PHYTOCHEMICAL STUDIES OF LEAVES AND PEELS OF LEMON AND THREE SPECIES OF GRAPE AND THEIR BIOLOGICAL ACTIVITIES

by

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DECLARATION

I, Gugulethu M Miya, with the student number 210127074, hereby declare that the dissertation entitled, "PHYTOCHEMICAL STUDIES OF LEAVES AND PEELS OF LEMON AND THREE SPECIES OF GRAPE AND THEIR BIOLOGICAL ACTIVITIES." which I am submitting for the degree of MASTER OF SCIENCE (CHEMISTRY) at Walter Sisulu University is my own original work. It represents the original work by the author and has not been submitted in any form to another University. Where the works of other scholars were used, they have been duly acknowledged in the text and on reference list.

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LIST OF PUBLICATION

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DEDICATION

I dedicate this work to God who made it possible to finish, and to the rest of my family especially my mother Mrs C.N Miya, late father Mr Z.A Miya and late grandmother Mrs B.M Phungula.

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ABSTRACT

Citrus trees are evergreen trees that produce fruits of different shapes and sizes from round to oblong, which are full of fragrance, flavor and juice. Lemon (*Citrus limon*) is a useful medicinal plant effective in fighting a wide range of diseases including cold, pain and inflammation. Grapefruit (*Citrus paradisi)* is a subtropical tree known for its sour to semi sweet fruit, varying in taste, from acidic and even bitter to sweet and sugary. Although the peels and leaf from these plants are discarded as wastes, yet both are regarded as medicines, effective in fighting a wide range of diseases including cold, pain and inflammation. South Africa which happens to be a major producer of these fruits is faced with a huge volume of these waste which constitutes environmental problem. A need to source for the end use value chain for the waste generated by citrus industries especially in the Eastern Cape Province necessitated this study.

This study describes the methods used in isolating the essential oil from the two parts of the plant which are fruit peels and leaf of *Citrus limon* and three different cultivars of *C. paradisi* namely Rose grapefruit, Star Ruby grapefruit and Marsh grapefruit, the analysis of the essential oils using GC and GC-MS for quantitative and qualitative analysis and the phytochemical screening of the peel and leaf plant part. On the study also some biological activities were investigated namely acute toxicity, *Citrus paradis* and *Citrus limon* acute inflammation and two models of antioxidant bioassay known as Ferric reducing power (FRAP) and 2, 2 ’-diphenyl-1-picrylhydrazyl (DPPH).

Lisbon lemon leaf and fruit were collected from Addo River Bend Farm, Addo Park, Eastern Cape, while the three grape fruit leaves and fruits were obtained from Mystic Blue Farm Eshowe, KwaZulu-Natal, South Africa. Isotaion of essential oils conducted using hydro-distillation method, analysis carried-out by gas chromatograohy (GC) and gas chromatography-mass spectrometry (GC-MS) processes and biological studies were conducted on the essential oils using known methods from literatures.

Percentage yields of essential oils extracted, the dried material had more essential oil yield when compared to fresh material and this can be attributed to the quantity of water in form of moisture content that is present in the fresh material. Nonetheless, the fruit peels had higher percentage yields (w/w %) compared to the leaf regardless of fresh or dried materials. Also noticeable was the colour of the essential oils. The fresh materials were more or less colourless while most of dried ones gave pale yellow to yellow colouration.

Lemon oils were analyzed in two different GC-MS instruments and from two different Universities namely Stellenbosch University (2016) and Walter Sisulu University (2017). The 2016 results for fresh fruit peels of *C. limon* essential oil had β-pinene (10.6%), limonene (52.5%), γ-terpinene (8.8%) and geranial (5.7%) as the prominent compounds. Fresh leaf oil had limonene (31.0%), β-pinene (13.3%), Citral (10.3%) and Geranial (13.2%) as major compounds. On the other hand limonene (36.0%), alloocimene (5.6%) and L-carvone (5.9%) were the major compounds from dried fruit. Linalool (8.6%), limonene (12.0%), caryophyllene (17.7%) and limonene glyco (5.2%) were the dominant constituents of dried leaf.

While the 2017 results from Walter Sisulu University showed that C. limon fresh fruit peel essential oil collected in 2017 had D-Limonene (60.4%), p-cymene (6.3%) and

β-pinene (9.3%), as major constituents, while in the dried fruit peel chromatogram had D-limonene (67.9%) as the dominant compound, γ-terpinene (6.9%) and β-pinene (3.9%) were the prominent compounds in the oil profile. The fresh leaf D-limonene (32.0%), β-pinene (8.6%), z-citral (11.5%) and geranial (14.2%) were the significant compounds. D-limonene (32.8%), geranial (16.2%), z-citral (12.8%), β-pinene (10.3%) and linalool (8.6%) as the leading dominant compounds of dried leaf. Star Ruby grapefruit (*Citrus paradisi*) essential oils had β-phellandrene (91.0%) as the prominent compound on the fresh leaf. The dried leaf essential oil had β-phellandrene (82.39%) and β-ocimene (7.0%) as major compounds. Fresh peels of *C. paradisi* Star Ruby grape had D-limonene (86.7%) as a major compound. The D-limonene (74.2%) and Terpinene (6.0%) were found to be the prominent components in dried peels.

A total of 32 compounds were identified in the fresh peel oil accounting for (99.5% of the total oil composition), dried peel oil had 20 compounds (99.6%), fresh leaf 12 compounds (96.5%) and the dried leaf oil 10 compounds (97.5%). The GCMS analyses of Rose grapefruit oils, showed that the most prominent constituent in the dried and fresh leaf oils is β-phallendrene (74.9 and 90.0%) respectively while dried and fresh fruit peels had D-limonene (79.3 and 89.9%) as the major compound. Marsh cultivar of *C. paradisi* fresh leaf oil analysis presents β-phellandrene as the only major compound (90.1%) while dried leaf had β-phellandrene (92.4%) as the major compound, with the fruit peels of Marsh *C. paradisi* presenting D-limonene as a major compound, though fresh peels having (88.1%) and dried peels (81.2%) respectively as predominating compounds in both oils.

Essential oils proved to be non-toxic and safe for orally when there was no mortality in all mice during acute toxicity tests, these oils also showed some significant results, though less active compared to the controls in the DPPH and ferric reducing power bio assays while the fruit peels of rose grapefruit and fruit peels of lemon essential oils showed an very promising results compared with all other essential oils this can be father be explained by the fact that the phytochemical screening of secondary metabolites of lemon fruit peels essential oil together with those of rose grape essential oil showed a very high percentage of flavonoids compared with other essential oils and these compounds96+ are well known by their antioxidant power.

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Abbreviations

ANOVA: Analysis of Variances

$CHCl_3$: Chloroform

CH_3CO_2H: Acetic acid

CHD: Conventional hydrodistillation

DMAPP: Dimethylallyl diphosphate

DPPH: (2,2-Diphenyl-1-picrylhydrazyl)

$FeCl_2$: Iron (II) chloride

$FeCl_3$: Ferric chloride

FID: Flame ionization detector

FRAP: Ferric-reducing power

GC: Gas Chromatography

GC-MS: Gas Chromatography-Mass Spectrometry

GC-MSMS: Gas Chromatography-Triple Quadrupole mass spectrometry

HCl: Hydrogen chloride

HNO_3: Nitric acid

H_2SO_4: Sulfuric acid

HMG-CoA: CoA-3-hydroxy-3-methylglutaryl

HMGR: CoA-hydroxyl-3-methylglutaryl reductase

K.I: Kovats index

$K_4[Fe(CN)_6]3H_2O$: Potassium ferricyanide

LD_{50}: 50% Lethal dose

MAHD: Microwave assisted hydrodistillation

NaCl: Sodium chloride

NH_3: Ammonia

NH_4OH: Ammonium hydroxide

RT: Retention time

SEM: Standard mean of error

SIM: Selected ion monitoring

TNF-alpha: Tumor necrosis factor-alpha

μgmL^{-1}: micro grams per millilitre

α **: Alpha**

β **: Beta**

γ **: Gamma**

δ **: Delt**

Chapter 1

INTRODUCTION

1.1 Medicinal Plants

Medicinal plants are plants used as natural medicine and whose use has been documented over a period by people in a particular locality. Interest in medicinal plants as sources of medicine to manage existing diseases has largely increased (Mohamoodally, 2013; Verma and Singh, 2008). It has been reported that this new trend and interest in herbal medicines can be endorsed to many factors including affordability, availability, acceptability, efficacy, lower adverse effects, traditional beliefs and prohibitive cost of orthodox drugs (Biljana, 2012; Denwick, 2002). Medicinal plants have distinct chemicals that confer specific biological properties thus making them a more readily accessible source of therapeutic materials (DeWeft *et al.*, 2005; Fennel *et al.*, 2004). Furthermore, medicinal plants have played significant roles in drug discovery and several drugs currently in use globally are traceable to plants used in ethno medicine (Balunas and Kinghorn, 2005). In many of these plants, their leaves, fruit, stem, bark or root (rhizomes) are often used as the source of medicine derived substance. Citrus is one of such plants whose juice has been used for years as natural medicine.

Medicinal plants are important aspect of the daily lives of many people and a cultural heritage especially in South Africa (Van Wyk, 2003). The parts of the plant used vary from plant to plant and from one traditional healer to another, depending on the nature and state of the disease (Thring and Weitz 2006).

1.1.1 Citrus fruits

Citrus plants are known to originate from Asia (where they were first domesticated), Europe, and Florida (Hartmann *et al.*, 1997; Jackson, 1991). Citrus belongs to the flowering trees and shrub of the Rue family called *Rutaceae*. Plants in the genus includes grapefruit, lemons, oranges and limes. (Liu *et al.*, 2012). Citrus was divided into two major groups by Hagerty in 1923 which are the Monograph on the Oranges of Wen-chou and Chekiang. These approximations are made by means of genetic recording of plant (Araujo *et al.*, 2003), Mandarin, orange and pummelo are the three original species in the citrus genus that have been hybridized into most modern commercial citrus fruit that we now have (Ross, 1949). Within the last few decades all common citrus fruits (sweet oranges, lemons, grapefruit, limes, and so on) were cultivated by crossing those original species. The grapefruit was initially believed a type of pummelo (q.v.) citrus. James Macfadyen who confirmed that grapefruit was a hybrid between the pummelo and the orange and was given the botanical name, *Citrus paradisi* Macfad (Malik, 1994). The botanical name has been altered to reflect this view, and it is now generally accepted as *Citrus* X *paradisi* (Kumamoto *et al.*, 1987). Thereafter, this new fruit was approved into cultivation and the name "grapefruit" originated into a common name which was known till date.

1.1.1.1 Descriptions and Economic importance of Citrus

Citrus fruit plant is cultivated in all continent of the world and is also one of the most important commercial fruit crops (Tao *et al.*, 2007). Citrus trees are evergreen trees that produce fruits of different shapes and sizes from round to oblong, which are full

of fragrance, flavor and juice. Citrus fruits are usually rough, robust and brightly colored from green to yellow skin or rind known as epicarp or flavedo, which covers the fruits and protects it from damages (Okwu, 2008). World's total production of Citrus fruit grew excessively during the last four decades of the twentieth century (Boelens, 1991). Citrus has been used medicinally for treatment of fever, arthritis and heart related diseases. It is known to be the source of distinctive flavours that have been esteemed by people throughout the world for centuries (Schieber, 2001). Citrus production used in medicine preparation like medlemon is estimated around one third of total Citrus production (Imran *et al.*, 2013).

South Africa is a major producer of citrus, with 2015/16 production season of the industry contributing R14.8 billion to total gross value of South African agricultural production annually. The industry has been reported to be an important foreign exchange earner, which comprises of four broad export categories; oranges, easy peelers (soft citrus), grapefruit, lemons and limes (Figure 1). (AgricOrbit, 2018; Uys, 2016)

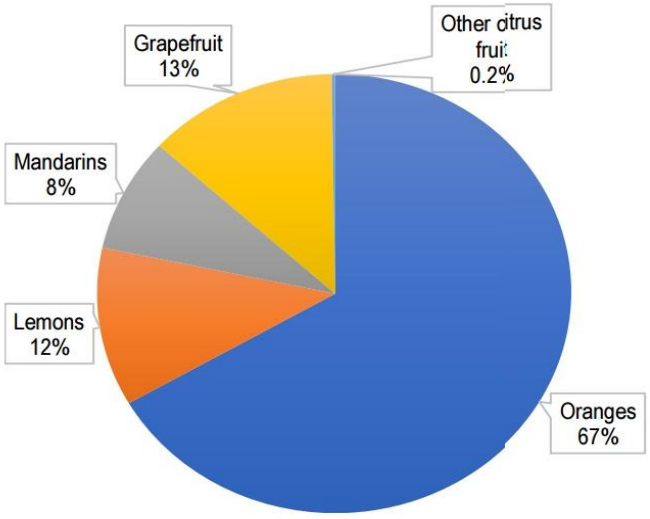


Figure 1: Composition of South African Overall Citrus Export Volumes per percentage 2010-2016 (Source: AgricOrbit, 2018)

Citrus cultivation represents one of South Africa's most important fruit group by value and volume. It is cultivated mainly in six provinces which are Limpopo, Western Cape, Mpumalanga, Eastern Cape, KwaZulu-Natal and Northern Cape. The cool climate in the Western Cape and Eastern Cape are most suitable for the cultivation of Navel oranges, mandarins and lemons, while grapefruit and Valencia oranges needs warmer climate which is obtained from Mpumalanga, Limpopo and KwaZulu-Natal (AgricOrbit, 2018; DAFF, 2017; Uys, 2016).

1.1.1.2 *Citrus limon*

Citrus limon commonly known as lemon is a strong tree is stron, branch spreading standing tree of about 3.1–6.1 m in height. The leaves are auxiliary, long-ovate 6.25–11.25 cm long with serrated boundaries and slight aerial petioles (Figure 2(a)). As tender Leaves they are reddish in colour but turn green as they become matured. The fruit is oval, naturally with a nipple-like top at the stylar end, and ranges from 5–12 cm in length. The peel is light-yellow to yellow with thickness ranging between 6–10 mm smooth or rough, and scattered with oil glands (Davies and Albrigo, 1994; Jackson, 1991; Morton, 1987; Tucker and Wardowski, 1976) (Figure 2(b).

Figure 2: (a) Lemon tree and (b) lemon fresh fruit and fruit peels

Lemon (*C. limon*) is a useful medicinal plant effective in fighting a wide range of diseases including cold, pain and inflammation (Mohanapriya *et al.*, 2013), and regulate blood pressure (Singh and Singh, 2016). Lemon oil has been used extensively to flavour beverages, especially carbonated beverages and to aromatize household products, imparting a clean, light citrus/lemon fragrance (Kawaii *et al.*, 2000).

1.1.1.3 *Citrus paradisi* Macfad

Citrus paradisi (Grapefruit) have three cultivars that were used in this study which are Marsh grapefruit, Star ruby grapefruit and Rose grapefruit (Figure 3). They are differentiated by color of the fruit which is initiated by the skin-color of the fruit. The rose grape cultivar is white inside with a pale yellow skin (Figure 3c) and round in shape having big leaf with a strong acidic taste. The star ruby grape cultivar is a red fruit with reddish skin (Figure 3b). It is a big fruit (in size) with sharp leaf and, it is the most available grapefruit in South African supermarkets with a sour taste. Marsh grape cultivar is not white nor red it has a weak orange coloration (Figure 3d), with a yellow skin and it`s leaf is tall, sharp shaped. The fruit is slightly sweet compared to the other

two described above. Generally, all the fruits have a diameter ranging from 10-15 cm (Malik, 1994). Mercaptan with IUPAC name methanethiol (**1**), a sulfur-containing terpene found in grapefruit, is reported to have a strong impact on the flavor and odor of grapefruit (Subramanian *et al.*, 2014).

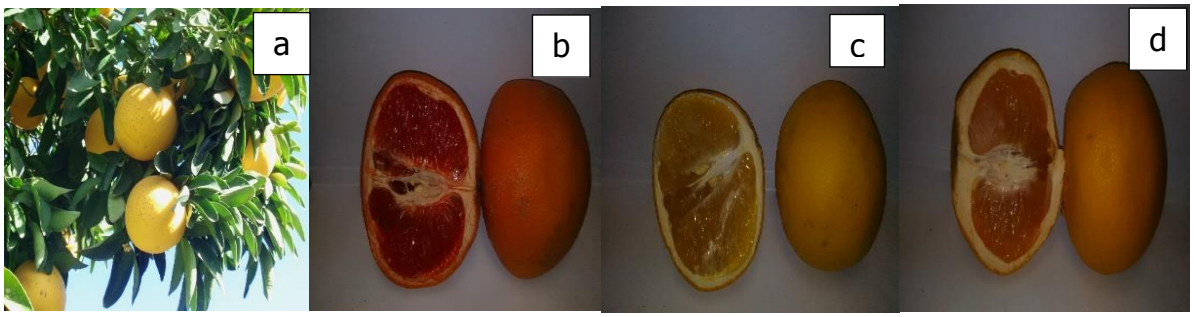
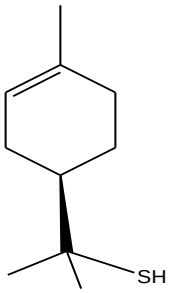


Figure 3: (a) Grapefruit tree (b, c, d) Grapefruit half cut fruits of the three cultivars in the order Star Ruby, Rose and Marsh grapefruit.



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1.1.2 Secondary metabolites

1.1.2.1 Terpenoids

Terpenoids at times called isoprenoids, are a huge and most diverse class of naturally occurring secondary metabolites whose precursor is derived from five membered

carbon isopentenyl diphosphate (IPP) and its allelic isomer dimethylallyl diphosphate (DMAPP) units, combined mostly in an head to tail format to form complex structures. Terpenoids are therefore said to be naturally occurring compounds which are made up of a building block of 5-carbon isoprene unit. They are mostly presented as acyclic, monocyclic, bicyclic or multi-cyclic compounds with vary functional groups despite the basic skeletal structure. Terpenoids perform several physiological and ecological functions in plants` life through direct and indirect plant defenses as well as in human society because of their enormous applications in the pharmaceutical, food and cosmetics industries. Terpenoids can be generally classified as volatile and non-volatile terpenoids. Volatile terpenoids (isoprenes, monoterpenes and sesquiterpenes) constitute the largest class of plant volatile compounds. Of most interest in this study is the volatile terpenoids which is made up of mono- and sesquiterpenoids in essential oils composition. These lipids (essential oils) are found mostly in plants terpenoid class of secondary metabolites of living things (Tholl, 2015; Firn, 2010; Pichersky *et al*., 2006).

1.1.2.2 Essential oils

Floral volatiles also known as essential oils are lipophilic liquids in nature, having high vapor pressure and low molecular weight at ambient temperatures. These properties allow them to freely pass through the cellular membranes for release into the adjacent environment (Pichersky *et al*. 2006). Essential oils are volatile substances present in plant oil sags with strong odor. Essential extracted through various distillation and pressing methods from leaves, flowers, seeds, fruit peel, roots, rhizomes and resins.

The distinctive technique of essential oil collection ensures that the essential oils retain its purity and unique odor (Pophof *et al*., 2005; Guenther, 1948).

Essential oils are generally used as pharmaceutical components in nutritious supplements, medicinal purposes and as odor enhancer in the cosmetic industry and aromatherapy, give flavor to drinks and foods in the food industry while being used as a major constituent during the preparation of some drugs, soaps, perfumes and other cosmetics as well as for home cleaning products (Maria *et al*. 2012; Okwi and Emenike, 2006).

1.1.2.3 Citrus Essential oils

Citrus fruits essential oils are identified as one of the by-products attracting the interest of researchers (Njoroge *et al*., 2006). Citrus essential oils are present in great quantity in epicarp portion than in any other parts of citrus fruit (Sawamura *et al*., 2004; Bocco *et al*., 1998). Mostly all the investigated citrus fruit species had shown that limonene is the dominant compound across the world. (Kamal *et al*., 2011; Okwu and Emenike, 2006).

Essential oils from citrus have pleasant refreshing smell and rich aroma, hence they are used in air-fresheners, household cleaning products, perfumes, cosmetics, and medicines. They are also of great value as aromatherapy and medicinal agents (Dosoky and Setzer, 2018; Sawamura et al., 2004). Citrus essential oils are used as natural preservatives due to their broad spectrum of biological activities. These citrus

essential oils are also highly active against various bacteria strains including *Staphylococcus aureus*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Escherichia coli* (Imran et al., 2013). A detailed review of some report is in section 1.2 below.

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Chapter 2

2.0 Literature review

2.1 Extractive methods of citrus essential oil

Methods of essential oil extraction differs, in this section three methods mostly used in the citrus extraction are discussed. The first method is the pressing method which has been in use for ages but was modified in 2013 by Ahmad et al., where hydraulic press was used to press the material to produce the heterogeneous mixture of oily and aqueous liquids. This was followed by using refrigerated centrifuge to rotate and completely separate the mixture. Separating funnel was then used to obtain the two layers in other to separate and be able to collect the oil without mixing. It was reported that a high yield was obtained in this process (Ahmad *et al.*, 2013).

Another method reported is the steam distillation which is often used on large scale especially in essential oil producing industries. It is not often use in laboratory protocol because of cost. In the steam distillation process, steam passes through the plant material, the combination of heated steam and gentle pressure causes the essential oil to be released. As the vapour mixture flows through a condenser and cools, it yields a layer of oil and a layer of water (Nasardin *et al.*, 2018). The essential oil rises to the top and is separated from the hydrosol (floral water) and collected.

Hydro-distillation is a method used in the laboratory for essential oil isolating from plant material. Here the plant material is immersed in water and brought to boil. The heat forces the essential oils out of the oil sag and it is then carried by steam through a condenser where it is cooled and separated into an oil phase on top and water layer

below. This is less costly than the steam distillation and the apparatus can be easily obtained or fabricated (Kamal *et al*., 2011; Oyedeji *et al*., 2010). The last two methods are common in literature as extractive technique for isolating essential oils in general, often the names are misused when reporting.

2.2 Ethno-medicinal uses

Lemon peels has been used as traditional medicine in almost all nations in the world for relieving scurvy, colds, flus and fevers and as anti-viral agent. Lemon juice is used to treat indigestion as it has a soothing effect on the gastrointestinal tract, and for curing colic or heart spasms (Pharmanews, 2018). Citrus leaves are reputed to possess antihypertensive effect (Duthie *et al*., 2000).

Grapefruit is consumed as a fruit, juice, and is used as a flavoring component. Although most grapefruit now are seedless but it was reported that the seed extract is good for killing bacteria and fungus, fight mold growth, kill parasites in animal feeds, preserve food and disinfect water (Sakamoto *et al*., 1996). Grapefruit juice and fresh fruit has remained useful as a folk medicine in many nations for its antibacterial, anti-fungal, anti-inflammatory, antimicrobial, antioxidant and anti-viral activities. It has also been used for cancer prevention, cellular renewal, reducing cholesterol level, purgative, detoxification, arthritis and weight loss (Imran *et al*., 2013).

2.3 Chemical constituents of *C. limon* and *C. paradisi*

It has been reported that *C. limonum* and *C. paradisi* peels are rich source of active compounds that are beneficial for human health. Some of the compounds from these species are vitamin C, carotenoids, flavonoids, limonoids, essential oils, acridone alkaloids, minerals and vitamin B complex. Flavonoids especially polymethoxyflavones, flavanone glycosides and limonoids are natural secondary metabolite compounds found in citrus (Ladaniya, 2008). Flavonoids and phenolic acids are reportedly common in leaves, flowering tissues and woody plant parts (Larson, 1988), however, they have reportedly been found in the peels of citrus fruit.

Chemical composition of the essential oil of the fruit peels of *C. limon* from Nigeria was reported to be dominated mainly by monoterpenes with limonene (**1**) (37.2%) camphene (**2**) (12.3%), α-terpineol (**3**) (11.2%), α-phellandrene (**4**) (6.5%) and 4-terpineol (**5**) (6.4%). The only significant sesquiterpenoid present was α–selinene (**6**) (3%) (Mahalwal and Ali, 2003). Lemon leaf oil from Iran had Linalool (**7**) (30.63%), α-terpinene (**8**) (14.52%), geraniol (**9**) (15.91%) and linalyl acetate (**10**) (13.76%) as the prominent constituents of the essential oil (Hojjati and Barzegar, 2017).

A study on the fruit peel and leaf oils of lemon growing in France was investigated by Lota *et al.*, (2002). The results of the study showed that the major compounds identified in the leaf essential oil were limonene (**1**) (19.1%), Nerial (**11**) (11.6%), β-pinene (**12**) (23.8%) and Geranial (**13**) (15.9%), while the fruit peel oil had limonene (**1**) (62.6%), β-pinene (**12**) (14.2%) and γ-tepirnene (**14**) (11.1%) as major

compounds (Lot *et al*., 2002). Investigation of peel essential oil of *Citrus limon* cultivated in Tunisia revealed that limonene (**1**) (39.74%) and β-pinene (**12**) (25.44%) were the only two predominant compounds followed by nerolidol (**15**) (6.91%) and farnesol (**16**) (4.28%). It was similary observed that the peel oil had oxygenated sesquiterpene in an appreciable amount in the oil composition (Housna *et al*., 2017). Two cultivars of lemon namely Meyer lemon and Interdoneto lemon growing in Turkey were studied and were observed that the two oils both had similar major compounds with only variation in percentages of the identified compounds. Limonene (**1**) (75.49% and 54.64%), α-pinene (**12**) (7.98% and 8.20%), 4-terpiniol (**5**) (2.17% and 2.99%), linalool (**7**) (3.67% and 2.65%), β–pinene (**12**) (5.91% and 17.95%), m-cymene (**19**) and β-myrcene (**20**) (8.64% and 7.44%) respectively (Bozkurt *et al*., 2017).

