyte viability in the presence of arachidonic and minimise its cytotoxicity. After all these assays, the best performing phytochemical was further analysed in comparison with the best performing whole plant extracts via annexin V-FTIC apoptosis test with flow cytometry and MTT to serve as a foundation for potentiation studies and further deepen understanding into their mechanism of action.

4.2 Aim

This chapter aimed to investigate whether or not the enhanced cell viability demonstrated by the Gentiana spp. extracts in Chapter 3 could be attributed to inherent phytochemicals and if their effects were synergistic in nature. The mechanism by which phytochemicals in Gentiana spp. extracts conferred hepatocyte protection via cell viability enhancement was also studied in this chapter.

4.3 Materials and Methods

4.3.1 Cell Line, Cell Culture and Passaging

For this study, human hepatocellular (HepG2) cells were utilised. The HepG2 cells were obtained from (ATTC, Middlesex UK). All cell lines were cultured in Dulbecco's modified eagle media (DMEM) with 4 g/L glucose (Lonza, Slough, UK) supplemented with 10% foetal bovine serum (FBS) (Biosera, Sussex, UK), 1% sodium pyruvate (*Sigma-Aldrich*, UK), 1% l-glutamine (*Sigma-Aldrich*, UK), and 1% penicillin-streptomycin (BioWest, USA). When thawing cells from liquid nitrogen, vials were quickly defrosted at 37 °C in a water bath containing distilled water, washed in 5 mL of DMEM containing foetal bovine serum (FBS) 10 % and seeded in to suitable culture flask. Cells were maintained in a 37°C incubator (Binder APT, Germany), and media changes made every three days or earlier if needed. DMEM with 1 g/L glucose (Lonza, Slough, UK) supplemented with 1% FBS was used during each assay. When the cells reach the required confluency (70-80%) they were passaged or frozen for storage. During passage, cells were washed once with Dulbecco's phosphate buffered saline (DPBS) free from calcium and magnesium (*Sigma-Aldrich*, UK), trypsinised with trypsin 0.25% (1X) solution, and neutralised with DMEM containing FBS 10%. Cells were centrifuged at 500 rpm for 5 min, re-suspended in DMEM containing FBS 10 % and seeded in to a new flask. THLE-2 hepatocytes were cultured as shown in 3.3.1.

4.3.2 Single Compounds and Arachidonic Acid Preparation

Single compounds: gentiopicroside (Abcam, UK), sweroside (Sigma-Aldrich, UK), swertiamarin (Sigma-Aldrich, UK), and silymarin (Abcam, UK) were prepared by making 8mM stock solutions in DMSO and then diluted with DMEM containing FBS 10 % as needed to obtain 20 µM final concentration. An 8mM stock of arachidonic acid was prepared in DMSO and diluted to 10, 30, 50 and 80 µM with DMEM as per the requirements of each assay.

4.3.3 MTT Assay for Measuring Cell Viability of cells pre-treated with, Single Compounds: Gentiopicroside, Sweroside, and Silymarin in the Presence of Arachidonic Acid

HepG2 cells were trypsinized and seeded at a concentration of $25x10^3$ /200 µL DMEM per well for 24 h and kept in an incubator (Binder APT, Germany) at 37°C. The media was then removed and three different types of single compound treatments were applied. Cells were pre-treated with (gentiopicroside, sweroside or silymarin) 20 µM and incubated at 37°C (Binder APT, Germany) for 24 h and then treatment removed and replaced with arachidonic acid (10, 30, 50 and 80 µM) and incubated again for 37°C and then assayed via MTT as described earlier in section 3.2.4. MTT assay was also performed to compare the best performing (most hepatoprotective) single compound with the best performing plant extract deepen understanding on effects and prepare for future potentiation studies.

4.3.4 Seahorse Assay for Assessing Mitochondrial Function of cells Pre-treated with Gentiana species and Single Compounds: Gentiopicroside, Sweroside, Swertiamarin and Silymarin in the Presence of Arachidonic Acid

Seahorse assay was performed seeding HepG2 cells in a seahorse XF24 plates at a concentration of $5x10^3$ /250 µL DMEM per well and kept for 24 h in an incubator (Binder APT, Germany) at 37°C. Media was removed and cells pre-treated with single compounds: (gentiopicroside, silymarin, swertiamarin or sweroside) 20 µM and incubated for another 24 h at 37°C. Media containing treatment was discarded after the incubation period and replaced with media containing 30 µM AA and then incubated at 37°C for 24h. After incubation, seahorse assay was initiated by removing media and washing thrice with 400 µL of seahorse media containing 1 % sodium pyruvate and 4.4 g/L glucose and media stabilized at ph 7.4. After washing, 500 µL of seahorse media was placed in each well and then incubated in a non-CO_2 incubator (to minimalize the influence of incubation of conditions) pending completion of calibration plate running. The calibration plate was prepared by placing oligomycin (5 µM), FCCP (5 µM) antimycin and rotenone (5 µM) after which it was placed in the seahorse XFe 24 analyser (Aglient/Seahorse Bioscience, USA). After calibration, the assay plate was removed from the non-CO_2 incubator and placed in the seahorse XFe 24 machine which measured oxygen consumption rate (OCR) in pmol/min at oligomycin, FCCP, antimycin and rotenone injection points. The hepatocytes in the plate were normalized after reading via the BCA protein assay. Taking normalisation

results, basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration were calculated as follows shown in section 4.1.

4.3.5 DCF Assay for Assessing ROS Produced by cells Pre-treated with Gentian spp and Single Compounds: Gentiopicroside, Sweroside, Swertiamarin and Silymarin in the Presence of Arachidonic Acid

HepG2 cells were trypsinized, seeded and treated as earlier explained in 4.3.3. In this instance, however, pre-treatment included *Gentiana macrophylla* (0.01 mg/mL) in addition to the single compounds in section 4.2.3. Also, dark clear bottom 96 well plates optimized for fluorescence-based application (Thermo Fisher Scientific, UK) were utilised. After arachidonic treatment and 24 h incubation at 37°C, DCF assay was performed by removing arachidonic acid treatment and washing each well with 100 µL of 1X buffer supplied with DCFDA-cellular reactive oxygen species detection assay kit (Abcam, UK). Prior to the DCF assay, positive control HepG2 cells were treated with tert-butyl hydrogen peroxide (TBHP) 50 µM for 2hours. This treatment, as well as the 100 µL of 1X buffer were removed and DCFDA assay reagent 100 µL of 20 µM added to each well and incubated for 30 min at a temperature of 37°C away from light. DCFDA was then removed from each well and replaced with 100 µL of 1X buffer followed by the measurement of fluorescence with (Fluostar Optima, BMG Labtech, UK) at excitation 485 nm and emission 535 nm.

4.3.6 Annexin V-FITC PI Assay for Investigating Apoptosis in Hepatocytes Pre-treated with Gentiana macrophylla and Single Compounds: Gentiopicroside, Prior to Arachidonic Acid exposure.

HepG2 cells were trypsinized and seeded in a 12-well plate at a concentration of $20x10^4$ cells/mL DMEM per well for 24 h and kept in an incubator (Binder APT, Germany) at 37°C. The media was then removed after which single compound gentiopicroside (20µM) and *Gentiana macrophylla* (10 µg/mL) pre-treatments were applied and incubated at 37°C (Binder APT) for 24 h and then treatment removed and replaced with arachidonic acid (30 µM) and incubated again for 24 h. Prior to annexin V assay, apoptosis was induced in the positive control group by adding 1µg/mL actinomycin whereas the negative control had cells with DMEM without any apoptosis inducing agent. Cells were harvested and washed in cold phosphate-buffered saline (PBS), recentrifuged and then re-suspended in 100 µL of 1x binding buffer after

discarding the supernatant. Annexin V-FITC (5 µL) and propidium iodide (PI) (5 µL) from the annexin V-FITC apoptosis detection kit (Stratech, UK) were added to each 100 µL of cell suspension. The cells were then incubated at room temperature for 15 minutes followed by the addition of 400 µL of 1x buffer. Flow cytometric measurements of the samples at a fluorescence 530 nm (emission) and 575 nm. Apoptotic cells showed green fluorescence whereas necrotic cells showed both red and green fluorescence.

4.3.7 Statistics

Results refer to mean ± standard deviation and are average values from three to seven values per experiment; which were also repeated at least thrice. Evaluation of hepatocyte protection conferred by single compounds at different concentrations of AA was performed via the two-way ANOVA with Tukey multiple comparison test (detailed test results in appendix). Differences at $p<0.05$ were considered significant.

4.4 Results

4.4.1 A Comparison of the Cytotoxic Effects of Fatty Acid on Single Compounds: Gentiopicroside, Sweroside, and Silymarin Pre-treated Hepatocytes (HepG2)

This experiment investigated whether pre-treating cells with gentiopicroside, sweroside and silymarin prior to fatty acid exposure conferred a degree of hepatocyte protection to the cells. In order to establish this, HepG2 cells were treated with the above-listed compounds (20 µM) for 24 h after which treatment was replaced with media containing AA (10, 30, 50 and 80 µM) for another 24 h (Fig 4.3). Cell viability was then studied via MTT assay. Consistency in reduced AA cytotoxicity was observed in all pre-treated hepatocytes with percentage viabilities ranging from (60-159%). Hepatocytes pre-treated with gentiopicroside had the highest range of cell viability (85-159 %) across all doses of fatty acid exposure compared to untreated hepatocytes. This was followed by silymarin with a range of (73-145%) and then sweroside with a range of (60 to 135%). Vehicle control cells (Fig. 4.3) which had been not exposed to any arachidonic after phytochemical pre-treatment had the highest viabilities recorded for each treatment. The lowest cell viability of 28% was recorded for hepatocytes exposed to arachidonic acid without any phytochemical pre-treatment.

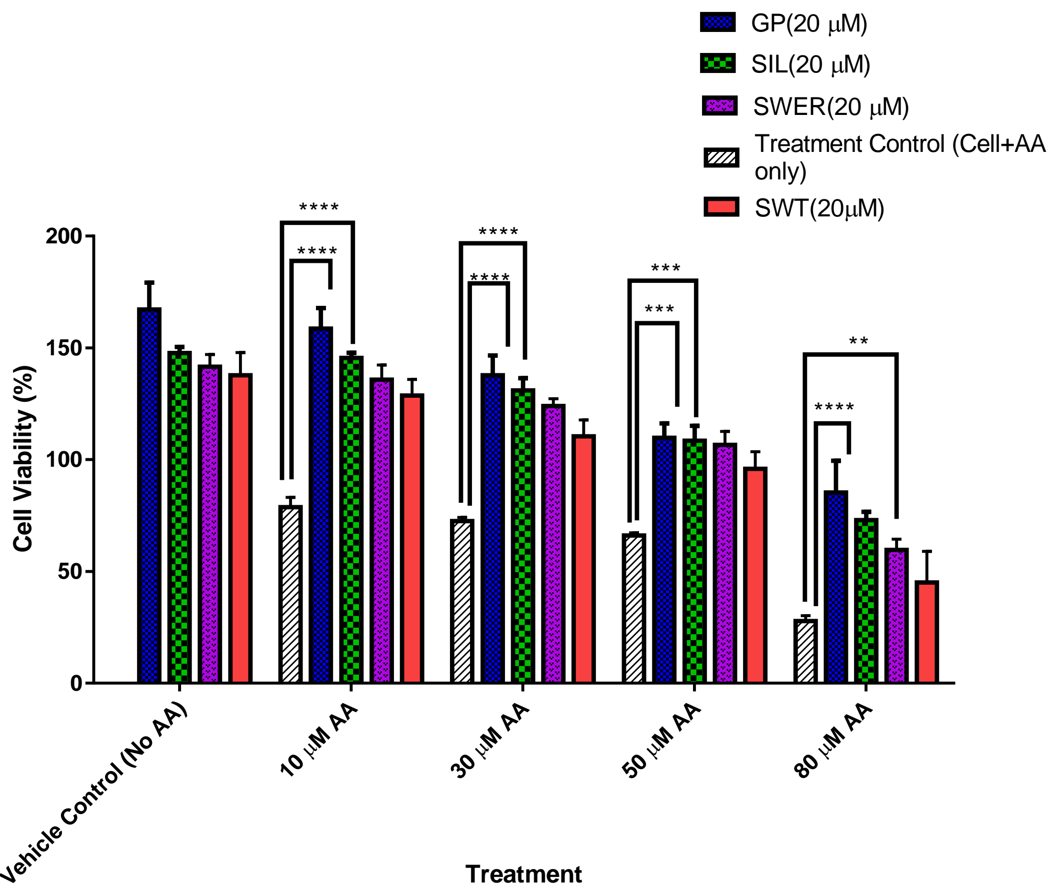


Fig. 4.3. MTT assay results showing hepatocyte protection conferred by phytochemicals. *MTT assay results showing hepatocyte protection conferred by gentiopicroside (GP), silymarin (SIL) and sweroside (SWER) pre-treatment for 24 h. For all phytochemical pre-treated hepatocytes (hepG2), AA cytotoxicity decreased compared with untreated cells. GP-treated hepatocytes presented the highest viabilities* (85-159 %) *in the presence of AA (10-80 μM) Two-way ANOVA with Tukey Multiple Comparison of phytochemical treatment factor and control (**p=0.0060), (***p=0.0002) and (****p<0.0001)*

4.4.2 A Comparison of the Cytotoxic Effects of Fatty Acid on Single Compounds: Gentiopicroside, Sweroside, and Silymarin Pre-treated THLE-2 cells (THLE-2)

In a similar fashion to HepG2 cells, THLE-2 cells treated with phytochemicals: gentiopicroside, sweroside and swertiamarin showed reduced AA cytotoxic effects in terms of diminished cell viability compared to control cells which had not been primed with phytochemicals. Using THLE-2 cells helped to determine if cell growth enhancement was only limited to HepG2 cells or could be seen in other cell types such as THLE-2 cells which are hepatocytes transformed with SV40 large T antigen. Cell viability was within the range of 77 to 153 % for gentiopicroside which elicited the highest hepatocyte viability among the phytochemicals tested when compared to

control. There was a general trend of cell viability reducing with increase in AA concentration. Cells which were devoid of priming with phytochemicals but exposed to AA (10-80 μM) yielded viabilities of 35-76 %. Other phytochemicals including sweroside, swertiamarin and silymarin enhance cellular viability as well by up to 137 %. The treatment of hepatocytes with phytochemicals alone did not appear to diminish cell viability of hepatocytes but rather enhanced it with viabilities of 127, 134, 140, 153 % recorded for swertiamarin, sweroside, silymarin and gentiopicroside respectively as shown in Fig 4.4.

