AN INVESTIGATION OF COMPOUNDS ISOLATED FROM *GLYCYRRHIZA GLABRA* (LIQUORICE ROOT)

by

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DEDICATION

To my husband and family

DECLARATION

I, Carike Raubenheimer (student number 58525831), declare that the dissertation entitled: **An investigation of compounds isolated from *Glycyrrhiza glabra* (liquorice root),** which I hereby submit for the degree of MSc in Life Sciences at the University of South Africa, is my own work and has not previously been submitted by me for a degree at this or any other institution.

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ABSTRACT

Introduction: Dark spots appearing on the skin caused by hyperpigmentation results from the action of tyrosinase, an enzyme whose activity leads to the production of the skin pigment melanin. Extracts of the plant *Glycyrrhiza glabra*, also known as liquorice, are commonly used to treat a range of conditions including skin hyperpigmentation. This study aimed at isolating and identifying compounds in extracts from South African liquorice root and assaying these compounds as to their antioxidant activity, their ability to inhibit the tyrosinase enzyme and their level of cytotoxicity.

Methods: The ability of plant extracts to scavenge free radicals was tested using the 2,2-diphenyl-1-picrylhydrazyl (DPPH), [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid)] (ABTS) and the ferric ion reducing power (FRAP) tests. The polyphenolic content of extract fractions was determined and extract compounds were identified using UHPLC-QToF-20 MS. *In vitro* anti-tyrosinase activity was also investigated as well as cytotoxicity in HepG2 liver and SK-MEL-1 melanoma cells using the MTT cell viability assay.

Results: Of the four fractions prepared from the 70% methanolic extract of liquorice root, fraction 3 (F3) showed increased polyphenolic content and antioxidant properties with IC_{50} of 56.1 ± 6.32, 39.14 ± 1.1 and 66.34 ± 1.4 μg/ml against DPPH, ABTS and FRAP, respectively. The anti-tyrosinase activity of this fraction showed an IC_{50} of 358.54 μg/ml compared to Kojic acid (0.75 mM) used as the control. In addition, this fraction showed reduced liver toxicity as a higher percentage cell viability was noted in the HepG2 cells compared to the SK-MEL-1 skin melanoma cells. However, both cell types showed higher percentage viability compared to acetaminophen that was used as cytotoxic control. The LC-MS analysis revealed the presence of a wide variety of compounds including 4-azido-3-benzyl-coumarin, ferulic acid, glycyrrhizin, quercitrin, cirsilineol, gentioflavine and 4",6,7-trihydroxyisoflavone. The literature indicates the use of these compounds regarding antioxidant and anti-tyrosinase activity. Significantly, cularidine was identified in this study, a compound not previously reported in studies involving liquorice root.

Conclusion: The results from this study concur with previous reports as to the anti-tyrosinase and antioxidant activities associated with liquorice roots, activities perhaps due to the relatively high polyphenolic content in extracts from South African liquorice root.

KEY WORDS

Glycyrrhiza Glabra (Liquorice); polyphenolics; tyrosinase activity; glabridin; antioxidants

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF GRAPHS

LIST OF ABBREVIATIONS

ABTS:	2,2′-Azino-bis (3-Ethylbenzothiazoline-6-Sulfonic Acid)
DMEM:	Dulbecco's modified essential medium
DPPH:	2,2-diphenyl-l-picrylhydrazyl
EC:	Enzyme Control
FBS:	Fetal bovine serum
FRAP:	Ferric reducing/antioxidant power
HepG2:	Cultured liver cancer cells
HPLC:	High Performance Liquid Chromatography
h:	Hour(s)
IC:	Inhibitor Control
IC_{50}:	The concentration at which 50% of experiment is inhibited
LC:	Liquid Chromatography
LCMS:	Liquid chromatography mass spectrometry
min:	minute(s)
ml:	millilitre
mm:	millimetre
MS:	Mass Spectrometry
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR:	Nuclear Magnetic Resonance
SK-MEL-1:	Cultured skin melanoma cells
TAC:	Total antioxidant capacity
TLC:	Thin Layer Chromatography
TPTZ:	2,4,6-Tri (2-pyridyl)-s-triazine

CHAPTER 1 BACKGROUND

1.1 Cosmetic products

To feel more attractive and to be more confident, people use cosmetics to improve their skin (Lall & Kishore, 2014; Krishnan *et al.*, 2017). In response to this need, the global cosmetics and personal care industry makes a significant social and economic contribution to national and regional economies.

Since 2012, there have been similarities between cosmetic sales in Europe and America. France and Germany have shown a marked increase in the cosmetic sector while, as more knowledge on skin functions and new ingredients become available, Asia appears to show more of an interest in developing specialized products (Secara & Sasu, 2013). Africa, particularly in countries such as South Africa and Nigeria followed by Kenya, Ethiopia, Tanzania, Ghana and Cameroon, is an emerging cosmetics market due to a rapidly increasing population, a fast-growing middle class, increasing urbanisation and improved business regulation (Mungai, 2014). These economies are experiencing economic growth, in part because of an increase in the middle class, where the youth are buying a wide range of cosmetics and beauty products (Beauty Africa, 2016). As an example, in 2016, the South African cosmetics industry experienced an estimated compound annual growth of 4.6% involving approximately 250 companies that employ more than 60,000 people, and export cosmetics to the value of R7.84bn. In addition, the South African Direct Selling Association represents the interests of more than 1.13 million independent direct sellers (Cision, 2017).

Within this rapidly growing sector, there is an increasing preference towards naturally derived cosmetic products and numerous cosmetics companies are increasing their global presence in the market for organic products. (Secara & Sasu, 2013). While the use of plants for medicinal purposes is well established, the modern use of plant extracts in skin care products highlights consumer demand for ecologically friendly products (Ribeiro *et al.*, 2015). As more and more people are informed about the link between exposure to sunlight and pigmentation, it becomes more feasible to manufacture a natural and cost-effective cosmetic that provides protection from the sun and is directed at improving skin quality by reducing skin pigmentation. In answer to this, the South African cosmetics industry has developed products from sources such as liquorice (Mungai, 2014).

1.2 Melanin and hyperpigmentation

Melanin is the pigment in skin produced by specific cells called melanocytes. Its production is triggered by an enzyme called tyrosinase which is located in vesicles called melanosomes, inside melanocytes (Deckner, 2014). The color of skin, eyes, and hair originates in the melanin pigment-producing cells

called melanocytes (Nerya *et al.*, 2003). The role of melanin is to protect the skin against UV light damage by absorbing UV sunlight and removing the resulting reactive oxygen species. Melanin is formed by a combination of enzymatically catalysed and chemical reactions (Chang, 2009) and melanogenesis has been defined as the entire process leading to the formation of dark macromolecular pigments. According to Pastirino (2018), tyrosinase is essential for skin pigmentation due to its role in melanin biosynthesis and is, therefore, an important target in the cosmetic and medicinal industries. When the key enzyme in melanin biosynthesis namely tyrosinase is overactive, it leads to the overproduction of melanin (Lall & Kishore, 2014).

Ribeiro *et al.* (2015) stated that there are two types of melanin pigments produced by melanocytes, eumelanin which is black or brown and pheomelanin which is red or yellow. Tyrosinase is a multifunctional, glycosylated, and copper-containing oxidase enzyme, which catalyzes the first two metabolic steps in human skin pigmentation and is also responsible for enzymatic browning reactions in damaged human skin (Vidyalakshmi & Dhamodharan, 2016). According to Chang (2009), melanin has mainly a photoprotective function in human skin. However, the accumulation of an abnormal amount of melanin in different specific parts of the skin results in more pigmented patches that might become an esthetic problem. This has prompted research into new tyrosinase inhibitors.

1.3 Treating hyperpigmentation

Tyrosinase is the enzyme that initiates the conversion of tyrosine, via various intermediates, to the pigment melanin. This enzyme contains two copper ions, coordinating with histidine residues in the active site (Chen *et al.*, 2015). These two copper ions are critical for the catalytic activities of this enzyme and are present in different tyrosinases regardless of their source. Because tyrosinase-catalyzed reactions are strongly associated with hyperpigmentation, the discovery of tyrosinase inhibitors are of great importance in cosmetic products. This prompted one of the objectives of this study that was to characterise liquorice extract as a source of tyrosinase inhibitor.

Ebanks *et al*. (2009) reviewed many of the biochemical mechanisms involved in the development of hyperpigmentation as well as the means of halting or reversing these mechanisms. Hydroquinone was used for many years to successfully treat skin pigmentation but this compound requires the presence of active tyrosinase and, therefore, is not useful in altering the colour of melanin present in the skin. However, arbutin is a derivative of hydroquinone that may be useful in treating hyperpigmentation disorders. Although required at high concentrations, arbutin displays a lower melanocyte cytotoxicity compared with hydroquinone. Deoxyarbutin is a synthetic form of arbutin that shows reversible inhibition of tyrosinase activity with associated skin lightening in the human skin and does not

permanently destroy melanocytes. Other tyrosinase inhibitors appear to be less useful in treating hyperpigmentation. These include Kojic acid that is associated with sensitization, contact dermatitis and erythema. In addition, patients treated with azelaic acid reported skin irritation, a burning sensation, mild erythema, scaling and pruritus 2-4 weeks after treatment. Gentisic acid is obtained from the root of *Gentiana* and is a natural derivative that is less cytotoxic and mutagenic than hydroquinone.

Another means of reducing hyperpigmentation is to break down the cytosomal structure in which melanin is produced. Mequinol is enzymatically oxidized by tyrosinase to produce melanocytotoxic quinones that results in safe pigment cell destruction and skin depigmentation. N-Acetyl-4-S-Cysteaminylphenol is an alternative tyrosinase substrate and has been reported to be a depigmenting agent that is less irritating and more stable than hydroquinone, with specificity for melanin-synthesizing cells. The literature indicates that there are many beneficial activities in addition to depigmenting activity associated with liquorice extracts and, therefore, liquorice extract should be used to formulate cosmetic formulations (Damle, 2014). Activities of liquorice root extracts are wide-ranging including inactivation of the major human drug metabolizing P450 enzyme, antioxidant activity against LDL to, thus, show a protective effect against the human lipoprotein oxidative system as well as anti-tumorigenic, antimicrobial, antiviral, anti-inflammatory and anti-oxidative activities (Kent *et al.*, 2002).

As liquorice root is a major source of cosmetic products, it needs to be fully explored so as to realise its full potential in reducing hyperpigmentation. Thus, the aim of this research study was to identify compounds in extracts from liquorice root, *Glycyrrhiza glabra* and to assay these extracts, particularly regarding their antioxidant and cytotoxicity potential.

