MSc by Research Thesis

The Isolation and Purification of Chemical Constituents of *Croton megalocarpus* Hutch Husks

By

Mohammed Danyaal

School of Life Sciences. Pharmacy and Chemistry

FACULTY OF SCIENCE, ENGINEERING AND COMPUTING

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ABSTRACT

Croton is a genus that is part of the Euphorbiaceae family. *Croton* is widely used in traditional medicine in a number of African countries such as Kenya, Tanzania, Uganda, Sudan, Mozambique and Zambia. Members of the *Croton* have been reported to treat malaria, diabetes, intestinal worms, influenza and arthritis along with other conditions. However, *Croton megalocarpus* Hutch is a species that is predominately found in Kenya. *C. megalocarpus* is regularly processed in the *Croton* factory that is situated in Kenya. The company that processes the nuts of *C. megalocarpus* is named Eco Fuels Kenya. The by-products from *C. megalocarpus* seed oil extraction includes fruit husks from the de-husking process and seed cake resulting from cold pressing of the oil.

The purpose of this study is to see whether the husks of *C. megalocarpus* possess interesting compounds and to determine the safety of these compounds for alternative use. Chemical constituents found in the husks of *C. megalocarpus*, and various extracts obtained from the husks, seeds and fruit cake were tested for their cytotoxic effects against FM-55 human melanoma cells and mushroom tyrosinase inhibition effects. Extracts were prepared using hexane, dichloromethane and methanol solvents.

The hexane extract of the husks produced an oil that had a similar profile to the cold pressed *C. megalocarpus* seed oil, the dichloromethane extract mainly exhibited diterpenoids of the labdane, cembrane and kaurane classes of compounds whereas methanol extract was mainly sugars with traces of magnoflorine and *trans*-4-hydroxy-*N*-methyl-L-proline. The dichloromethane (DCM) extract was chosen for further analysis including additional extracts such as ethyl acetate and dichloromethane extract of *Croton* vinegar oil and tested for cytotoxic and anti- melanogeni9c properties.

Seven compounds were successfully isolated from the dichloromethane extract of the husks of *C. megalocarpus* and characterised using nuclear magnetic resonance (NMR), high resolution mass spectrometry (HRMS) and infrared spectroscopy (IR). The seven isolated compounds are: *trans* ozic acid, sartone A, epoxykaurane, epoxychiromodine, vanillin, (-)-(1*S**,4*S**,10*R**)-1,4-dihydroxycembra-2*E*,7*E*,11*Z*-trien-20,10-olide and (+)-[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide.

In this study, the ethyl acetate extract of croton vinegar oil exhibited a tyrosinase inhibition of 59.33% at 20mM concentration. Epoxychiromodine exhibited an inhibition of 26.82% at 20µg/ml and showed to have a higher inhibition rate than the other isolated compounds. The dichloromethane extract of the husks was shown to have a cell viability of 45.62% ($P<0.05$) at 50µg/ml concentration. Sartone A was shown to have a cell viability of 86.08% ($P<0.05$) at 50µg/ml. The cytotoxicity and tyrosinase inhibition assay have helped determine the safety and effectiveness of the compounds and extracts that may play a vital role in skin care, skin lightening treatments and cancer treatment.

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LIST OF ABBREVIATIONS

WHO – World Health Organisation

EFK – Eco Fuels Kenya

CVO – *Croton* Vinegar Oil

FM-55 HMC – FM-55 Human Melanoma Cells

CMO – *Croton megalocarpus* Oil

AChE – Acetylcholinesterase

NMR – Nuclear Magnetic Resonance

HRMS – High Resolution – Mass Spectroscopy

LC-MS – Low Resolution-Mass Spectrometry

IR – Infrared Spectroscopy

HQ - Hydroquinone

CRE- *Croton roxburghii*

CSE - *Croton sublyratus*

CI – Confidence Interval

DMSO – Dimethyl Sulfoxide

DMEM – Dulbecco Modified Eagle Medium

DCM - Dichloromethane

1. Introduction

The World Health Organization (WHO) estimate that 80% of the world population utilise native medicinal plants as a source of healthcare (Salatino *et al.,* 2007). This is because of the constituents present within the plants that exhibit medicinal effects. *Croton* is a plant genus which is part of the Euphorbiaceae family. In general, Euphorbiaceae species have been found to be either toxic or medicinal due to the chemical nature of their constituents. The *Croton* genus is a large sector of the Euphorbiaceae family containing of 1,300 species of shrubs, herbs and trees (Salatino *et al.,* 2007). Phytochemistry of *Croton* has revealed the discovery of 399 novel compounds, including 339 diterpenoids (Xu *et al*., 2018). *Croton* is found in different continents of the world such as Africa, Asia, South America and North and Central America.

It has been reported that 39 species of *Croton* have been identified in Rio de Janeiro alone and they have been used to treat a variety of diseases including cancer (Salatino *et al.,* 2007). Thorough examination of *Croton* has revealed it contains constituents that have biological effects, including compounds such as phorbol esters, clerodanes, labdanes and kaurane (Salatino *et al.,* 2007). These chemical constituents are known as diterpenoids with a minimum 20-Carbon structure. Also, *Croton* has been reported to contain aromatic compounds and fatty acids (oil) (Salatino *et al.,* 2007). In many inhabiting countries, diverse parts of *Croton* have been used to treat an array of complications.

A review by Salatino *et al* (Salatino *et al*., 2007) lists the studies conducted on the biological properties of different Croton species. In the West Indies, the bark of *Croton eluteria* Bennett has been used to treat various diseases such as fever, malaria, bronchitis and dysentery. In the Atlantic forest of southern Brazil, bark and leaf decoctions of *Croton celtidifolius* Baill have been found to treat leukaemia, ulcers and rheumatism. In Mexico, aerial parts of *Croton arboreous* Millsp are used to produce an anti-inflammatory drink to treat respiratory ailments. Also, in North America, *Croton californicus* Mull found in the Mojave Desert is used as a pain reliever, for rheumatism. This is made from powdered leaves of the plant.

In Tanzania, the roots of *Croton macrostachys* Hochst. ex Rich is widely used as an anti-diabetic whilst the seeds of the tree are used as purgative in Somalia. In Thailand, the plant *Croton kongensis* Gagnep is used for medicine for dysmenorrhoea. In India,

diverse parts of *Croton roxburghii* NP Balakr are widely known to be used to treat snake poisoning, infertility, fever and wounds (Salatino *et al.,* 2007). *Croton* has been shown to exhibit anti-inflammatory, antioxidant and antibacterial properties. However, very little work has been done on the anticancer effects of these species. A better understanding of *Croton* chemistry and its bioactivity could lead to novel compounds being discovered which may be useful in treating a variety of diseases. This study focuses on the chemistry and biological assays of the husks of Kenyan *Croton megalocarpus* Hutch. The chemistry of several Kenyan *Croton* plants has been reported, including the chemistry of *Croton alienus* (Nyawira Muchane, 2019) *Croton dichogamus* (Aldhaher *et al*., 2017), *Croton megalocarpoides* (Ndunda *et al*., 2016) and *Croton sylvaticus* (Ndunda *et al*., 2015). These Kenyan *Croton* taxa have been reported to mainly yield diterpenoids.

1.1. *Croton megalocarpus* Hutch

Croton megalocarpus Hutch is a species that has gained interest due to its diverse applications, such as biofuel, organic fertilizer and poultry feed. A company that utilises the processing of *C. megalocarpus* nuts is called Eco Fuels Kenya (EFK). **Table 1** taken from a review article by Maroyi (Maroyi, 2017) represents a diversity of diseases that are treated by *C. megalocarpus,* the part of the plant used and the countries that practice this approach.

Table 1: The ethnomedicinal uses of *C. megalocarpus* in Africa
http://www.prota4u.org/search.asp

Diseases/Conditions	Part of plant used	Country practiced
Malaria	Bark or root decoction	Kenya, Tanzania
Pneumonia	Bark or leaf decoction	Kenya
Arthritis	Bark decoction	Kenya
Influenza	Bark and leaf decoction	Kenya
Intestinal worms	Bark or leaf decoction	Kenya, Tanzania
Induced labour	Root decoction	Uganda
Diabetes	Leaf decoction	Kenya
Dysentery	Bark decoction	Kenya
Respiratory problems	Leaf decoction	Kenya
Typhoid	Bark and leaf decoction	Kenya

EFK is a company that takes great interest in utilizing *C. megalocarpus* trees and transforming them for use as biofuel. This is due to the rise in air pollution that is caused by fossil fuel and therefore adds to global warming. Various studies have investigated the effects of fossil fuel diesel and biodiesel on the environment. A study by Kibet *et al* (Kibet *et al*., 2018) states fossil fuel diesel is known to produce high particulate matter, nitrogen oxides and greenhouse gases. The study also states that biodiesel blend of ~ 20% reduced about 15% in particulate matter, carbon monoxide and polyaromatic hydrocarbon combustion (Kibet *et al.,* 2018).

Climate change reporter David Kariuki (Kariuki, 2017) reports that biodiesel burns cleaner than fossil fuel diesel due to its engine lubricating effects and non-sulfuric content. The company aims to replace diesel with the *Croton* biofuel. The oil found inside the *Croton* is squeezed and put through a chemical process to convert it for suitability for use in cars. However, an report in The Guardian by Laura Secorun (Secorun, 2017) named “This is our future – Kenya’s *croton* tree touted as new biofuels crop” states that as Kenya usually import all their oil, some rural communities are unable to afford diesel for their water pumps and vehicle fuel.

One advantage of *Croton* nuts biofuel is that it can be directly used in water pumps and agricultural machinery like tractors (except for cars). The benefits of this led the company to process 1000 tons of *Croton* nuts for biofuel production in 2016 and motivated local farmers to plant the *Croton* tree on the scale of 100,000 trees. This would become more affordable for the local communities and therefore generate a good revenue. However, the company has identified the benefits of the by-products of the *Croton* plant such as the seedcake paste that is the end result of pressed *Croton nuts*. The seedcake paste is found to be high in protein levels and used as poultry feed for farmers.

The husks of the *Croton* plant are found to be used for fertilisers of depleted soil. This is cost effective for the agricultural industry in Kenya. However, Eco Fuels face issues such as marketing and knowledge for the development and growth of *Croton.* Therefore, this is deterring future investors, alongside the low oil price that is good for the consumers. By conducting a thorough investigation of *Croton* and its flexible use may interest future investment to carry out production of biofuels on a much larger scale. The research on Croton has focused mainly on various parts of the plant such as the

root, stem bark and leaves. Very little work has been done on the fruit husks of C. megalocarpus and therefore the husks may contain compounds that could possess properties of interest. The safety of the compounds found in the husks can be tested and validate the handling of C. megalocarpus for safety and toxicity. This project aims to provide recognition of the husks for the EFK.

The *Croton megalocarpus* tree is mainly found in central and eastern Africa including Kenya, Mozambique, Tanzania, Congo and Zambia (Maroyi, 2017). It is able to grow up to 40m in height and reach a diameter of 120 cm **(Figure 1)**. The bark of the tree is identified to be grey and the leaves of the tree are oval in shape (Maroyi, 2017). The tree is known to prefer light and well drained soils (Maroyi, 2017). *Croton megalocarpus* tree has many uses. The wood of the tree has been used in the construction industry for things like flooring, stools, mortars and plywood (Maroyi, 2017). The wood is also used for firewood and charcoal production (Maroyi, 2017).

Figure 1: *Croton megalocarpus* leaves (left) and the tree (right) Courtesy of KEW-Gardens library: http://epic.kew.org/index.htm

1.2. Traditional Use and Bioactivity of *Croton* Species and Its Isolates

Studies have shown that the roots of *C. megalocarpus* is commonly used by people in Kenya as it has been shown to treat a variety of health complications such as malaria and gastric problems (Bussmann, 2006). However, the leaf decoction has been shown to treat pneumonia, diabetes and respiratory complications (Kokwaro, 2009) and the bark decoction has been used as an herbal infusion to treat fever, colds and coughs (Fratkin,1996). This would indicate that different parts of *C. megalocarpus* possess unique compounds that are able to treat different conditions. Studies have shown and demonstrated the isolation of phytochemical compounds found in *C. megalocarpus* leaves and stem bark such as tannins, alkaloids, flavonoids, reducing sugars, sterols, terpenoids and clerodane diterpenoids (Maroyi, 2017) as shown in **Figure 2**. In Tanzania, the bark of the tree has been shown to treat diseases such as constipation, fever and malaria (Johns T *et al.,* 1994). The medicinal use of *C. megalocarpus* varies in different regions of Africa.

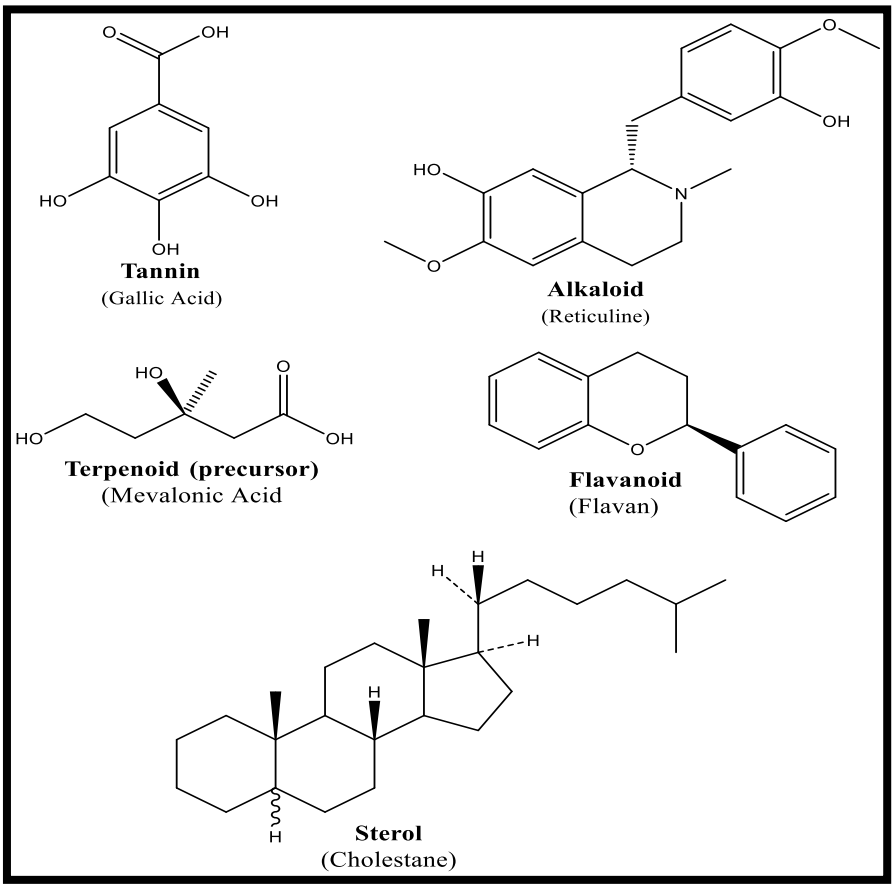


Figure 2: General class of compounds isolated from *C. megalocarpus*

Diterpenoids are most commonly found in oceanic organisms, higher plants and fungi. The following are different types of diterpenoids: *ent*-kaurane, labdane, cembrane, clerodane, halimane and abietane **(Figure 3)**. The research conducted on the *Croton* genus has shown the abundance of diterpenoids present. Diterpenoids have been reported as the predominant class of compounds extracted from various *Croton* species. Diterpenoids are a class of compounds that consist of a 20-Carbon structure. The online Dictionary of Natural Products Edition 28.2 states that diterpenoids are derived from geranylgeranyl pyrophosphate which is the biosynthetic precursor of the diterpenoid class of compounds **(Figure 3a)**

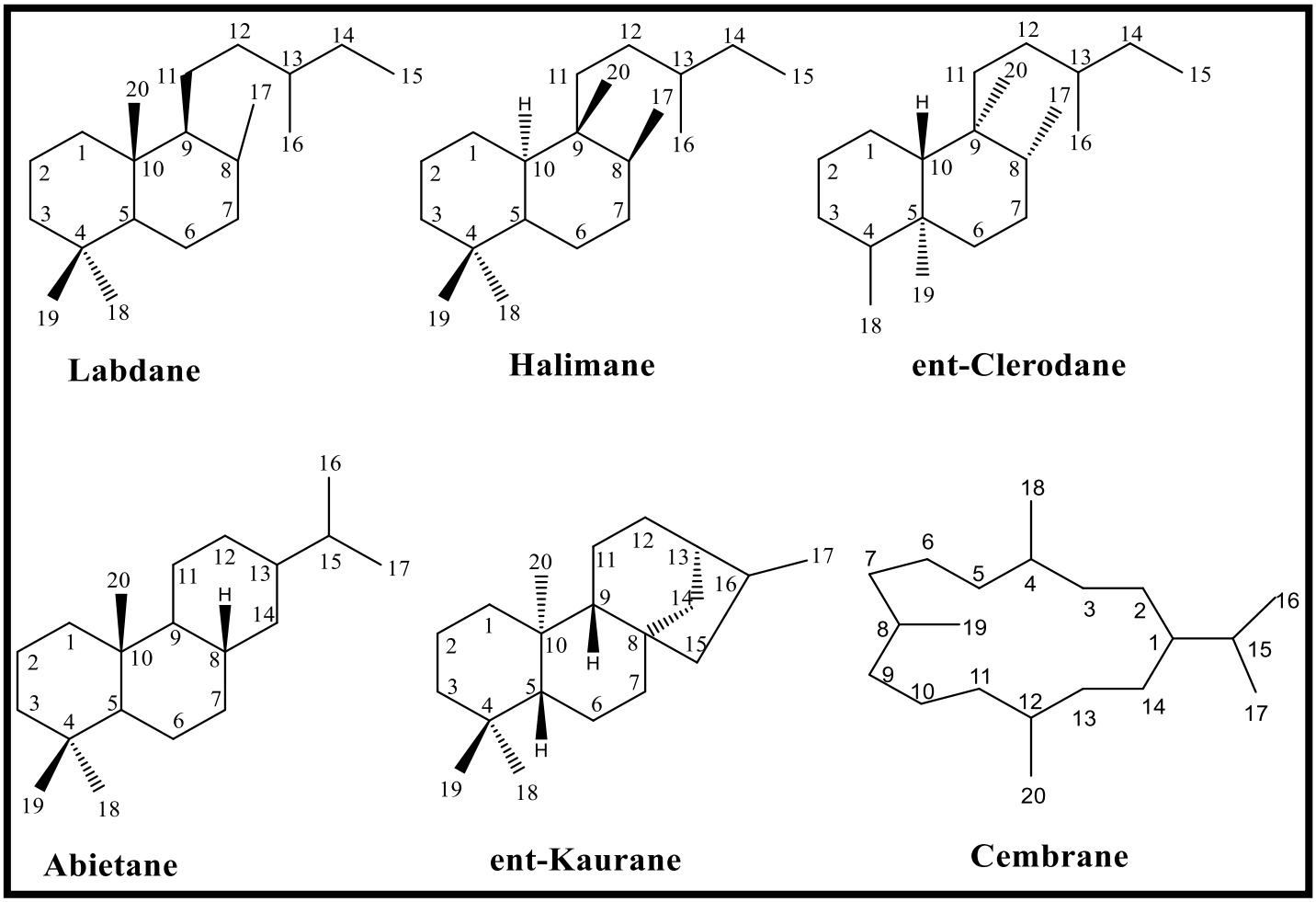


Figure 3: Different types of diterpenoids associated with *Croton* species

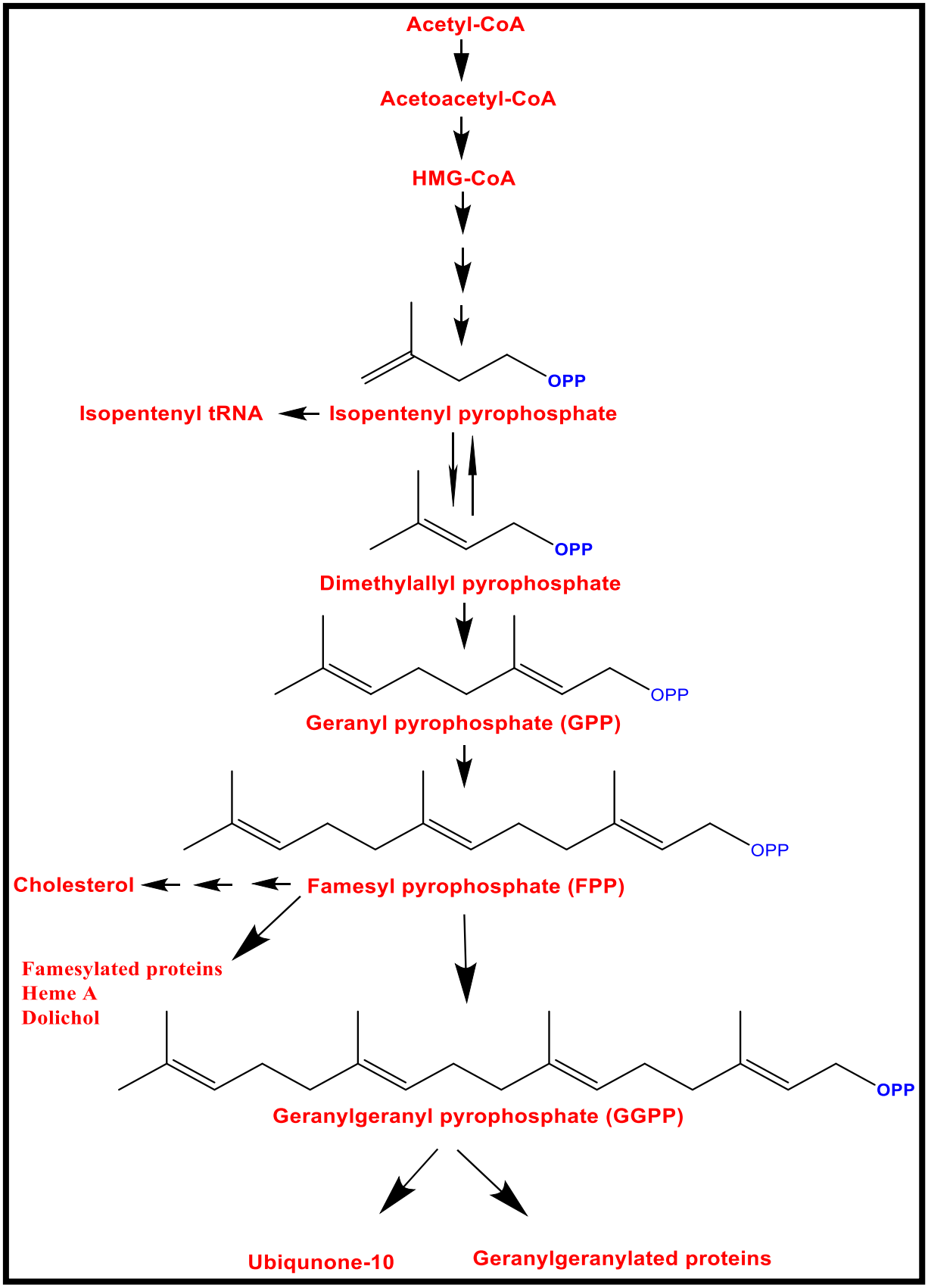


Figure 3a: **Biosynthetic route of geranylgeranyl pyrophosphate (GGPP) the precursor of diterpenoids. (Chhonker *et al*., 2018)**

Furthermore, there have been studies conducted on *Croton* phytochemical analysis. It has been speculated that studying the chemistry of *Croton* may provide a better understanding of potential medicinal use. A study by Aderogba *et al* (Aderogba *et al*., 2013) found that the ethyl acetate crude leaf extract of *Croton sylvaticus* contained acetylcholinesterase inhibitory activity alongside with other isolated compounds. The article determines that crude extract, solvent fractions and isolated compounds had high acetylcholinesterase (AChE) activity. The isolated compounds 2′-(3″,4″-dihydroxyphenyl)-ethyl-4 hydroxybenzoate, quercetin and kaempferol showed an IC_{50} range against AChE from 60.7- 415.0 µg/mL while crude and solvent fractions IC_{50} ranged from 235.0 – 4695 µg/mL. These findings demonstrated the potential of the isolated compounds and crude extract of *Croton sylvaticus* for further evaluation in the treatment of neurodegenerative disorders (Aderogba *et al.,* 2013*)*.

