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Responses of selected metabolites in *Moringa oleifera* Lam. leaves to seasonal variations

by

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

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RESEARCH OUTPUTS

Papers to be submitted:

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Poster presentation: Responses of selected metabolites of *Moringa oleifeira* L. to seasonal variation. South African Association of Botanists conference. 2019; University of Johannesburg.

Declaration

I declare and that this dissertation is my own independent and original work, and it has not been submitted or presented for any degree at any other higher institution of learning. It is submitted to fulfil the requirements of a Master of Science at the University of the Witwatersrand, Johannesburg. The research work that was conducted by other researchers has been acknowledged by reference and citations.

The experimental work described in this dissertation was executed in the School of Animal, Plant, and Environmental Science, at the University of the Witwatersrand, Johannesburg, South Africa. This research was conducted under the supervision of Dr Ida Risenga (School of Animal, Plant, and Environmental Science, University of the Witwatersrand) and Professor Luke Chimuka (School of Chemistry, University of the Witwatersrand).

Signature: 

Floyd Maseye Ralepele

Date: 19/08/20

Dedication

I dedicate this dissertation to my beloved family that mean so much to me.

To my mother I would like to dedicate this dissertation to you, it is through your sacrifices that I am who I am today.

To my late father, thank you for all that you did for me. You did not get to see me graduate, but I just want you know that I am completing my third degree and I will keep on achieving more, because I was the first one to graduate in my whole family line. So thank dad wherever you are.

Amen.

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Abbreviations and symbols

±	-	plus/minus
C_{18}	-	Carbon 18
°C	-	Degrees Celsius
DPPH	-	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DNA	-	Deoxyribonucleic acid
et al.	-	et alia
g	-	gram
GAE	-	Gallic Acid Equivalent
HPLC	-	High-performance liquid chromatography
MeOH	-	Methanol
µ	-	microliter
µm	-	micrometre
mg/100g	-	milligrams per 100 gram
mg/mL	-	milligrams per millilitre
mL	-	millilitre
mV.se	-	Milli volts per second
M. oleifera	-	*Moringa oleifera* Lam.
Nm	-	nanometre
ppm	-	parts per million
QE	-	Quercetin Equivalent
ROS	-	Reactive Oxygen Species
TPC	-	Total Phenolic Content

TFC	-	Total Flavonoid Content
UHPLC	-	Ultra High-performance liquid chromatography
UV-Vis	-	Ultraviolet–visible
wt/vol	-	Weight/Volume

Abstract

Moringa oleifera Lam. is significant in the livelihood of many populations in the tropics and subtropics region. It was discovered centuries ago in India and has since become one of the most popular medicinal plants due to its therapeutic and nutritional properties. The high antioxidant activity in *M. oleifera* has highlighted the phytochemical compounds that are attributed to the medicinal and nutritional benefits. The species is regarded as the 'Miracle tree' and its cultivation is thus essential due to the potential in providing the necessary essential nutrients and herbal compounds. Due to climate change, environmental conditions are gradually changing in all climatic seasons, and as a result, the warmer and cooler seasons are exhibiting signs of extreme conditions. Hence, a research study on the responses of secondary metabolites in medicinal plants to seasonal variation is needed to assess the impact of these stress conditions on the pathways of the phytochemical production. Therefore, the present study was aimed at investigating the responses of selected secondary metabolites in the *M. oleifera* leaves to South African calendar-based seasons (summer, autumn, winter, and spring) from March 2018 to February 2019. The leaf samples were collected from a research farm at Hammanskraal, Pretoria (Gauteng province, South Africa). The quantification of the selected bioactive compounds was performed by UV-VIS spectrophotometry and UHPLC-DAD-qTOF-MS. In addition, 2,2-diphenyl-β-picrylhydrazyl assay and leaf morphology were used to investigate the anti-oxidant activity of the extract and plant leaf colour changes, respectively. The results from the leaf morphology have shown that *Moringa oleifera* Lam. has the potential to adapt under chilling temperatures however the plant has also shown high sensitivity to extremely cold temperatures and consequently becomes dormant under such conditions, particularly during the last two months of the winter season (July and August). As a result, the leaf sample collection for the winter season was performed only in the first month of winter (June) as well as in the last two months of spring (October and November for spring collection) for the phytochemical analysis. Interestingly, the species remains evergreen during all the seasons in its native regions such as Northern India. However, it has shown to be deciduous in South Africa, particularly in the Hammaskraal region.

The phytochemical results showed a gradual increase in the total phenolic and flavonoid content during autumn (4123±1516.97 mg GAE/100g and 1097.29±55.18 mg QE/100g, respectively) which later decreased in winter (3467.85±175,11mg GAE/ 100g and

439.10±12.29mg QE/100g, respectively) as compared with the spring and summer seasons. Total phenolic content increased to an average of 1253.59±258.34 mg GAE/100g during the last two months of spring and 3889.61±389.64 mg GAE/100g in summer. Similarly, the total flavonoid content increased during the last two months of spring and decreased in summer with an average of 734.14±38.10 mg QE/100g and 322.25±53.74 mg QE/100g, respectively. Quercetin and kaempferol content remained fairly constant with slight fluctuations in autumn and summer. However, Quercetin content remained constantly higher (258.11±73.47 mg/100g) as compared to the kaempferol content (13.47±5.73 mg/100g). The antioxidant activity in *M. oleifera* leaves was significantly high in the winter and summer seasons. This response was indicated by low EC_{50} values of 1.61±0.18 mg/mL in winter and 1.98±0.23 mg/mL in summer. This data correlates with total phenolic and total flavonoid content, particularly in summer. On the contrary, the EC_{50} high values indicated lower antioxidant activity during autumn (2.049±0.13 mg/mL) and spring (4.03±0.57 mg/mL). The data obtained from the current study, illustrated the impact of seasonal variations on the selected metabolites and antioxidant activity in *Moringa oleifera* Lam. leaves. It may be concluded that the species exhibited great sensitivity to the winter season by demonstrating a state of dormancy and subsequent abscission. Furthermore, the high antioxidant activity in the first month of winter and the entire summer season indicated possible oxidative stress that the species was eradicating by potentially increasing the total phenolic and flavonoid content. The present study serves as a meaningful contribution towards the farming, harvesting and ultimate consumption of *Moringa oleifera* Lam..

Keywords: *Moringa oleifera* Lam., seasonal variation, total phenolic content, total flavonoid content, kaempferol, quercetin, antioxidant activity

CHAPTER ONE: INTRODUCTION

1.1. Background on *Moringa oleifera* Lam.

Many research studies have demonstrated that parts of *M. oleifera* such as pods, seeds, stem, fruits, leaves, roots and flowers are valuable sources of bioactive compounds that are used for human consumption in many parts of world (Pandey et al., 2019; Alegbeleye, 2018; Liyongo et al., 2017; Rockwood et al., 2013; Anwar et al., 2007; Stohs and Hartman, 2015). The leaves of the *Moringa oleifera* Lam. have high contents of proteins, vitamins and phenolic compounds. Furthermore, the mature seeds contain lipids, vitamins and proteins (Pandey et al., 2019; Okorie et al., 2019; Lui et al., 2018; Liyongo et al., 2017). The value of *M. oleifera* leaves has increased the plant's commercial production in various parts of the world. In South Africa, the demand of *M. oleifera* for industrial, nutritional and medicinal benefits has significantly increased (Gopalakrishnan et al., 2016). Therefore, the South African government has essentially supported the commercial cultivation of the *Moringa oleifera* Lam. in some provinces of the country such as Gauteng and Limpopo, through stakeholders such as MDASA (Moringa Development Association of South Africa). In 2011, they found that about 13.8 million South Africans experiemce inadequate access to full nutrious food and according to the Food and Security Policy which is to ensure access to food and nuitrition, the efforts of including medicinal plants such as *Moringa oleifera* Lam. are important to assist the government to ensure food security. In 2011, Gauteng launched a food security project where they planted 69000 *Moringa oleifera* Lam. saplings. However, the growth rate and production of the Moringa in some provinces is negatively affected by extreme climatic conditions (Mabapa et al., 2017). Moreover, the climatic seasonal variation in South Africa, Gauteng launched a food security project where they planted has been shown to have enormous impact on the concentration of the secondary metabolites in the species (Ahmed et al., 2019; Sintayehu, 2018; Scopings et al., 2015). Consequently, the purpose of this study was to evaluate the responses of *M. oleifera* leaves to climatic season variations by assessing the content of total phenolics, total flavonoids, quercetin, kaempferol, and antioxidant activity in autumn, winter, spring and summer from March 2018 to February 2019.

It has also been reported that the leaves of *M. oleifera* contain various medicinal properties that includes antioxidant, antimicrobial, diuretic, anti-inflammatory, antihypertensive, antihyperlipidemic, antidiabetic and hepatoprotectant (Anwar et al., 2007; Stohs and Hartman, 2015). The leaves of *M. oleifera* were found to have high antioxidant properties as a result of compounds such as ascorbic acid and secondary metabolites such as phenolics,

flavonoids and carotenoids (Alhakmani et al., 2013; Vongsak et al., 2014; Stohs and Hartman, 2015). Extensive research from various disciplines has revealed that this plant has numerous properties or benefits which led to suggestions that *M. oleifera* can be used as a preventative orthodox medicine against various ailments (Pandey et al., 2019). The production of secondary metabolites has been reported to be influenced by a shift in environmental conditions, a trait used by plants to adapt to certain environments under stressful conditions (Edvera et al., 2008; Galasso et al., 2014). Secondary metabolites in medicinal plants are affected by seasonal variations (Lemos et al., 2017). The content of polyphenols in medicinal plants is affected by factors such as endogenous, exogenous, biotic factors, and the collection and storing processes (Pacifico et al., 2015). Medicinal plants are also vulnerable to extreme weather events and the global temperature fluctuation (Lepetz et al., 2009). These changes in conditions affect the distribution and production of organic compounds in medicinal plants (Zobayed et al., 2005).

The purpose of this study was to evaluate the responses of *M. oleifera* leaves to seasonal variations by assessing the content of total phenolics, total flavonoids, quercetin and kaempferol, and antioxidant activity. There is limited research that explores how the production of secondary metabolites in leaves of *M. oleifera* is affected by calendar based seasonal conditions. In the context of commercialising *M. oleifera*, it is important to determine the season in which the optimum production of secondary metabolites such as phenolics and flavonoids and the highest antioxidant activity can be achieved. This will then indicate the most suitable season to harvest the leaves of *M. oleifera*.

1.2. Literature review

1.2.1. *Botanical overview*

Moringa oleifera Lam., also known as the ‘drumstick tree’, horseradish tree, ben oil tree or benzolive tree belongs to the family Moringaceae (Anwar et al., 2007). It is native to the northwest region in India on the south region of the Himalayan Mountains. *M. oleifera*’s existence dates back as far as 150 A.D, it was used in royal families for maintaining healthy skin and mental alertness (Anwar et al., 2007). It was also used by soldiers to enhance physical energy and wound healing. *M. oleifera* is one of the highly sought after species within the Moringaceae family and has various uses that range from being a medicinal plant treating ailments such as skin infections, asthma, and fever, food and cooking oil to combating malnutrition (Alegbeleye, 2018). *M. oleifera* is vastly cultivated and distributed in

countries such as Pakistan, India, Bangladesh, Nepal, Afghanistan, Sri Lanka, West Asia, the Arabian Peninsula, Ethiopia and Nigeria, Brazil, Venezuela, Southern Florida, Mexico Peru and Paraguay (Mughal et al., 1999; Mabapa et al., 2017). The mature *M. oleifera* tree can grow up to 10 to 12 m in height. The species grows best in tropical and subtropical climate conditions (25-35˚C). It grows well on sandy or loamy soil with slight acidic to alkaline pH. It is a drought resistant species and can withstand a variety of annual rainfalls ranging from a 250 to 3000 mm (Anwar et al., 2007; Alegbeleye, 2018).The leaves are bipinnate and are 45 mm long, with 1-2 cm long and 0.6-1 cm wide leaflets. The leaflets have a form of an ellipse and the side-end leaflets are narrow at the base. *M. oleifera* leaves have few hairs on the abaxial side and completely hairless on the adaxial side (Figure 1). The plant bears 20-50 cm long linear fruits (pods) under optimum conditions, the pods can grow to up 1 m (Roloff et al., 2009).