There are very few reports on the leaf and peel essential oil composition of *C. paradisi* in literature. Furthermore, it was observed that most of the report did not specify which of the three cultivar the report is on except for Bozkurt *et al*., (2017). In their study on Ruby red grapefruit peel essential oil from Turkey, limonene (**1**) (71.57%) α–pinene (**12**) (5.51%) and β-myrcene (**20**) (8.59%) were the major compounds (Bozkurt *et al*., 2017).

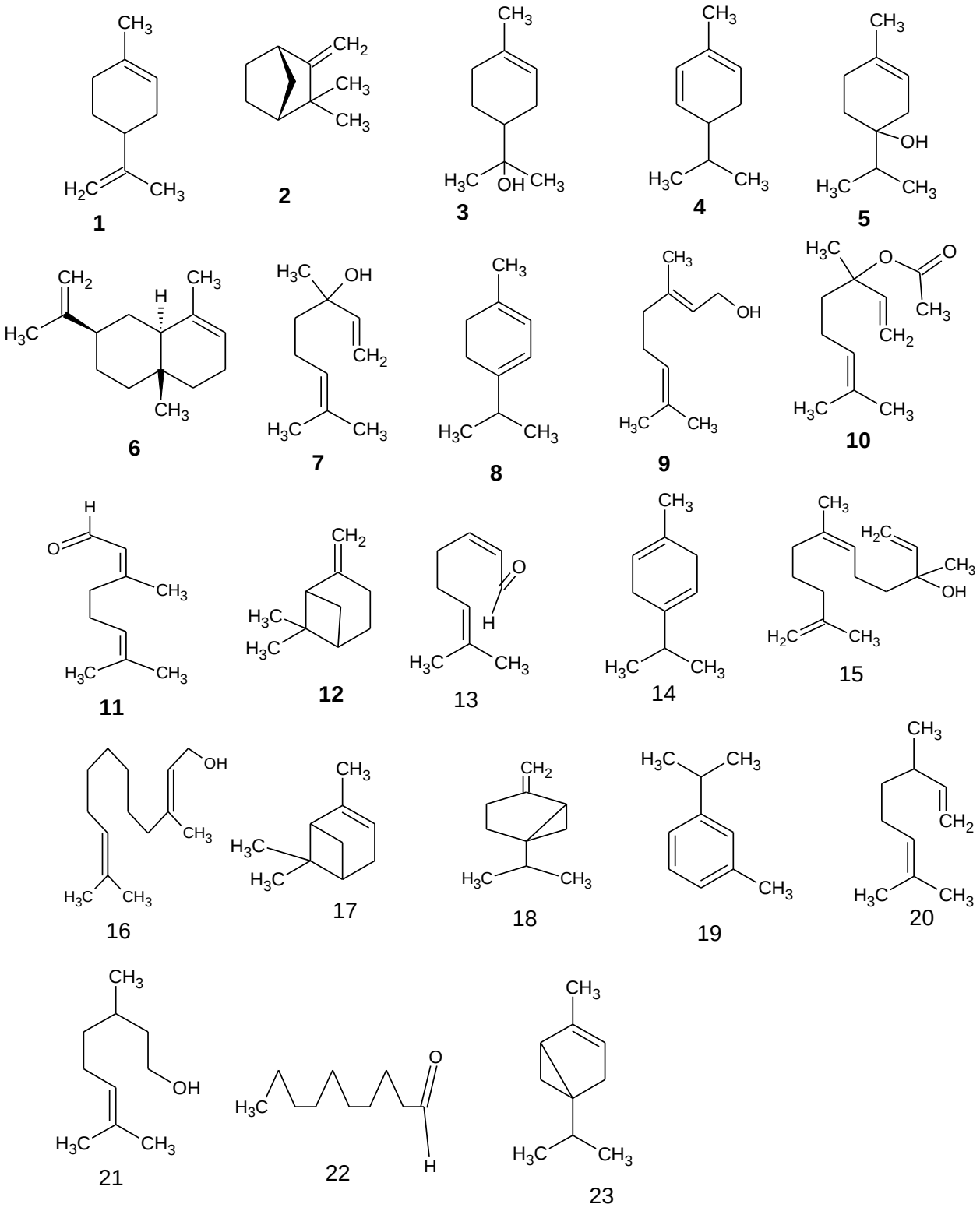

Figure 4: some major compounds found in Lisbon lemon and Grapefruit

Fruit peels of *C. paradisi* essential oil from Pakistan was reported to contain limonene (**1**) (86.27%) and myrcene (**20**) (6.28%) as the major compounds of the citrus specie studied (Ahmad *et al.*, 2013).

Chemical composition of Nigerian *C. paradisi* essential oil investigated had limonene (**1**) (75.05%) and β-myrcene (**20**) (7.25%), as its major compounds from the GC-MS analysis (Wahab *et al.*, 2013). This report however did not specify whether it is the peel or the leaf oil. Also, the study from Argentina *C. para*disi reported only limonene (**1**) (92.60%) as the main compound in the oil but failed to specify the plant part (Vasek *et al.*, 2015). (Figure 4).

The Nigerian grapefruit essential oil was found to contain the following compounds through the results from the GC-MS analyses of the essential oil and D-Limonene had the highest composition (75.05%), followed by β-myrene (7.25%), α-pinene (2.11%), caryophyllene (1.88%), octanal (1.68) and β-phellandrene (1.18%). Minor constituents of Nigerian grapefruit essential oil included δ-cadinene (0.89%), copaene (0.82%), methyl phthalate (0.54%), linalool (0.48%) and 3- δ -carene (0.21%) (Okwu, 2008). The Pakistan Grapefruit peel oil investigated in 2013 revieled some minor constituents to be α-thujene (0.15%), α-terpinene (2.11%), α-pinene (1.26%) citronellol (0.50%) and caprinaldehyde (0.31%) (Ahmad *et al.*, 2013)

There are very little reports on chemical composition of grapefruit essential oils as compared to other types of citrus such as clementine, lemon, lime and oranges.

2.4 Biological studies

Citrus fruits are highly consumed worldwide as fresh produce, the leaves and most often the peels are discarded as waste without consideration of the value due to the wide variety of secondary components present (Manthey and Grohmann, 2001). It has been reported that citrus fruits, or its extracts and flavonoids present in citrus exhibit a wide range of promising biological properties due to their phenolic profile and antioxidant properties (Montanari *et al*., 1998; Samman *et al*., 1996).

Citrus flavonoids have a large spectrum of biological activities including antibacterial, antifungal, antidiabetic, anticancer and antiviral (Burt, 2004; Ortuno *et al*., 2006). Flavonoids can function as direct antioxidants and free radical scavengers, and also have the capacity to modulate enzymatic activities and inhibit cell proliferation (Duthie and Crozier, 2000). Citrus leaf and peel essential oils have been bio-assayed for antioxidant activity and result shows significant reducing power and scavenging activity against DPPH free radicals (Federica *et al*., 2011; Khizar *et al*., 2009).

The effect of lemon peel oils on clinical bacterial strains; *Micrococcus aureusr, Salmonella typhimurium* and *Pseudomonas aeruginosa* has been studied and the results showed that lemon peels are good antimicrobial agents against these pathogens (Kawaii *et al*., 2000). Maruti *et al*. (2011), evaluated the effect of aqueous lemon extracts and phytochemicals on standard microorganism strains using routine antibacterial assay techniques and the report revealed that even the aqueous extract possess antimicrobial activities.

The essential oil of grapefruit peel possesses antimicrobial activities against *Staphylococcus aureus*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Escherichia coli*, *Salmonella typhimurium*, *Serratia marcescens* and *Proteus vulgaris* (Imran *et al.*, 2013).

2.5 Research Gap

Citrus is one of the South Africa's most important fruit crop. In the gross value, citrus industry in the country is the third largest horticultural industry after deciduous fruits and vegetables. Cultivated mainly in Limpopo, Western Cape, Mpumalanga, Eastern Cape, KwaZulu-Natal and Northern Cape provinces. The volume of the waste (fruit peels and leaf) generated in South Africa from the citrus industries directly or indirectly is enormous and do constitute environmental air pollution before decay process final takes place. Generally, the sting odor produced by the citrus peel is hazardous and offensive. However, lemon peels have been reported to effective in treating cold and flu ethno-medicinally. Grapefruit juice has been used in folk medicine in the management and treatment of infections, inflammation and as immunosuppressant. Industries uses lemon and grape fruits for many things such as flavoring, food spices, cosmetics and pharmacological, while a huge heap of leaves are left to rot without consideration to their medicinal and economical value.

These citrus waste are a good source of bioactive compounds. Although in some countries when dried and mixed with dried pulps are sold to feed cattle, nonetheless, the amount of waste generated is too large to be consumed alone by the animal farms. Moreover, there is a no literature on the study or investigation on the importance of secondary metabolites from grapefruit and lemon peel and leaf from South Africa.

Hence the need for this study, so that the citrus waste can be converted to useful end product for human consumption and health care.

Furthermore, during essential oil analysis, level of uncertainty in the identification of compounds is relatively high and because of the similarity of monoterpenoid compounds, chemical constituents are misrepresented. This study will try to unravel this discrepancy through the use of a newly acquired two dimension Gas chromatography equipped with quadrupole mass spectrometer (GCMS/MS) equipment in the natural product research laboratory.

2.6 Research questions

- What are the secondary metabolites present in grapefruit and lemon extracts?
- Which solvent of extraction is best for extractive solvent in extracting the secondary metabolites from citrus peels and leaves?
- Does South African *C. limon* and *C. paradisi* peels and leafs have medicinal and economic potential?
- What is the cytotoxicity profile of different species of citrus viz. lemon and grapefruit citrus?
- Which *Citrus* species or morphological part of *Citrus* plant possesses higher anti-inflammation and anti-oxidant properties?

2.7 Aim

The aim of this research is to investigate the chemical composition and biological activities of the peels and leaves (fresh and dried) of two *Citrus* fruits lemon and grapefruits essential oils.

2.8 Objectives

- To extract essential oil of leaf and fruit peels (both fresh and dried) of Lisbon lemon and Grapefruit using Clevenger-like apparatus.
- To determine the chemical compositions of the essential oils obtained using GC and GC/MS spectrometric techniques.
- To carry out phytochemical screening (qualitative and quantitative) of the three fruits using ethanolic and infusion extracts of their leaves.
- To evaluate the biological potentials such as cytotoxicity profile, anti-oxidant and anti-inflammatory activities of the essential oils.

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Chapter 3

Phytochemical studies of *Citrus limon*

3.1 Introduction

South Africa is one of the largest lemon producing country and of which Eastern Cape Province is the leading province where lemons are grown on commercial basis. This is followed by the Mpumalanga, Limpopo and KwaZulu-Natal Province. Lemon and lime account for 13 % of all citrus produced in South Africa between 2015-2016 cultivation year, 67% of this are exported in its raw fruit form while 29% are process by South Africa food and pharmaceutical industries for local consumption and export (DAFF, 2016).

The compound citral an aromatic compound used as perfume enhancer used in the perfume or flavouring industry. This compound has been found as the main component for the aroma and quality of citrus essential oils (Ladaniya, 2008). The E-isomer is known as geranial or citral A. The Z-isomer is known as neral or citral B. The two compounds are double bond isomers.

Eureka, Lisbon, limoneira and Meyer are the prominent varieties of *C. limon* which are mostly cultivated across South Africa. In this current study only Lisbon lemon fruit peels and leaf were studied. This chapter describes the methods used in isolating the essential oil from the two parts of the plant, *Citrus limon*. The analysis of the essential oils using GC and GC-MS for quantitative and qualitative analysis and the phytochemical screening of the peel and leaves plant parts were done.

3.2 Materials and Methods

3.2.1 Plant collection

Fresh Lisbon lemon leaf and fruit were collected from Addo River Bend Farm, Addo Park, Eastern Cape, South Africa, on the 23 May 2016 as shown in Figure 5; and the voucher specimen was deposited in KIE herbarium, Botany Unit, Walter Sisulu University with voucher spacemen MGM 002 for Lisbon lemon, for future reference.

3.2.2 Isolation and analysis of essential oil

3.2.2.1 Isolation of essential oil

Essential oils from *Citrus limon* leaf and fruit peels both fresh and dried were extracted at separate times through hydro-distillation method using Clevenger-like apparatus (Figure 6). About 600 g of fresh leaves was introduced into a round bottom flask and distilled water added to level above the citrus material. The mixture was brought to boil on a heating mantle at 100℃, then decreased to 70℃ and ran for 4 h. The above mentioned procedure was repeated for 600g fresh peel and 300 g of air-dried leaf and fruit peels of *C. limon* which (Ferhat *et al.*, 2007). The extracted oil was then stored in sample vials at a controlled temperature until the time of analysis.

Figure 5: Clevenger-like apparatus

3.2.3 Analysis of essential oil

The essential oils were analysed using Gas Chromatography (GC), Gas Chromatography/ Flame Ionization detector (GC-FID) and Gas Chromatography/ Mass Spectrometry (GC/MS) instruments.

3.2.3.1 GC Analysis

Two different Gas Chromatograph analysing instruments were used for comparison purposes, these were from University of Stellenbosch and Walter Sisulu University. Their methods were described below respectively

GC-FID Perkin-Elmner 8500

Gas Chromatographic analysis on the essential oils was carried out on a Perkin-Elmner 8500 gas chromatography with a non-polar column SGE BP X5 column and FID detector. Column length was 30 m with film thickness of 0.25 µm and diameter of

0.25 mm. The operation conditions were as follows: Carrier gas, nitrogen with flow rate of 3.0 mi/min; column temperature, 60-270 ^{0}C at 4 ^{0}C/min; injector and detector temperature, 280 ^{0}C; volume injected 0.1 µl of the essential oil; split ratio, 1:50.

GC-FID Bruker 450

FID Gas Chromatography analysis was carried out on a Bruker 450 gas chromatography equipped with an FID detector and BR HP-5 column (non-polar column) 30 m in length with film thickness of 0.25 µm and diameter of 0.25 mm. The operation conditions were as follows: Carrier gas, helium with follow rate of 1.0 ml/min; column oven temperature, 50-250 ^{0}C at 3 ^{0}C/min; injector and detector temperature, 300 ^{0}C; volume injected 0.1 µl of the essential oil; split ratio, 1:50.

3.2.3.2 GC/MS Analysis

The two different Gas Chromatograph equipped with Mass Spectroscopy analysing instruments were used for comparison purposes these were from University of Stellenbosch and Walter Sisulu University. Their methods are described below.

GC-MS Perkin-Elmner 890

The Gas Chromatography / Mass Spectrometry from University of Stellenbosch was an Agilent Gas Chromatography 890 equipped with a capillary column (30 m x 250 µm x 0.25 µm calibrate) attached with Agilent mass spectrometer system (5975C VL MSD with Triple Axis Detector) (Figure 6). The oven temperature was programmed from 50 ^{0}C - 310 ^{0}C. Helium was used as a carrier gas with follow rate of 5 ml/min at a split

ratio of 1:200. About 1 µl of essential oils were diluted with hexane and 0.5 µl of the diluted solution were manually injected into the GC/MS. The chemical composition of essential oils of both fresh and dried material of citrus fruits were determined according to their retention time and spectrometric electronic libraries (WILEY NIST).

Figure 6: Agilent GCMS at University of Stellenbosch

GC-MS Bruker 750

The Walter Sisulu University GC-MS is a Bruker 450 Gas Chromatography-300 mass spectrometer system operating in EI mode at 70 eV, equipped with a HP-5 MS fused silica capillary system with 5% phenylmethylsiloxane stationary phase, capillary column parameter was 30 m by 0.25 mm, film thickness 0.25 µm (Figure 7). The initial temperature of the column was 70°C and was heated to 240°C at a rate of 5°C/min; the final temperature was kept at 450°C and run time of 66.67 min. Helium was used as the carrier gas at a flow rate of 1min/min. The split ratio was 100:1. Scan time was 78 min with a scanning range of 35 to 450 amu. One microliter (1µl) of the diluted oil (in hexane) was injected for analysis. N-Alkane of C_8 to C_{30} was run under the same

condition of Kovat indices determination. The constituents were identified by GC using retention indices with those of literature. The retention indices were determined in relation to a homologous series of alkanes under the same operating conditions. The components of the oils were identified by matching their spectra and retention indices (Kovat Index) with those of the authentic samples and literature values (WILEY NIST) (ESO, 2000 and Adams, 2007).

Figure 7: Bruker GCMS at Walter Sisulu University

3.2.4 Phytochemical screening

3.2.4.1 Preparation of the plant materials for phytochemical screening

Fruit peel was peeled out manually and cut into small pieces while leaves were spread and both were air-dried at room temperature for two weeks, then grounded into powder. The powdered peel and leaf materials were weighted using electric balance and transferred to two 1000 ml conical flasks. Aqueous (Aq) and Ethanolic (EtOH) extracts were obtained by soaking the peel and leaf materials in large flasks containing various solvents for 48 h. The flasks were covered so that solvent evaporation does

not occur and then placed on a shaker for 48 h. The extracts were filtered. A portion of the filtrate for each sample was used separately for qualitative phytochemical screening while the rest of the filtrates were evaporated to form crude extract which were used for quantitative phytochemical screening.

3.2.4.2 Qualitative phytochemical screening of Aqueous and Ethanolic extracts

The presence of some classes or groups of phytochemicals from fruit peels and leaf were evaluated using modified existing method for phytochemical detection of tannins, flavonoids, steroids, terpenoids, saponins, alkaloids and glycosides, proteins and amino acids and phenolic compounds (Miya *et al*., 2016; Harbone, 2001; Ghani, 1998; and Sofowora, 2005).

Tannins

Ferric chloride test**:** 0.5 g of each extract was dissolved in 5 ml of distilled water and filtered. A few drops of a 10% ferric chloride solution was added to the filtrate. A greenish black colour or a precipitate was taken as an indication of the presence of tannins.

Flavonoids

Shinoda Test: small piece of magnesium ribbon followed by few drops of concentrated hydrochloric acid were added to a small amount of each extract of the sample material. Immediate development of a pink scarlet or crimson red colour indicated the presence of flavonoids.

Steroids/ Terpenoids

Liebermann-Burchardt test: 1 ml of each extract of the plant extract was boiled with 3 ml of acetic anhydride, and then cooled; 2 drops of concentrated sulphuric acid was added slowly through the wall of the tube. Appearance of dark green coloration of the solution indicated the presence of steroids, and formation of dark pink or red coloration in the interface indicated the presence of terpenoids.

Saponins

Emulsion Test**:** 2 drops of olive oil was added to the frothing solution (which was obtained by extracting plant material for 48 hours using distilled water and ethanol as solvents and filtrated) and shaken vigorously the formation of emulsion was taken as an indication of the presences of saponins.

Alkaloids

About 0.2 g of the plant extract was transferred into a conical flask; 20 ml of dilute sulphuric acid in 10 % methanol was added and then heated in water bath to boil for 5 min. The mixture was filtered (vacuum pump) and the filtrate was separated and treated with 2 drops of Mayer`s and Dragendorff's reagents in test-tubes. Development of creamy and an orange color respectively indicated positive result.

Glycosides

Liebermann's test: 2 ml of the plant extract was dissolved in 2 ml of 1:1 chloroform and acetic acid. This solution was cooled in ice then 2 ml of concentrated sulfuric acid was added. The color change from violet to blue to green indicated the presence of glycosides.

Phenolic compounds

1 g of the extract was dissolved in 5 ml of distilled water. Few drops of 5% ferric chloride solution were added. A dark green color indicates the presence of phenolic compounds.

Test for Protein and amino acids

Biuret test: To 0.5 mg of plant extract equal volume of 40% NaOH solution and two drops of 1% copper sulphate solution was added. The appearance of violet color indicates the presence of protein.

Ninhydrin test: About 0.5 mg of extract will be taken and 2 drops of freshly prepared 0.2% ninhydrin reagent was added and heated slightly. The appearance of pink or purple color indicates the presence of proteins, peptides or amino acids.

3.2.4.3 Quantitative phytochemical screening

The following secondary metabolites saponins, tannins, flavonoids and alkaloids, were quantified using methods described by Miya *et al*., (2016) and Dyayiya *et al*., (2016).

Determination of total Saponins content

The ground samples of *C. limon* fruit peels and leaf (20 g) were placed into separate conical flask and 100 ml of 20% aqueous ethanol was added to each. The sample was heated in water bath for 4 h with continuous stirring at about 55℃. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90℃. The concentrate was then transferred into a 250 ml separator funnel and 20 ml of diethyl

ether added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were then washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples was dried in the oven to a constant weight; the saponins content was calculated using the formula:

% yield = (mass obtained/starting material) × 100%.

Determination of total Flavonoid content

The dried powered plant material (10 g) was extracted repeatedly with 80% of aqueous methanol solution, at room temperature. The combined solution was filtered and the filtrate was transferred into a crucible and solvent evaporated over a water bath. The solid content was dried and weighed continuously to a constant weight and flavonoid content was calculated as the percentage yield obtained using the formula:

% yield = (mass obtained/starting material) × 100%.

Determination of total alkaloids

The ground sample (5 g) was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 h. This was then filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation is complete. The whole solution was allowed to settle, the precipitate was collected and washed with dilute ammonium hydroxide and filtered. The residue

which is the alkaloid was dried and weighed. The yield was calculated using the formula

$$\% \text{ yield} = (\text{mass obtained/starting material}) \times 100\%.$$

Determination of total tannins

About 0.5 g of the sample was weighed into a 50 ml bottle. 50 ml of distilled water was added and shaken for an hour on a mechanical shaker. This was filtered into a 50 ml volumetric flask and the total volume was filled up to the mark with distilled water. Then 5 ml of the filtered was transferred into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 M HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min. The tannin content was calculated using a standard curve of Gallic acid.

3.3 Results and discussion

3.3.1 Physiochemical analysis

Table 3.1 below shows the results of essential oils percentages in (w/w) for fresh and dried material of Lisbon lemon fruit peels and leaves. It was observed that the fruit peels gave higher percentage yield when compared to leaf (w/w) even though the starting material for fresh were almost double the starting material of the dried material, it can be concluded that, the low percentage yield which is not proportional to the weight of the starting material could be due to the water quantity in the fresh material compared to the dried material.

Table 3.1: Physiochemical analysis of *C. limon* essential oil (w/w)

Plant parts	Smell	Colour	Starting material/g	Mass of oil/g	% (w/w)
Leaf fresh	Lemon	Colourless	658.43	2.59	0.39
Leaf dried	Sharp lemon	Light yellow	302.09	2.09	0.69
Fresh peels	Citrus	Colourless	577.60	4.79	0.83
Dried peels	Sharp citrus	Light yellow	251.26	3.17	1.62

The smell and the colour of essential oils were common in a sense that they had a citrus odour although the smell of the leaf (fresh and dried) was more distinct with sharp lemon odour. The fresh leaf and fruit peels were colourless while the dried materials had light yellow coloration.

3.3.2 Essential oils analyses

3.3.2.1 Chemical composition of *C. limon* essential oils by GC and GC-MS (2016) from Stellenbosch University

Table 3.2 below shows results of GC-MS for *C. limon* essential oils analysis done in Stellenbosch University.

The fresh fruit peels of *C. limon* essential oil had a total of 26 compounds identified accounting for (81.9% of the total oil composition). β-pinene (10.6%), limonene (52.5%), γ-terpinene (8.8%) and geranial (5.7%) were the prominent compounds. The fresh leaf oil had 29 compounds identified from the GCMS chromatogram which is (96.4% of the total oil composition). Limonene (31.0%), β-pinene (13.3%), Citral (10.3%) and Geranial (13.2%) were the major compounds. On the other hand, the dried fruit peel oil had more compounds from the GCMS analysis as a total of 44

compounds were identified in the dried peel oil (with 86.3% of total oil composition). Limonene (36.0%), alloocimene (5.6%) and L-carvone (5.9%) were the major compounds. Twenty-one (21) compounds were identified in the dried leaf oil (82.4% of oil composition) with linalool (8.6%), limonene (12.0%), caryophyllene (17.7%) and limonene glyco (5.2%) as dominant constituents.

Table 3.2: Chemical composition of the essential oils of the leaf and peel of *Citrus limon*.