On the other hand, another study conducted by Mulholland *et al* (Mulholland *et al*., 2010) found that stem bark of *Croton gratissimus* contained cembranolides and was shown to have moderate activity against ovarian cancer and to treat malaria as it is used by traditional healers for this purpose. In this study, compounds (1) and (3) **(Figure 4)** was tested against PEO1 and PEO1TaxR (taxane resistant) ovarian cancer cell lines and were shown to have a lower potency than paclitaxel (chemotherapy drug used to treat ovarian cancer). However, after repeated attempts, both compounds (1) and (3) **(Figure 4)** had no significant effect on PEO1 ovarian cancer line cells. It was stated that the tubulin binding site of the compounds were different to paclitaxel and was not recognised by the MDR (multidrug resistance) transporter that was abundant in PEO1TaxR cells (Mulholland *et al.,* 2010).

The paper reported compounds that were isolated from the stem bark of *C. gratissimus* were four novel cembrane compounds (1, 2, 5, 6) (+)-[1R*,2S*,7S*,8S*,12R*]-7,8-epoxy-2,12-cyclocembra-3E,10Z-dien-20,10-olide (1), (+)-[1R*,10R*]-cembra-2E,4E,7E,11Z tetraen-20,10-olide (2), (+)-[1R*,4S*,10R*]-4-hydroxycembra-2E, 7E,11Z-trien-20,10-olide (5) and (−)-[1R*,4S*,10R*]-4-hydroxycembra-2E,7E,11Z-trien-20,10-olide (6). These compounds were isolated from the hexane and DCM extract of the plant. However, Langat *et al.* (2011) reported 10 compounds isolated from the leaves of *C. gratissimus* including novel cembrane diterpenoids (3, 4, 7-14) (+)-(10R*)-cembra- 1Z,3Z,7E,11Z,15-pentaen-20,10-olide (3), (+)-(1S*,4R*,8S*,10R*)-1,4,8- trihydroxycembra-2E,6E,11Z-trien-20,10-olide (4), (−)-(1S*,4S*,10R*)-1,4-

dihydroxycembra-2E,7E,11Z-trien-20,10-olide (7), (−)-(1R*,4R*,10R*)-4- methoxy-cembra-2E,7E,11Z-trien-20,10-olide (8), (−)-(1S*,4R*,10R*)-1-hydroxy-4- methoxy-cembra-2E,7E,11Z-trien-20,10-olide (9), (−)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide (10), (+)-(1S*,4S*,7R*,10R*)-1,4,7- trihydroxycembra-2E,8(19),11Z-trien-20,10-olide (11), (−)-(1S*,4S*,7S*,10R*)-1,4,7-trihydroxycembra-2E,8(19),11Z-trien-20,10-olide (12), (+)-(10R*)-cembra- 1E,3E,7E,11Z,16-pentaen-20,10-olide (13) and (+)-(5R*,10R*)-5-methoxycembra- 1E,3E,7E,11Z,15-pentaen-20,10-olide (14). Also, α glutinol (15), lupeol (16) and 4 (15)-eudesmene-1b,6a-diol (17) were isolated (Mulholland *et al.,* 2010). Compounds were identified and characterised using ^{13}C NMR, ^{1}H NMR and Infrared spectroscopy (IR). **Figure 4** represents the compounds isolated from *C. gratissimus*.

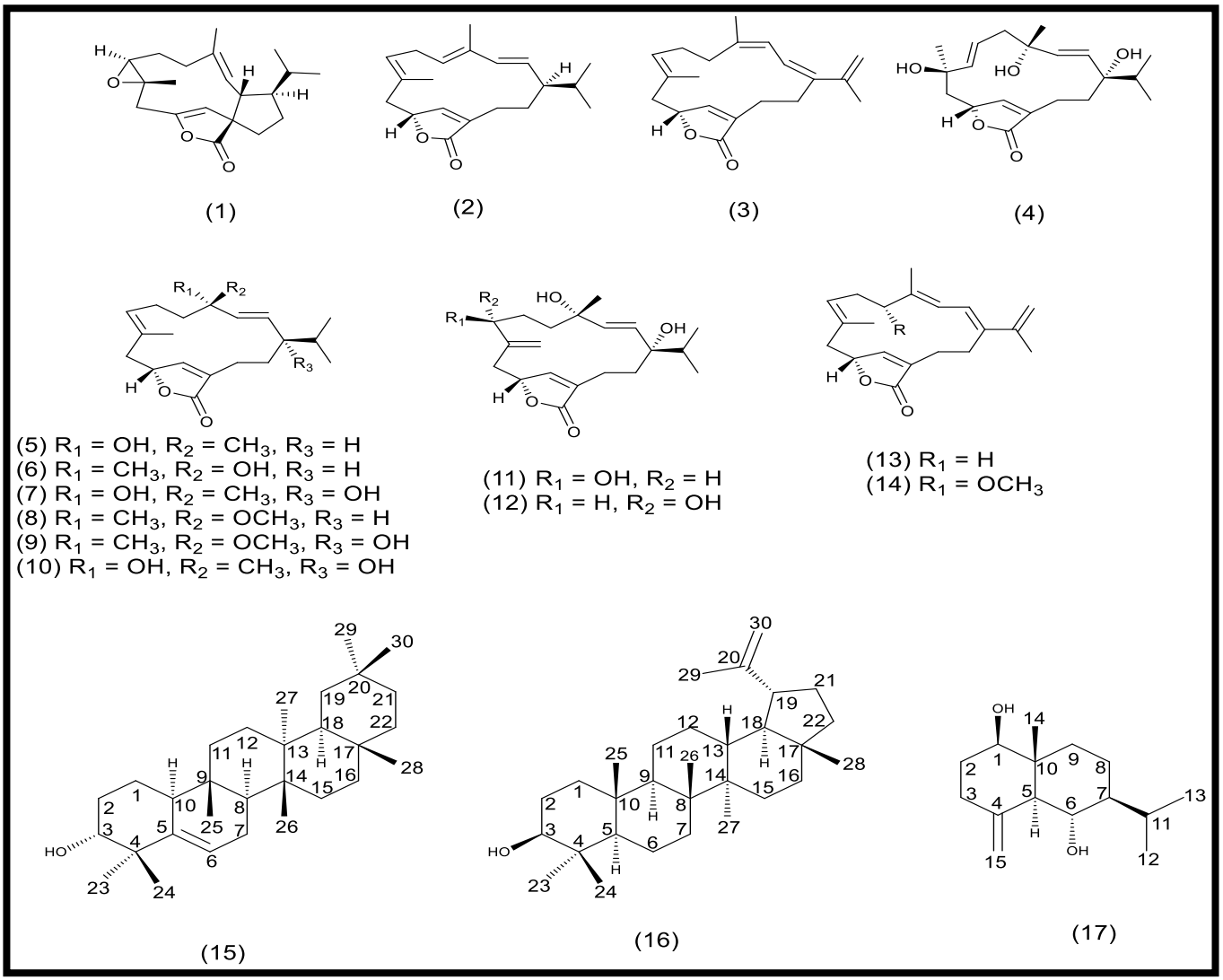


Figure 4: Compounds isolated from stem bark and leaves of *C. gratissimus*

A review by Alfred Maroyi. (Maroyi, 2017) reported the extraction and isolation of compounds from the stem bark of *C. megalocarpus* conducted by Addae-Mensah *et al* (Addae-Mensah *et al.* 1992*)*. In this study, a range of classes of compounds were successfully extracted and isolated. Two clerodane diterpenoids chiromodine (18) and epoxychiromodine (19), four triterpenoids lupeol (16), 3β-acetoacetyl lupeol (20) and ferulic acid ester derivatives (21-23), acetyl aleuritolic acid (24) and betulin (25) were isolated from stem bark of *C. megalocarpus* (Maroyi. 2017) as shown in **Figure 5**. Maroyi (Maroyi, 2017) also reported the range of impressive properties that *C. megalocarpus* was found to contain such as: anti-inflammatory, antifungal, healing, antibacterial, toxicity, molluscidal and antinociceptive activities. These properties raise great interest in testing *C. megalocarpus* for medicinal uses.

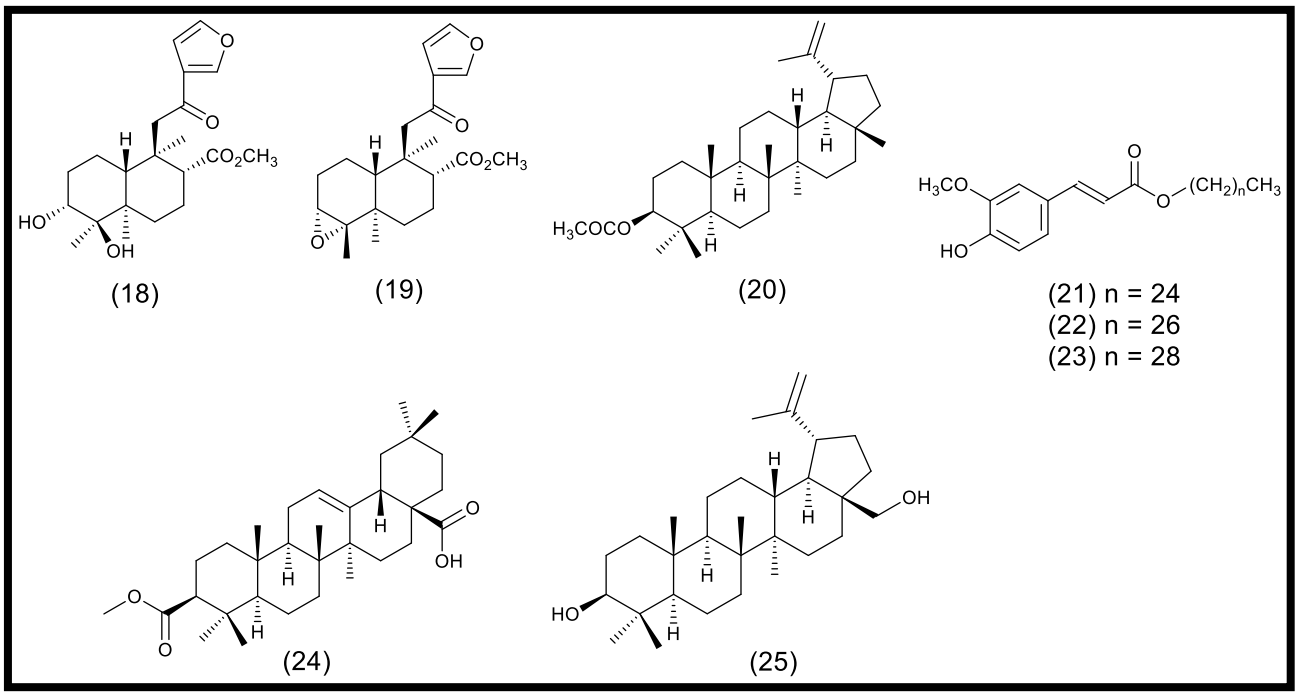


Figure 5: Compounds isolated from stem bark of *C. megalocarpus*.

A study by Alqahtani (Alqahtani, 2015) analysed compounds that have been extracted from the root of the *C. megalocarpus* plant. In a previous study by Addae-Mensah *et al* (Addae-Mensah *et al.,* 1992) the stem bark of *C. megalocarpus* was studied and had revealed two clerodane diterpenoids chiromodine (18) and epoxychiromodine (19) and 4 triterpenoids lupeol (16), 3β-acetoacetyl lupeol (20) and ferulic acid ester derivatives (21-23), acetyl aleuritolic acid (24) and betulin (25). However, Alqahtani ((Alqahtani, 2015) analysed the root of *C. megalocarpus* to see any comparison of

compounds found in stem bark and root of the plant. The dichloromethane/methanol root extract yielded the following compounds: 6 clerodane diterpenoids 3-keto-15,16-epoxy-13(16),14-entclerodadien- 17,12S-olide (crotonolide E) (26), 15,16-epoxy-3,4-dihydroxy-13(16),14-entclerodadien-17,12S-olide (furocrotinsulolide A) (27), 3β,4β:15,16-diepoxy-13(16),14-entclerodadien-17,12S-olide (28), epoxychiromodine (19), 3β,4β:15,16-diepoxy-13(16),14-entclerodadiene (29), trans-annonene (30), triterpenoid lupeol (16), halimane crotohalimaneic acid (31), two abietane 7,13-abietadien-2-one (32) and 7,13-abietadien-2-ol (33). Therefore, it transpires that more compounds were isolated from the root of *C. megalocarpus* than the stem bark. The clerodane diterpenoids have been reported previously in *Croton* genus as stated by Alqahtani (Alqahtani, 2015). **Figure 6** represents compounds 26-33.

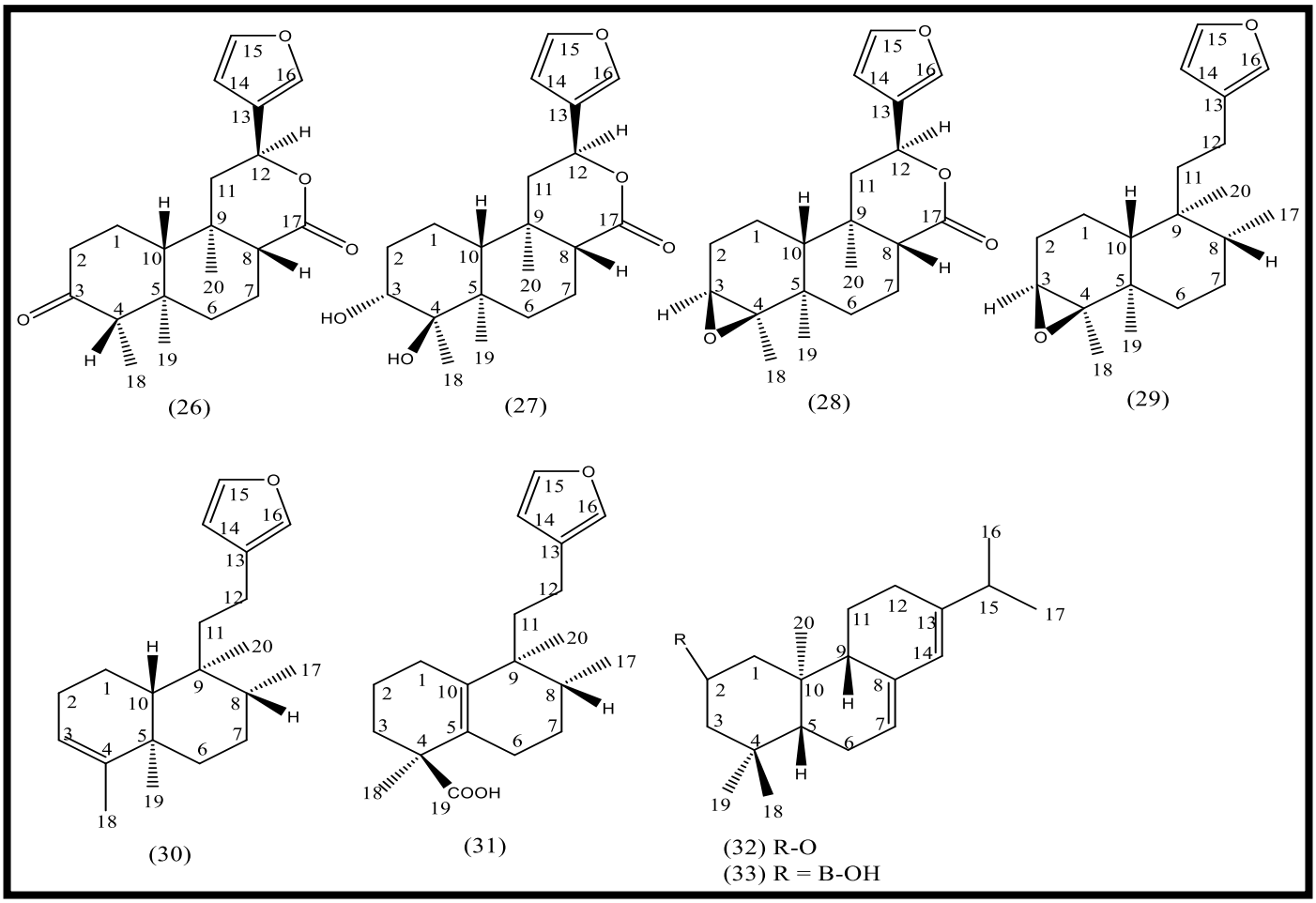


Figure 6: Compounds isolated from dichloromethane/methanol extract of the root of *C. megalocarpus* by Alqahtani (2015).

Moreover, anticancer screening of NCI60 human tumour cell lines was conducted on selected compounds by Alqahtani (Alqahtani, 2015). The compounds isolated from *C. megalocarpus* and selected for anticancer screening were compounds (19) **(Figure 5)**, 26-29 **(Figure 6)** and 31-33 **(Figure 6).** The methodology required 60 different human tumour cancer cell lines to be tested in two phases: phase 1 single dose injection of 10μM and phase 2 was testing compound against cancer cell line at a concentration of 5 μM (Alqahtani, 2015). Phase 2 was only possible for compounds that showed interesting cancer growth inhibition at phase 1. It was reported that all diterpenoids tested had no significant effect on the NCI60 cancer cell lines at a single dose of 10μM.

In addition to the anti-cancer activities of diterpenoids, a paper by Jian *et al* (Jian *et al*., 2018) reported the sub-types of diterpenoids that had a positive effect on several cancer lines. In this study, 24 diterpenoids were successfully extracted from *Euphorbia fischeriana* a plant that is known for treating cancer, edema and tuberculosis (Jian *et al.,* 2018). Diterpenoids isolated from *Euphorbia fischeriana* consisted of six *ent*-abietane jolkinolide B (34), jolkinolide A (35), 17-hydroxyjolkinolide B (36), 17-acetoxyjolkinolide B (37), euphorin E (38), euphorin H (39), yuexiandajisu E (40), three tigliane prostratin (41), 13-O-acetylphorbo (42), 12-deoxyphorbol 13-palmitate (43), daphnane langduin A (44), four ingenane ingenol-6,7-epoxy3-tetradecanoate (45), ingenol-3-myristinate (46), ingenol 3-palmitate (47), ingenol-20-myristinate (48), four *ent*-atisane ent-1β,3β,16β, 17-tetrahydroxyatisane (49), ent-1β,3α,16β,17-tetrahydroxyatisane (50), ent-3β-hydroxyatis-16-ene-2,14-dione (51), 19-O-β-D-glucopyranosyl-ent-atis-16-ene-3,14-dione (52), three *ent*-rosane euphorin C (53), ebractenoid C (54), ebractenoid F (55), *ent*-kaurane ent-kaurane-3-oxo-16β, 17-acetonide (56) and lathyrane jolkinol A (57) (Jian *et al.,* 2018) as seen in **Figure 7**.

The study discovered that four *ent*-abietane, three ingenane, two *ent*-atisane, three *ent*-rosane and lathyrane had inhibitory action of cell proliferation of human breast cancer MCF-7 cancer cell line (Jian *et al.,* 2018). This was due to the direct disruption of mammosphere formation in MCF-7 cancer cells. However, *ent*-kaurane-3-oxo-16β-17-acetonide was discovered to have inhibited cell proliferation of liver cancer cell line Hep-3B. The results of these diterpenoids seem promising for further analysis as they exhibit potential for development. The classes of compounds extracted by Alqahtani

(Alqahtani, 2015) have been found in the root of *C. megalocarpus* and therefore, would be interesting to compare any similarity or new compounds in *C. megalocarpus* husks.

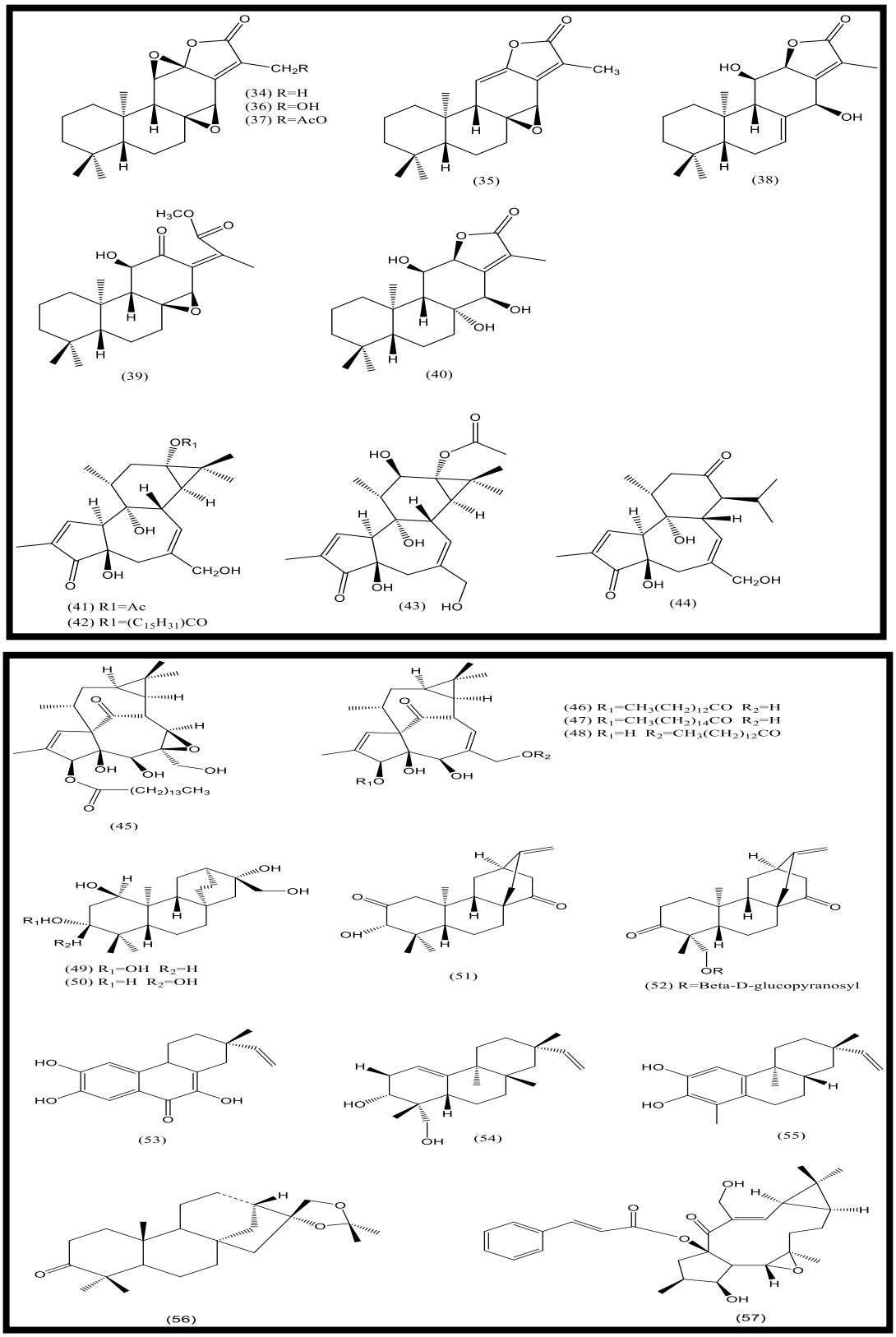


Figure 7: Compounds isolated from Euphorbia fischeriana by Jian *et al*. (2018).

1.3. Commercial Use of *C. megalocarpus*

In agriculture, a report by Rik Thijssen in the book “Domestication and Commercialization of Non-timber Forest Products in Agroforestry Systems” (Leakey, 1996) states that the seed of *Croton megalocarpus* has inspired farmers of Kenya to use the *Croton* seeds contained within the plant for poultry feeding. The article states that the seeds contain a large amount of oil and protein in the ratio of 32:18. However, chickens have been fed *Croton megalocarpus* seeds which has shown no adverse effects and even saved food costs of farmers. This breakthrough has reformed the agricultural industry in Kenya as the seed has shown to be one example of food benefit. Moreover, whilst the nutritious elements of the seed have benefited agricultural purposes, the seedcake has also been used for production of biofuels.