Figure 1: *M. oleifera* leaves. The leaflets have the form of an ellipse and the leaflets (closest to the petiole) are narrow at the base.

1.2.2. Nutritional benefits

M. oleifera has been reported to be a valuable food source due to its medicinal and nutritional properties (Busani et al., 2011). The nutritional benefits of *Moringa oleifera* Lam. have been documented in several research studies that highlighted its uses in human nutrition and the development of diets in animals (Nouman et al., 2014; Moyo et al., 2012). Most parts of *M. oleifera* are edible and most importantly the leaves were found to have medicinal and nutritional properties and other parts such as roots, bark, stem, fruit (pods) and seeds possess these properties (Pandey et al., 2019; Okorie et al., 2019; Lui et al., 2018; Liyongo et al.,

2017). The leaves, fruits or pods and flowers of *M. oleifera* are part of ingredients in many traditional dishes in India (Sohaimy et al., 2015), thus reflecting its nutritional importance within the Indian community. *M. oleifera* is also used, particularly in developing countries that are faced with famine and malnutrition. For example, it is a valuable food source in the West African region, because it is used to combat specifically protein malnutrition in infants and nursing mothers (Dhakar et al., 2011; Sohaimy et al., 2015). *M. oleifera* is a source of many organic and inorganic compounds that are essential to the dietary requirements in both human beings and livestock (animals) (Moyo et al., 2011; Sohaimy et al., 2015). The organic compounds and inorganic metals include: Protein, iron, vitamins, amino acids, calcium, potassium, carotenoids, trace metal ions, and antioxidants (Moyo et al., 2011; Taha et al., 2015). The leaves have two important amino acids such as Histidine and arginine that are critical to the nutrition of infants (Alegbeleye, 2018). The leaves have high levels of compounds such as minerals, vitamins, fatty acids and amino acids (Stohs and Hartman, 2015). Therefore, *M. oleifera* can serve as a substitute for nutritional supplements (Alegbeleye, 2018). The species contains more nutrients (grams per plant material) as compared with some Fruits and vegetables (Rockwood, Anderson, & Casamatta, 2013). Its leaves, either in fresh or dry form, were reported to have seven times higher vitamin C than oranges, fifteen times higher potassium than in bananas, seventeen times higher calcium than milk, ten times higher vitamin A than in carrots, nine times more protein than in yogurt, and twenty-five times more iron than in spinach (Fuglie, 2001; Thurber & Fahey, 2009; Rockwood et al., 2013). Further, β-carotene, carbohydrates, fat, fiber, magnesium, phosphorus, copper, sulphur are found in all parts of the plants. Additionally, antioxidants such as phenolics, flavonoids and ascorbic acid are contained in the leaves and other specific parts such as the stem bark and flowers (Gilani et al., 2007; Kumar et al., 2016; Gupta et al., 2018).

1.2.3. Medicinal uses

Moringa oleifera Lam. has been reported to have the ability to treat more than 300 ailments (Gopalakrishnan et al., 2016). For instance, it used to treat tumours, ulcers, skin infections, anxiety, asthma, scurvy, fever, bronchitis, diarrhea, diabetes, semen deficiency, headaches, wounds, malaria, cardiovascular disorder and blood impurities (Mahmood et al., 2010). Aqueous leaf extracts of *M. oleifera* have demonstrated antimicrobial properties against various infectious bacteria such as *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa* (Saadabi & Abu Zaid, 2011) as well as the potential to reduce

damage in the liver and kidneys in animal test subjects (Brilhante et al., 2017; Toppo et al., 2015; Sharifudin et al, 2013). *In vitro* studies by Guevara et al., (1999) showed that the ethanol seed extracts demonstrated the potential of an anticancer activity with compounds such as niazimicin present able to inhibit tumours. Methanolic leaf extracts have shown to act against *Hepatocellular carcinoma* and breast adenocarcinoma which were reported not to have a negative effect on the fibroblasts (Charoensin, 2014). Anti-inflammatory properties were detected in different parts of *M. oleifera* including the leaves (Brilhante et al., 2017). The leaf extracts were found to regulate the expression of compounds such as IFN-ɣ, iNOS, and C-reactive protein, reducing TNF-α and IL-6 in animal test subjects such as Swiss strain male albino mice (Das et al., 2012). The extracts significantly reduced the production of pro-inflammatory mediators such as iNOS and TNF-α (Waterman et al., 2014). Other studies have shown that *M. oleifera* has anti-diabetic properties that can act against Type 1 and Type 2 diabetes (Gopalakrishnan et al., 2016). Divi et al (2012) illustrated that aqueous leaf extracts could treat streptozotocin-induced Type 1 diabetes and Type 2 diabetes (insulin resistant) in male albino Witstar rats. Al-Malki and El Rabey (2015) treated rats with STZ-induced diabetes and also were fed *M. oleifera* seed powder, and a decrease in blood glucose was recorded. Ghasi et al (2000) fed rats the crude leaf extract and recorded a reduction in the cholesterol levels, this response highlighted hypocholestrerolemic activity in the leaves. Overall, this 'miracle tree' has shown to have anti-inflammatory, antipyretic, antiepileptic, antispasmodic, antihypertensive, diuretic, hepatoprotective, antioxidant, antibacterial, antifungal and circulatory stimulant properties (Anwar et al. 2006, Chen et al., 2007, Anwar et al. 2007; Gilani et al., 2007; Mahmood et al., 2010).

1.3. Antioxidant activity

Antioxidants are chemical compound that have the ability to reduce oxidative stress that is usually enhanced by high quantities of reactive oxygen species (ROS) (Pisoschi and Negulescu, 2011). Antioxidants play a role in the defence mechanism of plants and animals scavenging the free radicals that lead to oxidative stress (Pisoschi and Negulescu, 2011; Mbikay, 2012). There are two types of antioxidants: endogenous and exogenous. Endogenous antioxidants, in particular, are enzymes that include superoxide dismutase, catalase and some non-enzymatic compounds like albumin and uric acid. When the endogenous antioxidants are fail to protect the organism against the effects of high ROS, exogenous antioxidants in the form of pharmaceuticals supplements and nutritional supplements can aid in scavenging the free radicals (Pisoschi and Negulescu, 2011; Gopalakrishnan et al., 2016). Examples of

antioxidant compounds include ascorbic acid, vitamin E, D, and K3, phenolics, flavonoids and β-carotene (Pisoschi and Negulescu, 2011). Antioxidants are derived from either synthetic compounds or natural compounds such as phenolics, flavonoids, vitamins, and anthocyanins (Litescu et al., 2011).

All organisms have mechanisms that are able to maintain free radicals and keep them from being overproduced. Oxidative stress is usually caused by adverse biotic and abiotic factors which results in high production of ROS. High ROS may course cellular and tissue damage and denatures the proteins and DNA (Farber, 1994). Oxidative stress is therefore linked to many ailments and accelerated ageing effect in organisms (Tsao et al., 2004). According to literature, under normal conditions ROS remain supressed by the antioxidants in an organism as result, the level of antioxidants matches that of the ROS produced (Sreelatha & Padma, 2009). Medicinal plants such as *M. oleifera* serve as a significant source of antioxidants that neutralize free radicals (Zengin et al., 2011). Natural antioxidants are plant-derived and regarded as the safer antioxidants as compared with synthetic antioxidants (Wang et al., 2003). Consumption of natural antioxidants such as phenolics and flavonoids may have a positive effect in reducing ailments such as cancer and coronary diseases (Marchioli et al., 2001Pong, 2003; Gutteridge, 1995). *M. oleifera* contain most of the above mentioned antioxidants that aid in the defence mechanism in plants, and for medicinal and nutritional purposes. *M. oleifera* is rich in various antioxidants, and more relevant to this study, phenolic compounds, flavonoids, kaempferol and quercetin (Brilhante et al., 2017). The high antioxidant content and activity can be attributed to the presence of phenols and flavonoids in the leaves (Singh et al., 2009; Sreelatha & Padma, 2009; Sasikala et al., 2010). Jaiswal et al (2013) conducted a study where rats (normal and diabetic) were treated with aqueous leaf extracts of *M. oleifera*. The study showed a gradual increase in the activity of enzymes such as superoxide dismutase, glutathione S-transferase, catalase and a reduction in lipid peroxidation. This was attributed to high flavonoid content and phenolic content that played a role in protecting the cells against oxidative damage in both rats. In another study, 60 women who had gone through a menopausal stage used *M. oleifera* leaf powder as supplements. The results showed a reduction in malondialdehyde production over a period of three months which was attributed to antioxidants such as ascorbic acid, glutathione peroxidase, and superoxide dismutase (Kushwaha et al., 2012). The antioxidant activity attributed to the phenolics and flavonoids in *M. oleifera* was shown to protect beta cells that control hyperglycemia by scavenging ROS (Kamalakkannan & Prince, 2006; Al-Malki & El Rabey,

2015). Such studies highlight the importance high contents of antioxidants such as phenolics and flavonoids and subsequent antioxidant activity which is attributed to the scavenging of ROS.

1.4. Secondary metabolites

Primary metabolites are compounds that previously defined and described according to the roles they play in plant cell division and growth, storage, respiration, and reproduction (Bourgaud et al., 2001; Kossel, 1891). Secondary metabolites are compounds that are produced by plants and have no direct function in plant's primary growth and development. They contain many biological by-products that explain the vast interest in the traditional medicine systems (Hussein & El-Anssary, 2018). As the advancement in technology continued, the role of secondary metabolites was clearly correlated to the plants ability to adapt to the constantly changing environmental conditions (Bourgaud et al., 2001). Secondary metabolites essentially increase the fitness and survival of plants and ability to interact with different ecosystems (Bourgaud et al., 2001). According to literature, secondary metabolites have been shown to contain properties such as antifungal, antiviral, antibiotic, and as a result they play a role in the defence mechanisms against parasites and pathogens (Djinni et al., 2019; Stock et al., 2017; Wink, 2015). Therefore, they are mostly produced according to the specific needs of plants (Kebera et al., 2014). These compounds have been extensively used by humans on day to day basis as antidepressants, stimulants, aphrodisiacs, flavourings, medicines and essential oils (Kebera et al., 2014). There are three main groups of secondary metabolites that are found in plants which include alkaloids, terpenoids and phenolics.