Compounds	Kovat Index (KI)value	Oil percentage				Method of identification of A, B, and C
		C. limon (peel)		*C. limon* (leaf)		Fragment ions
		LLFP	LLDP	LLFL	LLDL	
Thujene	932	0.3	-	-	-	136, 91,77, 41
α-Pinene	934	1.5	0.2	1.1	-	136, 93, 79, 41
Sabinene	957	0.3	0.3	-	-	136, 93, 77, 41
δ-3-Carene	974	-	-	1.0	-	136, 93, 79, 41
β-Pinene	980	10.6	2.2	13.3	-	136, 93, 41, 79
Myrcene	991	1.4	-	1.4	-	136, 41, 93, 69
6-Methyl-5-hepten-2-one	989	-	0.3	2.7	1.8	126, 93, 41, 69
Octanal	1019	0.1	0.5	-	-	129, 41, 56, 69
Eucalyptol	1022	-	0.2	-	-	154, 43, 81, 108
Limonene	1034	52.5	36.0	31.0	12.0	136, 67, 93, 79
Limonene glyco	1035	-	-	-	5.2	170, 71, 67, 108
1,8 Cineole	1036	-	-	1.2	2.1	154, 43, 68, 93
Trans-β-ocimene	1047	0.1	-	2.3	-	136, 91, 79, 41
Linalool oxide	1056	-	-	-	3.8	170, 43, 59, 93

ɣ-Terpinene	1066	8.8	-	0.5	8.6	136, 93, 77, 41
Cyclo-octane	1071	-	0.1	-	-	112, 56, 41, 55
Epoxylinalool	1079	-	0.7	-	-	170, 59, 98, 115
α-Terpinolene	1084	0.5	-	0.3	-	136, 93, 121, 79
Trans-linalool oxide	1088	-	0.7	0.2	4.2	164, 43, 59, 93
p-Cymene	1092	-	0.2	-	-	134, 119, 91, 65
Linalool l	1099	1.2	1.3	2.3	-	154, 43, 71, 55
Nonanal	1104	0.2	0.2	-	-	142, 57, 41, 98
Fenchol	1117	-	0.1	-	-	154, 81, 80, 43
Cis-limonene oxide	1135	-	1.7	0.1	-	152, 43, 67, 109
Trans-Pinocarveol	1152	-	0.8	-	-	152, 41, 55, 70
Citronellal	1155	-	0.2	0.3	-	154, 41, 69, 55
Camphor	1159	-	0.2	-	-	152, 95, 41, 81
p-Menth-1-en-9-al	1162	-	0.2	-	-	154, 43, 71, 81
Pinocarvone	1163	-	0.8	-	-	150, 108, 81, 53
Uknown	-	-	1.3	-	-	-
Terpinene-4-ol	1182	1.1	1.1	1.0	1.5	154, 71, 43, 93
α-Terpineol	1192	2.7	-	1.5	-	152, 59, 43, 93
Unkown	-	-	-	-	1.1	-
Alloocimene	1207	-	5.6	-	-	136, 121, 79, 105
2-Butenal, 3-methyl	1209	-	0.5	-	-	84, 55, 41, 83
Citronellol	1211	-	0.7	-	1.8	154, 41, 69, 81
Nerol	1228	1.7	-	0.7	2.3	152, 41, 69, 81
Carveol	1233	0.3	-	-	1.0	152, 119, 91, 134
Trans-(+)-carveol	1235	-	3.5	-	-	152, 109, 41, 55
Z-Citral	1240	4.3	0.7	10.3	-	154, 69, 41, 85

Cis-carveol	1242	1.0	-	-	-	152, 41, 109, 55
Geraniol	1245	1.3	4.1	1.3	1.3	152, 41, 69, 81
(S)-Carvone	1249	0.3	-	0.2	1.3	152, 82, 54, 93
Piperitone	1251	-	0.2	-	-	152, 82, 110, 39
Geranial	1258	5.7	-	13.2	4.1	152, 41, 69, 95
Methyl geraniate	1323	-	-	0.2	-	182, 69, 41, 114
Citronellyl acetate	1338	-	-	0.3	-	198, 43, 81, 67
Farnesol	1345	-	0.3	-	-	222, 41, 69, 81
α-Humulene	1443	-	-	0.1	-	204, 93, 67, 80
Neryl acetate	1470	1.3	2.7	4.6	2.6	196, 41, 69, 93
Spiro(5.6)dodecane	1496	-	-	-	1.8	166, 96, 81, 67
d-Nerolidol	1539	-	0.2	-	-	222, 69, 41, 93
1,10-decanediol	1548	-	-	0.2	-	174, 55, 68, 41
Geranyl acetate	1560	0.5	-	4.4	-	196, 41, 69, 93
(+) Spathulenol	1579	-	-	-	3.9	205, 43, 91, 79
β-Caryophyllene	1594	0.3	-	0.5	-	204, 41, 91, 79
(-)-Humulene epoxide II	1603	-	-	-	1.3	220, 41, 79, 91
Zingiberene	1611	0.6	0.2	-	-	204, 93, 119, 43
d,l-trans-Sobrerol	1623	-	0.1	-	-	170, 109, 59, 79
4-methyl-5-vinyl thiazole	1673	-	0.2	-	-	125, 125, 97, 58
Unkown	-	-	0.2	-	-	-
Tetradecane, 5-methyl-	1710	-	1.2	-	-	212, 43, 57, 85
Valencene	1726	-	0.3	-	-	204, 105, 161, 91
Dimethyl-2,6-octadienoic acid	1730	-	-	-	2.8	182, 69, 114, 83

β-Bisabolene	1788	1.0	0.4	-	-	204, 69, 93, 41
Caryophyllene oxide	1962	-	0.5	0.2	17.7	220, 41, 79, 91
Isonicotinic acid	2088	-	0.1	-	-	123, 123, 78, 51
3,5-dimethyladamantan-1-ol	2102	-	0.3	-	-	180, 109, 123, 180
(+,-)-(Z)-Dihydrofarnesal	2166	-	0.1	-	-	222, 204, 197, 81
Trans-Chrysanthenol	2174	-	0.2	-	-	152, 119, 69, 41
Total percentage of compounds		**81.92**	**83.80**	**96.41**	**82.44**	

Keynote: LLFP (Lisbon lemon fresh fruit peels), LLDP (Lisbon lemon dried fruit peels), LLFL (Lisbon lemon fresh leaf) and LLDL (Lisbon lemon dried leaf). A- Cas Number, B- Kovat Index, C-Fragmentation pattern. t-Trace amount less than 0.1

The above table gives all the information about the essential oils of leaf and fruit peels of Lisbon lemon extracted from fresh and dried material. It also gives the KI values and percentage composition of each compound and final the total percentage of each essential oil sample of compounds identified by the GC-MS machine.

3.3.2.2 Chemical composition of *C. limon* essential oils by GC-MS (2017) from Walter Sisulu University.

The GCMS analysis of the C. limon fresh fruit peel essential oil collected in 2017 gave 16 constituents of which 14 were identified depicting (93.4% of the total oil composition. D-Limonene (60.4%), p-cymene (6.3%) and β-pinene (9.3%), were the major constituents, while in the dried fruit peel chromatogram, 16 compounds were identified accounting for (95.0% of the oil composition). Similarly in the fresh peel oil

profile, D-limonene (67.9%) was the dominant compound, including γ-terpinene (6.9%) and β-pinene (3.9%) as prominent compounds in the oil profile. The fresh leaf of essential oil had 25 constituents of which 22 identified presented (96.6% of the oil composition) from the GC-MS chromatogram. D-limonene (32.0%), β-pinene (8.6%), z-citral (11.5%) and geranial (14.2%) were the significant compounds. Twenty-three (23) compounds were identified from the dried leaf essential oil chromatogram (97.1%) with D-limonene (32.8%), geranial (16.2%), z-citral (12.8%), β-pinene (10.3%) and linalool (8.6%) as the leading dominant compounds.

Table 3.3: Chemical compounds from leaf and peel of *Citrus limon* essential oil collected in 2017

Compounds	KI Values	Percentages				Method of identification of A, B, and C
		LLDP	LLDL	LLFP	LLFL	Fragment ions
Hexanal	798	-	0.6	-	-	100, 44, 56, 41
(+)-α-Pinene	937	1.4	1.4	1.4	0.8	136, 93, 41, 79
Sabinene	974	1.4	2.2	1.1	2.0	136, 93, 77, 41
(-)-β-Pinene	981	3.9	10.3	9.3	8.6	136, 93, 79, 41
6-Methyl-5-hepten-2-one	985	-	1.5	-	2.4	126, 93, 41, 69
β-Myrcene	990	1.8	1.3	1.2	1.2	136, 41, 93, 69
3-Carene	1011	-	0.6	0.3	0.7	136, 93, 79, 41
α-Terpinene	1015	0.9	-	-	-	136, 93, 121, 77
p-Cymene	1026	0.8	-	6.3	-	134, 119, 91, 65
D-Limonene	1030	67.9	32.8	60.4	32.0	136, 67, 93, 79
Eucalyptol	1033	-	1.0	-	2.5	154, 43, 81, 108

β-Ocimene	1041	0.2	2.3	-	1.8	136, 91, 79, 41
γ-Terpinene	1056	6.9	0.5	2.4	0.1	136, 93, 77, 41
α-Terpinolene	1086	0.6	-	-	-	136, 93, 121, 79
Linalool	1103	1.4	1.8	1.1	2.1	154, 43, 71, 55
Citronellal	1151	-	1.9	-	1.3	154, 41, 69, 55
L-α-Terpineol	1172	-	0.5	-	1.0	154, 59, 43, 93
Terpinen-4-ol	1175	2.6	-	1.3	1.2	154, 71, 43, 93
Unknown	-	-	1.4	-	-	-
α-Terpineol	1189	2.3	0.5	3.1	-	154, 59, 43, 93
Nerol	1228	-	2.1	0.5	3.4	152, 41, 69, 81
Thymol	1235	1.5	-	-	-	154, 135, 150, 91
Citral	1240	0.7	-	-	-	154, 69, 41, 85
Unknown	-	-	0.2	-	1.3	150, 82, 39, 54
Z-Citral	1247	-	12.8	1.6	11.5	154, 69, 41, 85
Geraniol	1266	-	0.7		2.4	152, 41, 69, 81
Geranial	1270	0.9	16.2	2.2	14.2	152, 41, 69, 95
Citronellyl acetate	1354	-	0.3		0.2	198, 43, 81, 67
Neryl acetate	1365	**-**	4.7	-	3.2	196, 41, 69, 93
Geranyl acetate	1383	-	-	-	3.0	196, 41, 69, 93
Total Percentages of compounds		**95.02**	**97.10**	**93.41**	**96.63**	

Keynote: LLFP (Lisbon lemon fresh fruit peels), LLDP (Lisbon lemon dried fruit peels), LLFL (Lisbon lemon fresh leaf) and LLDL (Lisbon lemon dried leaf). A- Cas Number, B- Kovat Index, C-Fragmentation pattern. t-Trace amount less than 0.1

A comparative review of the major compounds in the *C. limon* oils is presented in Table 3.4 below. The prominent compounds from the essential oils is limonene except

for the dried leaf oil from 2016 collection which had caryophyllene oxide as the major compound.

Table 3.4: Major compounds from fresh and dried leaf and peels of *C. limon* collected at various times

Library/ID	**2016 %**				**2017 %**			
	LLFP	**LLDP**	**LLFL**	**LLDL**	**LLFP**	**LLDP**	**LLFL**	**LLDL**
β-Pinene	10.6	-	13.3	-	9.3	3.9	8.6	10.3
Citral	-	-	10.3	-	-	-	11.5	12.8
Limonene	52.5	36.0	31.0	12.0	60.4	67.9	32.0	32.8
Limonene glycol	-	-	-	5.2	-	-	-	-
γ-Terpinene	8.8	-	-	-	-	6.9	-	-
Linalool L	-	-	-	8.6	-	-	-	-
Alloocimene	-	5.6	-	-	-	-	-	-
Geraniol	-	4.1	-	-	-	-	-	-
L-Carvone	-	5.9	-	-	-	-	-	-
Geranial	5.7	-	13.2	-	-	-	-	16.2
caryophyllene oxide	-	-	-	17.7	-	-	-	-
Total percentage	**77.64**	**51.50**	**67.81**	**43.51**	**96.70**	**78.73**	**52.12**	**72.11**

Keynote: LLFP (Lisbon lemon fresh fruit peels), LLDP (Lisbon lemon dried fruit peels), LLFL (Lisbon lemon fresh lead) and LLDL (Lisbon lemon dried leaf). t-Trace amount less than 0.1

It is noteworthy the presence of citral and geranial in high concentration in most of the oils. The South African leaf and peel oils resembles the other African lemon oils from Tunisia and Nigeria reported in the literatures (Housna *et al*., 2017; Mahalwal and Ali, 2003) with the limonene as the major dominating compound, however, it can be said that the South African lemon oil follows the France chemotype pattern with the presence of geranial and citral (Lota *et al*., 2002).

3.3.3 Phytochemical screening

3.3.3.1 Qualitative phytochemical screening

Table 3.5: Qualitative Phytochemical screening of the ethanolic and aqueous extracts of Lisbon lemon leaf and peels.

Phytochemicals	L.L Aq	L.L EtOH	L.P Aq	L.P EtOH
Tannins	+	+++	++	+++
Saponins	+	-	++	-
Flavonoids	+	++	++	+++
Terpenoids	++	++	++	++
Glycosides	+++	+++	+++	+++
Phenolic compounds	++	++	+++	++
Alkaloids	++	+	+++	++
Steroids	++	+	++	+
Proteins and Amino acids	++	+	++	++

Foot note: +++; detected in rominent amount, ++; Detected in ordinary amount, +; Trace amount of detection and -; Not detected, L.L Aq= Lisbon lemon leaves aqueous extract, L.L EtOH= Lisbon lemon leaves ethanolic extract, L.P Aq= Lisbon lemon peels aqueous extract and L.P EtOH= Lisbon lemon peels ethanolic extract.

The Table 3.5 above represented results for leaf and fruit peels from two extracting polar solvents. It was observed that saponins were not detected from ethanolic extracts in both leaf and fruit peels while they were present in aqueous extracts. This could be inferred that perhaps the percentage is quite small to such an extent that it dissolves readily in water than alcohol. Thus it can be recommended that the use of water in extracting phytochemicals is the best compared to alcohol from the above results and due to the level of group of compounds detected in the both extracts.

3.3.3.2 Quantitative phytochemical screening

The results for quantitative screening is represented in a Table 3.6 below showing the powdered material, obtained phytoconstituents and the percentage yield per weight of each secondary metabolites.

Table 3.6: Quantitative phytochemical screening of Lisbon lemon fruit peels.

Quantified Phytochemicals	Initial material(g)		Recovered material (g)		Percentage w/w	
	Peels	Leaf	Peels	Leaf	Peels	Leaf
Flavonoids	10.0	10.0	0.74	0.50	7.40	3.69
Alkaloids	5.02	5.30	0.36	0.37	2.17	6.94
Saponins	20.0	20.0	0.086	0.37	0.43	0.25
Tannins	0.53	0.51	0.012	0.010	2.26	1.96

From the Table 3.6 above it was observed that leaf of *C. limon,* are rich in alkaloids compared to the fruit peels, while the fruit peels are rich in flavonoids content compared to the leaf. Moreover tannin is present in both plant materials in an appreciable quantity. The decreasing trend of the four phytochemicals (in terms of quantity) for the leaf is: Alkaloids followed by flavonoids, then tannins and lastly saponins, while in the fruit peels the trend was flavonoids, tannins, alkaloids and saponins being the lowest. The low content of saponins in the quantitative analysis correlates the earlier results of its little or no detection qualitatively.

3.4 Conclusion

The outcomes of this work revealed that the yields (w/w) of the essential oils from the dried materials doubled that of the fresh material even though the weight of the

initial fresh material was twice that of the dry material. It was also observed that fruit peels had higher oil (w/w) yields than leaf this was observed from both fresh and dried materials which is in agreement with literature reports of citrus peels having more oils than the leaves. There is an assumption that the oils of citrus are yellow in color and this is usually represented as such in advertisement of the commercial oils but this study showed that not all citrus oils especially *C. limon* is yellow in color. The color of *C. limon* fresh fruit peels, and fresh leaf essential oils were observed to be colourless, while dried fruit peels and leaf were pale yellow.

The compound citral was reported to be present in significant amount in the Brazilian lemon essential oil (13.3%) (Marcos *et al.*, 2014) while in the France lemon peel oil, neral (11.6%) and geranial (15.9%) were found in high concentrations (Lota *et al.*, 2002). Although limonene is the predominant compound in all of South African peels and leaf oils reported in this study which is in line with essential oil lemon report in the literatures. Nonetheless, the presence of citral (neral), geranial, linalool and β-pinene confirms the *C. limon* from Addio River farm to be more of France chemotype than others.

The high percentage of alkaloid in the leaf extract can relate to the use of citrus lemon to relive stress and bring about relaxation as alkaloids are known to be precursors of psychoactive drugs and used as stimulants of the central nervous system (Hesse, 2002). Flavonoids on the other hand, are wide range of phytochemicals with various pharmacological effects including antioxidant, anti-inflammation, anti-platelet, anti-allergic, cytotoxicity and reduced risk of heart diseases or cancer (Asif and Khodadadi,

2013). According to the above observations, it can be concluded that fruit peels of Lisbon lemon can possible have higher anti-oxidant potential as compared to the leaf due to the flavonoids quantity from the phytochemical screening result. However, both the leaf and the fruits can be used as a source of flavonoid intake to reduce associated free radical disease(s). Tannins in fruits serve as a natural defence mechanism against microbial infections and also known to be of medicinal value. The anti-carcinogenic and anti-mutagenic potentials of tannins may be related to their antioxidative property, which is important in protecting cellular oxidative damage, including lipid peroxidation. Tannins have also been reported to exert other physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipids level, produce liver necrosis, and modulate immune-responses (Chung et al., 1998).

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Chapter 4

Phytochemical studies of *Citrus paradisi* Macfad

4.1 Introduction

C. paradaisi is a subtropical tree known for its sour to semi sweet fruit, it is widely distributed in China, United States of America, Thailand and South Africa (Ahmad et al., 2013). Grapefruits differ in colour from white or yellow to pink and red which results in various varieties or cultivars. *C. paradisi* varies in taste, from acidic and even bitter to sweet and sugary (Isgro et al., 1983). The Limpopo Province is the leading grower of grapefruit followed by the Mpumalanga Province, Kwazulu-Natal Province and Northern Cape. In Mpumalanga, Limpopo and KwaZulu-Natal, the climate is warmer and better suited for the cultivation of grapefruit compared to the Eastern Cape Province (Zekri, 2011). The most cultivated grapefruit cultivar across South Africa is Star Ruby followed by Marsh and lastly Rose grapefruit. In the cultivating year 2015-2016, 59% of total cultivated grapefruit was exported (DAFF, 2016)

In this chapter, we shall report the investigation on the phytochemical studies on the three different cultivars of *C. paradisi* namely Rose grapefruit, Star Ruby grapefruit and Marsh grapefruit in terms of essential oils isolation from leaf and fruit peels.

4.2 Methodology

The methods described in chapter 3 were also employed in this chapter for *C. paradisi* plant regarding the isolation of essential oils, solvent extractions and phytochemical (qualitative and quantitative) determinations.

4.2.1 Plant collection

The three grape fruit leaves and fruits were obtained from Mystic Blue Farm Eshowe, KwaZulu-Natal, South Africa (Figure 8) and the voucher specimens were deposited in KIE herbarium, Botany Unit, Walter Sisulu University with voucher numbers MGM 003 for marsh, MGM 004 for rose and MGM 005 star ruby grapefruits, for future reference.

Figure 8: Collection of grapefruits.

4.2.2. Isolation of essential oil

The essential oils were isolated from fresh leaf and fruit peels and also from dried leaf and fruit peels of three grapefruit. The detailed methods were described in **section 3.3.1**.

4.2.3 Analysis of essential oil

Gas chromatography-flame ionization detector (GC-FID) and gas chromatography equipped with mass spectrometer (GC-MS), were used for the analyses of the oils and the methods along with the operating conditions were described in **section 3.3.2**.

4.3 Phytochemical screening

Aqueous and ethanolic extracts were prepared for both leaf and fruit peels for phytochemical screening and a detailed method of plant preparation was described in **section 3.2.4**.

Both qualitative and quantitative phytochemical screening were carried out on the leaf and fruit peels of the three cultivars of grapefruit using water (aqueous) and ethanolic solvent as extracting solvents for the qualitative phytochemical screening while the crude extracts together with raw materials were used in quantitative phytochemical screening using well known methods.

4.3.1 Qualitative phytochemical screening

Nine (9) phytochemicals (saponins, tannins, steroids, terpenoids, protains ans amini acids, alkaloids, phenolic compounds, glycosides and flavonoids) were tested for their presence in *C. paradisi* leaf and fruit peels qualitatively. The same procedures that were used and described in **section 3.2.4.1** were also utilized in this section.

4.3.2 Quantitative phytochemical screening

Flavonoids, alkaloids, tannins and saponins were the only four phytochemicals subjected to quantitative analysis from the secondary metabolites screened qualitatively. The methods used in **section 3.2.4.2** were also applied here.

4.4 Results and discussion

4.4.1 Physiochemical analysis

The percentage yield, color and odor analysis of the isolated essential oils from the three cultivars of *C. paradisi* are presented in Table 4.1.

Table 4.1: Physiochemical analysis of *C. paradisi* essential oil (w/w)

Plant name and plant part	**Smell**	**Colour**	**Starting material /g**	**Mass of oil/g**	**% (w/w)**
Star Ruby grape leaf fresh	Mild sweet citrus	Colourless	318.6	1.4	0.44
Star Ruby grape leaf dried	Herbaceous	Colourless	240.5	3.6	1.50
Star Ruby grape peel fresh	Sweet citrus	Pale yellow	885.6	3.0	0.34
Star Ruby grape peel dried	Sweet citrus	Yellow	352.3	6.3	1.79
Marsh grape leaf fresh	Strong lemonade	Colourless	386.2	0.8	0.21
Marsh grape leaf dried	Herbaceous	Colourless	275.2	2.3	0.84
Marsh grape peel fresh	Sweet Lemonade	Colourless	904.6	3.1	0.34
Marsh grape peel dried	Sweet Lemonade	Pale yellow	315.0	2.6	0.83
Rose grape leaf fresh	Mild citrus	Colourless	405.2	2.3	0.57
Rose grape leaf dried	Mild citrus	Pale yellow	265.7	1.3	0.49
Rose grape peel fresh	Mild citrus	Colourless	612.1	1.9	0.31
Rose grape peel dried	Mild citrus	Pale yellow	409.8	2.7	0.66

From the result above, the dried peel and leaf essential oil of Star Rudy grapefruit had highest percentage yields (1.50 and 1.77% respectively). Similar patterns were observed for the Marsh and Rose dried oil samples. The colour of the fresh oil plant material are colourless and pale yellow when dried except for Marsh dried leaf oil, Star Rudy dried leaf oil and Star Rudy fresh peel oil.

4.4.2 Essential Oil analysis of the three *C. paradisi* species

4.4.2.1 Chemical composition of the *C. paradisi* Rose cultivar essential oil

Table 4.2: Chemical composition of *C. paradisi* Rose cultivar essential oil.

Compound name	KI value	Percentage				Method of identification A, B and C
		C. paradisi (peels)		*C. paradisi* (leaf)		Fragment ions
		RGFP	RGDP	RGFL	RGDL	
Toluene	762	-	0.3	t	-	92, 91, 92, 65
2-Buten-1-ol,2-methyl	766	-	t	-	-	86, 71, 86, 43
Hexanal	780	-	0.1	1.2	-	100, 44, 56, 41
(Z)-3-Hexan-1-ol	827	0.1	-	-	-	100, 67, 41, 39
Furfural	830	-	t	-	-	96, 96, 95, 39
Heptanal	902	-	t	-	-	114, 70, 41, 44
α-pinene	935	0.5	0.5	-	-	136, 93, 79, 41
3-Carene	974	-	t	-	t	136, 93, 79, 41
β-pinene	980	0.1	0.1	t	t	136, 93, 41, 79
5-Heptene-2-one-6-methyl	985	-	t	-	-	126, 93, 41, 69
β-myrcene	991	2.5	2.0	t	0.2	136, 41, 93, 69
Octanal	1019	0.8	5.2	-	-	128, 43, 44, 41
β-phelllandrene	1005	-	t	-	-	136, 93, 91, 77
β-phellandrene	1031	0.6	0.4	90.0	75.0	136, 93, 91, 79

D-limonene	1034	89.9	79.3	t	t	136, 68, 93, 79
β-ocimene	1047	0.1	0.1	0.7	22.0	136, 91, 79, 41
Hexyl chloroformate	1055	t	-	-	-	164, 56, 55, 43
γ-Terpinene	1066	1.9	0.1	-	-	136, 93, 77, 41
Trans-linalool oxide	1088	0.1	5.9	2.0	-	170, 59, 94, 43
Linalool	1099	t	1.3	t	t	154, 43, 71, 55
Nonanal	1104	t	0.3	-	-	142, 57, 41, 98
Limonene oxide	1136	t	-	-	-	152, 94, 43, 108
Citronellal	1155	0.2	-	-	0.1	154, 41, 69, 55
Terpinen-4-ol	1182	0.3	0.5	0.2	0.1	154, 71, 43, 93
Decanal	1185	0.1	0.6	-	-	156, 41, 43, 57
α-terpineol	1192	t	1.1	-	-	152, 59, 43, 93
Citronellol	1211	0.2	t	t	0.1	154, 41, 69, 81
Trans-Carveol	1235	0.1	0.2	-	-	152, 109, 41, 55
Citral	1240	0.1	0.1	-	-	154, 69, 41, 85
Geraniol	1245	0.1	0.4	-	-	152, 41, 69, 81
(-)-Carvone	1249	-	t	-	-	150, 82, 54, 39
γ-elemene	1339	0.1	-	-	-	204, 121, 93, 107
Caryophyllene	1445	0.1	0.3	2.3	---	204, 41, 91, 79
Humulene	1456	-	0.1	-	-	204, 93, 80, 121
γ-muurolene	1477	0.5	-	-	-	204, 161, 105, 119
Generayl acetate	1560	t	0.1	-	-	196, 41, 69, 93
γ-Copaene	1585	t	0.2	-	-	204, 161, 119, 105
Cis-α-bisabolene	1783	0.2	---	-	-	204, 93, 41, 91
Caryophyllene oxide	1962	-	0.1	-	-	220, 41, 79, 91
Dodacanal	2217	0.1	-	-	-	184, 57, 41, 55
TOTAL %		**98.61**	**99.00**	**97.42**	**96.50**	

Keynote: RGFP (Rose grape fresh fruit peels), RGDP (Rose grape dried fruit peels), RGFL (Rose grape fresh leaf) and RGDL (Rose grape dried leaf). A- Cas Number, B- Kovt Index, C-Fragmentation pattern. t-Trace amount less than 0.1

Analysis of the GCMS chromatogram of the Rose cultivar oils is presented in Table 4.2 above. The GCMS chromatograms of the four oil samples afforded the identification of various number of compounds. The fresh and dried fruit peel oils of rose grape had more constituents compared to leaf oils. A total of 32 compounds were identified

in the fresh peel oil accounting for (99.5% of the total oil composition), dried peel oil had 20 compounds (99.6%), fresh leaf 12 compounds (96.5%) and the dried leaf oil gave 10 compounds (97.5%) (Table 8). From the GCMS analyses of Rose grapefruit oils, it was observed that the most prominent constituent in the dried and fresh leaf oils is β-phellendrene (74.9 and 90.0% respectively) while dried and fresh fruit peels had D-limonene (79.3 and 89.9%) as the major compound. Other significant compounds in the oil samples are β-myrcene (2.5%) in the fresh peel oil, octanal and *trans*-linalool oxide (5.2% and 5.9% respectively) in the dried peel oil, β-caryophyllene (2.3%) in the fresh leaf oil and β–ocimene (22.01%) in the dried leaf oil.