A study by Kafuku *et al* (Kufaku *et al*., 2010) identified the use of biodiesel which was derived from the seed oil of *Croton megalocarpus*. The study had aimed to create and utilise non- edible oil feedstock such as *C. megalocarpus* oil to produce biofuel. Currently, biodiesel is produced from edible oil feedstock such as vegetable oil and is affecting the food industry and may increase food price (Kafuku *et al.,* 2010). This is because vegetable oil is a worldwide used oil and a large amount of vegetable oil crops is used for production of biodiesel. In the world today, it is estimated that 1.4 billion cars are on the road. Therefore, processing a large amount of edible oil means less edible oil for the food industry and therefore the price of oil may increase. Crops that contain non-edible oils such as *Croton megalocarpus* can be produced in large quantities and processed for production of biofuels and replace the use of edible oil feedstock (Kufaku *et al*., 2010). The advantage of using edible oil for livestock such as sunflower oil is an easy and simple transesterification due to their lower free fatty acids (Kafuku *et al.,* 2010). The study went on to illustrate and explore how a one-step transesterification is required to convert the seed oil of the *Croton megalocarpus* into fatty acid methyl esters (Kafuku *et al.,* 2010). Also, the report had highlighted the key parameters that determined the efficiency production of biofuel such as: catalyst concentration, methanol to oil ratio, reaction temperature, agitation speed and reaction time (Kafuku *et al.,* 2010).

A study by Wu *et al* (Wu *et al*., 2012) investigated the use of *C. megalocarpus* biofuel for an alternative sector. The study goes on to describe the lack of electricity in sub-Saharan Africa for home owners as it is an under developed region. This makes it

difficult for the people to be able to run household operations that require electricity, heat, light and water. The study by Wu *et al* (Wu *et al*., 2012) discovered the direct use of *C. megalocarpus* oil (CMO) in a 6.5 kWe micro-trigeneration prototype a machine that is able to distribute energy and electricity through a sustainable and cleaner source. This study showed that direct use of CMO reduces the carbon footprint compared to the transesterification process of the conversion of CMO into biofuel (Wu *et al.,* 2012). The results confirm that pure CMO had produced maximum power of 6.5 kWe and around 13 kW heating output (Wu *et al.,* 2013). This changed the view of the potential of *C. megalocarpus* as its further application of being able to generate power for a region in sub-Saharan Africa and this has the potential to help reduce poverty. Moreover, a study by Kibet *et al* (Kibet *et al*., 2018) also stated how biodiesel is a cleaner alternative as the particulates emitted are reduced from using *C. megalocarpus* biodiesel oil compared to petroleum diesel.

1.4. Tyrosinase

As stated by a review article by Maroyi (Maroyi, 2017), *C. megalocarpus* is used for wound healing and possesses anti-inflammatory, antifungal and antioxidant properties. The use of *C. megalocarpus* for treating skin conditions requires prior safety checks as compounds and chemicals within *C. megalocarpus* could be toxic. The potential of *C. megalocarpus* being used to treat skin conditions opens a sizeable market for the cosmetic and beauty industry as it may be cost effective and more effective than products currently being used today. Skin lightening or skin bleaching is a treatment plan used and contains chemical constituents' corticosteroids, hydroquinone, mercury and other skin lightening agents (Yusuf *et al*., 2019).

The purpose of skin lightening is the treatment of pigment disorders such as melasma and enrichment of the skin in terms of colorization. Since tyrosinase is a key enzyme in the production of melanin, tyrosinase is used as an effective platform for discovering new ways to administer compounds with fewer adverse effects (Chatatikun *et al*., 2017). It has been reported that adverse effects of skin lightening agents include the following complications; acne vulgaris, hypertrichosis, perioral dermatitis, dischromias and ochronosis (Yusuf *et al*., 2019). This is why agents such as hydroquinone are administered in small amounts ranging from 2-5% concentration. Skin lightening

agents have been recognised as a potential health threat which has motivated a large number of countries to ban skin lightening products such as Ghana, Rwanda, South Africa and Sudan (Yusuf *et al.,* 2019). Therefore, it comes to interest on seeing the effect of compound of husks of *C. megalocarpus* against tyrosinase. The potential of compounds being able to inhibit tyrosinase may provide alternative treatment to the skin lightening technique being used today. The action of hydroquinone is shown in **Figure 8.**

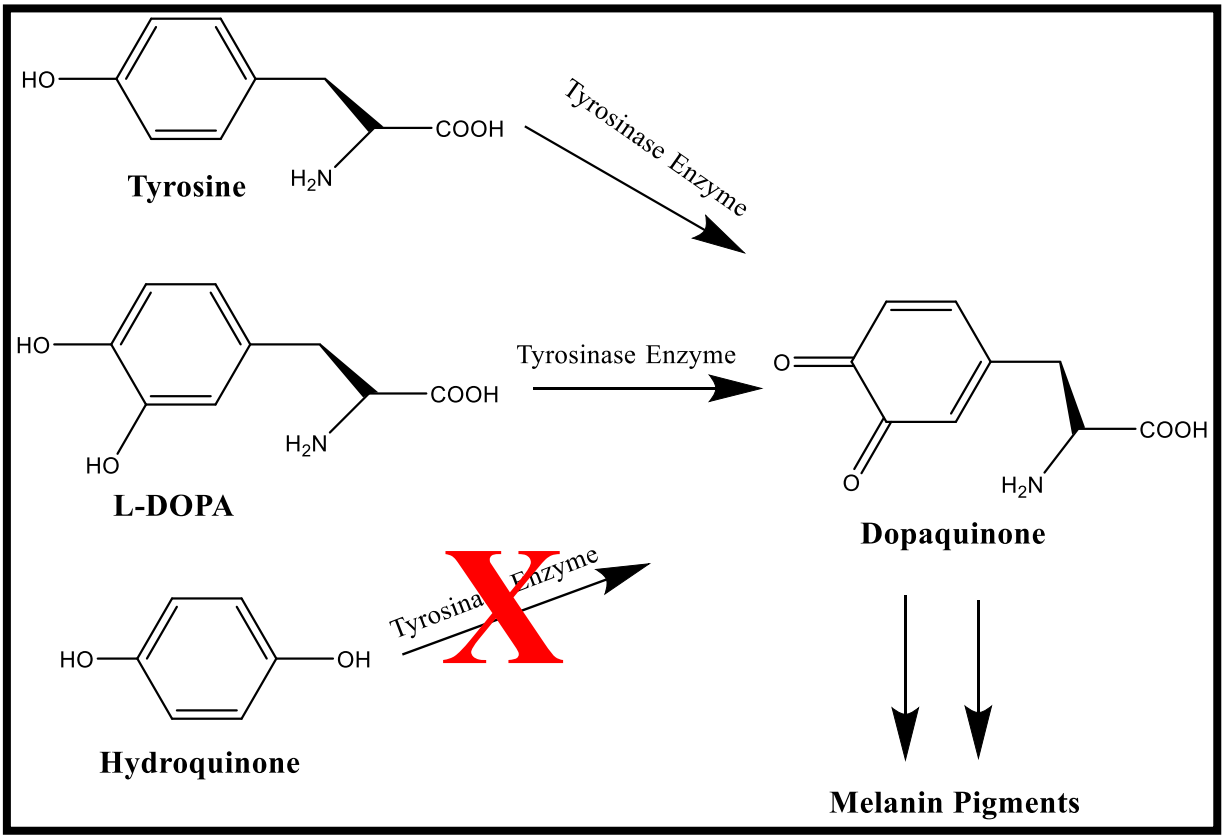


Figure 8: The action of hydroquinone inhibiting tyrosinase. This results in decreased production of dopaquinone. Therefore, this reduces melanin pigments produced for skin lightening treatment. **Figure 8** was illustrated from http://www.becarre-natural.com/lightening.php

Tyrosinase plays an important role with respect to skin cosmetics as testing the bioactivity of tyrosinase may introduce new applications for the cosmetic industry. In the cosmetic industry today in Africa, skin lightening practices are very common. Hydroquinone (HQ) is used mainly to treat skin conditions such as hyperpigmentation, freckles, and melasma (Shin *et al*., 2014). The ability for HQ to strongly inhibit tyrosinase thus reducing hyperpigmentation has enabled it to be used as an effective treatment. However, it can also be noted that whilst HQ is shown to be effective, it has also been

shown to cause cancer and ochronosis. According to a survey study by Dlova *et al* (Dlova *et al*., 2015), six hundred women aged 18-70 years were given a questionnaire on whether they use skin lightening products. The results showed that 32.7% reported using skin lightening products. Of the 32.7% that use skin lightening products, 66.7% said for skin problems and 33.3% for skin lightening. Therefore, skin lightening products have influenced repeated use in South African women. HQ is toxic and so testing the safety of compounds of husks of *C. megalocarpus* may provide a new application as an HQ replacement treatment. Therefore, having a compound that has low toxicity and have the ability to inhibit tyrosinase in the same manner as HQ may be of interest.

A study by Chatatikun *et al* (Chatatikun *et al*., 2017) investigated the anti- melanogenic effect of the ethanolic extracts of *Croton roxburghii* (CRE) and *Croton sublyratus* (CSE) leaves. The study states that both CRE and CSE are known for their ability to be used for medicinal purposes and treatments such as skin diseases, wounds, fever and scabies (Chatatikun *et al*., 2017). However, Chataikun *et al* (Chatatikun *et al*., 2017) also stated that no known reports have been published on any anti-melanogenic effects of extract of CRE and CSE. Similar properties are included with *C. megalocarpus* which is also part of the Euphorbiceae family. The toxicity of CRE and CSE were tested against α- melanocyte stimulating hormone (α-MSH) B16F10 mouse melanoma cells and showed to be non-toxic (Chatatikun *et al*., 2017). The study conducted a tyrosinase inhibition assay to see the quantity of both CRE and CSE that is able to inhibit the enzyme from converting L-DOPA to melanin. Kojic acid was used as a positive control.

The study found that tyrosinase activity had lowered dramatically with 117.16% at 50µg/ml and 159.33% inhibition at 100µg/ml for both CRE and CSE (Chatatikun *et al*., 2017). Kojic acid had inhibited tyrosinase at 150% at a concentration of 250µg/ml. The results also show that both CRE and CSE lower tyrosinase activity at lower concentrations of 6.25-50µg/ml as opposed to kojic acid at 250µmg/ml. These findings show much promise for tyrosinase inhibition of *C. megalocarpus* as CRE and CSE have not previously been reported for anti- melanogenic effects. Therefore, *C. megalocarpus* can be considered for future development in cosmetics and skin care if chemical constituents are found to be non-toxic and able to inhibit tyrosinase (the enzyme responsible for melanin production) for skin care treatment. The most occurrent reaction

mechanism of current skin care agents involve the disruption of tyrosinase. Similarly, the same process can be done for the compounds of the husks of *C. megalocarpus* and seeing if any compound can exhibit the same effect as current agents such as hydroquinone's with fewer adverse effects. This would therefore, introduce new ways of treating skin conditions using natural products.

1.5. Cancer Activity and Statistics

Cancer is a disease that has been one of the biggest challenges in the medical industry. Cancer is the occurrence of cells that begin to divide uncontrollably and create a cluster of cells that become malignant. Cancer cells have the ability to spread to other parts of the body if not treated. According to Cancer Research UK, 17 million new cases of cancer were recorded and 9.6 million deaths have been caused by cancer since 2018. Cancer Research UK also states that 33% of cancer diagnoses were caused by smoking (Cancer Research UK, 2019). However, the four most common cancers reported are bowel, prostate, lung and female breast cancer (Cancer Research UK, 2019). Mortality statistics state that lung, stomach, bowel and liver are the most common causes of cancer deaths accounting for more than four in ten of all cancer deaths (Cancer Research UK, 2019).

According to the World Health Organisation (WHO), breast cancer is the most common form of cancer in females. The WHO state that 50% of breast cancer cases and 58% of mortality happens in less developed countries due to the lack of an efficient healthcare system. However, the WHO determine that breast cancer survival rates vary from different parts of the world such as >80% in Japan, Sweden and North America, 60% cases in middle income countries and <40% in low income countries (World Health Organisation, 2019). Countries that have low income are more vulnerable as breast cancer cannot be detected early due to poor facilities and equipment.

A study by Wong *et al* (Wong *et al*., 2017) found that 1.8 million new cases of lung cancer were discovered and a total of 1.6 million died of lung cancer in 2012. The constituents isolated from *Croton* species may show significant cytotoxicity and as such could provide leads for novel chemotherapies. A study by Chatatikun *et al*. (Chatatikun *et al*., 2017) reported the cytotoxic effect of ethanolic extract of *Croton roxburghii* (CRE) and *Croton sublyratus* (CSE) leaves on B16F10 cells. The cells were

treated and incubated for 48hrs. The study found that both CRE and CSE showed no cytotoxic effects from concentrations ranging from 3.125-100µg/ml for both CRE and CSE. The results were significant ***P<.001. Another study by Mulholland *et al* (Mulholland *et al*., 2010) reported the action of compounds (1) and (4) **(Figure 4)** against PEO1 and PEO1TaxR (taxane resistant) ovarian cancer cell lines. The results showed that both compound **(**1) and (4) were found inactive against PEO1 ovarian cancer cells. This goes to show that cytotoxicity effect has been tested for various compounds and extracts and shows the gap in the market for validating a compounds ability to have a positive effect on cancer.

Aims & Objectives

The aim of this study was to extract and isolate the phytochemical compounds from *C. megalocarpus* husks, characterise the isolates, and carry out a tyrosinase inhibitory activity assessment and CellTiter MTS assay. Extract phytochemical compounds using cold solvent on a shaker, isolate compounds using column chromatography and thin-layer chromatography (TLC), characterise the isolates using Nuclear Magnetic Resonance (NMR), High Resolution Mass Spectrometry (HRMS) and Infrared (IR), determining cell cytotoxicity of FM-55 Human Melanoma Cells using CellTiter Assay and determine the tyrosinase inhibitory activity using a mushroom tyrosinase inhibition assay on a 96 well plate.

2. Experimental

2.1. Materials and chemicals

Analytical grade methanol, hexane, dichloromethane, ethyl acetate, ethyl ether, acetonitrile, p-anisaldehyde, chloroform, deuterated chloroform, deuterated methanol, silica gel 60 (0.015-0.040 mm), pre-coated TLC sheet ALUGRAM SIL G/UV_{254} 0.20 mm silica gel 60 with fluorescent indicator UV_{254}, Dulbecco modified eagle medium, feline bovine serum (FBS), streptomycin penicillin, PrestoBlue, hydrogen peroxide (30% solution), dimethyl sulfoxide (DMSO), kojic acid, L-tyrosine, mushroom tyrosinase, Nunclon 96 well plates, FM-55 human melanoma cells (Public Health England PHE) and Etoposide were all purchased from Sigma-Aldrich LTD, The Old Brickyard, NEW

ROAD, GILLIGHAM, DORSET, SP8 4XT, UK. The *C. megalocarpus* plant husks provided by EFK commercially as *C. megalocarpus* nuts and kept in the freezer for preservation. Croton vinegar oil was provided by EFK. Analytical instruments used include NMR Bruker Avance 400 MHz Coventry, UK, NicoLet IS5 I ID1 transmission IR machine Paisley, UK, TECAN Infinite M200 microplate reader Theale, UK and HPLC-MS (Velos-Pro, Thermo Fisher Scientific; Phenomenex C18 Column: 150 x 3mm Particle Size.

2.2. Preparation of husks of *C. megalocarpus*

The *C. megalocarpus* plant husks (650g) were grounded into fine powder using a Blendor produced by Waring. Once grounded, the husk powder was stored in a 1000 mL conical flask.

2.3. Determining of water content of the husks of *C. megalocarpus*

The water content of *C. megalocarpus* was determined to measure the moisture content. To begin with, ground *Croton husks* (1.0345g) were weighed and placed in an oven that was set at 120^{o}C for 60 minutes. Once dehydrated, the husks were left to cool for 10 minutes. Once cooled, the dried husks were reweighed to see the difference in mass.

2.4. Extraction

The husks were extracted successively with hexane, dichloromethane and methanol using the method outline by Mfotie Njoya *et al* (Mfotie Njoya *et al*., 2018). The husks were soaked in hexane overnight and followed by filtration. Compounds were extracted according to their polarity from non-polar to polar. This process was repeated twice for each solvent. Once filtered, the crude extract for each solvent was concentrated by evaporation using Buchi Rotavapor R-114 and Buchi Waterbath B-480 at 50^{o}C. The Genevac EZ-2 plus HCI compatible Winchester, UK was used to evaporate any remaining solvent for each of the crude extracts of hexane, dichloromethane and methanol. The parameters for the evaporation was set at 45^{o}C. After evaporation, the following were yielded: hexane (27.90g), dichloromethane (8.30g) and methanol (50.80g). The dichloromethane extract was chosen for further analysis. This is

because dichloromethane is a standard solvent used in extracting a wide range of organic compounds due to its semi-polar properties and upon examination of the ^{1}H NMR, the dichloromethane extract showed the most promising compounds.

2.5. Thin-Layer chromatography

Thin-Layer chromatography was used to separate the compounds for each crude extract of hexane, dichloromethane and methanol. The aim of this technique was to determine the range of compounds present in each extract based on their different polarities. A pre-coated TLC sheet ALUGRAM SIL G/UV_{254} was used to spot 10µL of each crude extract hexane, dichloromethane and methanol. Different solvent systems (mobile phase) were tested to determine best separation of compounds. Some systems tested were made up of a mixture of two solvents.

The different solvent systems tested were: hexane (100%), chloroform (100%), dichloromethane (100%), dichloromethane with hexane, dichloromethane with ethyl acetate and ethyl acetate (100%). The solvent system that gave best separation of the three crude extracts was a combination of dichloromethane and ethyl acetate at a ratio of 1:1. A total volume of 20ml of solvent system was poured into the chamber tank and left to sit for 20 minutes to saturate the tank. The aluminium backed silica gel plate was placed in the chamber tank and allowed for separation until reaching solvent front. The plate was viewed under UV light of 365 nm and 254 nm. Visible bands were circled using a pencil and plate was then visualised using p-anisaldehyde spray reagent. The p-anisaldehyde reagent was sprayed evenly upon the TLC plate, covering the entire plate. The plate was then heated using a blow dryer until turning pink. Rf values were not measured as this was a visual procedure to have an idea of compounds present in the three crude extracts.

2.6. Flash Chromatography – Column

Column chromatography was used to isolate the compounds of the dichloromethane. To begin with, dichloromethane extract was mixed with silica gel and left overnight. The purpose of the mixture was to absorb it onto the silica gel to make it easier to load onto the column. A teaspoon of sand was placed into the column to form the bottom

base followed by silica gel slurry. The silica gel slurry was made by mixing silica gel with dichloromethane in a 250 mL beaker. The silica gel slurry was the stationary phase of the column. However, the powdered dichloromethane extract was then placed inside the column to rest on top of the silica gel. The remainder of the column was then filled with the elution solvent that started off with hexane. Once compounds had started to separate through the column, fractions were collected in small 100 mL beakers. Column chromatography was used in conjunction with TLC and NMR. This was to determine which fractions from the column contained compounds and any similar compounds which was used for further analysis. After 100 fractions, two solvent systems were used for the column in alternating ratio percentages of dichloromethane and hexane. A total of 214 fractions were collected for the dichloromethane extract roughly collecting 3ml per vial.

2.7. Compound Purification

Identical fractions identified using NMR and Thin-Layer chromatography were then combined. Combined fractions of interest were purified using column chromatography. Several combined fractions were analysed as these fractions contained compounds of interest. The method required setting up the column and filling with silica. However, two different solvent systems were used for each fraction as Thin-Layer chromatography analysis showed that the compounds within each fraction had travelled up the TLC plate to various different polarity solvents. A combination of dichloromethane and ethyl acetate (29:1) were used for fractions 69-74, dichloromethane and hexane (12:8) was used for fraction 34-36 and dichloromethane and ethyl acetate (28:2) was used for fraction 80-81 from the 214 fractions collected. Once the column was set up, small fractions were collected and analysed using NMR Bruker Avance 400 MHz Coventry, UK to check for purity and identification of the compound molecular structure. The following compounds were isolated and purified: (19), (+)-[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide (5), (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide (7), Epoxykaurane (56), Ozic acid (58), Vanillin (59) and Sartone A (60) **(Table 2)**

2.8. Infra-red Spectroscopy (IR)

The isolated compounds were further analysed using IR. IR was used to produce peaks of the different functional groups of the compound. A small amount of dichloromethane was added to each compound to dissolve. Once done, a few drops of the compound were pipetted onto the Sodium chloride disc and allowed for DCM to evaporate. Once evaporated, the disc was placed in a NicoLet IS5 I ID1 transmission IR machine Paisley, UK and spectra were produced **(Appendix E)**.

2.9. Optical Rotation

Isolated compounds were further analysed using optical rotation. Optical rotation determined the angle of rotation plane polarised light for chiral compounds. The solvent used was dichloromethane and the optical rotation was observed in degrees of rotation at a concentration of 1mg/ml.

2.10. Cell Viability

DAY 1 – The assay methodology was conducted according to a study by Shahbazi-Gahrouei *et al* (Shahbazi-Gahrouei *et al.,* 2016). In preparation for cell culture, media from a T-25 flask containing FM-55 cells were aspirated and added with 1 ml trypsin. The flask was placed in incubator for 5 minutes to allow for detachment of cells from flask surface. Once incubated, flask was viewed under microscope to check for 75-80% confluency. The cell suspension was transferred into a small conical tube and added with 10 ml fresh media and centrifuged for 5 minutes at 1000 rpm using Centrifuge 5430. Once centrifuged, media was decanted and 5 ml fresh media was added. 20µl cell suspension was viewed under microscope for cell counting. Cells were counted using a haemocytometer to calculate cells needed for seeding. Finally, Nunclon 96 well plates were treated with FM-55 cells for 24 hrs.

DAY 2 – Stock solutions were prepared for each compound being tested. Stock solutions were made up to 20 mg/ml. Serial dilutions were prepared for each sample according to Appendix B and B1. The first step involved the addition of 50µl of each sample stock solution to wells A1-A12. The positive control in the assay was Etoposide (well E11) and the negative control was DMSO (well E12). Once samples were added, 50µL DMSO was added to each sample to produce a 10mM concentration (x2 fold)

and in sequence to row A and E. Then 100µL DMSO was added to each sample to make a 5mM concentration. Once done, the samples were diluted with DMEM at 50, 25, 12.5 and 6µg/ml as shown in Appendix C. Once serial dilutions were prepared, 2µl of each dilution was added to 198µl FM-55 cells using epMotion 96 automatic pipette dispenser and incubated for 24hrs.

DAY 3 – After 24hrs incubation, cells were treated with 10 µL CellTiter reagent and a first reading was taken 2 hours after addition of CellTiter reagent using TECAN Infinite M200 microplate reader Theale, UK at 490 nm.

2.11. Tyrosinase Inhibition Assay

2.11.1. Preparation

The compounds, *C. megalocarpus* plant extracts and reagents associated with the assay was prepared as follows. Firstly, pure compounds and *C. megalocarpus* plant extracts were prepared respectively at 20mg/ml (crude extract) and 20mM (pure compounds) with dimethyl sulfoxide (DMSO). A phosphate buffer pH 6.5 was prepared by mixing 8 ml 0.2M Na_2HPO_4 (2.84g/100ml) with 17ml 0.2M NaH_2PO_4 (2.40g/100ml) and 75ml H_2O to produce a 50mM working buffer. Kojic acid (positive control) was prepared with 284.22mg in 100ml deionized water to give 20mM. L-tyrosine was prepared with 1.087mg of substrate with 6ml phosphate buffer and sonicated for 30 minutes. Mushroom tyrosinase was prepared freshly on day of assay. 1mg of mushroom tyrosinase was added to 20.53ml phosphate buffer producing 50 units/ml.