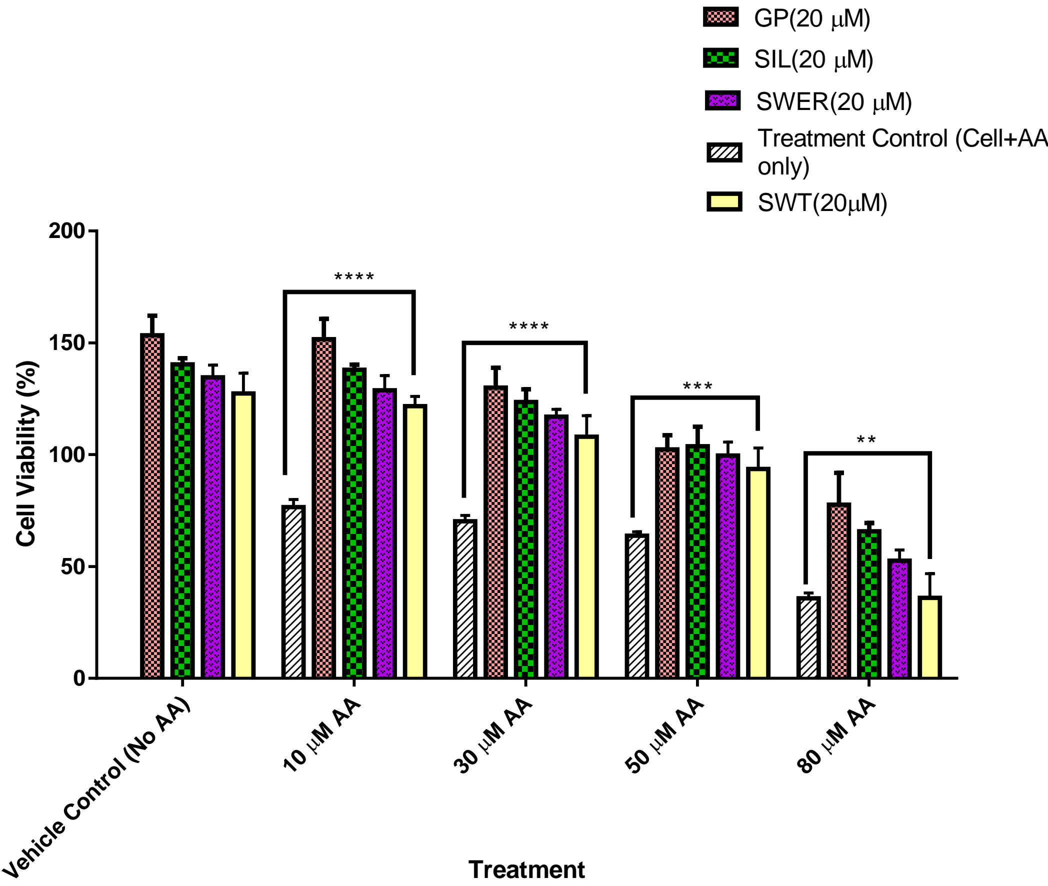


Fig. 4.4 Hepatocyte protection conferred on THLE-2 cells by phytochemical pre-treatment for 24 h. *For all phytochemical pre-treated hepatocytes, AA cytotoxicity decreased compared with untreated cells. GM-treated hepatocytes presented the highest viabilities* (77-151%) *in the presence of AA (10-80 μM). Hepatocytes treated with only phytochemicals (i.e. vehicle control) yielded viabilities up to 153 %. The four phytochemicals Data presented as mean±SD Two-way ANOVA with Tukey Multiple ****p=0.0001, ***p=0.0003 and **p=0.001.*

4.4.3 A Comparative Assessment of Hepatoprotective Effects of Pre-Treatment with Gentiana lutea and Gentiana macrophylla compared to Single Compounds: Gentiopicroside and Silymarin against Cytotoxic Effects of Arachidonic Acid

Prior to completing MTT assays, a comparative MTT assay was performed to compare the two leading Gentiana species (macrophylla and lutea) as per MTT assay results in chapter 3 with the top two performing single compounds (gentiopicroside and silymarin) based on MTT assay results from section 4.3.1. This was aimed at forming a basis for potentiation studies and also to investigate in part, the possible synergistic effects which could be obtained by combining the dominant single compounds in the plant extracts. Furthermore, this comparison was also aimed at forging a foundation for studies comparing the possible use of the whole root extract in a future wellness hepatoprotective agent against using the single compounds individually as hepatoprotectives. The results of these comparisons were preliminary and further in-vivo studies and full scale clinical trials would need to be conducted in order to properly establish the usage of these extracts as a wellness product and also draw a conclusive comparison between the phytochemicals and the Gentiana species extracts studied. The results showed that gentiopicroside pre-treated hepatocytes had the highest viability of up to 124% when treated with (10 to 30 µM AA), however, at 80 µM AA, lutea and macrophylla pre-treated hepatocytes had a higher viability (78% and 72%) than silymarin treated hepatocytes (70%) and also at par with gentiopicroside at the 80 µM AA treatment with gentiopicroside pre-treated cells viable at 80.2% (Fig 4.5). These results suggest possible influence of synergistic effect by different phytochemicals since the levels of gentiopicroside present in the 10 µg/mL lutea and macrophylla refluxed extracts (i.e. 1µg/mL (2.8 µM) and 0.4µg/mL (1.2 µM)) as per HPLC analysis were lower than the 20 µM found in the single compound. These scenarios were further analysed in more details in the discussion.

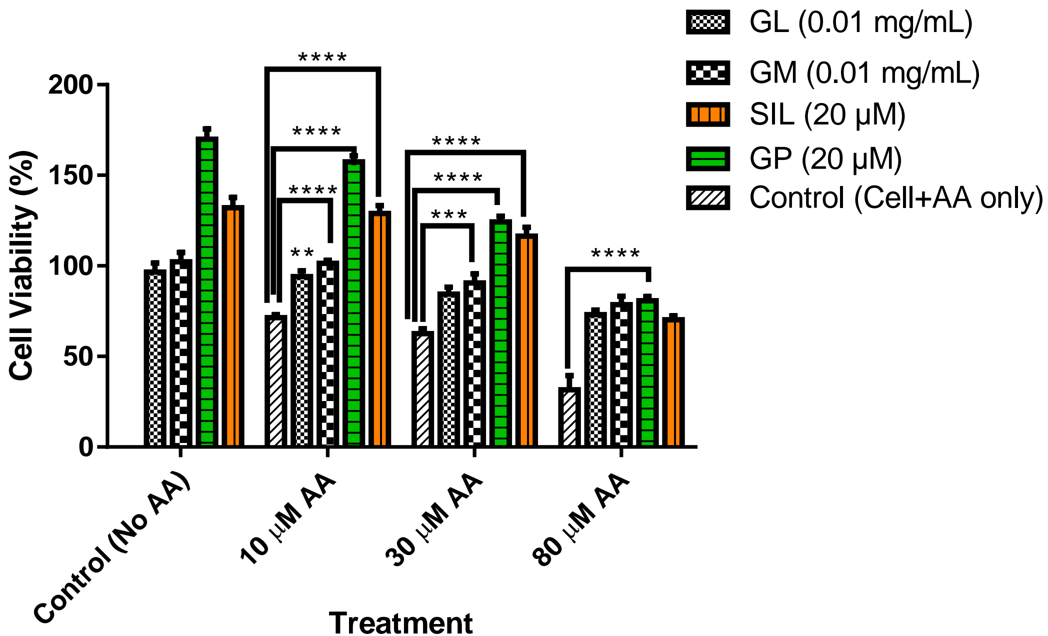


Fig 4.5 Comparative Assessment of Hepatoprotective Effects of Pre-Treatment with Gentiana lutea and Gentiana macrophylla compared to Single Compounds*. Graph shows highest viability in gentiopicroside treated hepatocytes over 150% in the presence of fatty acids. Two-way ANOVA with Comparison of phytochemical/extract treatment factor and control (***p=0.0002) and (****p<0.0001).*

4.4.4 A Comparison of the Effects of G. lutea, G. macrophylla and Single Compounds: Gentiopicroside, Sweroside, and Silymarin pre-treatment on Hepatocyte Mitochondrial Function in the Presence of Arachidonic Acid

The seahorse mitochondrial stress test enabled the measurement of basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration in hepatocytes pre-treated with *Gentiana lutea (0.01 mg/mL)*, *Gentiana macrophylla* (0.01 mg/mL), gentiopicroside, sweroside and silymarin (20 μM) before being exposed to arachidonic acid (30 μM). By injecting oligomycin, FCCP, antimycin and rotenone, the various complexes were inhibited as shown in (Fig 4.2) and explained in section 4.2.4 to enable the measurement of basal respiration, ATP production, spare respiratory capacity, non-mitochondrial respiration, maximal respiration and proton leak. A typical seahorse trace for gentiopicroside, sweroside and swertiamarin is shown in Fig 4.6. The concentration of ATP produced by phytochemical pre-treated hepatocytes appeared to increase compared to untreated hepatocytes exposed to arachidonic acid (Fig 4.8.). Gentiopicroside pre-treated hepatocytes caused an ATP production of 75 pmol/min followed by sweroside with 75 pmol/min. Basal respiration was also enhanced in pre-

treated hepatocytes compared to untreated hepatocytes exposed to fatty acids (Fig 4.7). Sweroside pre-treated hepatocytes presented the highest basal respiration of 114 pmol/min followed by gentiopicroside with 109 pmol/min. Pre-treating hepatocytes with phytochemicals also enhanced the maximal respiratory capacity of the cells even after they were exposed to arachidonic acid (Fig 4.8). This effect was mostly seen with sweroside pre-treatment up to 281 pmol/min followed by gentiopicroside up to 192 pmol/min. Gentiopicroside pre-treated hepatocytes presented the highest non-mitochondrial respiration of 115 pmol/min followed by sweroside with 80 pmol/min (Fig 4.10.). Spare respiratory capacity of hepatocytes was markedly increased by sweroside up to 115 pmol/min followed by gentiopicroside up to 95 pmol/min (Fig 4.11.). As far as proton leak is concerned, it was observed in all the phytochemicals used but markedly seen in gentiopicroside and gentiopicroside followed by sweroside up to 49 pmol/min (fig 4.12). Considering the effect of whole plant extracts on mitochondrial function, ATP production was increased by *Gentiana macrophylla* and *Gentiana lutea* pre-treatment up 79 pmol/min with the highest increase seen in macrophylla species (Fig 4.13b). Following a similar pattern, basal respiration increment was seen upon the application of both extracts with macrophylla species enhancing it up to 109 pmol/min which was higher than control cells exposed to fatty acids without any Gentiana extract pre-treatment (Fig 4.12a). Maximal respiration, spare respiratory capacity, non-mitochondrial respiration and proton leak were all increased by lutea and macrophylla treatments with the highest increase seen with macrophylla in each case, up to (202, 77,76 and 52 pmol/min) respectively (Fig4.12c-f). In the case of control cells with DMSO as well as negative control cells with only AA treatment reduced OCR rates were recorded for all parameters studied.