1.4.1. Phenolic and flavonoid compounds

There are four main classes of phenolic compounds, namely; phenolic acids, flavonoids, stilbenes and lignans (Kebera et al., 2014; Harborne, 1984). Medicinal plants such as *M. oleifera* mostly contain phenolics and flavonoids (Cai et al., 2004; Abdeltaif et al., 2018). Phenolic compounds are the largest group of phytochemicals in plants (Harborne, 1984; Bhebhe et al., 2016), and flavonoids are the most common and widespread group of phenolic compounds (Bhebhe *et al*., 2016; Matamela, 2014). Phenolic compounds are produced by plants through the phenylpropanoid, phosphate and shikimate pathways (Randhir et al., 2004; Abdeltaif et al., 2018). They are usually derived from phenylalanine with an aromatic ring bearing one or more hydroxyl groups (Figure 2A) (Sulaiman & Balachandran, 2012; Matshediso et al., 2015). Phenolic compounds consist of lignins, lignans, simple phenols,

tannins, coumarins, flavonoids and phenolic acids (Pereira et al., 2009). These compounds are involved in different plant defence mechanisms and physiological processes such as Ultraviolet Radiation (UV), infections and wounding (Alonso-Amelot et al., 2004; Bennett and Wallsgrove, 1994). According to a study conducted by Alonso-Amelot et al. (2004; 2007), plants that occur at higher altitudes generally contain more phenolic compounds due to the need for protection from the UV-B radiation. Phenolic acids in plants are edible and found in foods such as teas, berries, fruits, and coffee, which contain up to 103 mg/100 g fresh weight (Manach et al., 2004).

Flavonoids, on the other hand, are the largest group of naturally occurring phenolic compounds, and are widely spread in plants such as *M. oleifera* (Panche et al., 2016). Flavonoids consist of catechin, iso-flavonols, flavones, flavonols, anthocyanidins, proanthocyanidins and anthocyanins (Matamela, 2014; Panche et al., 2016). These groups all share a basic structure called 2-phenylchromane, and consist of a fundamental structural skeleton called 2-phenylchromane (Figure 2B) (Harborne, 1984). The phenyl ring varies in its position; it is either attached to the C-2 or C-3 bond (Matamela, 2014). They are natural products that are well known for their health benefits, and they are abundant in foods and are widely consumed (Gottlieb, 1989). Flavonoids are considered an essential aspect in a range of pharmaceutical, nutraceutical and medicinal applications (Panche et al., 2016). They are a cluster of phenolic compounds that are studied alongside phenolic acids because they are an indispensable component due to their vast medicinal properties (Sulaiman and Balachandran, 2012). Therefore, they have become an essential subject of medicinal research (Nagavani & Rao, 2010; Sulaiman and Balachandran, 2012; Dzoyem & Eloff, 2015).

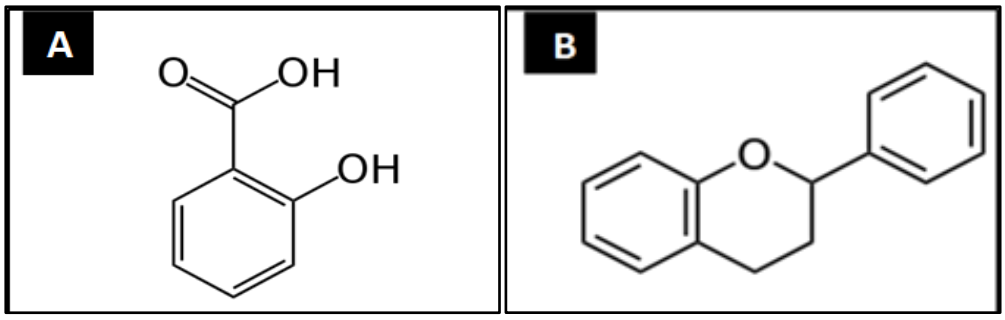


Figure 2: Phenolic compounds structure: phenolics (A), flavonoids (B).

1.4.2. Quercetin and kaempferol

Kaempferol and quercetin (Figure 3) are the most common flavonoids in *M. oleifera* leaves, and exist in abundance as glycosides attached to a wide spectrum of sugar moieties (Chen & Chen, 2013). Kaempferol is a natural flavonol that forms part of the flavonoid group. Like other antioxidants it has the ability to act against oxidative stress by scavenging free radicals. Furthermore, it can disrupt and prevent cancer cells from spreading by triggering apoptosis and modify the cell signalling pathways of the host (Ramos, 2007; Chen & Chen, 2013; Luo et al 2008; Zhang et al 2008). As reported by Zhang et al (2008), Kaempferol is found to have fewer effects on normal cells as compared to the present cancer therapy drugs. Quercetin is also a natural bioflavonoid that has gained attention in the scientific communities due to its medicinal properties such as antioxidant activity and anti-inflammatory effects (Gupta et al., 2016). Quercetin is a flavonoid that is found in various vegetables and also in red wine (D'Andrea, 2015; Beecher et al., 1999; Formica & Regelson, 1995; Hertog et al., 1993). In some parts of the world, quercetin is readily available as a dietary supplement (D'Andrea, 2015; Böhm et al., 1998; Linseisen et al., 1997). Similar to kaempferol, quercetin has excellent ability to scavenge free radicals and has great health benefits (Bors et al., 1994; Chorpa et al., 2000; Hayek et al., 1997). It is a strong antioxidant and as such it acts against the effects of neurodegenerative and cardiovascular diseases (Hollman & Katan, 1997; Balazs & Leon, 1994; Kahl & Hildebrandt, 1986). The high scavenging activity of free radicals reduces inflammation (Shoskes et al., 1999) prevents cell death and reduces oxidative stress (Hollman & Katan, 1997). In literature, quercetin has shown medicinal properties such as anticarcinogenic (Pereira et al., 1996; Deschner et al., 1991; Verma et al., 1988), anti-aggregatory (Pignatelli et al., 2000), and anti-inflammatory properties (Ferry et al., 1996). In a study performed by Pakade et al (2013), both quercetin and Kaempferol in *M. oleifera* that is grown in South Africa were found in high concentrations. The high concentrations of both compounds could also account for high antioxidant activity in *M. oleifera* leaf extracts.

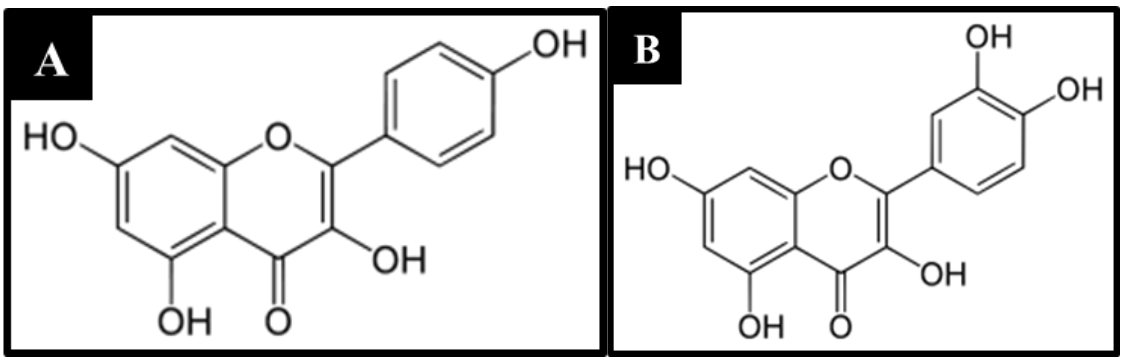


Figure 3: Individual flavonoid compounds structure: Kaempferol (A), Quercetin (B).

1.5. Farming and commercialisation

The commercial cultivation of *M. oleifera* usually occurs in the tropics such as Eastern Ethiopia, Somalia and northern Kenya (Mabapa et al., 2017; Foidl et al., 2001). As previously mentioned, *M. oleifera* is known to thrive under unfavourable conditions such as drought due to its drought resistant nature. It is also cultivated in regions such as the Caribbean, Central America, Africa and South America. The vegetative propagation method is commonly used in Indonesia, India, and in some parts of Africa, whereas in Sudan the traditional seed method is usually preferred (Leone et al., 2015; Palada, 1996). The seed germination has been reported to take approximately two weeks (Leone et al., 2015; Ojiako et al., 2011). Ramachandran et al. (1980) reported that the trees produced from traditional seed method resulted in poor fruit quality, while Animashaun et al. (2013) found that seed- cultivated trees form longer roots and thus providing an advantage as water becomes easily accessible for the root system. In contrast, the vegetative propagation method was reported to result in shorter roots (Mabapa et al., 2017). Currently limited reports exist on the different worldviews amongst farmers which indicates different perceptions on farming, production, utilization, and commercializing of *M. oleifera* (Mabapa et al., 2017). In South Africa, the cultivation of *M. oleifera* is recommended in the Limpopo Province due to its drought resistant properties. In addition, it may contribute to food security due to high nutritional value and tender green leaves and pods (Mabapa et al., 2017). The harvesting of *M. oleifera* begins three months after the planting period and usually continues throughout the year on an annual basis. Approximately 35.2% of leaves are harvested after five months and additional 32.3% after six months (Mabapa et al., 2017). Currently, 96.8% of farmers are in the active production business of *M. oleifera* products in all districts within the Limpopo province. Most farmers process the leaves into various products for their target market and other household users. Farmers usually process the leaves in dry powder form, whilst some percentage is processed into various products such as nutritional supplements. *M. oleifera* products are available in the market in various forms such as capsules, leaf powder, fresh leaves, seeds as well as teabags (Mabapa et al., 2017). Some farmers that can afford product processing equipment have established a market for products. Such products are sold to major supermarkets and pharmacies by the well-established farmers. (Mabapa et al., 2017).

1.6. South African seasons

South Africa's weather pattern is composed four distinct seasons. Winter is the coldest and driest season and covers the period May to July. Most vegetation shed leaves during this dry

winter period. Following winter is the hot, windy and dry spring season which is characterised by flowering in most of the plant species and occurs from mid-August to late-October. Summer succeeds spring and is the longest of the seasons stretching from November to February. Summer is the rainy season and is characterised by high temperatures. All the vegetation is green in summer with a predominantly rich geophyte component. Autumn marks the transition from summer into winter and usually falls between March and April. Plant leaves begin to go through a process of senescence during this season. Inland areas may experience slightly a variation in the seasonal pattern as compared to coastal areas, because though there areas are in South Africa, but it important that some areas experience summer rainfall patterns whilst others experience winter rainfall patterns. In the context of the chosen area, the climatic conditions are such that there are wet summer seasons and drier cold winter seasons.

South Africa is one of the countries in the world that has experienced drastic increased temperatures over the last 40 years (Kruger and Sekele, 2012). Several provinces such as Mpumalanga, Limpopo, Gauteng, North West, Eastern Cape and Kwazulu Natal in South Africa have since doubled the average global temperature patterns, meaning that increased temperatures have been evident over the last part of the 20th century (Lugina et al. 2005; Smith & Reynolds 2005; Kruger et al., 2012). According to Kruger and Sekele (2012), increased temperatures have augmented, whereas cold temperatures have declined with evident patterns in the north-eastern, eastern and western parts of South Africa.