2.11.2. Tyrosinase inhibition (96 well plate)

A Nunclon transparent 96 well plate was used for the assay. To begin with, 45µl phosphate buffer was added to control and sample wells whilst 145µl was added to blank wells. Once added, 5µl of DMSO (negative control), kojic acid (positive control) and sample was respectively added to each well according to Appendix A. Moreover, 50µl L-tyrosine was added to all wells using a multichannel pipette. The 96 well plate was

then shaken for 5 seconds and left to incubate at room temperature for 2 minutes. After 2 minutes, the plate was read using TECAN Infinite M200 microplate reader for the background optical density at 475nm. This pre reading was sample + substrate excluding tyrosinase enzyme (Tyro-1st). Once reading was recorded, 100µl tyrosinase enzyme was added to control and sample wells only. This was done using a multi-channel pipette to start the reaction at the same time. Once added, the plate was shaken for 5 seconds and left to incubate for 15 minutes at room temperature (25^{o}C). After 15 minutes, optical density was read at 475nm (Tyro-2nd). The tyrosinase inhibition activity percentage was calculated using the following formula. Data for both CellTiter assay and tyrosinase inhibition was process using GraphPad Prism 8.3.1 (549). Analytcial tools used include One-way ANOVA and a t test unpaired test.

$$\% \text{ inhibition} = [(A_{ctrl} - A_{sample}) / A_{ctrl}] \times 100$$

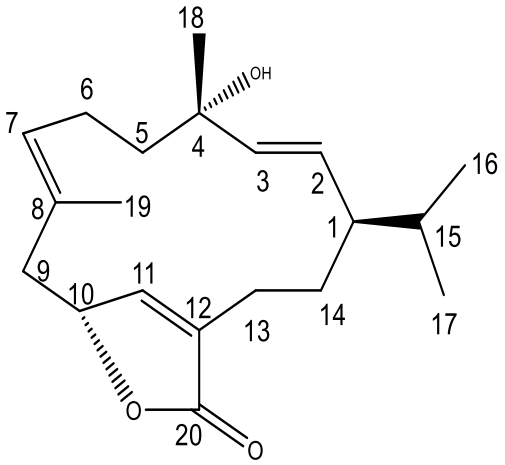
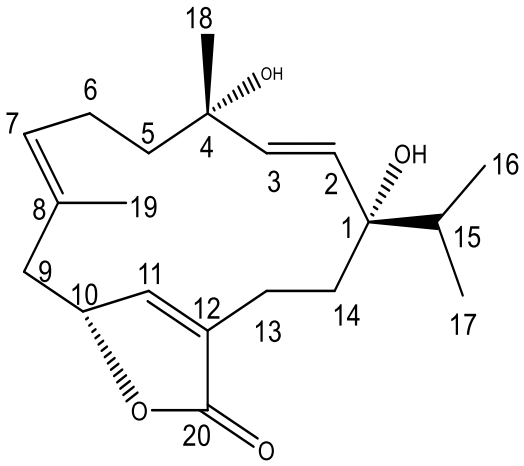
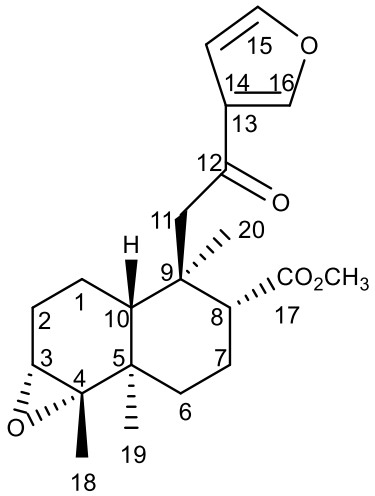
A_{ctrl} = average optical density of DMSO + substrate with tyrosinase

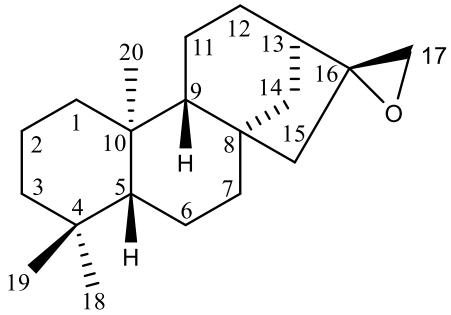
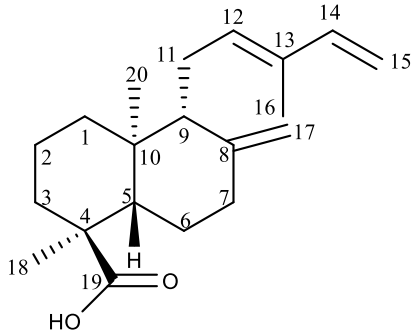
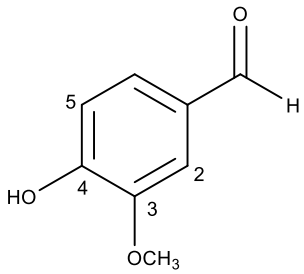
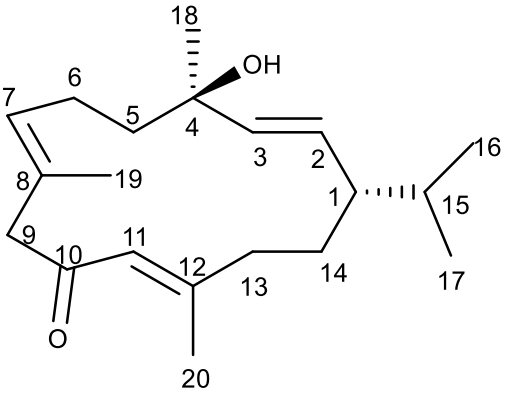
3. Results

3.1. Extraction and Isolation

The *C. megalocarpus* husks were ground to powder form and were subject to a series of solvent based extractions using hexane, dichloromethane and methanol. The following solvent extractions were produced: hexane (27.90g), DCM (8.30g) and methanol (50.80g). The water content that was found in the husks of *C. megalocarpus* was 9.17%. The dichloromethane extract was chosen for further analysis due to its ability to extract a wide range of organic compounds and promising compounds that was determined by 1H NMR, and the compounds isolated and purified using column and thin layer chromatography (TLC) techniques were (+)-[1R*,4S*,10R*]-4-hydroxy-cembra-2E, 7E,11Z-trien-20,10-olide (5), (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide (7), Epoxychiromodine (19), Epoxykaurane (56), *Trans* Ozic acid (58), Vanillin (59) and Sartone A (60) **(Table 2)**. Moreover, pure compounds were fully characterised using NMR, HRMS and IR. Processed ^{1}H and ^{13}C spectra can be seen in **Appendix F**.

Table 2: HRMS of Compounds Isolated from Dichloromethane Extract of Husks of *C megalocarpus*

ID	*Name*	*Class*	*Accurate Mass (g)*	*Chemical Formula*	*Structure*
5	**(+)-[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide.**	**Cembranolide (Diterpenoid)**	**357.20 (LCMS)**	**$C_{20}H_{30}O_2$**	
7	**(-)-(1S*,4S*,10R*)-1,4- dihydroxy-cembra-2E,7E,11Z-trien-20,10-olide**	**Cembranolide (Diterpe-noids)**	**357.20 (LCMS)**	**$C_{20}H_{30}O_3$**	
19	**Epoxychiromodine**	**Clerodane (Diterpenoid)**	**346.1780**	**$C_{20}H_{26}O_5$**	

56	**Epoxykaurane**	**Kaurane (Diterpenoid)**	**288.2453**	**$C_{20}H_{32}O$**	
58	**8(17),12, 14-Labdatrien-18-ol (Ozic Acid)**	**Labdane (Diterpenoid)**	**302.458**	**$C_{20}H_{30}O_2$**	
59	**Vanillin**	**Phenolic Aldehyde**	**152.149**	**$C_8H_8O_3$**	
60	**Sartone A**	**Cembrane (Diterpenoid)**	**304.2402**	**$C_{20}H_{32}O_2$**	

3.2. Compounds Isolated

Table 3: Correlation Table for NMR Data of Compound 56: *Ent*-16*S*,17-epoxykaurane.

Carbon No.	^{13}C NMR (100 MHz) in $CDCl_3$	^{13}C NMR (100 MHz) in $CDCl_3$ (Lopes *et al*., 1990; Aljancic *et al*., 1996)	^{1}H NMR (400 MHz) $CDCl_3$ (*J* in Hz)
1α	40.6 CH_2	40.4	1.79 m
1β			0.76 m
2α	18.8 CH_2	18.6	1.58 m
2β			1.40 m
3α	42.2 CH_2	42.0	1.36 m
3β			1.11 m
4	33.4 C	33.2	-
5	56.4 CH	56.2	0.79 m
6α	20.4 CH_2	20.2	1.54 m
6β			1.33 m
7α	41.3 CH_2	41.1	1.54 m
7β			1.54 m
8	45.6 C	45.4	-
9	56.1 CH	55.9	1.04 m
10	39.5 C	39.3	-
11α	19.5 CH_2	19.3	1.71 m
11β			1.60 m
12α	29.4 CH_2	29.2	1.59 m
12β			1.55 m
13	42.9 CH	42.7	1.75 m
14α	38.8 CH_2	38.6	2.02 dd *J* = 2.0, 11.5 Hz
14β			1.42 m
15α	49.1 CH_2	48.9	1.66 m
15β			1.60 m
16	66.6 C	66.4	-
17A	50.6 CH_2	50.4	2.86 d *J* = 5.0 Hz
17B			2.78 d *J* = 5.0 Hz
18	21.8 CH_3	21.6	0.80 s
19	33.8 CH_3	33.6	0.84 s
20	18.0 CH_3	17.8	1.02

Table 4: Correlation Table for NMR Data of Compound 58: *Trans* Ozic Acid.

Carbon no.	^{13}C NMR in $CDCl_3$	^{13}C NMR in $CDCl_3$ (Du *et al*.,2001)	^{1}H NMR
1	38.3	38.1	1.84 m
			1.21 m
2	18.6	18.4	1.63 m
			1.63 m
3	37.8	37.6	2.37 m

			2.09 m
4	47.6	47.5	-
5	57.2	57.0	1.86 m
6	26.8	26.6	1.48 dd *J* = 4.3, *J* = 12.7 Hz
			1.37 m
7	37.3	37.1	1.79 m
			1.66 m
8	148.0	147.8	-
9	49.6	49.4	1.99 dd *J* = 2.8, *J* = 12.7 Hz
10	39.0	38.8	-
11	23.2	23.0	2.36 m, 2.16 m
12	133.9	133.6	5.41 t *J* = 6.4 Hz
13	133.8	133.5	-
14	141.8	141.6	6.33 dd *J* = 10.8, *J* = 17.3 Hz
15	110.2	109.9	5.05 d *J* = 17.3 Hz
			4.89 d *J* = 10.8 Hz
16	16.6	16.4	117 s
17	108.4	108.2	4.83 *J* = 1.0 Hz
			4.48 *J* = 1.0 Hz
18	184.2	185.3	-
19	14.9	14.7	0.76 s
20	12.1	11.6	1.75 *J* = 0.5 Hz

Table 5: Correlation Table for NMR Data of Compound 19: Epoxychiromodine

Carbon No.	**^{13}C NMR (100 MHz) in $CDCl_3$**	**^{13}C NMR (100 MHz) in $CDCl_3$ (Addae-Mensah et al., 1992)**	**^{1}H NMR (400 MHz) $CDCl_3$ (*J* in Hz)**
1α	16.4 CH_2	16.17 CH_2	1.78 m
1β			1.23 s
2α	27.9 CH_2	27.65 CH_2	1.55 m
2β			2.01 d *J* = 3.2 Hz
3	62.4 CH	62.13 CH	2.87 brs $W_{1/2}$ = 2.8 Hz
4	66.3 C	66.01 C	-
5	40.6 C	40.39 C	-
6α	35.7 CH_2	35.49 CH_2	1.67 m
6β			1.45 d *J* = 4.0 Hz
7α	22.4 CH_2	22.16 CH_2	1.99 d *J* = 3.0 Hz
7β			1.70 m
8	48.8 CH	48.59 CH	3.21 dd *J* = 3.8, 13.2 Hz
9	37.2 C	37.04 C	-
10	47.0 CH	46.82 CH	1.80 m
11α	47.4 CH_2	47.20 CH_2	2.86*
11β			
12	194.7 C	194.44 C	-
13	129.2 C	129.0 C	-
14	108.8 CH	108.56 CH	6.73 d *J* = 1.8 Hz
15	144.3 CH	144.10 CH	7.40 s

16	147.3 CH	147.04 CH	8.02 brs $W_{1/2}$ = 3.0 Hz
17	175.1 C	174.86 C	-
18	19.8 CH_3	19.83 CH_3	1.15 s
19	16.9 CH_3	16.64 CH_3	1.08 s
20	20.0 CH_3	19.61 CH_3	0.88 s
17-OMe	51.4 CH_3	51.13 CH_3	3.59 s

Table 6: Correlation Table for NMR Data of Compound 60: Sartone A

Carbon no.	**^{13}C NMR in $CDCl_3$**	**^{13}C NMR in $CDCl_3$ (Iwagawa et al., 1996)**	**^{1}H NMR**
1	46.5	46.3	1.52 m
2	130.2	130.0	5.28 dd *J* = 8.6, 16.1 Hz
3	137.6	137.4	5.63 d *J* = 16.1 Hz
4	72.6	72.4	-
5	42.6	42.4	2.02 m
			1.56 m
6	22.6	22.3	2.58 m
			2.14 m
7	133.7	133.4	5.38 m
8	129.0	128.8	-
9	57.3	57.1	3.03 d *J* = 14.0 Hz
			2.86 d *J* = 14.0 Hz
10	200.3	199.9	-
11	122.3	122.1	6.23 s
12	158.0	157.8	-
13	38.9	38.6	-
14	27.8	27.6	6.23 s
15	33.0	32.8	1.50 m
16	19.4	19.4	0.81 d *J* = 7.0 Hz
17	20.8	20.8	0.83 d *J* = 7.0 Hz
18	28.6	28.4	1.40 s
19	16.8	16.5	1.65 s
20	18.0	17.7	2.09 s

Table 7: Correlation Table for NMR Data of Compound 5: (+)-[1*R,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide**

Carbon No.	**^{13}C NMR (125 MHz) in $CDCl_3$**	**^{13}C NMR (100 MHz) in $CDCl_3$ (Mulholland et al., 2010)**	**^{1}H NMR (400 MHz) $CDCl_3$ (*J* in Hz)**
1	48.4 CH	48.4 CH	1.57 m
2	131.4 CH	131.4 CH	5.43 dd *J* = 8.7, 15.7
3	138.0 CH	138.0 CH	5.61 d *J* = 15.7 Hz
4	72.5 C	72.5 C	-
5α	43.7 CH_2	43.7 CH_2	1.93 m
5β			1.51 m

6α	25.6 CH_2	25.6 CH_2	2.33 m
6β			1.59 m
7	131.1 CH	131.1 CH	5.37 t *J* = 7.5 Hz
8	129.4 C	129.4 C	-
9α	44.7 CH_2	44.7 CH_2	2.80 dd *J* = 2.6, 12.7 Hz
9β			2.07 dd *J* = 2.6, 12.7 Hz
10	80.3 CH	80.3 CH	5.04 br s $W_{1/2}$ = 19.6 Hz
11	149.6 CH	149.6 CH	6.97 d *J* = 3.4, 1.7 Hz
12	133.6 C	133.6 C	-
13α	22.7 CH_2	22.7 CH_2	2.20 m
13β			2.20 m
14α	28.1 CH_2	28.2 CH_2	1.94 m
14β			1.49 m
15	33.9 CH	33.8 CH	1.60 m
16	20.4 CH_3	20.4 CH_3	0.84 d *J* = 6.5 Hz
17	19.6 CH_3	19.7 CH_3	0.89 d *J* = 6.5 Hz
18	28.2 CH_3	28.1 CH_3	1.37 s
19	16.5 CH_3	16.5 CH_3	1.67 s
20	174.2 C	174.2 C	-

Table 8: Correlation Table for NMR Data of Compound 7: (-)-(1S*,4S*,10R*)-1,4- dihydroxy-cembra-2E,7E,11Z-trien-20,10-olide

Carbon No.	**^{13}C NMR (100 MHz) in $CDCl_3$**	**^{13}C NMR (100 MHz) in $CDCl_3$ (Langat *et al*., 2011)**	**^{1}H NMR (400 MHz) $CDCl_3$ (*J* in Hz)**
1	78.9 C	77.9 C	-
2	132.7 CH	131.9 CH	5.65 br s $W_{1/2}$ = 1.8 Hz
3	136.8 CH	135.8 CH	5.64 br s $W_{1/2}$ = 1.8 Hz
4	74.9 C	72.9 C	-
5α	43.3 CH_2	42.4 CH_2	1.71 m
5β			1.73 m
6α	24.1 CH_2	23.2 CH_2	2.16 m
6β			2.08 m
7	133.2 CH	131.8 CH	5.15 m
8	129.1 C	128.1 C	-
9α	41.9 CH_2	42.4 CH_2	2.54 m
9β			2.58 m
10	82.8 CH	80.6 CH	5.26 m
11	150.3 CH	148.5 CH	7.20 br s $W_{1/2}$ = 1.8Hz
12	136.0 C	135.3 C	-
13α	21.5 CH_2	21.3 CH_2	2.30 m

13β			2.32 m
14α	34.5 CH_2	33.7 CH_2	2.01 m
14β			1.92 m
15	38.9 CH	38.9 CH	1.74 m
16	17.9 CH_3	17.1 CH_3	0.96 d *J* = 6.8 Hz
17	17.6 CH_3	17.5 CH_3	0.92 d *J* = 6.8 Hz
18	29.7 CH_3	28.8 CH_3	1.29 s
19	18.9 CH_3	17.9 CH_3	1.59 s
20	176.6 C	174.5 C	-

Table 9: Correlation Table for NMR Data of Compound 59: Vanillin

Carbon No.	**^{13}C NMR (100 MHz) in $CDCl_3$**	**^{13}C NMR (100 MHz) in $CDCl_3$ (Bai et al. 2018)**	**^{1}H NMR (400 MHz) $CDCl_3$ (*J* in Hz)**	**^{1}H NMR (400 MHz) $CDCl_3$ (*J* in Hz) (Mukonyi and Ndiege)**
1	133.3 C	135.7	-	-
2	108.9 CH	111.3	7.41 d *J* = 2.0 Hz	7.41 m
3	149.6 C	152.0	-	-
4	147.5 C	149.9	-	-
5	114.5 CH	122.9	7.04 d *J* = 8.5 Hz	7.03 d *J* = 8.6 Hz
6	127.7 CH	124.7	7.37 d *J* = 2.0, 8.5 Hz	7.41 m
7	191.1 CH	190.1	9.82 s	9.81 s
3-OCH_3	56.3	56.4	3.97, s	3.96, s
4-OH	-	-	6.21, s	6.21, s

Table 10: Correlation Table for IR and LC-MS Data of The Five Compounds Isolated

Compound	**Absorption Band Frequency cm^{-1}**	**Molecular Ion Peak [M]$^{+}$ m/z**
3,4-Epoxide-15,16-epoxy-3,13(16),14-clerodatriene (19)	3452 and 2848 C-H stretch 1713 C=O stretch	346.1
Ent-16*S*,17-epoxykaurane (56)	2848 C-H stretch 1461 C-H bend alkane group 1110 C-O stretch epoxide	288.2
8(17),12, 14-Labdatrien-18-ol trans ozic acid (58)	2930, 2867 and 1693 C-H stretch for CH_2 and CH_3 1642 and 891 stretch C=C	302.4
Vanillin (59)	3417 OH 1714 C=O stretch 1714 and 1264 C=O and R-O-R	152.14
Sartone A (60)	3054 dicative OH group 1712 and 1614 β unsaturated C=O moiety	304.2

3.3. Mushroom Tyrosinase Inhibition Assay

The *C. megalocarpus* compounds isolated from the husks were subject to bioassays including mushroom tyrosinase inhibition assay **(Figure 9)**. Several extracts of *C. megalocarpus* were tested to see any variance of properties that each extract may have. The husks, seedcake and fruit cake were subject to different solvent extracts of hexane, dichloromethane and methanol. For the assay, all isolated compounds were prepared at 20mM whereas extracts were made at 20mg/ml. The *Croton* vinegar oil is an organic fertiliser and was tested in the same manner as the other samples. After the assay was conducted, several compounds and extracts that showed interesting inhibition were repeated for validation **(Figure 10)**.

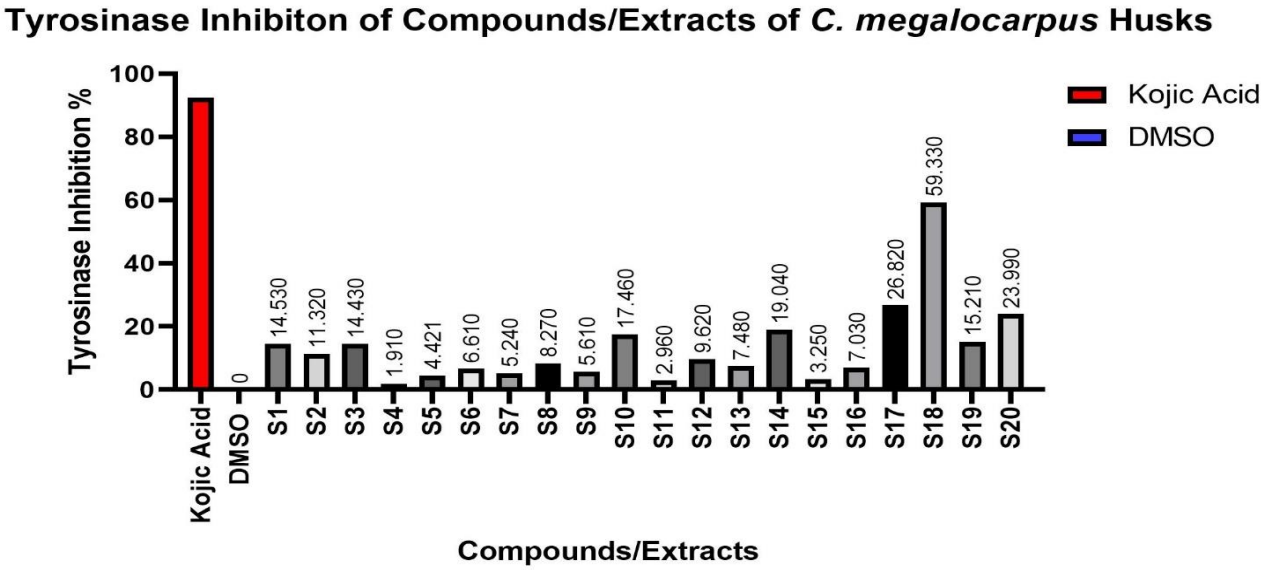


Figure 9: Tyrosinase inhibition of compounds/extracts of husks of *C. megalocarpus*. Hexane extract of husks (S1), hexane extract of husks with mould (S2), hexane extract of fruit cake (S3), hexane extract of seeds (S4), hexane extract oil (S5), methylene chloride extract of husks (S6), methylene chloride extract of husks with mould (S7), methylene chloride extract of fruit cake (S8), methylene chloride extract of seeds (S9), methylene chloride extract of *Croton* vinegar oil (S10), methanol extract of husks (S11), methanol extract of fruit cake (S12), methanol extract of seeds (S13), *trans* ozic acid (S14), sartone A (S15), epoxykaurane (S16), epoxychiromodine (S17), ethyl acetate extract of *Croton* vinegar oil (S18), pure Croton vinegar oil (S19) and magnoflorine (S20).