1.4 *Glycyrrhiza glabra*

The liquorice plant, *Glycyrrhiza glabra*, grows to 2.5 m in height and the leaves can grow from 7 to 15 cm in length. The plant has flowers that vary in colour from purple to a pale blue that are arranged as hermaphrodite inflorescence and the oblong legume fruit of the *Glycyrrhiza glabra* contains several seeds (Pastorino, 2018). According to Kaur *et al*. (2013), the main root can grow to 1.5 cm in length and from there it subdivides into roots that are about 1.25 cm long. From these subdivided roots, the horizontal woody stolons arise that can reach 8 meters in length that, when dried and cut, are used together with the root as commercial liquorice. When these roots are cut it shows a yellowish interior with the familiar liquorice odour and sweet taste. The only place in South Africa where these plants are found is in the Western Cape near Oudtshoorn on the farm Waaikraal at Dysseldorp. That is also where the liquorice roots used in this study were purchased.

A B

Figure 1: Photographs of liquorice plants (A) and liquorice root material (B) (Adopted from Kaur, 2013)

1.5 Research problem

Skin pigmentation is a significant problem for many people. Therefore, there is an urgent need to search for more potent, natural skin depigmenting agents. The root of the liquorice plant appears to be a source of compounds that display potent tyrosinase inhibitory activity that may be useful in treating skin pigmentation. Although studies have been done in South Africa on plants for skin care (Lall & Kishore, 2014), the impact of compounds isolated from liquorice root has not been extensively explored. Thus, this study was designed to determine whether extracts of liquorice roots could be useful in treating skin pigmentation.

1.6 Research question

The research question for this study was whether compounds in extracts of liquorice root could be identified and assayed as to their antioxidant, anti-tyrosinase and anticytotoxic potential so that such liquorice plant extracts could be considered as being suitable for inclusion in skin cosmetics effective in treating skin pigmentation.

1.7 Research aims and objectives

It is important to study the effects of natural plant compounds as skin cosmetics and so the aim of this study was to isolate and characterize compounds from extracts of liquorice root using liquid chromatography and then to screen liquorice root extracts regarding their cytotoxic, antioxidant and anti-tyrosinase activities.

To achieve the aims of the study, the following objectives were addressed:

- To chemically analyse methanolic extracts of commercially available, powdered liquorice root
- To conduct bioassays of these extracts using commercial antioxidant and anti-tyrosinase assays
- To conduct cytotoxicity assays on these extracts

1.8 Research output

The following article manuscript entitled: "The antioxidant, anti-tyrosinase and hepatoprotective activity of compounds isolated from *Glycyrrhiza glabra* (Liquorice root)" authored by C Raubenheimer, S Odeyemi & J Dewar, was generated from the results of this research and accepted for publication in the African Journal of Biomedical Research.

CHAPTER 2 LITERATURE REVIEW

2.1 Skin pigmentation

Visible pigmentation depends on the presence and metabolism of pigment cells such as melanocytes (Yamaguchi *et al.*, 2007). Melanocytes produce the pigment melanin and are the pigment factories for the skin (Lees, 2007). Skin colours in humans range from extremely fair/light to extremely dark depending on racial/ethnic background but the density of melanocytes in a given area is virtually identical in all types of skin (Yamaguchi *et al.*, 2007). Although different skin colour types have the same number of melanocytes, some people carry genes that make the melanocytes produce more melanin or pigment while sunlight, injuries to the skin and hormones can stimulate melanin production (Lees, 2007).

2.1.1 Skin hyperpigmentation

Hyperpigmentation refers to any condition that has more than the normal amount of melanin and it is both a long- and short-term cosmetic problem caused by the sun. Different pigmentation conditions are associated with variations in skin coloration and most pigmentation conditions are caused by abnormalities in pigment production in the melanocytes and affect any skin type (Lees, 2007). Skin discolouration conditions include hyperpigmentation (over production of melanin, resulting in dark splotches in the skin); solar lentigenes (skin freckles caused by sun exposure); mottling (speckled hyperpigmentation from cumulative sun exposure); vitiligo (a medical condition resulting in absence of skin pigment in splotches); post-inflammatory hyperpigmentation (dark splotches or spots related to skin trauma) and melasma (commonly referred to as pregnancy mask).

2.1.2 Treatment of hyperpigmentation

According to Prakash & Majeed (2009), skin pigmentation is influenced by several factors including haemoglobin in skin blood vessels, carotenoids in the dermis and, particularly, melanin, the dark pigment in the epidermis. Most skin lighteners target and inhibit tyrosinase as their main mechanism of action and studies have shown that the compound glabridin inhibits melanogenesis via a tyrosinase inhibitory activity and has demonstrated efficacy in reducing UV radiation-induced hyperpigmentation (Deckner, 2014; Sansidharan *et al.*, 2014). In addition to inhibiting tyrosine, extracts of licorice root may disperse melanin and inhibit cyclooxygenase activity to decrease the production of free radicals. The majority of people in Africa use plant-based traditional medicines for their health care and the use of medicinal plants to treat dermatological conditions and skin diseases occurs across southern Africa (Lall & Kishore, 2104).

2.1.3 Plants used for skin treatment

Most people in developing countries rely mainly on plants for their primary health care (Mabona & van Vuuren, 2013). In the rural areas in South Africa however, customers prefer to buy their cosmetic products from herbal shops that supply traditional medicines manufactured from a plant base (Lall & Kishore, 2014). Dlova & Ollengo (2018) reported that most African traditional healers use either the whole plant or just parts of it, which they boil in water or other solvents to extract the active ingredients.

Recently, traditional medicine has gained popularity and approval and the use of traditional ethnomedicine for skincare is a widespread practice in Africa. In South Africa alone, more than 250 plants are used for skin lightening, as sunscreens and for the treatment of skin diseases (Dlova & Ollengo, 2018). In rural areas, this may be the only such remedy and there should be an ongoing search for alternative, available and affordable treatments for common skin infection (Mabona & van Vuuren, 2013). Examples of plants used for the treatment of skin-related diseases are:

a. ***Achyranthes aspera***

   This belongs to the *Amaranthaceae* family and is commonly called the devil's horsewhip or prickly chaff flower. It is an annual or perennial, stiff erect or procumbent herb, 1-2 m in height, often with a woody base, commonly found as a weed on roadsides (Hasan, 2014). *A. aspera* has been reportedly used to treat boils, scabies, eruptions of skin and other skin diseases. The antibacterial activities against different types of bacteria such as *P. vulgaris* at higher doses of 4000 and 5000 ppm has been reported however, the aqueous leaf extract did not show any activity against *E.coli*, *K. aerogenes* and *P. aerogenes* at lower doses (Chakraborty *et al.*, 2002; Perumal Samy *et al.*, 1998).

b. ***Allium sativum***

   This plant is commonly called garlic belonging to the family of *Liliaceae* and was revealed as having a very effective chemo-preventive action against skin abscesses and the induction of skin cancer where the formation of skin papillomas in animals was delayed along with a decrease in their size and number (Kuete, 2013).

c. ***Aloe vera***

   Aloe vera, commonly called Barbados aloe, belongs to the *Xanthorrhoeaceae* family and has shown very good results when treating skin diseases, wrinkles, stretch marks, pigmentations and is often taken as health drink (Olsen *et al.*, 2001; Tabassum & Hamdani, 2014).

d. ***Beta vulgaris***

   Beta vulgaris (commonly called Beetroot) belonging to the family of *Brassicaceae* is an erect, biennial plant producing a rosette of leaves from a swollen rootstock. Beetroot contains a group of highly bioactive pigments known as betacyanin, a red-violet pigment with a chemo-

preventive effect in skin cancer cells. The high antioxidant properties of this plant are linked to its high phenolic content (Kapadia *et al.,* 2003).

e. ***Calendula officinalis***

Calendula officinalis (commonly called Marigold) belonging to the *Asteraceae* family is used as a remedy for burns (including sunburn), bruises and cutaneous and internal inflammatory diseases of several origins. The methanolic extract has revealed significant acceleration of wound healing by producing epithelialization (Duran *et al.,* 2005).

f. ***Althaeae radix***

Althaeae radix (marshmallow) root is used for the treatment of furunculosis, eczema and dermatitis. The ointment containing an aqueous marshmallow root extract (20%) has been reported to reduce irritation induced by UV radiation or tetrahydrofurfuryl alcohol *in vivo* (Dawid-Pać, 2013).

g. ***Cannabis sativa***

Cannabis sativa (commonly called Charas or Ganja) belongs to the family *Cannabinaceae*. The leaf powder is used for sores and wound dressing. Ganja is used to relieve pain in itchy skin diseases while hemp seed oil is useful for treating eczema and other skin diseases like dermatitis, seborrhoeic dermatitis/cradle cap, varicose eczema, psoriasis, lichen planus and acne roseacea (Tabassum & Hamdani, 2014).

h. ***Glycyrrhiza glabra* Linn**

Glycyrrhiza glabra (Fabaceae) is commonly known as liquorice and is a small perennial herb native to the Mediterranean region and central and southwest Asia. Liquorice has been recognized since ancient times for its ethnopharmacological value, especially for its skin lightening properties (Pastorino *et al.,* 2018).

2.2 The use of liquorice root in traditional medicine

Liquorice has been used in Greece, China, and Egypt for stomach inflammation and upper respiratory problems (Pastorino *et al.,* 2018). It was documented as a medicine used in Europe since prehistoric times (Fiore, 2005) and is still widely used in southern Europe and parts of Asia. In traditional Chinese medicine, it is often combined with other herbs in a single prescription, as a unique "guide drug" to enhance the effectiveness of other ingredients, to reduce toxicity and to improve flavour (Wang *et al.,* 2013). Berry (2018) reported the therapeutic use of liquorice root extract to cure skin infections as well as stomach disorders and to prevent liver disease. Irani *et al.* (2013) screened the antimicrobial activity of root and leaf extracts of liquorice against a panel of seven common bacterial pathogens and showed, particularly, anti-*Staphylococcus aureus* activity. Hajiaghamohammadi *et al.* (2016) reported on the use of liquorice extract to treat stomach disorders involving *Helicobacter pylori* while Matsumoto *et al.*

(2013) showed *in vitro* inactivation of hepatitis C by liquorice extracts containing glycyrrhizin. While extracts of liquorice root may be useful in treating a range of skin conditions and infections, importantly, it may be used to treat melasmas, or skin pigmentation (Sarkar *et al.*, 2013).

2.2.1 Depigmentation mechanisms associated with liquorice plant

Zhu & Gao (2008) reviewed the active compounds in liquorice extracts affecting melanogenesis. The main component of the hydrophobic fraction of liquorice was glabridin, shown to prevent UVB-induced pigmentation by inhibiting tyrosinase activity. It also inhibited tyrosinase in cultured B16 murine melanoma cells. Other active compounds isolated from liquorice extracts include glabrene, isoliquiritigenin licuraside, isoliquiritin, and licochalcone A that were also shown to inhibit tyrosinase activity (Zhu & Gao, 2008).