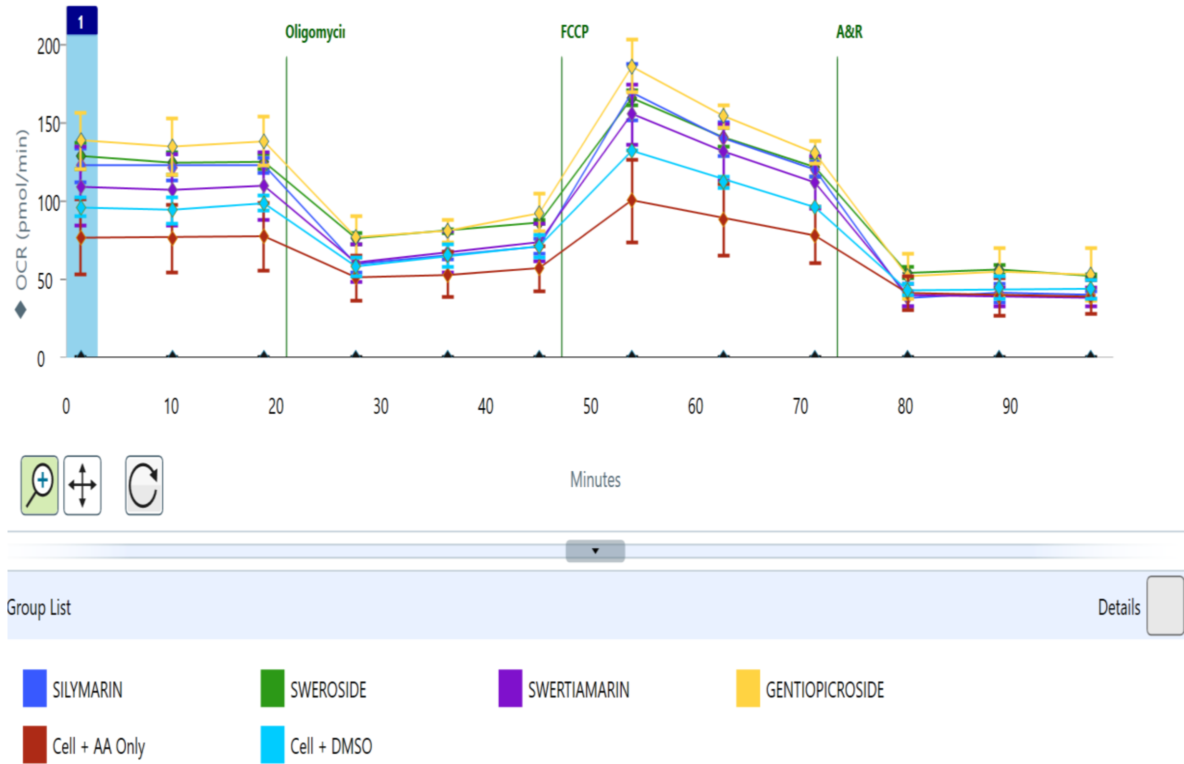


Fig. 4.6. Typical seahorse mito stress test trace for phytochemicals. *Typical seahorse mito stress test trace for gentiopicroside, silymarin, swertiamarin and sweroside showing the injection points of oligomycin, FCCP, antimycin and rotenone and the resultant effect on oxygen consumption rate (OCR) of hepatocytes after injection.*

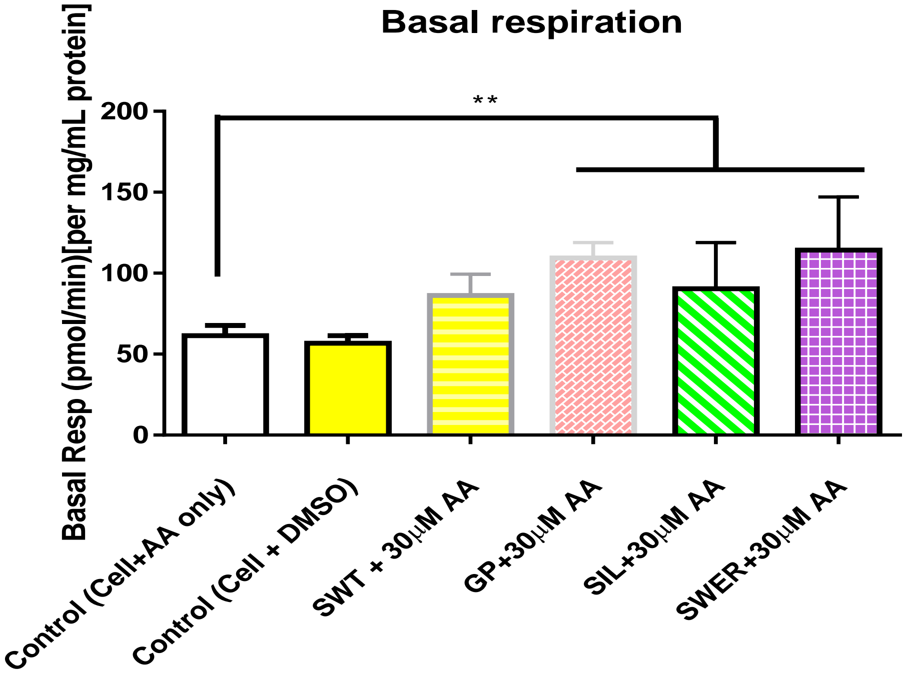


Fig. 4.7*. *Basal respiration graph. *Basal respiration from the seahorse mito stress test showing reduced basal respiration in control hepatocytes but increased basal respiration up to 114 pmol/min in pre-treated hepatocytes. Two-way ANOVA with Tukey Multiple Comparison of data shows significance of the effect of treatments with **p=0.0055*

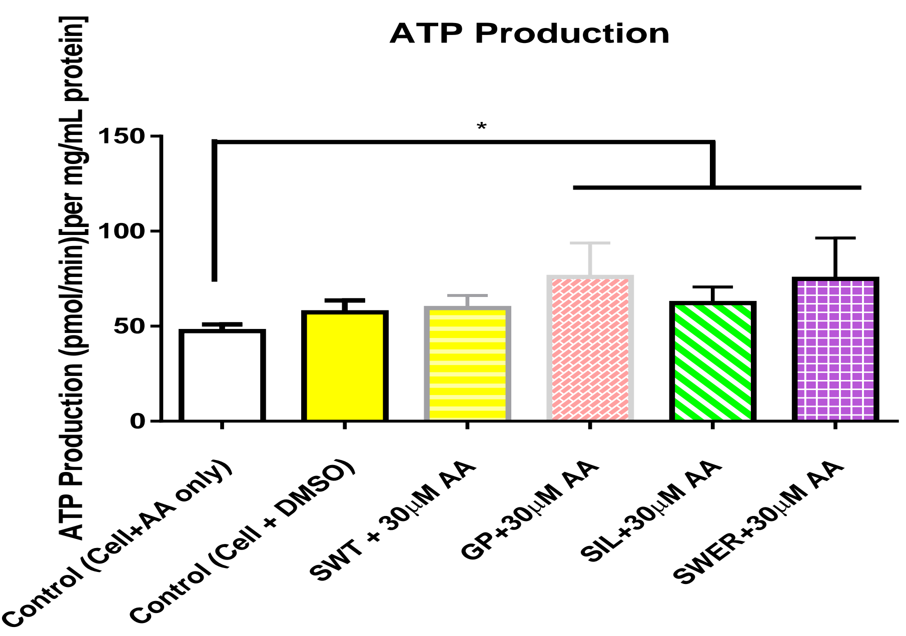


Fig. 4.8. ATP production graph. *ATP production calculated from the seahorse mito stress test showing reduced ATP production in control hepatocytes but increased ATP production up to 75.9 pmol/min in pre-treated hepatocytes. Two-way ANOVA with Tukey Multiple Comparison of data shows significance of the effect of treatments with *p<0.05*

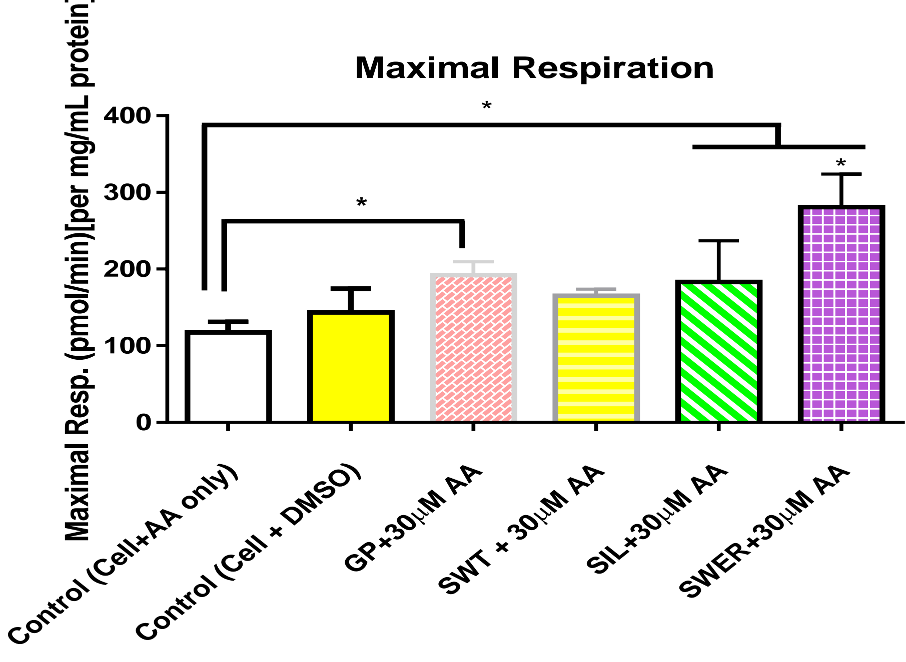


Fig. 4.9. Maximal respiration graph. *Maximal respiration calculated from the seahorse mito stress test showing maximal respiration in control hepatocytes but increased up to 281 pmol/min in pre-treated hepatocytes. Two-way ANOVA with Tukey Multiple Comparison of data shows significance of the effect of treatments with *p<0.05*

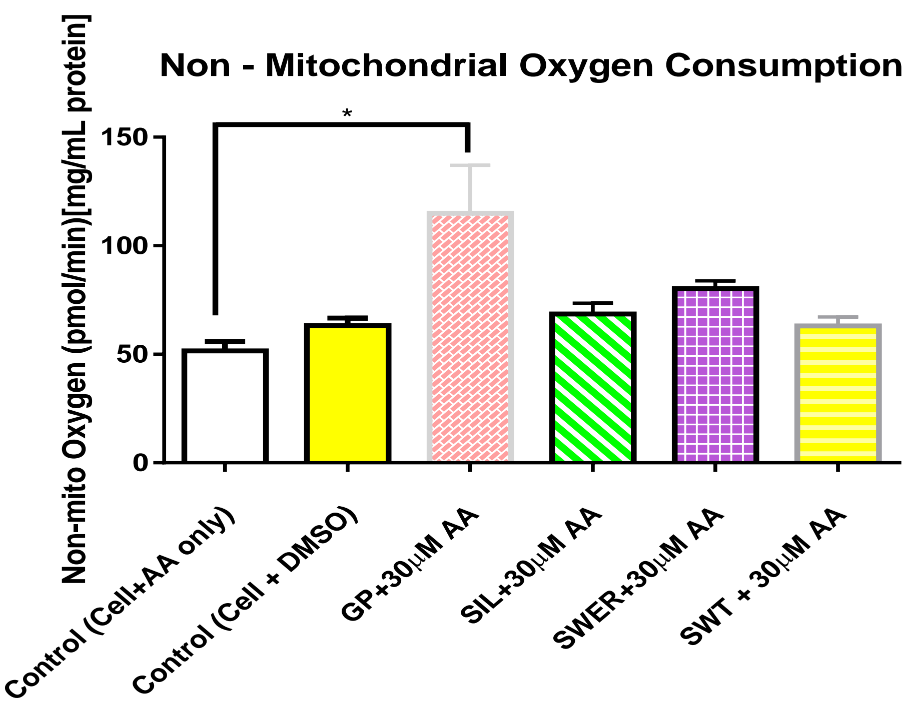


Fig. 4.10. ***Non-mitochondrial oxygen consumption graph****. Non-mitochondrial oxygen consumption calculated from the seahorse mito stress test showing reduced non-mitochondrial Oxygen Consumption in control hepatocytes but increased up to114.9 pmol/min in pre-treated hepatocytes. Two-way ANOVA with Tukey Multiple Comparison of data shows significance of the effect of treatments with *p<0.05*

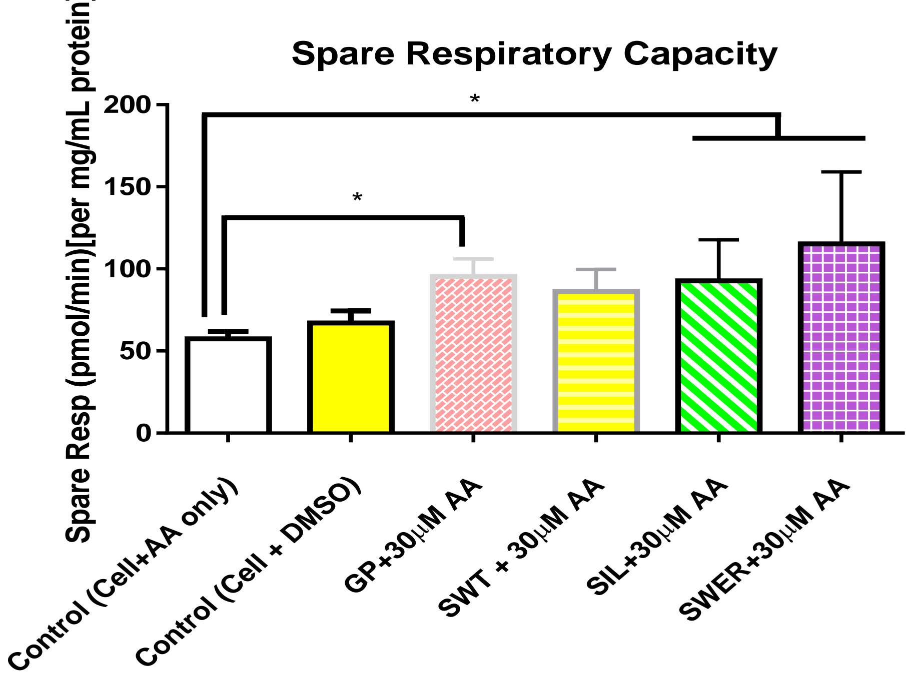


Fig. 4.11. Spare respiratory capacity graph*. Spare respiratory capacity calculated from the seahorse mito stress test showing reduced spare respiratory capacity in control hepatocytes but increased up to 115.2 pmol/min in pre-treated hepatocytes. Two-way ANOVA with Tukey Multiple Comparison of data shows significance of the effect of treatments with *p<0.05*