Generally, the oils are dominated by monoterpenoids with less than 5% sesquiterpenoid composition in each of the oils.

Analysis of the GCMS chromatograms of the four essential oils from leaf and peels of Star Rudy cultivar afforded the identification of the compounds presented in Table 4.3 below.

Table 4.3: Chemical composition of *C. paradisi* Star Ruby cultivar essential oil.

Name of compound	KI value	Oil percentage				Method of identification A, B and C Fragment ions
		Peels *C. paradisi*		Leaf *C. paradisi*		
		GSRFP	GSRDP	GSRFL	GSRDL	
Toluene	770	0.1	-	-	-	92, 91, 92, 65
Pentylcyclopropane	813	0.7	-	-	-	112, 56, 55, 42
(Z)-3-Hexen-1-ol	827	0.1	-	-	-	100, 67, 41, 39
Heptanal	896	t	1.9	-	-	114, 70, 41, 44
α-pinene	933	0.3	-	t	t	136, 93, 79, 41

β-pinene	981	0.1	2.0	0.4	1.4	136, 93, 41, 79
β-myrcene	992	2.4	3.0	0.9	3.0	136, 41, 93, 41
Octanal	987	1.5	-	-	-	128, 43, 44, 41
5-Hepten-2-one,6 methyl	994	t	-	-	-	126, 93, 41, 69
3-Carene	1011	t	t	t	t	136, 93, 79, 41
β-Phellandrene	1031	0.7	t	91.0	82.4	136, 93, 91, 79
D-limonene	1034	86.7	74.2	1.2	2.4	136, 68, 93, 79
β-Ocimene	1050	0.2	1.0	2.0	7.0	136, 93, 91, 79
Hexyl Chloroformate	1055	t	-	-	-	164, 56, 55, 43
γ-Terpinene	1062	t	6.0	0.1	t	136, 93, 77, 41
Trans linalool oxide	1088	0.7	0.3	-	-	164, 43, 59, 93
Linalool	1098	1.8	1.4	t	0.4	154, 43, 71, 55
Nonanal	1104	0.1	-	-	-	142, 57, 41, 98
Trans limonene oxide	1138	t	-	-	-	152, 43, 67, 109
Citronellal	1155	t	1.1	0.4	0.2	154, 41, 69, 55
Terpinen-4-ol	1182	0.2	t	0.8	0.1	154, 71, 43, 93
Decanal	1185	0.5	-	-	-	156, 41, 43, 57
α-terpineol	1192	0.4	0.2	-	-	152, 59, 43, 93
Trans-Carveol	1235	0.1	-	-	-	152, 109, 41,55
Citral	1240	0.4	t	t	0.1	154, 69, 41, 85
Geraniol	1245	0.4	-	-	0.1	152, 41, 69, 81
1-Decanol	1263	0.4	-	-	-	158, 70, 55, 56
p-Mentha-1(7),8(10)-dien-9-ol	1287	t	-	-	-	152, 93, 79, 92
Decane,1-Ethenyloxy	1297	0.1	t	-	-	184, 43, 57, 41
p-Metha-1,8-dien-7-ol	1330	t	-	-	-	152, 68, 79, 93
α-terpinyl acetate	1334	t	-	-	-	196, 43, 121, 93

Cis-limonene oxide	1135	t	0.7	-	-	152, 43, 67, 109
γ-Elemene	1339	0.1	0.1	-	-	204, 121, 93, 107
Caryophyllene	1445	0.5	2.8	0.6	0.5	204, 41, 91, 79
Humulene	1456	0.1	-	-	-	204, 93, 80, 121
γ-muurolene	1477	0.2	-	-	-	204, 161, 105, 119
Germacrene D	1480	0.1	1.1	-	-	204, 161, 105, 91
Geranyl acetate	1560	0.2	2.6	-	-	196, 41, 91, 69, 93
Trans-Nerolidol	1564	t	-	0.1	-	222, 41, 69, 43
γ-Copaene	1585	0.2	-	-	-	204, 161, 119, 105
Cyclohexane methanol	-	t	-	-	-	114, 55, 83, 67
Tau-Muurolol	1640	t	-	-	-	222, 95, 121, 43
Cis-β-farnesene	1696	t	-	-	-	204, 69, 93, 67
TOTAL %		**99.62**	**99.70**	**97.63**	**97.71**	

Keynote: GSRFP (Star ruby grape fresh fruit peels), GSRDP (Star ruby grape dried fruit peels), GSRFL (Star ruby grape fresh leaf) and GSRDL (Star ruby grape dried leaf). A- Cas Number, B- Kovt Index, C- Fragmentation pattern. t- Trace amount less than 0.1

In the fresh leaf essential oil, 17 compounds were presented in the GCMS chromatogram and only 14 compounds were identified and accounted for (97.6%) of the total oil composition. The major compound present in the chemical profile was β-phellandrene (91.0%). β-Ocimene (2.0%) and D-limonene (1.2%) were other prominent compounds. The dried leaf essential oil on the other hand presented 14 compounds (97.6%) identified from 16 compounds with β-phellandrene (82.39%) and β-ocimene (7.0%) as major compounds. Interestingly, D-limonene (2.4%) was the only significant compound in an appreciable amount in the chemical constituents. In

the leaf of *C. paradisi* Star Ruby grapefruit cultivar it was observed that β-phellandrene was a major compound in the range of (91.0-82.4%) fresh to dry respectively while in the dried leaf β-ocimene was also a major compound with (6.99%) though the fresh leaf showed β-phellandrene as the only prominent component.

The chemical profile of the peel oils was quite different from the leaf oils with almost all the compound given by the GCMS chromatogram being identified (99.6% and 99.7%). Fresh peels of *C. paradisi* Star Ruby grape had D-limonene (86.7%) as a major compound and only β-myrcene (2.4%) as the other significant compound. The dried peels had D-limonene (74.2%) and Terpinene (6.0%) were found to be the prominent components including β-myrcene at (3.0%) and β-pinene at (2.0%) composition.

Table 4.4: Chemical composition of *C. paradisi* Marsh cultivar essential oils

Compound name	KI value	Percentage				Method of identification A, B and C
		GMFP	GMDP	GMFL	GMDL	Fragment ions
Toluene	762	-	t	0.1	-	92, 91, 92, 65
2-Buten-1-ol,2-methyl	766	-	t	-	-	86, 71, 86, 43
Hexanal	780	-	t	1.0	-	100, 44, 56, 41
(Z)-3-Hexan-1-ol	827	0.3	-	-	-	100, 67, 41, 39
Furfural	830	-	t	-	-	96, 96, 95, 39
Heptanal	902	-	0.1	-	-	114, 70, 41, 44
α-Pinene	935	0.6	0.5	-	-	136, 93, 79, 41
3-Carene	974	-	t	-	t	136, 93, 79, 41
β-pinene	980	0.1	0.1	t	t	136, 93, 41, 79
5-Heptene-2-one-6-methyl	985	-	t	-	-	126, 93, 41, 79
β-myrcene	991	3.6	2.9	t	0.2	136, 41, 93, 69

Octanal	1019	0.5	1.2	-	-	128, 43, 44, 41
α-phelllandrene	1005	-	t	-	-	136, 93, 91, 77
β-Phellandrene	1031	0.6	0.4	90.1	92.4	136, 93, 91, 79
D-Limonene	1034	88.1	81.2	t	t	136, 68, 93, 79
β-Ocimene	1047	t	0.1	0.7	3.1	136, 91, 79, 41
Hexyl chloroformate	1055	t	-	-	-	164, 56, 55, 43
γ-Terpinene	1066	0. 6	0.1	-	-	136, 93, 77, 41
Linalool oxide	1088	0.1	2.1	0.6	-	170, 59, 94, 43
Linalool	1099	-	0.8	t	t	54, 43, 71, 55
Nonanal	1104	t	0.3	-	-	142, 57, 41, 98
Limonene oxide	1136	t	-	-	-	152, 94, 43, 108
Citronellal	1155	0.2	-	-	0.1	154, 41, 69, 55
Terpinen-4-ol	1182	0.1	0.5	0.2	0.1	154, 71, 43, 93
Decanal	1185	0.1	0.6	-	-	156, 41, 43, 57
α-Terpineol	1192	t	1.1	-	-	152, 59, 43, 93
Citranellol	1211	0.2	t	t	0.1	154, 41, 69, 81
Trans-Carveol	1235	0.1	0.2	-	-	152, 109, 41, 55
Citral	1240	0.1	0.1	-	-	154, 69, 41, 85
Geraniol	1245	t	0.4	-	-	152, 41, 69, 81
(-)-Carvone	1249	-	t	-	-	150, 82, 54, 39
1-Decanol	1263	0.4	-	-	-	158, 70, 55, 56
p-Mentha-1(7),8(10)-dien-9-ol	1287	t	-	-	-	152, 93, 79, 92
Decane,1-Ethenyloxy	1297	0.1	t	-	-	184, 43, 57, 41
p-Metha-1,8-dien-7-ol	1330	t	-	-	-	152, 68, 79, 93
α-terpinyl acetate	1334		-	-	-	196, 43, 121, 93
Cis-limonene oxide	1135	t	0.7	-	-	152, 43, 67, 109
γ-elemene	1339	0.1	0.1	-	-	204, 121, 93, 107
Caryophyllene	1445	0.4	1.3	t	0.5	204, 41, 91, 79
Humulene	1456	0.1	-	-	-	204, 93, 80, 121
γ-muurolene	1477	0.2	-	-	-	204, 161, 105, 119
Germacrene D	1480	0.1	0.1	-	-	204, 161, 105, 91
Geranyl acetate	1560	0.1	1.6	-	-	196, 41, 69, 93
Trans-Nerolidol	1564	t	-	0.1	-	222, 69, 41, 43
γ-Copaene	1585	0.7	-	-	-	204, 161, 119, 105
TOTAL %		**97.51**	**96.41**	**92.80**	**96.33**	

Keynote: GMFP (Marsh grape fresh fruit peels), GMDP (Marsh grape dried fruit peels), GMFL (Marsh grape fresh leaf) and GMDL (Marsh grape dried leaf). A- Cas Number, B- Kovt Index, C- Fragmentation pattern. t- Trace amount less than 0. 1

Table 4.4 above presented the findings from Marsh cultivar of *C. paradisi* where the leaf essential oils had β-phellandrene as a prominent constituent taking similar pattern as other cultivars described above. In the fresh leaf oil analysis, β-phellandrene was the only major compound (90.1%) while dried leaf had β-phellandrene (92.4%) as a major compound but β-ocimene (3.1%) as one of the minor constituents. It was interesting to find out that the fruit peels of Marsh *C. paradisi* had D-limonene as a major compound with fresh peels having (88.1%) and dried peels (81.2%) respectively as predominating compounds in both oils. These fruit peels also had β-myrcene as one of the minor constituents (3.6% and 2.9%) for fresh and dried fruit peels respectively.

4.4.3 Phytochemical screening

4.4.3.1 Qualitative phytochemical screening

Phytochemical screening in an aqueous and ethanolic solvents extraction is presented in Table 4.5 and, while Table 4.5 presents the aqueous and ethanolic extracts of Marsh and Star Ruby grapefruits, Table 4.6 contained that of Rose grapefruit.

Table 4.5: Qualitative phytochemical screening of Marsh grapefruit and Star Ruby grapefruit peels and leaves of aqueous.

Phytochemicals	G.M.L Aq	G.M.L EtOH	G.M.P Aq	G.M.P EtOH	G.S.L Aq	G.S.L EtOH	G.S.P Aq	G.S.P EtOH
Tannins	+++	++	+++	++	+++	++	+++	++
Saponins	++	-	+	-	++	-	+	-
Flavonoids	++	++	+++	++	++	++	+++	++
Terpenoids	++	++	++	++	++	++	++	++
Glycosides	+++	+++	+++	+++	+++	+++	+++	+++
Phenolic compounds	++	+++	++	+++	++	+++	++	+++
Alkaloids	++	++	++	++	++	++	++	++
Steroids	+	++	+	++	+	++	+	++
Proteins and Amino acids	++	+	++	+	++	+	++	+

Foot note: +++; detected in rominent amount, ++; Detected in ordinary amount, +; Trace amount of detection and -; Not detected, G.M.L Aq= Grapefruit Marsh leaves aqueous extract. G.M.L EtOH= Grapefruit Marsh leaves ethanolic extract, G.M.P Aq= Grapefruit Marsh peels aqueous extract, G.M.P EtOH= Grapefruit Marsh peels ethanolic extract, G.S.L Aq= Grapefruit Star Ruby aqueous extract, G.S.L EtOH= Grapefruit Star Ruby leaves ethanolic extract, G.L.P Aq= Grapefruit Star Ruby peels aqueous extract and G.L.P EtOH= Grapefruit Star Ruby peels ethanolic extract.

Table 4.6: Qualitative phytochemical screening of Rose grapefruit peels and leaves of aqueous and ethanolic extract.

Phytochemicals	G.R.L Aq	G.R.L EtOH	G.R.P Aq	G.R.P EtOH
Tannins	++	+++	++	+++
Saponins	++	-	++	-
Flavonoids	++	++	++	+++
Terpenoids	+++	+++	+++	+++
Glycosides	++	++	++	++
Phenolic compounds	++	+	++	++
Alkaloids	++	+++	++	+++
Steroids	++	++	++	++
Proteins and Amino acids	+	++	+	++

Foot note: +++; detected in rominent amount, ++; Detected in ordinary amount, +; Trace amount of detection and -; Not detected, G.R.L Aq= Grapefruit Rose leaves aqueous extract. G.R.L EtOH= Grapefruit Rose leaves ethanolic extract, G.R.P Aq= Grapefruit Rose peels aqueous extract and G.R.P EtOH= Grapefruit Rose peels ethanolic extract.

Saponins was not detected in the leaf and fruit peel ethanolic extracts of Rose, Marsh and Star Ruby grapes, though the aqueous extracts of three species had positive results in moderate amount. Glycosides, tannins and flavonoids showed to be prominent in the three extracts both leaf and fruit peel while phenolic compounds were majorly detected only in the ethanolic extracts as well as in both leaf and fruit peels of Marsh and Star Ruby. Alkaloids and tannins were prominent in the ethanolic extract in both leaf and fruit peels of Rosy cultivar, while terpenoids were highly detected in both extracts leaf and fruit peels.

4.4.3.2 Quantitative phytochemical screening

Table 4.7: Quantitative phytochemical screening of Rose, Star Ruby and Marsh grapefruit peels.

Quantified phytochemicals	Powdered material (g)			Obtained constituents (g)			Percentage (w/w)		
	Marsh	Star Ruby	Rose	Marsh	Star Ruby	Rose	Marsh	Star Ruby	Rose
Flavonoids	10.21	10.12	10.11	0.27	0.42	0.67	2.64	4.15	6.63
Alkaloids	5.031	5.100	5.240	0.126	0.089	0.173	2.50	1.75	3.30
Saponins	20.02	20.11	20.01	0.068	0.101	0.146	0.34	0.50	0.73
Tannins	0.530	0.510	0.501	0.005	0.012	0.011	0.96	2.35	2.20

Table 4.7 above shows that *C. paradisi* fruit peels from all the three species had the highest concentration for flavonoids (2.64-6.63%) followed by alkaloids, tannins and lastly saponins. The high concentration of flavonoids in *C. paradisi* can be related to its antioxidant activities, because flavonoids are said to be responsible for antioxidant

properties (Di-Moja *et al.*, 2005). The Rose cultivar had the highest concentration of flavonoids, alkaloids and saponins followed by Star Ruby in flavonoids and saponins while Marsh was the second highest only in alkaloids. Tannins were more concentrated in Star Ruby followed by Rose and Marsh was the lowest.

The leaf extracts followed the same trend as the fruit peel extracts (Table 4.8), with flavonoids being the class with the furthermost concentration in all the three leaf crude extracts followed by alkaloids, tannins then saponins were the list concentrated phytochemicals.

Table 4.8: Quantitative phytochemical screening of Rose grapefruit leaf, Star Ruby grapefruit leaf and Marsh grapefruit leaf.

Quantified phytochemicals	Powdered material (g)			Obtained constituents (g)			Percentage (w/w)		
	Marsh	Star ruby	Rose	Marsh	Star ruby	Rose leaf	Marsh	Star ruby	Rose
Flavonoids	10.07	10.22	10.31	0.14	0.18	0.21	1.41	1.75	2.01
Alkaloids	5.007	5.001	5.004	0.04	0.05	0.07	0.87	1.08	1.31
Saponins	20.21	20.00	20.08	0.004	0.004	0.008	0.02	0.02	0.04
Tannins	0.540	0.504	0.500	0.0007	0.00096	0.0001	0.13	0.19	0.23

4.5 Conclusion

Physiochemical analysis showed that all grapefruit essential oil had a range of percentage yield of 1.78%-0.21% (w/w), which is in agreement with the results found from Pakistan where reported on their percentage yield was high with 0.73% w/w in

the fruit peels (Ahmad et al., 2013). In our results, Star Ruby had the highest yield of 1.78% w/w and Marsh grape with the minimum 0.21% w/w.

From the GC-MS results, it is evident that grapefruit fresh and dried peel contain greater number of compounds than the leaf oils. Major volatile compounds from the peel oil of all the three varieties were identified as D-Limonene (90%-87%), β-myrcene (4%-2%) and γ-terpinene (2%-0.1%). Whereas β-phellandrene (90%-91%), was the major volatile compounds from the leaf oil of all the three varieties. Caryophyllene (2.8%-0.6%) was found to be the only major sesquiterpene found in Star Ruby dried peel oils, other leaf and peel oils had the compound in low concentration less than 1%. The presence of these compounds indicates that the South African species of grapefruit closely resembles those growing in Nigeria and Pakistan (Buettner, 1999; Imran et al., 2013). D-Limonene, the major monoterpene hydrocarbon in the peel oil of all grapefruits varieties this gives indications that they can be used medicinally whilst the presence of β-phellandrene a cyclic monoterpene as the major compound in leaf oil of all grapefruit varieties is indicative of the fact that the leaf oil possesses antimicrobial and antifungal activities.

Phenols, phenolic glycosides and volatile aromatic compounds such as limonene, α-thujene, myrcene, α-terpinene, α-pinene have been reported as the major compounds of these three varieties of grapefruit (Okwu, 2008; Okunowo et al., 2013).

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Chapter 5

Biological studies of *C. limon* and *C. paradisi* essential oils

5.1 Introduction

Toxicity is the degree to which a substance can harm humans or animals although the severity differs in degree and dosage. Nonetheless, toxicity can be broadly divided into acute and chronic. Acute toxicity involves harmful effects in an organism through a single or short-term exposure. Sub chronic toxicity is the ability of a toxic substance to cause effects for more than one year but less than the lifetime of the exposed organism. Chronic toxicity is the ability of a substance or mixture of substances to cause harmful effects over an extended period, usually upon repeated or continuous exposure, sometimes lasting for the entire life of the exposed organism (Kasper et al., 2015). Folklore medicine users neglect the importance of toxicity test in their medical usage and dispensary, hence the need to test every plant used traditionally for its toxicity level. In this study an acute toxicity test was conducted with oral administration essential oils to mice since the medicine prepared from the plant material used in the study is usually taken orally.

Inflammation is a process whereby body white blood cells protect the body from infection with foreign organisms, such as bacteria and viruses. Some signs of inflammation include heat, pain, redness and swelling. Inflammation has three stages namely acute, sub-acute and chronic inflammation and all these differs due to their strength and time taken. The acute stage of inflammation includes changes in the concentrations of many plasma proteins, which are known as the acute phase proteins, as well as numerous behavioural, physiological, biochemical and nutritional

changes (Esmon, 2006). Minimal inflammation could lead to continuation of tissue damage by the dangerous bacteria and compromise the survival of the organism (Abbas *et al*., 2009). The chronic stage is a long term- inflammation which is categorised by longer period of condition which can lead to more complex conditions and diseases such as arthritis (Pahwa and Jialal 2018; Mateen *et al*., 2016). Though there are quite some few non-steroidal drugs (NSD) to treat inflammation, a lot of people in the rural communities still rely mostly on traditional medicine especially in South Africa. This necessitated the chemical and biological studies on *Citrus paradisi* and *Citrus limon* waste from South Africa so as to evaluate their effectiveness or significant potential in treating acute inflammation.

Antioxidant are agent/substances which slows down the activity and or damage free oxygen radicals. Recent studies shows that superoxide dismutase and vitamin E in experimentally induced arthritis are good antioxidant therapy strategies for the prevention and treatment of arthritis (Bandt *et al*., 2002, Behaska *et al*., 2002; Salvemini *et al*., 2002). In another study, measured Mediterranean dietary intervention study in patients with arthritis was conducted, it was observed that vitamin C, retinol and uric acid in reverse interrelated with variables associated to these disease activity (Hagfors et al., 2003). The compounds mentioned above are found in most citrus plant (González-Molina *et al* 2010). Equally antioxidants and few fatty acids are recommended for patients with arthritis and related disorders (Darlington and Stone, 2001).

There are many methods used to determine free oxygen radical scavenging activity of a substance in the literature. This current study however, focuses on two models namely Ferric reducing power (FRAP) and 2, 2 ’-diphenyl-1-picrylhydrazyl (DPPH).

5.2 Experimental animals

Mice (20-30g) and rats (150-260g) were obtained from the South African Vaccine Initiative, Johannesburg and kept at Animal Holding Facility, Walter Sisulu University, NMD campus, Mthatha. They were acclimatized to the laboratory environment for 1 week, while being maintained under 12 h light/dark circle at temperature of 22 ± 2 ℃ and housed 5/6 animals per cage in a Plexiglas cage with wood shavings as beddings. The animals were fed with standard laboratory food for rodents and water was provided freely except during the experiment.

Materials used for the *in vivo* biological studies were: Essential oils of *C. limon* and *C paradisi* tween 80, distilled water and Diclofenac (Sigma Chemical Co., USA). This study was approved by the Department of Higher Education, WSU and ethical clearance approval was obtained from Walter Sisulu University Ethics Committee with Reference No. DVC (AA&R) DRD/SREC: FNS 01/02/2017

5.2.1 *In vivo* Bioassay

5.2.1.1 Acute toxicity test

Acute toxicity of the volatile oils of lemon and the three grapefruits were assessed in mice using oral route (p.o) according to Lorke's method (Lorke, 1983). Each oil was tested for acute toxicity (LD_{50}) effect, orally using 12 animals each. The procedure was divided into two phases, in phase I, 3 animals per dose of 10, 100 and 1000 mg/kg body weight (bw) were used. Phase II used one animal per dose levels of 1000, 1600 and 2600 mg/kg bw. Each animal after treatment was observed for a period of

one hour initially to check for immediate effect that could arise due to the introduction of foreign substance and thereafter for 24 h for case of mortality. Animal that survived for more than 24 h is scored as no mortality. Animal that survived beyond 24 h is assumed to have LD_{50} of 2600 mg/kg bw (Lorke, 1983). The LD_{50} of the essential oil was estimated as the geometric mean of the lowest dose causing death and the highest dose causing no death according to the formula below:

$$LD_{50} = \sqrt{(A \times B)}$$

Where A is the maximum dose producing 0% death and B is the dose that produces 100% death (Lorke,1983).From the result of LD_{50},the working doses was chosen such that the highest working dose is below half of the LD_{50} according to the equation: Working dose $\leq$ ½ (LD_{50}).