Liquorice extracts have been tested for the treatment of melasma to provide good results with only minor irritation of the skin. It is a good source to treat melasma because liquorice inhibits the biosynthesis of melanin and tyrosinase. (Ebanks *et al*., 2009). These authors also reported other mechanisms of reducing melanin by the direct inactivation of tyrosinase following the chelation of copper within the enzyme's active site. In addition, antioxidant quenching of free radicals and peroxides prevents melanin formation and modulates the depigmenting capabilities of melano-cytotoxic agents. Lastly, another depigmenting mechanism in liquorice extracts may involve removal of epidermal melanin.

Zolghadri *et al.* (2019) recently reported on other natural sources of anti-tyrosinase activity including fungi, bacteria and plants. Thus, these agents may not only produce bioactive compounds for food but also for medicinal use and cosmetic application. Semisynthetic and synthetic sources of simple phenol inhibitors of tyrosinase have at least one aromatic ring and one (or more) hydroxyl groups. One example of these are the flavonoids, the best-studied group of plant polyphenol benzo-γ-pyrane derivatives. More than 4000 members of the flavonoids have been identified in plants classified into six major groups, flavanols, flavones, flavonols, flavanones, isoflavones and anthocyanidins (Solano *et al.*, 2006).

2.3 Analysis of compounds in extracts from *Glycyrrhiza glabra* (Liquorice root)

2.3.1 Compounds in extracts from *Glycyrrhiza glabra*

According to Kaur *et al*. (2013), *Glycyrrhiza glabra* (liquorice) contains a number of compounds that may have antiviral and antimicrobial activities including commonly detected compounds such as isoliquiritigenin, liquiritigenin, glabrene, liquiritin, licocaumarin and glycyrrhizin while other important compounds are glycyrrhizic acid and glabridin.

Tian *et al.* (2008) used various solvents such as water, methanol, ethanol, acetonitrile and chloroform to extract compounds from liquorice roots and showed that an hour-long extraction at 50°C with a mixture of ethanol/water (30:70, v/v) optimally extracted glycyrrhizic acid and glabridin from liquorice roots.

Li *et al.* (2016) quantified 14 liquorice components (liquiritin, isoliquiritin, liquiritin apioside, isoliquiritin apioside, licuraside, liquiritigenin, isoliquiritigenin, glycyrrhizin, glycyrrhetinic acid, glabridin, glycycoumarin, licoricidin, licochalcone A, and p-hydroxybenzylmalonic acid), representing several natural product classes including chalcones, flavanones, saponins, and isoflavonoids and triterpenes. The triterpenes include compounds such as glycyrrhizic acid and glycyrrhetinic acid monoglucuronide that are the main pharmacoactive agents in liquorice responsible for antioxidant, anti-allergic, antiviral, and anti-neoplastic characteristics. Pastorino *et al.* (2018) also conducted a comprehensive study on liquorice root and confirmed its phytochemical composition and pharmacology activities. These authors reported that liquorice roots contain compounds such as flavonoids with antioxidant, anticancer, anti-inflammatory, antiviral and antimicrobial activity. In addition, few side effects or toxicity related to liquorice root were noted apart from hypertension and fluid retention.

2.3.2 Screening for tyrosinase inhibition

Liquorice extract has an inhibitory effect on tyrosinase activity because of the high concentration of glabridin, a known tyrosinase inhibitor (Yokota *et al*., 1998). In studies reported by Nerya *et al.* (2003), glabridin was found at a concentration of 0,4% (w/w) and to have an isoflavane structure as indicated in Figure 3. Glabridin also appears to have antiseptic activity and antimalarial, antispasmodic, anti-inflammatory and anti-hyperglycemic properties (Roshan *et al.,* 2012) and is not associated with cytotoxicity (Alobaidi *et al.,* 2015). However, it has relatively poor skin-penetration properties and is unstable in formulations (Nerya *et al.*, 2003). The presence of an *a*-keto group in flavonoids is responsible for this activity (Pastorino *et al*., 2018).

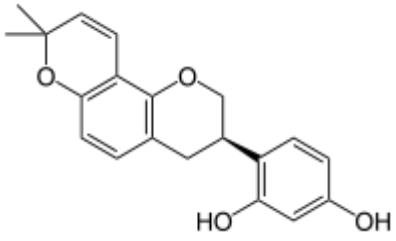


Figure 2: Structure of glabridin.

2.3.3 Oxidative stress and antioxidant activity of liquorice root

An antioxidant is a chemical that prevents the oxidation of other chemicals to protect a cell against the damaging effects of free radicals that are natural by-products of cell metabolism (Shalaby & Shanab,

2013). According to these authors, the human body uses an antioxidant defence system to neutralize excessive levels of reactive oxygen species. This leads to a dynamic balance between the amounts of free radicals generated in the body and levels of antioxidants to quench and scavenge them and protect the body against their deleterious effects. *In vitro,* antioxidants inhibit free radical chain oxidation reactions.

Pastorino *et al.* (2018) stated that the antioxidant activity of *Glycyrrhiza glabra* L is one of the major reasons for its general use and its success. Liquorice extract formulations can inhibit microsomal lipid peroxidation and maintain skin homeostasis by providing skin protection against ultraviolet (UV) light so that these extracts may be of value in innovative dermal and cosmetic products to counteract oxidative stress damage. According to Damle (2014), liquorice flavonoids have exceptionally strong antioxidant activity at over 100 times the level than that of antioxidant activity of vitamin E and are, therefore, currently the strongest natural antioxidants known. In addition, liquorice extract can scavenge DPPH free radicals with an inhibition of 80% and protect fibroblasts against oxidative stress (Pastorino *et al.,* 2018). Thus, natural antioxidants with strong fee radical-scavenging activity are in demand because of their safety and effectiveness against many types of free radicals (Sohail, 2017) and could be used as a substitute for commercial antioxidants in oxidation-sensitive formulations (Morteza-Semnani *et al.,* 2003).

2.4 Techniques used to assay plant compounds

2.4.1 2,2-diphenyl-l-picrylhydrazyl (DPPH) antioxidant assay

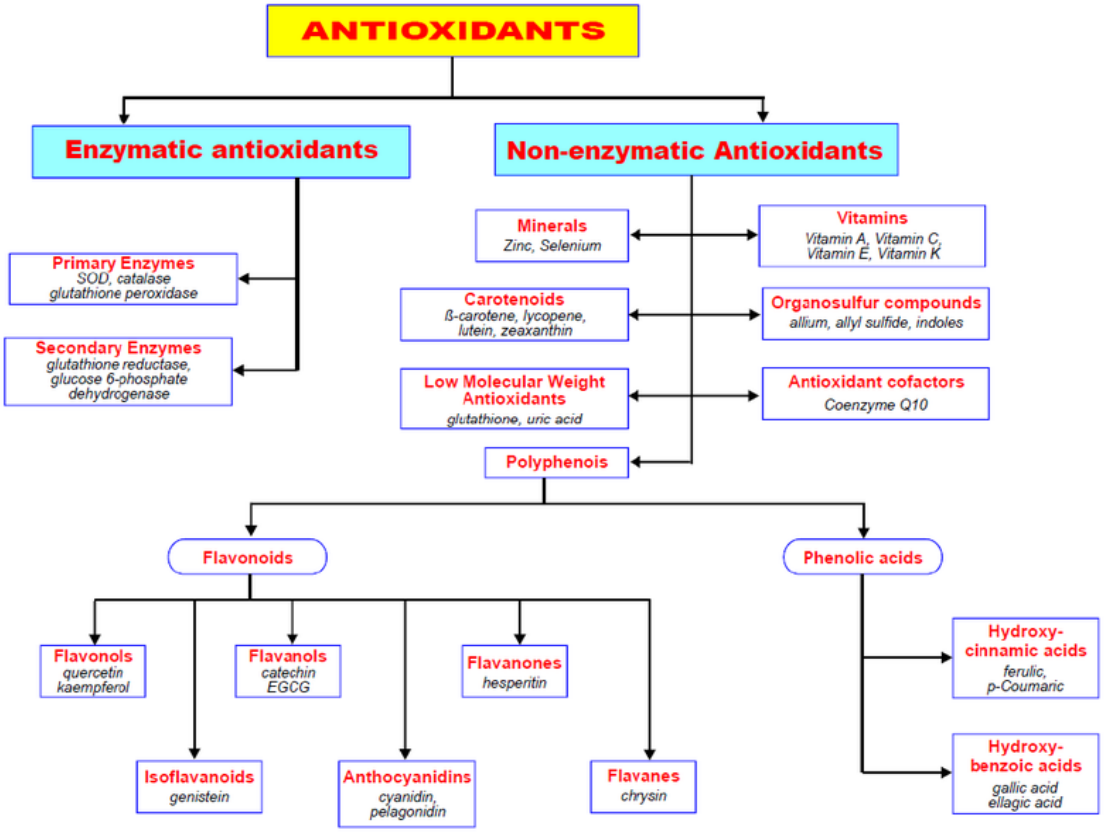
Shalaby & Shanab (2013) reported that the DPPH antioxidant assay is based on the measurement of the scavenging capacity of antioxidants towards a stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M = 394.33). The delocalisation of the free hydrogen gives DPPH its deep violet colour, with an absorption in ethanol solution at around 520 nm and its reduced form in the presence of antioxidants has a reduced violet colour (Kedare & Singh, 2011).

2.4.2 2,2′-Azino-bis 3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) antioxidant assay

The 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS•+) is a stable free radical used to estimate the total antioxidant capacity (TAC) of natural products. The ABTS method is based on spectrophotometric monitoring of the decay of this radical-cation following oxidation of ABTS caused by the addition of an antioxidant present in a sample. The ABTS strongly absorbs at longer wavelengths such as 415, 645, 734, and 815 nm but 734 nm is used by most investigators (Rajurkar & Hande, 2011).

2.4.3 Ferric reducing/antioxidant power (FRAP) antioxidant assay

This assay is based on the reducing power of an antioxidant to reduce the ferric ion ($Fe3^{+}$) to the ferrous ion (Fe^{2+}). The Fe^{2+} then forms a blue complex (Fe^{2+}/TPTZ), which increases absorption at 593 nm (Benzie & Strain, 1996).



2.4.4 Total phenolic content determination

The determination of the total phenolic content (TPC) of a sample is determined quantitatively using the Folin-Ciocalteu reagent that is measured spectrophotometrically at 725 nm. Gallic acid is used as standard (Ainsworth & Gillespie, 2007).

2.4.5 Thin Layer Chromatography (TLC)

Chromatography is the collective term for a set of laboratory techniques used to separate mixtures into their components (Kumar *et al.*, 2013). All forms of chromatography work on the same principle, that a liquid or a gas, carries the mixture that is dissolved in a fluid through the stationary phase which may be a solid or a liquid supported on a solid (Kumar *et al.*, 2013). During this process, separate, individual molecules dissolved in the mobile phase separate can be detected (Sherma & Fried, 2005).

