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Although natural ingredients have been traditionally used since human civilisation for skin care purposes, they are becoming more prevalent in modern-day formulations. The term “natural” is defined as ‘something or an ingredient that is produced by the nature or found in nature and is directly extracted from plants or animal products’. Sources of natural ingredients can be herbs, fruits, flowers, leaves, minerals, water and land. The effect of natural ingredients in skin care products depends on their *in vitro* and *in vivo* efficacy and the form of dermatological base where they are integrated (Ribeiro, *et al.*, 2015)

## **2.5 Plants used for skin disorders in South Africa**

Herbal medicines take central stage to African tradition and are also deeply rooted in the treatment of skin ailments and boosting physical appearance. The use of herbs in Africa is as old as the first settlers, but many of the African dialects had no written form, which is why there is no written record of the plants that were used at that. Documentation of plants started to take shape when white settlers arrived even though they considered herbal remedies as primitive and inferior to contemporary medicine (Shumba, *et al.*, 2009).

Ethnobotanical studies have documented the use of plants by traditional healers for the treatment of various skin ailments. Different plant parts commonly used as cosmetics or face

masks, known as umemezis, are widely used in southern Africa for skin problems like inflammation, wounds, burns, eczema and puberty acne (Lall and Sharma, 2014)

De Wet, *et al.*, 2013 conducted a study in Northern Maputaland where 87 individuals were interviewed for plants used for dermatological diseases. The skin diseases included abscesses, acne, burns, boils, incisions, ringworms, rashes, shingles, sores, wounds and warts. The 47 plant species belongs to 35 families, with Fabaceae (*sensu lato*) (eight species) being the most frequently represented family, followed by Asteraceae and Solanaceae (three species each), Anacardiaceae (two species) and the remainder, had one species each. Plant species from the family Fabaceae (*sensu lato*) are well known world-wide for the treatment of wounds. The following nine plant species have been recorded for the first time globally for the use to treat skin disorders; *Acacia burkei*, *Brachylaena discolor*, *Ozoroa engleri*, *Parinari capensis*, subsp. *capensis*, *Portulacaria afra*, *Sida pseudocordifolia*, *Solanum rigescens*, *Strychnos madagascariensis* and *Drimia delagoensis*. Although *A. burkei*, *B. discolor*, *O. engleri*, *P. capensis*, *P. afra*, *S. madagascariensis* and *D. delagoensis* have been recorded for other medicinal uses; *S. pseudocordifolia* and *S. rigescens* have no other documented medicinal uses. The indigenous herb *Senecio serratuloides* was by far the most frequently used species by the interviewees to treat skin disorders (17 interviewees). This is a well recorded medicinal plant in South Africa for the treatment of various wounds and dermatological ailments. The second most mentioned species was the indigenous tree *Tabernaemontana elegans*, followed by *Sclerocarya birrea* and *S. madagascariensis*, and *Dialium schlechteri*.



## 2.6 Tyrosinase inhibitors

The main causative agent for over-expressed melanogenesis is UV radiation of solar light, UV may directly or indirectly act on melanocytes through the release of keratinocytes derived factors named  $\alpha$ -MSH ( $\alpha$ -Melanocyte stimulating hormones). Melanogenesis can be inhibited, at least partially, through tyrosinase deactivation (Khan, 2007). A number of tyrosinase inhibitors, especially from natural sources have become increasingly important (Chang, 2009). Plants are very rich in bioactive chemicals and consist mostly of no side effects, hence the increased interest in identifying tyrosinase inhibitors (Nithitanakool, 2009).

Below, tyrosinase inhibitors from natural sources are discussed.

Hydroquinone, kojic acid, arbutin, magnesium ascorbyl phosphate, licorice extract, aloesin, azelaic acid, soybean extract, and niacinamide

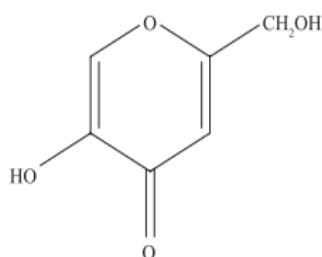


### 2.6.1 Kojic acid

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) is a secondary metabolite that is produced in aerobic conditions by various microorganisms, viz. *Aspergillus*, *Acetobacter* and *Penicillium* (Briganti, *et al.*, 2003). Kojic acid was discovered by K. Saito in 1907 in the strain of *Aspergillus ozyrae* that was grown on steamed rice. is a potent and mostly studied inhibitor of tyrosinase and is used widely on a commercial scale as a cosmetic skin whitening agent; it also acts as an antioxidant, bacteriostat, chelates metal ions and prevents photodamage (Ho, *et al.*, 2007). Additionally, kojic acid's application is widespread in food, medicine, cosmetics, chemical and agricultural industries. In the medical industry, it is reported to treat antibiotic, treating bacterial, fungal, leukemic and microbial problems. In the food industry, the application of kojic acid include prevention of undesirable browning of crabs, crustaceans, vegetables and fruits during storage. In the chemical field, since kojic acid forms a complex of deep red when

reacted with traces of  $\text{Fe}^{3+}$ , it can be used as an analytical tool for determining cations, also, Kojic acid (Zirak & Eftekhari-Sis, 2015). In the cosmetic field, it is used at concentrations ranging from 0.1% to 2% as a skin-whitening agent; it chelates the copper ion in the tyrosinase through the 4 carbonyl and 5-hydroxyl group

Kojic acid has a molecular mass of 142.11 (Burnett, *et al.*, 2010).



(Burnett, *et al.*, 2010)

**Figure 2.4:** Chemical structure of kojic acid



### 2.6.3 Hydroquinone

Hydroquinone-o- $\beta$ -D-glucopyranoside is a potent skin-whitening agent with high melanocyte-specific cytotoxicity (Gasparetti, 2012). It is regarded as the gold standard in treatment of pigmentation disorders. Hydroquinone is a phenolic compound which is found in coffee, tea, beer, berries, wheat but the liver detoxifies it into an inert compound (Ebanks, *et al.*, 2009).

Hydroquinone is an effective and widely used skin lightening agent for the treatment of skin pigmentation such as melasma, postinflammatory hyperpigmentation, and other hyperpigmentation disorders. It acts by inhibiting the conversion of tyrosine to melanin.

Hydroquinone is available over the counter in the United States in strengths up to 2% and by prescription in strengths of 3 to 4%. Higher concentrations are available through

compounding pharmacies. Four to 6 weeks of monotherapy with hydroquinone is generally required before whitening effects are visible. Several products are available in the market which contain hydroquinone in combination with vitamin C, tretinoin, retinol, glycolic acid, and fluorinated steroids. The period necessary for these products to take effect varies.

While it is used in depigmenting medication, its use in cosmetic preparations has been discouraged due to numerous side effects, including skin irritation (Gasparetti, 2012)

The most common side effects associated with hydroquinone are skin irritation and contact dermatitis, which can be treated with topical steroids. A rare side effect is the development of exogenous ochronosis, a sooty hyperpigmentation in the treatment area, which can be extremely difficult to reverse. Although this adverse event is uncommon with normal use, it may result from hydroquinone use for extended periods of time. Alternating the use of hydroquinone in 4-month cycles with one of the natural depigmenting agents listed below can prevent or reduce side effects, such as irritation or even exogenous ochronosis (Rendon and Gaviria, 2005).



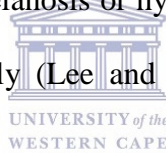
#### **2.6.4 Arbutin**

Arbutin (hydroquinone-O- $\beta$ -D-glucopyranoside) is a derivative of hydroquinone, a plant derived compound found in several plants including blueberries, cranberries, pears and wheat. Arbutin is used as an effective treatment of hyperpigmentary disorders, and demonstrates less melanocyte cytotoxicity compared to hydroquinone. The compound inhibits melanin synthesis by competitively and reversibly binding tyrosinase without influencing the mRNA transcription of tyrosinase. It also inhibits melanosome maturation, possibly by its influence on DHICA polymerase activity and Pmel-17 protein (Ebanks, 2009).

## 1.6.5 Liquorice

## 2.7 Pigmentation disorders

The colour of the skin is principally determined by melanin pigment whose synthesis occurs in melanocytes and is restricted to melanosomes. Melanosomes contain key enzymes, such as tyrosinase and tyrosinase-related proteins (TRPs). Racial and ethnic differences in skin colour are related to the number, size, shape, distribution, and degradation of melanosomes. Changes in skin pigmentation, which induces significant cosmetic problems with effect on quality of life, could result from abnormalities in the formation of melanosomes, melanosome melanisation, melanosomes transfer to keratinocytes with/without degradation in lysosome-like organelles, and the number of melanocytes. Abnormal pigmentation conditions can be divided into two types, that is, hypermelanosis or hypomelanosis, which involve excessive or insufficient melanin in skin, respectively (Lee and Noh, 2013). Some of the pigmentation disorders are discussed below.



### 2.7.1 Hyperpigmentary disorders

#### 2.7.1.1 Melasma

Melasma (chloasma) is pigmentation disorder that is acquired due to melanogenesis dysfunction. It is more common in females than in males. The disease is also known as the mask of pregnancy or chloasma. The cause is still unknown, although there are known to be triggering factors, such as pregnancy, menopause and oral contraceptive use. Clinically, brown macules with distinct and irregular margins are seen. These are generally symmetrical and often on the face when exposed to the Sun. There are two subtypes of facial melasma depending on the site of involvement, centofacial and peripheral. It may also be seen in extrafacial regions such as the arms, forearms or cervical and sternal regions. The course

involves irregular, symmetrical hyperchromic skin discoloration. Involvement in these regions is generally seen in advanced age in patients undergoing the menopause or receiving hormone replacement. Diagnosis is generally clinical. Wood's light and histopathology may be employed on rare occasions (Engin, 2015).

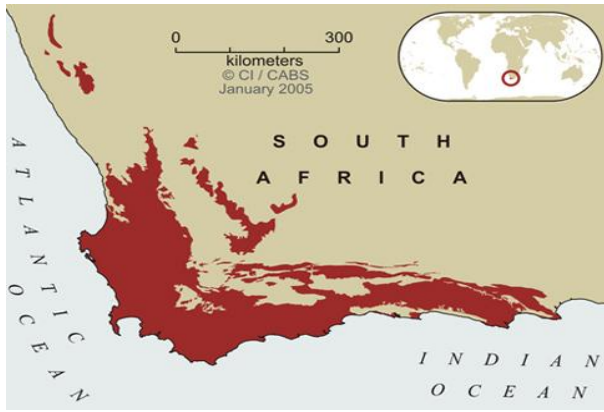
#### **2.7.1.2 Cafe au lait macules.**

Cafe au lait macules Cafe au lait macules present as uniformly pigmented macules or patches with sharp margins. Size varies from small confetti macules to large irregular plaques of numerous centimeters. Cafe au lait macules are often present at birth. In normal individuals, only 1 or 2 lesions are usually observed. Light microscopy examination reveals increased epidermal melanin with normal number of melanocytes. Ultrastructural examination shows increased pigment. Giant pigment granules (macromelanosomes) that are a feature of cafe au lait macules of the neurofibromatosis are absent in sporadic cafe au lait macules (Passeron, *et.al.*, 2005).



#### **2.8 Cape Floristic Region (CFR)**

Extending from 31° and 34°30'S latitude is the biogeographic region located at the South-western tip of South Africa known as the CFR (Manning and Goldblatt, 2012). The CFR boasts one of the richest botanical diversity with approximately 9000 species vascular plants which are native to this area. When compared to other floras of the African region, the Cape flora is unexceptionally species rich especially for its size (it comprises land area of 90 000 km<sup>2</sup>). It comprises three of the seven largest plant families in the CFR. For an arid region, the largest families are Asteraceae and Fabaceae which make-up 20% of the total species combined (Manning and Goldblatt, 2000).



**Figure 2.6** Map showing geographical distribution of the Cape Floristic region biodiversity hotspot.



## 2.9 Myricaceae

### 2.9.1 Biological description

The family Myricaceae is a member of the Division Magnoliophyta, Class Magnoliopsida, Subclass Hamamelidae, and is placed in the order Myricales. Several authors placed Myricaceae in the order Myricales alone due to distinctive characteristics including single orthotropous ovule, entire leaves with resinous balloon-like glands and the unilocular ovary (Thorne, 1973). Subclass Hamamelidae is comprised of Myricales and six other orders, Fagales, Juglandales, Leitneriales, Urticales, Hamamelidales and Casuarinales. This subclass is phylogenetically grouped in an order characterised by strongly reduced, typically unisexual flowers which can lack or produce a poorly developed perianth.

Initially, the taxonomic treatment of Myricaceae was monographed by Chevalier in 1901, the family was divided into three genera namely, *Myrica*, *Gale*, and *Comptonia*. The genera was classified mainly on basis of the nature of the fruit exocarp and different morphological developments of the pistillate flower bracts during fruit maturation. (Lutzow-Felling *et. al.* 1995). Currently, taxonomist recognises four genera of the family including, *Morella* Loureiro, *Myrica* L, *Comptonia* L'Heritier ex Aiton and *Canacomyrica* Guillaumin (Knapp, 2002)

Myricaceae, a small sub-cosmopolitan consisting primarily of shrubs. The flowers of trees are unisex borne in catkins, entire leaves, peltate glands, single orthotropous ovule and a unilocular ovary. With the exception of two monotypic genera, *Comptonia* and *Canacomyrica*, the species of Myricaceae have traditionally been referred to the genus *Myrica* which is of Linnaean origin.

However, *Myrica* was split into two genera, *Myrica sensu stricto* and *Morella*, based on phylogenetic analysis of nuclear ITS and chloroplast trnL-F sequence data and morphological differences such as deciduous or evergreen, dry fruits or fleshy fruits; and sunken stoma or not. In Myricaceae, only one genus and four species including *M. adenophora*, *M. rubra*, *M. Nana*, *M. esculenta* and are located in China. These species are easily recognised by their distinct tomentose branchlets and petioles, whereas *M. rubra* and *M. nana* are glabrous or sparsely pubescent. Previous phylogenetic studies strongly supported the monophyly of these four species, but the relationships among *M. rubra*, *M. nana* and *M. adenophora* were not resolved. The genetic diversity and population structure of wild *M. rubra* populations are poorly known (Liu, *et. al.*, 2015).

### 2.9.2 Genus *Myrica*



When Linnaeus named the genus *Myrica* in 1753, the following five species were known to him: *Myrica gale*, *M. cerifera*, *M cordifolia*, *M quercifolia* and *M. asplenifolia*. There are significant morphological differences between *Myrica gale*, whose fruit is smooth and dry, and *M cerifera*, *M. cordifolia* or *M. quercifolia*, all of which have papillose waxy fruits. These differences were not recognised formally until the genus was split by Spach in 1984, adopting *Gale dumort* to accommodate *Myrica gale*, retaining *Comptonia*, and placing the remaining species in *Myrica*. In 1901, Chevalier published a monographic study of the family with the division of *Myrica* into three sections (see table 2.1). The majority of literature published since 1901 has recognised only *Myrica* and *Comptonia*.

Earlier taxonomists gave recognition to the differences between *M. gale* and the majority of other species, by recognising the subgenera *Gale* and *Morella*. Some authors (Baird, 1968; Wilbur, 1994) expressed the opinion that *Myrica* (s.l.) should be split into two genera in order

to distinguish between these two groups of species and stated that these should be named *Myrica* and *Morella*. However, this would entail transferring the majority of species to *Morella*. (Verdcourt and Polhill, 1997) made a proposal to conserve the generic names *Myrica* and *Gale*, in order to give these groups generic status whilst minimising the number of name changes that would be required. In line with nomenclatural rules their proposal would involve changing the type specimen of the genus *Myrica* from *M. gale* to *M. cerifera*. *Myrica gale* would then change its name to *Gale belgica* and become the type of the genus *Gale*. However, the proposal was rejected (Brummitt, 1999) and *M. gale* remains the type of the genus *Myrica*. Thus, the generic name *Morella* must be adopted for the majority of those species formerly treated as *Myrica*. Most of the new combinations have now been made. The nomenclature adopted herein follows Wilbur (1994) in recognising *Myrica* s.s., *Morella* and *Comptonia*, with the inclusion of *Canacomyrica*.



### **2.9.2.1 Distribution and ethnopharmacological uses of *Myrica***

The genus *Morella* is the largest of the Myricaceae family, it is described to consist of approximately 50 species with more than half of the species occurring in Africa, the remainder occurs in Europe, North America and Asia (Silva, *et. al.* 2015). The genus *Myrica* is well represented in South Africa, 15 of these species occur in South Africa in the Cape Peninsula region, 11 in tropical Africa, 6 in the Mascarene Island and the distribution of the remainder occurs throughout the Northern Hemisphere (Lutzow-Felling, *et. al.* 1995).

Seven of which were described from the Cape Peninsula (in the Cape Town region): *M. ovata*, *M. cordifolia*, *M. burmanni*, *M. humilis*, *M. diversifolia*, *M. dregeana*, *M. quercifolia*, *M. elliptica*, *M. kraussiana*, *M. zeyheri*, *M. conifera* and *M. braifolia* Burm. Eleven occur in tropical Africa, six in the Mascarene Islands, and the remainder extensively distributed

throughout the Northern Hemisphere. Distribution and ethnobotanical uses of *Morella* and *Myrica* is further discussed in table 2.1 below.

**Table 2.1** Distribution and ethnopharmacological uses of *Morella* and *Myrica* species

Species name	Distribution	Ethnobotanical uses	References
<i>Morella adenophora</i> (Hance) J. Herb.	China and Taiwan	Roots and bark to treat bleeding, diarrhea and stomach pain	Li, 2006
<i>Morella nana</i> (A. Chev.) J. Herb.	China	Roots are used to treat bleeding, diarrhea, stomach pain, burns, and skin diseases.	Wang and Zhang, 2009
<i>Morella serrata</i> (Lam.) Killick	South Africa and Southern African countries extending into tropical Africa	Used to treat asthma, coughing and shortness of breath . The decoction of the root is used to treat painful menstruation, cold, coughs and headaches and to enhance male sexual performance . It is also used in the management of sugar related disorder and as laxative to treat constipation. The stem bark is used to treat headache	Schmidt, 2002; Moffet, 2010; Ashafa, 2013
<i>Morella arborea</i> (Hutch.) Cheek	Cameroon	Bark decoction used to treat fevers and inflammation.	Tene, et al., 2000
<i>Morella cerifera</i> (L.) Small	North America	Herb decoction or tincture used as astringent, diaphoretic, as a circulatory stimulant, to treat irritable bowel syndrome, ulcerative colitis, digestive system disorders, diarrhea, dysentery, leukorrhea, mucous colitis, colds, stomatitis, sore throat,	Hofman and Hofman, 2003



<i>Morella salicifolia</i> (Hochst. ex A.Rich.) Verdc. & Polhill Myrica	Southeast Africa, Ethiopia and Saudi Arabia	measles and scarlet fever, convulsions, nasal catarrh and jaundice Roots infusion is used to treat gastro-intestinal disorders. while roots and bark used in the treatment of headache, pain, inflammation and respiratory diseases.	Kefalew, <i>et al.</i> , 2015; Teklay, <i>et al.</i> , 2013; Schlage, <i>et al.</i> , 2000
<b>Myrica</b>			
<i>Myrica rubra</i> (Lour.) Siebold & Zucc.	China, Japan, Taiwan and Korea	The various organs are used to treat gastrointestinal diseases, headaches, burns and skin diseases. Leaves are used to treat inflammatory diseases.	Sun, <i>et al.</i> , 2013;
<i>Myrica esculenta</i> Buch. Ham. ex D. Don	India, South China, Malaysia, Japan, Vietnam and Nepal	Ayurvedic medicine use decoction of bark to treat asthma, bronchitis, fever, lung infection, dysentery, toothache and wounds; leaf, root, bark and fruits juice for worms, jaundice and dysentery; Vietnamese folk medicine uses bark to treat catarrhal fever, cough, sore throat and skin disease	Kirtikar and Basu, 2009; Nadkarni, 2002; Laloo, <i>et al.</i> , 2006; Bich, 2004
<i>Myrica gale</i> L.	Europe, Siberia, Canada and Northern USA	Used in the treatment of ulcers, intestinal worms, cardiac disorders and aching muscles.	Small, 2014
<i>Myrica nagi</i> Thunb.	China, Malaya Islands, Pakistan and Nepal	Bark finds its application in reducing inflammations to treat cardiac diseases, bronchitis, gonorrhea, diuresis, dysentery, epilepsy, gargle, haemoptysis, hypothermia, catarrh, headache, menorrhagia, putrid sores, typhoid, face palsy and paralysis and	Panthari, <i>et al.</i> , 2012; Kumar and Rana, 2012

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wounds. Fruit wax or oil is used for treating ulcers, bleeding piles, body ache, toothache and for regulating the menstrual cycle

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### 2.9.2.2 Ecological distribution

Myricaceae occupy a range of habitats, mostly mesic, claims swampy habitats all eastern U.S. species except for *Comptonia*, mostly which thrives in dry sterile soil (Youngken, 1919). *Morella californica* occurs in canyons and moist slopes, whereas *Myrica hartwegii* is found on montane stream banks. The South African species (*Morella*) occur on sandstone or limestone slope (Goldblatt and Manning, 2000), but the sandstone water availability in these localities is not described by some authors.