5.2.1.2 Anti-inflammatory test

The anti-inflammatory activity of the volatile oils of *C. limon* and three *C. paradisi* cultivars were evaluated using egg albumin-induced edema model (Catorce and Gervekian, 2016). In this test, five groups of three rats per group randomly allocated were used. Group 1 was the negative control (administered 5% Tween 80), groups II, III and IV were administered volatile oil (50, 100 and 200 mg/kg bw respectively) and group V was the positive control (diclofenac 100 mg/kg bw). All treatments were by the oral route and pre-treatment was done 1 h prior to egg-albomin injection. Paw sizes were measured at time 0, 1, 2, 3, 4 and 5 h post-egg-albumin injection. Baseline paw size was measured before and after 1, 2, 3, 4 and 5 hours post injection of egg-albumin using a rope and a ruler (Cartorce and Gervekian, 2016).

5.2.2 *In vitro* Bioassay: Antioxidant studies on *C. limon* and *C. paradisi* essential oils

5.2.2.1 Ferric reducing power

The reducing power of each essential oil was evaluated according to the method of Kumar and Pandey, (2013). Essential oil (0.1 ml) was prepared in 166µL of 5% tween 80 in distilled water and was added to the mixture containing 0.25 ml of phosphate buffer (0.2 M; pH 6.6) and 0.25 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v). Standard compounds used were L (+) - Ascorbic acid ($C_6H_8O_6$) and 2,6–Di –*tert*–butyl–4–methylphenol [$(CH_3)_3C]_2C_6H_2(CH_3)OH$]. The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 0.25 mL of CCl_3COOH (10% w/v) which was then centrifuged at 3000 revolution per minute (rpm) for 10 min. The upper layer of the solution was mixed with 1 mL of 5% tween 80 in distilled water with same amount and 0.5 mL of $FeCl_3$ (0.1 %, w/v). The experiment was conducted in duplicate and absorbance measured at 700 nm (Helios Epsilon Thermo Spectronic, USA) against a blank sample of only phosphate buffer. Higher reducing power of the essential oil was indicated by the increased absorbance.

5.2.2.2 DPPH (2, 2 ’-diphenyl-1-picrylhydrazyl) radical test

The antioxidant activity of the citrus peels and leaf essential oils was assessed by measuring their scavenging ability on 2, 2 ’-diphenyl-1-picrylhydrazyl stable radicals (DPPH). The total antioxidant capacity of the extract was carried out using 2,2-

diphenyl-1-picrylhydrazyl (DPPH) radical as previously described by Okeleye *et al*., (2013). A solution of 0.135 mM DPPH in methanol was prepared and 180µL of this solution mixed with 180 µL of essential oil that was dissolved in 40µL of 5% tween 80 distilled water. DPPH in 5% tween 80 distilled water was used as a negative control. The combination was thoroughly mixed and left in the dark at room temperature for 30 min before the absorbance was measured at 517.5 nm (Helios Epsilon Thermo Spectronic, USA). L (+) – Ascorbic acid and DBPC*BHT were used as the reference (control). The ability of extract (oils or control) to scavenge DPPH radical was calculated using the following equation: % Scavenged [DPPH] = $[(A_0-A_1)/A_0] \times 100$ where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of extract and standards.

5.3 Results and discussion

5.3.1 Acute toxicity test

Table 5.1 showed the results of the acute toxicity tests of essential oils of the peel and leaf of *C. limon*. The results showed that oral administration of up to 2600 mg/kg bw of the essential oils did not result in mortality after 24 h. The implication of these results suggests that habitual consumption of the citrus fruit and or its peels is safe and may not constitute severe health hazard in the short time. According to Lorke (1983) and Rodricks (1992), LD_{50} values above 2600 mg/kg bw indicate that the essential oil is non-toxic and safe.

Table 5.1: Acute toxicity profile of the essential oils of the fruit peels and leaf of *C. limon* in mice

Treatment mg/kg, per orall (p.o)	*C. limon* leaf essential oil Death pattern after 24 h	*C. limon* peels essential oil Death pattern after 24 h
Phase 1		
10	0/3	0/3
100	0/3	0/3
1000	0/3	0/3
Phase 2		
1000	0/1	0/1
1600	0/1	0/1
2600	0/1	0/1
LD_{50}	**≥2600 mg/kg bw**	**≥2600 mg/kg bw**

The results of the acute toxicity tests carried out on the essential oils of the fruit peel and leaf of *C. paradisi* are presented in Table 5.1. Similarly there were no mortalities up to the dose of 2600 mg/kg bw in the animals (mice) either in the fruit peel or leaf oil treated animals suggesting that these oils are non-toxic orally (Rodricks, 1992).

Table 5.2: Acute toxicity profile of the essential oils of the three fruit peels and three leaf of *C. paradisi* in mice

Marsh Grapefruit			**Rose Grapefruit**			**Star ruby Grapefruit**		
Treatment mg/kg, p.o.	Leaf	Peels	Treatment mg/kg, p.o.	leaf	Peels	Treatment mg/kg, p.o.	Leaf	Peels
	Mortality after 24h	Mortality after 24h		Mortality after 24h	Mortality after 24h		Mortality after 24h	Mortality after 24h
Phase 1			Phase 1			Phase 1		
10	0/3	0/3	10	0/3	0/3	10	0/3	0/3
100	0/3	0/3	100	0/3	0/3	100	0/3	0/3
1000	0/3	0/3	1000	0/3	0/3	1000	0/3	0/3
Phase 2			Phase 2			Phase 2		
1000	0/1	0/1	1000	0/1	0/1	1000	0/1	0/1

1600	0/1	0/1	1600	0/1	0/1	1600	0/1	0/1
2600	0/1	0/1	2600	0/1	0/1	2600	0/1	0/1
LD_{50}	**≥2600 mg/kg bw**	**≥2600 mg/kg bw**	**LD_{50}**	**≥2600 mg/kg bw**	**≥2600 mg/kg bw**	**LD_{50}**	**≥2600 mg/kg bw**	**≥2600 mg/kg bw**

5.3.2 Anti-inflammatory test

5.3.2.1 *Citrus limon* anti-inflammatory studies

Figure 9 below shows the results of swelling of hind paw of rats after they were administrated egg albumin into the hind paw, there were progressive increase in the paw sizes. Lisbon lemon fruit peel essential oil in the first and second hours, 100 and 200 mg/kg bw and Diclofenac were significantly ($p<0.05$, $p<0.01$) lower compared to control, except for 50 mg/kg bw probably due to delayed absorption. However, from 3rd - 5th hour, all the concentrations were effective compared to control, even better than Diclofenac, a well-known non-steroidal anti-inflammatory drug (NSAID).

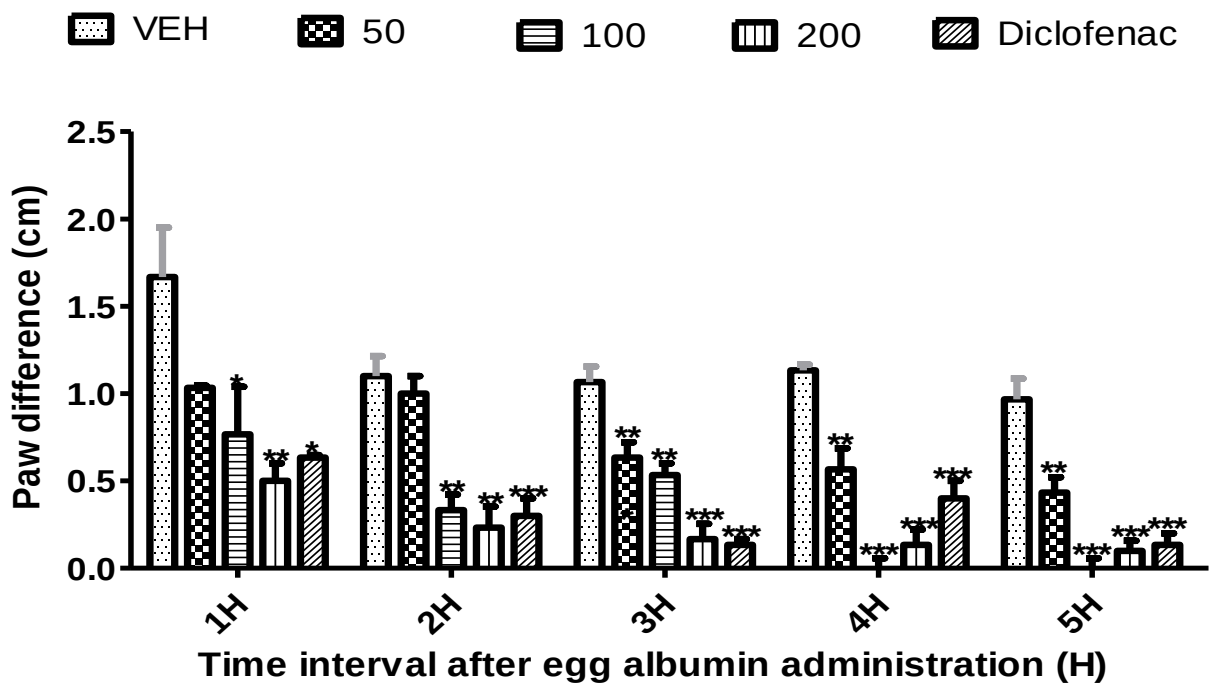

Figure 9: Effect of essential oil of *C. limon* fruit peels in egg albumin-induced inflammation in Wistar rats.

Control Diclofenac, 50, 100 and 200 represent negative control (tween 80 and distilled water), Diclofenac (100 mg/kg), 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw respectively. *p<0.05, ***p<0.001 statistically compared to control (ANOVA, Tukey).

Below there is figure 10 with results of anti-inflammatory effects of Lisbon lemon leaf essential oil on hind paw of rats. It was observed that in the first hour, none of the three concentrations 50, 100 and 200 mg/kg had significant results on ($p<0.05$ and $p<0.001$). However, form second, third, fourth and fifth hours all concentrations showed significant results at $p< 0.001$ although lower than the control Diclofenac (100 mg/kg bw). Thus leaf essential oil of *C. limon* can be of use for treatment and management of swelling due to inflammation as it had significant results on all the concentrations from second hour to the fifth hour. The delay in showing significant results can be associated with essential oil or drug being reacting with the blood system before reaching the affected part of the body in the rat.

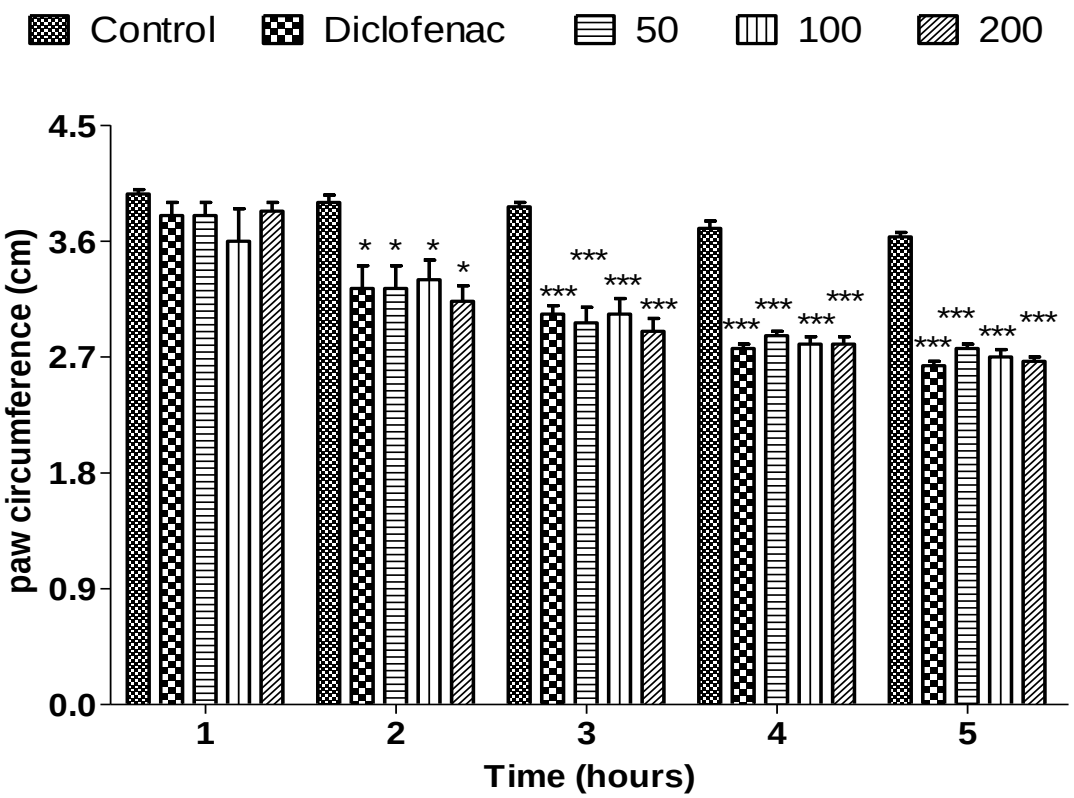

Figure 10: Effect of essential oil of *C. limon* leaf in egg albumin-induced inflammation in Wistar rats.

Control Diclofenac, 50, 100 and 200 represent negative control (tween 80 and distilled water), Diclofenac (100 mg/kg), 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw respectively. *p<0.05, ***p<0.001 statistically compared to control (ANOVA, Tukey).

5.3.2.2 *Citrus paradisi* Anti-inflammatory studies

Grapefruit essential oils were evaluated based on their anti-inflammatory potential using rats for both leaf and fruit peels in the dose levels 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw. A positive control used during the experiments was 100mg/kg bw Diclofenac, the negative control (VEH) used was tween 80 in distilled water in the ratio (5% is to 90% respectively).

In the first hour only the peel oils at the 100 mg/kg bw was significantly ($p<0.05$, $p<0.01$) higher compared to control, except for 50 and 200 mg/kg probably due to delayed absorption. However, from 2nd - 5th hour, all the concentrations were effective compared to control. In the 3rd hour 100 mg/kg gave significant effects on reducing swelling on rats paw even better than Diclofenac, a known non-steroidal anti-inflammatory drug (NSAID). As shown in figure 11 below

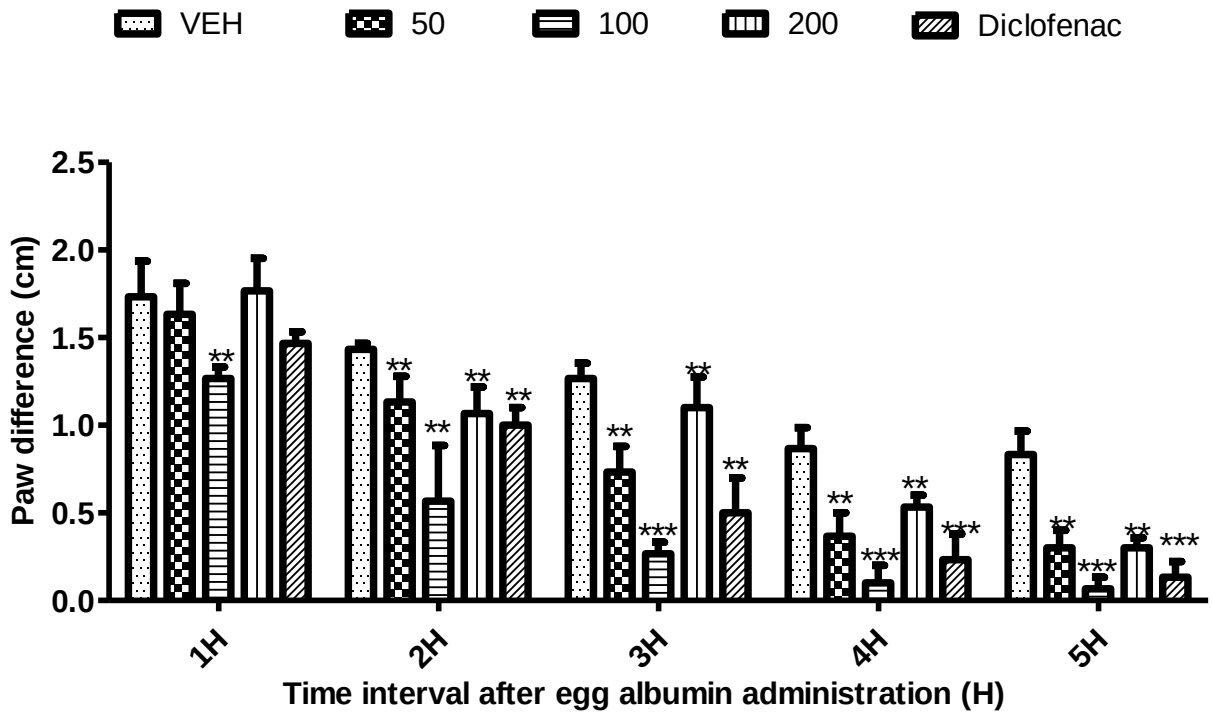

Figure 11: Effect of essential oil of Marsh grapefruit (*C. paradisi*) fruit peel in egg albumin-induced inflammation in Wistar rats.

Control Diclofenac, 50, 100 and 200 represent negative control (tween 80 and distilled water), Diclofenac (100 mg/kg bw), 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw respectively. *p<0.05, ***p<0.001 statistically compared to control (ANOVA, Tukey).

Marsh *C. paradisi* leaf essential oil at 200 mg/kg bw significantly ($p < 0.05$-0.01) reduced paw oedema of rats in the 2, 3, 4 and 5h of observation period also the leaves oil at 100 mg/kg significantly ($p < 0.05$-0.01) reduced paw oedema of rats in the 3, 4

and 5h of observation period compared to vehicle. Diclofenac also reduced significantly ($p < 0.05$-0.01) paw oedema of rats in the 2, 3, 4 and 5h of assessment period as shown in Figure 12 below.

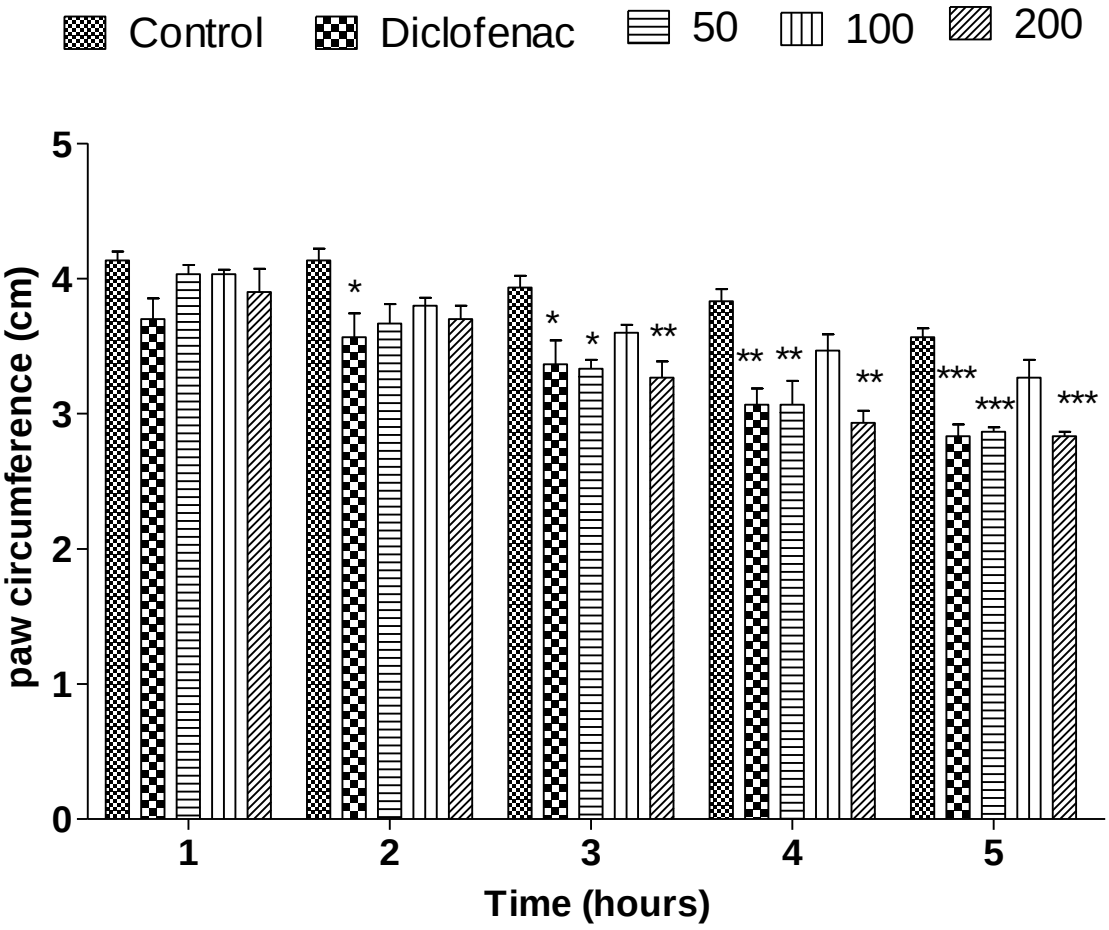

Figure 12: Effect of Marsh grapefruit (*C. paradisi*) leaf in egg albumin-induced inflammation in Wistar rats.

Control Diclofenac, 50, 100 and 200 represent negative control (tween 80 and distilled water), Diclofenac (100 mg/kg bw), 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw respectively. *p<0.05, **p<0.01, ***p<0.001 statistically compared to control (ANOVA, Tukey).

Rose grapefruit (*C. paradisi*) fruit peel essential oil in Figure 13 below showed in the first hour the oil at 50 and 100 mg/kg only were significantly compared to the negative

controlled ($p<0.05$, $p<0.$), except for 200 mg/kg bw and positive control probably due to delayed absorption by the higher concentration and control. However, from 3rd - 5th hour, all the concentrations were effective compared to negative control.

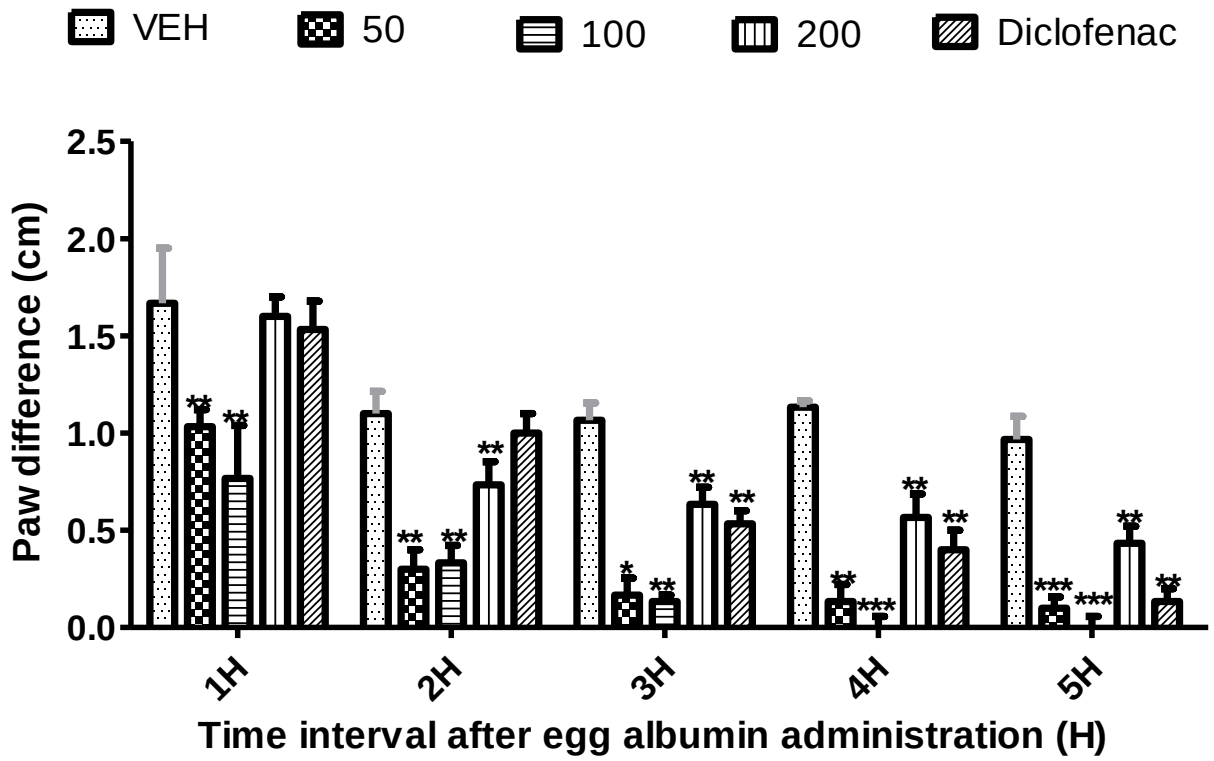

Figure 13: Effect of Rose grapefruit (*C. paradisi*) fruit peels in egg albumin-induced inflammation in Wistar rats.

Control Diclofenac, 50, 100 and 200 represent negative control (tween 80 and distilled water), Diclofenac (100 mg/kg bw), 50 mg/kg bw, 100 mg/kg and 200 mg/kg bw respectively. *p<0.05, **p<0.01, ***p<0.001 statistically compared to control (ANOVA, Tukey).