Thin layer chromatography (TLC) is a type of liquid chromatography that can be used to separate and precisely quantitate many different compounds from a wide variety of biological samples (Preethi *et al.,* 2017). In this technique, the stationary phase is a thin uniform layer on a glass plate, aluminium foil or a plastic sheet. A solution of the sample is applied to the lower part of the plate and the plate is developed by placing it in a closed chamber in which the base is covered with the mobile phase, which is a mixture of solvents. After capillary movement of the solvent phase up through the gel, sample compounds will partition between the solvent and the stationary phases upwards from the application mark. Under UV light, the relative mobility of the various compounds can be determined from measuring the position of each compound in the gel relative to the start point and the migration of standard molecules (Sherma and Fried, 2005).

2.4.6 High Performance Liquid Chromatography (HPLC)

The high performance liquid chromatography (HPLC) technique is one of the most widely used analytical techniques employed for the quantification and characterization of biologically active components according to differences in their structure and/or composition. It is used to isolate and purify valuable products in the chemical and pharmaceutical industry. The hardware associated with this technique is shown in Figure 3 and consists of a pump, injector, column, detector and integrator or acquisition and display systems (Kupiec, 2004).

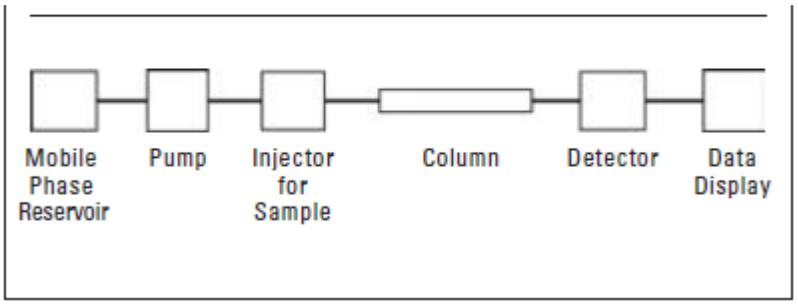


Figure 3: Schematic of an HPLC instrument. (Adopted from Kupiec, 2004).

The technique of HPLC works on the principle of the separation of the material according to their molecular weight and polarity. It is a form of liquid chromatography where separation (or partition) occurs between a mobile phase (the solvent) and a stationary phase (the column packing). The column is the most important part because that is where the separation occurs (Kupiec, 2004). Bendini *et al.* (2007) extensively reviewed HPLC analysis of polyphenolics in olive oil while Viswanathan & Mukne (2016) studied the practical development and validation of HPLC and HPTLC methods for analysis of glabridin in liquorice plants grown in India. The latter authors showed that glabridin is a prenylated isoflavonoid and a major constituent in the hydrophobic extracts of liquorice.

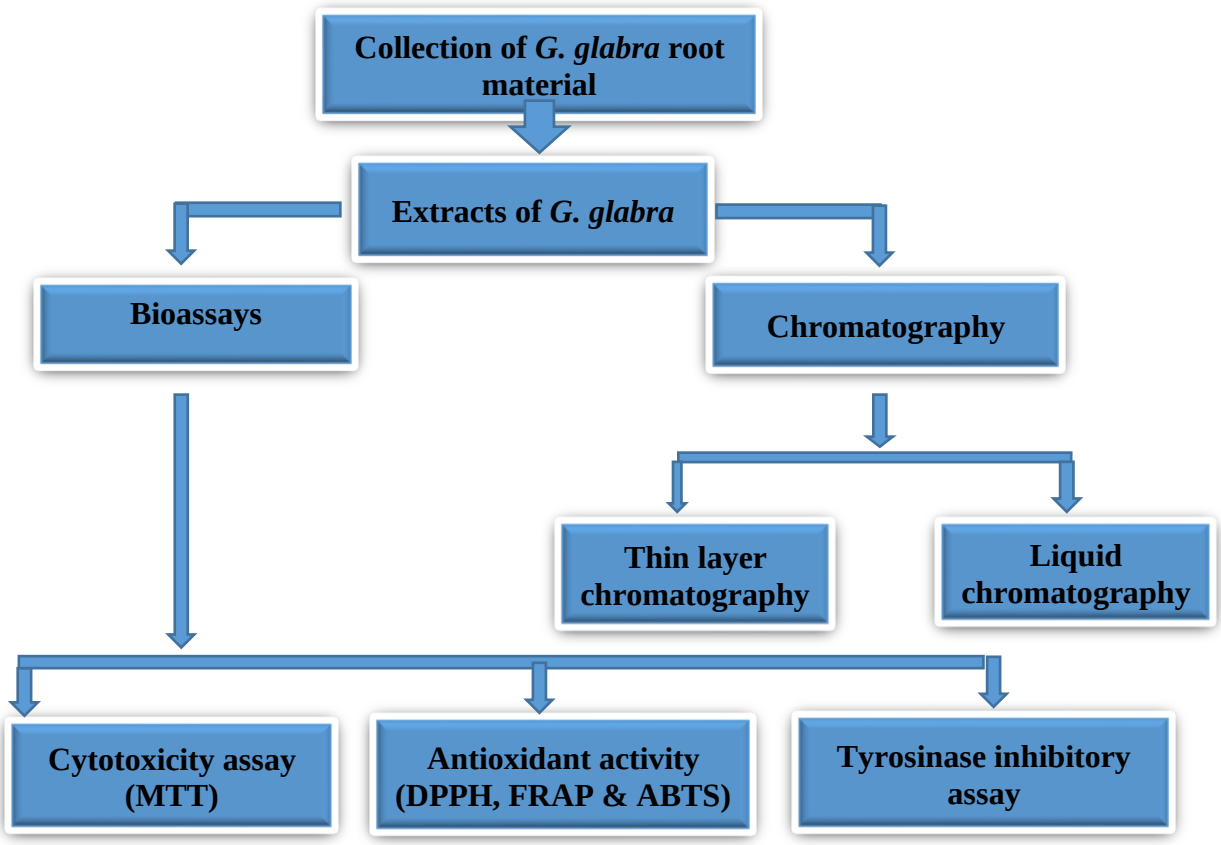
CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

Glycyrrhiza glabra was purchased from the farm Waaikraal near Oudtshoorn (Western Cape Province, South Africa). All laboratory solvents were HPLC grade, and other reagents and assay kits were purchased from Sigma-Aldrich (South Africa). These included ethanol, dichloromethane, ethyl acetate, ethylacitate, methanol, glacialacetic buffer, ascorbic acid, TPTZ, $FeSO_4$, DPPH, dichloromethane, 2,2‐diphenyl‐1‐picrylhydrazyl, 2,2'‐azino‐bis (3‐ethylbenzthiazoline‐6‐sulfonic acid), ascorbic acid, DMEM, FBS, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ) and glabridin. Thin-layer chromatography (TLC) plates were purchased from Inqaba (South Africa). The HepG2 cells were purchased from Sigma-Aldrich (Johannesburg, South Africa and the SK-MEL-1 cells were purchased from Cellonex (Johannesburg, South Africa).

3.2 Research Design

The research design encompassed the methods and procedures employed to conduct mixed but mainly qualitative scientific research. A study aim was used to identify any causal links between the factors or variables that pertain to the research problem and such research is very structured in nature (Van Wyk, 2013). Thus, before beginning each experiment, both data and methods and the way in which these were configured in the research project needed to be optimised in producing the answers to the research questions and to contribute towards addressing the study aim. The flow diagram below indicates the sequence of experiments used to address the objectives of the study.



3.3 Liquorice root specimens

The liquorice root material was purchased from the farm Waaikraal, near Oudtshoorn in South Africa. This farm is situated in the only area in South Africa where liquorice is grown. The roots are the most commonly used parts and are shown in Figure 4.

Figure 4: Photograph of liquorice root material.

3.4 Plant Extraction

The liquorice plant material was initially extracted into 70% ethanol as described (Tian *et al.*, 2008). The dried roots of *Glycyrrhiza glabra* (100 g) were thinly cut and placed in 70% ethanol for 60 minutes at 50°C. The ethanolic extract was then dried under vacuum using a Genevac EZ-2 rotary evaporator (Thermo Fisher Scientific, Johannesburg, South Africa) and then freeze-dried to afford 35.6 mg of the crude sample. This was stored at 4°C.

3.5 Fractionation

The crude extract sample was loaded onto a glass column containing silica gel 60 (0.063 – 0.200 nm) previously washed with methanol and eluted using solvent systems consisting of hexane:ethylacetate:methanol:water that differed according to increasing polarity, to afford a total of 10 fractions. These fractions were then combined to give four fractions, labelled F1, F2, F3 and F4. These were then dried using a Genevac EZ-2 series evaporator (Thermo Fisher Scientific, Johannesburg, South Africa).

3.6 Thin layer chromatography (TLC)

The fractions were chromatographed using TLC with ethyl acetate/acetone/water prepared at a ratio of 10:2:3. The resulting chromatogram as observed under UV light at 254 nm and 365 nm and sprayed with DPPH solution (20 g/L) for UV enhancement.

3.7 Ultra-high-performance liquid chromatography mass spectrophotometry

An Agilent Ultra high-performance liquid chromatography mass spectrophotometer (Compass 445 QToF Series 1.9, Bruker Instrument: Impact II) system was used to identify the extract compounds.

Column separation was carried out using an Acquity UPLC BEH C18 column 1.7 um, diameter 2.1 × 100 mm (Microsep Waters, Johannesburg, South Africa). The mobile phase consisted of 0.1% formic acid (FA) in water and acetonitrile. The column flow was set at 0.3 ml/min, column oven temperature at 35°C, draw speed at 3 µl/s with a total injection volume of 2 µl. The parameters for the mass spectrometer (MS) were as follows: capillary voltage of 4500 V, drying gas 8 l/m, gas temperature set at 200°C, ionization energy of 4.0 eV, collision energy of 7.0 eV with a cycle time of 0.5 s. Data analysis was done using Bruker Compass Data Analysis 4.3 software (Bruker Daltonik GmbH, Bremen, Germany, 2014). Study data were compared against the annotated data stored in the National Institute of Standards and Technology (NIST 2005) library.

3.8 Determination of total phenolic content

The total phenolics in the extracts were determined using the Folin–Ciocalteau assay as previously described by Ainsworth & Gillespie (2007) and as modified slightly by Odeyemi & Afolayan (2018). An aliquot of 50 µl of freshly prepared Folin–Ciocalteau's reagent (1N) and 50 µl of Na_2CO_3 (20% *w/v*) was added to 7.5 µl of the sample (250 µg/ml) and incubated at 40°C for 20 min. Quercetin in distilled water (2–10 µg/ml) was used to prepare a calibration curve. The absorbance was measured at 725 nm. The total phenolic content was expressed as µg quercetin equivalent/mg of dry weight of extract. All determinations were performed in triplicate.