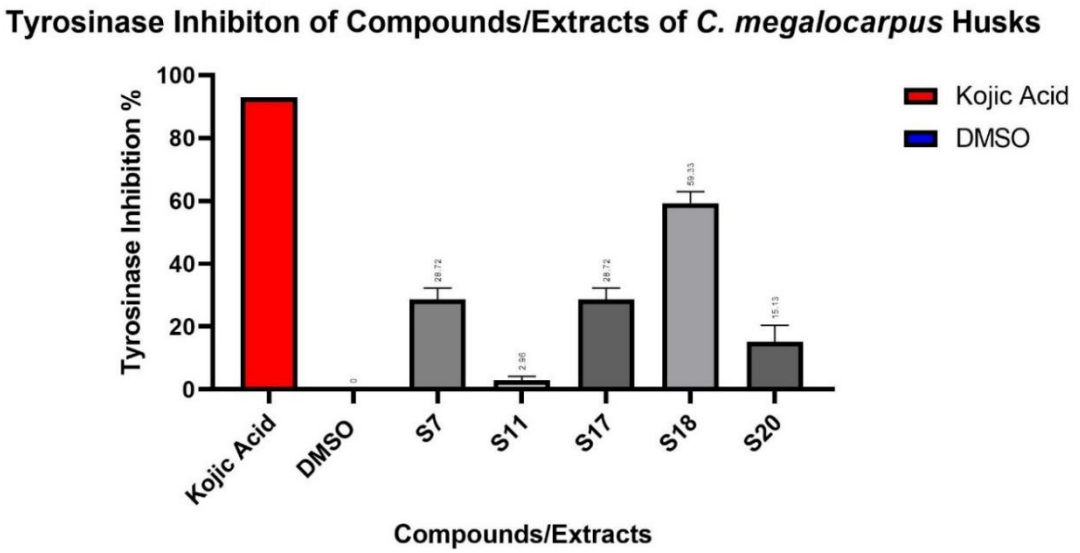


Figure 10: Tyrosinase inhibition of the five compounds/extracts of interest. epoxychiromodine (S17), magnoflorine (S20), methanol extract of husks (S11), methylene chloride extract of husks with mould (S7) and ethyl acetate extract of croton vinegar oil (S18) n=3. Compounds/extract (S7) ($p<0.0052$), (S17) ($p<0.0052$), (S18) ($p<0.0012$) and (S20) ($p<0.0393$) were significant $p<0.05$ compared to kojic acid. Methanol extract of husks (S11) showed to not be significant with $p<0.0542$.

3.4. FM-55 Human Melanoma Cells (Cytotoxicity Assay)

The *C. megalocarpus* compounds isolated from the husk were subject against FM-55 human melanoma cells. The same compounds and extracts were tested in the same manner as the tyrosinase assay. In this assay, the results provide a varied analysis for the effect of compounds and extracts on FM-55 human melanoma cells. Isolated compounds were made at 20mM and extracts were made at 20mg/ml. The compounds and extracts were done in triplicate repeats. Absorbance data were converted to cell viability %.

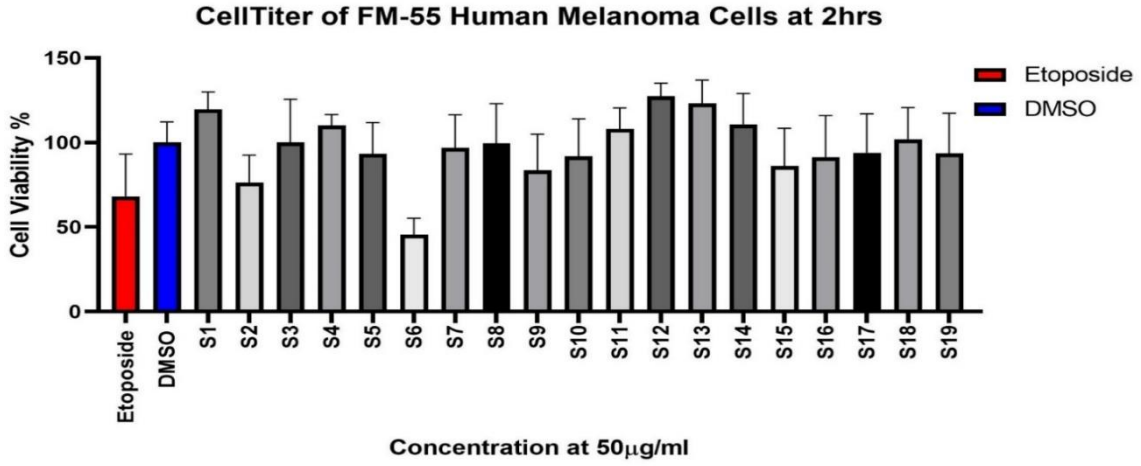


Figure 11: Cell Viability of compounds and extracts at 50µg/ml

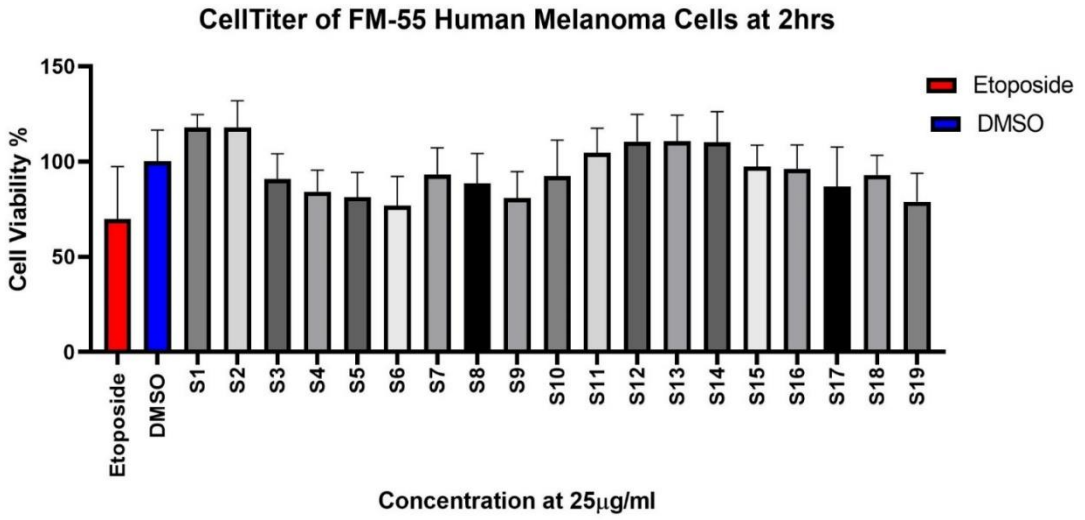


Figure 11A: Cell Viability of compounds and extracts at 25µg/ml

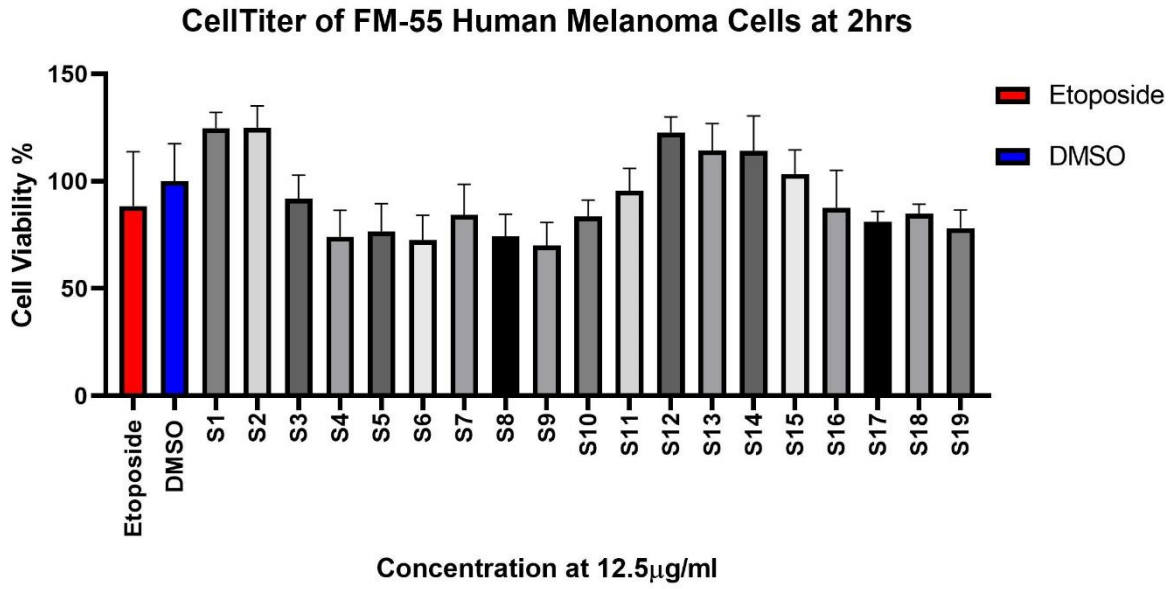


Figure 11B: Cell Viability of compounds and extracts at 12.5µg/ml

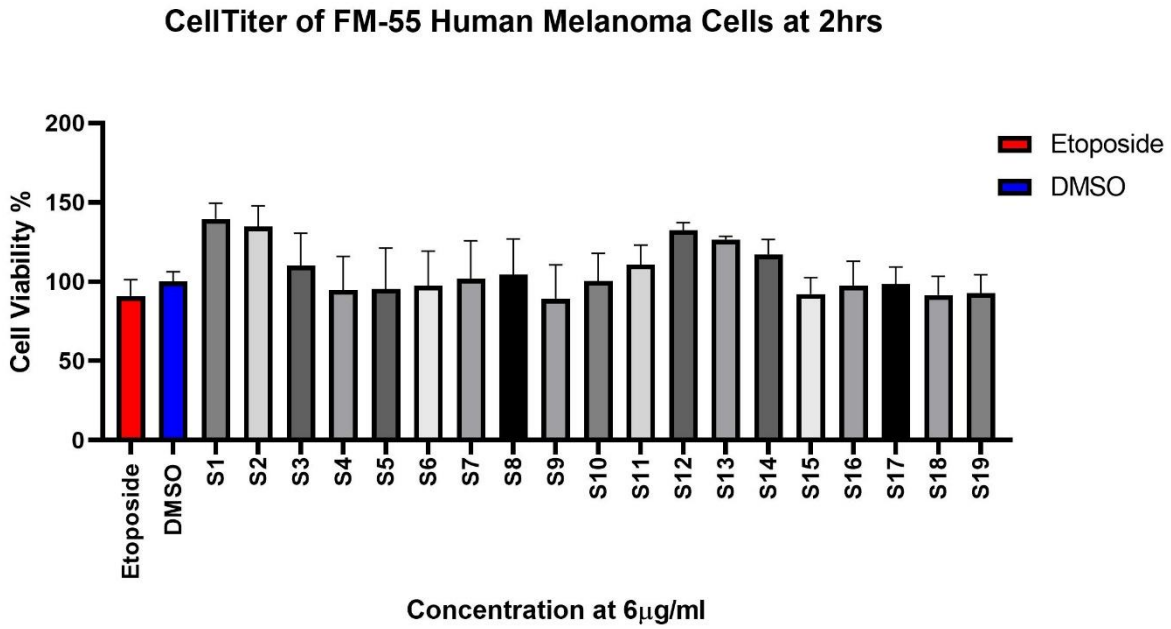


Figure 11C: Cell Viability of compounds and extracts at 6µg/ml

Figures 11, 11A, 11B, 11C: Data accumulated from mean and standard deviation of each extract, n=3 from GraphPad Prism 8. Etoposide used as positive control and DMSO used as negative control. Hexane extract of husks (S1), hexane extract of husks mould (S2), hexane extract of fruit cake (S3), hexane extract of seeds (S4), hexane extract oil (S5), methylene chloride extract of husks (S6), methylene chloride extract of husks with mould (S7), methylene chloride extract of fruit cake (S8), methylene chloride extract of seeds (S9), methylene chloride extract of *Croton* vinegar oil (S10), methanol extract of husks (S11), methanol extract of fruit cake (S12), methanol extract of seeds (S13), *trans*-ozic acid (S14), sartone A (S15), epoxykaurane (S16), epoxychiromodine (S17), ethyl acetate extract of *Croton* vinegar oil (S18) and magnoflorine (S19).

4. Discussion

4.1. Compounds Isolated

4.1.1. *Ent*-16*S*,17- epoxykaurane (56)

Compound (56) was isolated from the dichloromethane extract of husks of *C. megalocarpus*. Compound (56) was identified by analysis of the 1H, 13C and 2-D NMR spectra as well as the mass spectrum and the IR spectrum as the known ent-16S,17-epoxykaurane, previously isolated from Aristolochia triangularis (Lopes *et al*., 1990) and Achillea clypeolata (Aljancic *et al*., 1996). The LC-MS data for compound (56) gave a molecular ion peak $[M]^+$ at 288.2453 m/z which indicated a molecular formula

of $C_{20}H_{32}O$. IR of compound (56) showed an absorption band at 2848 cm^{-1} for C-H stretch, a 1461 cm^{-1} for C-H bend alkane group, a 1110 cm^{-1} C-O stretch for epoxide. The ^{1}H and ^{13}C NMR spectroscopic data for compound (56) are shown in **Table 3**. The ^{13}C NMR chemical shifts of compound (56) were identical to those reported in the literature for *ent*-16*S*,17- epoxykaurane. The optical rotation value for compound (56) was + 0.07^{o} whilst the value obtained from literature is -55^{o} (Aljančić *et al*., 1996). In consideration of NMR, HRMS and IR, compound (56) was confirmed to be the known *ent*-16*S*,17- epoxykaurane.

4.1.2. 8(17),12, 14-Labdatrien-18-ol (ozic acid) (58)

Compound (58) was isolated from the dichloromethane extract of husks of *C. megalocarpus*. Compound (58) was identified by analysis of the 1H, 13C and 2-D NMR spectra as well as the mass spectrum and the IR spectrum as the known 8(17),12, 14-Labdatrien-18-ol, previously isolated from *Cunninghamia lanceolate* (Du *et al*., 2001) and *Daniellia* species (Bevan *et al*., 1968). The LC-MS data for compound (58) gave a molecular ion peak $[M]^{+}$ at 302.458 m/z which indicated a molecular formula of $C_{20}H_{30}O_2$. IR of compound (58) showed an absorption band at 2930, 2867 and 1693 cm^{-1} for C-H stretch for CH_2 and CH_3 and a C=C double bond at 1642 and 891 cm^{-1}. The ^{1}H and ^{13}C NMR spectroscopic data for compound (58) are shown in **Table 4**. The ^{13}C NMR chemical shifts of compound (58) were identical to those reported in the literature for *trans* ozic acid. The optical rotation value for compound (58) was + 0.09^{0} whilst the value quoted in the literature is -47^{o} (Du *et al*., 2001). In consideration of NMR, HRMS and IR, compound (58) was verified to be *trans* ozic acid.

4.1.3. 3,4-Epoxide-15,16-epoxy-3,13(16),14-clerodatriene (19)

Compound (19) was isolated from the dichloromethane extract of the husks of *C. megalocarpus*. Compound (19) was identified by analysis of the 1H, 13C and 2-D NMR spectra as well as the mass spectrum and the IR spectrum as the known epoxychiromodine, previously isolated from *C. megalocarpus* (Addae-Mensah *et al*., 1992). LC-MS analysis gave a molecular ion peak $[M]^{+}$ reading of 346.1780 m/z which indicated a molecular formula of $C_{20}H_{26}O_5$. IR of compound (19) showed absorption bands at 3452 and 2848 cm^{-1} for C-H stretch, 1713 cm^{-1} for C=O stretch. The ^{1}H and ^{13}C NMR

spectroscopic data for compound (19) are shown in **Table 5**. The ^{13}C NMR chemical shifts of compound (19) are similar to those reported in the literature for epoxychiromodine. The optical rotation value obtained for compound (19) was + 0.01^{o} whilst the value obtained in the literature was -31^{o} (Addae-Mensah *et al*., 1992). With respect to the HRMS data, ^{1}H and ^{13}C NMR, it can be confirmed that compound (19) is the known epoxychiromodine.

4.1.4. Sartone A (60)

Compound (60) was isolated from the dichloromethane extract of husks of *C. megalocarpus*. Compound (60) was identified by analysis of the 1H, 13C and 2-D NMR spectra as well as the mass spectrum and the IR spectrum as the known sartone A, previously isolated from soft coral *Sarcophyton* (Iwagawa, T *et al*., 1996). LC-MS data for compound (60) revealed the molecular ion peak $[M]^{+}$ of 304.2402 m/z which indicated a molecular formula of $C_{20}H_{32}O_2$. IR of compound (60) showed absorption bands at 3054 cm^{-1} for indicative hydroxy group and a 1712 C=O and 1614 cm^{-1} double bond that forms a α, β unsaturated keto carbonyl group (van Zandvoort *et al*., 2015). The ^{1}H and ^{13}C NMR spectroscopic data for compound (60) are shown in **Table 6**. The ^{13}C NMR chemical shifts of compound (60) were identical to those reported in the literature for sartone A. The specific rotation value for compound (60) was + 0.0^{o} whilst the value obtained from the literature is +134.6^{o} (Iwagawa, T *et al*., 1996). In consideration of NMR, HRMS and IR, compound (60) was confirmed to be the known sartone A.

4.1.5. (+)-[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide (5)

Compound (5) was isolated from the dichloromethane extract of husks of *C. megalocarpus*. Compound (5) was identified by analysis of the 1H, 13C and 2-D NMR spectra as well as the mass spectrum and the IR spectrum as (+)-[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide, previously isolated from stem bark of *Croton gratissimus* (Mulholland *et al*., 2010). NMR analysis of compound (5) indicated a molecular formula of $C_{20}H_{30}O_3$ with a molecular mass of 357.20 m/z. Literature confirmed the IR absorption band of compound (5) showed presence of carbonyl stretch at 1738 cm^{-1} which indicates a α, β unsaturated 5 membered lactone and O-H stretch at 3411 cm^{-1} (Mulholland *et al*., 2010). The ^{1}H and ^{13}C NMR spectroscopic data for compound

(5) are shown in **Table 7**. The ^{13}C NMR chemical shifts of compound (5) were identical to those reported in the literature for (+)-[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide. Therefore, compound (5) is confirmed to be (+)-[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide.

4.1.6. (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide (7)

Compound (7) was isolated from the dichloromethane extract of husks of *C. megalocarpus*. Compound (7) was identified by analysis of the 1H, 13C and 2-D NMR spectra as well as the mass spectrum and the IR spectrum as (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide, previously isolated from leaves of *Croton gratissimus* (Langat *et al*., 2011). NMR analysis of compound (7) indicated a molecular formula of $C_{20}H_{30}O_4$ with a molecular mass of 357.20 m/z. Literature confirms IR absorption bands of compound (7) was 3421 cm^{-1} for hydroxy group and 1757 cm^{-1} for α, β unsaturated γ lactone (Langat *et al*., 2011). The α, β unsaturated γ lactone was determined as the C=O stretching is shifted between 15-25 cm^{-1} lower frequency in unsaturated environment than an unconjugated C=O (Pavia *et al*., 2009) The ^{1}H and ^{13}C NMR spectroscopic data for compound (7) are shown in **Table 8**. The ^{13}C NMR chemical shifts of compound (7) were identical to those reported in the literature as (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide. Therefore, compound (7) is confirmed to be (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide.

4.1.7. Vanillin (59)

Compound (59) was isolated from the dichloromethane extract of husks of *C. megalocarpus*. Compound (59) was identified by analysis of the 1H, 13C and 2-D NMR spectra as well as the mass spectrum and the IR spectrum as the known vanillin, which is a common isolate from a range of plant species (Bai De *et al*., 2018). LC-MS data showed the molecular ion peak $[M]^+$ of 152.149 m/z which confirmed the molecular formula of $C_8H_8O_3$. IR of compound (59) showed absorption bands at 3417 cm^{-1} broad peak for hydroxyl group OH, a 1714 cm^{-1} for carbonyl group stretch, a 1714 and 1264 cm^{-1} for carbonyl groups and ether groups. The ^{1}H and ^{13}C NMR spectroscopic data for compound (59) are shown in **Table 9**. The ^{13}C NMR chemical shifts of compound

(59) were identical to those reported in the literature for vanillin. Therefore, both IR and NMR analysis confirm that compound (59) is vanillin.

4.1.8. Mushroom Tyrosinase Inhibition Assay

Tyrosinase inhibition of isolated compounds and extracts of husks of *C. megalocarpus* showed varied results. The tyrosinase assay is a technique used to determine the inhibition rate of tyrosinase. Tyrosinase is an important enzyme in the role of melanin production in the skin. Tyrosine reacts with tyrosinase to produce melanin. In this assay, the compounds being tested are placed in a well of a 96 well plate along with tyrosinase and tyrosine in their respective quantities (see section 2.11.2). The reaction of tyrosine and tyrosinase turns a reddish/brown colour when reacted over time. This colourisation indicates the production of dopaquinone. The 96 well plate is then placed in the micro plate reader before addition of tyrosinase and post addition of tyrosinase. The difference is calculated and the inhibitory rate is determined.

Tyrosinase inhibition assay was chosen in this project as tyrosinase is an important enzyme that is targeted by current skin lightening agents such as hydroquinone and mercury. Applying the same protocol for the compounds/extracts of husks, seed and fruit cake of *C. megalocarpus*, it can be determined whether any of the compounds or extracts may exhibit the same mechanism as hydroquinone. The successful compound would then be tested for its cytotoxic effect on cells to determine its safety. The combination of tyrosinase inhibition and cytotoxicity enables the characterisation of successful compounds with further analysis and an alternative to the current skin lightening agents that are being used. Kojic acid was used as the positive control and DMSO as the negative control.

Kojic acid has the ability to inhibit tyrosinase and is a known positive control against tyrosinase. Kojic acid is also used for skin care as it is a most common ingredient in creams and lotions in the cosmetic industry. Kojic acid was used in the study by Chatatikun *et al* (Chatatikun *et al*., 2017). Kojic acid was shown to inhibit tyrosinase at 150% at 250µg/ml. The results also showed that both *Croton roxburghii* (CRE) and *Croton sublyratus* (CSE) lowered tyrosinase activity at lower concentrations of 6.25-50µg/ml as opposed to kojic acid at 250µmg/ml. The use of current skin lightening such as hydroquinone's, mercury and corticosteroids while shown to be effective, have

also been shown to have adverse effects on people such as acne vulgaris, hypertrichosis, perioral dermatitis, dischromias and ochronosis (Yusuf *et al*., 2019).

This has led African nations such as Sudan, Ghana and South Africa to ban the use of skin lightening products that contain these ingredients. For this assay, the template was carried out according to **Appendix A**. Using a Nunclon transparent 96 well plate, 145µL of phosphate buffer was added to blank wells and 50µL was added to control and sample wells. 5µL of kojic acid, DMSO and compound/extract was added to their respective wells. 50µL of tyrosine substrate was added to all wells and the 96 well plate was shaken for 5 seconds and left to incubate at room temperature (23^{o}C) for 2 minutes. The plate was placed in the TECAN Infinite M200 microplate reader (Theale, UK) and a pre reading was noted. 100µL of freshly made mushroom tyrosinase was added to control and sample wells.

The plate was incubated at room temperature for 15 minutes and a second reading was taken. The results for the tyrosinase inhibition assay are shown in **Figure 9**. Compounds/extracts that showed a high or promising inhibition rate were repeated to validate the inhibition activity. Repeats were done for epoxychiromodine (S17), magnoflorine (S20), methanol extract of husks (S11), methylene chloride extract of husks with mould (S7) and ethyl acetate extract of croton vinegar oil (S18) (n=3). Significance of the results was not analysed as only 5 compounds/extracts of interest were repeated.

Hexane extract of husks (S1), hexane extract of husks with mould, (S2), hexane extract of fruit cake (S3) and *Croton* vinegar oil (S19) showed similar inhibition effects at 14.5, 11.3, 14.4 and 15.2%. Hexane extract of the seeds (S4), hexane extract oil (S5), methylene chloride extract of husks (S6), methylene chloride extract of husks with mould (S7), methylene chloride extract of fruit cake (S8), methylene chloride extract of seeds (S9), methanol extract of husks (S11), methanol extract of fruit cake (S12), methanol extract of seeds (S13), sartone A (S15) and *ent*-16*S*,17-epoxykaurane (S16) showed significantly low inhibition activity against tyrosinase at 1.9, 4.4, 6.6, 5.2, 8.3, 5.6, 3.0, 9.6, 7.5, 3.3 and 7.0% in comparison to kojic acid at 92.44%.