Most species are commonly found in habitats such as sand-dune, recent volcanism areas, waterlogged areas where nutrients are deficient. They are frequently components of early-successional communities, but also occur in climax vegetation where environmental conditions are not suitable for taller trees. Mostly, the species, if not all, have low tolerance to drought. Several species grow with their roots waterlogged: for example, *Myrica gale* is found in bogs and swamps (Gorham, 1957), *Morella faya* grows in wet forests on the volcanic soils. *M. serrata* occurs in riparian habitats in Cape Town, South Africa. It has been reported that *M. javanica* can grow as an epiphyte on other forest trees in Borneo, this observation is illustrative of the high light requirements that the family has and can thrive in nutrient-poor substrates.

*Canacomyrica* and most species of *Morella* occur in the tropics but they are found in montane habitats at altitudes where the climate is more moderate, the soil often thin and the cloud layer

provides almost constant moisture. *Canacomyrca* grows on the serpentine soils of southern New Caledonia which are rich in toxic elements, such as iron and nickel, and notoriously poor in essential minerals. Several *Morella* species are also endemic to meandering areas such as those found in Cuba and Malesia. Other species are endemic to the mediterranean regions of South Africa and California, where they are exposed to periods of drought. The requirements of Myricaceae species for abundant water supply cannot, however, be over-emphasised and in these summer-drought regions *Morella* species are mostly restricted to riparian habitats or altitudinal zones where a semi-constant cloud layer provides regular precipitation; two species that may have less stringent requirements for water are *M. cordifolia* and *M. quercifolia*, both South African Cape species. Two notable species are found near sea level; *M. cordifolia* is an important dune stabilising species on coastal sand dunes in the Cape region of South Africa and *M. cerifera* is frequently found in coastal habitats in North America. The latter species is the most wide-ranging *Morella* species, occurring throughout southern North America, Central America and some Caribbean islands. It is possible that this species has a higher tolerance to drought than its congeners, allowing it to populate a wider range of habitats. However, deep root systems are a feature of the family and permit species to tap deep groundwater (Parra-O, 2002).

## 2.10 *Myrica quercifolia*

The genus name is *Myrica* from the family Myricaceae and Order Fagales. Dioecious small spreading shrub up to 60 cm, it has obovate leaves, attenuate below, usually pinnatifid, gland dotted. Flowers in axillary spikes. It has wart-textured fruits which are 3-4mm in diameter. It flowers between July and September, grows mostly in coastal sand limestone flats and slopes. NW, SW, AP, SE, LB (Namaqualand to Eastern Cape).

**Table 2.2:** Comparison between three genera of the Myricaceae family.

	<i>Morella</i>	<i>Myrica</i>	<i>Camptonia</i>	References
Terminal buds	Present	Lacking	Lacking	Wilbur, 1994
Leaves	Thick, usually persistent, toothed or incised.	Thin, deciduous, entire or weakly serrate distally.	Thin, deciduous, roundedly pinnatifid.	Wilbur, 1994
Stipules	Lacking	Lacking	Present	Wilbur, 1994
Aments	Inserted on old wood mainly below the leaves	Inserted at the summit of the branchlets		

### 2.10.1 Biological activities and chemical composition of the species from the genus *Myrica*

Various species from *Myrica* have many ethnobotanical, the fruits are rich in anti-oxidants and thus bear health promoting properties (Zhang *et.al.* 2015). Few properties are discussed below.



### 2.10.2 Anti-cancer properties

A study was conducted in 2005 by Sylvestre, *et.al.* on *M. gale* to assess its potential against growth of human lung carcinoma cell line A-549 and human colon adenocarcinoma DLD-1, cell line. A total of 53 components were identified, the major compounds isolated were: myrcene (23.18–12.14%), limonene (11.20–6.75%),  $\alpha$ -phellandrene (9.90–6.49%) and  $\beta$ -caryophyllene (9.31–10.97%) in the 30 and 60 minutes fractions, respectively, whereas higher caryophyllene oxide content was detected in the 60 min fraction (9.94%) than in the 30 min fraction (3.47%). The 60 min fraction showed higher anticancer activity against both tumor cell lines. The higher cell growth inhibition induced by the 60 minute fraction, as compared to the 30 min fraction, could be due to sesquiterpene enrichment in which anti-cancer properties were detected.

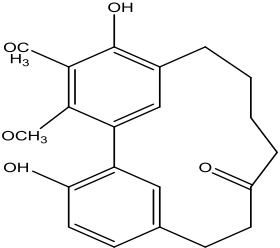
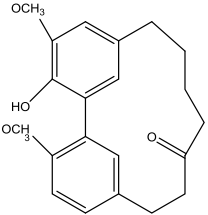

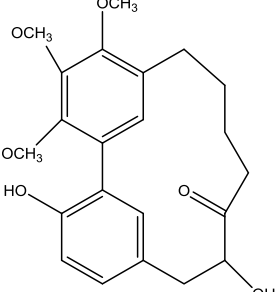
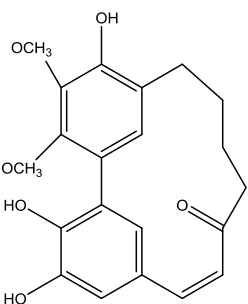
### 2.10.3 Anti-tyrosinase and radical scavenging properties

In 1995, Matsuda *et.al.*, investigated *in vitro* effects of 50% ethanolic of dried leaves and bark of *M. rubra*. The extracts exhibited tyrosinase inhibition, additionally, production of melanin from dopachrome by autooxidation was inhibited. The extracts also demonstrated superoxide dismutase like (SOD) activity.

In another study, ten cyclic diarylheptanoids, three new compounds including; myricanone 5-O-a-L- arabinofuranosyl-(1→6)-b-D-glucopyranoside, myricanone 17-O-b-D-(6'-O-galloyl)-glucopyranoside, and 16-methoxy acerogenin B 9-O-b-D-apiofuranosyl-(1Æ6)-b-D-glucopyranoside, along with two flavonoids, were isolated from the extracts of *M. rubra* bark. On evaluation of compounds 1-12 against the melanogenesis in the B16 melanoma cells, six compounds, 3, 5, 7, 8, 10, and 12, exhibited inhibitory effects with 30-56% reduction of melanin content at 25 mg/ml with very weak toxicity to the cells (82-103% of cell viability at 25 mg/mL). In addition, upon evaluation of compounds 1-12 against the scavenging activities of free radicals [against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical], seven compounds, (Akazawa, *et.al.* 2010)

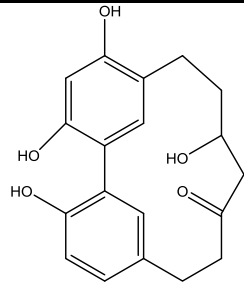


**Table 2.3:** Compounds isolated from the members of the *Myrica* and *Morella* genus.

Compound name	Chemical structure	Name of species (plant part(s))	References
Diarylheptanoids			
Myricanone		<i>Mo. adenophora</i> ; <i>Mo. arborea</i> ; <i>Mo. cerifera</i> ; <i>My. gale</i> ; <i>My. rubra</i>	Ting, <i>et al.</i> , 2014
5-Deoxymyricanone		<i>Mo. adenophora</i>	Ting, <i>et al.</i> , 2014
Myricananin C		<i>Mo. nana</i>	Wang <i>et al.</i> , 2008
Porson <sup>b</sup>		<i>My. Gale</i>	Nagai <i>et al.</i> , 1995
Myricananin D		<i>Mo. adenophora</i> ; <i>Mo. nana</i>	Ting, <i>et al.</i> , 2014; Wang <i>et al.</i> , 2008

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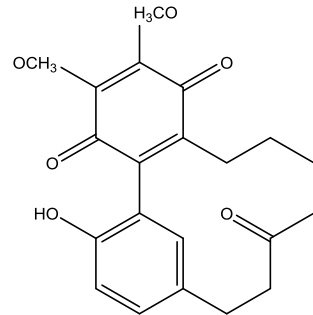
Alnusonol



*Mo. nana*

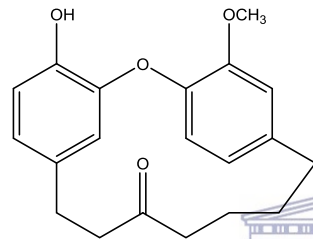
Wang *et al.*, 2008

Actinidione



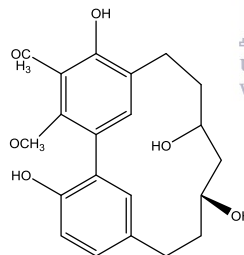
*Mo. adenophora*; *Mo. nana* Ting, *et al.*, 2014; Wang *et al.*, 2008

Galeon



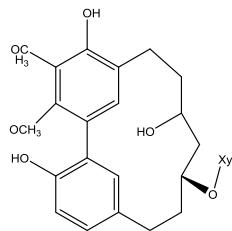
*Mo. adenophora*; *My. gale* Ting, *et al.*, 2014; Morihara, *et al.*, 1997

Myricanol



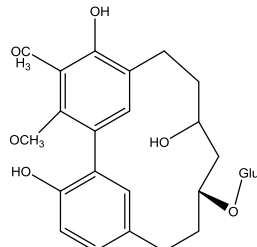
*Mo. adenophora*; *Mo. arborea*; *Mo. Cerifera* Ting, *et al.*, 2014; Tene, *et al.*, 2000; Joshi *et al.*, 1996

Myricanol 11-O- $\beta$ -D-xylopyranoside



*Mo. adenophora*; *Mo. arborea* Ting, *et al.*, 2014; Tene, *et al.*, 2000

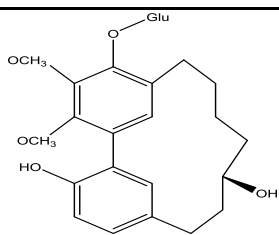
Myricanol 11-O- $\beta$ -D-glucopyranoside



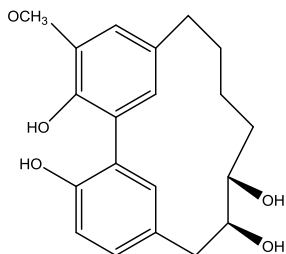
*Mo. adenophora*; *My. Rubra* Ting, *et al.*, 2014

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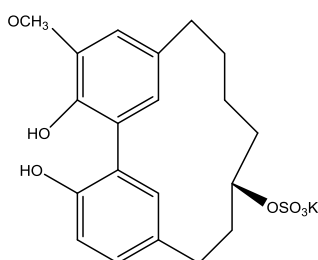
Myricanol 5-O-β-D-glucopyranoside *My. rubra* Akazawa *et al.*, 2010



Myricananin A *Mo. nana* Wang *et al.*, 2008

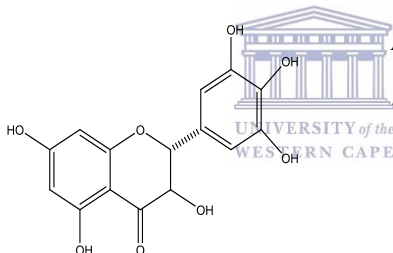


Juglanin B-11(R)-O-sulphate *My. rubra* Kim *et al.*, 2014

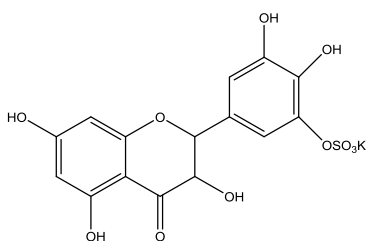


### Flavonoids

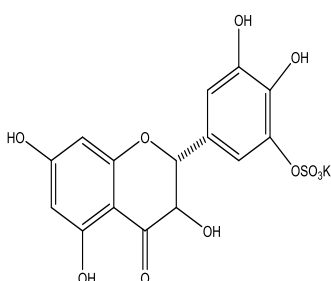
Myricetin *Mo. adenophora; My. rubra; My. esculenta.* Ting, *et al.*, 2014; Kim *et al.*, 2014; Nhiem, *et al.*, 2013



Myricetin-3'-O-sulfate *My. rubra* Kim, *et al.*, 2014

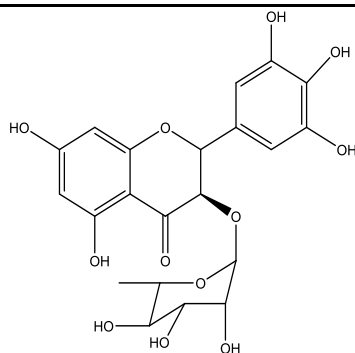


Ampelopsin 3'-O-sulfate *My. rubra* Kim, *et al.*, 2014



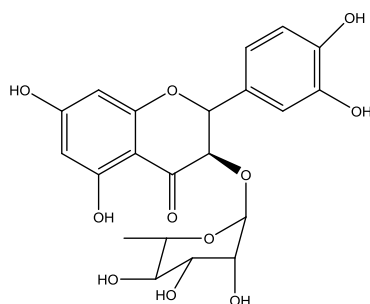
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Myricitrin



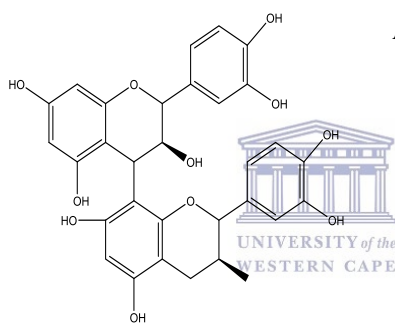
*Mo. adenophora*; *My. rubra*; *Mo. cerifera* Ting, *et al.*, 2014; Kim *et al.*, 2014; Nhiem, *et al.*, 2013  
*My. esculenta*.

Quercitrin



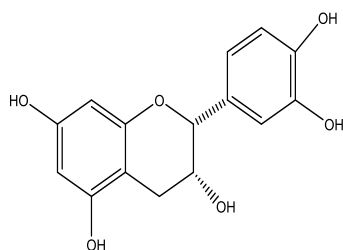
*Mo. adenophora* Ting, *et al.*, 2014

Procyanidin B2



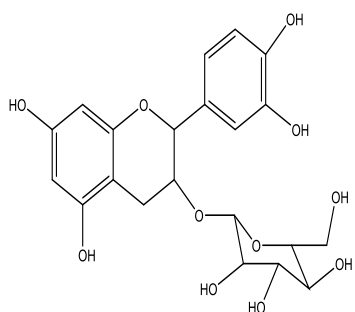
*My. rubra* Zhang, *et al.*, 2013

(-)-Epicatechin



*My. rubra* Zhang, *et al.*, 2013

Cyanidin  
3-O-glucopyranoside



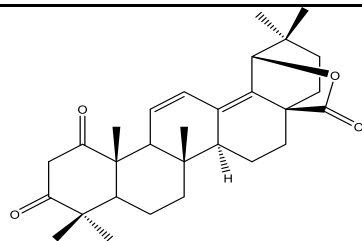
*My. rubra* Sun, *et al.*, 2013

Miscellaneous compounds

Myricalactone

*Mo. adenophora*; *My. gale* Ting, *et al.*, 2014; Morihara, *et al.*, 1997

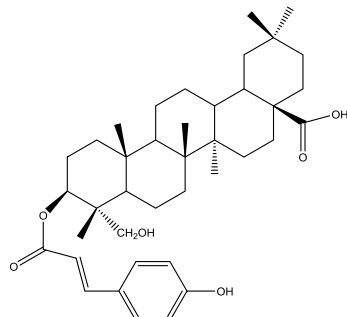
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3 $\beta$ -Trans-p-coumaroyloxy-2 $\alpha$ ,23-dihydroxyolean-12-en-28-oic acid

*Mo. adenophora*

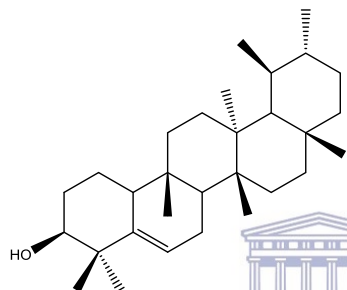
Ting, *et al.*,2014



Rhoiptelenol

*My. rubra*

Tao, *et al.*, 2002

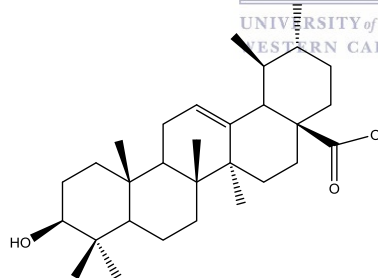


Ursolic acid



*My. rubra*

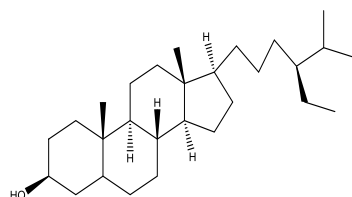
Tao, *et al.*, 2002



$\beta$ -Sitosterol

*Mo. adenophora*; *My. esculenta*

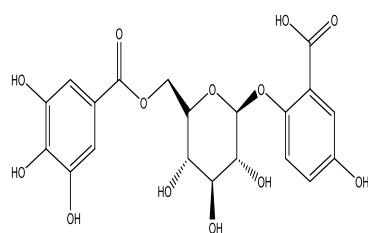
Ting, *et al.*,2014;  
Bamola, *et al.*, 2009



6'-O-galloyl orbicularin

*Mo. adenophora*

Ting, *et al.*,2014

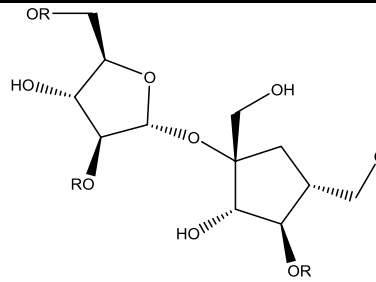


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Myricadenin A

*Mo. adenophora*

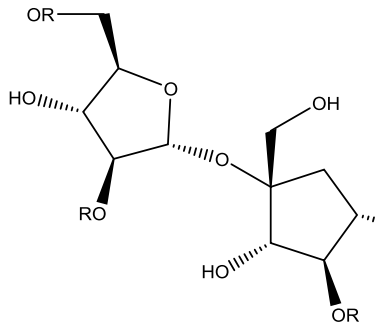
Ting, *et al.*,2014



Myricadenin B

*Mo. adenophora*

Ting, *et al.*,2014



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#### 2.10.4 Nitrogen fixation

Myricaceae have evolved two important adaptations for survival on nutrient poor soils: an actinorhizal association with a bacterium which provides the ability to fix nitrogen; and cluster roots which improve availability of other essential nutrients, especially phosphorus.

The characteristics of the myricaceae family as a whole is to inhabit moist areas, including habitats where water has been standing for prolonged periods, this results in nitrate deprivation and this phenomena has been repeatedly observed (Carlquist, 2002). Previous study on symbiotic nitrogen fixation have emphasised the role of leguminous plants, particularly in agricultural practice. Such symbioses have been described in some 13,000 species of the family Leguminosae. In contrast, nitrogen fixation by symbiotic bacteria in non leguminous plants has been reported in only 13 genera representing 118 species. The importance of non-leguminous symbiosis have been indicated by the studies in the world's nitrogen cycle and emphasised its significance for plants of pioneer community succession. (Tiffney, 1974)



Myricaceae has evolved two important adaptations to thrive on soils with nutrient deficit: an actinorhizal association with a bacterium which has the ability to fix nitrogen and cluster roots which improve availability of other essential nutrients, especially phosphorus.

Myricaceae are one of only nine angiosperm families known to form a symbiotic relationship with Frankia. Frankia are widespread, filamentous, gram-positive, non-endospore forming, mycelial bacteria (Schwintzer, 1979). Traditional classifications based upon morphological characters split actinorhizal plants among four subclasses of dicotyledons



## Chapter 3

### Screening of crude extracts for their inhibitory potential against tyrosinase enzyme

#### 3.1 Introduction

Hyperpigmentation, overproduction of melanin, which may be caused by chronic exposure to the sun is not desirable on the human skin. Melanin production is facilitated by the enzyme tyrosinase, consequently, a number of tyrosinase inhibitors have been developed for such skin condition (Wang, *et.al.*, 2006). The discovery of novel inhibitors of tyrosinase becomes attractive due to their wide potential applications such as improving quality of foods, insect pest control and prevention and treatment of human health problems related to melanin production (García & Furlan, 2015).

The latter is the goal of this project, where a bioactive ingredient from plant species of the Cape Flora that possesses depigmenting properties would be identified. This compound should ideally have the ability to selectively depigment melanoma cells without being toxic to them. A total of 37 plants were screened from different families growing in the Cape region for their anti-tyrosinase potential, These species were extracted and assessed for their ability to possess skin-whitening/lightening properties.

#### Chapter description

- Detection of tyrosinase inhibitory effects of plant extracts using TLC Bioautography
- Compare tyrosinase inhibitory activity using mushroom tyrosinase inhibition method on various plant extracts using ELISA
- Investigate *in vitro* melanin biosynthesis inhibitory effects of active plant extracts spectrophotometrically (ELISA) on melanoma B16-F10 cells
- Evaluate Cytotoxicity effects of active crude extract on melanoma cells.