3.9 Antioxidant assays

Spectrophotometric methods used to determine antioxidant capacity included the ABTS, DPPH and FRAP assays. The ABTS and DPPH assays were conducted as described by Floegel *et al.* (2011).

3.9.1 2,2′-Azino-bis(3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) assay

Radical scavenging activity using the 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay was determined as previously described (Re *et al.*, 1999; Thaipong *et al.*, 2006; Floegel *et al.*, 2011). Briefly, the reaction stock solutions including 7 mM ABTS and 2.4 mM potassium persulfate solutions were reacted together for 12 h at room temperature in the dark. The reaction was diluted by mixing 60 ml of methanol with 1 ml of the reaction stock and the absorbance adjusted to 0.708 ± 0.001 units at 734 nm. The samples (1 ml) were then reacted with 1 ml of the ABTS radical solution and the absorbance measured using the spectrophotometer at 734 nm after 10 min. The results were expressed in µM ascorbic acid equivalent per ml of the sample. All determinations were performed in triplicate.

3.9.2 2,2‑diphenyl‑1‑picrylhydrazyl (DPPH) assay

The DPPH radical-scavenging activity was determined using the method described by Odeyemi & Afolayan (2018). Briefly, 100 µl of different concentrations of the samples or standard were reacted with 100 µl of freshly prepared solution of 0.135 mM DPPH radical in methanol. The resulting solution was then vortexed and the decrease in absorbance was measured at 517 nm after 10 min. The percentage inhibition was calculated, and results were expressed in µM ascorbic acid equivalent/ml. All determinations were performed in triplicate.

3.9.3 Ferric reducing/antioxidant power (FRAP) assay

The antioxidant potential ferric reducing/antioxidant power (FRAP) assay was conducted as described by Odeyemi & Afolayan (2018). Briefly, the FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPµTZ (10mM) and FeC13. 7H2O (20 mM) at 10:1:1 (v/v/v). This was followed by the addition of 100 µl of the FRAP reagent to different concentrations of the samples. The absorbance was read at 593 nm after 10 min of incubation. All determinations were performed in triplicate and the IC_{50} values were calculated using Finney software and the results were expressed in µM ascorbic acid equivalent/g.

3.10 Tyrosinase inhibitory assay

The measurement of relative tyrosinase inhibition activity in the fractions was determined using a Tyrosinase Inhibitor Screening Kit (Colorimetric) (MAK257, Sigma-Aldrich, South Africa) according to the manufacturer's instructions. Briefly, the sample was dissolved in DMSO and diluted to 5 times the desired test concentration with tyrosinase assay buffer just before use. Tyrosinase enzyme solution was then added to 20 µl of different concentrations of the sample, inhibitor control (Kojic acid), or tyrosinase assay buffer in separate wells of a 96 well plate. The reaction was mixed and then incubated for 10 mins at 25°C. Thereafter, 30 µl of tyrosinase substrate was added before the absorbance was measured in kinetic mode for 30–60 minutes at 510 nm.

3.11 Cell culture and cytotoxicity assay

The cell culture and MTT assay were conducted according to Odeyemi & Dewar (2019). Briefly, the HepG2 and SK-MEL-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified incubator at 37°C supplemented with 5% CO_2. To conduct the MTT assay, the cells were cultured at a density of 5×10^6 cells in each well of a 96-well culture plate. After incubation at 37°C for 24 h, different concentrations of the extract samples were added to the confluent cell monolayer and incubated for another 24 h. The media from each well was removed and replaced with media containing 0.5% MTT and further incubated for 2–4 h at 37°C.

Finally, after centrifuging the plate at 400 rpm for 10 min, the absorption in each well was read at 570 nm. Acetaminophen was used as control.

CHAPTER 4 RESULTS

As the root of liquorice has so many beneficial effects and has been an excellent remedy for so many illnesses over the ages, the main aim of this study was to focus on some of the compounds in extracts of liquorice root that may assist in reducing skin pigmentation. Many cosmetic depigmenting compounds target the regulation of tyrosinase synthesis in humans (Dlova & Ollengo, 2018). In the current study, in addition to plant extracts from *Glycyrrhiza glabra* (liquorice) showing tyrosinase inhibition activity, a number of compounds in such extracts were identified using chromatographic techniques. Many of these were polyphenolics that were effective anti-oxidising agents and which were shown not to be toxic to cultured liver cells.

4.1 Phenolic content of plant extract fractions

Different solutions were used in proportions from the TLC plates so that those combinations with clear separations were then used to separate the samples/extracts on a silica gel-packed column. This afforded four fractions labelled F1 – F4.

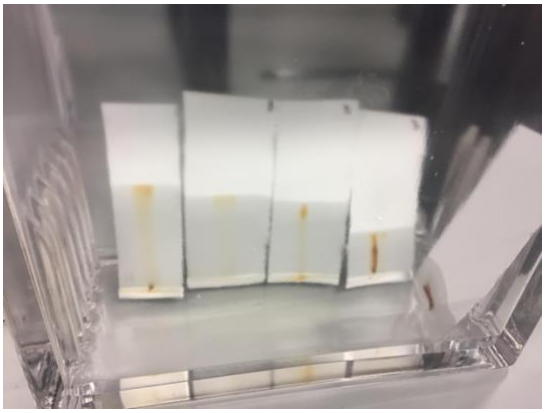


Figure 5: Photograph of TLC plates showing separation of four different liquorice root extracts. Photograph taken by candidate.

As indicated in Figure 6, high levels of total phenolic content was shown in the different fractions from the liquorice root and the phenolic content of these four factions was concentration-dependent. Of the four fractions tested, fraction 3 (F3) contained the highest phenolic content. Interestingly, there was no significant difference between the phenolic content of F1, F2 and F4 at an extract concentration of 500 mg/ml. In addition, F2 tended to show the lowest phenolic content compared to the phenolic concentrations in the other extracts.

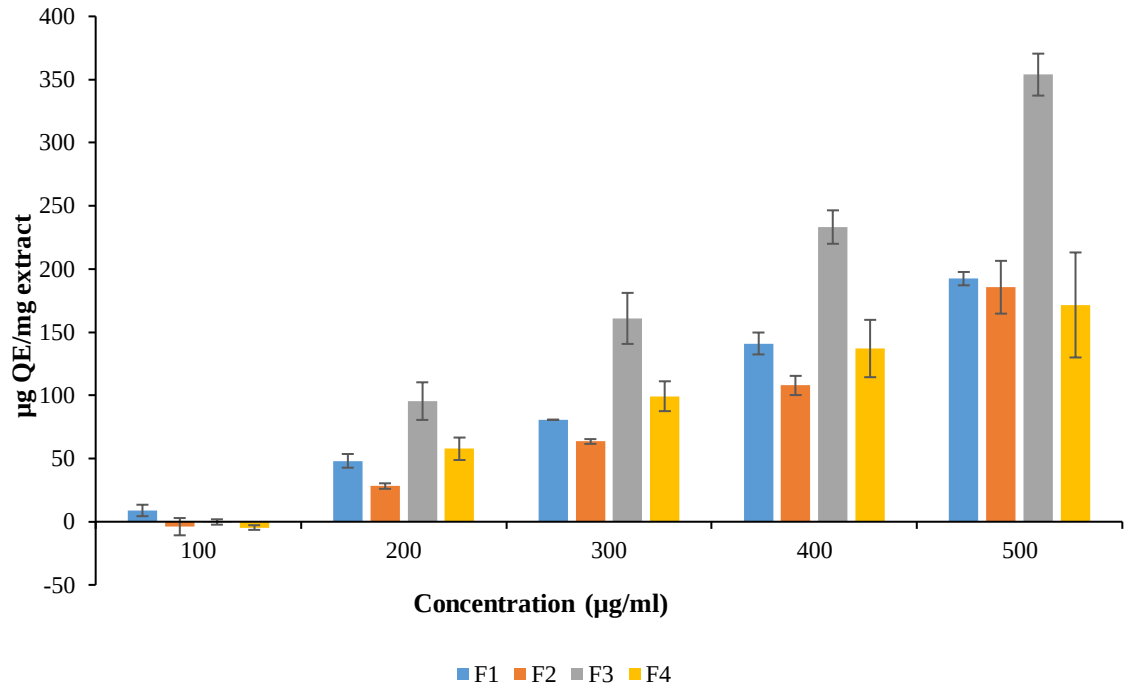


Figure 6: Phenolic content of different fractions of liquorice root. Results are means of triplicate (mean ± SD).

4.2 Antioxidant assay results

4.2.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging activity

The percentage inhibition of DPPH is shown in Figure 7. Fraction 3 showed the highest percentage inhibition of DPPH and is comparable with Vitamin C, the inhibition control in this experiment. Percentage inhibition was dependent on the concentration of the extracts. Compared to F4 and, particularly F3, F1 and F2 showed the lowest relative percentage inhibition of DPPH.

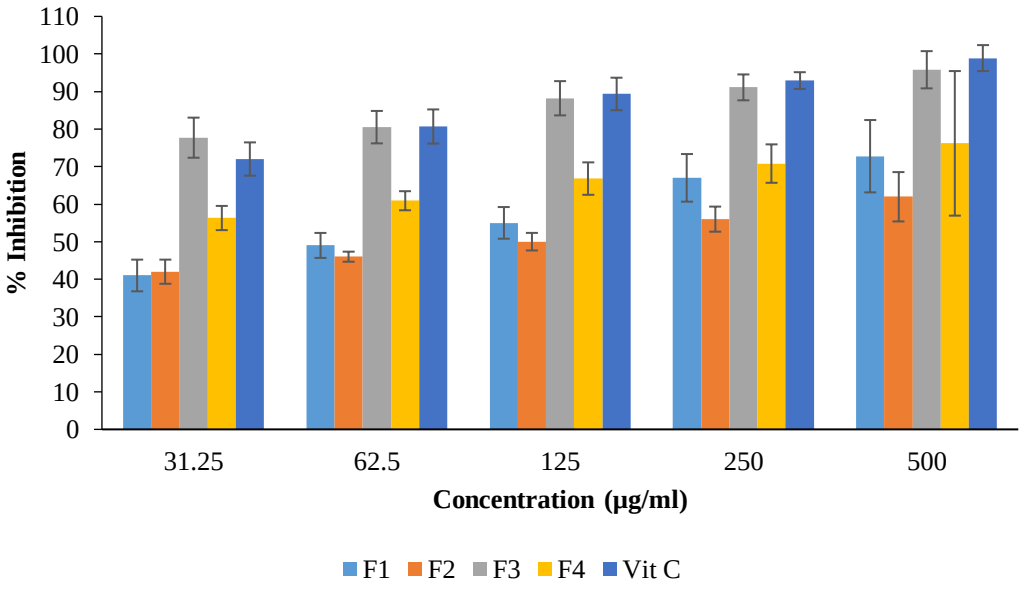


Figure 7: DPPH radical scavenging activity of different fractions of liquorice root. Results are means of triplicates.