The methylene chloride extract of *Croton* vinegar oil (S10) and *trans* ozic acid (S14) showed a slightly higher inhibition at 17.5 and 19.0%. Epoxychiromodine (S17) and magnoflorine (S20) inhibited nearly a quarter of the enzyme activity at 26.8 and 24.0%. The ethyl acetate extract of *Croton* vinegar oil (S18) showed an inhibition of 59.3%

therefore inhibiting over 50% of the enzyme's activity. According to a study by Chatatikun *et al* (Chatatikun *et al*., 2017), the leaves of *Croton roxburghii* and *Croton sublyratus* produced inhibition rates of 117.2% at 50µg/ml and 159.3% inhibition at 100µg/ml for both *Croton roxburghii* and *Croton sublyratus* (Chatatikun *et al*., 2017) with comparison to kojic acid at 150%.

The compounds/extracts for this experiment were made at 20µg/ml (extracts) and 20mM (pure compounds) respectively as opposed to the parameters used by Chatatikun *et al* (Chatatikun *et al*., 2017) that was using concentration from 3.125-50µg/ml for CRE and 3.125-200µg/ml for CSE. The CRE and CSE were ethanolic extracts of the leaves whilst methanol, methylene chloride and hexane were used for husks, seed and fruit cake of *C. megalocarpus*. As the study by Chatatikun *et al* (Chatatikun *et al*., 2017) was also based on *Croton*, this shows the applications of diterpenoids from *Croton* to be used for potential natural product cosmetic treatments as the results obtained showed promising results. Graphical representation for the tyrosinase inhibition can be seen in **Figure 9**. Moreover, graphical data was also produced for five compounds/extract of interest that were repeated due to their ability to inhibit tyrosinase **(Figure 10)**. T test analysis showed that compounds/extract (S7) ($p<0.0052$), (S17) ($p<0.0052$), (S18) ($p<0.0012$) and (S20) ($p<0.0393$) were significant $p<0.05$ compared to kojic acid. Compound/extract S11 showed to not be significant with $p<0.0542$.

4.1.9. FM-55 Human Melanoma Cells (Cytotoxicity Assay)

Cytotoxicity of compounds/extracts of *C. megalocarpus* showed some varied results. The cytotoxicity assay that was conducted was an MTS assay of CellTiter 96 Aqueous One Solution Cell Proliferation Assay. CellTiter involves the quantification of viable cells after addition of the desired test substance and seeing a decrease in the number of cells that would indicate the test substance ability to kill cells. The compound used in this assay is [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Barltrop, J.A. *et al*., 1991). The assay is based on the abundancy of formazan that is produced when CellTiter is reduced **(Figure 12)** by viable cells and then measured by the absorbance (Barltrop, J.A. *et al*., 1991).

The cells are treated with varied concentrations of the test substance and a positive and negative control are assigned. The positive and negative control are kept at a constant concentration. After 24 hours incubation, the cells are treated with CellTiter reagent and incubated for a further 2hrs. The plate is then read in the microplate reader and absorbance is noted. CellTiter luminescent cell viability assay was chosen as it is a fast and easy assay to conduct. The assay only requires up to 30hrs of experimental time. The mechanism of CellTiter assay involves the ability for viable active cells to convert MTS into formazan (Riss *et al*., 2013). Formazan is a purple coloured chemical that is detectable at 570nm on the microplate reader (Riss *et al*., 2013).

Dead cells no longer are able to convert MTS to formazan and therefore colourisation serves as an indicator for viable cells (Riss *et al*., 2013). On the other hand, some factors that were considered for the outcome of the assay such as the ability for the compounds/extracts to reduce the MTS CellTiter reagent rather than the viable cells. This would result in higher cell viability and therefore give a false reading. Natural products are isolated in small quantities and is another reason for not being able to have enough to make up to the desired stock concentration.

Administrating low stock concentrations of the compounds/extracts may not give it enough strength to kill the cells. The FM-55 cells used in this assay were found to be sensitive when subjected to centrifugation. Centrifugation revealed to have disturbed the cells in the form of large clumps. Therefore, centrifugation was avoided to allow proper and equal distribution of cells into the 96 well plates. In this MTT-type assay, 3 separate 96 well plates were prepared and seeded with approximately 10,000 FM-55 cells in each well. On the stock solution 96 well plate, 50µL of each compound/extract was added to their designated wells. Etoposide was used as the positive control and DMSO (1%) as the negative control. A total of 100µL DMSO was added to each compound/extract and the following concentrations were produced by addition of DMEM: 50, 25, 12.5 and 6µg/ml. Each compound/extract was added to the FM-55 cells and incubated for 24hrs. After 24hrs, CellTiter reagent was added to the cells and absorbance was measured after 2hrs. Cell viability was calculated using the following formula.

[(Sample absorbance average / Negative Control absorbance average)] x 100

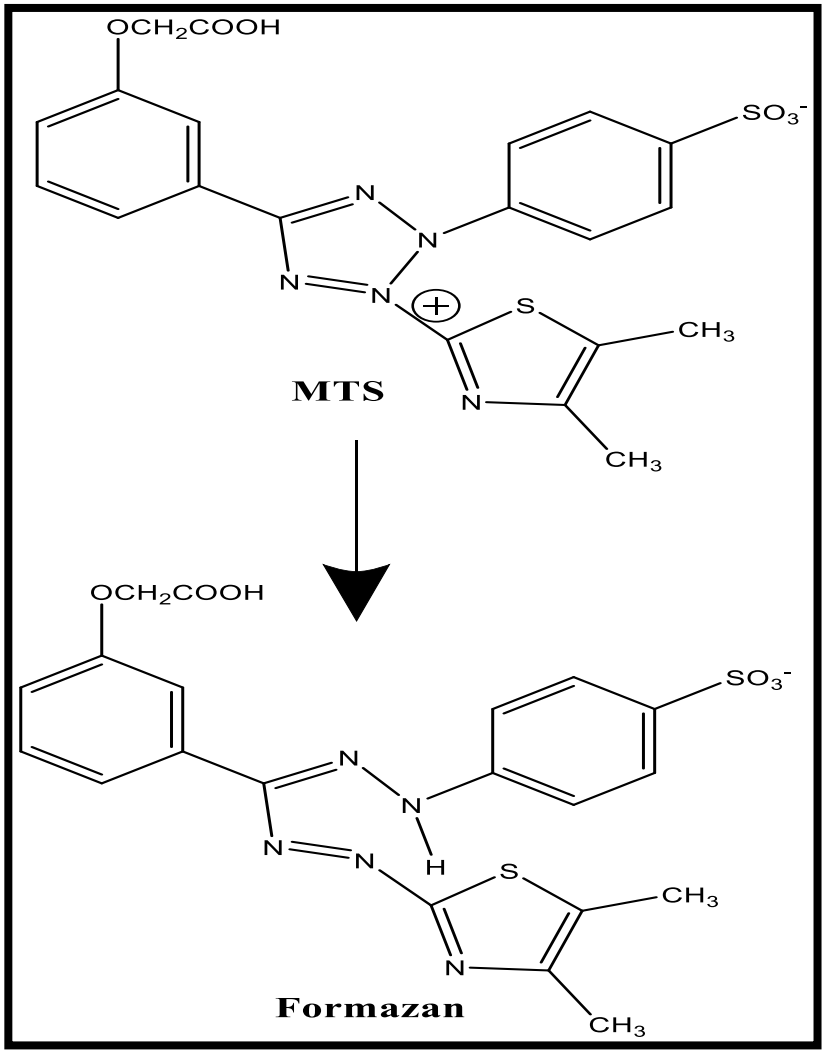


Figure 12: MTS CellTiter reduction of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium into formazan. The CellTiter reagent is reduced by active viable cells and the quantity of formazan is measured by the absorbance.

Cytotoxicity analysis is important as a range of different cells can be tested on compounds of interest. The cytotoxic potential of a compound can be tested against healthy skin cells and cancerous melanoma cells. This would provide the behavioural characteristics of the compound of interest and enable and encourage more use of natural products in treatment for cancer. A study by Chatatikun *et al* (Chatatikun *et al*., 2017) reported the results of *Croton roxburghii* (CRE) and *Croton sublyratus* (CSE) leaves on B16F10 cells. Compounds (1) and (4) **(Figure 4)** were tested against PEO1 and PEO1TaxR (taxane resistant) ovarian cancer cell lines. The results showed that both compound (1) and (4) were found inactive against PEO1 ovarian cancer cells.

The CellTiter assay results were found to be significant $P<0.05$. **Figure 11** represents the isolated compounds and extracts at a concentration of 50µg/ml. The results at 50µg/ml revealed that compounds/extracts hexane extract of husks (S1), hexane extract of seeds (S4), methanol extract of husks (S11), methanol extract of fruit cake (S12), methanol extract of seeds (S13) and *trans* ozic acid (S14) showed cell viability above 100% with a CI ranging from 65.04-157.5%. This indicated that these compounds and extracts induced cell growth rather than killing the FM-55 cells. The methylene chloride extract of husks (S6) showed to kill the FM-55 cancer cells with a cell viability of 45.62% with a CI of 21.78-69.47% compared to etoposide that produced a cell viability of 68.13% with an CI at 5.853-130.4%.

Therefore, the methylene chloride extract of husks (S6) showed to kill the cells more effectively than etoposide which is an approved drug used for treatment of cancer. Compounds/extracts hexane extract of husks with mould (S2), hexane extract of fruit cake (S3), hexane extract oil (S5), methylene chloride extract of husks with mould (S7), methylene chloride extract of seeds (S9), methylene chloride extract of *Croton* vinegar oil (S10), sartone A (S15), epoxykaurane (S16), epoxychiromodine (S17), ethyl acetate extract of *Croton* vinegar oil (S18) and Magnoflorine (S19) showed to kill a small amount of FM-55 cell with no major effect and produced CI from 30.43-163.4%.

At 25µg/ml **(Figure 11A)**, the results showed to be varied. Hexane extract of husks with mould (S2) was shown to have increased in cell viability above 100% with a 95% CI from 82.89-152.9% and indicates that at a lower concentration, it may have aided in cell growth rather killing the cells. Hexane extract of fruit cake (S3), hexane extract of seeds (S4) and hexane extract oil (S5) showed to have killed a small number of FM-55 cells compared to concentrations at 50µg/ml with a CI ranging from 49.04-123.3%. Moreover, methylene chloride extract of husks (S6) showed to increase in cell viability thus not being able to kill the FM-55 cells compared to the concentration at 50µg/ml.

The remaining compounds/extracts hexane extract of husks (S1), methylene chloride extract of husks with mould (S7), methylene chloride extract of fruit cake (S8), methylene chloride extract of seeds (S9), methylene chloride extract of *Croton* vinegar oil (S10), methanol extract of husks (S11), methanol extract of fruit cake (S12), methanol extract of seeds (S13), *trans* ozic acid (S14), sartone A (S15) epoxykaurane (S16), epoxychiromodine (S17), ethyl acetate extract of *Croton* vinegar oil (S18) and

magnoflorine (S19) did not show much difference in cell viability compared to 50µg/ml with a CI at 35.25-150%. At 12.5µg/ml **(Figure 11B)**, the results appear to be virtually identical to the concentration at 25µg/ml with a small amount of variance.

The methylene chloride extract of the husks (S6) had showed to be higher in cell viability than 50µg/ml. At this stage of the experiment, it is indicated that the methylene chloride extract of husks (S6) is more effective at higher concentrations on FM-55 human melanoma cells and not so effective at lower concentrations. The concentration at 6µg/ml **(Figure 11C)** was shown to be virtually identical to the concentration at 12.5µg/ml with small variance. T test analysis can be seen in **Appendix D**. Overall, the results showed to be varied but most effective at 50µg/ml. The effectiveness and ability of the compounds/extracts to kill FM-55 cells is reduced as the concentration is lowered. The methylene chloride extract of the husks (S6) showed to kill the FM-55 cells with a cell viability of 45%.

In comparison to the isolated compounds of the methylene chloride extract, the extract is shown to have more positive effect on the FM-55 melanoma cells rather than the individual compounds themselves. This analysis indicates synergism as the compounds increase their activity when used together. On the other hand, the tyrosinase assay data **(Figure 9)** shows that *trans* ozic acid (S14), epoxykaurane (S16) and epoxychiromodine (S17) inhibit tyrosinase with rates of 19.04, 7.03 and 26.82% in comparison of methylene chloride extract of husks (S6) 6.61% and therefore is better individually rather than as an extract. However, another possible explanation for the methylene chloride extract of husks (S6) cytotoxic effect is the presence of a compound that was not isolated.

The compound present in the methylene chloride extract may be killing the FM-55 cells rather than the isolated compounds. The ethyl acetate extract of *Croton* vinegar oil (S18) showed a tyrosinase inhibition of 59.30% and a weak toxicity with a cell viability of 91.87% and is a suitable subject for skin lightening and skin care. The tyrosinase inhibition of the ethyl acetate extract of *Croton* vinegar oil (S18) may also be synergistic as there may be compounds that are inhibiting tyrosinase together or an individual compound.

The analytical techniques used in this project were suitable as they are standard techniques used in the isolation and characterisation of natural products. Column chromatography (CC) was used to isolate and purify compounds subject to an array of solvent systems. CC was effective in producing a large production of samples which also contributed to greater quantity of compound. This technique was used in conjunction with TLC. TLC is a visual technique that indicates the presence of compounds within the extract.

TLC helped determine the quantity of compounds that separate after the addition of p-anisaldehyde. TLC is fast and reliable. Once determining the vial of interest, NMR was used to see the purity of the compound. NMR was also used to identify the number of hydrogens and carbons, confirming it to be a diterpenoid as diterpenoids were of interest in this project. Compounds that showed to be impure were subject to column again to purify the compound. NMR was the backbone in providing the characteristics of the compounds. LC-MS was a useful analytical technique and used as a confirmatory for the compounds by identifying the mass. The mass identified was then confirmed using an established chemical finder. IR was used to identify the functional groups present. IR was not time consuming and was fast. Polarimetry was used to help determine the angle of rotation for each chiral compound. Polarimetry was also fast and easy to use.

5. Conclusion

It can be concluded that the approach taken for this project provided an array of techniques for the isolation and characterisation of the compounds isolated from the husks of *C. megalocarpus*. The tyrosinase inhibition assay determined that the ethyl acetate extract of croton vinegar oil had the highest inhibition rate of 59.33%. This gives an indication that the ethyl acetate extract of croton vinegar oil is suitable for further work as it may play a vital role for the cosmetics and beauty industry for skin lightening or skin care as a melanin suppressor. CellTiter assay revealed that the dichloromethane extract of husks showed a cell viability of 45% and indicates that its potential to be able to kill FM-55 melanoma cells may also be effective in killing other types of cancer cells such as CaCo-2, Hela and MCF-7. This study has also shown that the

compounds isolated from the husks of *C. megalocarpus* have a relatively low toxicity (as determined by tyrosinase inhibition assay) and is safe to handle.

However, as the isolated compounds were derived from the dichloromethane extract, it can also be concluded that the compounds may be synergistic as the dichloromethane extract showed a cell viability of 45%. This study has shown the potential of *C. megalocarpus* to be studied for future research as its different types of diterpenoids and recognises the potential use of the husks. The husks are shown to be promising as diterpenoids have been extracted, isolated and tested for cytotoxicity and anti-cancer properties and these findings would enable EFK to begin utilising the husks instead of throwing away after the de-husking process.

6. Further work

The outcome for the investigation of the husks of *C. megalocarpus* appears it to be an area of interest for future work. The bioassays consisted of the CellTiter assay and mushroom tyrosinase inhibition that tested for anti-melanogenic and toxicity properties. The melanin assay and yeast inhibition would have given a dynamic and varied characteristic of the compounds and extracts and would be suitable for the continuation of the project. The melanin assay will be more cell-based work and will involve direct interaction with melanocytes unlike the tyrosinase assay which is non cell based.

The melanin and tyrosinase assay are inter-related and would have provided a more in-depth analysis and profiling of the compounds and extracts. The yeast inhibition assay is an additional characteristic on which compounds and extract is able to kill yeast cells. This may provide alternative methods for treating people that have a yeast infection such as the overuse of antibiotic and general infections that occur due to high amount of yeast production in the body. Other cell viability assays such as neutral red and PrestoBlue may produce a varied approach to the CellTiter assay. Analysing the compounds of the methanol extract of husks of *C. megalocarpus* as similar compounds may be extracted and isolated to the dichloromethane extract.

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8. Appendices

Appendix A: Tyrosinase Inhibition Assay Template

Pt-1	1	2	3	4	5	6	7	8	9	10	11	12
A	L-Ty + Kojic Blank Buffer	L-Ty + Kojic + Enzyme	L-Ty + **Methanol crude extract** + Buffer	L-Ty + **Methanol crude extract** + Enzyme	L-Ty + **CVO** + Buffer	L-Ty + **CVO** + Enzyme						
B												
C												
D												
E	L-Ty + DMSO Blank Buffer	L-Ty + DMSO + Enzyme										
F												
G												
H												

Appendix B: Plate 1 – CellTiter 96 Well Plate Template

Hexane Crude Extract Husk	Hexane Crude Extract Husk (mouldy)	Hexane Crude Extract F Cake	Hex-ane Crude Extract Seed	Hexane Crude Extract Seed Oil	DCM Crude Extract Husks	DCM Crude Extract (mouldy)	DCM Crude Extract F Cake	DCM Crude Ex-tract Seed	Croton Vinegar Oil (DCM Ex-tract)	Methanol Crude Extract Husks	Meth-anol Crude Ex-tract F Cake

Appendix B1: Plate 2 - CellTiter on 96 Well Plate Order of Compounds and Extracts Template

Methanol Crude Extract Seed	Ozic Acid	Sartone A	Epoxychiromodine	Lavix Deudua Resin	Croton Vinegar Oil (Ethyl Acetate Extract)	Magnoflorine	DMSO	Etoposide

Appendix C: CellTiter on 96 Well Plate Dilutions of Different Concentrations Template

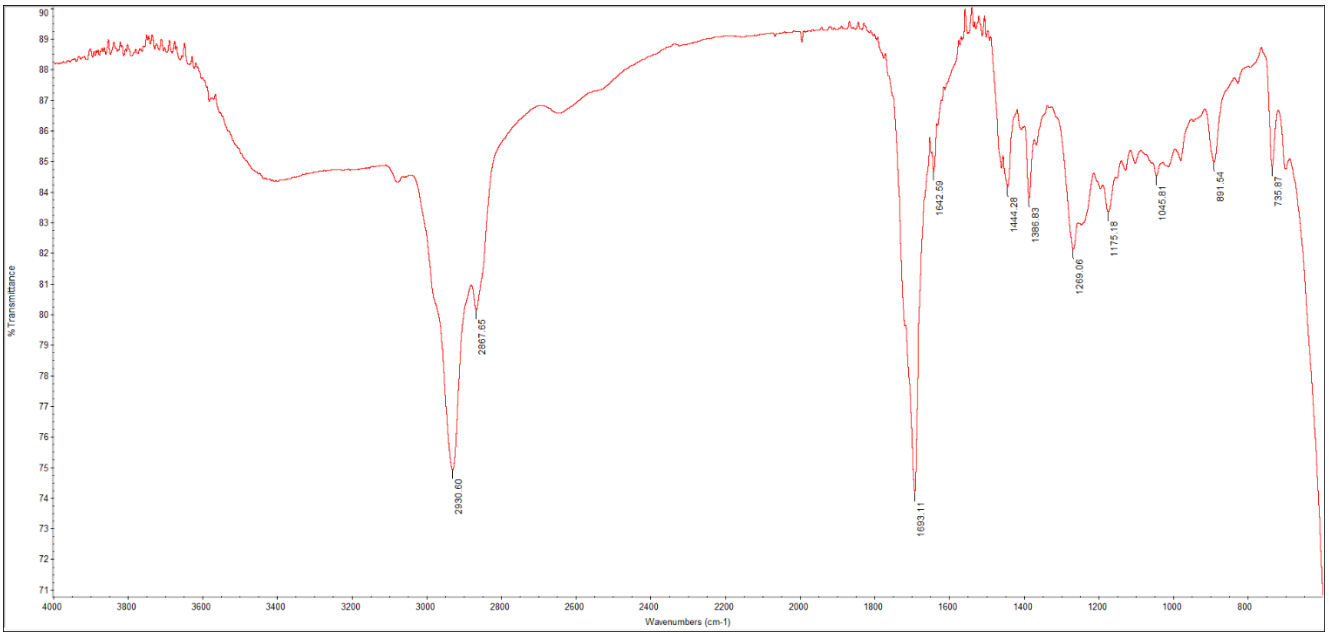
A	**50µg/ml**										
B	**25µg/ml**										
C	**12.5µg/ml**										
D	**6µg/ml**										
E	**50µg/ml**										
F	**25µg/ml**										
G	**12.5µg/ml**										
H	**6µg/ml**										

Appendix D: T test analysis of compounds/extracts of CellTiter MTS Assay

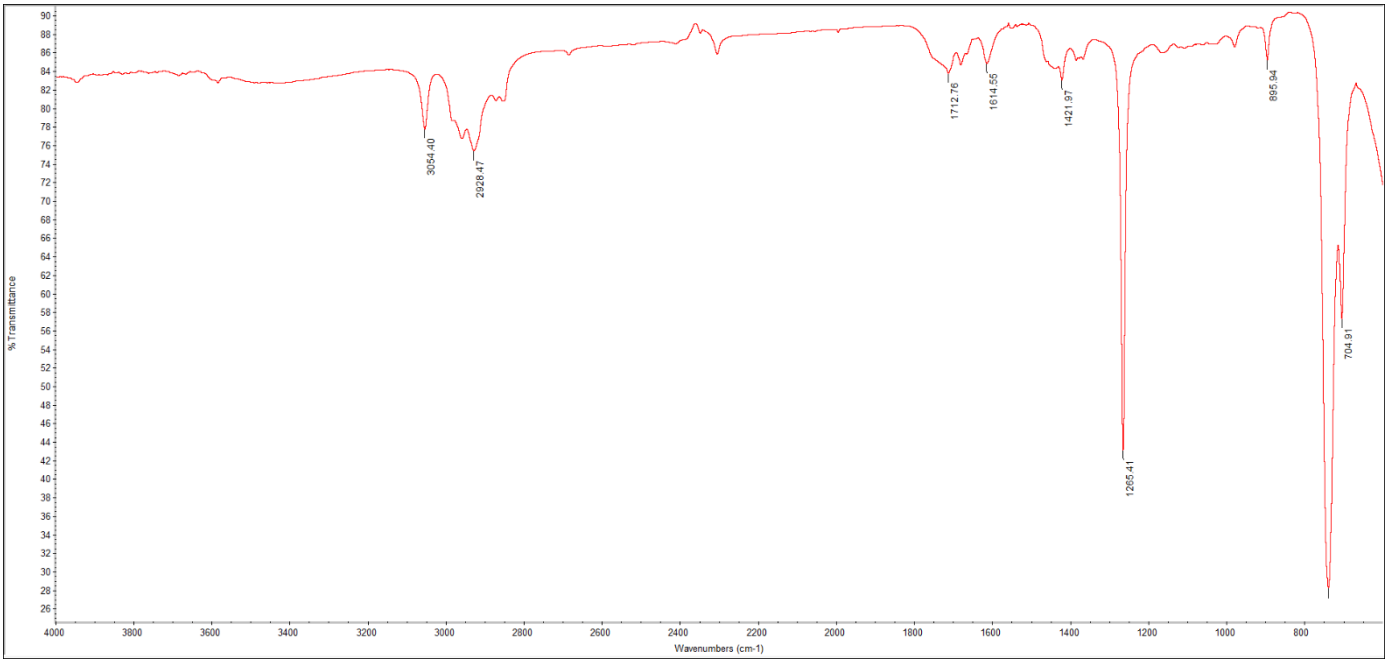
	50µg/ml			**25µg/ml**			**12.5µg/ml**			**6µg/ml**		
	df	***T***	**P**	***df***	***T***	**p**	***df***	***T***	**p**	***df***	***T***	**p**
S1	2	19.94	**0.0025	2	29.79	**0.0011	2	29.11	**0.0012	2	24.47	**0.0017
S2	2	8.202	*0.0145	2	14.49	**0.0047	2	21.45	**0.0022	2	18.17	**0.0030
S3	2	6.788	*0.0210	2	12.11	**0.0067	2	14.42	**0.0048	2	9.316	*0.0113
S4	2	28.96	**0.0012	2	12.94	**0.0059	2	10.51	**0.0089	2	7.729	*0.0163
S5	2	8.736	*0.0129	2	10.83	**0.0084	2	10.29	**0.0093	2	6.436	*0.0233
S6	2	8.231	*0.0144	2	8.677	*0.0130	2	10.93	**0.0083	2	7.715	*0.0164
S7	2	8.565	*0.0134	2	11.53	**0.0074	2	10.27	**0.0094	2	7.398	*0.0178
S8	2	7.387	*0.0178	2	9.799	*0.0103	2	12.66	**0.0062	2	8.044	*0.0151
S9	2	6.813	*0.0209	2	10.11	**0.0096	2	11.32	**0.0077	2	7.214	*0.0187