## **3.2. Materials and methods**

### **3.2.1 Plant collection**

The plant materials were collected randomly in and around the vicinity of the University of the Western Cape (UWC) campus in Cape Town, Western Cape province in the month of August, 2015. Herbariums of each plant species were created. The leaves of the plants which weighed approximately 100 gram were pulverised using an electric blender.



**Table 3.1:** List of plant species used for the present study.

Assigned code	Plant species	Family	Sampling location
BC-/31-30	<i>Acacia karroo</i> (Hayne.)	Fabaceae	CFNR
BC/35-1	<i>Acokanthera oppositifolia</i> (Lam.) Codd	Apocynaceae	UWC
BC/29-14	<i>Antizoma capensis</i> (L.f.) Diels	Menispermaceae	CFNR
BC/35-3	<i>Artemisia afra</i> (Jacq.) Ex Willd	Asteraceae	CFNR
BC/31-23	<i>Aspalathus hispida</i> (Thunb.)	Fabaceae	CFNR
BC/27-8	<i>Chrysanthemoides monilifera</i> (L.) Norlindh	Asteraceae	CFNR
BC/27-9	<i>Cineraria geifolia</i> (L.)	Asteraceae	CFNR
BC/27-5	<i>Euclea racemosa</i> (L.)	Ebenaceae	UWC
BC/29-13	<i>Euphorbia mauritanica</i> (L.) Var	Euphorbiaceae	CFNR
BC/29-19	<i>Ficus bengalensis</i> (L.) 1753	Fabaceae	CFNR
BC/29-22-1	<i>Helichrysum petiolare</i> Hilliard & B.L.Burt	Asteraceae	UWC
BC/31-27	<i>Hyobanche sanguinea</i> (L.)	Orobanchaceae	CFNR
BC/35-12	<i>Hypoxis hemerocallidea</i> Fisch. & C.A. Mey	Hypoxidaceae	Afriplex
BC/37-14	<i>Laurus nobilis</i> (L.)	Lauraceae	UWC
BC/31-26	<i>Leonotis ocymifolia</i> (Burm. F.)	Lamiaceae	UWC
BC/37-17	<i>Maytenus bachmanii</i> (Loes.) Marais	Celastraceae	UWC
BC/29-15-1	<i>Myrica quercifolia</i> (L.)	Myricaceae	UWC
BC/37-16	<i>Myrtus communis</i> (L.)	Myrtaceae	UWC
BC/29-16	<i>Nylandtia spinosa</i> (L.) Dumort	Polygalaceae	CFNR
BC/27-11	<i>Olea europaea</i> (L.)	Oleaceae	UWC
BC/27-4	<i>Otholobium fruticans</i> (L.) C.H.Stirt	Fabaceae	CFNR
BC/27-2	<i>Passerina rigida</i> Wikstr	Thymelaeaceae	CFNR
BC/27-3	<i>Phylica ericoides</i> (L.)	Rhamnaceae	CFNR
BC/37-21	<i>Plectranthus barbatus</i> Andrews	Lamiaceae	UWC
BC/37-20	<i>Plectranthus ecklonii</i> Benth	Lamiaceae	UWC
BC/37-25	<i>Salvia africana-caerulea</i> (L.)	Lamiaceae	CFNR
BC/37-24-1	<i>Salvia africana-lutea</i> (L.)	Lamiaceae	CFNR
BC/31-25	<i>Scirpus antarcticus</i> Willd	Cyperaceae	CFNR
BC/29-20	<i>Searsia laevigata</i> (L.)	Anacardiaceae	UWC
BC/29-12	<i>Searsia lucida</i> (L.) F.A.Barkley	Anarcadiaceae	UWC
BC/27-7	<i>Senecio halimifolius</i> (L.)	Asteraceae	CFNR
BC/31-32	<i>Serruria furcellata</i> R.Br	Proteaceae	CFNR
BC/39-27	<i>Sutherlandia frutescens</i> (L.) R.Br	Fabaceae	Afriplex
BC/39-29-1	<i>Tetragonia riparia</i>	Lamiaceae	KBG
BC/31-24	<i>Trachyandra revoluta</i> (L.) Kunth	Asphodelaceae	CFNR
BC/27-10	<i>Viscum capense</i> (L.f.) Tiegh	Viscaceae	CFNR
BC/29-17	<i>Zygophyllum fulvum</i> (L.)	Zygophyllaceae	CFNR

KBG., Kirstenbosch Botanical Garden, CFN., Cape Flats Nature Reserve, UWC., University of the Western Cape

campus.

### 3.2.2 Materials and reagents

Kojic acid was used a positive control and 5771 U/mg of mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma-Aldrich in Cape Town (South Africa). All organic solvents including methanol (MeOH), ethanol (EtOH), hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and dimethyl sulfoxide (DMSO) and normal-phase silica gel 60 PF254 pre-coated aluminium plates that were used to run thin layer chromatography (TLC) were supplied by Merck from Cape Town (South Africa).

Fetal calf serum (FBS) was purchased from Thermo Scientific , trypsin and EDTA were purchased from Gibco, penicillin/streptomycin and Phosphate saline buffer (PBS) were supplied by Lonza. The Cell Proliferation Kit II 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) were purchased from Roche and 0.4% trypan blue stain was purchased from Invitrogen.



### 3.2.3 Equipment and Instruments

All spectrophotometric readings were taken using the enzyme-linked immunosorbent assay (ELISA) on AccuReader M 965/965+, Metertech Inc. version 1.11 from Taipei (Taiwan). Cells were grown in a CO<sub>2</sub> incubator from Shel lab: they were counted using Countess<sup>TM</sup> automated cell couter and Countess<sup>TM</sup> cell counting chamber. Cells were span down using centrifuge 5417R.

Cells were cultured in Cell culture flasks 25 and 75 cm<sup>2</sup> from SPL Life Science. Both 24 and 96 well plates were supplied by Sigma-Aldrich.

### **3.3 Methods**

#### **3.3.1 Plant extraction**

The aerial part of the plant material was used in this study, all the dried material were pulverised using a blender, thereafter, the plant material (~ 100 g) were placed in a conical flask and methanol was added enough to cover the plant material at room temperature for 24 hours, this was repeated three more times. Filtration of the extract was carried out by using Whatman no 1 filter paper and a vacuum pressure.

Combined extracts were concentrated until dryness using Methanol from the extract was removed by rotary evaporator at 50 °C. Finally, the residues were collected and used for the experiment.

#### **3.3.2 Plant sample preparation**



All crude plant extracts were dissolved in DMSO to a concentration of 20 mg/ml stock, the working solutions were prepared from this DMSO stock into complete DMEM medium to desired concentrations (100, 50, 25, 12.5, 6.25 µg/ml).

#### **3.3.3 Tyrosinase enzyme preparation**

A working solution of the mushroom tyrosinase enzyme was prepared from 5771 U/mg of the stock solution to a concentration of 200 U/ml by diluting the enzyme in a phosphate buffer of pH 6.5. The enzyme was then placed in ice, the activity of the enzyme was checked prior to each assay by adding equal amount of enzyme to substrate, colour change would denote that the enzyme is active.

### 3.3.4 TLC bioautography assay of crude plant extracts

A tyrosinase solution of 250 U/ml was prepared by diluting 1000 U of the enzyme with the phosphate buffer. The plant extracts and the positive control were weighed and dissolved in methanol (1 mg/ml). Then 20  $\mu$ l of each extract was spotted using a glass capillary. Two copies of TLC plates were spotted; after development, plates were removed from the tank, they were left at room temperature to dry, one plate was sprayed with the 2 mM L-tyrosine then incubated at room temperature for 10 minutes then sprayed with the aforementioned tyrosinase solution. The plate was further incubated at room temperature for 30 minutes; the active compound was indicated by clear white spots or inhibition zones and the background of the plate indicated a purplish colour. The other plate was dipped in vanillin sulphuric acid and heated at a  $\sim$ 110°C. The results are shown in figure 3.1. The Retention factor ( $R_f$ ) of interesting spots were calculated using the following equation.



Equation 1: **Retention factor** ( $R_f$ ) =  $\left(\frac{A}{B}\right)$

A is the distance travelled by the sample from the origin, whereas B is the distance travelled by the solvent from the origin to solvent front

### 3.3.5 Spectrophotometric determination of tyrosinase inhibition of crude extracts using ELISA reader.

Tyrosinase assay was performed as described previously by Chen *et al.*, 2015 with slight modifications. All the plant extracts were weighed and dissolved in DMSO to a final concentration of 20 mg/ml, a working solution was then prepared by diluting a stock solution to 1000  $\mu$ g/ml in phosphate buffer with a pH of 6.5. The two concentrations of the extracts and the

positive controls, Kojic acid, were 50 and 200 µg/ml, these concentrations were achieved by adding 70 µl of each plant extract together with 30 µl of the tyrosinase enzyme (250 U/ml). The well containing the negative control, 70 µl of the buffer was added instead of the plant extract. Then 110 µl of 2mM L-tyrosine was added to each, the plate was incubated at room temperature for 30 minutes. The wells of the plate were read at an optical density of 490 nm with an ELISA well reader. The tyrosinase inhibition percentage was calculated using the formula below.

$$\text{Equation 2} \therefore \text{Tyrosinase inhibition (\%)} = \left( \frac{[(A-B) - (C-D)]}{(A-B)} \right) \times 100$$

where A is the absorbance of the control with the enzyme, B is the absorbance of the control without the enzyme, C is the absorbance of the test sample with the enzyme and D is the absorbance of the test sample without the enzyme. The results are tabulated in table 3.2.



### **3.4 Investigation of melanin inhibition and cytotoxicity in melanoma B16-F10 cell culture by crude plant extracts.**

#### **3.4.1 Preparation of test samples**

Samples were prepared as mentioned in section 3.3.2

#### **3.4.2 Preparation of cells**

Melanoma B16-F10 cell lines were grown in a 75 ml T-flask at a density of  $1 \times 10^6$  cells/flask. Cells were cultured at a humidified atmosphere of 5% CO<sub>2</sub> at 37°C Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin. After 24 hours of cultivation, the T-flask and the flow hood was sterilised by spraying it with ethanol, the medium was removed and the cells were rinse two

times with the phosphate buffer saline (PBS ) and cells were sub-cultured every 2 days to maintain logarithmic growth.

### 3.4.3 Cell count

A 1:10 dilution of the cell suspension in trypan blue solution (e.g. 10 µl cells in 90 µl trypan blue) in an eppendorf tube was prepared and well mixed. Ten microlitres of this dilution was transferred to two chambers of the hemacytometer. Cells were counted as per manufacturer's instructions using Countess™ automated cell counter.

### 3.4.4 Cell viability assay

Cytotoxicity was determined by a modification of the method of Uchida, *et al.*, 2014. Melanoma cells were seeded ( $1 \times 10^5$ ) in a 96 well plate, thereafter, they were incubated for 24 hours for adherence, cells were exposed to different concentrations of plant test samples (preparation described in 3.3.2) and the volume was aspirated; the attached cells were incubated with 100 µl MTT (0.3 mg/ml for 2 hours). A reference plate was prepared by adding 100 µl of the medium, 100 µl of samples and 50 µl of MTT and was also incubated for 2 hours. The absorbance at 570 nm was then measured together with the reference plate which was read at a wavelength of 630 nm using a micro-plate reader. The viability of cells were calculated in percentage using the control as a reference (medium with DMSO). Cell viability was calculated with the following equation:

$$\text{Equation 3} \therefore \text{Cell viability (\%)} = \left( \frac{OD \text{ of A}}{OD \text{ of B}} \right) \times 100$$

where A denotes the OD 570 absorbance of treated cells, and B represents the OD 570 absorbance of control. The dose-dependent inhibition experiments were performed in triplicates to determine the IC<sub>50</sub> of the test samples (See table 3.5 for results).

### 3.4.5 Melanin inhibition assay

#### 3.4.5.1 Preparation of plates

This melanin inhibition assay was previously described by Curto, *et al.*, 1999 with slight modification. After cells were (cell preparation described in 3.3.2) confluent, plates were removed from the incubator. The medium was aspirated and cells were rinsed twice with PBS, they were then trypsinised and centrifuged at 970 rpm. Cells were suspended in 2ml DMEM medium and the viability was determined by staining with trypan blue and were counted using an automated cell counter.



The medium with new DMEM medium containing test samples of various concentrations was replaced. After 72 hours of incubation, the adherent cells were washed with phosphate buffered saline (PBS) and detached from the T-flask by trypsinisation. The cells were collected in a test tube and washed twice with PBS.

Melanoma B16-F10 cells with density of  $1 \times 10^5$  cells/flask were incubated in 60 mm dishes with various concentrations of the test compounds. After treatment, the cells were washed twice with phosphate buffered saline (PBS)

The number of cells were determined by means of staining with trypan blue and were then counted using a cell counter. In order to determine melanin inhibition,  $1 \times 10^6$  B16 -F10 melanoma cells were pelleted in an eppendorf tube and were resuspended in 200  $\mu$ l of 1 M NaOH and were then solubilised on a heating block at 100°C for 30 minutes. Solubilised cells were then centrifuged at 16 000 rpm for 20 minutes at room temperature. After centrifugation,

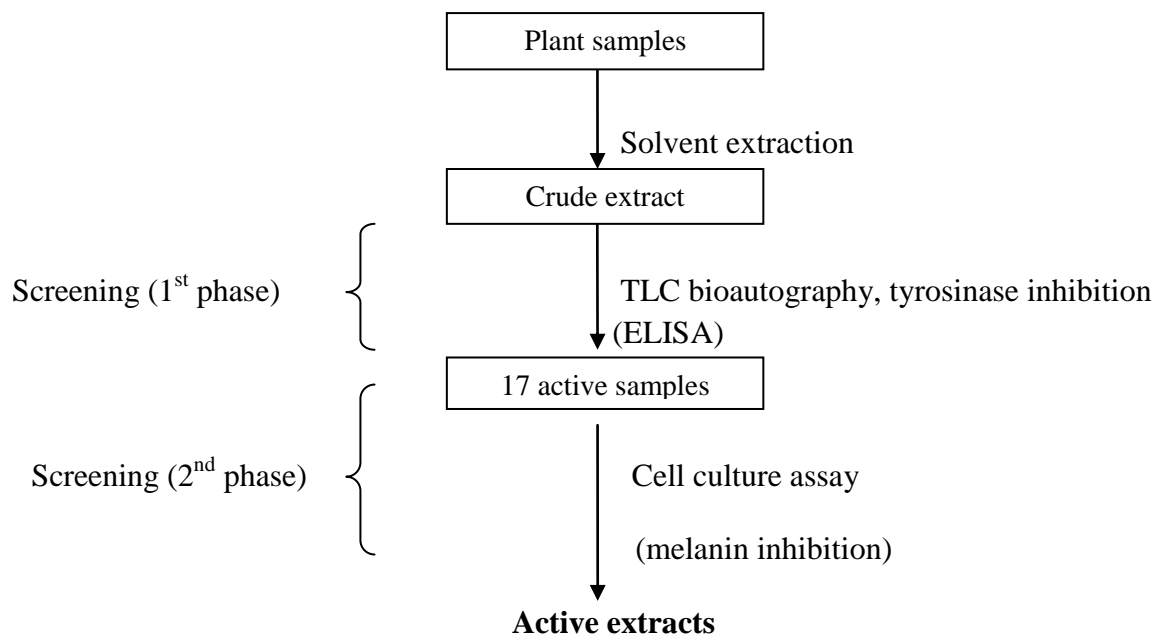
the supernatant was collected and 200 µl was aliquoted in 96 well plates and the melanin was determined by enzyme micro-plate readings at an absorbance of 400 nm on a spectrophotometer. The melanin inhibition was calculated and corrected for the concentrations of proteins, using control cells as 100 %.

Melanoma B16-F10 cells with density of  $1 \times 10^5$  cells/flask were incubated in 60 mm dishes with various concentrations of the test compounds. After treatment, the cells were washed twice with phosphate buffered saline (PBS), and lysed in 200 µl of 1 N NaOH for 1 hour at 100°C.

$$\text{Equation 4} \therefore \text{Melanin inhibition \%} = \left( \frac{A - B}{A} \right) \times 100$$

A is the total number of cells who were untreated, whereas B denotes the cells that were treated with test sample or positive control.





<i>E. racemosa</i>	<i>T. riparia</i>
<i>M. quercifolia</i>	<i>C. geifolia</i>
<i>A. karroo</i>	<i>S. africana-lutea</i>
<i>S. hamilifolius</i>	<i>P. ericoides</i>
<i>H. petiolare</i>	<i>S. africana-caerulea</i>
<i>S. furcellata</i>	<i>S. antarcticus</i>
<i>M. communis</i>	<i>S. lucida</i>
<i>P. rigida</i>	<i>A. afra</i>
<i>P. ecklonii</i>	



Cell viability	Melanin inhibition
----------------	--------------------

50	<i>C. geifolia</i>	36.9
>100	<i>S. antarcticus</i>	20.3
>100	<i>S. furcellata</i>	7.1
97.2	<i>M. quercifolia</i>	<6.25
>100	<i>P. ericoides</i>	27.7
46.9	<i>T. riparia</i>	43.9
>100	<i>M. communis</i>	>100
>100	<i>A. karroo</i>	>100

**Scheme 3.1:** General schematic representation of screening procedure for 37 crude extracts.

## 3.5 Results and discussion

### 3.5.1 Introduction

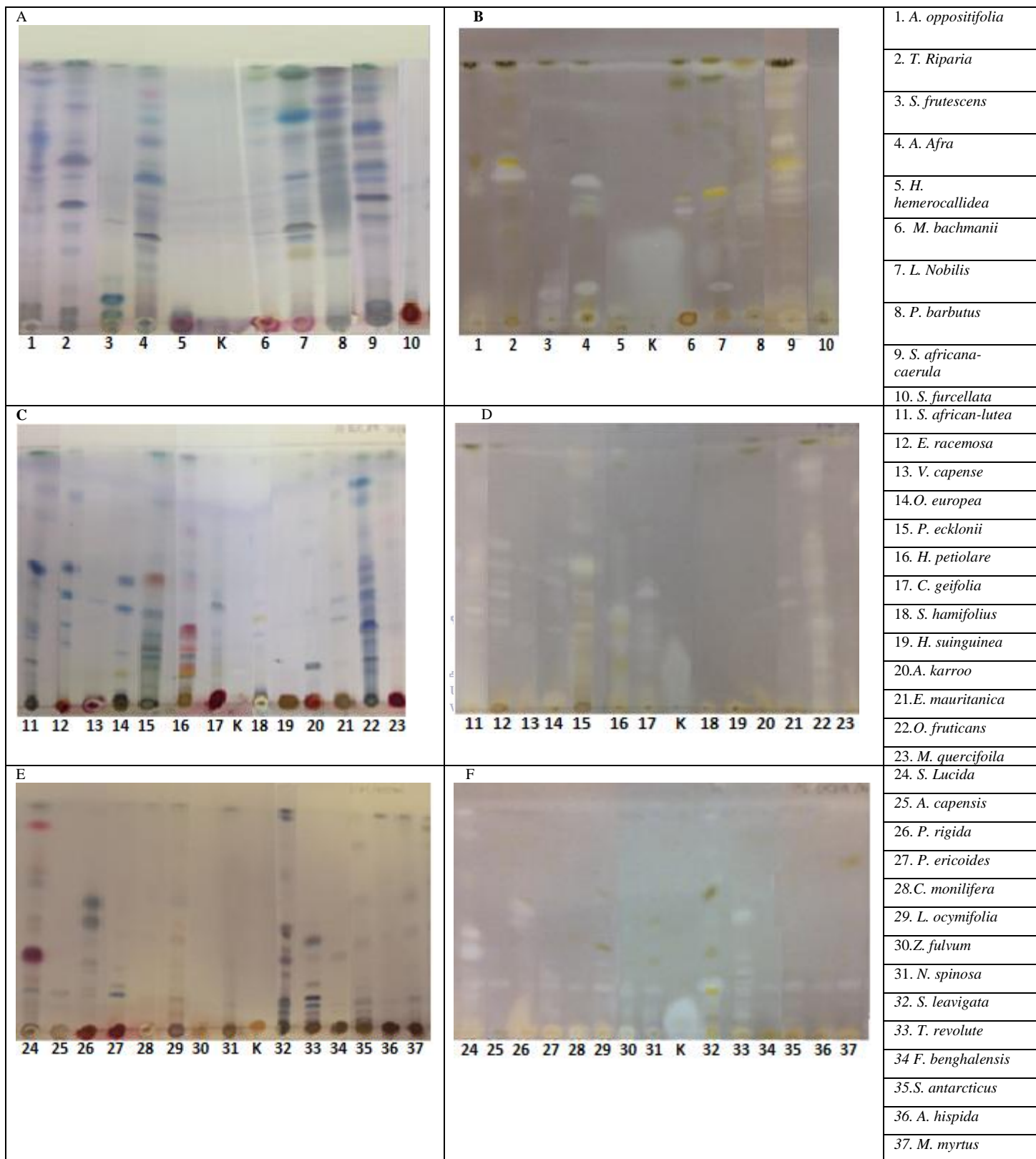
Plants are a rich source of bioactive compounds, many of which are free from unpleasant side effects, plant extracts usually occur as a combination of complex bioactive compounds or phytochemicals whose activity as a crude extract might not be sufficiently potent, thus screening and characterisation of bioactive molecules hold industrial importance (Sasidharan, *et al.*, 2011). Safe and effective tyrosinase inhibitors have become important for their potential applications in improving the quality of food, depigmentation, and preventing other melanin-related health problems in human beings, in addition to cosmetic applications (Quispe, *et al.*, 2017). Thirty nine species to be studied were selected which were collected randomly from the CFR and supported by the fact that there have been no previous studies on their effect on tyrosinase activity. Table 3.1 provides the scientific name, the family name, as well as sampling location of each plant. These 39 species belong to 22 botanical different families. Lamiaceae was the family with the largest species, followed by the Fabaceae, Asteraceae and other families.