4.2.2 2,2′-Azino-bis (3-Ethylbenzothiazoline-6-Sulfonic Acid radical scavenging activity

Figure 8 shows that the inhibition of ABTS was concentration-dependent and that, of the fractions, F3 showed the highest percentage inhibition of ABTS. This was particularly noticeable at an extract concentration of 31.25 µg/ml where there was no significant difference between F3, F4 and the control Vitamin C.

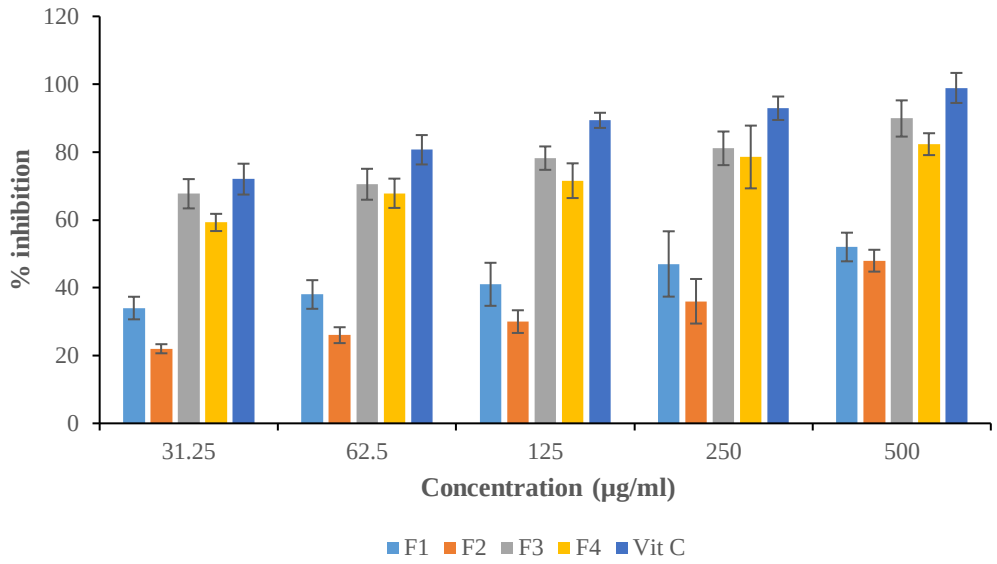


Figure 8: ABTS scavenging activity of different fractions of liquorice root. Results are means of triplicates.

4.2.3 Ferric reducing antioxidant power (FRAP)

The FRAP of the different fractions is shown in Table 1 and indicates that F3 showed the highest FRAP with 353.90 µg/ml when compared to the other fractions.

Table 1: FRAP of different fractions of liquorice root.

Concentration (µg/ml)	F1	F2	F3	F4	VIT C
			µg/ml		
0	-23.67	-19.77	-20.61	-14.98	-13.38
25	-13.89	-14.22	-15.68	-11.37	28.99
50	-12.15	-8.038	-2.43	-8.26	85.57
100	8.93	-3.91	-0.19	-4.59	186.15
200	48.19	28.23	95.47	57.75	332.07
300	80.71	63.52	160.94	99.36	485.2
400	141.09	107.85	233.24	137.09	614.66
500	192.41	185.61	353.90	171.59	769.68

The IC_{50} is the concentration at which 50% of experiment is inhibited and the concentration showing the lowest IC_{50} is the most active. Table 2 shows that F3 has the lowest IC_{50} of all the fractions against DPPH and ABTS, while F1 showed the lowest IC_{50} amongst the fractions against FRAP, ahead of F3.

Table 2: IC_{50} Scavenging activity of liquorice root and standard.

	DPPH	ABTS	FRAP
Samples	IC_{50} (µg/ml)		
F1	83.608 ± 2.34	403.06 ± 1.2	**56.32 ± 2.2**
F2	163.965 ± 3.32	897.16 ± 2.4	97.64 ± 1.4
F3	**56.1 ± 6.32**	**39.14 ± 1.1**	66.34 ± 1.4
F4	104.2 ± 11.12	86.6 ± 2.1	120.1 ± 1.4
Vit C	12.1 ± 1.12	26.3 ± 1.4	18.33 ± 1.4

4.3 Cytotoxicity

The cytotoxic activities of F3 against HepG2 and SK-MEL-1 cells were determined after 24 h treatment with different concentrations of F3 and APAP as shown in Figure 9. Fraction 3 showed a higher percentage cell viability compared to APAP that was used as the standard control. The HepG2 cells, however, showed a relatively higher percentage viability compared to the SK-MEL-1 cells.

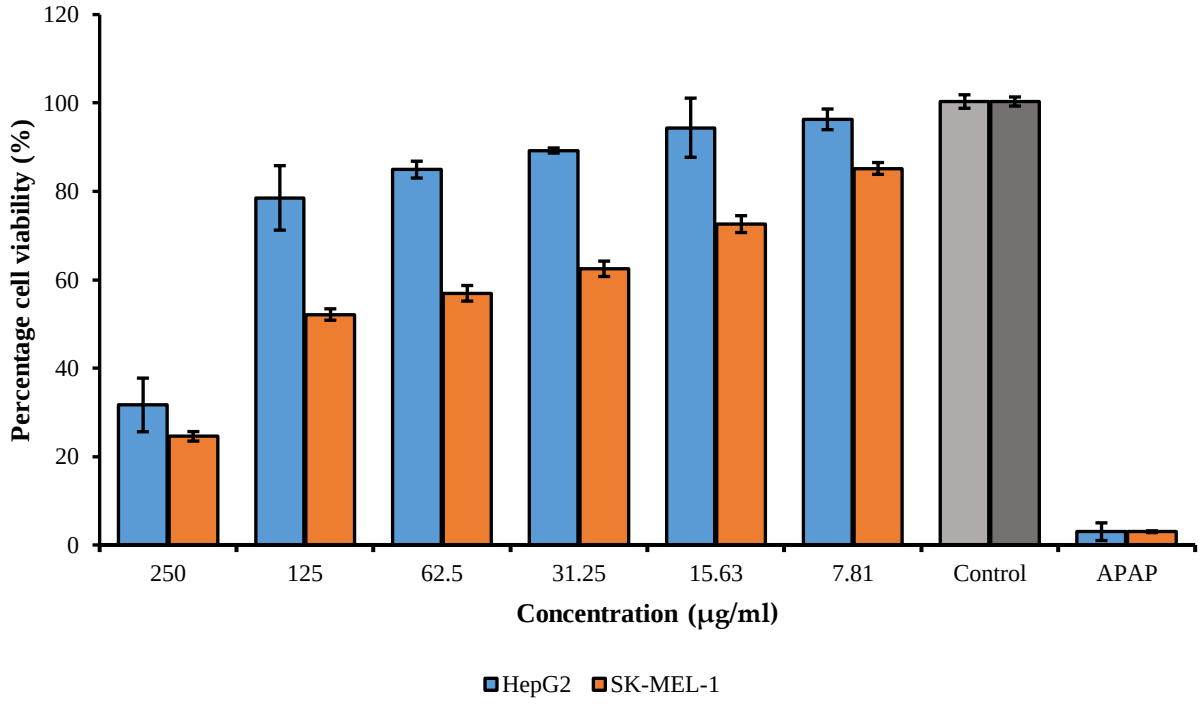


Figure 9: Cytotoxic effect of the active fraction of liquorice root on HepG2 and SK-MEL-1 cells after 24 h treatment. Values are means ± SD percentage of control from triplicate independent experiments. APAP = Acetaminophen (100 mM).

4.4 Tyrosinase activity

The anti-tyrosinase activity of F3 extract from liquorice root is shown in Table 3. This activity is concentration-dependent with the highest activity observed at the highest concentration of 500 µg/ml compared to kojic acid used as control. The IC_{50} is 358.54 µg/ml. The data from Figure 10 appears to show a linear increase in tyrosinase inhibition with increase in extract concentration. However, graphical representation of these data indicate a curve with a plateau. Thus, the greatest relative tyrosinase inhibition occurs at an F3 extract concentration of 250 µg/ml.

Table 3: The effect of Liquorice root extract F3 on tyrosinase activity. Kojic acid was used as control.

Samples	**% Relative inhibition**	**IC_{50}** (µg/ml)
500 µg/ml	65.98	
250 µg/ml	42.59	358.54
125 µg/ml	19.4	
62.5 µg/ml	4.04	
31.25 µg/ml	-18.44	
Kojic Acid (0.75 mM)	91.45	

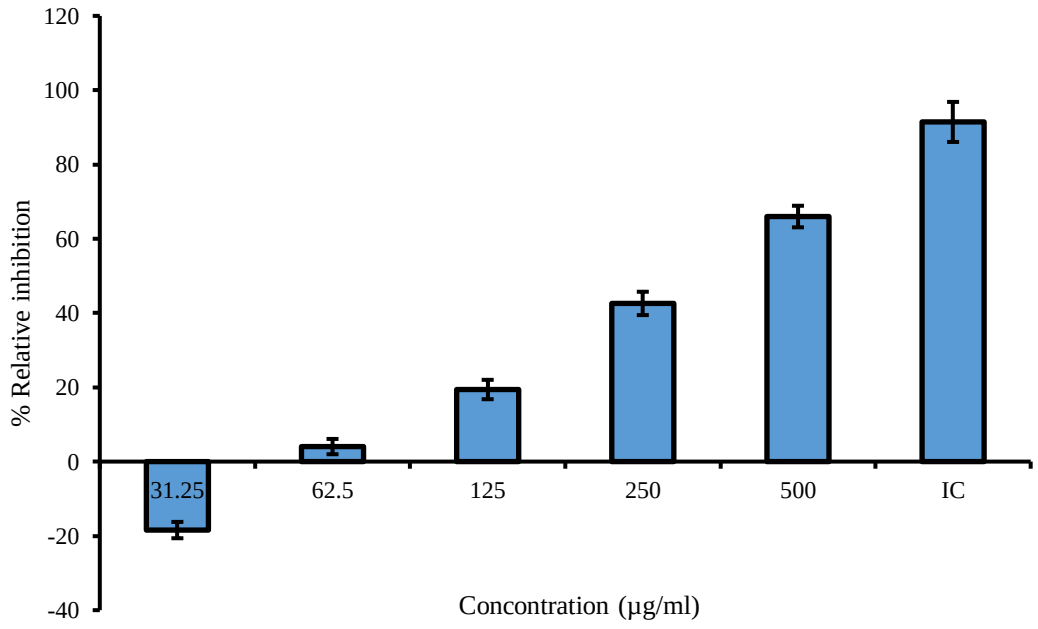


Figure 10: The effect of liquorice root extract F3 on tyrosinase activity.