S10	2	7.217	*0.0187	2	8.445	*0.0137	2	19.47	**0.0026	2	9.946	*0.0100
S11	2	15.22	**0.0043	2	14.08	**0.0050	2	15.89	**0.0039	2	15.41	**0.0042
S12	2	28.27	**0.0012	2	13.28	**0.0056	2	29.07	**0.0012	2	47.59	***0.0004
S13	2	15.42	**0.0042	2	13.91	**0.0051	2	15.81	**0.0040	2	105.9	****<0.0001
S14	2	10.44	**0.0091	2	11.87	**0.0070	2	12.12	**0.0067	2	21.58	**0.0021
S15	2	6.680	*0.0217	2	14.87	**0.0045	2	16.01	**0.0039	2	15.63	**0.0041
S16	2	6.450	*0.0232	2	13.35	**0.0056	2	8.784	*0.0127	2	11.01	**0.0082
S17	2	7.044	*0.0196	2	7.244	*0.0185	2	29.40	**0.0012	2	15.54	**0.0041
S18	2	9.401	*0.0111	2	15.59	**0.0041	2	34.23	***0.0009	2	13.37	**0.0056
S19	2	6.841	*0.0207	2	9.249	*0.0115	2	16.10	**0.0038	2	13.74	**0.0053
Etoposide	2	28.27	**0.0012	2	4.424	*0.0475	2	6.075	*0.0260	2	15.25	**0.0043
DMSO	2	15.42	**0.0042	2	10.55	**0.0089	2	9.960	**0.0099	2	27.71	**0.0013

Appendix E: IR spectra of isolated compounds: *trans* ozic acid, sartone A, epoxykaurane, epoxychiromodine, (+)-[1*R,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide, (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide and vanillin**



Appendix E1- *Trans* ozic acid

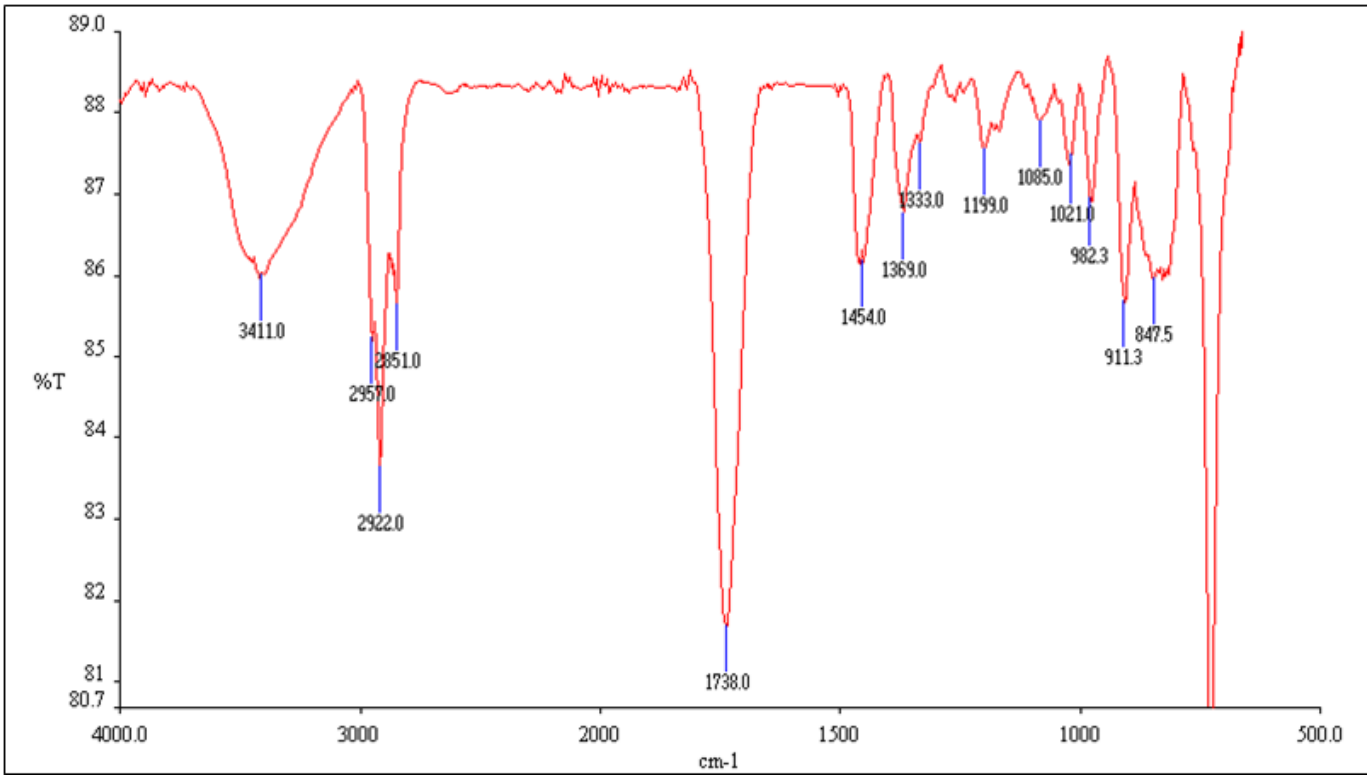


Appendix E2- Sartone A

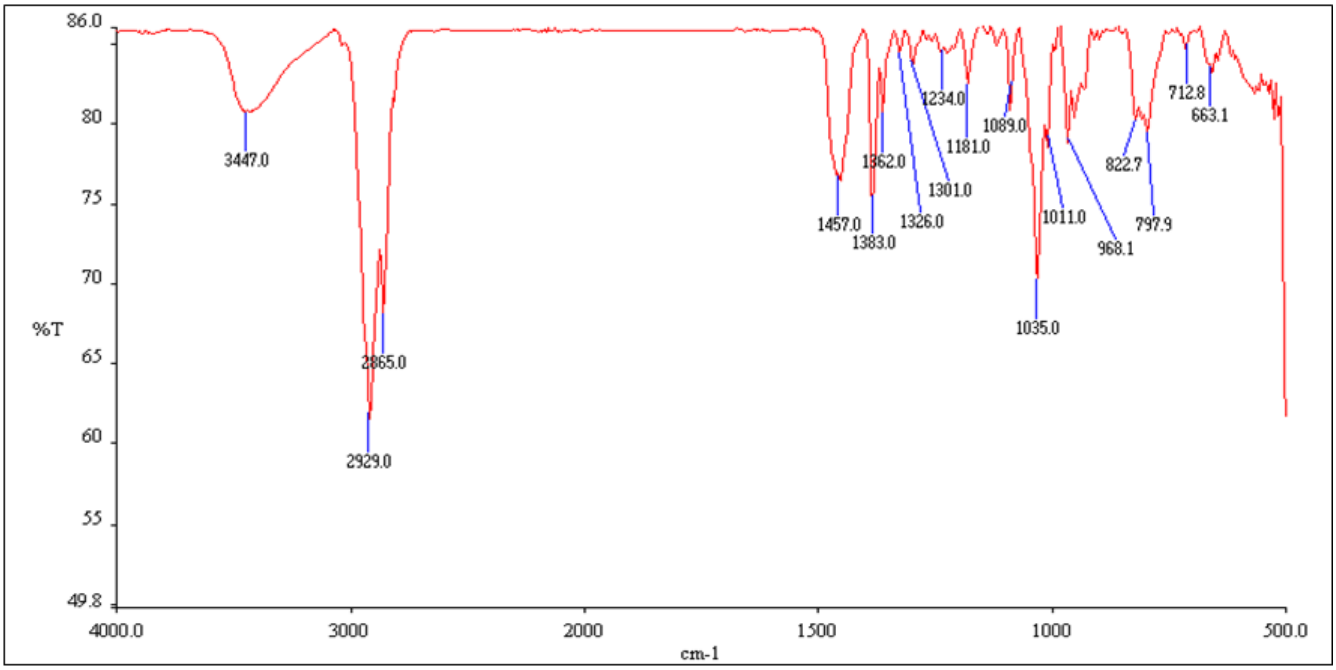
Appendix E3- Epoxykaurane

Appendix E4- Epoxychiromodine

Appendix E5- Vanillin

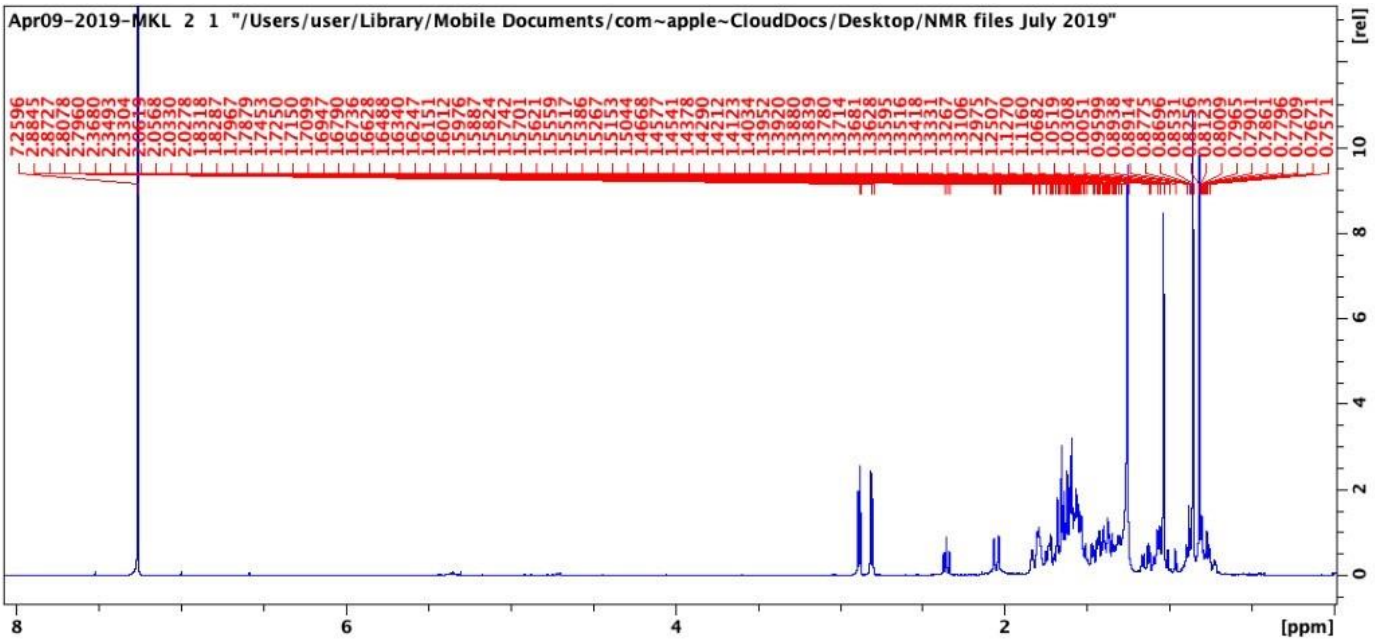


Appendix E6- (+)-[1*R,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide**

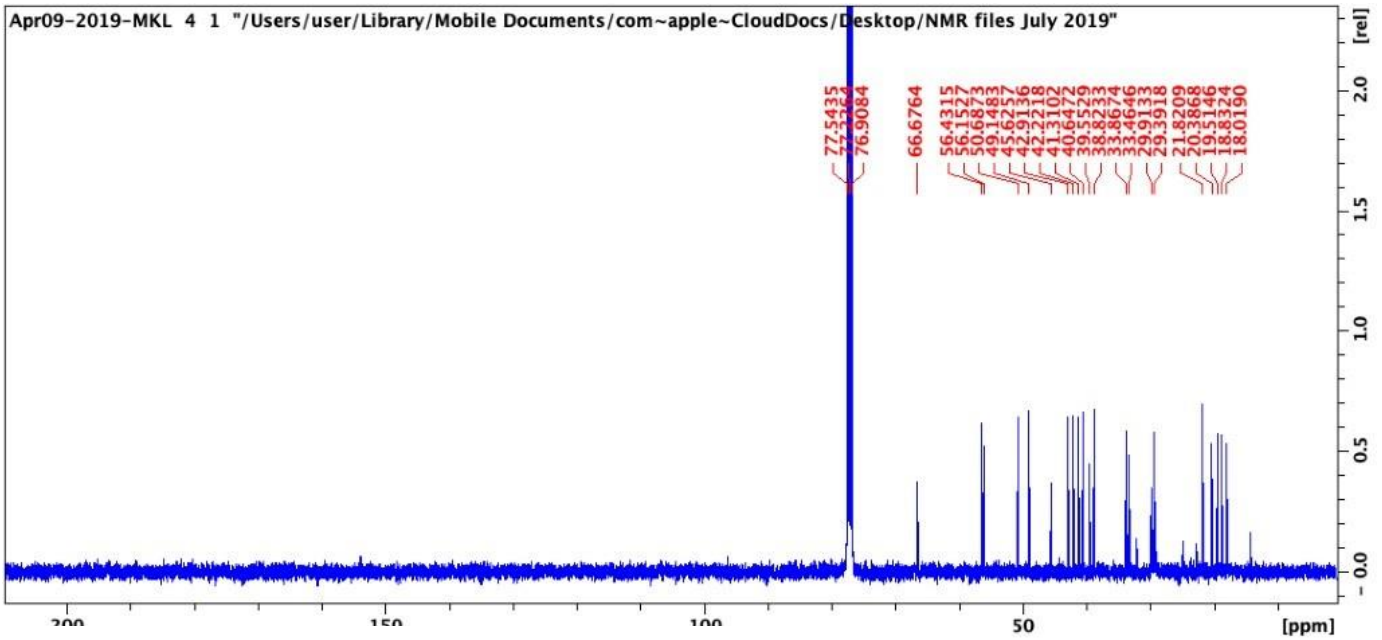


Appendix E7 (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide

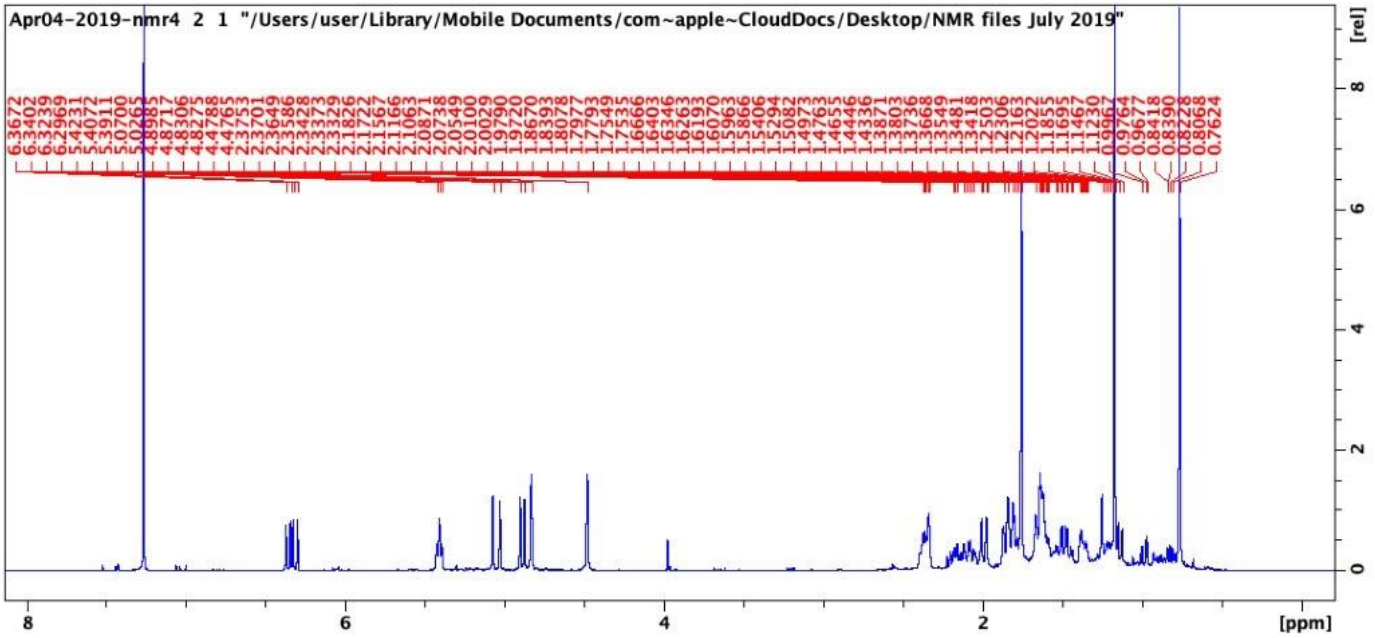
Appendix F: Processed ^{1}H and ^{13}C of the seven isolated compounds of the husks of *C. megalocarpus*: Ent-16S,17-epoxykaurane, *Trans* ozic acid, sartone A, epoxychiromodine, (+)-[1*R,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide, (-)-(1S*,4S*,10R*)-1,4- dihydroxy-cembra-2E,7E,11Z-trien-20,10-olide and vanillin.**



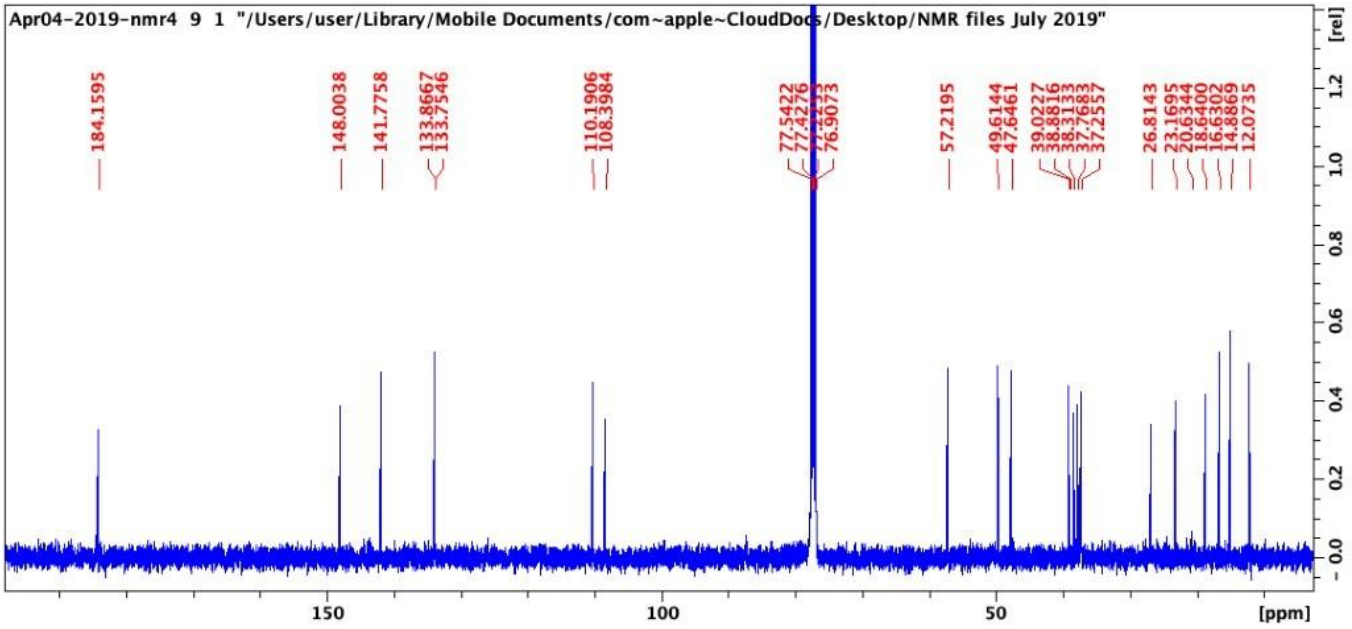
^{1}H NMR for Compound 56: Ent-16S,17-epoxykaurane



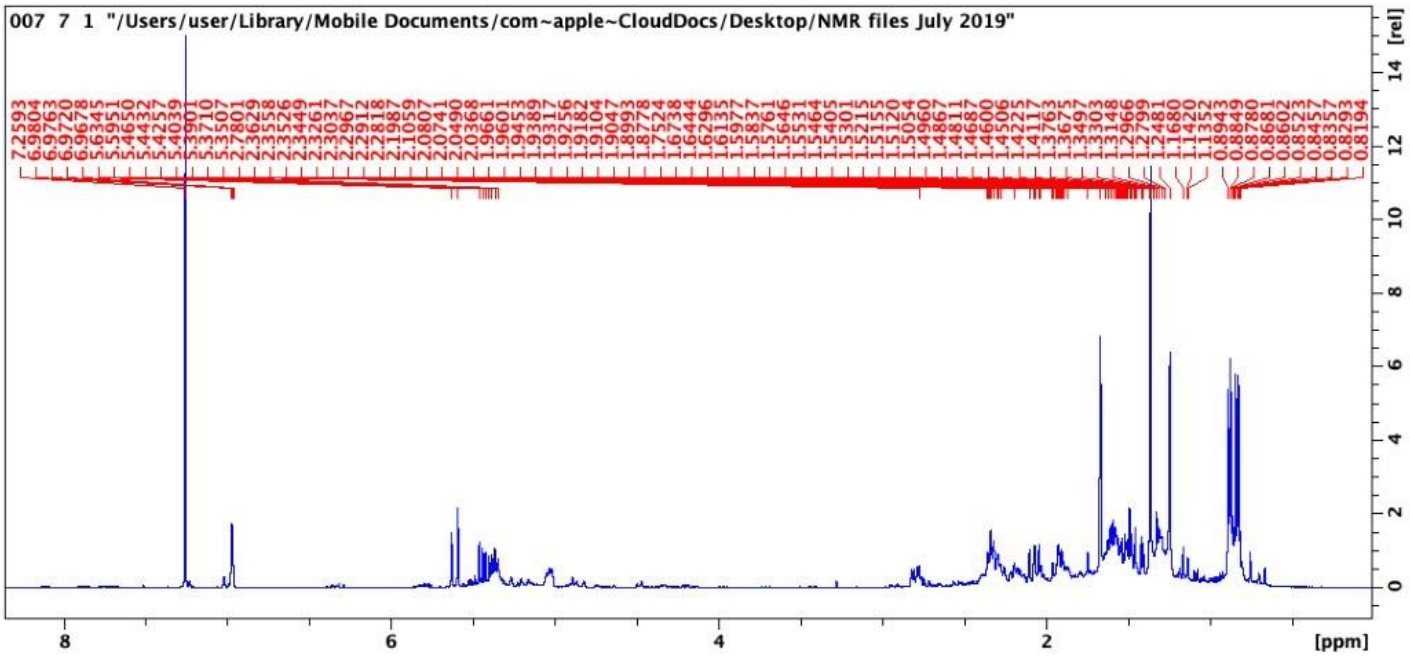
^{13}C NMR for Compound 56: Ent-16S,17-epoxykaurane