1.7. The effect of seasonal variations

Climate change is defined as any notably change in climate parameters such as wind, precipitation, and temperature over a longer period of time (Das et al., 2016). Studies show that the climate is changing rapidly and this leads to a change in species diversity and richness and thus affecting biodiversity because there is not enough time for plants to adapt to the changing environmental conditions (Manish, 2010; Lindzen, 1990). Food security and livelihoods are threatened by climate change (Kikon and Angami, 2018). Global temperature are expected to increase by 1.8 – 4.0°C and this will increase the intensity of hot seasons, drought, floods, cyclones, and forest fires (Kikon and Angami, 2018). Medicinal plants are also threatened by the effects of global climate change (Das et al., 2016). It is obvious that climate change effects are affecting the distribution and the flowering patterns of plants and medicinal plants specifically (Das et al., 2016). The African region is more vulnerable to climate change which puts medicinal plants in these regions under threat because it affects

their survival rate and genetic diversity (Neilsin et al., 2005). Studies have shown that there may be a great loss in medicinal plants species due to climate. Reports by Cavaliere (2008, 2009) show certain medicinal plants that may be at risk as a result of climate change are *Rhodiola rosea* and *Saussurea laniceps*. other reports demonstrates how a shift in seasonal timing for various plants and extreme weather events have affected the production and harvest yields of medicinal plants all over the globe (Das et al., 2016). In Germany and Poland, extreme hot summer season have been reported to affect the reseeding of chamomile (*Matricarcia recutita*) and extreme floods in Hungary has decreased the harvesting of *Pimpinella anisum* and *Foeniculum vulgare* (Pompe et al., 2008). Scientists have not yet established how climate change will have long term or immediate threat to medicinal plants, however it has been shown to have the potential of exerting pressures in the coming years (Das et al., 2016). Medicinal plants are very important in the traditional medicine systems in third world countries; as such the effects of climate change may pose a threat in the future. Seasonal variation is influenced by different factors such as temperature, sunlight, humidity, soil moisture, soil temperature and many other factors. The South African weather pattern has four distinct seasons. The winter season is the coldest and driest and occurs from June to August. Most plants shed leaves and become dormant during the dry winter period. This followed by a windy and dry spring season that occurs from September to November. This season is characterised by the emergence of new shoots and flowering in most plant species. The summer season commences from December to February and it is characterized by rain, high temperatures and favourable growing conditions plants such as *M. oleifera*. Autumn season serves as a transition season between summer and winter and occurs from March to May. Plants usually undergo senescence, and leaves change colour and start wilting. South Africa is one of the countries that have experienced drastic increases in average temperatures in the last 40 years (Kruger and Sekele, 2012). Some provinces such as Mpumalanga, Limpopo, Gauteng, North West, Eastern Cape and Kwazulu Natal have evidently shown the increase of the average global temperature patterns being doubled (Lugina et al. 2005; Smith and Reynolds 2005; Kruger et al., 2012). According to Kruger and Sekele (2012), increased temperatures have become more prominent, whereas cold temperatures have decreased with evident patterns in the north-eastern, eastern and western parts of South Africa.

Seasonal variation may influence the availability of secondary metabolites in medicinal plants and as a result affect the therapeutic potency (Soni et al., 2015). Variations in response patterns by medicinal plants to seasonal variation have been observed and this shown by the

production and fluctuation of secondary metabolites (Ahmad et al., 2011; Szakiel et al., 2011). Various assumptions have been brought forward to suggest the best time to harvest specific parts of medicinal plants to obtain the best concentration of medicinal and nutritional properties (Soni et al., 2015). Winter and summer seasons may likely influence the higher production of secondary metabolites (Skrzypczak-Pietraszek & Pietraszek, 2014; Sivaci & Duman, 2014; Patil et al., 2013; Moniruzzaman et al., 2013; Banerjee et al., 2013; Chaves et al., 2013; Kale, 2010; Qayoom et al., 2009), which might be due to low and high temperatures.

There are limited studies that highlight the responses of leaves of *M. oleifera* to seasonal variation on composition of secondary metabolites, medicinal and nutritional properties (Gore, 2006; Schar et al., 2004). *Moringa oleifera* Lam. is classified as an evergreenplant in India, but there is a possibility that it might behave as deciduous plant in South Africa, it is expected there might be morphological changes that might result from cold conditions. Medicinal plants produce high quantities of secondary metabolites under stress conditions, especially under extreme temperature conditions (Das et al., 2016). However, there are other factors that can influence the synthesis of secondary metabolites such as soil moisture, pathogens (diseases), solar radiation, grazing or browsing, and competition in between the plants (Dean, 2007).

There are limited studies on the effect of climate change on plant metabolites composition, therefore it is important to study the effect of seasonal variations (extreme weather condition, global temperature fluctuations, and precipitation) on medicinal plants such as *M. oleifera* (Gairola et al., 2010). The importance of medicinal plants and secondary metabolites is relevant in developing countries because they form part of the traditional medicine systems as they are known to treat various diseases (De Silva, 1997). As such, the effect of seasonal variations in *M. oleifera* leaves need to be studied in order to determine the quality and composition of secondary metabolites, particularly for species that occur in South Africa.

1.8. Rationale of the study

Many studies have outlined the importance of *M. oleifera* and its various medicinal and nutritional benefits which are essential to the livelihoods of human beings and animals. In South Africa, *M. oleifera* has relatively a high commercial value because the products are presently sold in various outlets and pharmacies as well as by street vendors across the country (Mabapa et al., 2017). However, with the potential negative effects of climate change

in South Africa, it is essential to understand the impact of changing seasonal variations on medicinal plants such as *M. oleifera*, as it has been predicted that there might be regular anomalies of extreme weather events (Harish, et al., 2013; Hoffman, et al, 2009). The composition of secondary metabolites is affected by climate change and geoclimatic conditions (Pakade et al., 2012). According to Pakade et al., (2012), the concentration of secondary metabolites such as total phenolics, total flavonoids, individual flavonoids (Kaempferol and quercetin) would differ across the calendar-based seasons. Iqbal et al., (2005) and Pakade et al., (2012) made similar observation where they established that TPC and TFC were affected by different seasons, with winter having the highest concentration and summer having the lowest. The responses of medicinal plants such as *M. oleifera* to seasonal variations are rarely studied. Furthermore, to date there is no available study that has been conducted on the response of *M. oleifera* on fluctuating seasonal conditions, particularly in South Africa.

1.9. Aim

The aim of this study was to investigate the effect of seasonal variation on total phenolics flavonoids, kaempferol, and quercetin and antioxidant activity in *Moringa oleifera* Lam.

1.10. Objectives

- To determine the effect of seasonal variation on antioxidant activity in *M. oleifera* leaves obtained at a farm in Hammanskraal, Pretoria during all calendar seasons.
- To determine the effect of seasonal variation on kaempferol and quercetin content in *M. oleifera* leaves obtained at a farm in Hammanskraal, Pretoria during all calendar seasons.
- To determine the effect of seasonal variation on total phenolic and flavonoid content in *M. oleifera* leaves obtained at a farm in Hammanskraal, Pretoria during all calendar seasons.

CHAPTER TWO: PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITY ANALYSIS

2.1. Materials and methods

2.1.1. Study site

The study site is located at Hammanskraal, Northern Tshwane, Gauteng province [Figure 4 (25°20'55.01"S; 28°15'52.93"E)], where *M. oleifera* is cultivated. The study took place from March 2018 until February 2019.



Figure 4: (A) An image of Moringa farm in Hammanskraal obtained from Google Earth, where there is ~1 hectare of *M. oleifera* plants. (B) *M. oleifera* trees which were one meter apart being cultivated at Hammanskraal.

2.1.2. Plant materials

Fresh *M. oleifera* leaves were collected at Hammanskraal farm during the calendar based seasons: summer, autumn, winter, and spring. The leaves were collected from March 2018 to February 2019. A specific sampling pattern was adopted, where the pinned points on Figure 5 were the collection points. In each collection 5-10 trees were chosen at random and the leaves were collected from those specific trees in the pinned spots. Leaves samples were collected at the end of each month during each season, and were only collected when plants had a full canopy (Figure 6**)**. The leave samples were harvested and packed in brown paper bags on site and then transported to the University of the Witwatersrand. Samples were immediately washed and dried at 25˚C for 3 to 5 days in the laboratory. After drying, the leaves were crushed and ground using a blender and after grinding, the blender was cleaned to avoid cross contamination, and then stored in foil-zip lock bags. The powder was kept at room temperature away from direct sunlight.



Figure 5: The pinned spots (blue) on the farm are where the leaves were harvested.

The winter season only comprised of one collection and this was due to the morphological changes that were observed from the plant. July and August collections did not commence because the leaves underwent senescence. In September, the leaves were not collected

because the *Moringa oleifera* Lam. leaves were immature and had not formed a canopy (see figure 6).



Figure 6: The leave samples were collected from the middle section of the canopy.

2.2. Extraction of total phenolic content

The extraction of total phenolics from *M. oleifera* leaves was carried out using a method from Siddhuraju and Becker (2003) with slight modifications (Pakade et al., 2013). The leaves were dried at 25˚C and ground into fine powder using blender. The extraction was composed of 20 mL 80% methanol and 3 g of the fine leaf powder. The mixture was placed in a sonication bath for 25 minutes. Then the extract was centrifuged at 6000 rpm for 10 minutes and the supernatant was isolated. The residue was extracted again to ensure all the phenolics were extracted and the resulting supernatant made a total volume of 50 mL with the 80%

methanol solution. The total phenolic content was determined using the Folin-Ciocalteu method with the aid of gallic acid which was used as a standard (Makkar et al., 1997).

2.2.1. Total Phenolic Content (TPC) analysis from the methanol extract

The determination of the TPC from the methanol extract of *M. oleifera* was conducted with the aid of the Folin-Ciocalteu reagent assay that was reported by Folin et al. (1927). A mixture that consisted of 200 μL of the extract, 750 μL 1:10 Folin-Ciocalteu of the reagent, 2 mL 7.5% sodium carbonate (Na_2CO_3) was placed inside an Erlenmeyer flask with a rubber stopper to initiate a reaction.Then the mixture was diluted with 7 mL of deionized water. For the completion of the reaction, the mixture was then placed in a dark cupboard for about two hours and thereafter, an ultraviolet-visible spectrophotometer was used to measure the absorbance of the TPC in the extract at a wavelength of 765 nm. The experiments were triplicated. Gallic acid working stock solutions was prepared to attain the concentration of the TPC and a calibration curve was plotted and this graph was used to accurately obtain the concentration of the total phenolics (Iqbal et al., 2006).

2.2.2. Extractions of total flavonoid content (TFC)

The extraction of total flavonoids was carried out using a method from Siddhuraju and Becker (2003) and Pakade et al., (2013). 3 g of dry powdered leaf sample was extracted in an apparatus that contained a reflex condenser and a round-bottom flask with 100 mL of 80% methanol (MeOH) for 3 hours. The extract was left to cool down for 2 mimutes and centrifuged for 15 minutes at 5000 rpm, and a 0.45-μm filter paper was used for filtering the extract. Extract volume was 100 mL with 80% MeOH (HPLC grade) and the extracts were used to estimate the total flavonoid content.

2.2.3. Total Flavonoid Content (TFC) analysis from the methanol extract

The method that was reported by Siddhuraju and Becker (2003) was followed to determine the total flavonoid content (Pakade et al., 2013). About 0.3 mL of the extract was placed in a 10 mL volumetric flask and deionized water was added in the volumetric and the total volume of the mixture was 4 mL. 0.3 mL of sodium nitrate was added to the mixture above and the contents were mixed. 3 mL of Aluminium chloride solution (1:100, wt/vol) was added to the mixture after 5 minutes. 2 mL of 1 M sodium hydroxide was added to the mixture after 6 minutes from adding the aluminium chloride and deionized water was used for dilution to get a total volume of 10 mL of the mixture. The mixture was stirred for 15 minutes. Measuring the TFC of the extract mixture required an ultraviolet-visible

spectrophotometer at a wavelength of 510 nm. With the aid of quercetin as a standard, it was used to plot a calibration curve, which was used to calculate the flavonoid content.

2.2.4. Extractions – kaempferol and quercetin

To obtain the total kaempferol and quercetin, a sonication method was used. *M. oleifera* dried leaves were crushed and ground into fine powder with the aid of a waring blender. 3 g of the dried leaf sample was mixed with 20 mL of 80% methanol (MeOH) and 20% distilled water in a conical flask (50 mL) and the mixture was covered with an aluminium foil and was sonicated in the darkness for 4 hours. The solution was filtered.