For the extraction of these plants, only aerial parts were used in this study, the effect on tyrosinase inhibition of all crude methanolic extracts were investigated. TLC bioautography assay was employed, extracts were spotted at a concentration of 1 mg/ml (results are shown in figure 3.1 in section 3.5.2) and using an ELISA reader, spectrophotometrically at 200 and 50 µg/ml.

### **3.5.2 Screening of tyrosinase activity of methanolic extracts using TLC bioautography.**

The thin layer chromatographic (TLC) plates were developed in a pre-saturated glass tank using MeOH/DCM (1:9), the developed TLC plates were viewed at  $\lambda 254/\lambda 366$  nm UV lamp, they were then sprayed with 250 U/ml of tyrosinase and 2 mM tyrosine (figure 3.1). The white spots, also referred to as inhibition zones on the plates in figure 3.1 (on plates A, C and E) indicate the presence of active compounds in the extract. The white spots appear against a purplish-brownish background and this is a result of formation of coloured quinones from L-tyrosinase.





**Figure 3.1:** TLC bioautography detection of tyrosinase inhibitors. From left to right are plant samples 1 to 39, k is kojic acid which was used as reference standard. Elution was carried out with MeOH/DCM (1:9); (A,C and E) vanillin-sulphuric acid stain used as a detection agent from 1-39; (B, D and F) bioautography for tyrosinase inhibitor detection.

A series of inhibition zones occurred at a number of points in the TLC chromatograms; *S. furcellata* showed spots at  $R_f$  0.39 and 0.62, the inhibition zones were also observed at  $R_f$  0.21 for *M. quercifolia* (L.) whereas *A. karroo* (Hayne.) did not show any spots of inhibition. *T. riparia* showed the clearest zone of inhibition out of selected active extracts at  $R_f$  0.66 followed by *C. geifolia* (L.) at  $R_f$  0.46 and  $R_f$  0.38. The TLC plate indicated by the letter B, the positive control, kojic acid (K) was spotted too much which led to streaking and slightly covered the next sample (Plate B in figure 3.1).

On the plates that were sprayed with vanillin-sulphuric acid (Chromatograms A,C and E), the various constituents within the samples were clearly distinguishable with *T. riparia* with  $R_f$  of 0.29, 0.39, 0.47, 0.55, 0.61, 0.69, 0.75 and 0.88 in TLC plate A, this extract showed clearest and indicating the presence of a number of extra constituents, when compared to the other crude extracts. *A. karroo* (Hayne.) showed the presence of a constituent when detected with vanillin sulphuric acid at  $R_f$  0.38. All the other active extracts showed the bands at the same  $R_f$  as on the bioautography plates.



The TLC bioautography assay was further confirmed by conventional spectrophotometric microplate reader, the results are tabulated below in 3.2 in section 3.5.3.

### **3.5.3 Screening of tyrosinase inhibitory activities of methanol crude extracts using ELISA reader.**

The effect on tyrosinase inhibition of all crude methanol extracts were assayed at a concentration of 200 and 50  $\mu\text{g/ml}$  and the results are reported in Table 3.2. Kojic acid was used as the positive control at the same concentrations as the plant samples. Crude extracts were analysed with the help of a spectrophotometer and the readings were taken at 490 nm. The percentage of inhibition were then determined using equation 2 mentioned under subtitle 3.3.5. and the results are reported in Table 3.2.



**Table 3.2:** Tyrosinase inhibition by crude plant extracts at two different concentrations.

Species	Tyrosinase inhibition (%)	
	200 µg/ml	50 µg/ml
<i>A. karroo</i>	77.08	45.07
<i>A. oppositifolia</i>	22.51	31.26
<i>A. capensis</i>	18.53	11.89
<i>A. afra</i>	58.28	69.18
<i>A. hispida</i>	23.58	13.62
<i>C. monilifera</i>	16.98	14.10
<i>C. geifolia</i>	85.06	74.83
<i>E. racemosa</i>	73.79	76.19
<i>E. mauritanica</i>	66.45	40.47
<i>F. bengalensis</i>	28.91	11.04
<i>H. petiolare</i>	59.22	44.28
<i>H. sanguinea</i>	50.51	41.58
<i>H. hemerocallidea</i>	26.78	43.91
<i>L. nobilis</i>	9.08	10.35
<i>L. ocyimifolia</i>	17.52	16.38
<i>M. bachmanii</i>	43.57	12.67
<i>M. quercifolia</i>	95.49	80.84
<i>M. communis</i>	71.50	72.56
<i>N. spinosa</i>	11.55	16.11
<i>O. europaea</i>	13.81	5.61
<i>O. fruticans</i>	29.11	18.29
<i>P. rigida</i>	88.25	60.07
<i>P. ericoides</i>	62.85	26.21
<i>P. barbatus</i>	42.49	21.53
<i>P. ecklonii</i>	39.40	46.27
<i>S. africana-caerulea</i>	68.71	70.83
<i>S. africana-lutea</i>	53.98	38.22
<i>S. antarcticus</i>	53.98	35.15
<i>S. laevigata</i>	25.69	9.19
<i>S. lucida</i>	86.61	91.24
<i>S. halimifolius</i>	59.25	59.47
<i>S. furcellata</i>	96.29	83.22
<i>S. frutescens</i>	3.38	16.04
<i>T. riparia</i>	7.60	0.88
<i>T. revoluta</i>	-3.59	1.09
<i>V. capense</i>	36.93	30.83
<i>Z. fulvum</i>	33.08	10.74



Of the 37 extracts assessed, 17 crude extracts demonstrated significant effects on tyrosinase inhibition with activities of >50 % at 50 µg/ml and >60 % at 200 µg/ml. The 17 plant extracts

which were the most active extracts according to tyrosinase inhibition assay (ELISA) were selected and further screened for their ability to inhibit melanin biosynthesis at 50 µg/ml and the results are demonstrated in table 3.3 in section 3.5.4.

### 3.5.4 Screening of melanin biosynthesis inhibitory effects of methanol crude extracts at 50 µg/ml.

**Table 3.3** Melanin inhibition activity of the most active crude extracts evaluated on melanoma B16-F10 cells at 50 µg/ml.

Species	Inhibition (%)
<i>A. karroo</i>	83.6±7.1
<i>A. afra</i>	37.7±12.5
<i>C. geifolia</i>	73.9±1.9
<i>E. racemosa</i>	59.9±4.3
<i>C. geifolia</i>	73.9±1.9
<i>E. racemosa</i>	59.9±4.3
<i>C. geifolia</i>	73.9±1.9
<i>E. racemosa</i>	59.9±4.3
<i>C. geifolia</i>	73.9±1.9
<i>E. racemosa</i>	59.9±4.3
<i>S. Africana-caerulea</i>	56.0±8.7
<i>S. Africana-lutea</i>	39.5±9.5
<i>S. antarcticus</i>	80.2±4.4
<i>S. lucida</i>	77.2±3.2
<i>S. hamilifolius</i>	48.8±6.9
<i>S. furcellata</i>	94.3±11.4
<i>T. riparia</i>	77.2±5.4



To investigate the effects of the crude extracts on melanin production, B16 melanoma cells were cultured in the presence of crude extracts and Kojic acid (positive control).

Out of 17 plants were evaluated for their ability to inhibit melanin biosynthesis in melanoma cells. The best inhibitory activities were observed in extracts of *M. quercifolia* (L.) which showed inhibitory activity of  $80 \% \pm 7.9$ , *A. karroo* (Hayne.) with an inhibition of  $83.6 \% \pm 7.1$ , *S. furcellata* R.Br with inhibition percentage of  $94.3 \% \pm 11.4$ , *T. riparia* ( $77.2 \% \pm 5.4$ ), *C. geifolia* (L.) ( $73.9 \% \pm 1.9$ ), *S. antarcticus* (Willd.) ( $80.2 \% \pm 4.4$ ) *P. eroides* (L.) ( $65.1 \pm 6.1$ ).

The active crude extracts were assayed further and tested for melanin inhibition and cytotoxicity at different concentrations and their  $IC_{50}$  were determined respectively. All the results are listed in table 3.4.



### 3.5.5 Screening of melanin biosynthesis inhibitory effects and cytotoxicity of active methanol crude extracts.

**Table 3.4:** IC<sub>50</sub> of melanin inhibition and cytotoxicity of crude extracts that were assessed on melanoma cells at 50 µg/ml.

Species	Inhibition IC <sub>50</sub> (µg/ml)	
	Melanin inhibition	Cytotoxicity
<i>T. riparia</i>	43.88	46.94
<i>S. furcellata</i>	7.13	>100
<i>C. geifolia</i>	36.88	>100
<i>A. karroo</i>	>100	>100
<i>M. quercifolia</i>	< 6.25	97.2
<i>P. eroides</i>	27.67	>100
<i>S. antarcticus</i>	20.25	50.00
<i>M. communis</i>	> 100	>100
<b>Kojic acid (positive control)</b>	>6.25	50.13



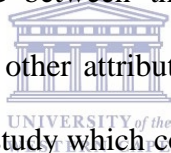
Methanolic extract of *M. quercifolia* (L.) showed potent melanin inhibition activity with an IC<sub>50</sub> of <6.25 µg/ml, subsequently, the cytotoxic effects of the extracts were evaluated which showed an IC<sub>50</sub> of >100 µg/ml. This extracts also showed significant tyrosinase inhibition with the value of 80.8 % at 50 µg/ml. *S. furcellata* R.Br. also showed significant melanin inhibition at an IC<sub>50</sub> of 7.13 µg/ml with no cytotoxic effects (>100 µg/ml) followed by *S. antarcticus* (Willd.) (20.25 µg/ml) with moderate cytotoxicity (50 µg/ml)

The plant extracts of *C. geifolia* (L.) and *P. eroides* (L.) showed moderate activity with IC<sub>50</sub> of 35.88, 27.67 µg/ml respectively followed by *T. riparia* (43.88 µg/ml) and had IC<sub>50</sub> of >100 µg/ml for cytotoxicity.

There is no information in the literature that has been found regarding the plant extracts in table 3.4, but there is a report on *M. communis* (L.). The inhibitory effects of DCM, EtOAc, EtOH and MeOH extracts of the aerial parts and berries were investigated against cholinesterase and tyrosinase. The highest tyrosinase inhibition was exhibited by EtOAc berry extracts (40.53±0.47%) (Tumen, *et al.*, 2012). These results are compare well with the finding of this study, *M. communis* (L.) showed no activity even then it showed significant effects on tyrosinase inhibition of 72.56 %. *M. communis* (L.) exhibited an IC<sub>50</sub> value of >100 µg/ml with no cytotoxic effects (>100µg/ml).

## Conclusion

Findings indicated that the extract of *M. quercifolia* (L.) consistent tyrosinase activity in the assays and a correlation was observed between the aforementioned bioactivities without cytotoxic effects. This plant might have other attributes such as potent antioxidant properties that were not investigated in the present study which could be useful in the treatment of general health promotion. Although the plant extracts of *A. karroo* (Hayne.), *S. furcellata* R.Br, and *S. antarcticus* (Willd.) showed melanin inhibition activity with; these plant materials also exhibited to melanoma cells without cytotoxicity. The plant extracts of *C. geifolia* (L.) and *P. eroides* (L.) also exhibited melanin biosynthesis inhibition activity and had no toxicity to the mouse melanomas; these extracts therefore have potential as anti-tyrosinase agents, and thus should be investigated further.



## Chapter 4

### Extraction, purification, characterisation and anti-tyrosinase activity of compounds from *Myrica quercifolia* (L.).

#### 4.1 Introduction

Natural products and its derivatives have been the basis of treatment of human illnesses since the beginning of time. The idea that effect of drug in human body are mediated by specific interactions of the drug molecule with biological macromolecules led scientist to the conclusion that pure compounds are the factors required for the biological activity of the drug. This led to the beginning of a completely new era in pharmacology, as pure compounds, instead of extracts, became the standard treatments for diseases. Consequently, bioactive compounds, responsible for the effects of crude extract drugs, and their chemical structure are elucidated in modern science (Lahlou, 2013).



However, natural products are diverse in nature and present distinct physicochemical properties thus, extraction of a pure compound from a crude natural product extract is a formidable task (Cannell, 1998) as a crude material consist of a cocktail of compounds. Therefore it is difficult to apply a single separation technique to isolate an individual compound from a crude extract (Gray *et.al.*, 2006).

Bioassay-guided isolation strategies connecting information on the chemical profiles of extracts and fractions with their activity data *in vitro* bioassays performed at micro-scale significantly reduced the time for hit discovery. The extract of *M. quercifolia* exhibited impressive anti-tyrosinase properties and thus it was studied further as well as full chemical studies for the most active compounds.

## Chapter description

- Fingerprint analysis of plant sample with the positive control in full panel of anti-tyrosinase-related assays *in vitro*.
- Elucidate the inhibition of melanin biosynthesis of the active plant extract.
- Bioassay-guided isolation of compounds from *M. quercifolia* (L.) using different techniques including HPLC and elucidate their structures.
- Investigation of inhibition of tyrosinase and melanin biosynthesis assays of isolated compound *in vivo*.



#### 4.2 Plant *Myrica quercifolia* (L.)

*Myrica quercifolia* (Myricaceae) known as *Morella quercifolia* and commonly known as Bayberry, Oak-leaved Myrica, Waxberry Bush or Waxberry ([https://toptropicals.com/catalog/uid/Myrica\\_quercifolia.htm](https://toptropicals.com/catalog/uid/Myrica_quercifolia.htm)) is a dioecious small spreading shrub that grows from 60 cm up to 1 meter, it has obovate leaves, attenuate below, usually pinnatifid, with dotted glands. Flowers are arranged in axillary spikes and has wart-textured fruits which are 3-4 mm in diameter. It flowers between July and September, grows mostly in coastal sand limestone flats and slopes (Wilcox and Cowan, 2016; Manning and Goldblatt 2012). This plant species is native to South Africa and is provincially distributed in Eastern Cape, Northern Cape, North West and Western Cape.



<https://www.ispotnature.org/node/530909>

**Figure 4.1:** Leaves of *M. quercifolia* (L.)

## 4.3 Materials and methods

### 4.3.1 Materials and Equipment

Pre-coated plates of silica gel 60 F254 (Merck, Germany) were used for TLC analysis; visualisation of TLC plates were observed using UV lamp from CAMAG, Switzerland.

Column chromatography was performed using silica gel 60 H (0.040-0.063 mm particle size, Merck, South Africa). Sephadex LH-20 purchased from Sigma-Aldrich, South Africa was used as stationary phase. The purity of isolated compounds was monitored with Agilent Technologies 1200 series, coupled with UV detector, manual injector, quaternary pump (G1311A), vacuum degasser (G1322A), column compartment (G1316A) and reversed phase C18 column SUPELCO (25 x 1.0 cm, 5  $\mu\text{m}$ ).



### 4.3.2 Reagents and Solvents

All organic solvents such as the HPLC graded methanol, ethanol, ethyl acetate, dichloromethane, hexane, deuterated chloroform were supplied by Merck (Darmstadt, Germany).

## 4.4 Methods

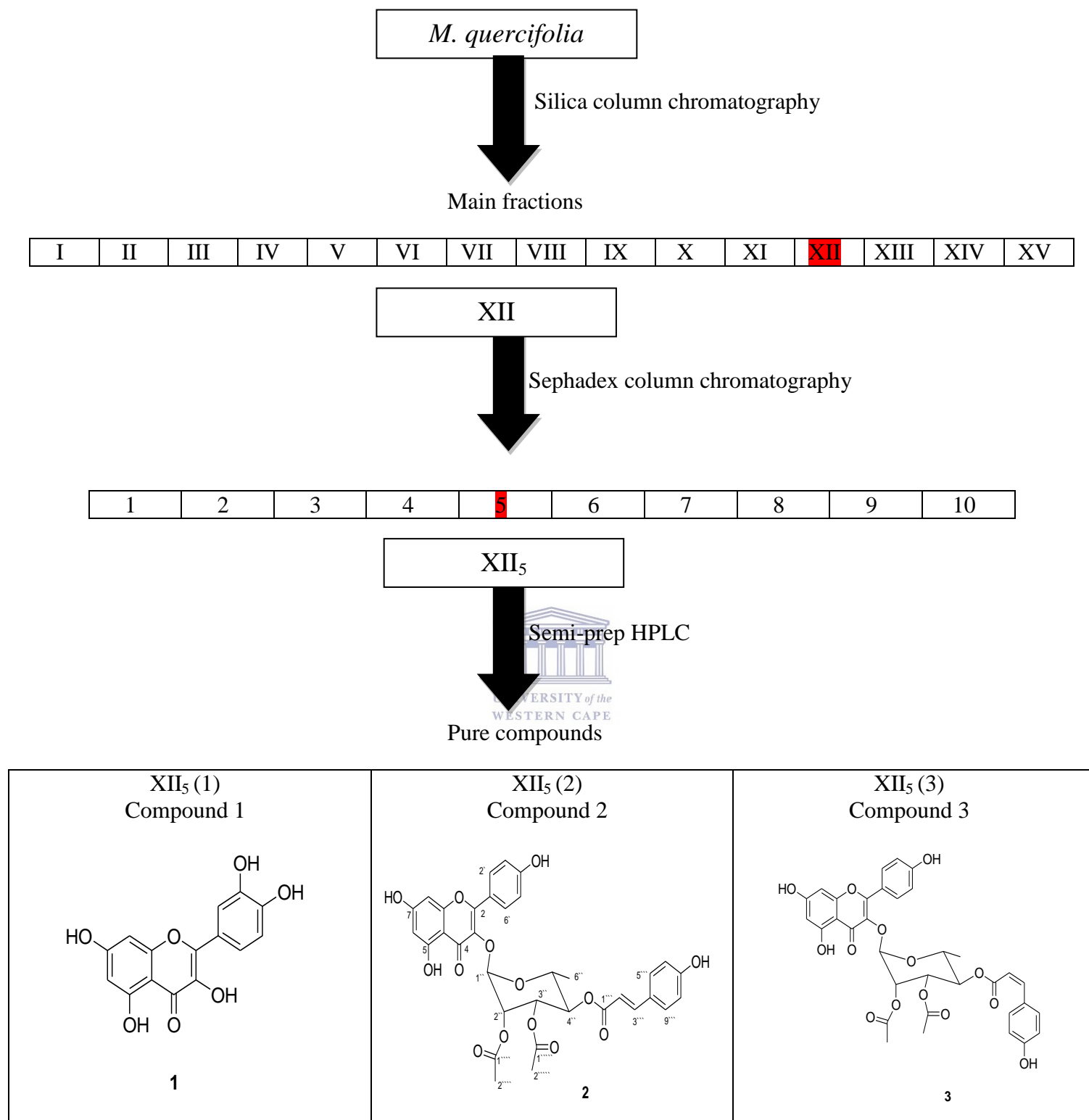
### 4.4.1 Extraction of *M. quercifolia*

The aerial parts of the fresh plant material (~1 kg) were blended and extracted with methanol (4.5 L) at room temperature (25 °C) for 24 hours. The methanol extract was filtered and evaporated to dryness under reduced pressure at 40 °C to yield 15,1 g (1.51 %). The extracts were kept under cold conditions for further use.

### 4.4.2 Thin Layer Chromatography (TLC)

Different plant extracts, fractions and compounds were spotted on TLC to detect their constituents and/or purity (See section 3.3.4.) Visualization of TLC plates was done by observing the bands after development under UV at wavelengths  $\lambda$ 254 and  $\lambda$ 366 nm using UV lamp, followed by phytochemical identification using vanillin/sulphuric acid as a detection agent (See figure 4.3).