Leaf essential oil of Rose *C. paradisi* caused reduction in oedema size of rats hind paws in the 3, 4 and 5h of observation period compared to vehicle group (Figure 14). The same also was observed for Diclofenac (standard drug) reducing significantly ($p< 0.01$) the oedema size in rats hind paws at 3, 4 and 5h post egg albumin injection

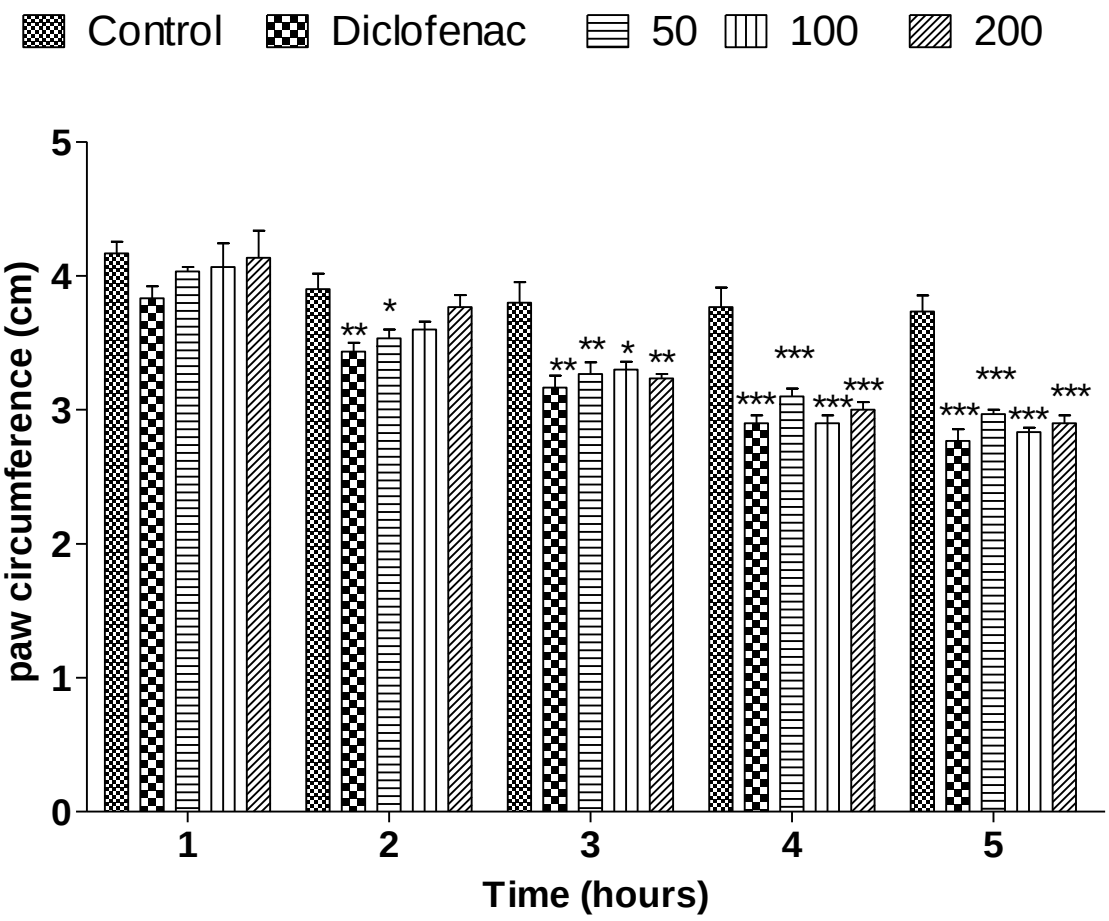

Figure 14: Effect of Rose grapefruit (*C. paradisi*) leaf in egg albumin-induced inflammation in Wistar rats.

Control Diclofenac, 50, 100 and 200 represent negative control (tween 80 and distilled water), Diclofenac (100 mg/kg bw), 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw respectively. *p<0.05, **p<0.01, ***p<0.001 statistically compared to control (ANOVA, Tukey).

Star Ruby *C. paradisi* fruit peels oil at doses 50 and 100 mg/kg bw showed significant ($p < 0.01$) reduction of rats paw oedema in 4 and 5 h compared to the negative control. Diclofenac positive control also reduced significantly ($p < 0.01$) rats paw oedema in the 3, 4 and 5h of observation period.Similarly essential oil at 200 mg/kg reduced significantly ($p < 0.01$) the rats paw oedema in the 2, 3, 4 and 5h of

assessment period compared to the vehicle group figure 15 below explain it graphically.

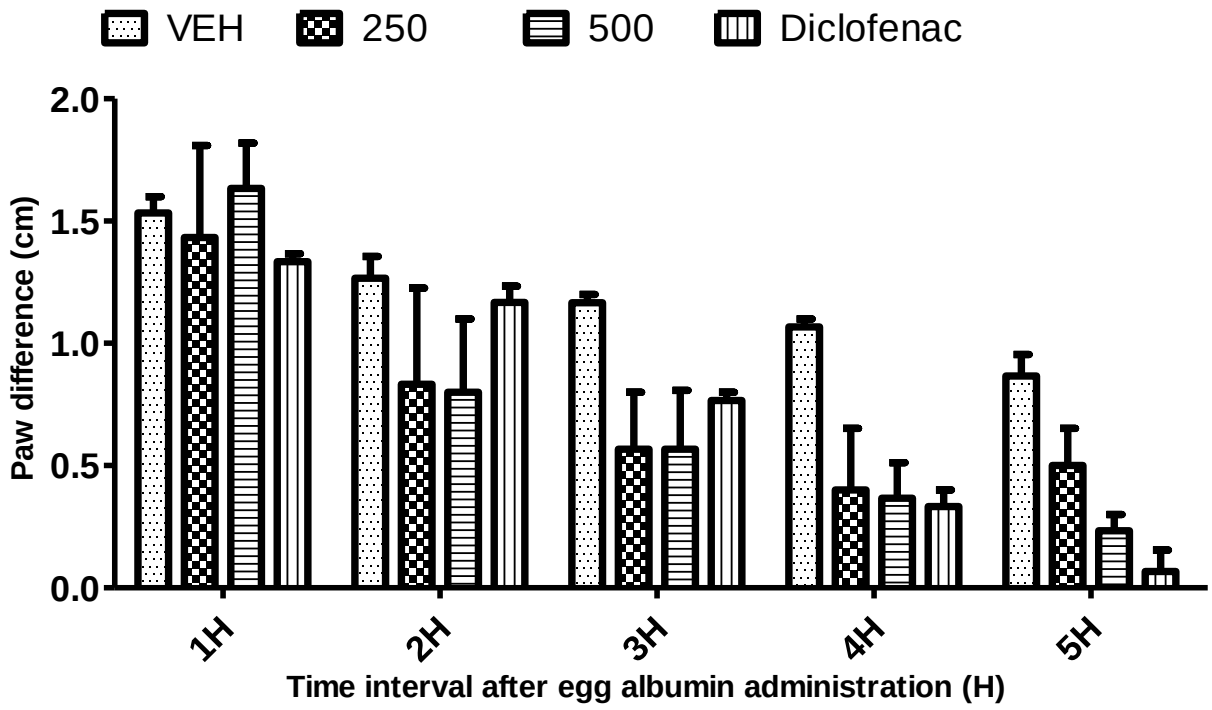

Figure 15: Effect of Star Ruby grapefruit (*C. paradisi*) fruit peels in egg albumin induced inflammation in Wistar rats.

Control Diclofenac, 50, 100 and 200 represent negative control (tween 80 and distilled water), Diclofenac (100 mg/kg bw), 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw respectively. *p<0.05, **p<0.01, ***p<0.001 statistically compared to control (ANOVA, Tukey).

In the first hour the oil at 50 and 100 mg/kg only were significantly compared to the negative control ($p<0.05$, $p<0.001$), except for 200 mg/kg bw and positive control probably due to delayed absorption by the higher concentration and control. However, from 3rd - 5th hour, all the concentrations were effective compared to control. As shown in Figure 16 below

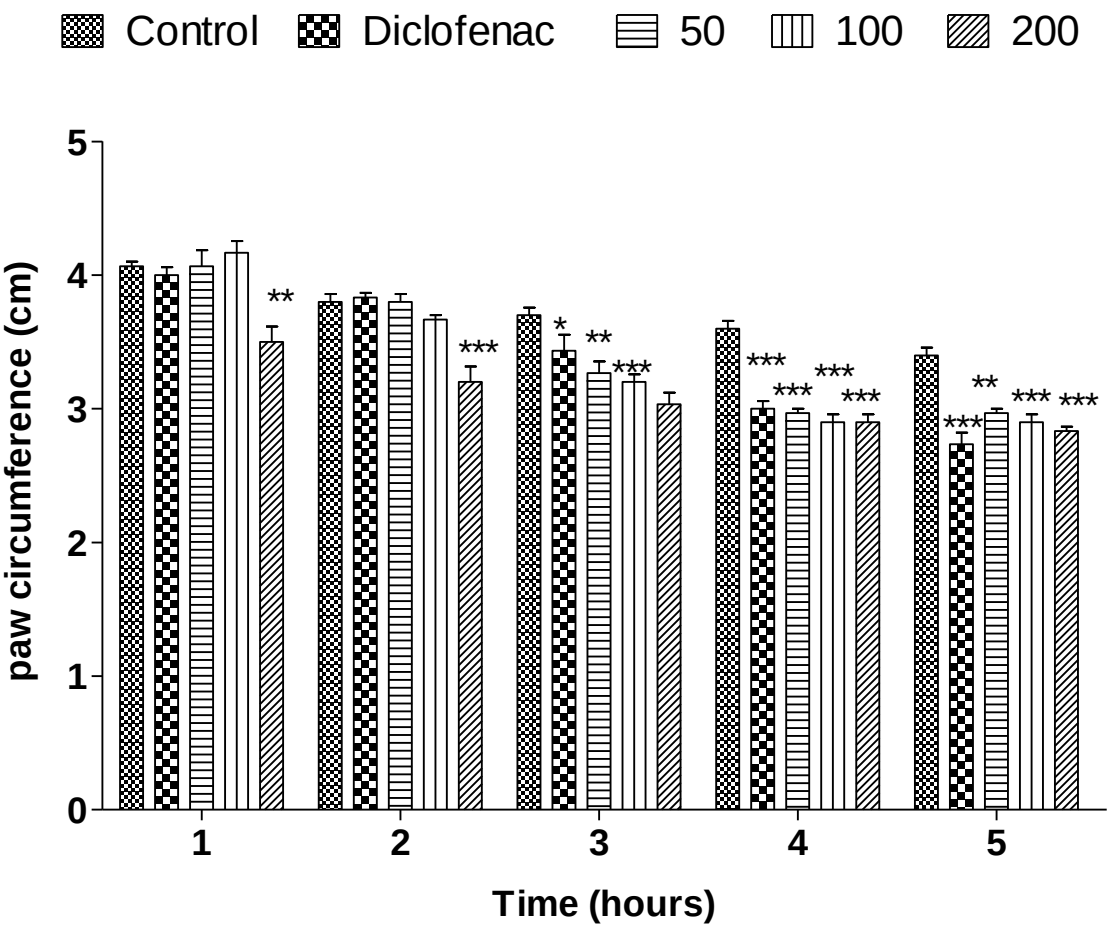

Figure 16: Effect of Star Ruby grapefruit (*C. paradisi*) leaf in egg albumin-induced inflammation in Wistar rats.

Control Diclofenac, 50, 100 and 200 represent negative control (tween 80 and distilled water), Diclofenac (100 mg/kg bw), 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw respectively. *p<0.05, **p<0.01, ***p<0.001 statistically compared to control (ANOVA, Tukey).

5.3.3 Anti-oxidant bioassay

The flavonoids and phenolic acids present in the medicinal plant exhibit strong antioxidant activity which is depending on their potential to form the complex with metal atoms, particularly iron and copper. This method is based on the principle of increase in the absorbance of the reaction mixtures, including that the absorbance increases the antioxidant activity increases as the antioxidant compound present in

the samples forms a colored complex with potassium ferricyanide, trichloroacetic acid and ferric chloride, which is measured at a known wavelength by UV-Spectrophotometer (Do *et al*., 2014). DPPH is a stable powder free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

The ferric reducing power and DPPH anti-oxidant activities were done only in the dried fruit peels and leaf of all the three grapes and lemon essential oils.

5.3.3.1 Ferric reducing power

This result obtained in the ferric reducing power (FRP) assay was analysed using UV/ visible instrument for absorbance measurements where common wavelength of 700nm for consistence and to obtain accurate results data a blank solution was use which was 5% tween 80 in distilled water. Microsoft excel was used to express the analysis in a graphical format

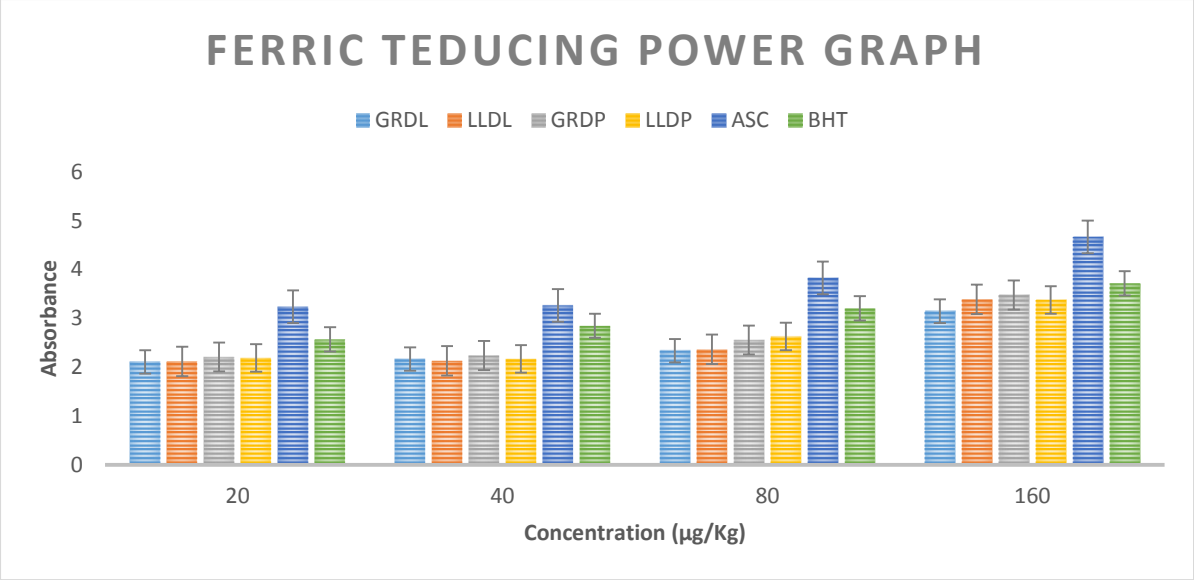

Figure 17: Ferric reducing power of Lisbon lemon and Rose grapefruit dried leaf and peels

Foot notes: GRDL (Grapefruit rose dried leaf), LLDL (Lisbon lemon dried leaf), GRDP (Grapefruit rose dried peel), LLDP (Lisbon lemon dried peel), ASC (Ascorbic acid) and BHT (2,6–Di –*tert*–butyl–4–methylphenol)

From Figure 17 above all the essential oils were significantly active in ferric reducing power though less effective as compared to the two positive controls. It was also observed that the dried essential oil of *C. limon* both leaf and fruit peel were more active compared to *C. paradisi* essential oils. These results can be aligned with the results of quantitative secondary metabolites screening which showed that these *citrus* species are rich in flavonoids known as a good source of anti-oxidant potential phytochemicals.

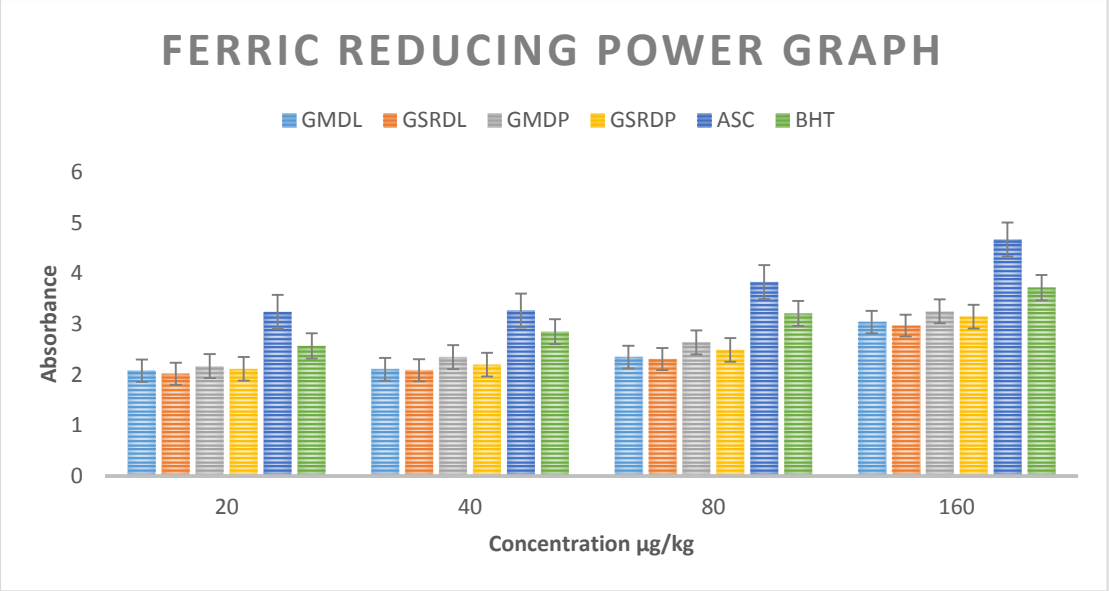

Figure 18: Ferric reducing power of Marsh grapefruit and Star Ruby grapefruit dried leaf and fruit peels.

Foot notes: GMDL (Grapefruit marsh dried leaf), GSRDL (Grapefruit star ruby dried leaf), GMDP (Grapefruit marsh dried peel), GSRDP (Grapefruit star ruby dried peel), ASC (Ascorbic acid) and BHT (2,6–Di –*tert*–butyl–4–methylphenol)

The essential oils showed a significant results but less active compared to the controls. The fruit peels essential oils showed significant results compared to the leaf essential oils. This can be further be explained by the fact that the phytochemical screening of secondary metabolites of fruit peels showed a very high percentage of flavonoids compared to the essential oils of leaf and these compounds are well known by their antioxidant power.

5.3.3.2 DPPH Scavenging Capacity

The DPPH was analysed based on the percentage inhibition (scavenged) calculated using the formulae % Scavenged [DPPH] = $[(A_0 - A_1)/A_0] \times 100$ which is fully described

in **section 5.2.2.2** Which indicate that the higher the scavenging percentage the higher the capacity to reduce the hydrogen free radicals.

The experiment was done using only the dried material due to the fact that it is easy to keep plants material to dry and to reduce the water quantity which can affect the results one way or the other.

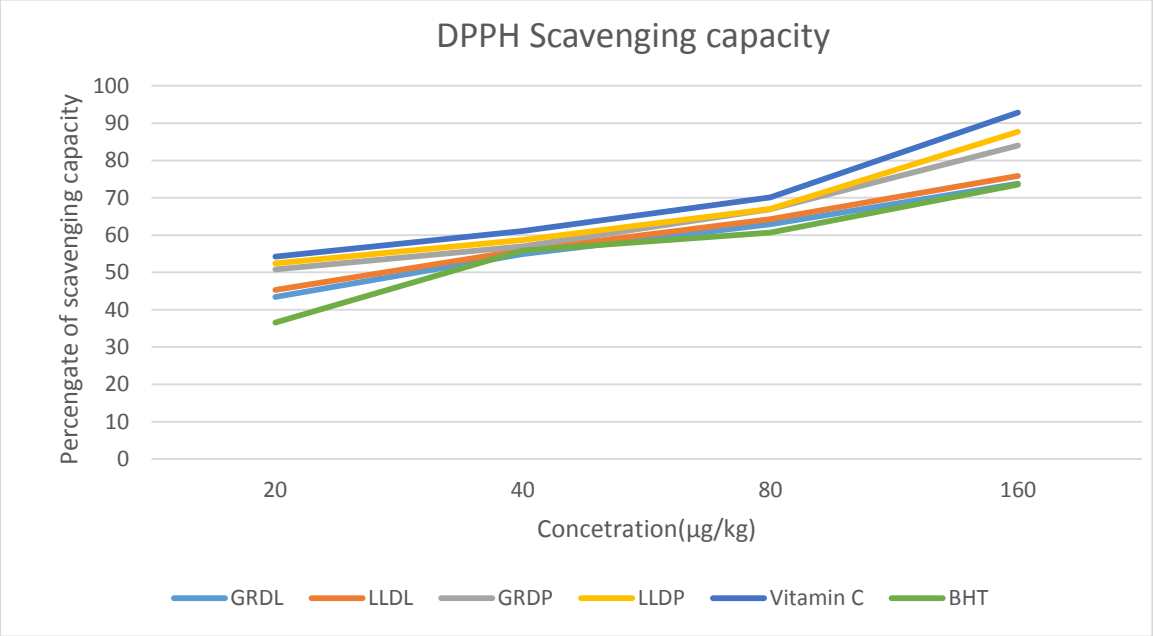

Figure 19: DPPH scavenging capacity for Lisbon lemon and Rose grapefruit dried leaf and fruit peels.

Foot notes: GRDL (Grapefruit rose dried leaf), LLDL (Lisbon lemon dried leaf), GRDP (Grapefruit rose dried peel) LLDP (Lisbon lemon dried peel) and BHT (2,6–Di –*tert*–butyl–4–methylphenol)

From the above figure it clearly shows that ascorbic acid known anti-oxidant drug has the highest hydrogen free radicals capacity compared to all the essential oils of the dried materials of lemon and the rose grape, though all the essential oils showed significant scavenging capacity even higher than the second control BHT. This may

then be associated to the results reflecting higher concentration of flavonoids in the quantitative phytochemical screening, because they are associated with anti-oxidant properties of citrus fruits (Di-Majo *et al.*, 2005).

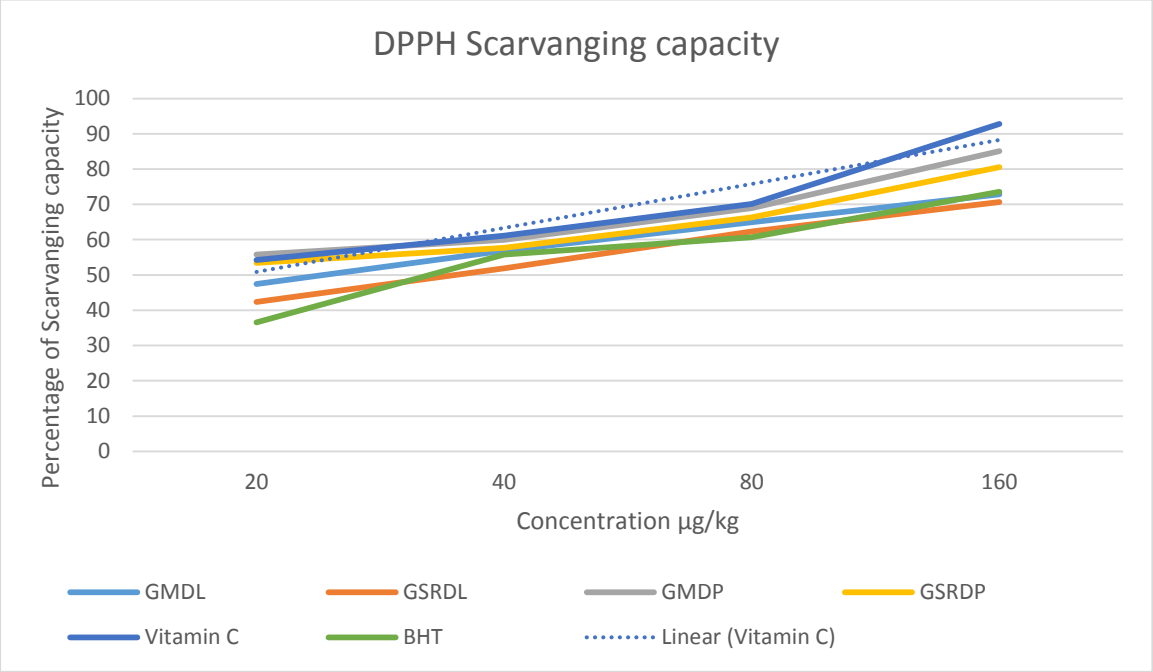

Figure 20: Scavenging capacity of Marsh grapefruit and Star ruby grapefruit dried leaf and fruit peels.

Foot notes: GMDL (Grapefruit marsh dried leaf), GSRDL (Grapefruit star ruby dried leaf), GMDP (Grapefruit marsh dried peel), GSRDP (Grapefruit star ruby dried peel) and BHT (2,6–Di –*tert*–butyl–4–methylphenol)

From Figure 20 above the dried fruit peels of marsh grape had the highest scavenging capacity in the 20 µg/kg concentration even higher than the ascorbic acid a well know anti-oxidant drug. From 40 µg/kg to 160 µg/kg the ascorbic acid had the highest capacity then followed by the marsh fruit peels, star ruby fruit peels and leaf were the lowest for both cultivars of grapefruit.