2.2.5. Determining individual flavonoid content using the UHPLC-DAD-qTOF-MS

For total kaempferol and quercetin content, a mass of 3 g of *M. oleifera* leaves powder was mixed with 20 mL of 80% methanol in a 50 mL conical flask covered with the aluminium foil on the top and sonicated for 4 h in darkness. After filtration, 10 mL of the extract was mixed with 20 mL of 1% (v/v) HCl in 80% methanol in a 50 mL round-bottomed flask to hydrolyze the flavanols. The flask was fitted with a reflux condenser. The mixture was refluxed at 90°C for 2 h in the water bath system to obtain aglycons of flavanols. The extract was cooled to room temperature and then sonicated for 5 min to remove air, and filtered through a 0.45 µm filter paper (Millipore, Bedford, MA, USA). The extract was then filtered and taken for analysis using UHPLC- DAD-qTOF-MS set 370 nm (Dionex ultimate 3000, Thermofisher, South Africa, Johannesburg). The extraction was repeated several times until the analytes could not be detected. The amount from each fraction was combined to estimate the total amount of quercetin and kaempferol in the powder. A calibration curve was established for both kaempferol and quercetin (Appendix 3 and Appendix 4) and this enabled us to obtain the concentration of kaempferol and quercetin from the graphs (Pakade et al., 2012).

2.2.6. DPPH scavenging activity assay

A slightly modified free radical 2,2 diphenylpicrylhydrazyl (DPPH) by Brand-Williams et al (1995) was followed to estimate the antioxidant activity of *M. oleifera* leaves (Chu et al., 2000; Garcia et al., 2012; Liyana-Pathirana and Shahidi, 2005). Scavenging activity is the rate at which natural antioxidants attack free radicals and stabilise the ROS (Pavithra & Vadivykkarasi, 2015). This method is used extensively in literature because it is accurate and its advantage is that it able to interact with whole sample extract and it is considered fast and accurate (Fitriana et al., 2016). The extract was composed 3 g of dried leaves that were

crushed and grinded into fine powder and 80% methanol. To qualify and quantify the scavenging potential of DPPH on the *M. oleifera* leaf extract, a stock solution (DPPH) was prepared by combining 50 mg of DPPH with 100 ml of 80% methanol. From the stock solution, a work solution was prepared by a dilution mixture with the ratio of 1:5 of stock solution to 80% methanol. For each extract there were five quantities (10-50 µL of the extracts of each month). A reaction mixture was composed of the 10-50 µL extract, 700 µL of the work solution and this was filled up with 80% methanol to make up 1 mL. A blank was setup using the 80% methanol and every time the control solution was prepared using the work solution and 80% methanol and the absorbance of the control sample was measured. The mixture was stored in the dark for 45 minutes. At a wavelength of 517 nm, a spectrophotometer was used to measure the absorbance. Inhibition (%) was quantified using the following equation:

$$\%\, scavenging/\, inhibition \;=\; [(Absample - Abblank) \,/\, Acontrol] \;*\; 100$$

Visual observation of the physical appearance of the plant samples were captured using a Canon digital camera (D5300)

2.2.7. Statistical analysis

Total Phenolic content, Total flavonoid content, Antioxidant activity, Kaempferol and Quercetin content were the mean ± SE of three replicates of the *M. oleifera* exracts. Data was analysed with RStudio version 3.4. One-way ANOVA was used to to observe significance in the seasonal content of antioxidant activity, TPC, TFC, kaempferol and quercetin. General linear model was followed by a multiple Post-hoc Tukey Honest significant difference, where $\alpha = 0.05$. This multiple Post-hoc Tukey Honest significant difference was used to observe significance in the seasonal content of antioxidant activity, TPC, TFC, kaempferol and quercetin.

CHAPTER THREE: RESULTS AND DISCUSSION

3.1.Results and Discussion

3.1.1. Total Phenolic content and Total Flavonoid content

3.1.1.1. Gallic acid and quercetin calibration curves

Appendix 1 and 2 (shown in figure 1.1 and 1.2, respectively) contains calibration curves that were used in the quantification of the total phenolic and total flavonoid contents acquired from the leaf extracts harvested in different seasons. Linear calibration curves were considered and the concentrations of the standards ranged between 5mg/L to 50mg/L and the correlation was significant as the R^2 was above 0.99. The absorbance of UV-Vis at the wavelength 768nm and 512nm of the different Gallic acid and quercetin concentrations are shown in the curves.

3.1.1.2.Total phenolic concentrations in different seasons

Represented in table 1 below are the monthly contents of total phenolics, total flavonoids. As previously stated in chapter 2, the leaf collection occurred in every month of the calendar-based seasons. The graphical representation of these monthly contents of total phenolics (Appendix 14), total flavonoids (Appendix 16), quercetin (Appendix 17) and kaempferol (Appendix 19) illustrates the changes throughout the seasons. During the transitional seasons (autumn and spring) an increase in the contents of total phenolics, total flavonoids, quercetin, and kaempferol was observed in spring, except for total flavonoids in autumn. These observations show that there are biochemical changes that are occurring throughout these transitional seasons and this might be attributed to fluctuations in the seasonal conditions and the changes that the plant has to undergo while adapting to the changing conditions.

Table 1: Amount of total phenolics and total flavonoids

	TPC (mg GAE/ 100g)	TFC (mg QE/ 100g)
Autumn		
March	2122,81 ± 103,85	1061,44 ± 64,16
April	5012,24 ± 530,62	1124,09 ± 22,69
May	5235,53 ± 470,82	1106,26 ± 78,71
Mean±SD	**4123.53±1516.97**	**1097.26±32.28**
Winter		
June	3467,85 ± 175,11	439,10 ± 12,29
July	-	-
August	-	-
Mean±SD	**3467,85 ± 175,11**	**439,10 ± 12,29**
Spring		
September	-	-
October	1335,99 ± 325,27	628,92 ± 47,65
November	1171,19 ± 191,50	839,36 ± 28,52
Mean±SD	**1253.59±323.18**	**734.14±148.81**
Summer		
December	3744,30 ± 300,31	304,61 ± 125,61
January	3526,33 ± 467,03	297,97 ± 9,35
February	4398,21 ± 401,59	364,19 ± 26,26
Mean±SD	**3889.61±477.84**	**322.27±36.46**

*mg GAE/ 100g- milligrams Gallic Acid Equivalent per 100 grams.
*mg QE/ 100g- milligrams Quercetin Equivalent per 100 grams.
*- represents the months that the leaves were not harvested.

Autumn – 2018

As previously mentioned in chapter one, the autumn season serves as a transition season between summer and winter and occurs from March to May. According the visual observations (figure 7), in the first month of autumn, plant samples looked healthy and green, and canopies had fully formed pods (figure 7A1). The plants seemed to adapt well in the beginning of this season. Similar appearance was observed in the second month (figure 7B-B1), however in the third month of autumn, signs of sensitivity to the changing season were visible on the older of leaves on the bottom sections of the canopies. Leaves were showing signs of senescence (figure 7C) by turning yellow (figure 7.1), wilting and falling off. The mid-section (the harvesting point) however still looked green and healthy (figure 7C1). The last months of this season certainly marked the onset of senescence and confirmed the

deciduous nature of *M. oleifera* that occur in South Africa particularly in the Gauteng province.

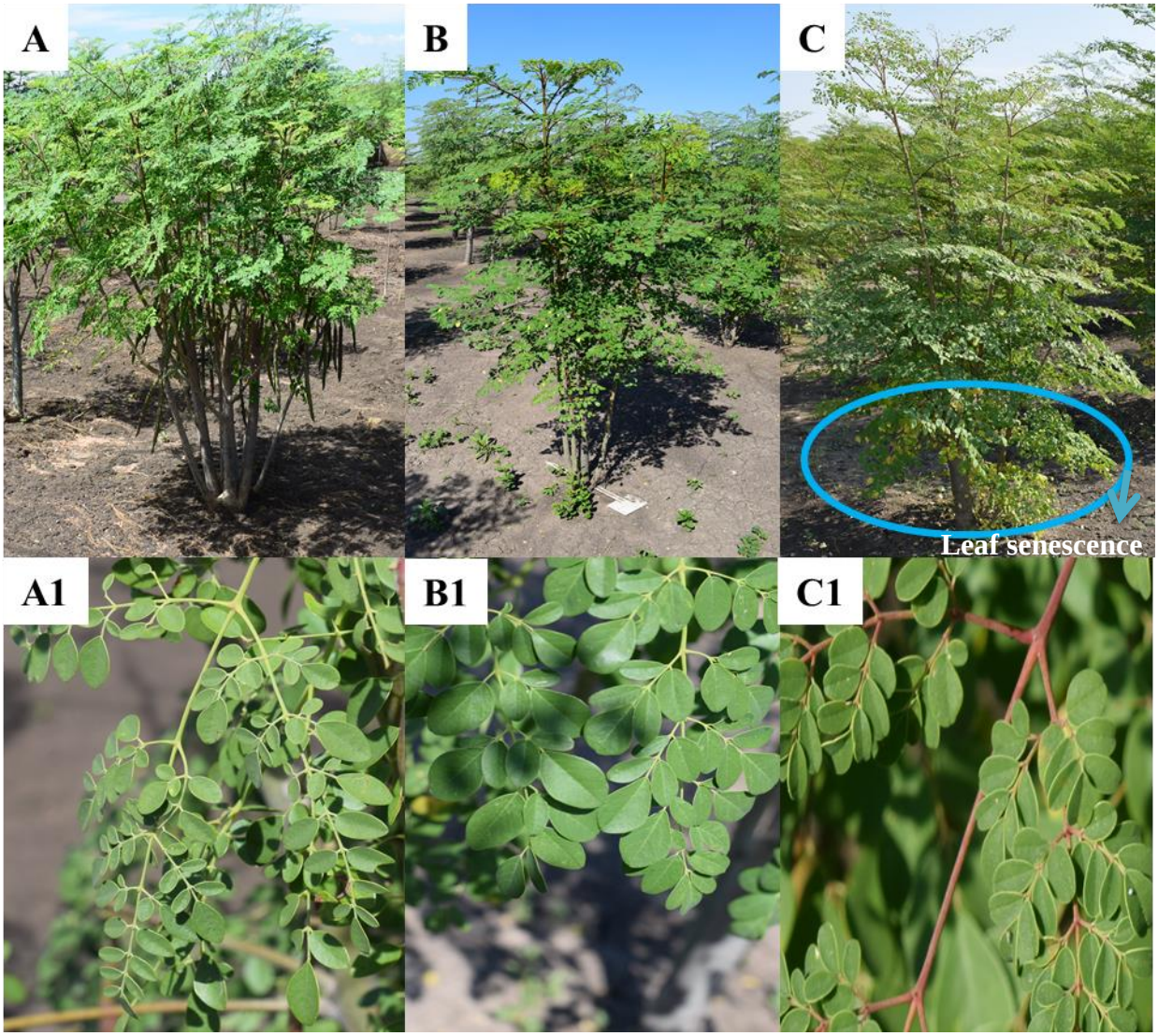


Figure 7: The physical appearance of *M. oleifera* samples during the autumn season – first (A-A1), second (B-B1) and third month of autumn (C-C1). Leaf senescence was observed on the older leaves as indicated with a blue circle (C).