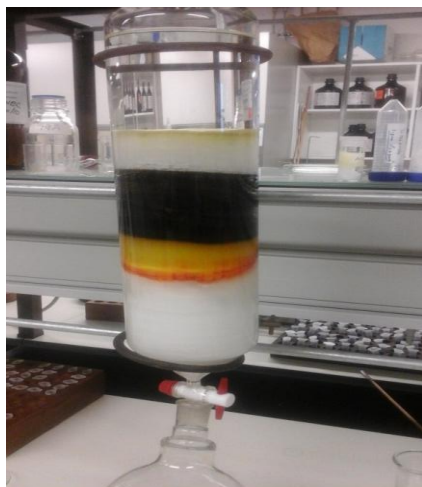




**Scheme 4.1:** A flow diagram of experimental procedure for the isolation of pure compounds from *M. quercifolia*.

#### 4.4.3 Fractionation of crude extract using column chromatography

The methanolic extract of *M. quercifolia* (L.), which showed the highest anti-tyrosinase activity dried and quantified (15.1 g). This extract was applied to a silica column chromatography and eluted with 100% hexane followed by Hex:EtoAc (9:1), 8:2, 7:3, 6:4, 5:5, 4:6, 2:8 and 100% EtOAc (Table 4.2). The elution process was further carried using EtOAc:MeOH (95:5), 70:30, 50:50. Thirty eight fractions (250 ml each) were collected and spotted on a TLC plate to check profile similarity. The fractions were combined according to their TLC profile to yield 21 main fractions (Table 4.1) and concentrated using a rotary evaporator. The fractions were spotted on TLC plates (Figure 4.3). The obtained fractions were coded by roman numbers (I - XXI) and the results are summarized in table 4.3, fraction XVI-XXI were ignored due their high tannin content. Scheme 4.1 shows the schematic representation of the column purification steps for the isolation of compounds from *M. quercifolia*.



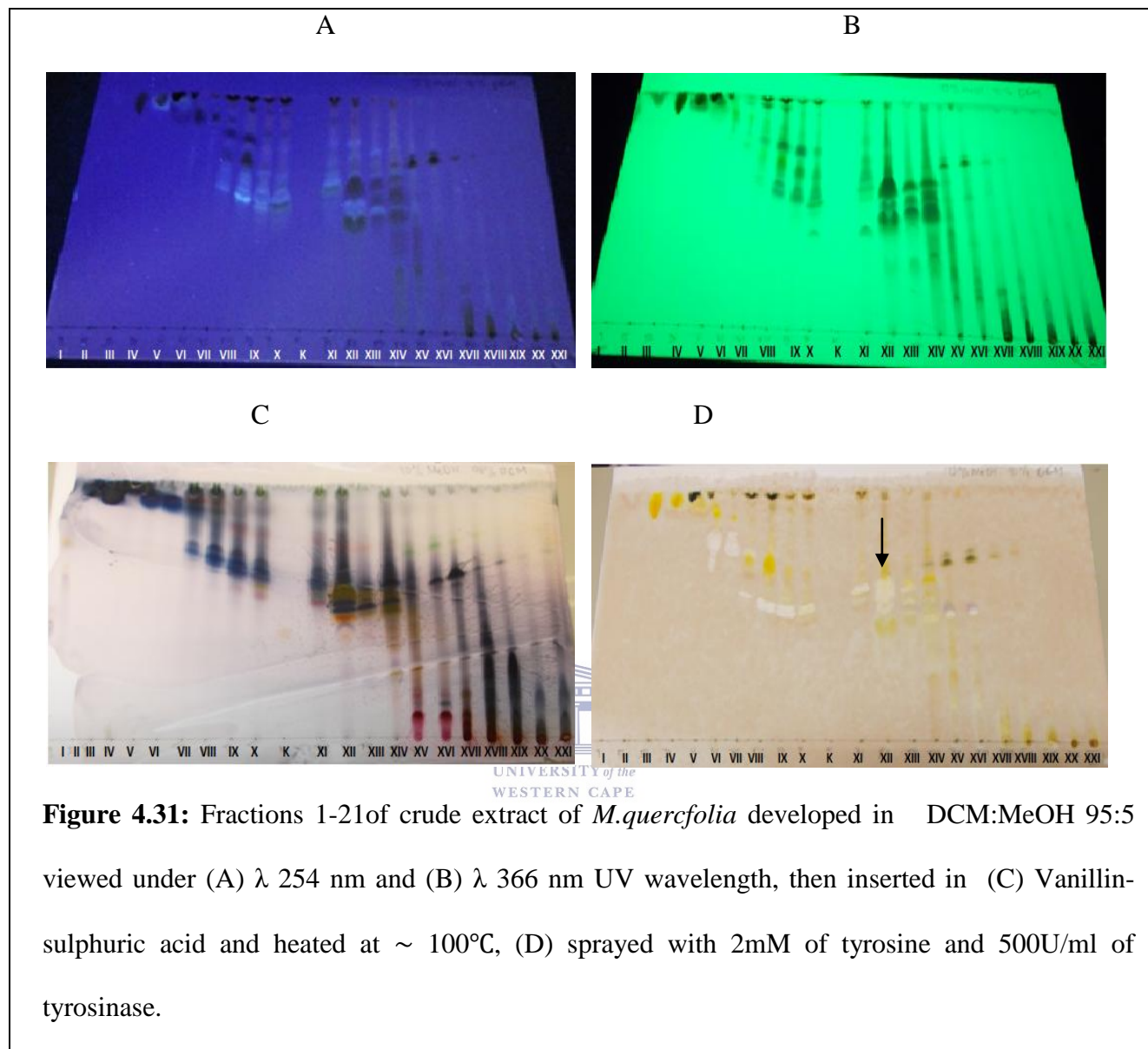
**Figure 4.2** Column chromatography of crude *M. quercifolia* on silica gel.

**Table 4.2** Solvent system used for fractionation of *M. quercifolia* on column chromatography.

Solvent system	Ratio	Volume (L)
Hex:EtoAc	10:0	1
Hex:EtoAc	9:1	1
Hex:EtoAc	8:2	2
Hex:EtoAc	7:3	2
Hex:EtoAc	6:4	2
Hex:EtoAc	5:5	2
Hex:EtoAc	4:6	1
Hex: EtoAc	2:8	1
Hex: EtoAc	0:10	1
EtoAc:MeOH	95:5	2
EtoAc:MeOH	70:30	2
EtoAc:MeOH	50:50	1

**Table 4.3** Fractionation of *M. quercifolia* extract.

Assigned Numeric no	Collected fractions	Weight (mg)
<b>I</b>	1-3	1794.3
<b>II</b>	4	1292.4
<b>III</b>	5	1650
<b>IV</b>	6	–
<b>V</b>	7	939.9
<b>VI-VIII</b>	8-12	2813.1
<b>IX-XI</b>	13-18	952.0
<b>XII</b>	19-20	1221.0
<b>XIII</b>	21	263.8
<b>XIV</b>	22-23	604
<b>XV</b>	24-25	931



**Figure 4.31:** Fractions 1-21 of crude extract of *M. quercifolia* developed in DCM:MeOH 95:5 viewed under (A)  $\lambda$  254 nm and (B)  $\lambda$  366 nm UV wavelength, then inserted in (C) Vanillin-sulphuric acid and heated at  $\sim 100^{\circ}\text{C}$ , (D) sprayed with 2mM of tyrosine and 500U/ml of tyrosinase.

#### 4.4.4 Anti-tyrosinase activity of different fractions

All fractions were subjected to biological evaluation against tyrosinase to determine the potent fraction (s). fractions were tested using TLC bioautography (see section 4.4.2), the results are shown in figure 4.3. Also, all fractions were tested for their inhibitory effects against tyrosinase activity at 100 and 50  $\mu\text{g}/\text{ml}$  (Table 4.1).

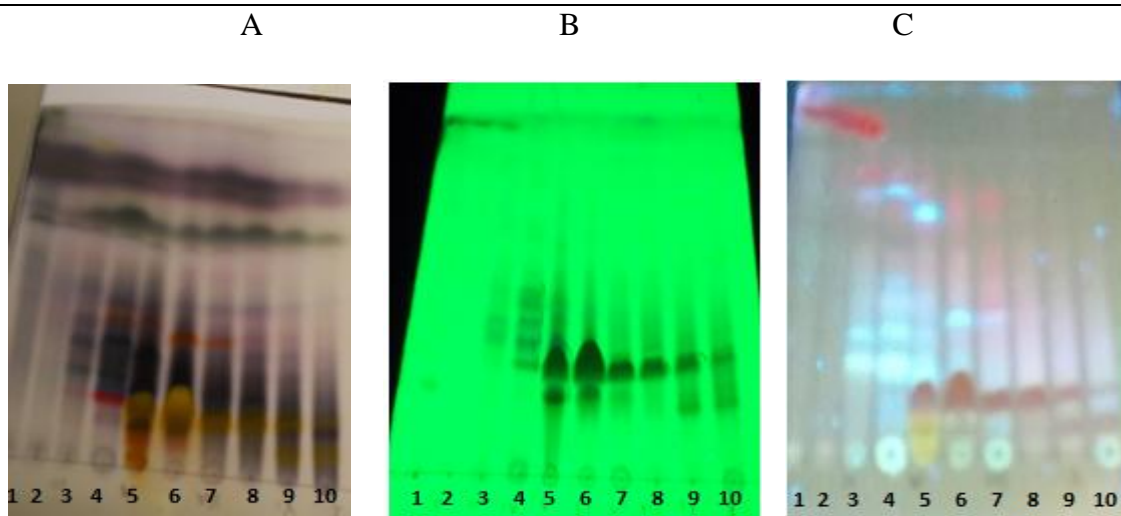
**Table 4.2:** Tyrosinase inhibitory effects of total fractions of *M. quercifolia* that were evaluated at concentrations of 100 and 50 µg/ml.

Fractions	Percentage of inhibition (%)		TLC bioautography Inhibition zone (Rf) (Relative intensity)
	100 µg/ml	50 µg/ml	
II	50.56	40.79	
III	82.40	45.07	
IV	46.67	26.49	
V	76.06	30.00	0.80 (+)
VI	99.03	46.69	0.78 (+)
VII	86.11	30.72	0.6 (+)
VIII	67.28	24.36	0.54 (+)
IX	51.36	30.61	0.50 (++)
X	59.24	28.28	0.49 (+)
XI	54.83	29.47	0.56 (+++)
XII	64.09	42.10	0.62 (+)
XIII	62.11	40.61	
XIV	69.44	69.74	
XV	86.28	74.10	

#### 4.4.5 Fractionation of fraction XII

According to table 4.1, fraction XII among the fractions which showed high inhibition as well as clear inhibition zones on the TLC bioautography, therefore, it was selected for further purification.

Fraction XII<sub>5</sub> of *M. quercifolia* was dissolved in methanol it was then applied to a Sephadex column and eluted with ethanol/distilled water mixture (1:1) followed by EtOH:H<sub>2</sub>O (9.5:0.5). As the column's elution started, compounds started to form bands and moved down the column at different rates. Ten main fractions were collected and concentrated separately using a rotor vapour (see figure 4.5).

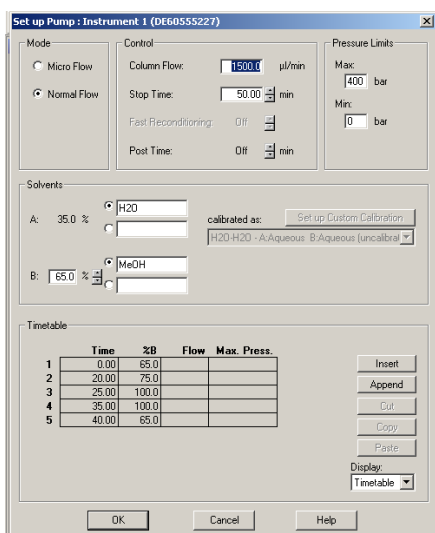


**Figure 4.32:** TLC plate of fractions I to X of fraction XII developed in DCM:MeOH and 95:5, the plate was then dipped in vanillin-sulphuric acid and heated at  $\sim 100\text{ }^{\circ}\text{C}$ , plates were viewed under (B) 254nm and (C) 366 UV wavelength.

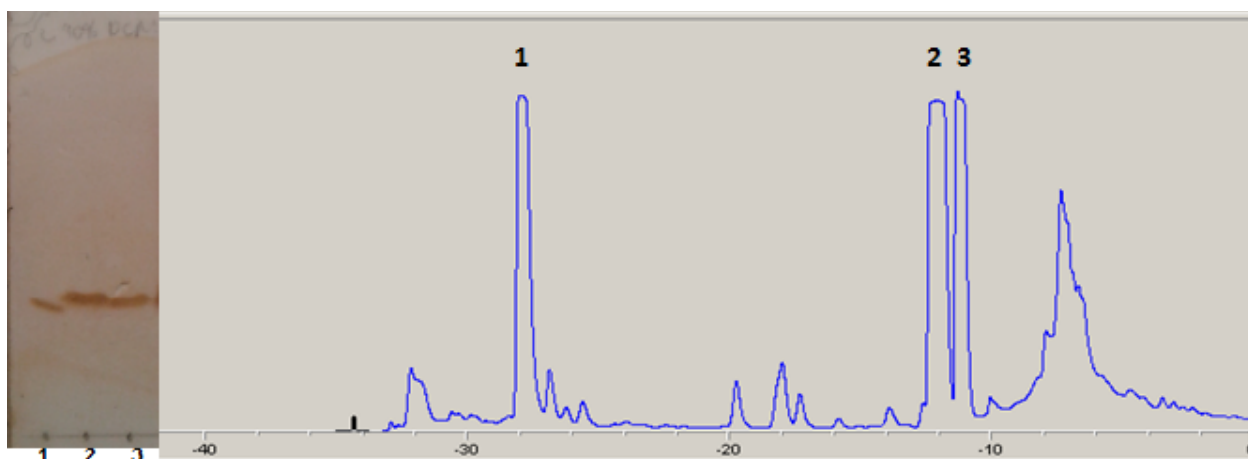


#### **4.4.6 Purification of compounds from fraction XII using Semi-preparative High Pressure Liquid Chromatography (HPLC)**

Fraction XII was selected because it showed interesting chemical profile. The final purification of the compound from fraction XII<sub>5</sub> (330 mg) was carried using semi-prep HPLC. The flow rate was set at 1.5 ml/min and 50  $\mu\text{l}$  was the sample injection volume. All experiments were done at room temperature (20°C). Briefly, the mobile phase was composed of 65% MeOH to 75% in 20 min, then to 100% in 25 min, keep isocratic 100% to 35 min. The detector monitored the eluent at wavelength  $\lambda$  254 nm. The peaks were observed at 14.2 min for compound 1, 30.17 min for compound 2 and 30.8 min for compound 3 (figure 4.61)



**Figure 4.5:** HPLC Conditions



**Figure 4.6:** TLC silica gel of isolated compounds, [DCM:MeOH (9:1)] and HPLC chromatogram of XII<sub>5</sub> (Fig. 4.5).

#### 4.4.7 Structure elucidation using Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded at 20 °C, using deuterated methanol on a Bruker Avance 400 MHz NMR spectrometer (Germany), Chemical shifts of <sup>1</sup>H (δH) and <sup>13</sup>C (δC) in ppm were determined relative to tetramethylsilane (TMS) as internal reference.

## 4.7 Results and discussion

### 4.7.1 Fractionation and purification of *M. quercifolia* constituents.

The preliminary screening of plants extracts indigenous to South Africa using TLC bioautography and ELISA assays indicated that *M. quercifolia* showed the best activity and it was selected for further chromatographic purification to isolate the bioactive compound.

The methanolic extract was subjected to silica gel column chromatography (see table 4.2 for solvent system) and separated into 38 fractions, subsequent to fractionation, all fractions were spotted and combined according to their TLC profile similarity to make 21 main fractions. Fractions 16-21 were not used because they had high tannin content.

A bioactivity-guided process was adopted to fractionate the leaf extract of *M. quercifolia*. The inhibitory activity of each fraction was measured spectrophotometrically (Table 4.1), the most powerful fraction (VI) [99% inhibition (100 µg/ ml)] and second most powerful fraction (VII) [86 % inhibition (100 µg/ml)]. Fraction XII [64 % inhibition (100 µg/ml)] was selected and submitted for further purification because of anti-tyrosinase activity both spectrophotometrically and on the TLC bioautography showing clear inhibition zone at  $R_f$  0.62.

Figure 4.6 shows the HPLC chromatograms of fraction XII<sub>5</sub>. All the major peaks were separated within 40 minutes, with retention factors depending mainly on structural hydrophobicity. After the isolation, three compounds were identified as candidates for tyrosinase inhibition, eluting in different retention times.

Compound **1** appeared at 14.2 min, compound **2** at 30.18 min and compound **3** at 30.8 min.

Compound **1** isolated from fraction XII and identified as quercetin from its NMR data.

$^1\text{H}$  and  $^{13}\text{C}$  NMR data showed typical 6-, and 8-H signals at 6.52/6.25 (1H each/bris) attached to carbons  $\text{C}_6$  and  $\text{C}_8$ .

1,3,4. Trisubstituted rings B was identified from the signals at 7.80 (bis) 7.68 (d) and 6.98 (d) and attached to carbons  $\text{C}_2$ ;  $\text{C}_5$ , and  $\text{C}_6$ . The remaining of carbon and careful analysis of 2D NMR data (HSQC and HMB) confirmed the structure of quercetin.