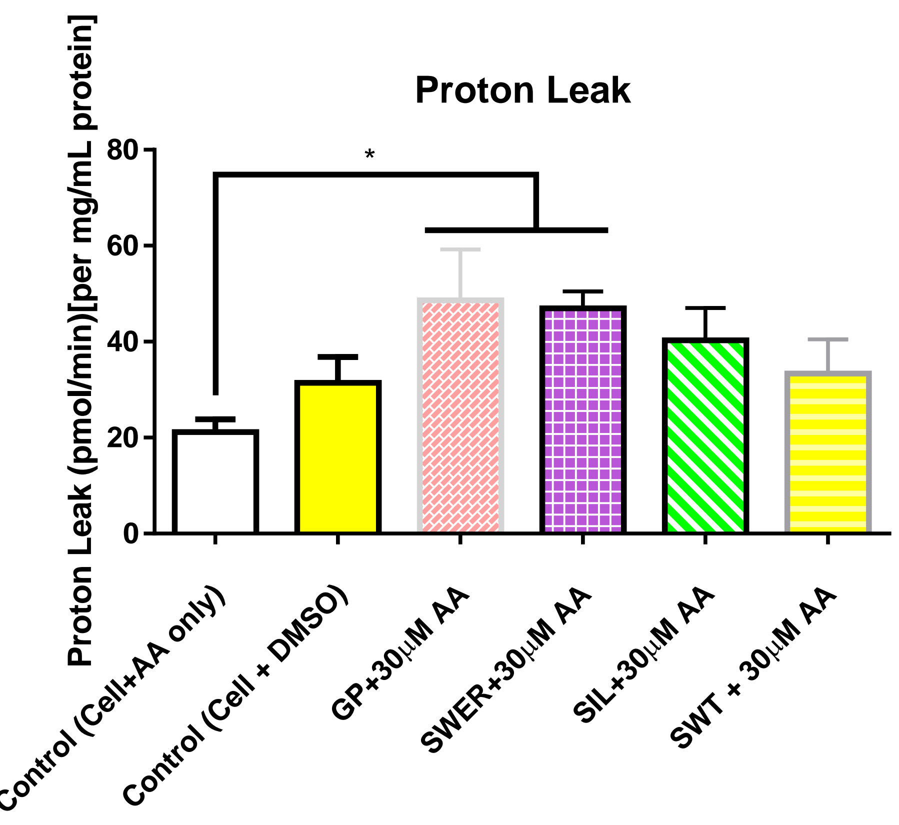


Fig. 4.12. Proton Leak graph. *Spare respiratory capacity calculated from the seahorse mito stress test showing reduced spare reduced proton leak in control hepatocytes but increased up to 48 pmol/min in pre-treated hepatocytes. Two-way ANOVA with Tukey Multiple Comparison of data shows significance of the effect of treatments with *$p<0.05$*

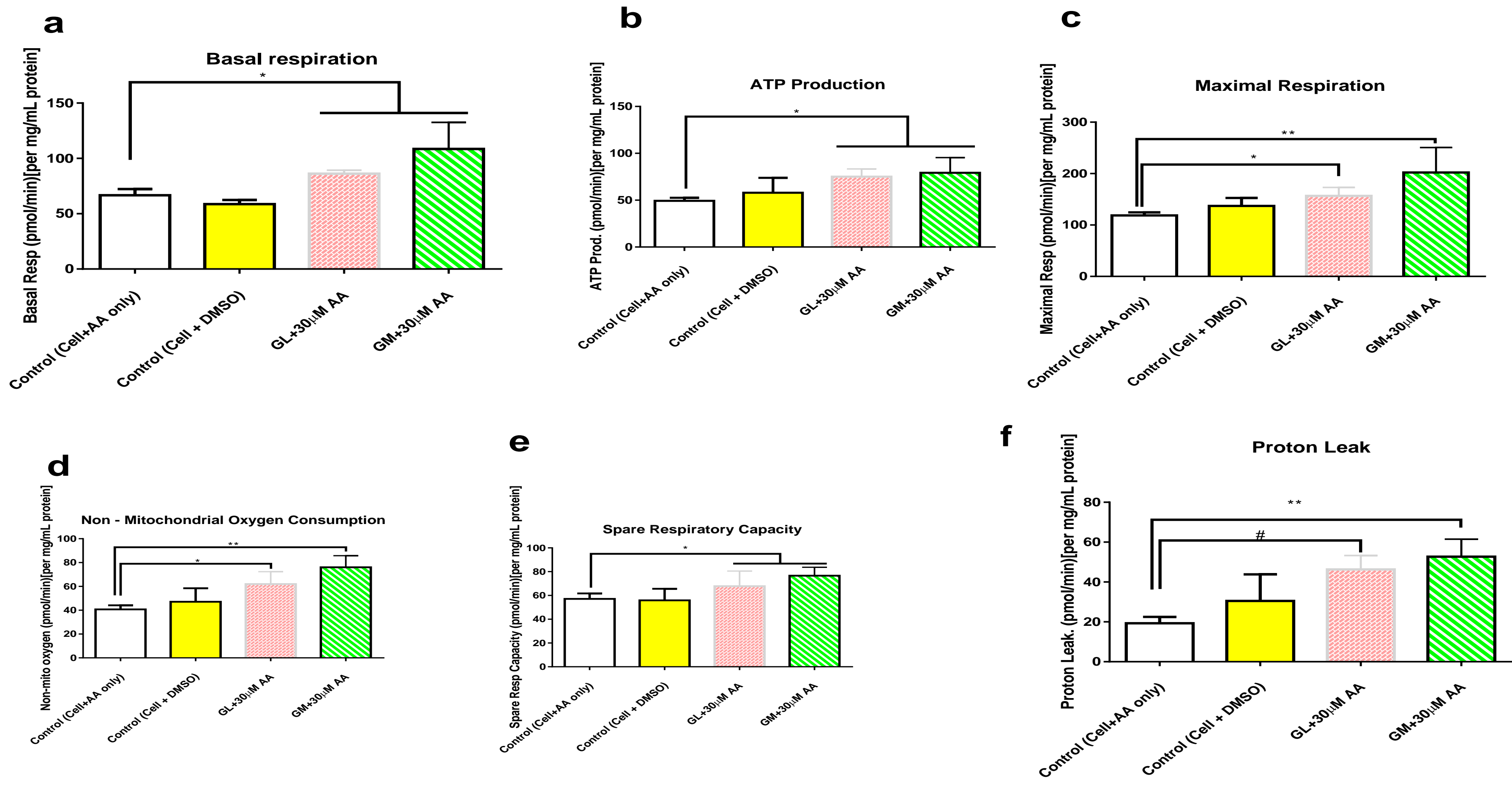


Fig. 4.13. Seahorse mito stress test of G. lutea and G. macrophylla. *Seahorse mito stress test of G. lutea and G. macrophylla showing increased: (a) Basal respiration in pre-treated hepatocytes up to 108 pmol *p=0.0439;(b)ATP production in pre-treated hepatocytes up to 79.2 pmol **p=0.00284;(c) Maximal respiration in pre-treated hepatocytes up to 202.1 pmol *p=0.0212, **p=0.0080;(d) Non-mitochondrial respiration in pre-treated hepatocytes up to 76 pmol *p=0.0132, **p=0.0024;(e)Spare respiratory capacity in pre-treated hepatocytes up to 76.6 pmol *p<0.05; (f) Proton leak up to 52 pmol **p<0.05 #p<0.05. All data analysed via two-way ANOVA assessing the significance of drug treatments*

4.4.5 Effect of Gentiana Macrophylla and Single Compounds: Gentiopicroside, Sweroside, Swertiamarin and Silymarin pre-treatment on Hepatocyte ROS Production in the Presence of Arachidonic Acid

This test evaluated the ROS scavenging effects of the above-listed phytochemicals in comparison to silymarin which is a well-known ROS scavenging phytochemical. In this instance, the presence of AA (10 µM) caused an increase in ROS by up to 112% which however decreased at higher doses of AA (30, 50 and 80 µM) (Fig 4.13). Although there were variations in the amounts of ROS scavenged by the different pre-treatments, sweroside and silymarin were most consistent and portrayed the best ROS scavenging capacity of up to 67 and 71 % respectively (Fig 4.13).

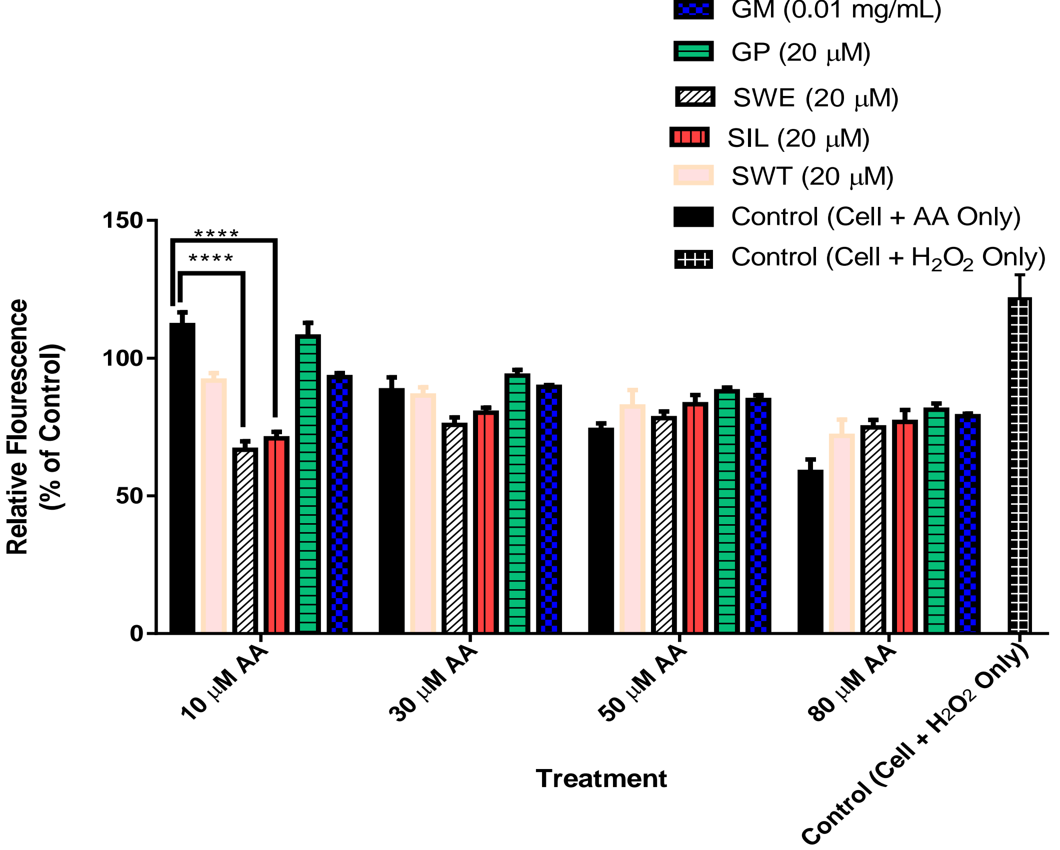


Fig. 4.13. ***DCF assay results of HepG2 cells exposed to AA****. Results of DCF assay showing relative fluorescence which depicts the amount of ROS produced at each instant. ROS is scavenged to a degree by pre-treatments but markedly in sweroside and silymarin (67 and 71%) respectively. Higher doses of AA (30, 50 and 80 µM) shows decrease in the amount of ROS produced. Results analysed by two-way ANOVA with Tukey multiple comparisons ****p<0.0001*

4.4.6 Comparative Assessment of Hepatocyte (HepG2) Protection via Apoptosis and Necrosis Prevention by Gentiana Macrophylla and Gentiopicroside

This study employed the annexin V-FITC-PI assay to assess whether or not pre-treating hepatocytes with gentiopicroside and *Gentiana macrophylla* prevented apoptosis and necrosis in the presence of 30 µM arachidonic acid. This study was a further validation of MTT assays carried out earlier which deduced that Gentiana *macrophylla* pre-treatment enhanced cell viability by up to 118% (section 3.3.5) which was the highest among the four Gentiana species tested whereas gentiopicroside produced viability up to 159% (section 4.3.3) which was also the highest among all the phytochemicals tested. However, the MTT assay was limited in terms of not differentiating between apoptotic and necrotic death, hence the need to perform the annexin V-FITC-PI assay. Scatter diagrams of the results showed a high degree of apoptosis (75%) and low necrosis (9%) in positive control cells exposed to 1 µg/mL actinomycin (Fig 4.14 (a) and Fig 4.15). Negative control cells seeded with DMEM and DMSO 0.1 % only, also showed a high proportion of live cells (97%) (Fig 4.14(b) and Fig 4.15). Treatment of hepatocytes with 30 µM AA increased apoptosis up to 56% as seen in Fig 4.14 (c) and Fig 4.15. Pre-treatment of hepatocytes with gentiopicroside and *Gentiana macrophylla* prior to arachidonic acid exposure increased the proportion of live cells up to 87 and 95 % respectively while reducing apoptosis to 10 and 3% Fig 4.14 (d-e) and Fig 4.15. Necrosis was also reduced significantly in the presence of both pre-treatments.