4.5 LC-MS/MS Analysis of *Glycyrrhiza glabra* fractions

Chromatographic spectral analysis of the phytochemical constituents within the fractions confirmed the presence of various compounds with different retention times. Further data relating to these compounds included each of their peak fragmentation, retention time and molecular formula. Some features of the secondary metabolites identified by the LC-MS/MS analysis are shown in Table 4. The compounds that were eluted at different times were analyzed by the mass spectrometer to identify their structures. These mass spectra are fingerprints of compounds that were identified from the data library. This is also possible due to the fragmentation of the large compounds into smaller compounds giving rise to appearance of peaks at different m/z ratios. The most abundant compounds are 4-azido-3-benzyl-coumarin, ferulic acid, Glycyrrhizin, Quercitrin, Cirsilineol, Gentioflavine and 4",6,7-Trihydroxyisoflavone, licoricidin, 2,3-Dihydroxy-p-cumate, 3-tert-Butyl-5-methylcatechol and licoisoflavone A.

Table 4: Retention time (RT), measured mass and calculated formula by elemental compositions for major compound identified in the liquorice roots.

Compound Number	**Major compounds**	**Fraction(s)**	**t_R (min)**	**Formula**	Measured mass (m/z)	Positive ESI Mode		
						Adduct Ions	Mass Error (ppm)	MS^n Fragment Ions
1	Diethyl phthalate	1,2,3,4	0.2182	$C_{12}H_{14}O_4$	222.24 182.18	221.089 [M + H]+	0.5	65.039 [C5H4]+H+, 93.034 [C6H4O]+H+, 121.028 [C7H4O2]+H+
2	Homovanillate	4	12.01	$C_9H_{10}O_4$		181.058 [M + H]+	1.1	68.9971 [C3H2O2-H]+, 81.0335 [C5H5O]+, 95.0491 [C6H4O+2H]+H+, 109.0648 [C7H6O+2H]+H+, 119.0491 [C8H8O-H]+, 137.0597 [C8H9O2]+, 165.0546 [C9H9O3]+
3	(R)-3-(4-Hydroxyphenyl)lactate	1	12.08	$C_9H_{10}O_4$	182.18	181.058 [M + H]+	0.2	91.0542 [C7H6]+H+, 109.0648 [C7H7O+H]+H+, 119.0491 [C8H8O-H]+, 137.0597 [C8H9O2]+, 165.0546 [C9H9O3]+
4	Licoisoflavone A	1,3	1.8622	$C_{20}H_{18}O_6$	354.11	353.35 [M + H]+	4.1	100.11214 [C6H12N+H]+H+
5	Glycyrrhizin	3	9.2	$C_{42}H_{62}O_{16}$	823.4111	822.404 [M + H]+	0.005	121.065 $[C8H11O-2H]^+$
6	2-Methoxy-4-vinylphenol	4	3.829	$C_9H_{10}O_2$	151.07	150.177 [M + H]+	0.6	103.054 [C8H6]+H+, 117.034 [C8H6O-H]+, 121.065 [C8H7O+H]+H+
7	4-azido-3-benzyl-coumarin	1	12.188	$C_{16}H_{11}N_3O_2$	278.29	277.18 [M + H]+	2.1	149.06 $[C9H9O2]^+$, 177.055 $[C10H9O3]^+$
8	Licoricidin	3,4	11.88	$C_{26}H_{32}O_5$	425.07	424.25 [M + H]+	7.1	117.034 $[C8H6O-H]^+$, 100.11214 [C6H12N+H]+H+

9	3-tert-Butyl-5-methylcatechol	1,3	4.2581	$C_{11}H_{16}O_2$	181.247	180.12 $[M + H]^+$	0.65	93.07 $[C7H10-H]^+$, 121.065 $[C8H11O-2H]^+$, 149.06 $[C9H10O2-H]^+$
10	Ferulic acid	2,3	6.242	$C_{10}H_{10}O_4$	195.18	194.0594 $[M + H]^+$	1.1	77.0386 $[C6H5]^+$, 117.034 $[C8H6O-H]^+$, 121.065 $[C8H6O+2H]+H^+$, 145.028 $[C9H6O2-H]^+$, 149.06 $[C9H9O2]^+$, 177.055 $[C10H9O3]^+$
11	4-chloroquinoline	3	6.020	C_9H_6ClN	164.55	163.60 $[M + H]+$	0.2	93.034 $[C6H4O]+H+$
12	Herniarin	2,3,4	6.0091	$C_{10}H_8O_3$	177.0546	176.047 $[M + H]^+$	−1.1	121.028 $[C7H6O2-H]^+$, 149.023 $[C8H6O3-H]^+$
13	Lophophorine	2,3	9.50	$C_{13}H_{17}NO_3$	236.11	235.121 $[M + H]^+$		148.1121 $[C10H12N+H]+H+$
14	Cirsilineol	1,3	8.99	$C_{18}H_{16}O_7$	345.01	344.09 $[M + H]^+$	1.1	123.0441 $[C7H7O2]+$, 147.0441 $[C9H8O2-H]+$, 163.039 $[C9H8O3-H]+$, 175.039 $[C10H8O3-H]+$, 285.0757 $[C16H10O5+2H]+H+$, 327.0863 $[C18H15O6]+$
15	2"-Hydroxyisoflavone	1,3	8.991	$C_{15}H_{10}O_3$	239.22	238.142 $[M + H]+$	2.2	145.028 $[C9H6O2-H]^+$, 149.06 $[C9H9O2]^+$, 177.055 $[C10H9O3]^+$
16	Androstan-3alpha,17beta-diol	1	7.065	$C_{19}H_{32}O_2$	293.56	292.4562 $[M + H]+$	1.3	145.028 $[C9H6O2-H]^+$, 149.06 $[C9H9O2]^+$, 177.055 $[C10H9O3]^+$
17	Daidzein	2,4	7.265	$C_{15}H_{10}O_4$	255.133	254.23 $[M + H]+$	1.1	103.054 $[C8H6]+H^+$, 117.034 $[C8H6O-H]^+$,
18	Coumarin 314	1,3	10.372	$C_{18}H_{19}NO_4$	314.1387	313.131 $[M + H]^+$	−1.3	77.0386 $[C6H4]+H^+$, 93.07 $[C7H6+2H]+H^+$, 103.054 $[C8H6]+H^+$, 117.034 $[C8H6O-H]^+$, 121.065 $[C8H9O]^+$, 145.028 $[C9H6O2-H]^+$, 149.06 $[C9H9O2]^+$, 177.055 $[C10H9O3]^+$
19	2-Hydroxymethylclavam	3	11.88	$C_6H_9NO_3$	144.153	143.058 $[M + H]^+$	-1.1	$[C4H5NO]+H+$
20	Melatonin	1,2,3,4	7.697	$C_{13}H_{16}N_2O_2$	233.057	232.121 $[M + H]^+$	2.2	145.028 $[C9H6O2-H]^+$, 149.06 $[C9H9O2]^+$, 177.055 $[C10H9O3]^+$
21	4",6,7-Trihydroxyisoflavone	1,3	11.68	$C_{15}H_{10}O_5$	271.44	270.053 $[M + H]^+$	0.2	121.065 $[C8H9O]^+$, 145.028 $[C9H6O2-H]^+$,

22	Apigenin	1,3,4	11.72	$C_{15}H_{10}O_5$	271.45	270.053 $[M + H]^+$	0.02	243.06522 [C14H11O4]+, 258.08871 [C15H13O4]+H+
23	Quercitrin	3	12.17	$C_{21}H_{20}O_{11}$	448.21	448.101 $[M + H]^+$	0.07	153.0196 [C7H4O4]+H+,
24	Cularidine	1,3	12.46	$C_{19}H_{21}NO_4$	328.03	327.147 $[M + H]^+$	0.01	77.0386 [C6H6-H]+, 121.0634 [C8H7O+H]+H+, 121.0634 [C8H7O+H]+H+
25	Gambiriin A2	1,3,4	12.30	$C_{30}H_{28}O_{12}$	581.1	580.158 $[M + H]^+$	0.4	109.0284 [C6H5O2]+, 125.0233 [C6H5O3]+, 135.0441 [C8H7O2]+, 167.0339 [C8H8O4-H]+
26	2,3-Dihydroxy-p-cumate	2,3	13.02	$C_{10}H_{12}O_4$	197.72	196.074 $[M + H]^+$	-0.5	68.9971 [C3H2O2-H]+, 91.0542 [C7H9-2H]+, 119.0491 [C8H9O-2H]+, 121.0284 [C7H7O2-2H]+, 151.0754 [C9H11O2]+, 155.0703 [C8H9O3+H]+H+, 179.0703 [C10H11O3]+,
27	Gentioflavine	3,4	13.05	$C_{10}H_{11}NO_3$	194.16	193.074 $[M + H]^+$	0.4	80.0495 [C5H6N]+, 106.0651 [C7H8N]+, 108.0444 [C6H4NO+H]+H+, 134.06 [C8H9NO-H]+
28	Vestitol	4	4.169	$C_{16}H_{16}O_4$	273.87	272.105 [M + H]+	-0.2	215.07031 [C13H12O3-H]+, 243.06522 [C14H11O4]+, 258.08871 [C15H13O4]+H+

CHAPTER 5 DISCUSSION

The liquorice plant has long been used as a traditional medicine and consists of three commonly used species, *Glycyrrhiza glabra L*., *Glycyrrhiza uralensis* Fish. ex DC and *Glycyrrhiza inflata* Batalin (Li *et al.,* 2017). These plants contain various metabolites that may be synthesised according to variables such as geographical location, seasonal variations and harsh environmental conditions (Corradi *et al.,* 2018). In this study, *Glycyrrhiza glabra L*. root material was collected for analysis.

Liquorice in traditional Chinese medicine is combined with other herbs in a single prescription and is known as a guide drug to enhance the effectiveness of other ingredients, to reduce toxicity and to improve flavour in almost half of Chinese herbal formulas (Wang *et al.,* 2013). These authors also reported that this guide effect of liquorice is partially through components transformed in liquorice-drug interactions. Some of the affected enzymes are cytochrome P450 enzymes (Qiao *et al*., 2014). In addition, these authors reported that liquiritigenin, isoliquiritigenin, together with seven isoprenylated flavonoids and arylcoumarins, viz. glycycoumarin, semilicoisoflavone B, licoisoflavone A, licoricone, glycyrol, licoflavonol and licoisoflavone B (C15) may be the key compounds responsible for liquorice–P450 interactions.

One of the primary aims of this study was to make use of a liquorice root extraction protocol that allowed for the extraction of a spectrum of compounds depending on their hydrophilic and/or hydrophobic nature (Tian *et al.,* 2008). Thus, various solvent fractions were generated that contained compounds with mainly hydrophobic features through to fractions containing compounds that possessed a combination of hydrophobic and hydrophilic features, and finally through to those extracts that contained compounds that were mainly hydrophilic in nature. We noted in the study that fraction 3 (F3) contained many polyphenolic compounds that possess both hydrophilic and hydrophobic features.