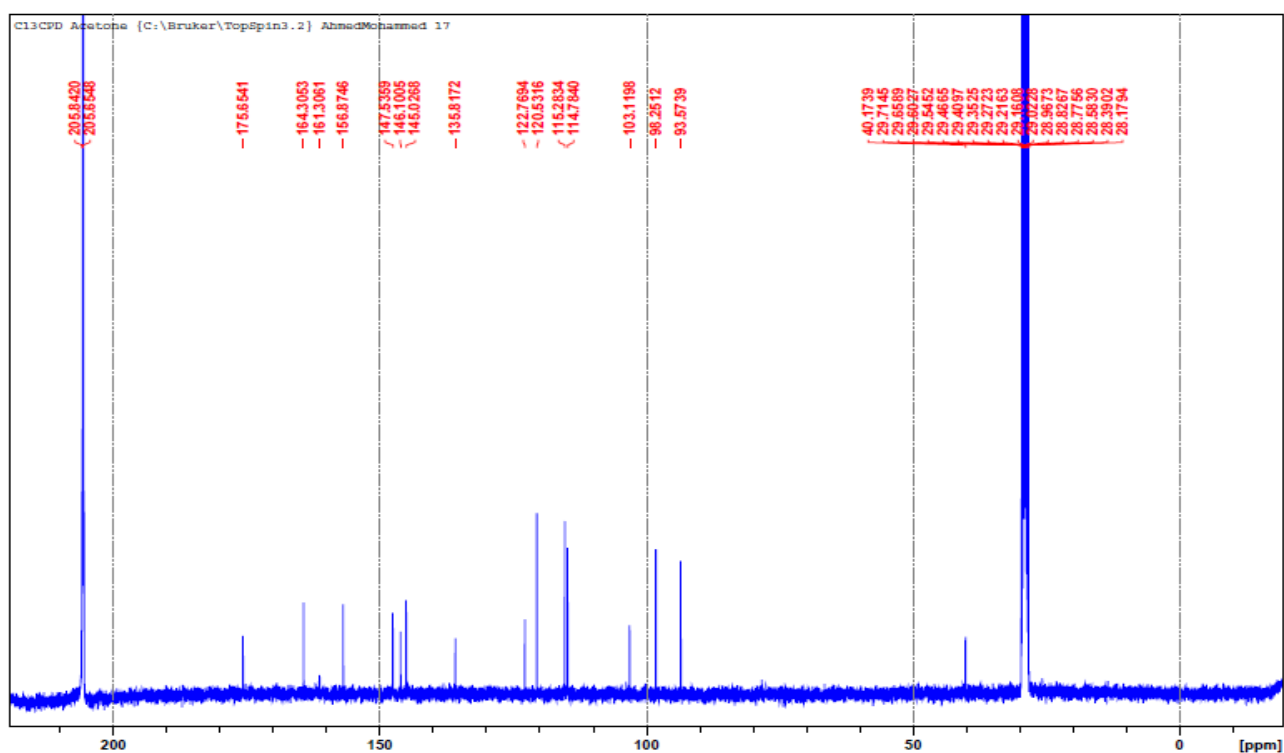


Figure 4.71:  $^{13}\text{C}$ -NMR of compound 1

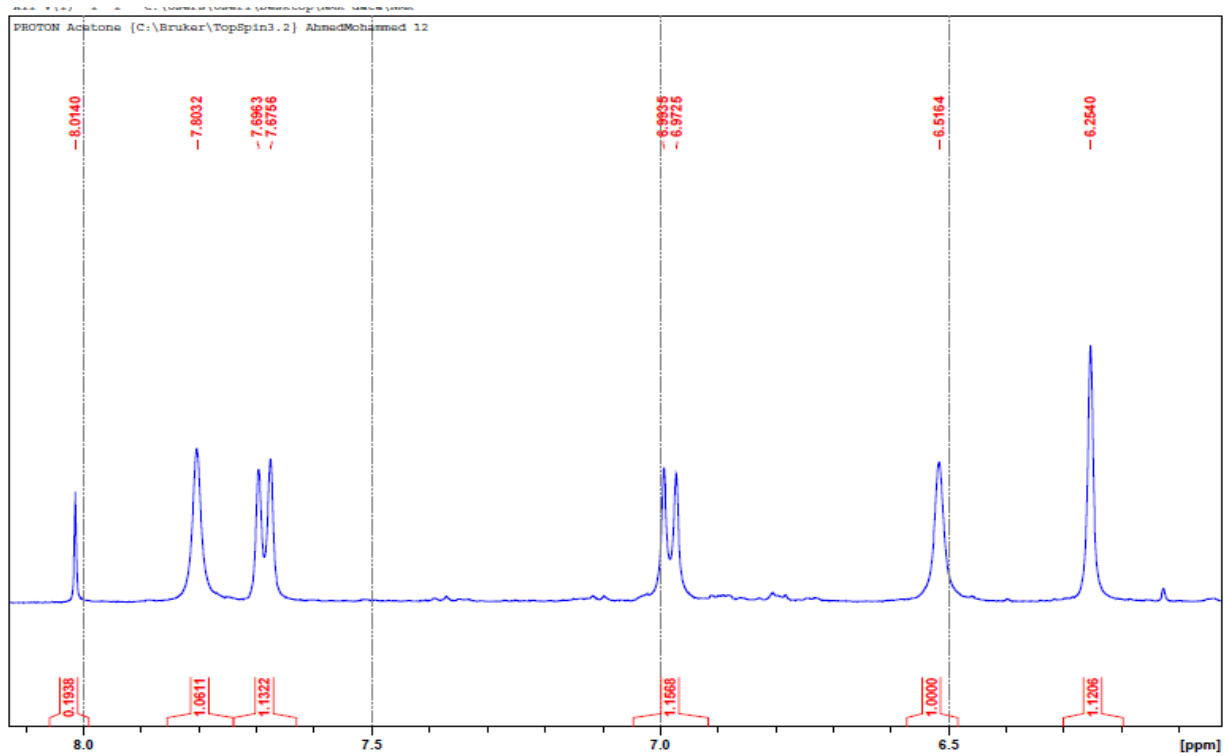


Figure 4.72:  $^1\text{H}$ -NMR spectrum of compound 1

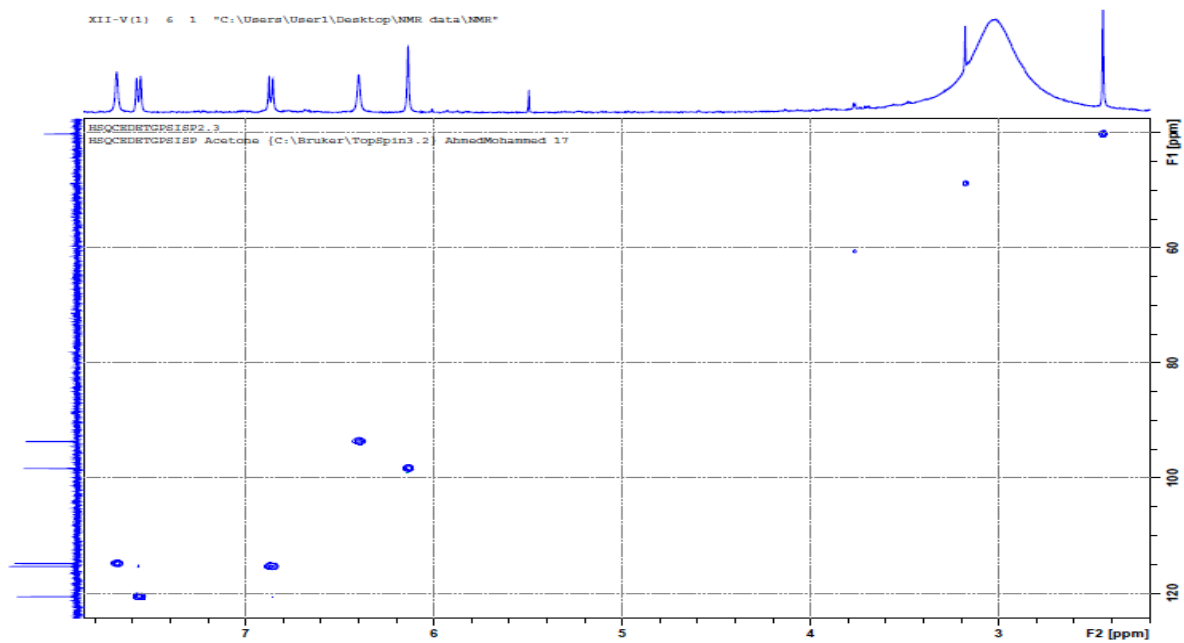


Figure 4.73: HSQC NMR spectrum of compound 1

Compound 2 was isolated as amorphous white-yellowish powder (6 mg). It was isolated using semi-prep HPLC from the polar fraction (XII<sub>5</sub>) and gives purple colour under  $\lambda$  254 nm which indicated unsaturated structure and more specifically, flavonoid. This was confirmed from the UV absorption peak at 265 and 310 nm.

<sup>1</sup>H- and <sup>13</sup>C-NMR showed three different regions, the first region showed a typical kaempferol. The typical 6 and 8-signals protons appeared at 6.28 and 6.48 ppm, and 1,4 disubstituted ring B at 7.89 and 7.11 (2H/each) for 2',6' and 3,5' respectively. <sup>13</sup>CNMR supported the kaempferol structure with 15 carbon signals as shown in table 4.4. Therefore, data indicated the presence of kaempferol nucleus attached to Rhamnose which was acylated with 2-acetate groups (C2 and C3) and p-coumaric acid at C-4. Thus compound 2, has the structure of kaempferol 3-(2,3-diacetoxy-cis-p-coumaroyl)rhamnose. The structure of kaempferol-3-(2,3-diacetoxy-4-p-coumaroyl)rhamnoside and was identified previously from *Myrica gale*.

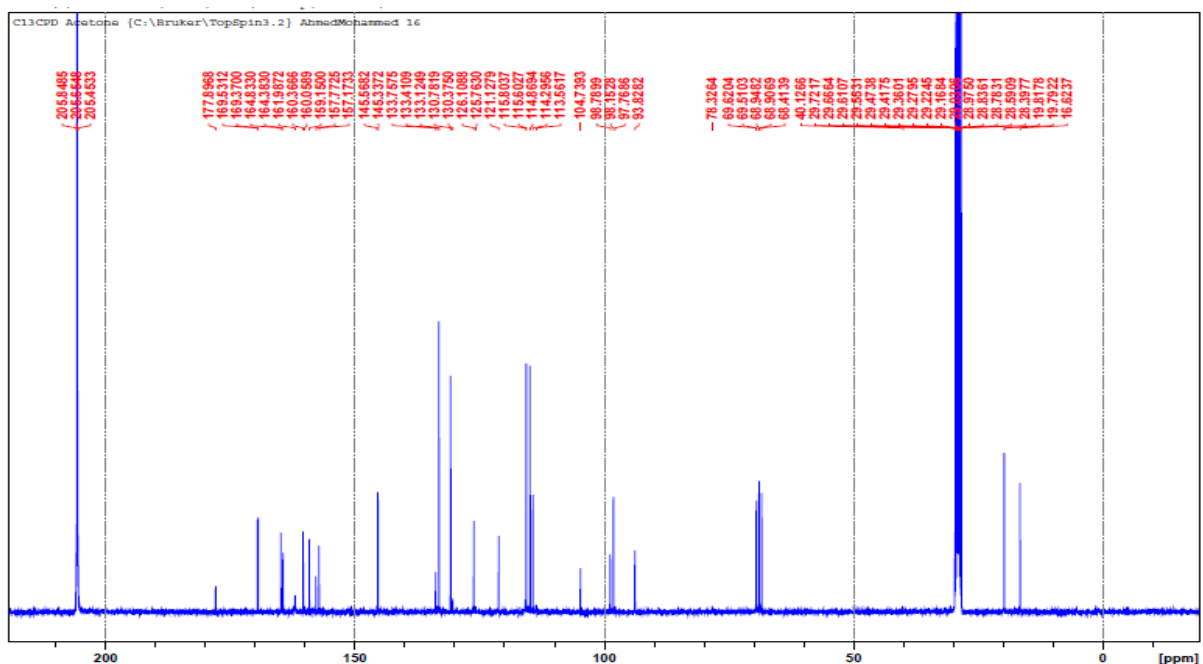


Figure 4.74:  $^{13}\text{C}$ -NMR of compound 2

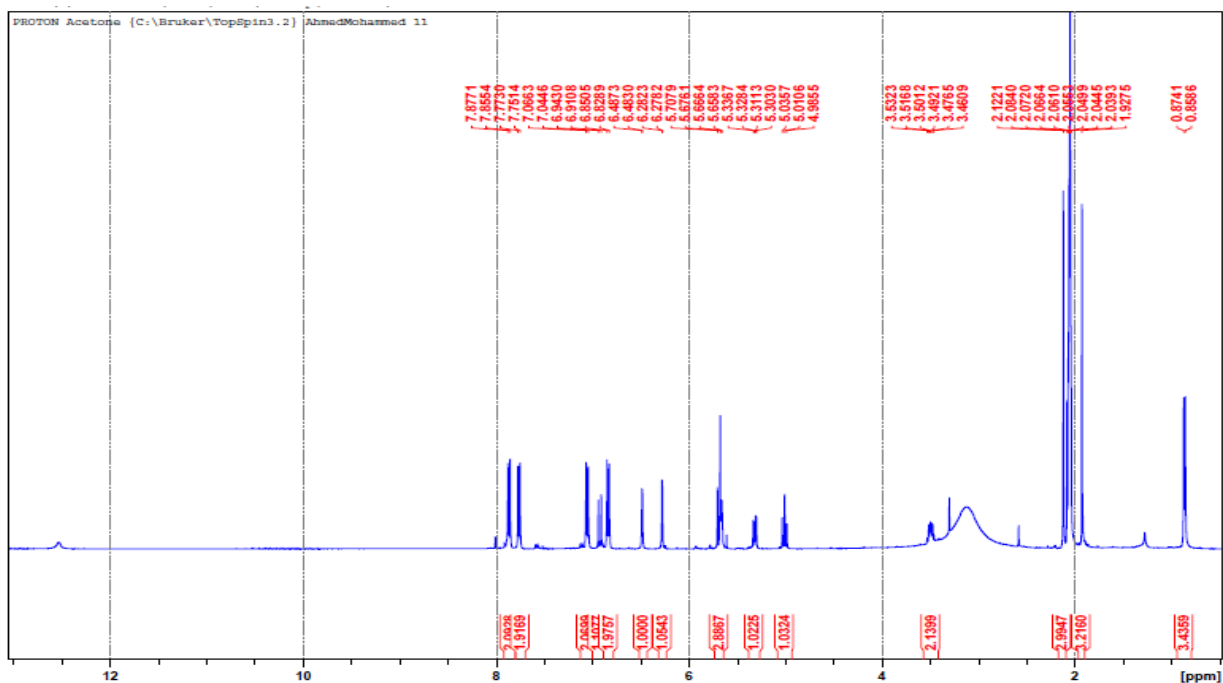


Figure 4.75:  $^1\text{H}$ -NMR spectrum of compound 2

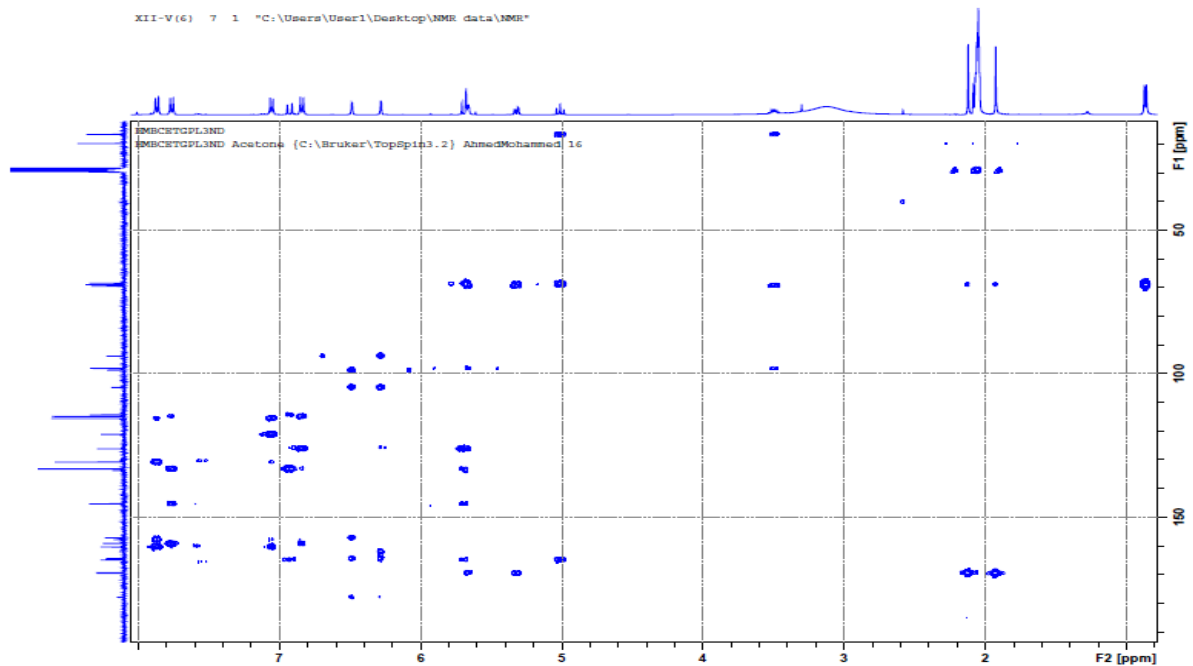
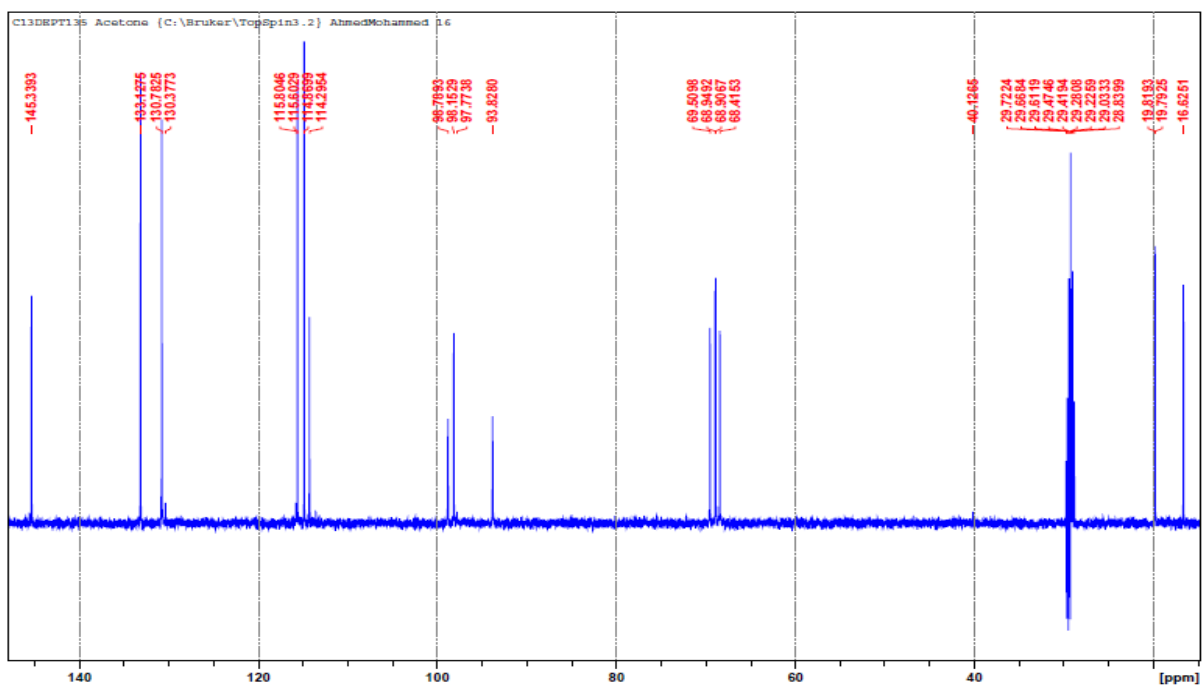


Figure 4.76: HMBC NMR of compound 2



**Figure 4.77:** DEPT NMR spectrum of compound 2

Region 2 showed a glycoside unit acylated at positions 2,3 and 4. The signal of the methyl group at 0.86 (d, 6.2Hz/  $\delta$ C16.6) indicated Rhamnose. The HMBC correlation (Fig..) showed a correlation between the two acetyl groups and C-2 and C-3 respectively, the C-4 was acylated with phenolic acid.

Region 3; showed a typical signal of *p*-coumaric acid (Table 4.4), it showed signals of trans coupled two protons at 7.57 and 6.26 (d, J=16.0 Hz), 1,4-disubstituted benzene ring at 7.58/130.4 and 6.90/115.8 (2H each; d, 8.5 Hz), in addition to carbonyl group at 165.6.

Compound 3 was isolated from the same fraction (XII<sub>5</sub>), and showed a typical flavonoidal skeleton on TLC and UV analysis.

The <sup>1</sup>H- and <sup>13</sup>C-NMR data were very similar to compound 2 (Table 4.4) except for the region of *p*-coumaric acid and especially C-1-C-4, and proton signals of the double bond

which showed clear shift from compound 2.

Careful analysis of the data including 2D (HMBC) indicated that the compound has a typical structure of compound 2 except the double bond geometry which showed different chemical shift and coupling identical with *cis p*-coumaric acid. This was further confirmed on typical and this confirmed by the coupling constant between (H-2 and H-3).