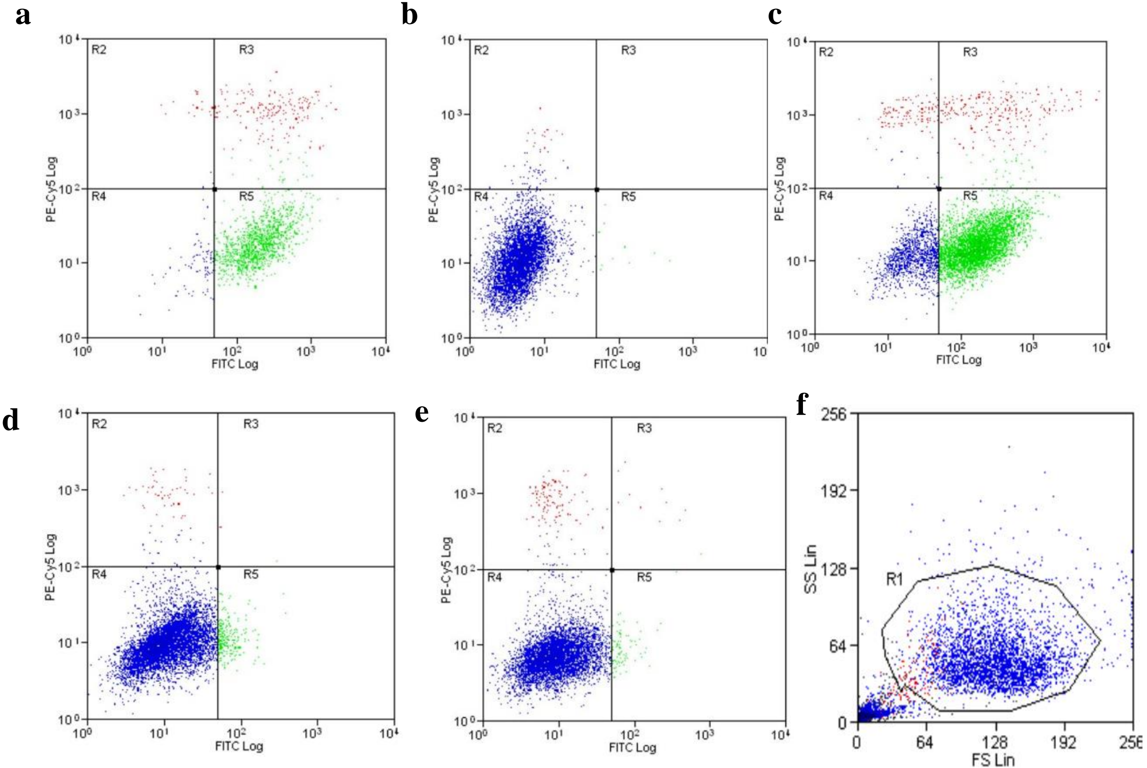


Fig. 4.14. ***Results of Annexin V-FITC and PI assay****. (a) Scatter diagrams of positive control cells exposed to 1µg/mL actinomycin showing a high level of apoptosis (b) Negative control cells seeded with DMEM and 0.1%DMSO only showing a high proportion of live cells. (c) Cells with 30 µM AA only and no drug pre-treatment presenting live, apoptotic and necrotic cells. (d) Cells with 20 µM GP pre-treatment for 24 h before 30 µM AA exposure. (e) Cells with 10 µg/mL GM pre-treatment for 24 h before 30 µM AA exposure and (f) flow cytometry gating strategy*

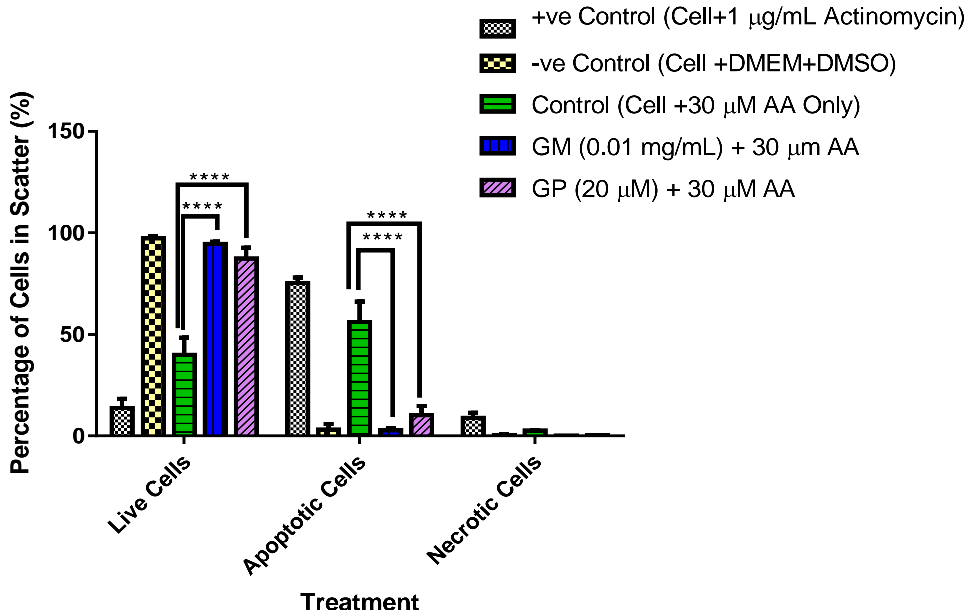


Fig. 4.15. Histogram showing level of apoptosis and necrosis in hepatocytes pre-treated with GP and GM. Apoptosis is reduced by up to 53.3% in GM pre-treated hepatocytes compared to control cells without *any pre-treatment prior to AA exposure. Two-way ANOVA with Tukey Multiple Comparison of data shows statistically significant difference between GM/GP pre-treated cells and control cells exposed to AA without any drug pre-treatment ****p<0.0001*

4.5 Discussion

After discovering gentiopicroside, sweroside and swertiamrine in all four Gentiana species, and determining that the extracts conferred a degree of hepatocyte protection, this study aimed to establish whether or not the phytochemicals found in the extracts conferred any form of hepatocyte protection via the pre-treatment method which proved most effective in assessing the hepatocyte protection provided by Gentiana species as seen in section 3.3.5 following an MTT assay. It was generally observed that phytochemicals: gentiopicroside, sweroside and swertiamarin conferred hepatocyte protection in terms of enhancing cell growth by promoting mitochondrial function in the presence of AA, preventing apoptosis and the build-up of ROS. These effects were in some cases greater than those elicited by silymarin.

In this study, gentiopicroside pre-treated hepatocytes emerged with the highest viability, followed by silymarin and then finally sweroside in order of decreasing cell viability in both HepG2 cells and THLE-2 cells. This result agreed with studies by (Zhao *et al.*, 2015) showing that after pre-treating chondrocytes with 50-150 µg/mL of gentiopicroside for 24 h followed by MTT, there was no toxic effects present but rather increased function. A mitogenic attribute of gentiopicroside was also observed with increased cell replication. This attribute needs to be further investigated to gain more understanding. Gentiopicroside is known to possess hepatoprotective effects on d-galactosamine and lipopolysaccharide induced hepatic failure (Lian, 2010). Furthermore, gentiopicroside was shown to exhibit hepatoprotective effects on IL-1β induced inflammation response in rat articular chondrocyte. Silymarin and glycyrrhizin have been shown to use a common hepatoprotective pathway in protecting the liver from primary biliary cirrhosis in tests using HepG2 cells (Karim, 2014). Furthermore silymarin has been found to be an effective hepatoprotective agent against fatty liver disease induced in rats (Zhang *et al.*, 2013). Bearing these in mind the phytochemicals were tested alongside silymarin in order to obtain a known and familiar point of reference. Sweroside, which was the third most effective phytochemical in terms of cell viability maintenance in this study has shown hepatoprotective properties against carbon-tetrachloride induced injury in rats (Mihailovic *et al.*, 2013). It was however observed that cell viability enhancement was more pronounced in HepG2 cells than THLE-2 cells. This could be because, HepG2

cells possess higher sensitivity for basic compounds whereas THLE-2 cells possessed higher sensitivity for acidic and neutral compounds (Shah *et al.*, 2014).

In studies comparing *Gentiana lutea* and *Gentiana macrophylla* to gentiopicroside via MTT it was observed that at 80 µM AA, lutea and macrophylla pre-treated hepatocytes had a higher viability (78% and 72%) than silymarin treated hepatocytes (70%) and also at par with gentiopicroside at the 80 µM AA treatment with gentiopicroside pre-treated cells viable at 80.2%. At 10 and 30 µM AA gentiopicroside produced the highest viabilities in both cases over 50 % above control showing a mitogenic effect. These results suggest possible influence of synergistic effect by different phytochemicals in the extracts especially when faced with higher concentrations of AA (80 30 µM AA) since the levels of gentiopicroside present in the 10 µg/mL refluxed extracts of *Gentiana lutea* and *Gentiana macrophylla* (i.e. 1 µg/mL (2.8 µM) and 0.4µg/mL (1.2 µM)) respectively as per HPLC analysis were lower than the 20 µM found in the single compound. The MTT assay results observed showed that pre-treated hepatocytes had substantial amounts of mitochondrial dehydrogenases which converted the MTT's yellow tetrazone to purple coloured formazan.

The next step entailed further studying mitochondrial function in terms of ATP production, basal respiration, maximal respiration, spare respiratory capacity, proton leak and non-mitochondrial respiration in pre-treated (i.e. primed) hepatocytes via the seahorse mitochondrial stress test. This was to determine if any of these parameters had any bearing on maintenance of cell viability by the named phytochemicals. The initial injection of 5 µM oligomycin suppressed complex V and ATP synthase, enabling the measurement of ATP production, after subtracting from basal oxygen consumption rate. Impaired mitochondrial respiration and hepatic ATP synthesis has been associated with the accumulation of fatty acids in hepatocytes (Paradies *et al.*, 2014). However, phytochemical pre-treated hepatocytes in this study had a higher rate of ATP production of up to 75 pmol/min observed with gentiopicroside compared to untreated hepatocytes which were exposed to 30 µM of arachidonic acid. Although, the rate of ATP production was increased across all pre-treated hepatocytes, a slightly higher increase was seen in *G. macrophylla* pre-treated hepatocytes compared to the single phytochemicals. Phytochemicals such as gentiopicroside, sweroside and amarogentin which are gastro-protective and hepatoprotective have been found in *G.*

lutea and *G. macrophylla* extracts (Singh, 2008). Bearing this in mind, there is a possibility of synergistic effect being demonstrated by a combination of the phytochemicals present in *G. macrophylla* responsible for the increased ATP production levels compared to the individual single compounds. This possibility requires further investigation in potentiation experiments aimed at determining whether or not combination of phytochemicals from Gentiana species can better enhance hepatoprotective action.

Mitochondrial respiration is essential due to the electrochemical gradient it generates which is utilised in the production of ATP (Paradies *et al*., 2014). The injection of antimycin A and rotenone suppressed complexes I & III respectively which enabled the measurement of non-mitochondrial respiration deducted from basal oxygen consumption rate to determine basal respiration. In this instance, the amount of mitochondrial respiration was decreased in hepatocytes which lacked phytochemical/extract pre-treatment compared to pre-treated hepatocytes up to 115 pmol/min for gentiopicroside. Following a similar pattern basal respiration, maximal respiration and spare respiratory capacity were all increased in phytochemical/extract pre-treated as described in the results at section 4.4.4. These results denote the possibility that phytochemicals: gentiopicroside, sweroside and swertiamarin may protect hepatocytes form arachidonic acid induced cytotoxicity by enhancing mitochondrial function in terms of ATP production, basal respiration of cells, increasing cellular respiratory capacity as seen in maximal respiration results and also broadening the spare respiratory capacity of hepatocytes which is required to meet rapid energy demands of the cells especially for dealing with a high influx of fatty acids (AA) as seen in Fig 4.6 to 4.12. However, gentiopicroside displayed mitogenic effects.

Gentiopicroside pre-treated cells had a very high non-mitochondrial respiration capacity of 115 pmol/min raising the possibility that the effects of gentiopicroside on hepatocytes extend beyond the mitochondria into other cellular organelles. This however needs to be confirmed through further investigations. There is evidence which indicates that hepatic mitochondrial dysfunction is crucial to the pathogenesis of NAFLD. This is because the resultant electron flow disruption associated with a dysfunctional mitochondrial respiration causes the preceding respiratory intermediates

to transfer electrons to molecular oxygen, hence producing superoxide anions and hydrogen peroxide in the process (Wei *et al.*, 2008). Hence the protection and enhanced function conferred by gentiopicroside, sweroside, swertiamarin and Gentiana extracts to the mitochondria could be a point of intervention in the pathogenesis of NAFLD.

Proton leak is one key factor which affects mitochondrial coupling efficiency and ROS production. It is cell-type specific, caused by mitochondrial anion carriers directly proportional to cellular metabolic rate (Jastroch *et al.*, 2010). This correlation between proton leak and cellular metabolic rate may have contributed to the increased amount of proton leak observed in pre-treated HepG2 as seen in Fig 4.12 and 4.13(f). The phytochemicals gentiopicroside and sweroside which produced the highest ATP productions also observed increased proton as also seen in the instance of *Gentiana macrophylla.* The site for proton leak is in the inner mitochondrial membrane of eukaryotes and accounts for about 20 % of standard metabolic rates in rats (Stuart *et al.*, 1999). As a result, lower levels of proton leak of up to 21 *pmol/min* was observed for control cells even though they had no phytochemical or plant extract pre-treatment.

Linked to the mitochondria is ROS production, since the mitochondria serves as a major intracellular source of ROS generated at complex I and III (complex I and III was assessed in section 4.3.4 via seahorse mito stress assay using antimycin and rotenone) of the respiratory chain. Increase in the amount of ROS has been linked to DNA mutations, ageing, apoptosis and necrosis (Orrenius, 2007). ROS has been successfully measured in HepG2 cells using a fluorescent probe 2,7-dichlotofluorescein diacetate (DCFH-DA) (Sohn *et al.*, 2005). The DCF-DA ROS assay performed in this study showed that the phytochemicals (sweroside, silymarin and swertiamarin) scavenged ROS produced. Secoiridoid glycosides inhibit free radical activity and prevent the onset of peroxidation reactions (Gülçin *et al.*, 2009). However, sweroside possessed the highest ROS scavenging effect, followed by silymarin and swertiamarin as shown in section 4.3.5. Sweroside has been found to possess reactive oxygen species scavenging effects (Nawa *et al.*, 2007). In HepG2 cells, silymarin showed antioxidant and hepatoprotective activity against tacrine-induced cytotoxicity (Jung *et al.*, 2004). A dose of 10-100 µM silymarin possessed antioxidant effects in HepG2 cells against bleomycin which is a known ROS generator

(Angeli *et al*., 2009). It was observed that upon treating hepatocytes with 10 µM arachidonic acid, ROS levels were increased up to 112%. This was to be expected because studies by (Cocco *et al*., 1999) indicate that arachidonic acid causes an increase in the production of ROS when it interacts with mitochondrial electron transport chain by causing an increased production of hydrogen peroxide in addition to the mitochondria respiring with pyruvate, malate or succinate as substrate. This increase in ROS production was reduced at a dose of 80 µM AA in, possibly as a result of increased cell death in untreated hepatocytes due to high oxidative stress. Oxidative stress leading to cell death can be caused by an imbalance between reactive oxygen species and antioxidant defenses(Klamt *et al*., 2002). Hence the lack of an active ROS scavenger can be detrimental to viability of hepatocytes exposed to ROS producing compounds.