5.4 Conclusion

The safety of any substance to be ingested either raw or refined is usually taken with care, hence the testing of the efficacy of medicinal product is of very great importance and this may not be substantiated without the toxicity test. From the bioassay done on the leaf and peel oil it can be concluded that all the oil extracts had LD_{50} values above 2600 mg/kg indicate that the essential oil is non-toxic and safe for oral consumption. Furthermore, the antioxidant assay of the oils were promising such that the oils can be said to be good antioxidants. This is further buttressed with the antiinflammatory studies. Thus it can be concluded that the oils of citrus either being leaves or fruit peels contain some biological activities potentials.

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Chapter 6
Conclusion and recommendations

6.1 Overall conclusion

This chapter is focusing on the summary of all the findings and observations on the chemical investigation and biological evaluation on the fruit peel and leaf of *C. limon* and *C. paradisi*. It also gives possible insight into the variation in results from the use of generic instrument from different institutions on same plants materials. Lastly the chapter also provides answers to the questions raised in the research questions in chapter two which are:

What are the secondary metabolites present in grapefruit and lemon extracts?

Quantitative screening of the plant materials (peel and leaves) shows that glycosides, phenolic compounds, flavonoids, tannins, terpenoids, sterols, alkaloids are secondary metabolites present in high concentration in the leaf and peel of *C. limon*. Saponin was found in very low concentration. Flavonoids were highest in concentration in the peel while in the leaf it was the alkaloids. Similar class of compounds were detected in the phytochemical screening of the peel and leaf of *C. paradisi*. Quantity of saponins were higher in the aqueous extracts of *C. paradisi* than in *C. limon*. Saponins are secondary metabolites known for wounding healing. The low saponins content in citrus leaf and peel could suggest the inability of the waste to be used in healing wounds as oppose to citrus juice which is widely acclaimed to be used for such treatment. However, Ahamed *et al*., (2013) proved that the citrus extracts do contain Vitamin C and Carotene which also acts as wound healing agents in rats. Thus, it can be

concluded that the *C. limon* and *C. paradisi* although low in saponins can still be used in wound healing management. The high percentages concentration of alkaloids in both plants materials is noteworthy as alkaloids are known to be precursors of psychoactive drugs and used as stimulants of the central nervous system (Hesse, 2002).

Which solvent is the best extractive solvent in extracting the secondary metabolites from citrus peels and leaves?

Ethanolic and aqueous extracts were used for qualitative phytochemical screening. The was concentration variation in the amount of phytochemicals present using these two extractive solvents, thus some secondary metabolites were present only in the aqueous extract for example saponins (although in small quantity) but was absent in ethanolic extract. It was also observed that when a secondary metabolite is present in both extracts, the ethanolic extract gave a higher detection concentration compared to the aqueous extract. Nonetheless, it can be concluded that for medicinal purposes, water extract showed more secondary metabolites than alcoholic solvents.

Does South African *C. limon* and *C. pardisi* peels and leaves have medicinal and economic potentials?

South African *C. limon* and *C. paradisi* are of medicinal and economic value as demonstrated from the chemical analysis of the essential oils and solvent extracts

alongside with the promising biological activities as anti-inflammatory and antioxidants agents.

The chemical profile evaluation of the essential oils of *C. limon* and the fruits peel of *C. paradisi* indicates high percentage composition of D-limonene. D-limonene is listed in the Code of Federal Regulations as generally recognized as safe (GRAS) for a flavouring agent. It is found in common food items such as fruit juices, soft drinks, baked goods, ice cream, and pudding. D-limonene is considered to have fairly low toxicity. It has been tested for carcinogenicity in mice and rats (Sun, 2007). Oral administration of D-limonene is rapidly and almost completely absorbed in the gastrointestinal tract in humans as well as in animals thereby rapidly distributed to different tissues in the body and readily metabolized (Sun, 2007; Igimi *et al*., 1974). Its use in the treatment of inflammation and tumour is well documented (Jessica et al., 2014; Crowell et al., 1992). Furthermore, Yu *et al* (2017) studied the anti-inflammatory effect of D‐limonene in an ulcerative colitis (UC) rat model and reported that D-limonene reduced MMP‐2 and ‐9 mRNA expression levels via regulation of the iNOS, COX‐2, PGE2, TGF‐β and ERK1/2 signaling pathways in a UC rat model, indicating its potential antioxidant and anti‐inflammatory properties. Limonene has also been reported to possess anti-ageing activity. Limonene is also a potent inhibitor of both gram-negative and gram-positive bacteria (Patriza *et al*, (2015).

The phellandrenes are used in fragrances because of their pleasing aromas, the leaf oils of *C. paradisi* has β-phellendrene as a major constituent indicates the economic potential of the South African citrus leaf waste in the flavouring industries. (National Centre for Biotechnology Information, 2018). Other major compounds have been

implicated to have medicinal potential of which citral one. The activities of citral and geranial was found to stimulate significantly in vivo anti-allergic and anti-inflammatory effects, suppressing an immunoglobulin E (IgE)-induced passive cutaneous anaphylactic reaction in mice and a 12-O-tetradecanoylphorbol-13-acetate-induced inflammatory mouse ear edema, (Mitoshi, et al., 2014).

From this study, the presence of some secondary metabolites indicates the medicinal potential of the leaf and peels. Flavonoids have a wide range of pharmacological potentials which includes its use as antioxidant, anti-inflammation, anti-platelet, anti-allergic, cytotoxicity, reduce risk for heart disease or cancer (Asif and Khodadadi, 2013). Tannins in fruits serve as a natural defence mechanism against microbial infections and also known to be of medicinal value. The anticarcinogenic and antimutagenic potentials of tannins has been related to their antioxidative property. Tannins have also been reported to exert other physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immunoresponses (Chung et al., 1998), which is important in protecting cellular oxidative damage, including lipid peroxidation. We can conclude that the evaluation of citrus waste in this study is of great medicinal and economical value.

What is the cytotoxicity profile of different species of citrus viz. lemon and grapefruit citrus?

The acute toxicity test conducted on mice using various concentrations of the oils and extracts of the leaf and peel of *C. limon* and *C. paradisi* shows no mortalities up to the dose of 2600 mg/kg in mice in either the fruit peel or leaf oil treated animals

suggesting that these oils are non-toxic orally. According to Lorke., (1983) and Rodricks., (1992), LD_{50} values above 2600 mg/kg indicate that the essential oil is non-toxic and safe.

Which *Citrus* species or morphological part of *Citrus* plant possesses higher anti-inflammation and anti-oxidant properties?

The anti-inflammation test of both the three cultivars of *C. paradisi* and *C. limon* essential oils potential anti-inflammatory agent at all the three dose levels tested. Although at the first hour only the *C. limon* failed to reduce inflammation while the higher dose of significantly reduced inflammation in the first hour. All other doses of both *C. limon* and three species of *C. paradisi* essential oils significantly inhibited diclofinic induced paw inflammation in the rat. Diclofinic induced inflammation occurs where involved the release of reactants such as histamine and serotonin.

All essential oils of *C. limon* and *C. paradisi* showed significant antioxidant results but were less active compared to the controls in the DPPH and ferric reducing power bio assays. Among the oils, the fruit peel of Rose grapefruit and fruit peel of lemon essential oils showed promising results when compared with all other essential oils. According to our observations, it can be concluded that fruit peels of Lisbon lemon do possible higher anti-oxidant potential as compared to the leaf due to the flavonoids quantity from the phytochemical screening result. However, both the leaf and the fruits can be used as a source of flavonoid intake to reduce free radical disease. This could be related further related to the results of the phytochemical screening where flavonoids and tannins are found in high concentration.

6.2 Recommendation

South Africa is among the world's most citrus producing country with total citrus production of 2102681 tonnes in year (2011/2012), of which, 441899 tonnes were processed into juice thereby generating large quantities of waste (citrus peel and leaf) (Khan et al., 2015). Although some of the waste has found its usage in animal feeds, majority of the waste are however left to decompose thereby generating toxic gases and environmental pollutants. Considering the medicinal potential in these waste, it is therefore recommended that further clinical trials should be conducted for the commercialization of citrus waste material into food and condiment for human benefit. This will help alleviate some health challenges, create job and increase South African economy.

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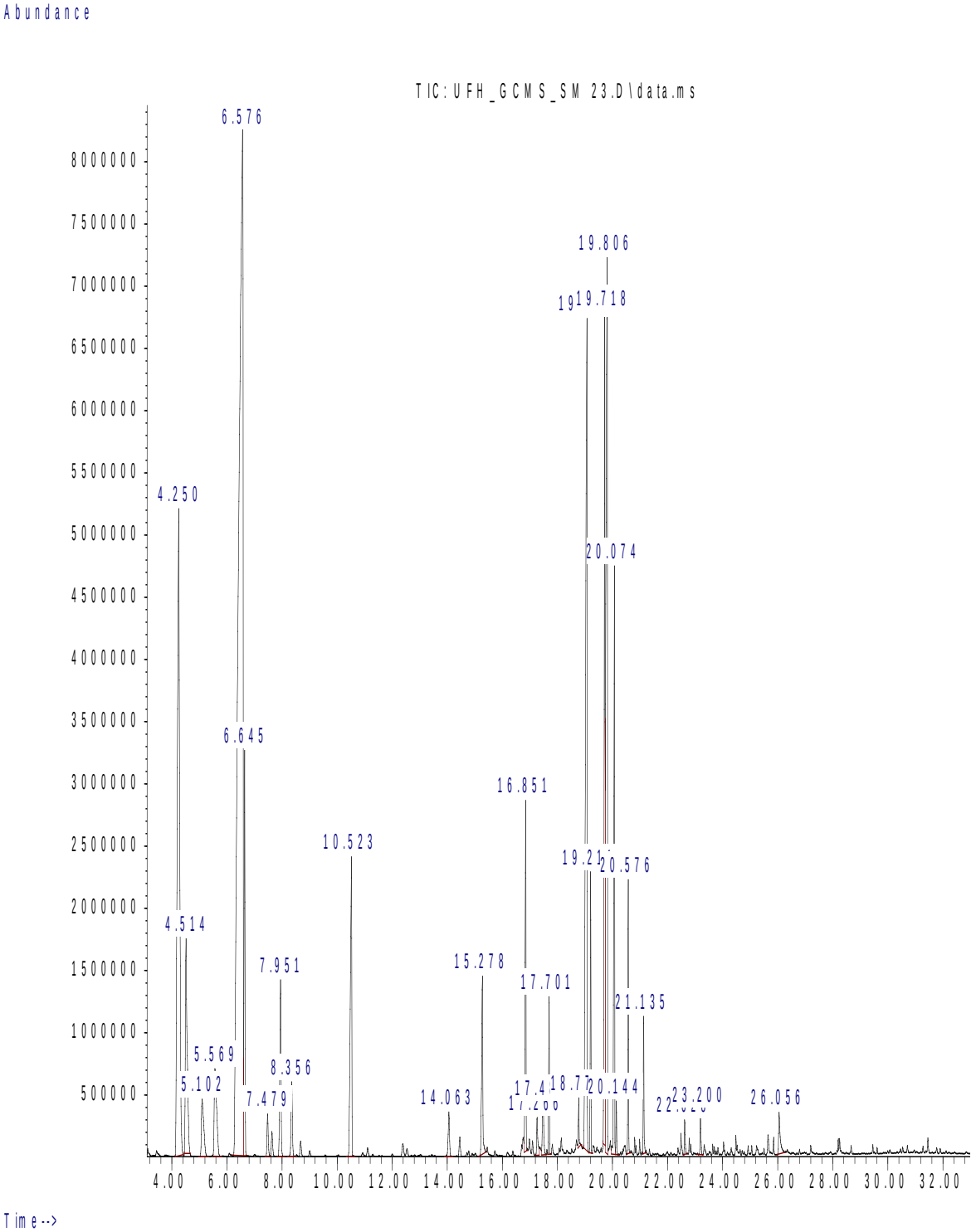
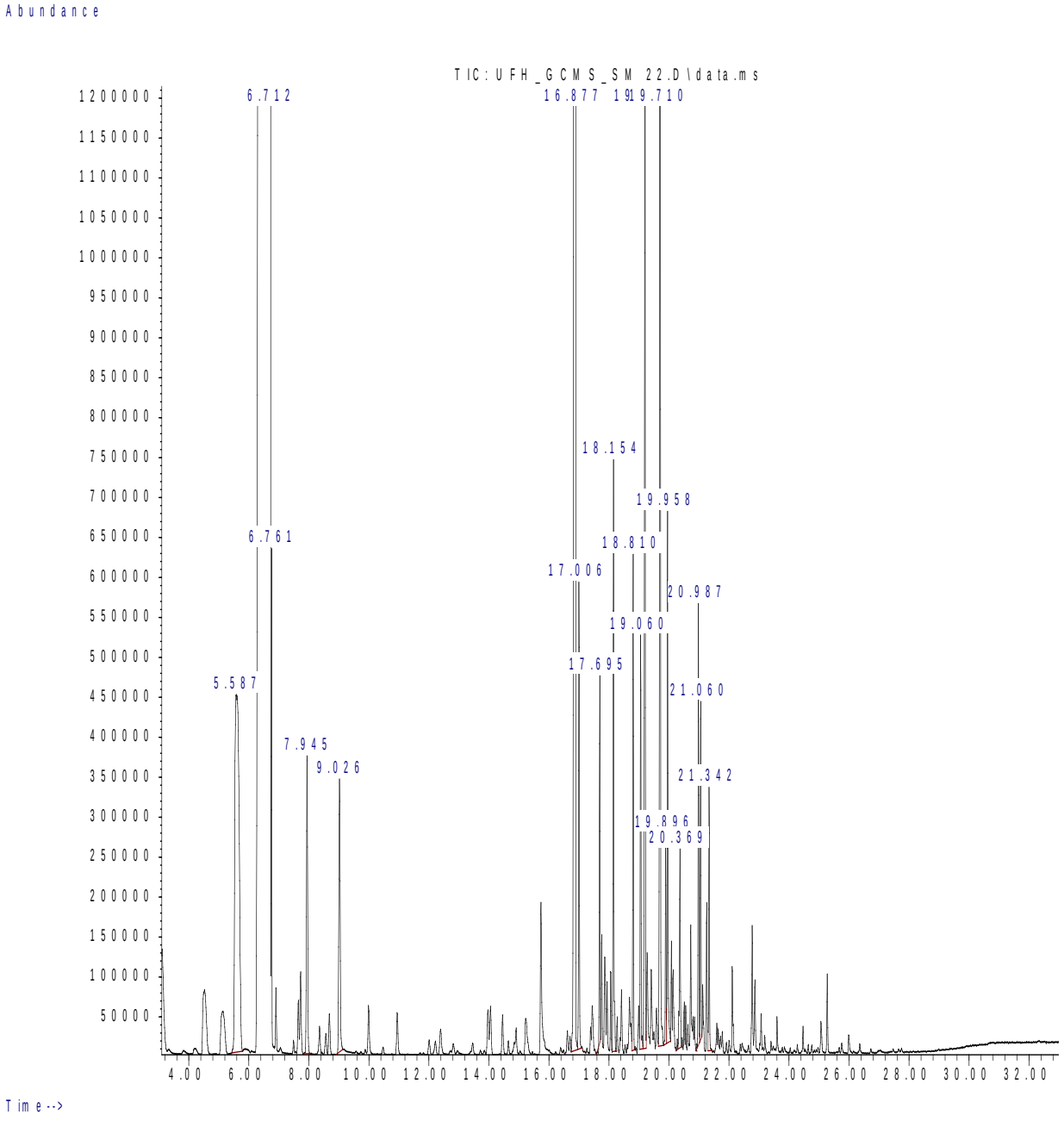
Appendix

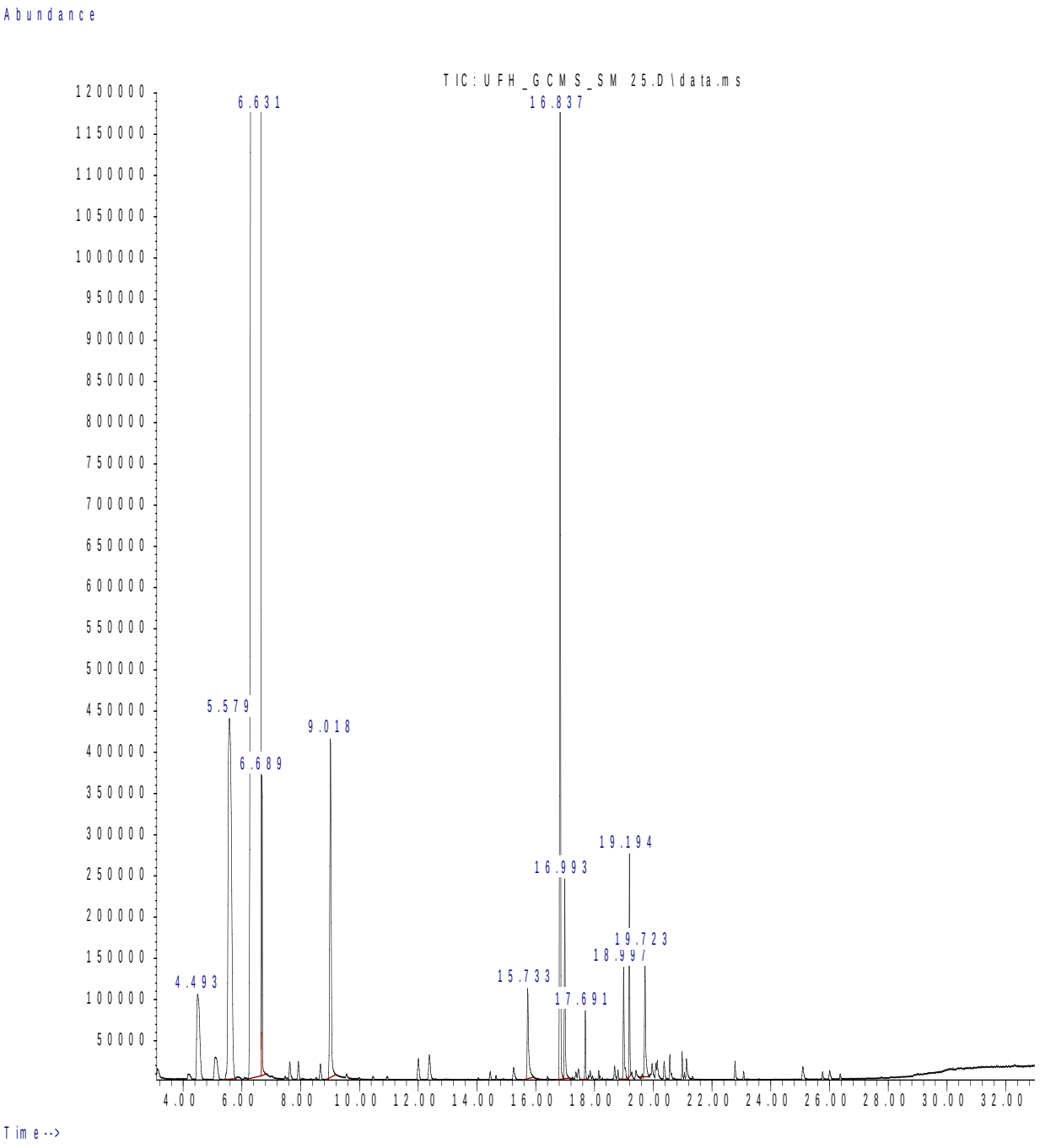
Appendix 1: GCMS Chromatogram of dried peels essential oil of Lisbon lemon *C. limon* (LLDP)



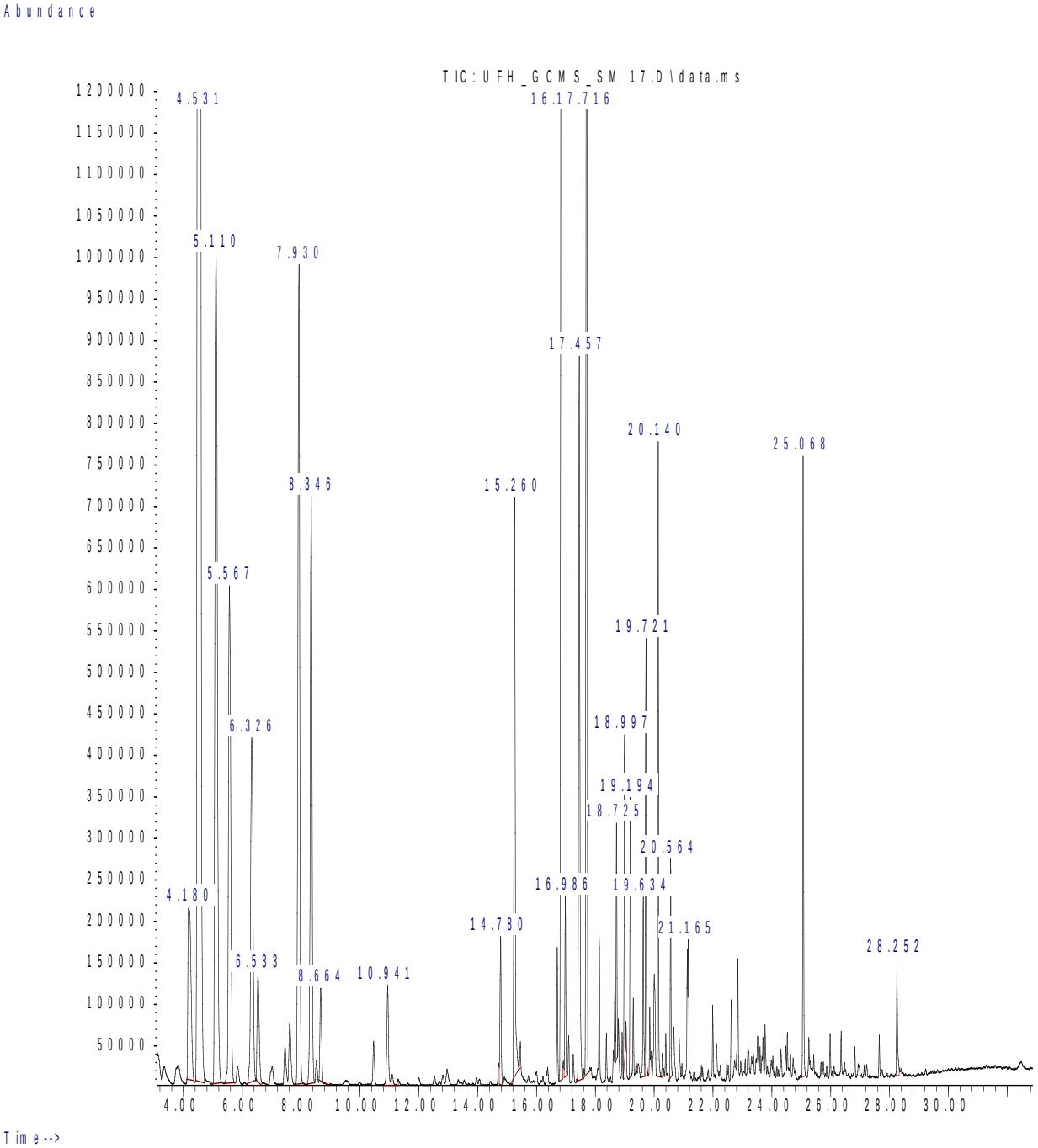
Appendix 2: GCMS Chromatogram of dried peels essential oil of Lisbon lemon *C. limon* (LLDP)



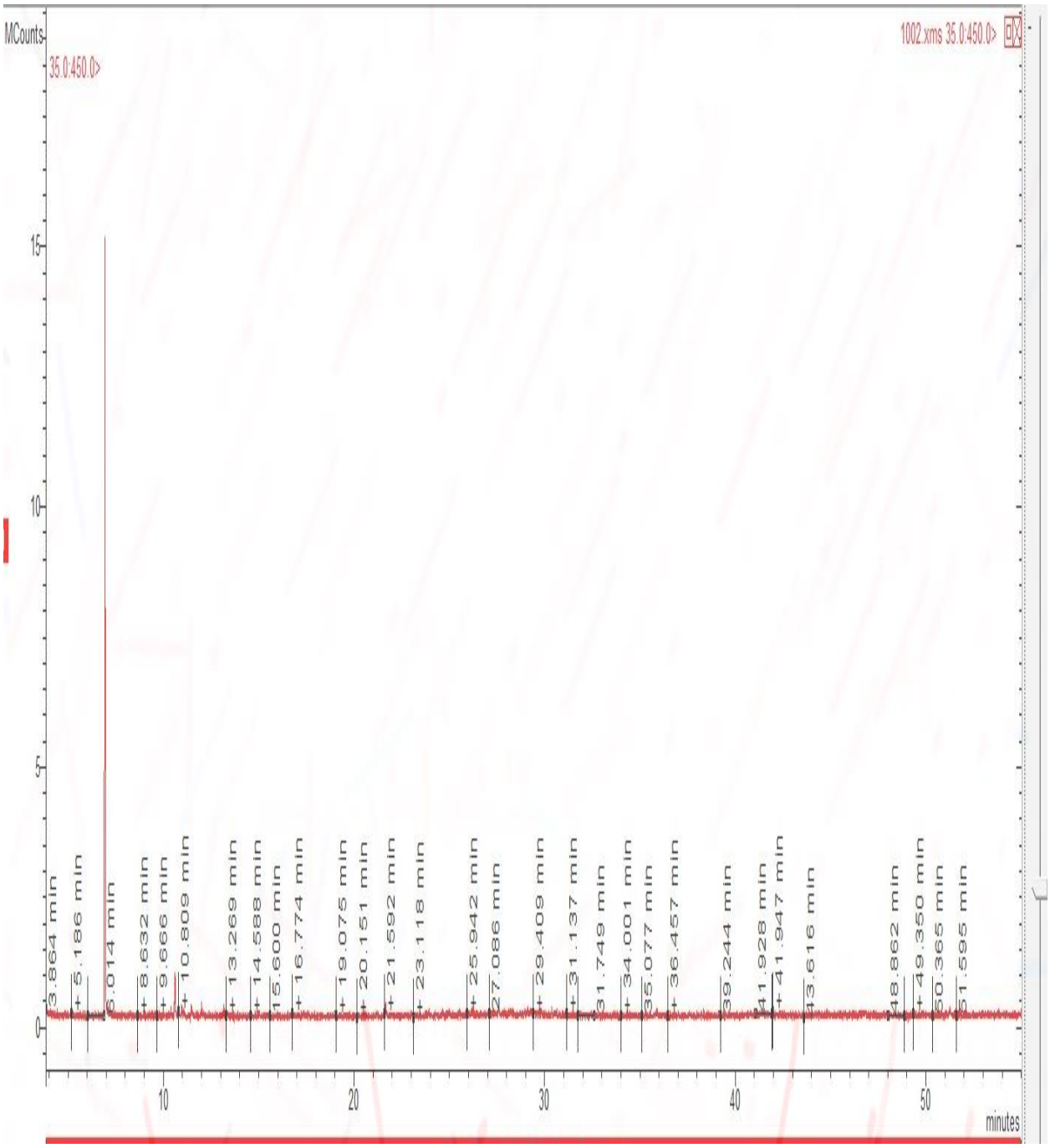
Appendix 3: GCMS Chromatogram of dried peels essential oil of Lisbon lemon *C. limon* (LLDP)