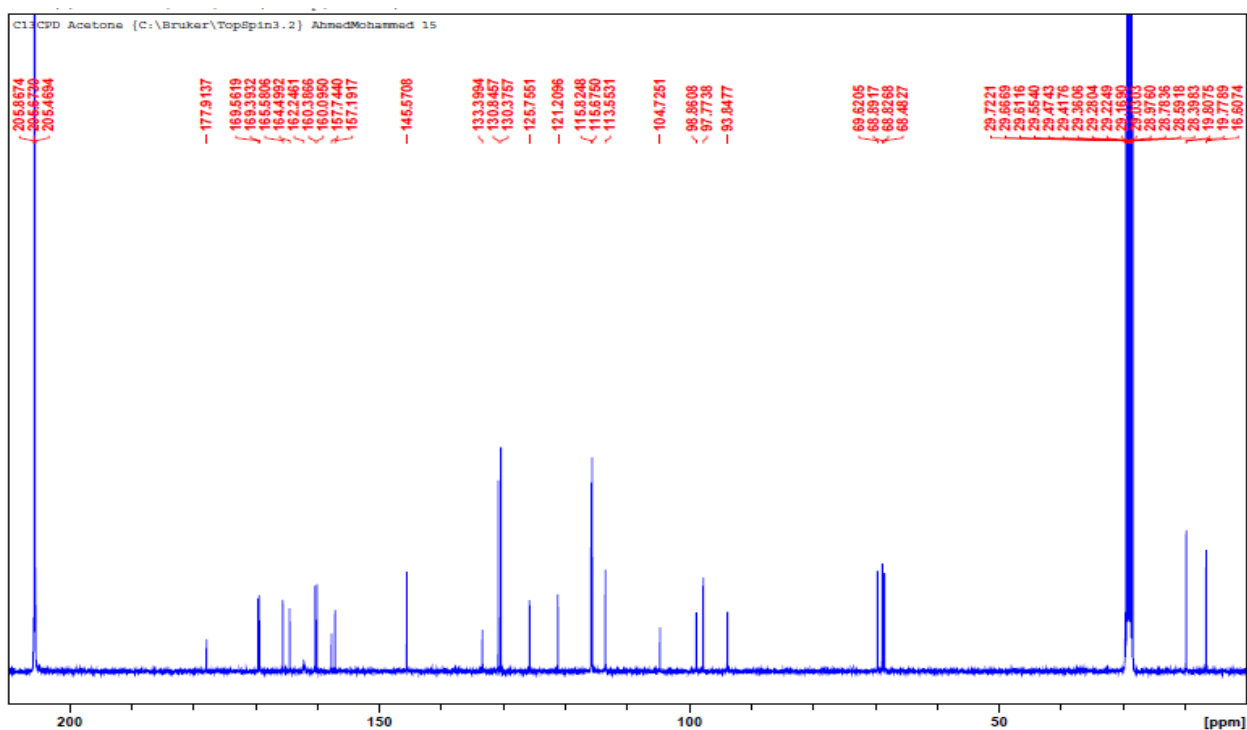


Figure 4.78: <sup>13</sup>C-NMR spectrum of compound 3

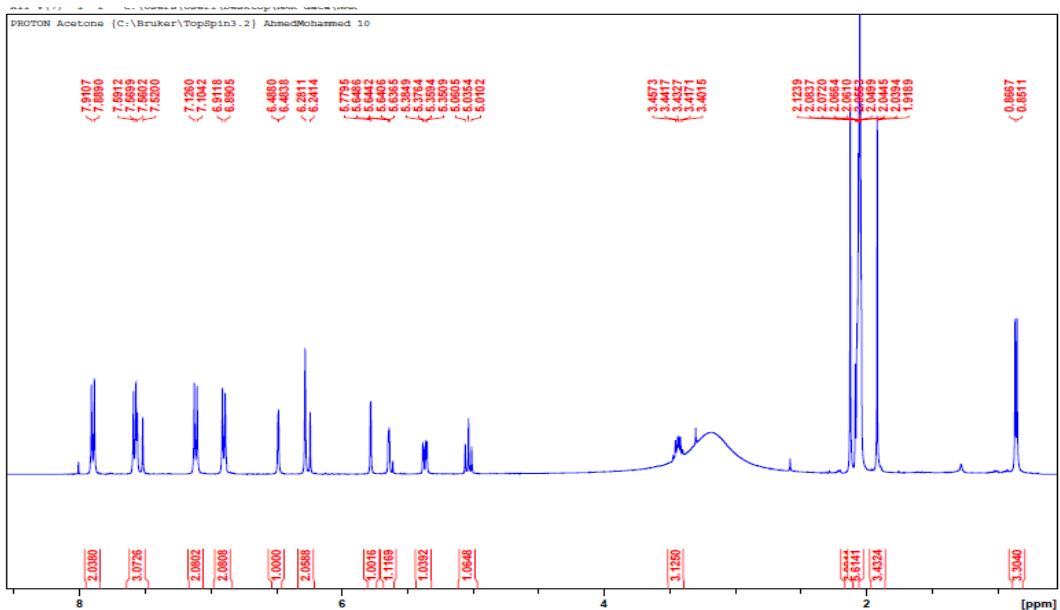


Figure 4.79: <sup>1</sup>H-NMR spectrum of compound 3

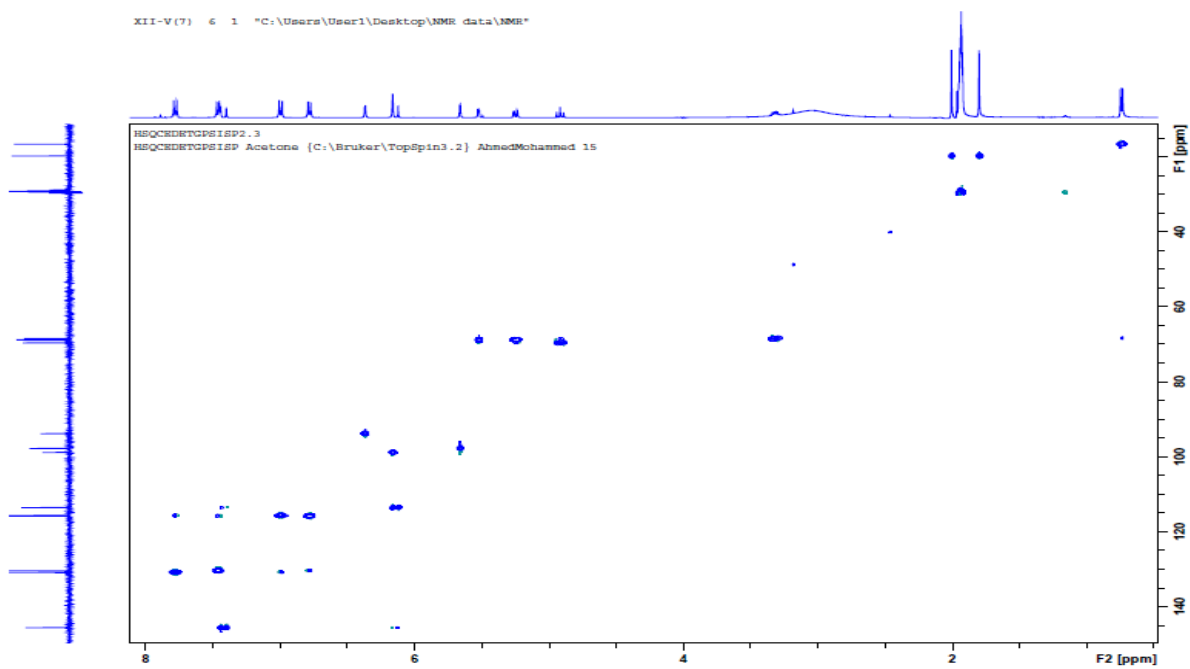


Figure 4.8: HSQC NMR spectrum of compound 3

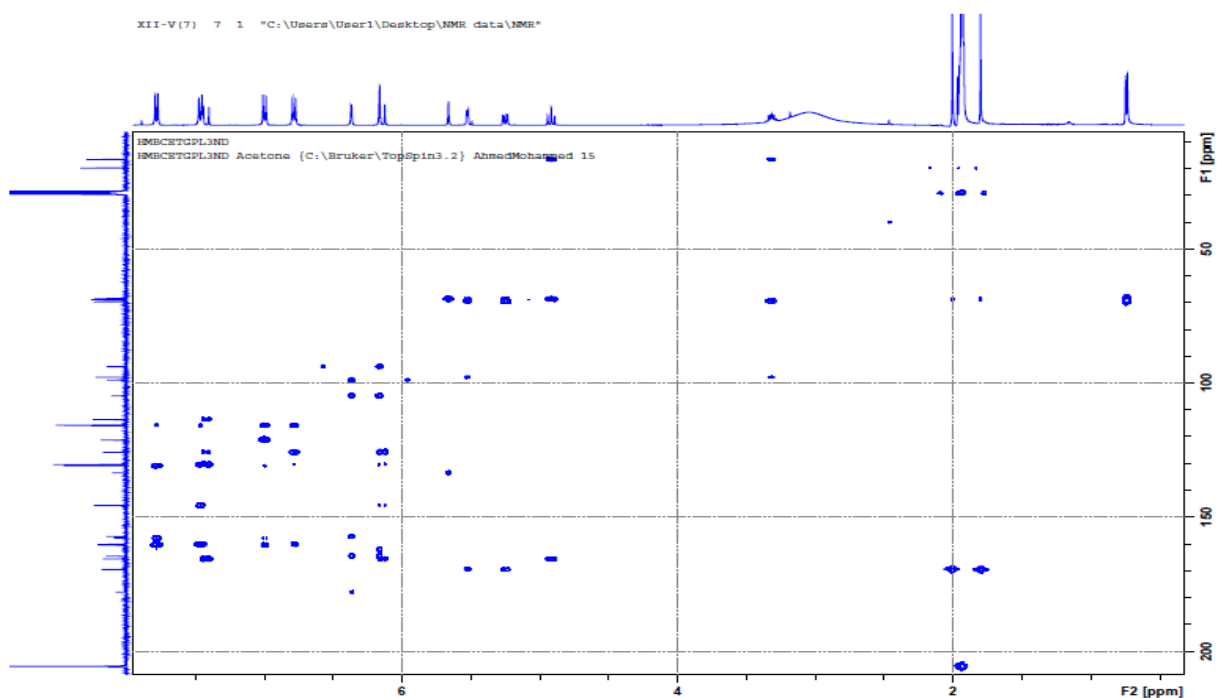


Figure 4.81: HMBC1 NMR spectrum of compound 3

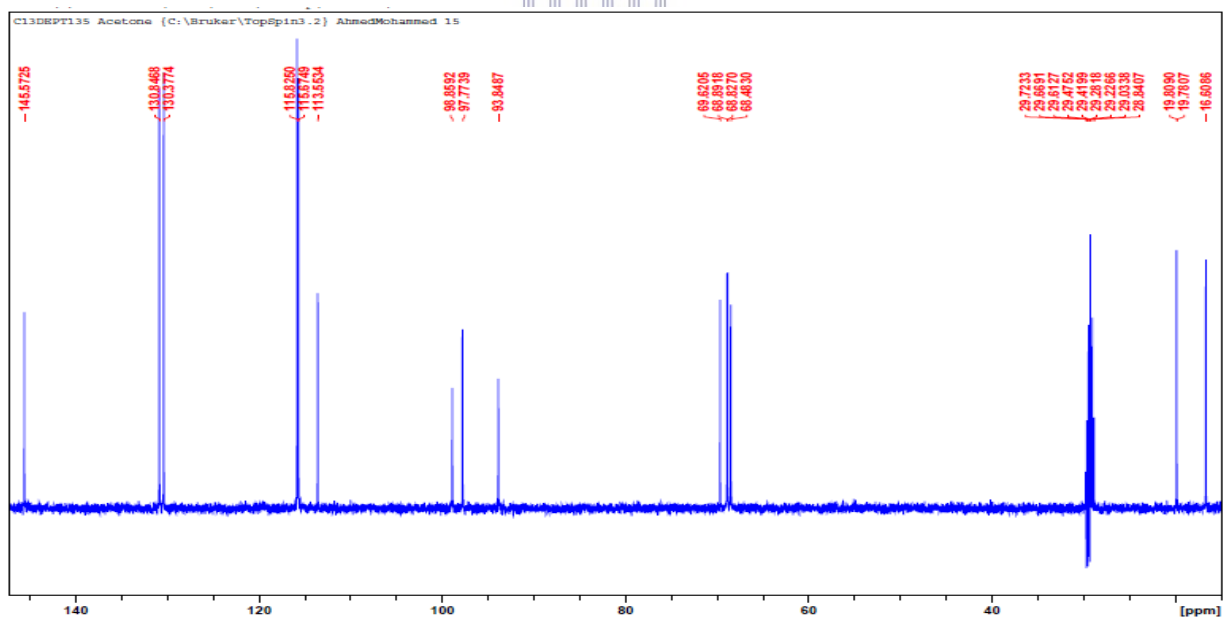


Figure 4.82: DEPT NMR spectrum of compound 3

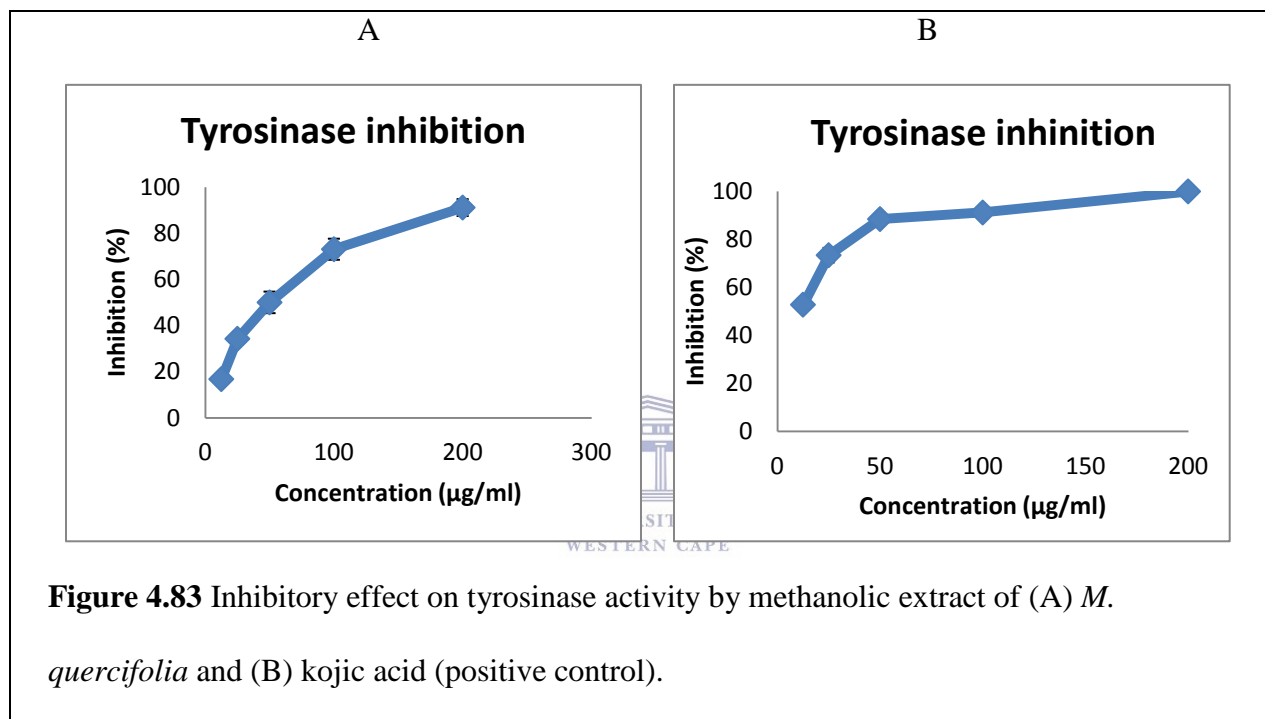
Table 4.4

No	XII-V(7)		C	H	C	H
	C	H				
2	157.2 s		157.8 s			
3	133.4 s		133.8 s			
4	177.9 s		177.9 s			
5	157.7 s		157.2 s			
6	98.9 d	6.28 d, 1.7	98.8 d	6.28 d, 1.7		
7	164.5 s		164.8 s			
8	93.9 d	6.48 d, 1.7	93.8 d	6.48 d, 1.7		
9	162.3 s		162.0 s			
10	104.7 s		104.7 s			
1 <sup>`</sup>	121.2 s		121.1 s			
2 <sup>,6</sup>	130.8 d	7.89 d, 8.7	130.8 d	7.89 d, 8.7		
3 <sup>,5</sup>	115.79 d	7.11 d, 8.7	115.6 d	7.11 d, 8.7		
4 <sup>`</sup>	160.4 s		160.4 s			
1 <sup>``</sup>	97.8 d	5.77 s	98.2 d	5.77 s		
2 <sup>``</sup>	68.6 d	5.64 t, 1.7	68.9 d	5.64 t, 1.7		
3 <sup>``</sup>	68.9 d	5.36 d, 3.4, 10.1	69.0 d	5.36 d, 3.4, 10.1		
4 <sup>``</sup>	69.9 d	5.04 d, 10.1	69.5 d	5.04 d, 10.1		
5 <sup>``</sup>	68.5 d	3.43 m	68.4 d	3.43 m		
6 <sup>``</sup>	16.6 q	0.86 d, 6.2	16.6 q	0.86 d, 6.2		
1 <sup>```</sup>	165.6 s		162.0 s			
2 <sup>```</sup>	145.6 d	7.57 d, 16.0	145.3 d	7.57 d, 16.0		
3 <sup>```</sup>	113.6 d	6.26 d, 16.0	114.9 d	6.26 d, 16.0		
4 <sup>```</sup>	125.6 s		126.1 s			
5 <sup>```</sup> , 9 <sup>```</sup>	130.4 d	7.58 d, 8.5	133.1 d	7.58 d, 8.5		
6 <sup>```</sup> , 8 <sup>```</sup>	115.8 d	6.90 d, 8.5	114.8 d	6.90 d, 8.5		
7 <sup>```</sup>	160.1 s		160.1 s			
1 <sup>````</sup>	169.4 s		169.4 s			
2 <sup>````</sup>	19.78 <sup>†</sup> q	2.12 s	19.78 <sup>†</sup> q	2.12 s		
1 <sup>`````</sup>	169.6 s		169.6 s			
2 <sup>`````</sup>	19.81 <sup>†</sup> q	1.92 s	19.81 <sup>†</sup> q	1.92 s		

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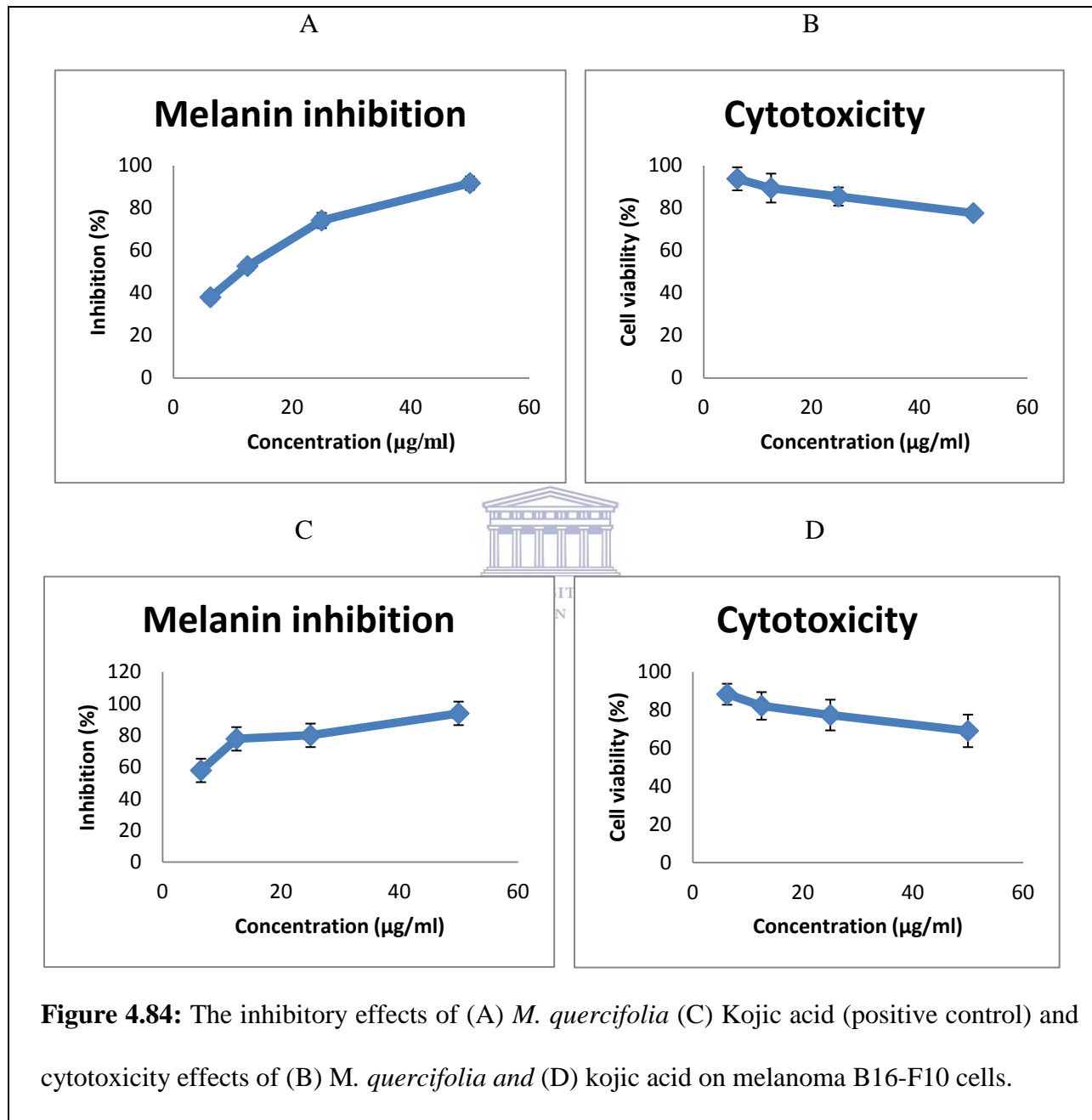
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**4.7.2 Inhibition of the enzyme tyrosinase by *M. quercifolia* extract and Kojic acid (positive control) using L-tyrosine as a substrate.**



According to the results obtained from the present study, the tyrosinase inhibitory effect of the methanolic leaves of *M. quercifolia* was evaluated using ELISA. As indicated in figure 4.3A, the MeOH extract of *M. quercifolia* showed inhibitory effects with an  $IC_{50}$  value of 66.9 µg/ml, which is comparable to kojic acid with an  $IC_{50}$  of <12.5 µg/ml. All experiments were repeated 3 times in triplicate, and the extract of *M. quercifolia* exhibited the same consistency throughout all the experiments.