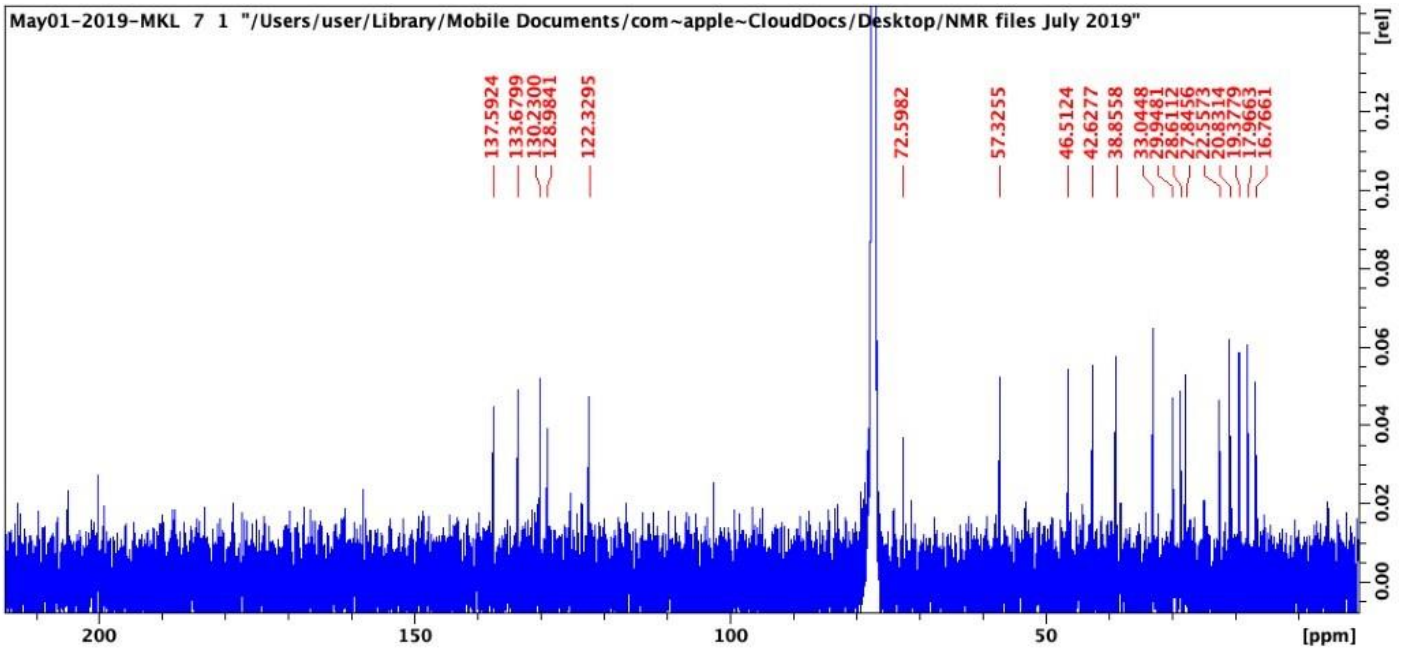
^{1}H NMR for Compound 58: *Trans* ozic acid



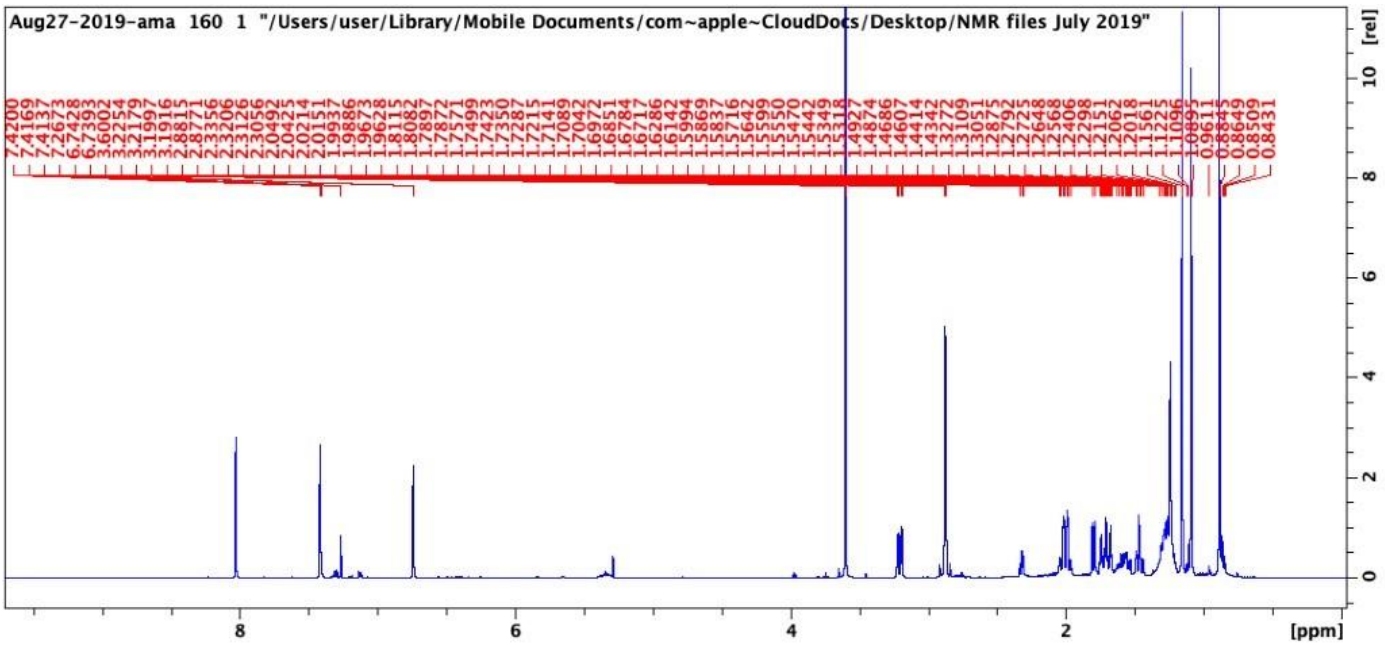
^{13}C NMR for Compound 58: *Trans* ozic acid



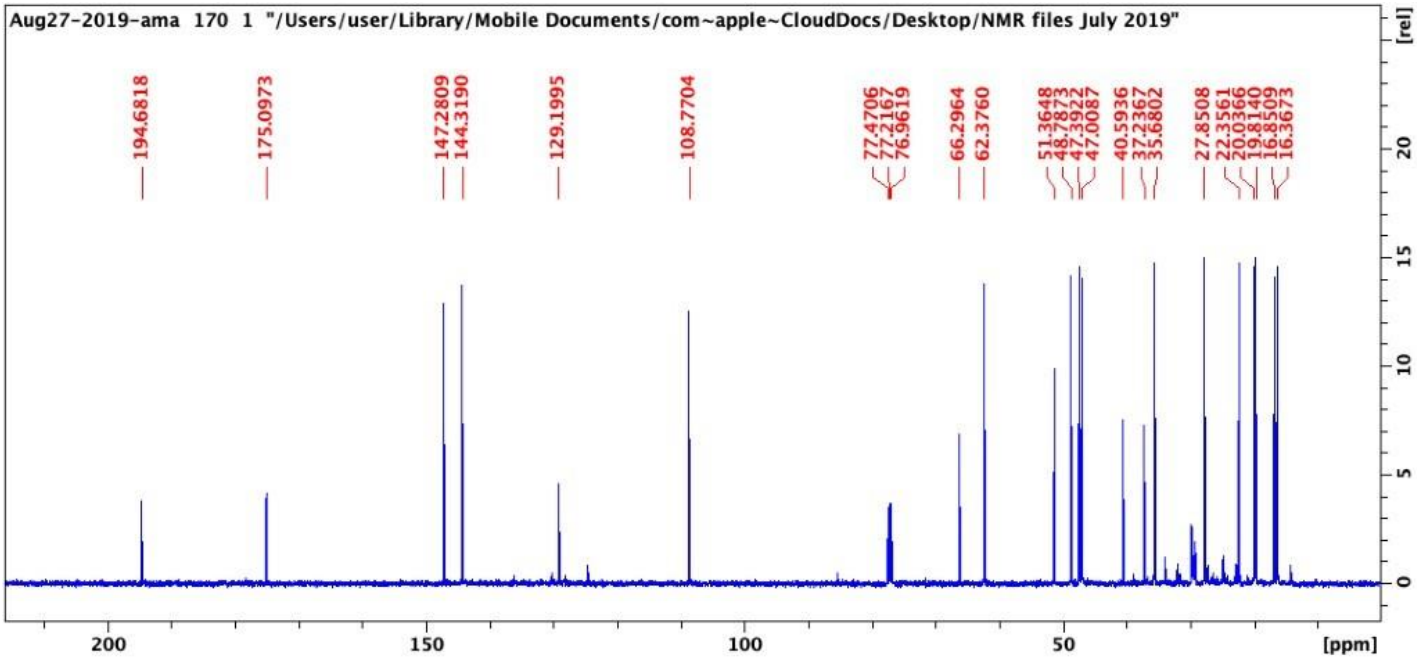
^{1}H NMR for Compound 60: Sartone A



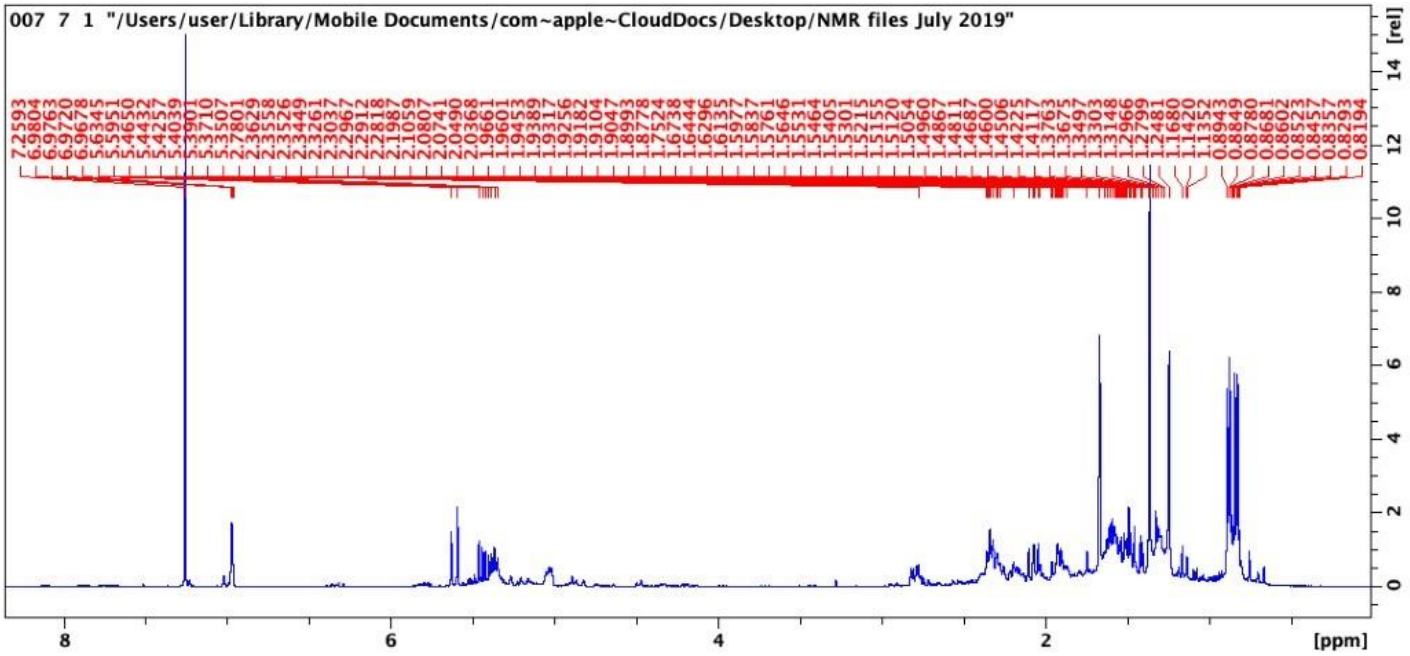
^{13}C NMR for Compound 60: Sartone A



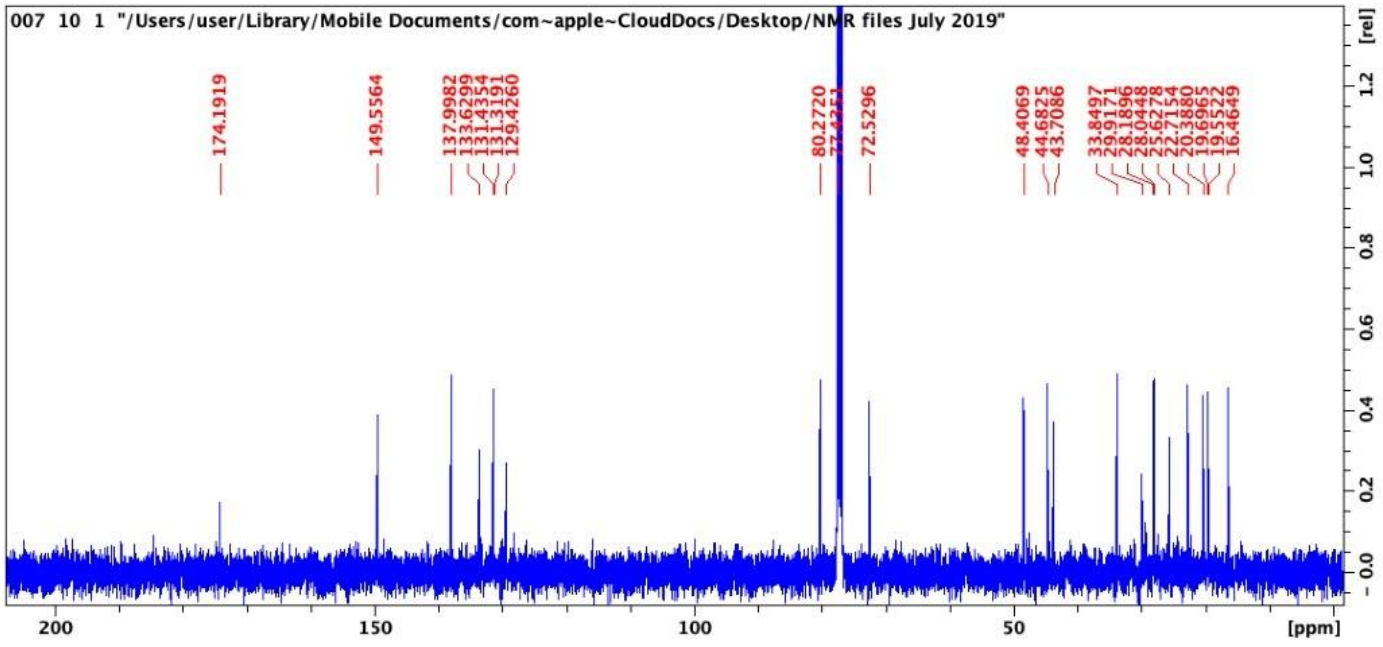
^{1}H NMR for Compound 19: Epoxychiromodine



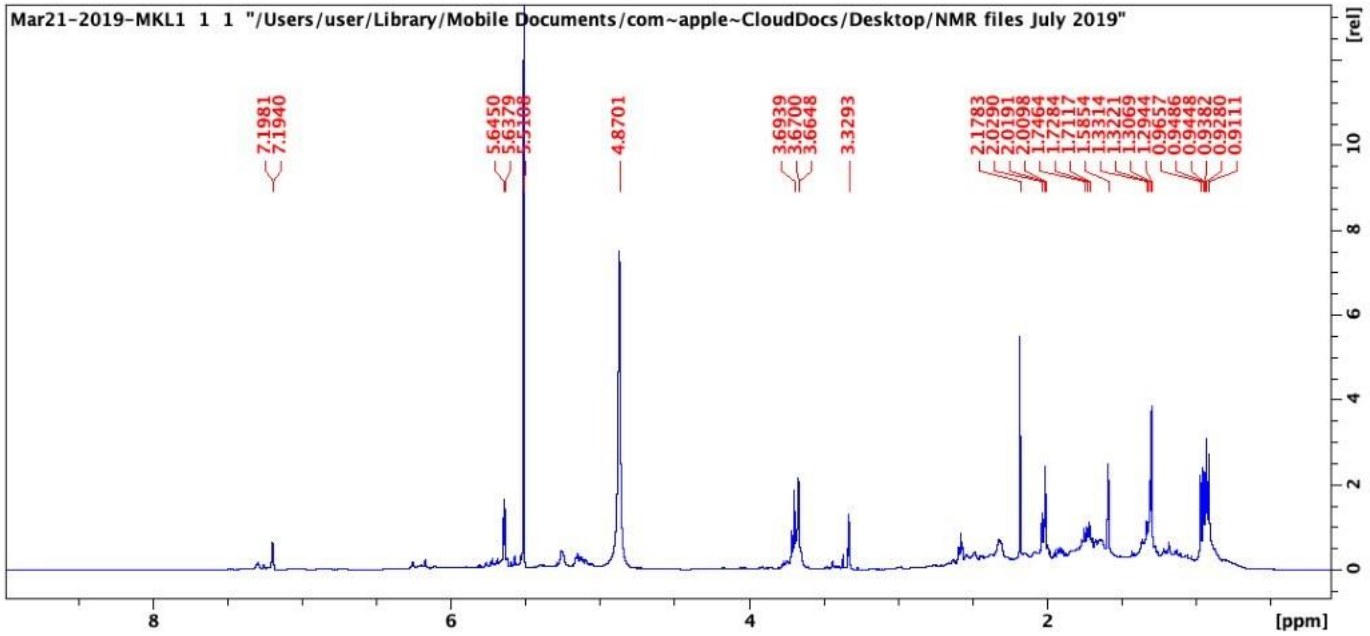
^{13}C NMR for Compound 19: Epoxychiromodine



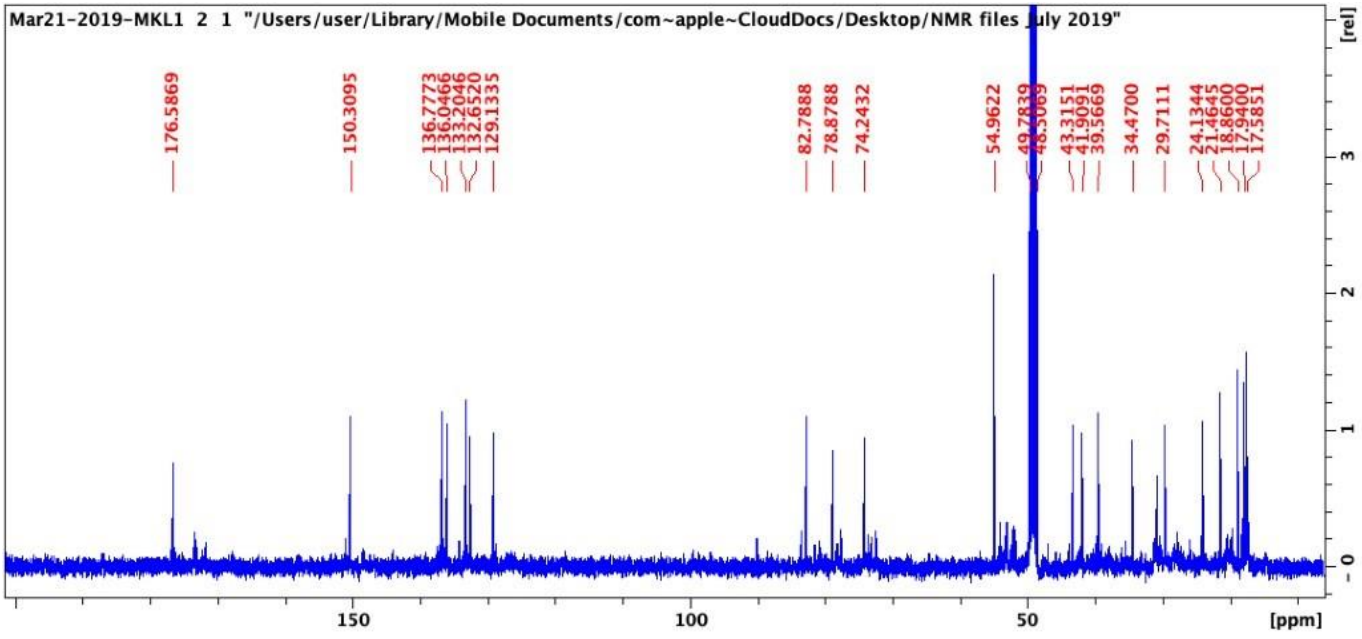
^{1}H NMR for Compound 5: (+)-[1*R,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide**



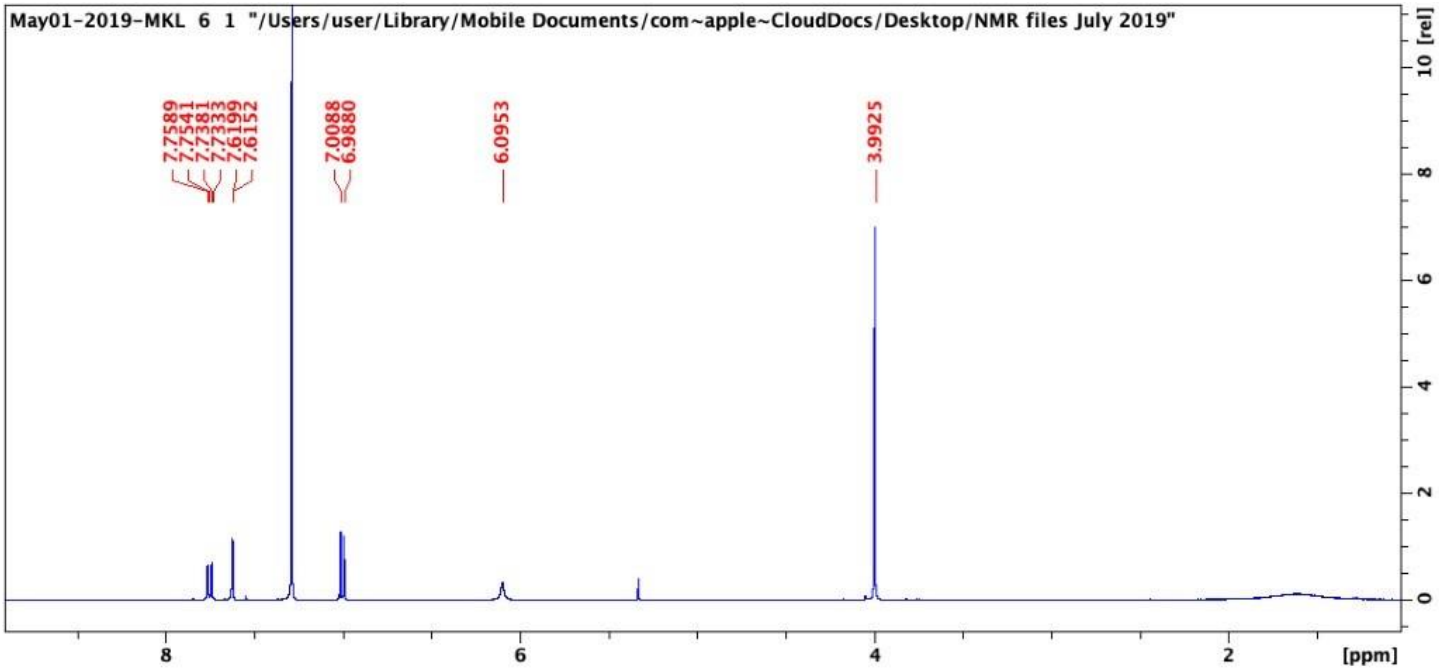
^{13}C NMR for Compound 5: (+)-[1*R,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide**



[1]H NMR for Compound 7: (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide



[13]C NMR for Compound 7: (-)-(1S*,4S*,10R*)-1,4- dihydroxycembra-2E,7E,11Z-trien-20,10-olide



^{1}H NMR for Compound 59: Vanillin