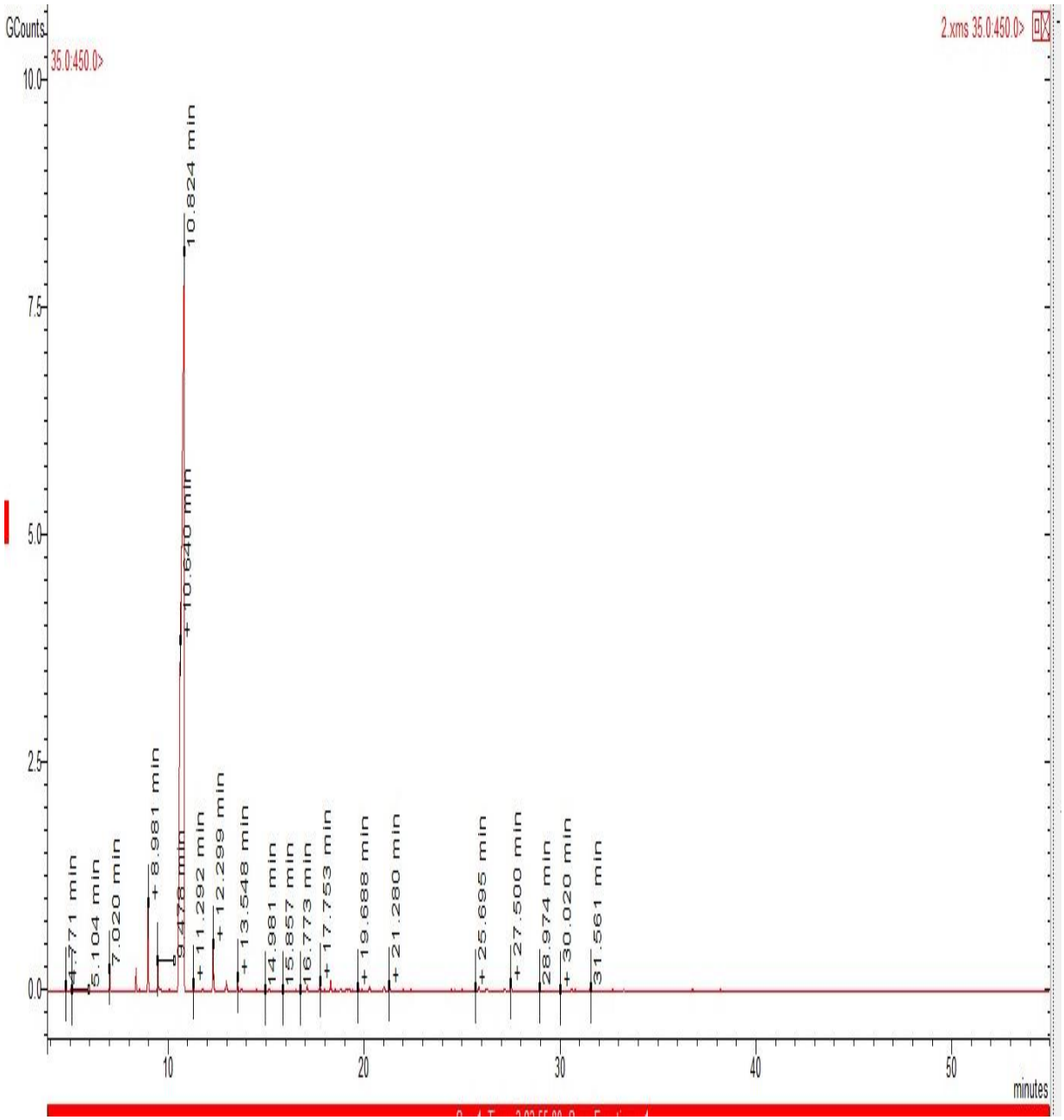
Appendix 4: GCMS Chromatogram of dried peels essential oil of Lisbon lemon *C. limon* (LLDP)



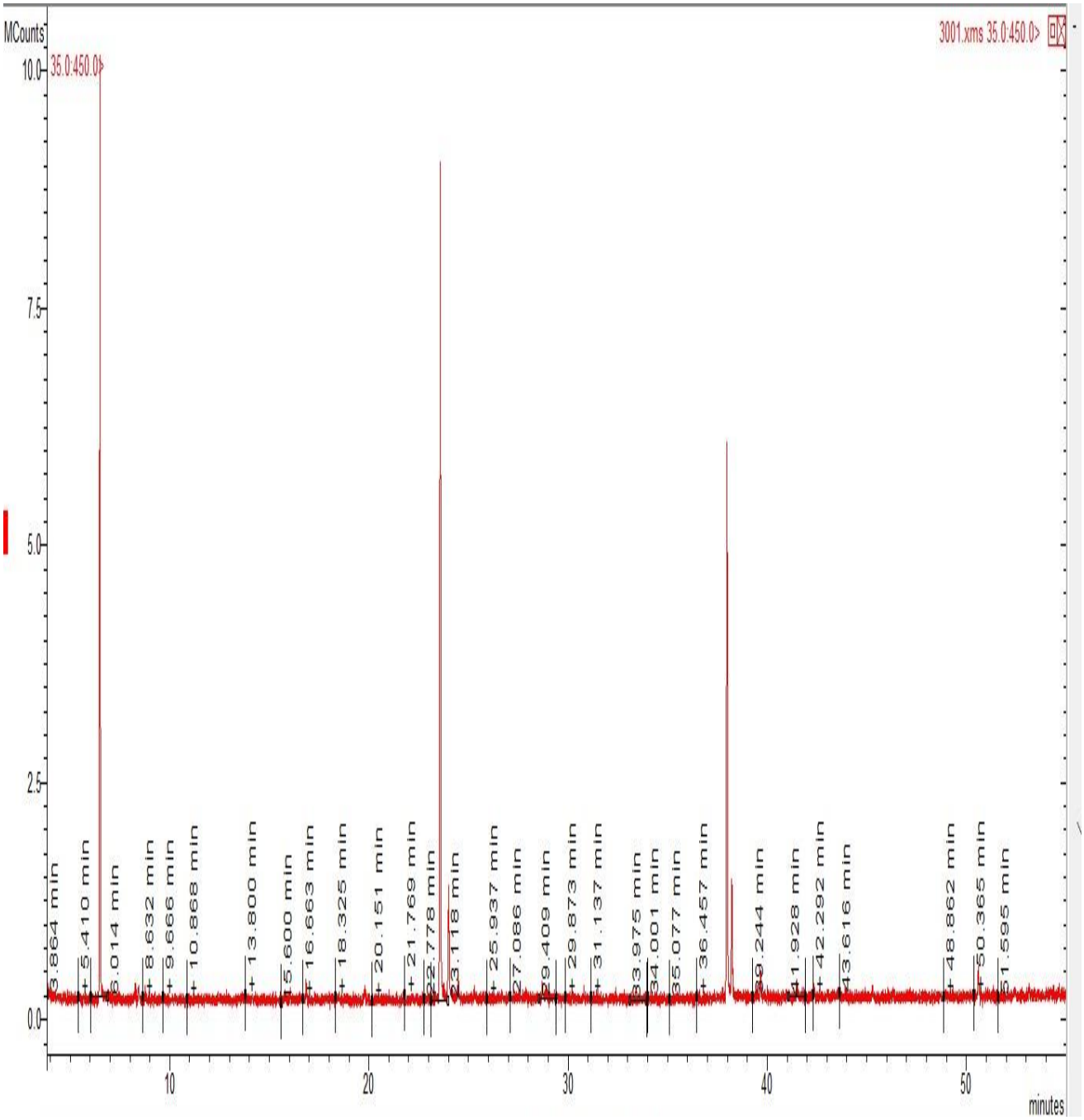
Appendix 5: GCMS Chromatogram of fresh leaf essential oil of Rose *C. paradise* cultivar (GRFL)



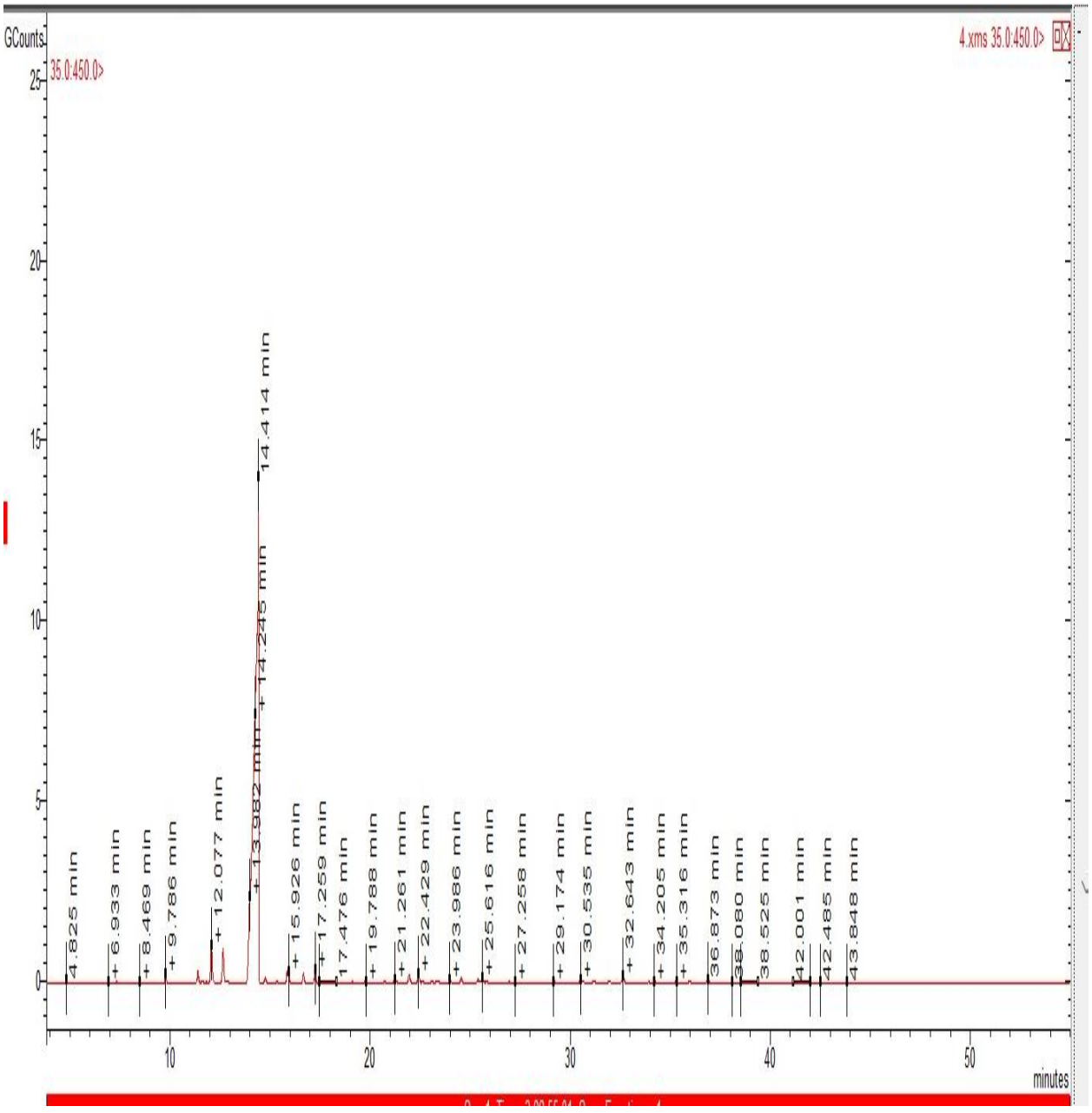
Appendix 6: GCMS Chromatogram of fresh peels essential oil of rose *C. paradisi* cultivar (GRFP)



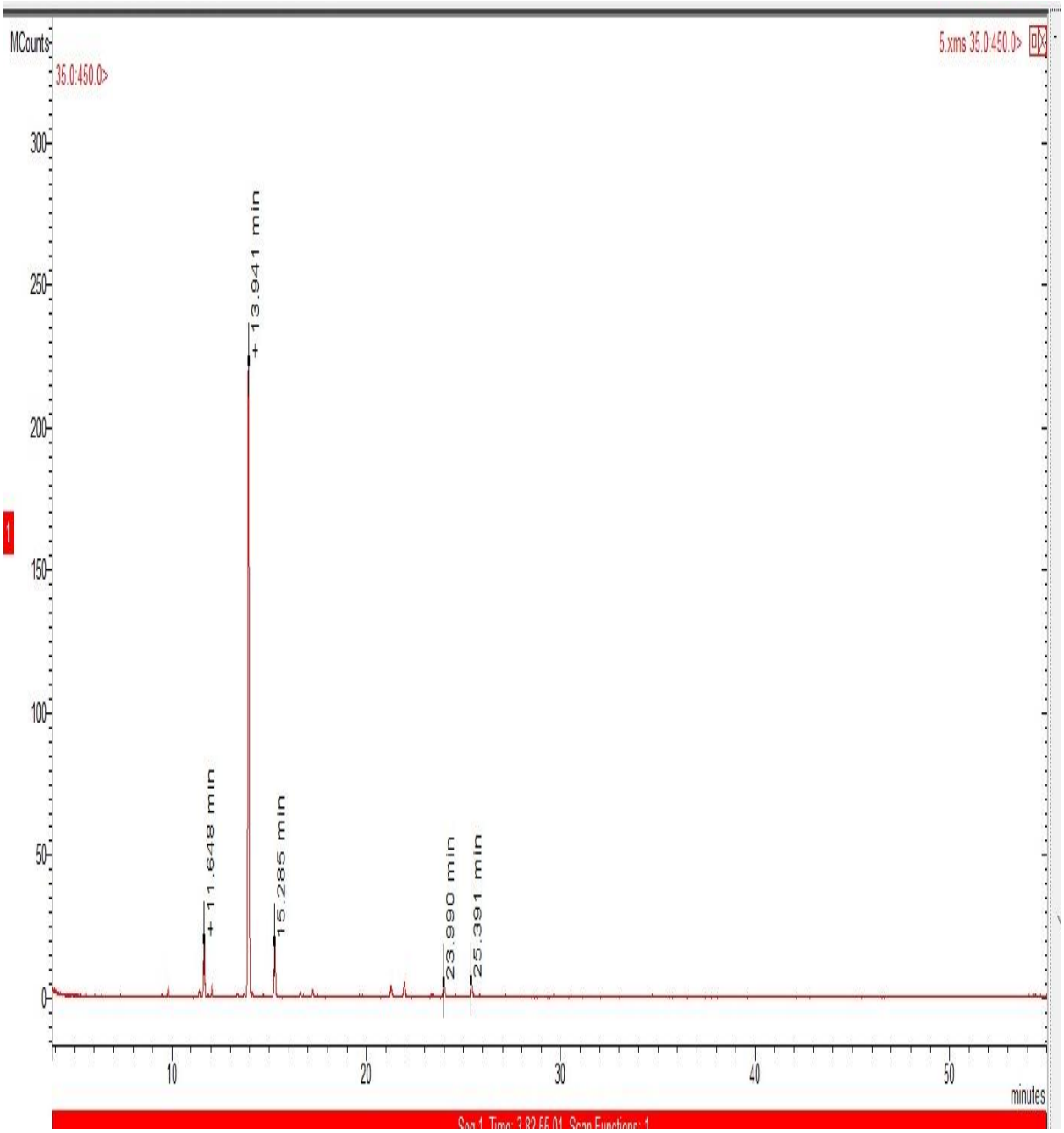
Appendix 7: GCMS Chromatogram of dried peels essential oil of rose *C. paradisi* cultivar (GRDP)



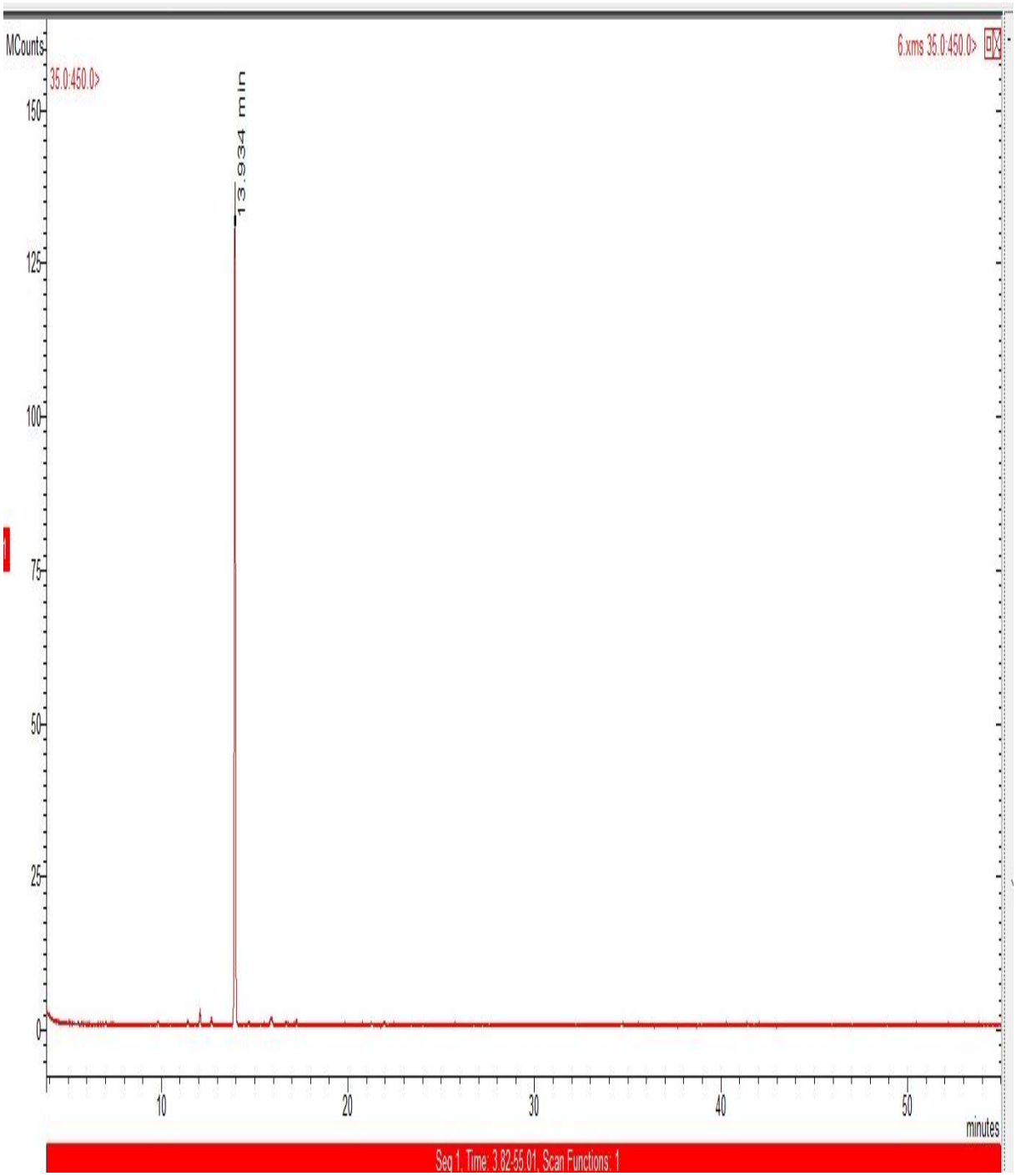
Appendix 8: GCMS Chromatogram of fresh peels essential oil of Star Ruby *C. paradisi* cultivar (GSRFP)



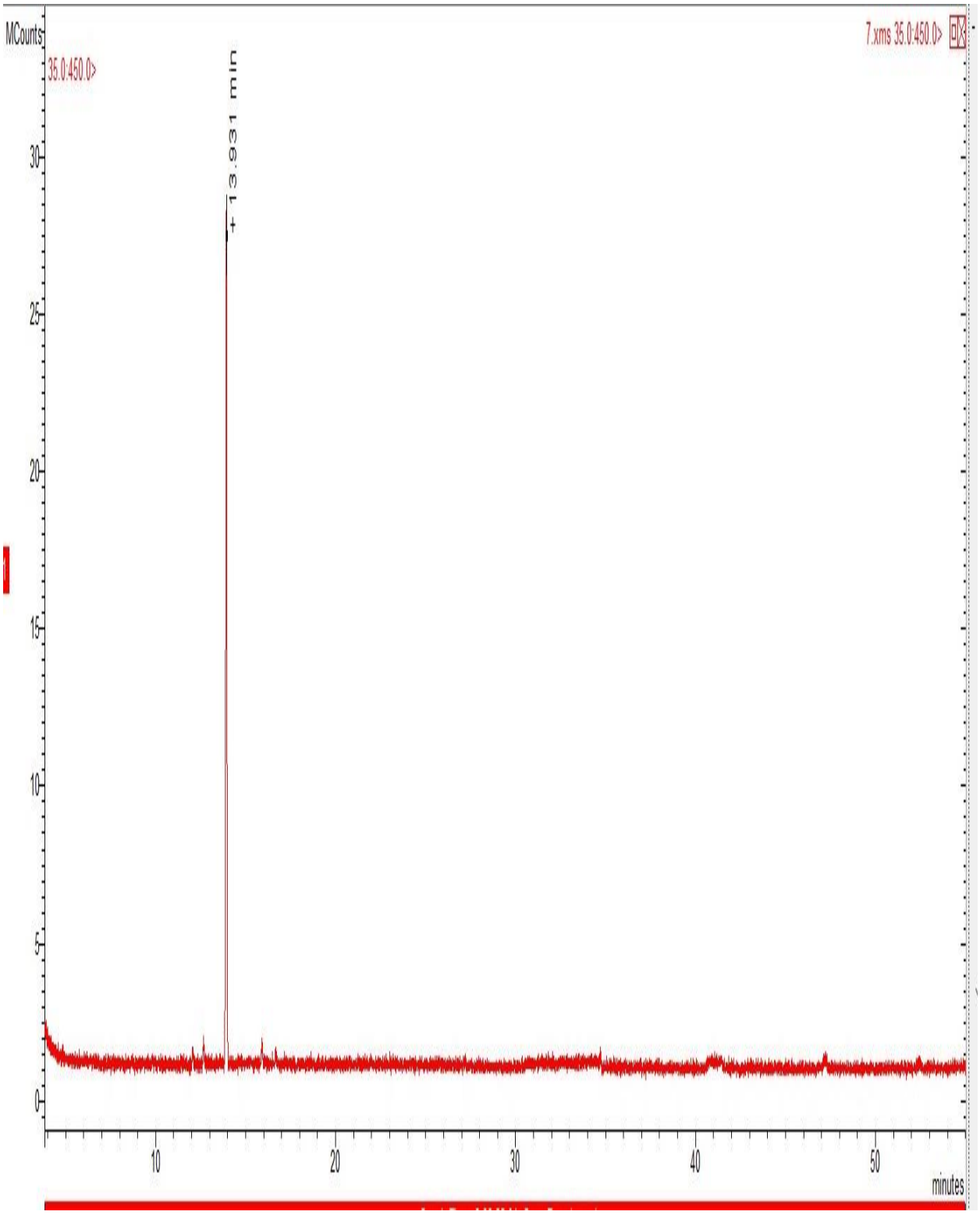
Appendix 9: GCMS Chromatogram of dried peels essential oil of Star Ruby *C. paradisi* cultivar (GSRDP)



Appendix 10: GCMS Chromatogram of fresh peels essential oil of Marsh *C. paradisi* cultivar (GMFP)



Appendix 11: GCMS Chromatogram of dried peels essential oil of Marsh *C. paradisi* cultivar (GMDP)



Appendix 12: GCMS Chromatogram of dried peels essential oil of Star Ruby *C. paradisi* cultivar (GMDL)