ROS levels in gentiopicroside pre-treated hepatocytes were quite high, although not as high as control cells treated with only 10 µM AA and not primed with gentiopicroside or other phytochemicals tested. Enhanced mitochondrial function produced higher amount of ROS via the mitochondrial electron transport chain than ROS produced by glutathione depletion (Tan *et al*., 1998b). Hence enhancing mitochondrial function without a concurrent increase in maximal respiratory capacity could lead to the production high amounts of ROS above cellular respiratory capacity. As seen in the seahorse mito stress assay, gentiopicroside and sweroside acted on mitochondrial complex I and III producing a very high basal respiration but sweroside pre-treated cells, apart from having a high basal respiration had the highest maximal respiration capacity whereas gentiopicroside pre-treated cells had a low maximal respiration capacity. This may account for the better performance of sweroside than gentiopicroside in managing ROS generated by hepatocytes. Under normal conditions, a percentage of oxygen consumed by the mitochondria of hepatocytes are changed into superoxides by complex I and III (Ligeret *et al*., 2008). Mitochondrial electron transport chain induced superoxide production is also linked to increased protein oxidation (Klamt *et al*., 2002) . This factor also explains the production of ROS by control hepatocytes which had neither been pre-treated nor exposed to arachidonic acid, since the mitochondria has an inherent ability to produce a level of ROS.

Loss of cell function and eventual apoptosis or necrosis are the end results of oxidative stress emanating from high ROS levels (Halliwell and Gutteridge, 2015). The annexin V-FITC-PI assay assessed the anti-apoptotic/anti-necrotic effect of gentiopicroside and *Gentiana macrophylla* pre-treatment against arachidonic acid induced apoptosis/necrosis. The presence of polyunsaturated fatty acids such as arachidonic acid coupled with the increased production of reactive oxygen intermediates by cells expressing CYP2E1 in HepG2 cells causes cellular toxicity leading to lipid peroxidation and eventually apoptosis (Chen *et al*., 1998). Arachidonic acid is also an intermediate in apoptosis signalling regulated by cytochrome c oxidase subunit 2 (COX-2) and fatty acid-CoA ligase 4 (FACL4) (Cao *et al*., 2000). These support the increased necrosis and apoptosis seen in control cells exposed to arachidonic acid 30 μM without any *Gentiana macrophylla* or gentiopicroside pre-treatment seen in section 4.4.6. (Fig 4.14c). Apoptosis was however markedly reduced in gentiopicroside and *Gentiana macrophylla* pre-treated cells by up to 53.3%. The antiapoptotic effect of gentiopicroside is credited with its hepatoprotective effects against D-galactosamine/lipopolysaccharide-induced hepatic failure (Lian, 2010). Mitochondrial dysfunction causes the release of cytochrome c and other pro-apoptotic proteins, which initiates caspase activation and apoptosis. This raises the possibility that the anti-apoptotic effect of gentiopicroside may also be linked with its ability to improve the efficiency of mitochondrial function in terms of mitochondrial ATP production and basal respiration as seen in the seahorse mito stress assay results. It has also been reported that *Gentiana macrophylla* has an apoptosis-inhibition effect (Huang *et al*., 2015). The level of antiapoptotic effect is slightly better for macrophylla than gentiopicroside alone, which could be as a result of synergistic effect of sweroside and swertiamarin working together with gentiopicroside found in macrophylla. Assessing the individual anti-apoptotic effect of sweroside and swertiamarin is a key further study area. As stated earlier and independently confirmed, this was the first research assessing the mitochondrial function of the four Gentiana species as well as their phytochemicals via the seahorse mito stress assay, hence further work in that regard will be most instrumental.

4.6 Conclusion

The mitochondria is a key organelle to NAFLD pathogenesis in terms of fatty acid oxidation, mitochondrial respiration, ATP production as well as fatty acid synthesis. These studies have shown that pre-treating hepatocytes with *G. macrophylla, G. lutea* and single compounds: gentiopicroside, sweroside and silymarin provides a degree of protection which may be attributed to enhancing mitochondrial function in terms of ATP production, basal respiration, spare respiratory capacity, maximal respiration, proton leak and non-mitochondrial oxygen consumption. This was best was gentiopicroside. It has also been observed that apart from enhancing mitochondrial function, *Gentiana macrophylla* and all the above-named phytochemicals most notably sweroside, silymarin and swertiamarin protected hepatocytes by scavenging ROS produced by arachidonic acid and the mitochondrial electron transport chain. Another key mechanism of hepatocyte protection observed was the antiapoptotic effect of gentiopicroside and *G. macrophylla* against arachidonic induced apoptosis and necrosis. These investigations have also pointed to the possibility of a synergistic action being responsible for elevated hepatocyte protection seen in *G. macrophylla.* A mitotic effect of gentiopicroside as well as Gentiana macrophylla also requires further investigation using primary hepatocytes. Further investigation and exploitation in potentiation studies are required to determine whether or not hepatocyte protection will be enhanced when by combining different active phytochemicals found in Gentiana spp in order to control mitogenic effect.

Chapter 5. Concluding Remarks

5.1 Overview

This study examined the hypothesis that the methanolic extracts and selected phytochemicals (gentiopicroside, sweroside and swertiamarin) of four Gentiana species: *lutea*, *macrophylla*, *rigescens* and *scabra* exhibit hepatoprotective effects in non-alcoholic fatty liver disease (NAFLD). In line with the set objectives, the first stage of this study entailed an assessment of methanolic extracts of Gentiana species via HPLC and HPTLC to identify and quantify the above-listed phytochemicals prior to bioactivity screening. The second stage involved an *in vitro* screening to determine the resistance of HepG2 and THLE-2 cells to fatty acid (arachidonic acid) induced cytotoxicity in the presence of methanolic extracts of Gentiana species. The third stage comprised of investigations into the effects of bioactive Gentiana spp. extracts and phytochemicals on mitochondrial function, apoptosis and reduction of oxidative stress on HepG2 cells in the presence of fatty acids.

5.2 Stage One – Assessment of Methanolic Extracts of Gentiana Spp.

This step served as a validation step for the four Gentiana species used throughout this study and served as a foundation for understanding the bioactivity of the extracts as well phytochemicals identified when used in cell the proceeding cell work. A review of literature showed that extraction via refluxing or sonication followed by HPLC or HPTLC were the first point of call for the qualitative and quantitative assessment of plant extracts prior to *in vitro* or *in vivo* screening. In this study however, both HPTLC and HPLC assessments of methanolic extracts obtained by both refluxing and sonication were performed concurrently to provide a robust verification of all plants species used and to aid in deciphering the most applicable for this study. Furthermore, the use of both gradient and isocratic methods served to provide a comparative assessment of the degree of sensitivity of both methods when used to assess the four Gentiana species, as well as phytochemicals and also shed more light on different outcomes which could be attained based on the method employed.

Higher amounts of phytochemicals were obtained from refluxed plants compared to sonicated ones. A broader spectrum of peaks were observed for gradient HPLC runs than the isocratic mode. These observations will be useful guide for studying these four Gentiana species in making an informed choice of methodology for qualitative

and quantitative assessment. This stage fulfilled the desired objective with the authentication of the four Gentiana species: *lutea*, *macrophylla*, *scabra* and *rigescens* and, the identification and quantitation of phytochemicals: gentiopicroside, sweroside and swertiamarin of which gentiopicroside was most abundant in all species. Methanolic extracts of *Gentiana lutea* presented the highest amount of each phytochemical quantitated. Having achieved this objective, the next step was to screen methanolic extracts (refluxed) of the four Gentiana species for their bioactivity on hepatocytes (HepG2, VA-13 and THLE-2) in the presence of fatty acid (arachidonic acid).

5.3 Stage Two – *Iv Vitro* Screening of Methanolic Extracts of Gentiana Spp

This stage was initialised by assessing the survival rate of hepatocytes (in terms of cell viability) under each plant species as well as the best mode of application (i.e. pre-treatment, co-administration or post-treatment) and time of exposure. This was done through trypan blue, MTT and LDH assays. At this stage, the focus was not yet on individual phytochemicals but to determine the best Gentiana species extract in terms of resisting the cytotoxicity of arachidonic acid. Arachidonic acid exhibited greater cytotoxicity in HepG2 cells in comparison with other fatty acids such as palmitic, hence the choice of arachidonic acid. This was further confirmed by assessing the cytotoxicity of arachidonic acid on hepatocytes in MTT, LDH and trypan blue assays.

The extent of cell viability maintained in hepatocytes treated with Gentiana plant extracts in the presence of fatty acids was the preliminary indication of hepatocyte protective effect. Checking cell viability of hepatocytes in different treatment timelines as well as treatment modes also aimed at portraying the most effective application of Gentiana plant extracts to obtain hepatocyte protection. This was found to be pre-treatment (priming) of hepatocytes with Gentiana extracts for 24 h prior to arachidonic acid exposure. *Gentiana macrophylla* was found to be the most effective species in conferring hepatocyte protection but showed a mitogenic effect. This was followed by *Gentiana lutea, Gentiana scabra* and *Gentiana rigescens* in decreasing order of hepatocyte protection. A similar pattern was seen in THLE-2 cells pre-treated with the above-named extracts in the presence of arachidonic acid. Attaining this objective raised further questions concerning the role played by the phytochemicals

identified in stage one in providing the hepatocyte protection seen in Gentiana extracts and also the most effective phytochemical (s). Furthermore, it was essential to further investigate the enhanced cell growth seen in stage two to determine the extent to which necrosis and apoptosis were prevented by Gentiana extracts and phytochemicals. These studies were conducted in the third stage of this study.

5.4 Stage Three – Effects of Bioactive Gentiana species extracts and Phytochemicals on Mitochondrial Function, Apoptosis and Reduction of Oxidative stress

In order to determine the means by which Gentiana species extracts and identified phytochemicals protected hepatocytes, their effects on mitochondrial function in terms of ATP production, basal respiration, maximal respiration, spare respiratory capacity, proton leak and non-mitochondrial oxygen consumption in the presence of arachidonic acid was performed in the first study of its kind for Gentiana spp. extracts and phytochemicals. Further insight into the mode of hepatocyte protection was obtained in flow cytometric annexin V-FITC and DCF ROS assays.

Assaying the three phytochemicals: gentiopicroside, sweroside and swertiamarin in comparison with positive control silymarin provided further understanding of the means by which hepatocyte protection was achieved by the Gentiana extracts. The identified phytochemicals enhanced cell viability of in varying degrees with gentiopicroside and *Gentiana macrophylla* showing the highest potency in this regard for both HepG2 and THLE-2 cells. Both *Gentiana macrophylla* and gentiopicroside were further analysed via annexin-V FITC to gain further understanding of cell viability promoted by them in terms of the prevention of necrosis and apoptosis. Although both enhanced cell viability, the 10 µg/mL *Gentiana macrophylla* (containing 0.4 µg/mL (i.e. 1.2 µM) gentiopicroside as determined by HPLC analysis portrayed higher anti-apoptotic activity than 20 µM gentiopicroside. This points to possible synergistic effects of other phytochemicals including (0.24 µM sweroside and 0.30 µM swertiamarin) found in 10 µg/mL *Gentiana macrophylla* working in combination with the 1.2 µM gentiopicroside to present a more enhanced anti-apoptotic effect in the hepatocytes. This observation has set a foundation for further work which could involve a combination of gentiopicroside, sweroside and

swertiamarin in varying proportions for testing on hepatocytes to determine their hepatocyte protection effect. Furthermore, gentiopicroside eliciting a synergistic effect at a dose as low as 1.2 µM opens up the possibility of it being combined with other mainstream hepatoprotective agents such as reducdyn (n-acetyl-d, l-homocysteine thiolactone) to enhance their effect as well as possibly reducing their side-effects. This assumption would however require further studies to validate it.

High fat diet, coupled with decreased ATP production, decreased mitochondrial respiration as well as reduced fatty acid oxidation constitutes the first hit phase leading to NAFLD proceeded by the second hit leading to NASH. It is expected that an effective hepatocyte protective agent would intervene at the first hit stage to prevent the onset of NAFLD. Based on results from this study, it can be deduced that Gentiana phytochemicals protect hepatocytes from the first hit in NAFLD by increasing ATP production (most markedly noticed with gentiopicroside pre-treatment), mitochondrial basal respiration, maximal respiration, spare respiratory capacity as well as non-mitochondrial oxygen consumption as summed up in Table 5.1. Hence, the hepatocyte's capacity to metabolise a high influx of fatty acids is increased by the phytochemicals, alongside an increased output capacity in ATP production (which implies increase beta oxidation) while scavenging ROS produced as a result of this increased rate of metabolism to prevent them from harming the liver cells. Furthermore, the Gentiana phytochemicals maintain the longevity of the hepatocytes by preventing necrosis and apoptosis in the presence of fatty acids. By instituting these counter-measures, hepatocyte protection is achieved and some of the effects of the first hit leading to NAFLD are minimised or prevented. Gentiopicroside and sweroside performed better than silymarin in most of the parameters tested in this study which presents both of them as leading candidates for combined usage as hepatocyte protectors when compared to swertiamarin and silymarin.