Regarding safety factors associated with liquorice extracts, Damle (2014) suggested that liquorice extract is the safest pigment-lightening agent known with fewest side effects - due to the presence of liquiritin that disperses melanin, thereby inducing skin lightening. Zolghadri *et al.* (2019) warned that phenolic compounds (simple phenols, polyphenols and their derivatives) and other compounds such as terpenoids, phenols, pyridine, piperidine, pyridinone, hydroxypyridinone, thiosemicarbazone, thiosemicarbazide, azole, thiazolidine, kojic acid, benzaldehyde and xanthate derivatives are potent tyrosinase inhibitors but that few of these compounds are safe to use. Of these compounds, phenolic acids and flavonoids from natural sources may vary in potency against tyrosinase but are clinically safe with fewer side effects (Odeyemi & Dewar, 2019).

In the current study, the phytochemical analysis of different solvent extractions of *Glycrrhiza gabra* showed high levels of polyphenolics that are beneficial due to their capacity to serve as electron donors to oxidize a broad spectrum of free radicals to more stable radical intermediates and to chelate metals by quenching singlet and triplet oxygen. The polyphenolics identified in the roots of *G. glabra* were glycyrrhizin, licoisoflavone A, glabridin, licoricidin and gentioflavine, consistent with previous reports that identified many of these compounds in *G. glabra* (Simmler *et al.,* 2013).

The current study also tested and established that polyphenolics are good antioxidants, as evidenced by the high percentage inhibition observed in the DPPH, ABTS and FRAP activities of the various experimental fractions, particularly the polyphenolic-rich fraction 3 (F3). Different antioxidant methods were used in this study to analyse the antioxidant capacity of each fraction because antioxidant agents with different compositions and contents present different mechanisms for antioxidant activities. For instance, polyphenolics scavenge free radicals from DPPH by donating hydrogen to the nitrogen-centered free radicals of DPPH converting it to a stable diamagnetic molecule known as diphenyl-picrylhydrazine whereas they scavenge free radicals from ABTS by single electron transfer to the ABTS radical (Odeyemi & Afolayan, 2018). Therefore, ABTS is an excellent way of determining the hydrogen-donating capacity of antioxidants. The FRAP assay measures the reducing potential with a ferric tripyridyltriazine (Fe3+-TPTZ) complex to produce a ferrous tripyridyltiazine (Fe2+-TPTZ) by the donation of an hydrogen atom at low pH of 3.6 (Rajurkar *et al.,* 2011). Therefore, the results from this study suggest that the polyphenolics isolated from *G. glabra* are potent hydrogen donors.

The identification of tyrosinase inhibitors from both natural and synthetic sources has gained more attention due to the critical role of tyrosinase in the synthesis of melanin and the browning process. The natural anti-tyrosinase is advantageous over the synthetic ones because of fewer side effects and toxicity concerns. Liquorice root was used for decades without any adverse effects and even the combination of liquorice roots with toxic compounds has been reported to reduce their toxicity (Wang *et al.,* 2013). Viswanathan & Mukne (2016) used HPLC and HPTLC to analyse glabridin in the roots of liquorice plants grown in India. They showed that glabridin is a prenylated isoflavonoid and a major constituent in the hydrophobic extracts of liquorice. Although inhibition of tyrosinase,activity was confirmed in the current study, analysis of the F3 extract from the liquorice root did not identify glabridin. This contrasted with previous reports (Simmler *et al.,* 2013; Zolghadri *et al.,* 2019). This may be due to the choice of solvent used in the extraction process, the geographical location of growth of the original liquorice plant or the method used to fractionate the plant material. The current study identified at least 26 compounds from F3 of *G. glabra*. Many of these compounds have been previously reported but (R)-

3-(4-hydroxyphenyllactate has not been reported as a tyrosinase inhibitor. However, its derivate (2S)-(O-hydroxyphenyl)lactate has shown tyrosinase inhibition in melanona cells (Li *et al.,* 2002; Chia-Ying *et al.,* 2002).

Some of the compounds found in high abundance in the current study were glycyrrhizin, licoisoflavone A, ferulic acid, quercitrin and melatonin, compounds thought to contribute significantly to many of the biological activities observed in this study. Glycyrrhizin has been reported to be an active ingredient of *G. glabra* responsible for the skin lightening effects of liquorice roots. Therefore, the identification of glycyrrhizin in this study corroborates previous reports (Tamura *et al.,* 2013). Ferulic acid and Daidzein have also been reported to be good anti-tyrosinase inhibitors (Georgiev *et al.,* 2013; Chang, 2009; Wang *et al.,* 2013).

Chang *et al*. (2005) reported that 6,7,4-trihydroxyisoflavone is a novel potent tyrosinase inhibitor with a six-fold higher IC_{50} compared to kojic acid and should be an excellent skin lightener. Apigenin is another flavonol reported to possess anti-tyrosinase activity (Ye *et al.,* 2010; Haliloglu *et al.,* 2017; Eghbali-Feriz *et al.,* 2018). Nguyen *et al.* (2012) investigated the presence of apigenin and nobiletin from the methanolic extract of the heartwood of *Artocapus altilis* with 11 other phenolic compounds for their inhibitory activities on tyrosinase. In another report, apigenin-7-glucoside and naringenin exhibited anti-proliferative activity against B16F10 melanoma cells and the authors suggested that apigenin-7-glucoside could be introduced into cosmetic products as natural tanning agents (Nasr Bouzaiene *et al.,* 2016). Quercetin and quercitrin have been shown to be potent antioxidants and, in addition, quercetin demonstrated anti-tyrosinase properties (Sirat *et al.,* 2010).

The higher cell viability observed in the cytotoxicity experiment shows that the active fraction is not toxic to either the hepatocellular or the melanoma cells at the tested concentrations. This contrasts with recent reports on the isoangustone A isolated from liquorice roots where it was suggested that isoangustone A has PI3-K, MKK4 and MKK7 as primary molecular targets that are involved in the suppression of cell proliferation in SK-MEL-28 cells (Song *et al.,* 2013).

The only alkaloid identified in this study was cularidine, a compound that was previously isolated from *Dicentra cucullaria (L.) Bernh*. and *Corydalis claviculata* (L.) DC. It is monophenolic and upon methylation with diazomethane is converted into cularine. Although, cularidine was described in the 1960s (Manske, 1965), it has not been previously reported in studies involving liquorice root. In addition, the antioxidant activity associated with this compound could be of great significance together with its inhibition of tyrosinase and requires further research.

CHAPTER 6 CONCLUSION

The cosmetics industry is a fast-growing industry and many new cosmetic products require the use of new ingredients to provide unique properties to the cosmetics. Skin lightening agents are currently on demand by the consumer as well as the cosmetic and pharmaceutical industries and, therefore, new compounds appear frequently in the cosmetic industry. There are very limited scientific studies done on liquorice as a skin care product, especially in South Africa, underscoring the need to conduct controlled studies such as described in this dissertation to ensure the safety of use of such compounds.

Different fractions of *G. glabra* showed high levels of total phenolic content confirming previous reports. Polyphenols in plants are beneficial due to their capacity to oxidize a broad spectrum of free radicals to more stable radical intermediates. In addition, they chelate metals, quenching singlet and triplet oxygen and serve as electron donors. The results of the current study identified polyphenols such as glycyrrhizin, Licoisoflavone A, Licoricidin and Kaempferol-3-O-β-D-glucoside. These results are consistent with previous reports that identified these compounds in *G. glabra* except that the abundance of glabridin detected in these fractions was low compared to other compounds, in contrast to previous reports. This may be due to the choice of solvent used for extraction, metabolic features of the liquorice plant associated with the geographical location where the plants grew and were collected (Oudtshoorn) or the method used to fractionate the particular extract. The presence of other polyphenols such as Licoricidin also contribute to the anti-tyrosinase activity associated with this plant. This suggests that further studies need to be conducted on extracts of liquorice root to optimise the characterization of the compounds in the plant extracts. Nonetheless, the high anti-tyrosinase activity observed in this study indicates that phenolic compounds (simple phenols and polyphenols) and their derivatives showed themselves to be potent melanin synthesis inhibitors (Zolghadri *et al.,* 2019). Likewise, the SOD activity and other assay results also supports the antioxidant activities of the isolated compounds.

Experiments on animals have been prohibited since March 2013. Nonetheless, European guidelines for cosmetic compounds - 1223/2009 (Regulation EC No. 1223/2009) require that any new substance or formulation rated as to sensitization activity by documenting the combination of active molecule(s) and passage enhancer(s) in that new cosmetic compound. Thus, the results of the current study support the need for continuing research to develop skin care and cosmetoceutical products such as creams, lotions and make-up for skin application. Further such exploration could lead to the discovery of more skin care compounds that use liquorice compounds as part of an anti-pigmentation formulation.

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ADDENDUM PAGE 1 OF ARTICLE MANUSCRIPT

The antioxidant, anti-tyrosinase and hepatoprotective activity of compounds isolated from Glycyrrhiza glabra L. roots (Liquorice root)

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Abstract:
The antioxidant, anti-tyrosinase and hepatoprotective activities of different compounds from *Glycyrrhiza glabra* L. were investigated. The ability to scavenge free radicals was tested against 2,2-diphenyl-1-picrylhydrazyl (DPPH), [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid)] (ABTS) and the ferric ion reducing power (FRAP). The compounds in the most active fraction were identified using UHPLC-QToF-20 MS. Hepatoprotective activity was then investigated in HepG2 cells using cell viability assay. The concentrations of alanine aminotransferase (AST), aspartate aminotransferase (ALT) and lactate dehydrogenase (LDH) released into the medium were evaluated. Among the four fractions, *G. glabra* fraction 3 (GGF3) showed the highest antioxidant properties with IC_{50} of 56.1 ± 6.32, 39.14 ± 1.1 and 66.34 ± 1.4 against DPPH, ABTS, and FRAP respectively. There was also decreased activities of liver superoxide dismutase and high anti-tyrosinase activity. The pretreatment with GGF3 also ameliorated APAP-the induced hepatocellular injury by significantly preventing the leakage of AST, ALT, and LDH into the medium. The LC-MS analysis revealed the presence of a wide variety of compounds such as 4-azido-3-benzyl-coumarin, ferulic acid, Glycyrrhizin, Quercitrin, Cirsilineol, Gentioflavine and 4'',6,7-Trihydroxyisoflavone among the dominant constituents. In conclusion, the isolated compounds showed low cytotoxicity, hepatoprotective activity and effective DPPH and ABTS radical scavenging ability.

Keywords: *Glycyrrhiza glabra*; antioxidant; hepatoprotectitive; acetaminophen; cell viability; anti-tyrosinase