It was observed that total phenolic content increased significantly from the beginning of autumn to the end of the season (Table 1). The TPC of *M. oleifera* leaves was measured during the autumn season, a significant increase from 2122.81 mg/100g to 5012.24 mg/100g from March to April, as well as a further increase from 5012.24 mg/100g to 5235.53 mg/100g towards the end of the season (April to May). An increase in TPC every month within a season indicates that plants experience more stress as the season the progressed, hence an

increase in TPC from the beginning of autumn to the end of autumn, where the climatic conditions are changing and we observe this through the onset of senescence in the last month of autumn (figure 7C). Iqbal & Bhanger (2006) studied the effect of seasons on *M. oleifera* leaves from different locations, however, the study only focused on one month per season, which does not give a complete response that plants undergo throughout the season. In this study, leaves obtained in May showed the highest concentration of total phenolics, which might indicate a transitioning of seasons and the leaves trying to acclimatize to the new season ahead. When plants acclimatize to specific stress such as temperature, which is one of the effects of seasonal variation, plants tend to produce large quantities of secondary metabolites to assist the plant to tolerate the stress (Janmohammadi et al., 2012). The collection of leaves occurred at the end of the season and the increase in TPC could be due to changes in temperature regimes from warmer to cooler temperatures. These findings are similar to a study by Chung et al., (2006) where they exposed *Rehmannia glitinosa* to cold temperature stress and observed an increased production of TPC.



Figure 7.1: Leaf senescence as a result of chlorosis.

Winter – 2018

Transitioning to winter, as previously mentioned that winter is one of the coldest and driest season, which occurs from June to August. The healthy green leaves had shed in the last two months of winter (figure 8B-B1 & 8C-C1). Leaf senescence took effect due to the extreme cold temperature and this is reflected in Figure 8. These observations show the previously

healthy green leaves undergo chlorosis turning yellow at the beginning of winter (Figure 8A & 8A1). The plant became dormant in the last two months of the winter season due to sensitivity to extremely low temperatures in South Africa, reflecting that it is indeed a deciduous plant. Morphologically, *M. oleifera* leaves responded to winter conditions by wilting and leaf rolling as observed in figure 8B-B1, and 8C-C1 and they changed colour to complete brown. These responses were also observed in plants such as soybean under extreme temperature conditions (Ashraf, 2004). These changes reflect the sensitivity of *M. oleifera* to South African winter conditions, especially in the Gauteng region. The change in plant leaf colour is usually due to chlorosis, which is the degradation or loss of chlorophyll due to limited light and changing environmental conditions. Chlorophyll is essential for the development and growth of plants because it manufactures food for the plant, and if chlorophyll is absent the plants suffer and cause the leaves to turn yellow (Wann et al., 1930). These low winter temperatures may promote the degradation or loss of chlorophyll and this directly affects the manufacturing mechanism of plant foods and leads to the plants dying (Ter Heerdt et al., 2017; Wang et al., 2012; Cramer et al., 2004).

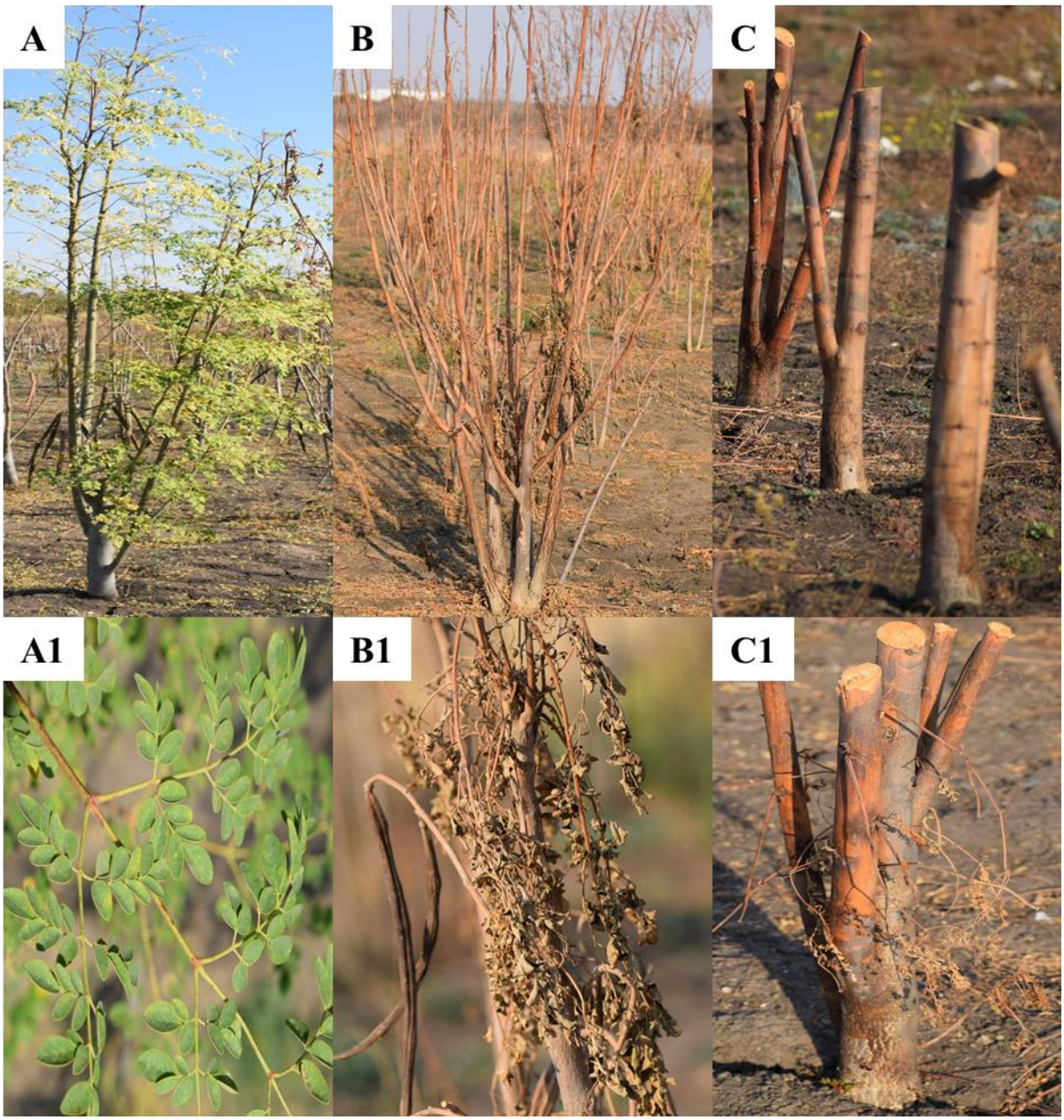


Figure 8: The physical appearance of *M. oleifera* samples during the winter season – first (A-A1), second (B-B1) and third month of winter (C-C1). Leaf senescence was observed from the first month of winter (A1).

A significant decrease from 5235.53 mg/100g to 3467.85 mg/100g in the concentration of total phenolics was observed from May to June, and this indicates that the leaves were undergoing some biochemical changes (Table 1). Generally, plants shed their leaves before winter starts (Axelrod, 1966), but the *M. oleifera* leaves seemed to survive some parts of the first month of winter in this study (figure 8A-A1), however, according to these results, a drop in TPC, indicate that *M. oleifera* could be redirecting its resources and energy towards

surviving winter than producing secondary metabolites (Table 1) and it also explains why plants undergo chlorosis in winter by redirecting resources for survival.

Figure 8B-B1 illustrates how seasonal changes can lead to the leaves drying out and shedding from the tree. Generally, the contributing factors to plant leaves changing colour to yellow and brown might be due to moisture stress, low light stress, and prolonged cold temperature stress (Woo et al., 2018; Thakur et al., 2016; Woo et al., 2013; Lim et al, 2003). Figure 8C-C1 the coppicing of plants for the upcoming growing spring season. In its native land (India), *M. oleifera* tree is considered an evergreen or deciduous or semi-deciduous indicating that its leaves are evergreen throughout the year (Roloff et al., 2009). With recent results, *M. oleifera* in South Africa could be shedding its leaves in response to climatic change since it grows in a different agro-climatic location where it is relatively cooler than its native habitat. In some parts of the northern hemisphere, it loses the leaves around the dry winter season (Popoola & Obembe, 2013).

Spring 2018

Spring season is a transactional season between winter and summer, and as previously mentioned it is a windy and dry season, usually, it associated with re-sprouting of new shoots from plants that were dormant during the cold season (reference?). According to the visual observations (figure 9), in the first month, new shoots began to sprout and the leaves appeared healthy and green (figure 9A-A1), but there was no harvesting because the plants did not have canopies, as mentioned in the methods that leaves were only be collected from plants with full canopies. As the season continued the plant began flourishing and in the second month, it was observed that the leaves were healthier and greener and a collection was made because the plants had formed canopies (Figure 9B-B1). The plants seemed to adapt to the warm temperatures and began flowering towards the end of the spring season (figure 9C-C1).

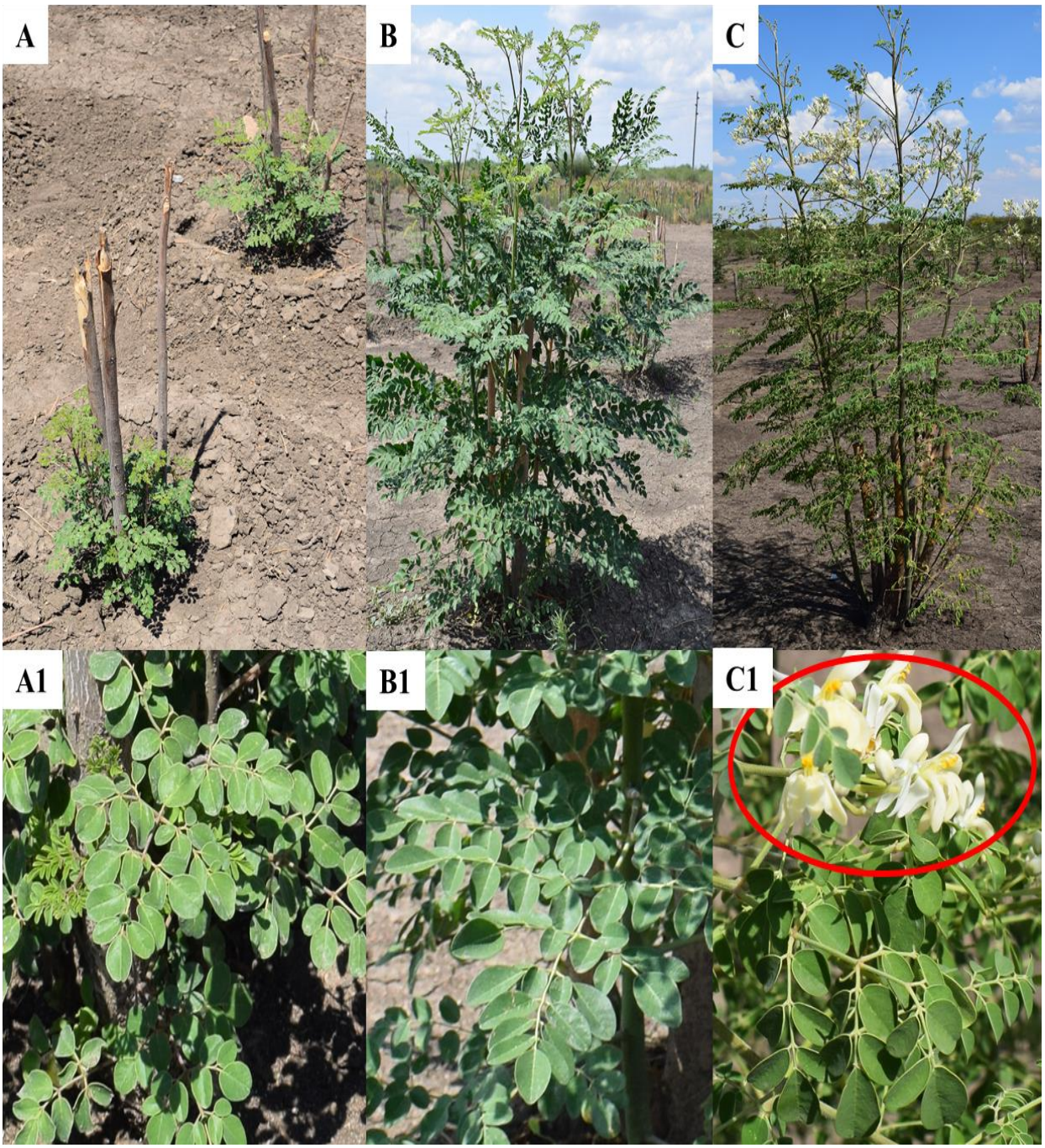


Figure 9: The physical appearance of *M. oleifera* samples during the spring season – first (A-A1), second (B-B1) and third month of spring (C-C1). The plants are observed to start flowering in late spring as shown in red (C1).