**4.7.3 Melanin biosynthesis inhibition by *M. quercifolia* and kojic acid and Kojic acid using L-tyrosine as a substrate.**



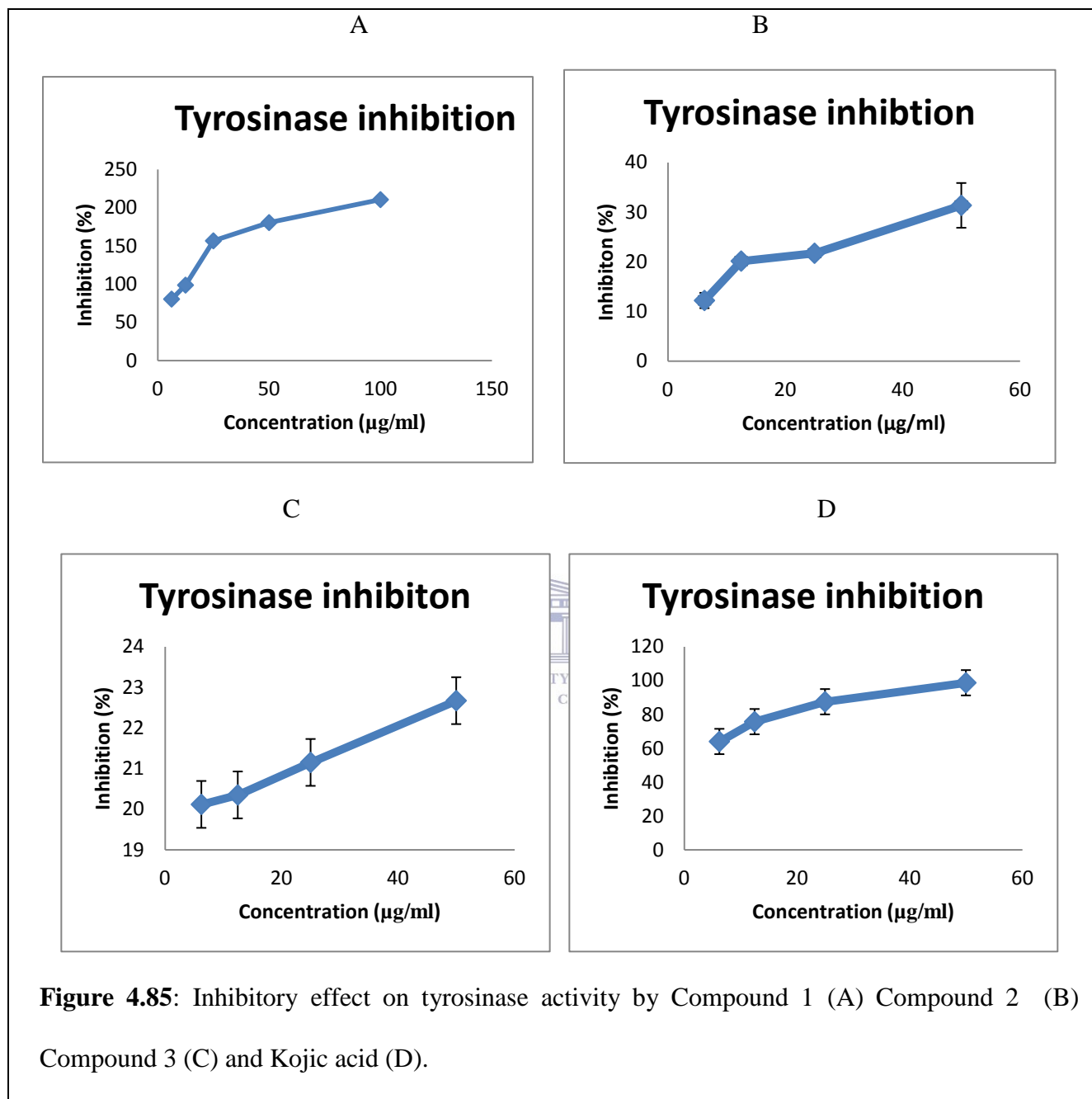
In the current study, melanoma B16-F10 cells were employed in assaying melanin inhibition as it contains moderate amount of the pigment and are suitable for observing the the melanin

pigment. Melanoma cells were treated with different concentrations of plant extracts ranging from 6.25 to 50 µg/ml; the activity was shown in a concentration dependent manner (see figure 4.9). *M. quercifolia* exhibited potent inhibitory effects on melanoma cells (<6.25 µg/ml) without cytotoxic effects (>100 µg/ml) kojic acid showed the same inhibitory and cytotoxic results as *M. quercifolia*.

#### **4.7.4 Effect of bioactive compounds on tyrosinase Inhibition.**

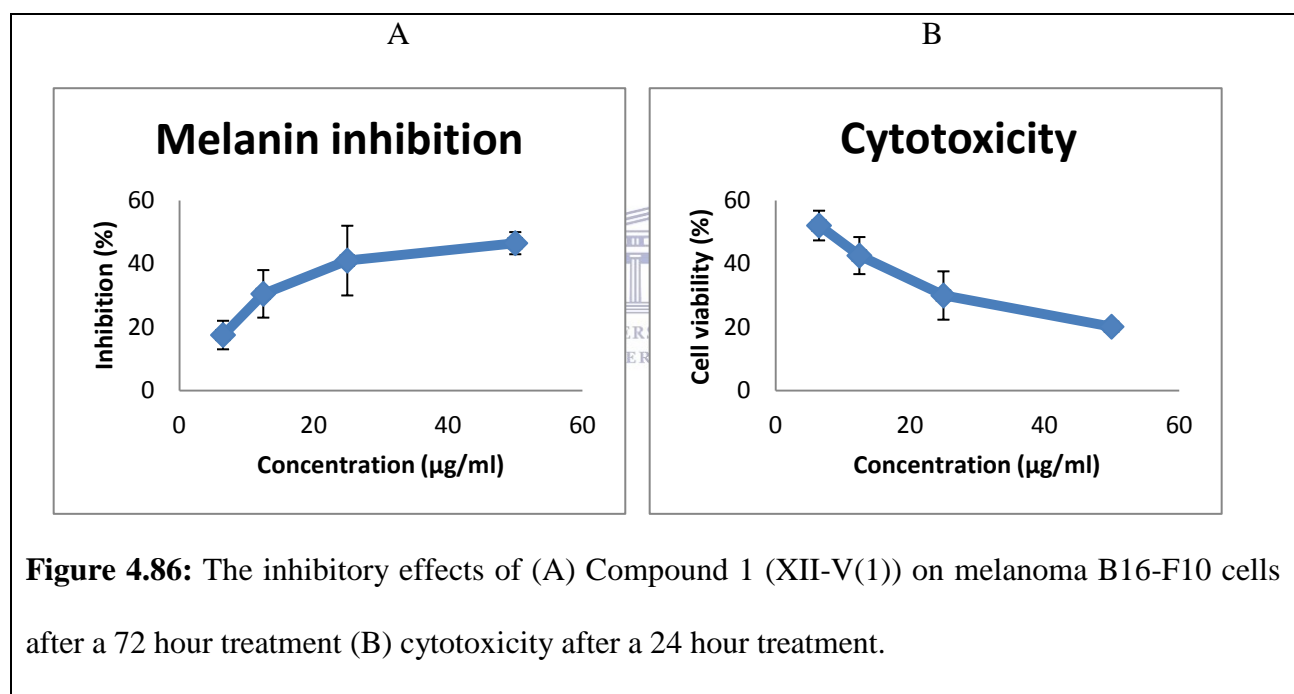
Methanolic extract of *M. quercifolia* was chosen for further examination as they displayed the highest and consistent tyrosinase inhibition activity among 37 plant species that were screened.

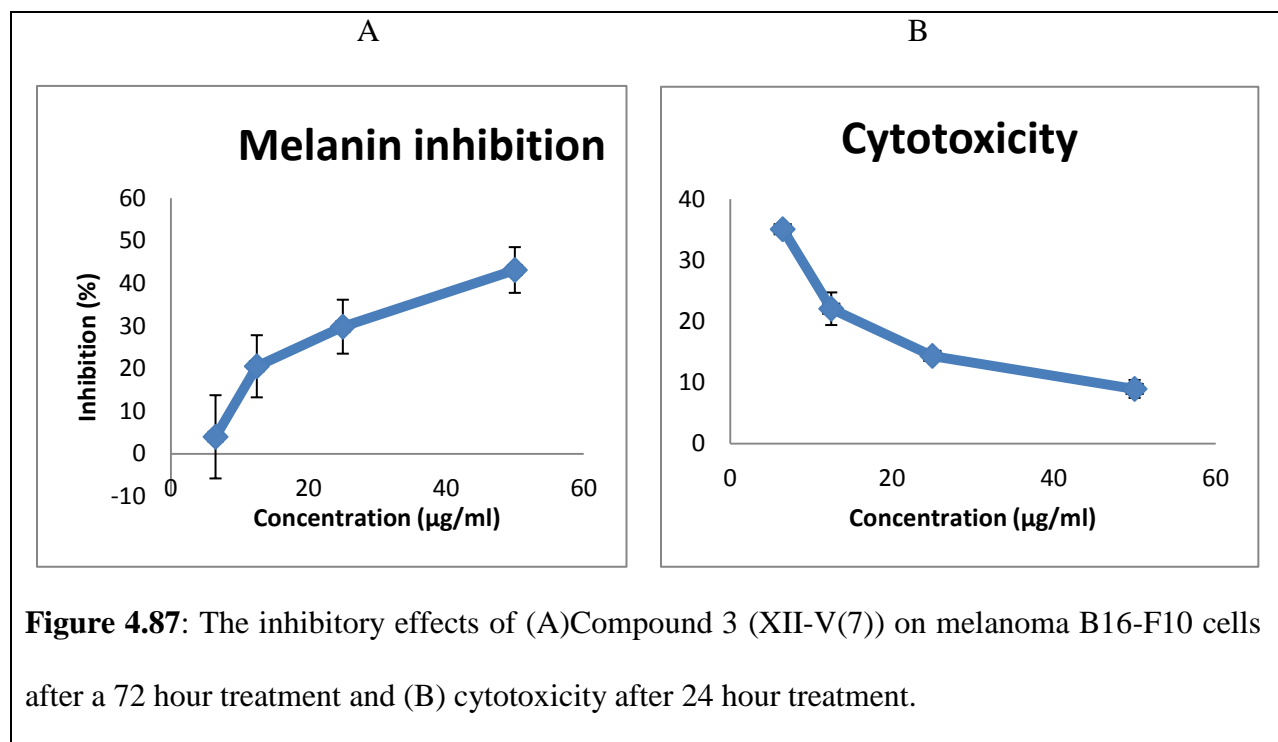




The tyrosinase inhibitory activities of the bioactive components of *M. quercifolia* (L.) were confirmed spectrophotometrically using a tyrosinase assay. As shown in Figure 4.6, all isolated compounds did not show any anti-tyrosinase activity except for compound 1 which showed potent inhibitory effects with an  $IC_{50}$  of  $<6.25 \mu\text{g/ml}$ ; compounds 2 and 3 had  $IC_{50}$  values of  $> 100 \mu\text{g/ml}$ . Kojic acid had a significant inhibitory effect with an  $IC_{50}$  of  $< 6.25 \mu\text{g/ml}$ .

#### 4.7.5 Effect of bioactive compounds on melanin inhibition





**Table 4.5.** IC<sub>50</sub> values of pure compounds isolated from *M. quercifolia* (L.)

Sample	IC <sub>50</sub> (µg/ml)		
	Inhibition (tyrosinase)	Inhibition (melanin)	Cytotoxicity
<i>Myrica quercifolia</i>	66.9	< 6.25	97.2
<b>Compound 1</b>	< 6.25	> 100	< 100
<b>Compound 2</b>	> 100	> 100	< 100
<b>Compound 3</b>	> 100	> 100	< 100
<b>Kojic acid</b>	< 6.25	< 6.25	50.3

Three compounds were isolated from the MeOH extracts, then evaluated for their melanogenesis inhibitory activity. By addition of these compounds at the concentration of 6.25, 12.5, 25 and 50 µg/ml, to an incubation medium of melanoma B16-F10 cells, all of the compounds showed weak inhibition of melanogenesis (melanin inhibition of 3.99-46.5%). However, all three compounds did not possess the same degree of activity when compared to the crude plant extract at comparable concentrations; this could be because the compounds in the plant matrix have a multi-factorial effect or they form a synergistic interaction to inhibit melanin or the compounds that are responsible for inhibition were not isolated.

Cell viability was assessed by the MTT reduction assay. Mouse melanoma cells were only resistant to compound 3 at a low concentrations (6.25 µg/ml) showing viability of 52.06%. All three of the isolated compounds, showed toxicity to the cells (cell viability 8.91-52.06%) compared to the toxicity of the crude plant extract that they were isolated from whose viability

was 77% at 50 µg/ml. In 2010 Akazawa and colleagues carried a melanogenesis inhibitory study where *Myrica rubra* was evaluated against mushroom tyrosinase; myricanol, myricanone, myricetin among other compounds were isolated and exhibited very high toxic effects to the melanoma B16 cells and thus induced cell death adversely with 1.9-17.7% cell viability at 25 µg/ml.

There is little literature on *M. quercifolia* but according to Plantzafrica it has been traditionally used for its wax to make candles and for stomach aches. Up to date, *M. quercifolia* has not been reported for tyrosinase inhibition, melanin inhibition or for any other scientific bioassays. However, there are reports of *Myrica* genera of which *M. rubra* is the most researched and is also popular for its rich phyto-nutritional content. The leaf consists of, proanthocyanidins, triterpenes, diarylheptanoids and flavonoids (Sun *et al.*, 2013) and flavonol glycosides (myricetin-3-O-β-D-glucoside, myricetin-3-O-α-L-rhamnoside, quercetin-3-O-α-L-rhamnoside, and quercetin-3-O-β-D-glucoside) has been isolated from the leaves and kernels of *M. rubra* (Wang *et al.*, 2010).

In 2010, Matsudi *et al.*, assessed methanolic extracts from leaves of *M. rubra* against tyrosinase activity. The extract showed potent tyrosinase activity; the IC<sub>50</sub> of the tyrosinase inhibitory effect was 0.23 mg/ml.

In 1995 Matsudi and colleagues, conducted a tyrosinase inhibitory study; ethanolic extract of the leaf and bark of *Myrica rubra* were evaluated. The bark which was tested at a concentration of 500 µg/ml exhibited the highest inhibition of 73% and the leaves showed activity of 70% at the same concentration. The inhibitory activity of all other plant species mentioned above are not reported. Figure 3.3 illustrates crude extracts which showed activity

higher (these extracts were selected from the 37 extracts that were screened for tyrosinase inhibition, see table 3.2). Crude extracts were further selected and re-evaluated for melanin inhibition, cytotoxicity and IC<sub>50</sub> determination.

One study by Ohguchi and Ozaki, 2017 , reported the stem bark of *Myrica esculenta* which was investigated for its ability to inhibit  $\alpha$ -MSH induced melanogenesis. When melanoma B16 cells were treated with  $\alpha$ -MSH in the presence of *M. esculenta* bark extracts,  $\alpha$ -MSH induced melanin production decreased significantly, compared to the cells that were not treated with *M. esculenta* bark extracts. The amount of melanin content decreased in a dose-dependent manner by *M. esculenta* bark extracts, with the maximal level at 10  $\mu$ g/ml.



## Chapter 5

### 5.1 Motivation of the study

As a contribution to the on-going search for novel, effective, stable, safe and affordable treatments to be used for depigmentation in southern Africa, it was necessary to advocate scientific research on plants from the CFR.

The analysis and identification of natural antioxidants with practical applications in food, medicine, and cosmetics, and the determination of anti-tyrosinase compounds become more attractive and of great interest scientific research on plants used for skin diseases.

### Conclusion

With its unsurpassed botanical diversity, the CFR holds unexplored potential natural resources of global significance. For that reason, 37 plants from the CFR were evaluated for tyrosinase inhibition using TLC bioautography in order to develop new potential skin whitening agents; kojic acid was used as a positive control. The activity was confirmed with tyrosinase assay spectrophotometrically; seventeen methanolic extracts including *A. karroo* (Hayne.), *A. afra* Jacq. Ex Willd, *C. geifolia* (L.), *E. racemosa* (L.), *H. petiolare* Hilliard & B.L.Burt, *M. quercifolia* (L.), *M. communis* (L.), *P. rigida* (Wikstr.), *P. ecklonii* (Benth.), *P. ericoides* (L.), *S. Africana-caerulea* (L.), *S. Africana-lutea* (L.), *S. antarcticus* (Willd.), *S. lucida* (L.) F.A.Barkley, *S. hamilifolius* (L.), *S. furcellata* R.Br and *T riparia* showed significant inhibition for mushroom tyrosinase. Melanogenesis inhibitory activity for the 17 active extracts was employed *in vivo* at 50 µg/ml. Of the 17 extracts, eight showed the best activity [*T. riparia*, *S. furcellata* R.Br, *C. geifolia* (L.), *A. karroo* (Hayne.), *M. quercifolia* (L.), *P. eroides* (L.), *S. antarcticus* (Willd.) and *M. communis* (L.)], consequently the active extracts were selected for

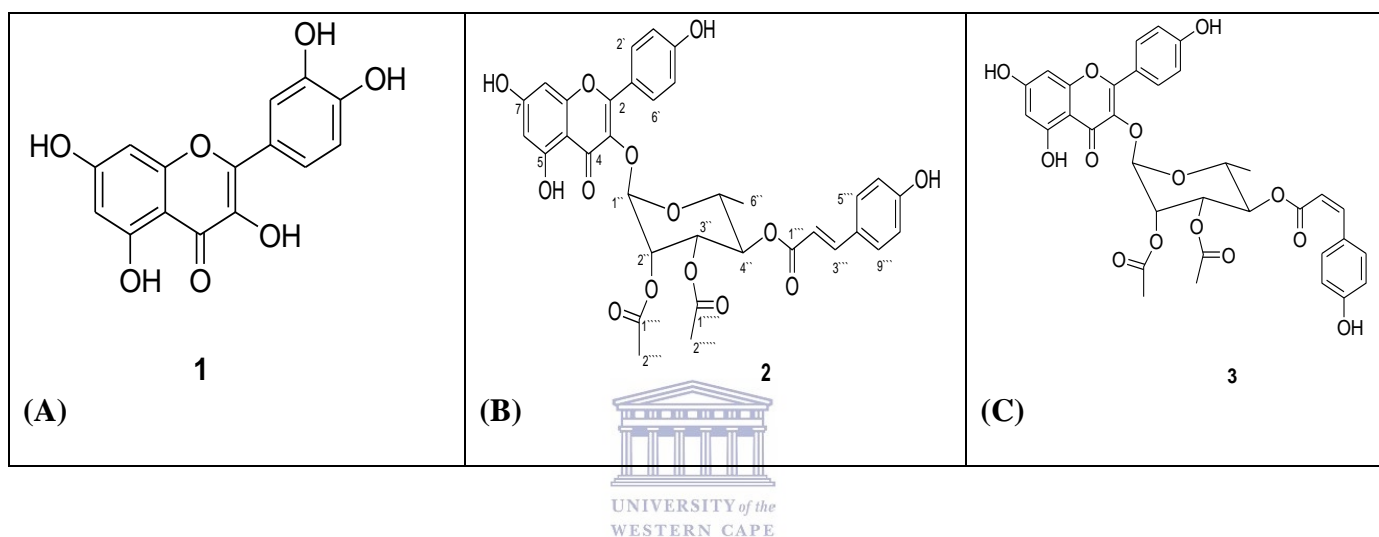
further *in vivo* studies; melanogenesis inhibitory and cytotoxic studies were employed. *M. quercifolia* exhibited the best inhibitory effects compared to other plant extracts and was selected for purification and characterisation of bioactive compounds. There is no information in literature to describe the inhibitory effects of *M. quercifolia*, however, it is known that the genus *Myrica* has a variety of molecules with different biological activities such as antioxidant, melanogenesis, activities from various species including as *M. rubra* and *M. esculenta*. The compounds that were isolated from *M. rubra* that have been attributed to melanin inhibition are myricanol 11-O-b-D-glucopyranoside, myricanol 5-O-b-D-(6'-O-galloyl)-glucopyranoside, Myricanone 5-O-a-L-arabinofuranosyl-(1→6)-b-D-glucopyranoside, myricitrin (myricetin 3-O-a-L-rhamnopyranoside and 16-Methoxy acerogenin B 9-O-b-D-apiofuranosyl- (1 →6)-b-D-glucopyranoside.



In this study, the bioactive compounds of *M. quercifolia* were isolated following bioactivity-guided isolation using chromatographic techniques and subsequently identified using spectropic techniques. Three compounds, such as quercetin, kaempferol-3-(2,3-diacetoxy-4-trans-*p*-coumaroyl)rhamnoside and kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside were isolated from the methanolic fraction (XII<sub>5</sub>) as the active principles.

Quercetin (compound 1) strongly inhibited tyrosinase activity but showed weak activity on B16 melanoma cells in comparison with a known melanogenesis inhibitor, kojic acid, the cells were only resistant to toxicity at the lowest concentration tested. Kaempferol and compound 3 showed weak activity both in tyrosinase and melanin inhibition assays and were toxic to cells at all tested concentrations.

This study gives some validation towards the use of some medicinal plants for the treatment of skin disorders. Moreover, selective plants from the CFR could be targeted for future study on melanin inhibition which plays an important curative role in the overall health of the skin. This study indicated that *M. quercifolia* is a suitable plant extract for isolation of suitable bioactive candidates for skin-whitening.



**Figure 4.88.** Chemical structures of (A) quercetin (B) Kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside, and (C) kaempferol-3-(2,3-diacetoxy-4-cis-*p*-coumaroyl)rhamnoside isolated from the methanol extract of *M. quercifolia*.


## Recommendations

This study is a starting point for further investigations on tyrosinase inhibitors with respect to CFR plant extracts. Simultaneously, more CFR plants should be explored in order to identify useful chemical compounds that exhibiting broad-spectrum activities against the tyrosinase activity. The present data would certainly help to determine the potency of the tested plant materials as potential source of tyrosinase inhibitors to be used for cosmetics, pharmaceutical, medical and functional food applications.

Further research is needed to identify individual components from *M. quercifolia* that have noteworthy inhibition against tyrosinase. It may be beneficial to test compounds together to address the synergistic effects on skin lightening, particularly when the active components influence distinct steps of melanogenesis. Further study including the evaluation of tyrosinase inhibitory activity is required to clarify the mechanisms of the active extracts and compounds.



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