Table 5.1 Summary table of mode and intensity of hepatocyte protection

	Gentiana macrophylla	**Gentiopicroside**	**Sweroside**	**Silymarin**	**Swertiamarin**	***Gentiana lutea***	***Gentiana scabra***	***Gentiana rigescens***
Cell viability	+++++	+++++#	+++	++++	++	++++	+++	++
ATP production	+++++	+++++	++++	+++	+++	++++	**N/A**	**N/A**
Basal respiration	+++++	++++	+++++	+++	+++	++++	**N/A**	**N/A**
Maximal respiration	+++++	++++	+++++	+++	++	++++	**N/A**	**N/A**
Non-mitochondrial oxygen consumption	+++++	+++++	++++	+++	+++	++++	**N/A**	**N/A**
Spare respiratory capacity	+++++	++++	+++++	+++	++	++++	**N/A**	**N/A**
ROS scavenging	+++	++	+++++	++++	+++	**N/A**	**N/A**	**N/A**
Anti-apoptosis	+++++	++++	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**

+++++ Showed excellent performance when compared with control

++++ Showed second best performance compared to control

+++ Showed third best performance compared to control

++ Showed fourth best performance compared to control

showed mitogenic effect

N/A Not applicable

The summary points are as follows:

- A mitogenic attribute markedly observed in gentiopicroside and Gentiana macrophylla should be further investigated using primary hepatocytes without any modifications. This would help to deepen the understanding of mitogenic effect being observed and provide further ways of addressing it.
- Cell growth may be promoted by Gentiana species phytochemicals which work in a synergistic manner to enhance mitochondrial function, scavenge ROS and prevent apoptosis hence maintaining cell longevity. Based on the results a promising synergistic combination which can be further investigated incudes a gentiopicroside: sweroside combination. Such a combination will bring together increased ATP production by gentiopicroside coupled with an increased maximal respiratory capacity enabling the hepatocyte to cope with the increased respiratory rate. Sweroside will also aid the scavenging of increased ROS which is associated with enhanced mitochondrial function whereas gentiopicroside will maintain cell survival rate by preventing apoptosis and promoting cell viability.
- *Gentiana macrophylla* which was the best performing specie as well as gentiopicroside both prevented necrosis and apoptosis as seen the annexin V-FITC PI assay. Even though the main focus was on the best performing Gentiana species and phytochemicals for annexin-V FITC, further work could be done by testing each of the remaining Gentiana species as well as other species of Gentiana not covered in this study.
- Cell viability is preserved by the scavenging of ROS particularly by sweroside followed by swertiamarin and gentiopicroside.
- Gentiana species extracts G. *lutea* and G. *macrophylla* as well as phytochemicals: gentiopicroside, sweroside and swertiamarin enhanced mitochondrial function in terms of increased maximal respiratory capacity most notably with sweroside, as well as increased spare respiratory capacity enabling the cell to respond to high energy demands.
- G. *lutea* and G. *macrophylla* as well as phytochemicals: gentiopicroside, sweroside and swertiamarin promoted ATP production with the highest ATP production seen in gentiopicroside treatment.
- G. *lutea* and G. *macrophylla* as well as phytochemicals: gentiopicroside, sweroside and swertiamarin increased the basal respiration in hepatocytes, hence

improving the efficiency with which they respire and consume oxygen while in a resting state. Furthermore, non-mitochondrial oxygen consumption was increased pointing to the possibility that other hepatocellular organelles were functionally enhanced by the phytochemicals. This needs to be further researched to obtain the full spectrum of effects associated with Gentiana phytochemicals.

5.5 Further Work

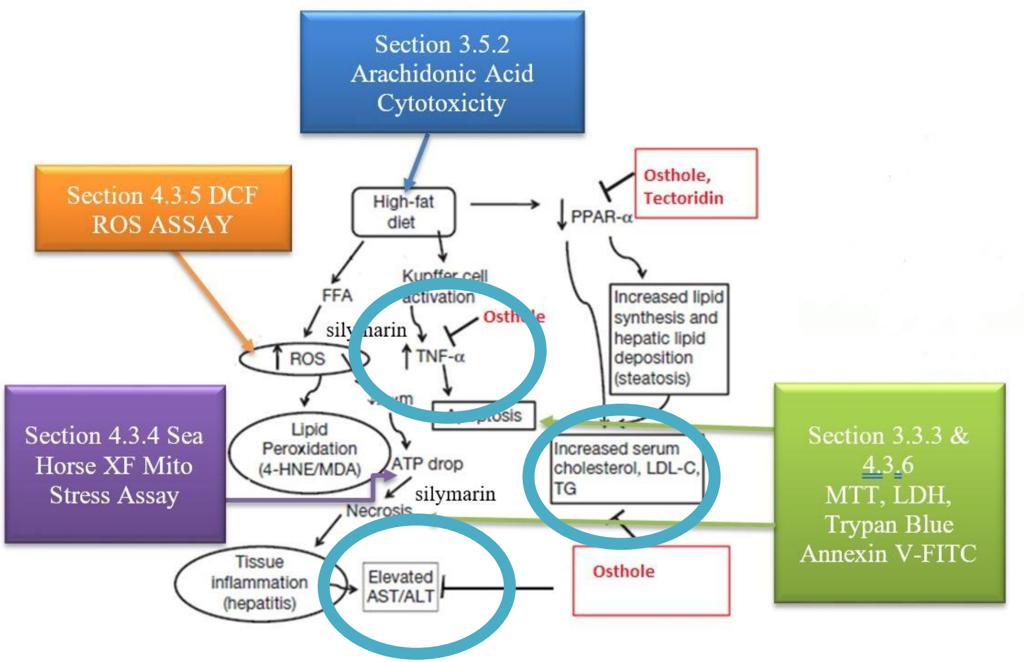


Fig 5.1 Metabolic pathways of a high fat diet leading to NAFLD. *This diagram depicts the metabolic pathways of a high fat diet and the sections of this thesis investigating possible points of intervention by Gentiana spp and phytochemicals in the fat metabolism pathway and potential points for further study. (Gyamfi, et al, 2009)(Song et al., 2007).*

This study has portrayed that the four Gentiana species: *lutea, macrophylla, scabra* and *rigescens* possess hepatocyte protection effects derived from their phytochemicals some of which are: gentiopicroside, sweroside and swertiamarin. Further work could be done on other phytochemicals found in Gentiana plants such as loganic acid and amarogentin to determine their hepatocyte protective effects in terms of mitochondrial function (seahorse mito stress test). As circled in (Fig 5.1), the role played by Gentiana species and phytochemicals (gentiopicroside, sweroside and swertiamarin) in preventing inflammation can also be assessed in a further study. *In-vitro* and *in-vivo* models can also be used to further investigate if the four Gentiana species and phytochemicals play any role in stabilizing liver enzymes AST and ALT which are elevated in inflamed liver tissue. Another scenario for further study is the role of Gentiana species and phytochemicals in preventing lipid accumulation in hepatocytes. This study has set a foundation for the assessment of hepatocyte protection derived from

Gentiana plants especially in terms of mitochondrial and non-mitochondrial respiration. It will not only deepen our understanding of hepatocyte protection but stimulate novel ideas in the screening of phytochemicals and further research in the management of liver diseases.

THE FIGHT TO PROTECT THE LIVER: GENTIAN PLANTS vs FATTY LIVER DISEASE

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My interest in researching phytochemicals (plant chemicals) which help to protect the liver began during my BSc Herbal Medicine degree in Ghana, which was done in a pharmaceutical and clinical setting where I assessed many patients with liver diseases. I also obtained an MSc in Pharmaceutical Science from London Metropolitan University during which I received a citation from Her Majesty Queen Elizabeth II for my Diamond Jubilee article titled: *Her Majesty's Diamond Jubilee; A Diamond in the Hearts of Many.*

After my first degree, I worked as a Medical Researcher at the Noguchi Memorial Institute for Medical Research (NMIMR) and Centre for Scientific Research into Plant Medicine, which undertook WHO-funded projects in phytochemistry, pharmacology, microbiology and toxicology. One key study that shaped my interest in researching phytochemicals for my PhD was done at NMIMR, in collaboration with Harvard and Yale, to investigate the anti-malaria action of phytochemicals and sulphadoxine-pyrimethamine using the haematin-polymerisation prevention mechanism of chloroquine as a template. I later worked as the Director for Research and Development at Tree of Life Inc, where I formulated and implemented research models for the development of medicinal plants. These varied experiences have enhanced my PhD research in a special way.

I have had the opportunity to interact with other researchers during my PhD, showcase my research findings at numerous conferences and also acknowledge the support received from my supervisory team and guidance obtained via the Graduate School's Doctoral Researcher Development Programme sessions.

The liver serves as an epicentre and paramount determinant of the health status of an individual. Due to the key role played by the liver in the sustenance of life, it is important to research into substances which can help to protect the liver from non-alcoholic fatty liver disease (NAFLD), which is a metabolic disorder associated with the accumulation of fat in the liver. NAFLD is also linked to obesity and a sedentary lifestyle. Data from Public Health England states that 61.7% of the UK population are obese. The NHS also states that 5% of the UK population are in the early stages of NAFLD. Furthermore, NAFLD has been found to be prevalent in the general population of North America (34%) and other developed countries such as China (15%).

My PhD research aimed at investigating the bioactivity of four Gentian plant species – Gentiana lutea, Gentiana macrophylla, Gentiana scabra and Gentiana rigescens – against NAFLD. The roots of Gentiana lutea were used in 180 BC as a tonic, listed in the British Pharmacopoeia as Gentian BP and also used as the principal plant species in a Chinese folkloric proprietary blend called Longdan Xiegan Tang (a liver tonic). The Chinese Materia Medica reports that Gentian causes a reduction in jaundice while promoting gall-bladder function. I aimed to determine whether or not Gentian conferred protection to the liver from the effects of fatty acids when liver cells were pre-treated with Gentian before fatty acid exposure and determine the active compounds responsible for this protection.

The results showed that the active compounds in Gentian helped to minimise the effects of fatty acids on the liver by over 70%. This research can be applied in producing wellness products which can be taken to protect the liver from the harmful effects of fat and enhance its function.

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APPENDIX

Appendix A: Intra-day Gentiopicroside Calibration Tables

GPS (0.5 μg/mL)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	12.413	0.0722	0.60
2	12.414	0.0723	0.59
3	12.413	0.0722	0.60
AV	12.4135	0.072233333	0.60
SD	0.000707107	7.07107E-05	0.001414214
RSD	0.005696272	0.097892032	0.23570226

GPS (1 μg/mL)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	12.413	0.1424	1.21
2	12.413	0.1444	1.22
3	12.412	0.1444	1.21
AV	12.413	0.143733333	1.215
SD	0	0.001414214	0.007071068
RSD	0	0.983914816	0.58198089

GPS (5 μg/mL)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	12.413	0.7572	6.02
2	12.413	0.7544	6.01
3	12.413	0.7580	6.02
AV	12.413	0.756533333	6.015
SD	0	0.001890326	0.007071068
RSD	0	0.249866882	0.117557237

GPS (10 μg/mL)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	12.413	1.4413	11.89
2	12.417	1.4457	11.97
3	12.413	1.4433	11.93
AV	12.415	1.443433333	11.93
SD	0.002828427	0.002203028	0.056568542
RSD	0.022782337	0.152624175	0.474170515

GPS (15 µg/mL)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	12.413	2.3261	19.52
	12.413	2.3193	19.60
	12.413	2.3245	19.75
AV	12.413	2.3233	19.56
SD	0	0.004808326	0.056568542
RSD	0	0.206961052	0.289205227

GPS (20 µg/mL)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	12.42	2.7029	23.05
	12.423	2.7041	23.00
	12.423	2.7037	23.10
AV	12.4215	2.703566667	23.025
SD	0.00212132	0.00061101	0.035355339
RSD	0.017077811	0.022600149	0.153551961

GPS (50 µg/mL)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	12.423	6.8915	58.20
	12.427	6.8816	58.25
	12.423	6.9010	58.30
AV	12.425	6.891366667	58.23
SD	0.002828427	0.009700687	0.035355339
RSD	0.022764001	0.140765798	0.060721922

Appendix B: Intra-day Sweroside Calibration Tables

SWE (0.5µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	12.94	0.1563	1.34
2	12.92	0.1565	1.35
3	12.94	0.1565	1.34
AV	12.93	0.156433333	1.345
SD	0.014142136	0.00011547	0.007071068
RSD (%)	0.109374599	0.073814226	0.525729949

GPS (1µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	12.94	0.3131	2.68
2	12.94	0.313	2.68
3	12.94	0.313	2.67
AV	12.94	0.313033333	2.68
SD	0	5.7735E-05	0
RSD (%)	0	0.018443731	0

SWE (5µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	12.947	1.5653	13.39
2	12.95	1.5645	13.40
3	12.95	1.5664	13.46
AV	12.9485	1.5654	13.395
SD	0.00212132	0.000953939	0.007071068
RSD (%)	0.01638275	0.060939006	0.05278886

SWE (10µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	12.94	3.0422	25.76
	12.94	3.0302	25.7
	12.94	3.0363	25.71
AV	12.94	3.036233333	25.73
SD	0	0.006000278	0.042426407
RSD (%)	0	0.197622419	0.164890816

SWE (15µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	12.94	4.69	32.34
	12.94	4.7	32.38
	12.94	4.69	32.3
AV	12.94	4.693333333	32.36
SD	0	0.005773503	0.028284271
RSD (%)	0	0.123014972	0.087405041

SWE (20µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	12.937	5.9993	42.07
	12.937	5.982	42.04
	12.94	5.9892	42.05
AV	12.937	5.990166667	42.055
SD	0	0.008690416	0.021213203
RSD (%)	0	0.145078036	0.050441573

SWE (50µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	12.95	15.2453	132.48
	12.953	15.2474	132.50
	12.947	15.233	132.48
AV	12.9515	15.2419	132.49
SD	0.00212132	0.007778817	0.014142136
RSD (%)	0.016378955	0.051035746	0.010674115