In the first month of spring (September), the leaves were not collected because the tress had not developed into a full canopy and they were still immature, as shown in figure 9A-A1. Comparing June and October, we observed a significant drop from 3467.85 mg/100g to 1335.99 mg/100g in TPC, and further November dropped from 1335.99 mg/100g (October)

to 1171.19 mg/100g (Table 1). There was an insignificant variation in the TPC in spring, which may highlight that the plants were adapted to the spring conditions.

Summer 2018-2019

The high temperatures and rain conditions in the summer seasons seemed to promote growth and development of *M. oleifera* plants because they continued to thrive and the leaves appeared healthier and greener at the beginning of the season (figure 10A-A1). *M. oleifera* originates in tropical regions and as such throughout the summer seasons, it showed morphological adaptations, because it continued flowering and the plant canopies grew bigger. The pods started to develop in the second month of the summer season and this continued to the last month of the season (figure 10B-B1 & 10C-C1). The plants have shown adaption to the summer season.

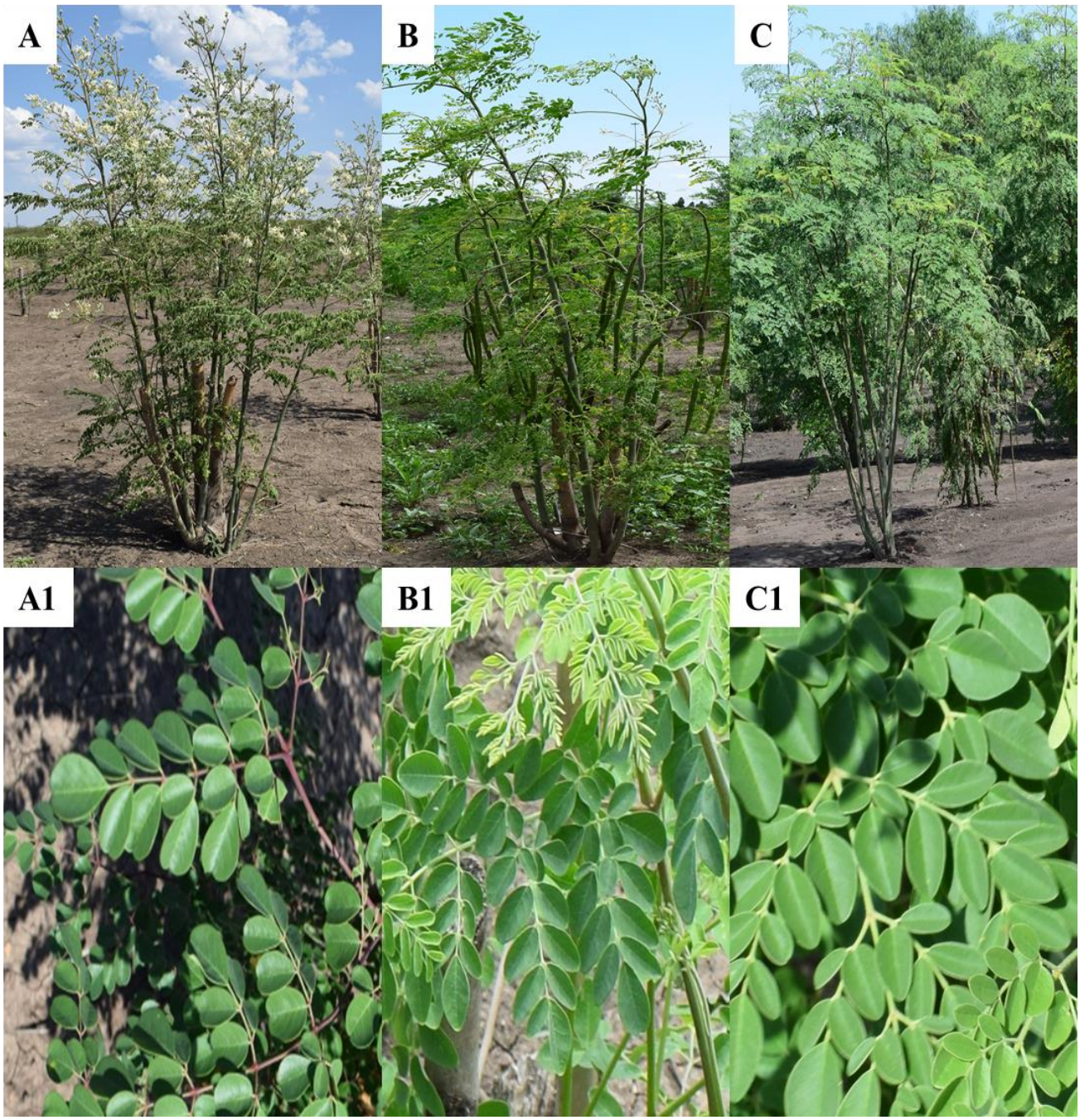


Figure 10: The physical appearance of *M. oleifera* samples during the summer season – first (A-A1), second (B-B1) and third month of summer (C-C1).

During summer, TPC increased significantly as compared with spring. The concentration of total phenolics increased from 1171.19 mg/100g to 3744.30 mg/100g in the first month of the season and then decreased from 3744.30 mg/100g to 3526.33 mg/100g in January (Table 1). However, the total phenolic content increased from 3526.33 mg/100g to 4398.21 mg/100g towards the end of summer in February. The increase in TPC towards the end may be due to the transitional period that the plants have to undergo due to changing seasons, thus they

become more stressed and this triggers the production of TPC. It was important to assess each month of every season because this clearly shows the fluctuation that occurs through each season and in this study we achieved that perspective of focusing on all the months in the seasons. When comparing the results of the first month in each season, it was observed that summer had the highest concentration of total phenolics and followed by winter (Table 1). Similar results were reported by Harbowy & Balentine (1997), where they found that the highest concentration of total phenolics was in summer, followed by winter in *Hypericum perforatum*.

The effect of different calendar-based seasons on TPC in *M. oleifera* leaf is shown in Figure 11 below. Observations from the effect of seasonal variations on leaves resulted in fluctuations in the TPC throughout the four calendar-based seasons. The collection of the leaves commenced in autumn and the TPC was at its peak as compared with other seasons. Overall from autumn to winter season, the TPC decreased from 4123.53 mg/100g to 3467.85 mg/100g. This was followed a further decrease from 4123.53 mg/100g to 1253.59 mg/100g observed between autumn and spring. A continued decline in concentration was observed between autumn and summer (4123.53 mg/100g to 3889.61 mg/100g). When winter was compared with the rest of the seasons, a decrease in TPC from 3467.85 mg/100g to 1253.59 mg/100g was observed between winter and spring. A gradual increase from 3467.85 mg/100g to 3889.61 mg/100g was observed in the TPC between spring and summer. The harvesting of the leaves occurred at random and this trend was maintained throughout the different seasons. These findings suggest that seasonal variations have an impact on the TPC of the *M. oleifera* leaves and these effects are not consistent because in some seasons a decrease in observed whilst in others, an increase is observed (Df= 3, F-value= 319.1, Pr(>F)= 2x10-16). This suggests that there are different factors influenced by seasonal variations that affect the content of total phenolic in the leaves. There is also an observable variation in each season because each month in the seasons presents a different factor that might come as a stress to the plant leaves and thus alter the production of the secondary metabolites. Abiotic stress is reported to alter the production of metabolites in plants and these metabolites are usually involved in the defense mechanism against stress (Obata et al., 2012). Increasing temperatures that may represent the summer season in this context, have been reported to affect the production of metabolites and a change in these can lead to proteins denaturing and impacting plant membrane structure, function and gene expression (Kravlova et al., 2012).

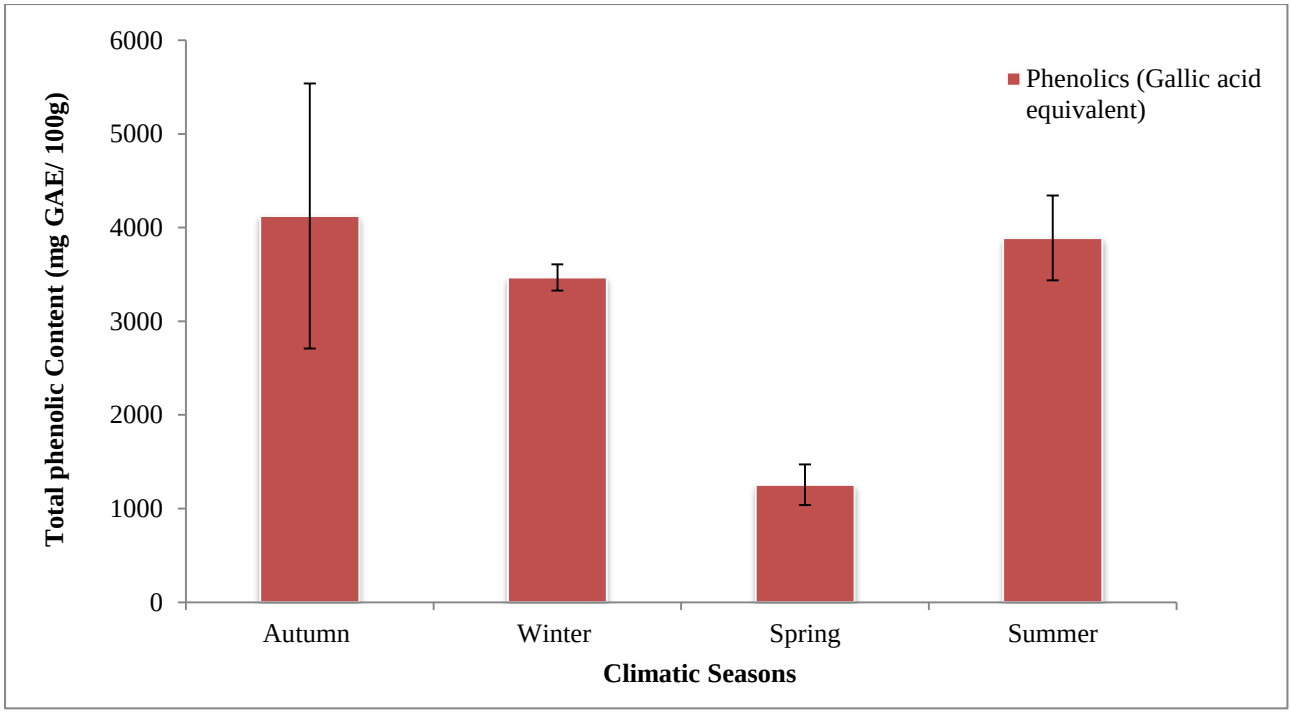


Figure 11: The effect of seasonal variation on Total Phenolic content of *M. oleifera* leaves.