Appendix C: Intra-day Swertiamarin Calibration Tables

SWT (0.5µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	11.717	0.15145	1.61
2	11.715	0.15143	1.63
3	11.717	0.1514	1.60
AV	11.716	0.151426667	1.62
SD	0.001414214	2.51661E-05	0.014142136
RSD (%)	0.012070788	0.016619341	0.872971335

SWT (1µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	11.717	0.303	3.22
2	11.717	0.3031	3.20
3	11.72	0.314	3.21
AV	11.717	0.3067	3.21
SD	0	0.006322183	0.014142136
RSD (%)	0	0.019052368	0.440564973

SWT (5µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	11.717	1.9791	16.11
2	11.72	1.9846	16.18
3	11.717	1.9793	16.11
AV	11.7185	1.981	16.145
SD	0.00212132	0.003119295	0.049497475
RSD (%)	0.01810232	0.157460615	0.306580828

SWT (10µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
1	11.717	3.1298	24.42
2	11.717	3.1282	24.4
3	11.713	3.1353	24.43
AV	11.717	3.1311	24.41
SD	0	0.003724245	0.014142136
RSD (%)	0	0.118943658	0.057935828

SWT (15µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	11.723	4.9139	40.36
	11.727	4.915	40.33
	11.727	4.9262	40.34
AV	11.725	4.918366667	40.345
SD	0.002828427	0.006806125	0.021213203
RSD (%)	0.024123046	0.138381807	0.05257951

SWT (20µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	11.72	6.2427	55.3
	11.717	6.2258	55.37
	11.717	6.2272	55.35
AV	11.7185	6.2319	55.335
SD	0.00212132	0.009379232	0.049497475
RSD (%)	0.01810232	0.150503576	0.089450573

SWT (50µg/ml)

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
	11.727	15.8972	15.32
	11.73	15.8879	15.35
	11.723	15.9233	15.3
AV	11.7285	15.9028	15.335
SD	0.00212132	0.018352384	0.021213203
RSD (%)	0.018086885	0.115403476	0.138331943

Appendix D: Intra-day and Inter-Day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed Gentiana scabra Based on Peak Areas with RSD

	Inter-day (n=3)			**Intra-day (n=3) (mAU*Min)**
Compound	**Day 1 (mAU*Min)**	**Day 2 (mAU*Min)**	**Day 3 (mAU*Min)**	
Gentiopicroside	1.0617 SD=0.0006 RSD =0.06 %	1.1098 SD=0.0007 RSD=0.06 %	1.0500 SD=0.025 RSD= 0.24 %	1.0386 SD=0.0021 RSD= 0.02 %
Sweroside	0.0435 SD=0.001 RSD = 3.08 %	0.0462 SD=0.001 RSD=2.57 %	0.0483 SD=0.002 RSD=3.70 %	0.0479 SD=0.001 RSD= 2.95 %
Swertiamarin	0.07245 SD=0.007 RSD = 1.27 %	0.0768 SD=0.001 RSD= 1.38 %	0.0733 SD=0.003 RSD= 3.36%	0.0752 SD=0.002 RSD= 2.70%

Appendix E: Intra-day and Inter-Day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated Gentiana scabra Based on Peak Areas with RSD (in parenthesis)

	Inter-day (n=3)			**Intra-day (n=3) (mAU*Min)**
Compound	**Day 1 (mAU*Min)**	**Day 2 (mAU*Min)**	**Day 3 (mAU*Min)**	
Gentiopicroside	0.2774 SD=0.0015 RSD = 0.54 %	0.2685 SD=0.0050 RSD= 0.37 %	0.2821 SD=0.0030 RSD= 1.06%	0.2557 SD=0.0010 RSD= 0.39 %
Sweroside	0.0102 SD=0.0003 RSD = 2.94 %	0.01131 SD=0.0049 RSD=0.37 %	0.01096 SD=0.00018 RSD=1.64 %	0.01162 SD=0.0001 RSD= 0.86 %
Swertiamarin	0.0130 SD=0.002 RSD = 1.64 %	0.01336 SD=0.0004 RSD= 2.99 %	0.01346 SD=0.0003 RSD= 2.23 %	0.01374 SD=0.0005 RSD=3.64 %

Appendix F: Intra-day and Inter-Day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed Gentiana rigescens Based on Peak Areas with RSD

	Inter-day (n=3)			**Intra-day (n=3) (mAU*Min)**
Compound	**Day 1 (mAU*Min)**	**Day 2 (mAU*Min)**	**Day 3 (mAU*Min)**	
Gentiopicroside	0.2798 SD=0.003 RSD =1.09 %	0.2725 SD=0.005 RSD= 0.30 %	0.2709 SD=0.003 RSD= 0.98 %	0.2803 SD=0.004 RSD= 1.42 %
Sweroside	0.0065 SD=0.0001 RSD = 1.53%	0.00632 SD=0.004 RSD=0.90 %	0.0067 SD=0.0002 RSD=2.99%	0.0069 SD=0.0001 RSD= 1.45 %
Swertiamarin	0.0085 SD=0.001 RSD = 3.24 %	0.0080 SD=0.0003 RSD= 2.97 %	0.0083 SD=0.002 RSD= 1.54 %	0.0087 SD=0.005 RSD= 3.04 %

Appendix G: Intra-day and Inter-Day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated Gentiana rigescens Based on Peak Areas with RSD (in parenthesis)

	Inter-day (n=3)			**Intra-day (n=3) (mAU*Min)**
Compound	**Day 1 (mAU*Min)**	**Day 2 (mAU*Min)**	**Day 3 (mAU*Min)**	
Gentiopicroside	0.0851 SD=0.0001 RSD =0.12 %	0.0832 SD=0.003 RSD= 0.84 %	0.0826 SD=0.002 RSD= 0.42 %	0.0811 SD=0.0005 RSD= 0.52 %
Sweroside	0.0050 SD=0.0003 RSD = 1.43%	0.0052 SD=0.001 RSD=3.30 %	0.0054 SD=0.0007 RSD=4.38%	0.0051 SD=0.0004 RSD= 2.49 %
Swertiamarin	0.0073 SD=0.001 RSD = 3.24 %	0.0080 SD=0.0003 RSD= 2.97 %	0.0083 SD=0.002 RSD= 1.54 %	0.0087 SD=0.005 RSD= 3.04 %

Appendix H: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed 100µg/mL Gentiana lutea Based on Peak Areas

GPS

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
R1	12.423	1.0693	9.13
R2	12.42	1.0728	9.08
R3	12.42	1.0716	9.06
AV	12.421	1.071233333	9.09
SD	0.001732051	0.001778576	0.036055513

SWE

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
R1	12.95	0.0515	0.4
R2	12.95	0.0512	0.4
R3	12.95	0.0535	0.4
AV	12.95	0.052066667	0.4
SD	2.17558E-15	0.001250333	6.7987E-17

SWT

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
R1	11.727	0.1537	1.14
R2	11.723	0.1617	1.15
R3	11.723	0.1615	1.13
AV	11.72433333	0.158966667	1.14
SD	0.002309401	0.004562163	0.01

Appendix I: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated 100µg/mL Gentiana lutea Based on Peak Areas

GPS

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
R1	12.42	0.5822	4.83
R2	12.42	0.5877	4.84
R3	12.42	0.5875	4.85
AV	12.42	0.5858	4.84
SD	0	0.003119295	0.01

SWE

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
R1	12.95	0.0264	0.2
R2	12.95	0.0275	0.2
R3	12.95	0.0271	0.2
AV	12.95	0.027	0.2
SD	2.17558E-15	0.000556776	3.39935E-17

SWT

	Ret. Time (Min)	Peak Area (mAU*Min)	Peak Height (mAU)
R1	11.727	0.0765	0.54
R2	11.723	0.0825	0.55
R3	11.727	0.0815	0.55
AV	11.72566667	0.080166667	0.546666667
SD	0.002309401	0.00321455	0.005773503

Appendix J: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed 500µg/mL Gentiana macrophylla Based on Peak Areas

GPS

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
R1	12.417	7.7461	65.21
R2	12.417	7.7598	65.33
R3	12.417	7.7433	65.16
AV	12.417	7.749733333	65.23333333
SD	0	0.008829685	0.087368949

SWE

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	12.947	0.3466	2.62
	12.943	0.3461	2.61
	12.943	0.3461	2.61
AV	12.94433333	0.346266667	2.613333333
SD	0.002309401	0.000288675	0.005773503

SWT

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	11.72	1.1899	8.22
	11.72	1.1916	8.19
	11.723	1.1668	8.16
AV	11.721	1.182766667	8.19
SD	0.001732051	0.01385364	0.03

Appendix K: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated 500µg/mL Gentiana lutea Based on Peak Areas

GPS

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
R1	12.42	1.8347	14.89
R2	12.423	1.8465	15.06
R3	12.42	1.8496	15.12
AV	12.421	1.8436	15.02333333
SD	0.001732051	0.007861934	0.119303534

SWE

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	12.947	0.0824	0.61
	12.947	0.0855	0.61
	12.947	0.0816	0.61
AV	12.947	0.083166667	0.61
SD	2.17558E-15	0.002059935	0

SWT

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	11.727	0.2521	1.68
	11.727	0.254	1.7
	11.727	0.2534	1.7
AV	11.727	0.253166667	1.693333333
SD	2.17558E-15	0.000971253	0.011547005

Appendix L: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed 1000µg/mL Gentiana lutea Based on Peak Areas

GPS

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
R1	12.417	15.3943	128.83
R2	12.417	15.4344	128.04
R3	12.42	15.4863	128.73
AV	12.418	15.43833333	128.5333333
SD	0.001732051	0.046125951	0.430155011

SWE

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	12.947	0.805	5.46
	12.943	0.7018	5.48
	12.947	0.8067	5.49
AV	12.94566667	0.771166667	5.476666667
SD	0.002309401	0.060079309	0.015275252

SWT

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	11.72	2.3619	16.32
	11.72	2.3622	16.06
	11.723	2.3767	16.13
AV	11.721	2.366933333	16.17
SD	0.001732051	0.008459511	0.13453624

Appendix M: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated 1000µg/mL Gentiana lutea Based on Peak Areas

GPS

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
R1	12.417	4.4721	36.04
R2	12.42	4.472	35.88
R3	12.42	4.4816	35.97
AV	12.419	4.475233333	35.96333333
SD	0.001732051	0.005513922	0.080208063

SWE

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	12.943	0.1955	1.42
	12.947	0.1933	1.42
	12.943	0.2087	1.47
AV	12.94433333	0.199166667	1.436666667
SD	0.002309401	0.008329066	0.028867513

SWT

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	11.723	0.618	4.04
	11.723	0.6165	4.02
	11.727	0.6186	4.04
AV	11.72433333	0.6177	4.033333333
SD	0.002309401	0.001081665	0.011547005

Appendix N: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed 500µg/mL Gentiana macrophylla Based on Peak Areas

GPS

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
R1	12.43	2.5764	19.8
R2	12.433	2.5849	19.91
R3	12.423	2.5788	19.75
AV	12.42866667	2.580033333	19.82
SD	0.005131601	0.004382161	0.081853528

SWE

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	12.95	0.2297	1.54
	12.957	0.2335	1.55
	12.953	0.232	1.56
AV	12.95333333	0.231733333	1.55
SD	0.003511885	0.001913984	0.01

SWT

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	11.687	0.3317	2.14
	11.687	0.3327	2.15
	11.683	0.3632	2.19
AV	11.68566667	0.342533333	2.16
SD	0.002309401	0.017904841	0.026457513

Appendix O: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated 500µg/mL Gentiana macrophylla Based on Peak Areas

GPS

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
R1	12.437	0.1677	1.27
R2	12.44	0.1675	1.27
R3	12.44	0.1658	1.26
AV	12.439	0.167	1.266666667
SD	0.001732051	0.001044031	0.005773503

SWE

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	12.963	0.0244	0.18
	12.97	0.0246	0.18
	12.967	0.0287	0.19
AV	12.96666667	0.0259	0.183333333
SD	0.003511885	0.002426932	0.005773503

SWT

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	11.7	0.0076	0.07
	11.703	0.0064	0.06
	11.7	0.0065	0.06
AV	11.701	0.006833333	0.063333333
SD	0.001732051	0.000665833	0.005773503

Appendix P: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Refluxed 1000µg/mL Gentiana macrophylla Based on Peak Areas

GPS

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
R1	12.427	5.168	39.57
R2	12.423	5.1826	39.72
R3	12.423	5.1715	39.65
AV	12.42433333	5.174033333	39.64666667
SD	0.002309401	0.007622554	0.075055535

SWE

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	12.95	0.4617	3.1
	12.947	0.5026	3.21
	12.947	0.4684	3.11
AV	12.948	0.477566667	3.14
SD	0.001732051	0.021936803	0.060827625

SWT

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	11.68	0.6589	4.43
	11.68	0.6604	4.43
	11.68	0.7774	4.44
AV	11.68	0.6989	4.433333333
SD	0	0.067987131	0.005773503

Appendix Q: Intra-day HPLC Precision of Gentiopicroside, Sweroside and Swertiamarin in Sonicated 1000µg/mL Gentiana macrophylla Based on Peak Areas

GPS

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
R1	12.44	0.3157	2.37
R2	12.44	0.3152	2.38
R3	12.443	0.3162	2.41
AV	12.441	0.3157	2.386666667
SD	0.001732051	0.0005	0.02081666

SWE

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	12.97	0.0428	0.34
	12.967	0.0495	0.36
	12.967	0.0465	0.34
AV	12.968	0.046266667	0.346666667
SD	0.001732051	0.003356089	0.011547005

SWT

	Ret. Time (Min)	**Peak Area (mAU*Min)**	**Peak Height (mAU)**
	11.7	0.0119	0.11
	11.7	0.0133	0.12
	11.7	0.0132	0.12
AV	11.7	0.0128	0.116666667
SD	2.17558E-15	0.000781025	0.005773503

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