Plants usually acclimatize under certain stress conditions which are due to changes in the metabolic pathways. High concentrations of phenolics may be expected when plants undergo stress. Fluctuations in the secondary metabolites are observed in plants such as the Australian wheat (Triticum aestivum) when it is grown under different temperature conditions (Jones et al., 2017). The results show that there is a change in the concentration of phenolics throughout different seasons. In summer, temperatures are expected to rise and therefore an increase in TPC is expected (Jones et al., 2017). Although it was not the highest, but a significant increase in TPC was observed in summer as compared to the spring season. The literature outlines that cold temperatures (winter) influence the production of secondary metabolites (Chung et al., 2006) and as demonstrated by the results, although there was a decline in TPC, it was insignificant because the autumn season had so much variation. Chung et al., (2006) and Posmyk et al., (2006) found that cold stress increases the concentration of total phenolics. In this study, autumn, winter, and spring exhibited similar responses in the production of total phenolics, because a decline was observed, it is statistically insignificant.

Environmental conditions do not only affect plant development and growth but also affects the production of secondary metabolites. Literature suggests that there is a noticeable variation in the secretion of metabolites in different seasons, because of certain

environmental factors such as temperature and precipitation, which are variable throughout the seasons (Muhammad et al., 2011; Szakiel et al., 2011). Literature also showed that there are assumptions on which part of the medicinal plants to collect in which seasons. The bark of the plant may be harvested in spring and winter for essential oils (Soni et al., 2015). With these assumptions, a conclusion can be drawn from the obtained concentration of total phenolics in spring, because they were at their lowest. It is then not advisable to harvest the *M. oleifera* leaves in spring because they are not mature and they show the lowest concentration of total phenolics. In a study by Anesini et al. (2008), they assessed TPC in *Camelia sinensis* at different harvest times. They found that TPC increased in warmer months and decreased in cooler months (Anesini et al., 2008). It shows that either a rise in temperature or sunlight may influence the biosynthesis of total phenolics. The conclusions drawn are that difference in TPC between warmer months and cooler months cannot only be attributed to temperature, but also other factors such as length of the day (Harbowy & Balentine, 1997). Harbowy & Balentine (1997) state that shaded tea flushes exhibits lower concentrations of total phenolics. *Hypericum perforatum* was harvested at different seasons; they found that summer seasons had the highest concentration of total phenolics as compared to winter (Gadzovska et al., 2013). Our results also show that summer had the highest concentration of total phenolics when compared to winter, although the variations were slight, they are statistically significant (Appendix 15). In another study, secondary metabolites of *Bacopa monneiri* were found to be high in the monsoon period and lower in the summer. These findings were attributed to the conditions in the monsoon period because they are favourable for the plant (humidity, water, and temperature) (Phrompttayarat et al., 2011). Ahmed et al. (2011) harvested *Menta longifolia* shoots in different seasons and found that that the production of total phenolics was enhanced in winter, followed by summer and then autumn. In our study, we observed that total phenolic content in *M. oleifera* leaves was high in the autumn season, followed by summer and the winter. The high total phenolic content in winter can be attributed to low-temperature stress. *Glycyrriza glabra* exhibited high TPC in the warmer season than in winter which can be attributed to TPC increasing with increasing light intensity (Aires et al., 2011; Teh & Mohamed, 2011; Slimstead & Verhuel, 2009). In another study, total polyphenols in the leaves of *Chelidonium majus* L. was the highest content in spring and this is due to the plant being in the rosette stage. In this study, they established that the concentration of polyphenols drops when the plants start flowering and peak when the fruit formation takes place (Javovljevic et al., 2013). Shi et al., (2017)

found the TPC of walnuts (Juglans sigillata Dode) increased in the summer season as compared to autumn.

Mohammed (2015) also reported the *M. oleifera* leaves had a spike increase in the TPC when exposed to high temperature, which was attributed to the summer season and according to this present study, leaves harvested during the summer season resulted in the second-highest concentration of total phenolics. However, it is important to note that the TPC will vary according to specific geographic locations because plants undergo different seasonal variations. Iqbal & Bhanger (2006) found the highest concentration of TPC to be in the cooler season (winter). Various authors reported that TPC is likely to increase with increasing temperature (Mohamed, 2015; Ahmed et al., 2012; Iqbal & Bhanger, 2006; Nour El-Din et al., 2004).

Winter was expected to have the highest concentration of total phenolics, however according to our results, autumn was higher, followed by summer and then winter, with spring being the lowest. The lower concentration of total phenolics in winter can be attributed to a decreased biosynthesis of phenolics as a result of low temperatures, which causes the plants to conserve their resources to survive the cold season and ensure re-sprouting in spring (Teh & Mohamed, 2011). Winter was expected to have the highest TPC because literature states that phenolic content is expected to peak with the maturity of leaves and low temperatures (Jukunen-Tiitto 1989; Wierwann, 1981).

3.1.1.3.Total flavonoid concentrations in different seasons

Autumn

Table 1 illustrates the monthly responses of *M. oleifera* leaves on the effect of seasonal variations on total flavonoid content within each season. In autumn, the concentration of total flavonoids increased from 1061.44 mg mg/100g to 1124.09 mg/100/100g from March to April and then decreased 1124.09 mg/100g to 1106.26 mg/100g towards the end of the season in May as compared to April. These results show less variation, as such, there was no significant difference in TFC as a result of seasonal variation during this season.

Winter

In winter we observed a significant decrease in the concentration of total flavonoids from 1106.26 mg/100g to 439.10 mg/100g from May to June. This indicates that indeed there was an effect on the TFC, because according to many researchers, cold temperature stress can

lead to fluctuations in the concentration of secondary metabolites (Oh et al., 2009; Zhang et al., 1997) and this might have been the case because the leaves were collected at the end of the June. During July and August, there were no leaves collected as indicated earlier on figure 8B and 8C, the leaves had shed and there was a morphological change on the plant leaves.

Spring

Figure 9A, as mentioned above, the leaves at the beginning of spring (September) were still immature and full canopy had not yet fully developed. The collection commenced in October as compared with June, we found an increase from 439.10 mg/100g to 628.92 mg/100g in the concentration of total flavonoids and a further increase was also observed from October to November from 628.92 mg/100g to 839.36 mg/100g. It is also important to outline that spring had the second-highest concentration of total flavonoids across all seasons.

Summer

A significant drop in TFC (from 839.36 mg/100g to 304.61 mg/100g) was observed from November to December. Throughout summer slight fluctuations were observed and thus leading to smaller variations within the season. A drop from 304.61 mg/100g to 297.97 mg/100g in TFC was observed from December to January, whereas an increase in TFC from 297.97 mg/100g to 364.19 mg/100g from January to February was oberved.

Figure 12 shows the effects of seasonal variations on *M. oleifera* which indicated a significant fluctuation of TFC throughout the seasons. There was a significant decrease from 1097.26 mg/100g to 439.10 mg/100g from autumn to the winter season. This was followed by a significant increase from 439.10 mg/100g to 734.14 mg/100g in TFC from winter to spring and finally, there was a decrease from 734.14 mg/100g to 322.27 mg/100g from spring to summer. Comparison across all seasons showed a statistically significant difference in the concentration of total flavonoids, which shows that seasonal variation does affect the composition of total flavonoids in the leaves of *M. oleifera* (Df= 3, F-value= 622.4, Pr(>F)= 2x10-16). Aoussar et al. (2018) studied *Pseudevernia furfueracea*, *Evernia prunastri* and *Ramalina farinacea* and they found a significant difference in TFC throughout the different seasons. The highest TFC in *P. furfueracea* was found in winter and followed by spring and summer and autumn had no significant difference. The present study does not show similar results, because autumn had the highest concentration of total flavonoids, followed by spring

and winter, and the lowest was summer. Seasonal variations such as climatic conditions, precipitation, and changes in temperature have been found to have a significant impact on the secondary metabolite composition in medicinal plants (Thakur & Kapila, 2016, Kapila & Thakur, 2016; Kapila et al., 2014). In a study by Thakur & Kapila (2017), they showed that TFC was significantly different throughout the seasons. Mature leaves seemed to contain high TFC because the end of the growing season recorded the highest flavonoid content (Thakur & Kapila, 2017; Pakade et al., 2013).

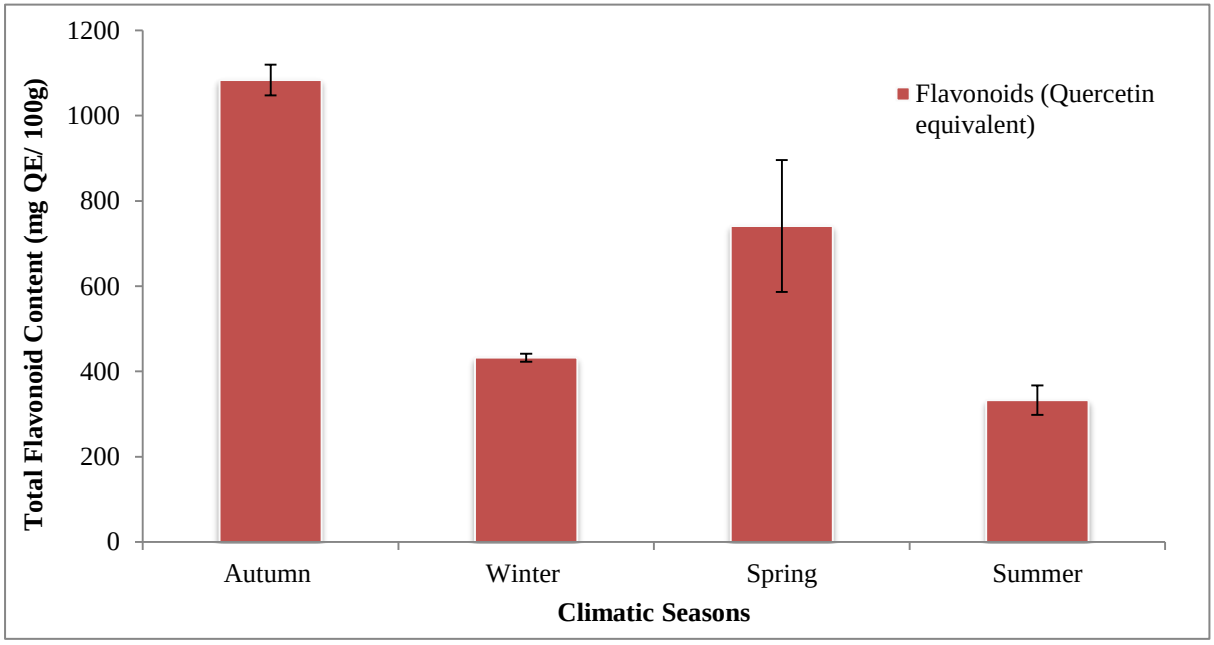


Figure 12: The effect of seasonal on Total Flavonoid content of seasonal variation of *M. oleifera* leaves.

It should be noted that there was no similar trend to the TFC, but the only correlation is in autumn because it is the highest in both. During the TPC assay, it should be noted that other non-phenolic compounds bind to phenolics such as organic acids (Fu et al., 2010; Medina-Remon et al., 2009), sugars (Singleton & Rossi, 1965; Kakáč & Vejdělek, 1977), ascorbic acid (Singleton & Rossi, 1965; Medina-Remon et al., 2009) and amino acids (Kakáč & Vejdělek, 1977; Roura et al., 2006), which results in the interference of the determination of the TFC, and it may lead to the overcompensation of the phenolics (Prior et al., 2005). Flavonoids are known to be natural antioxidants and they are not directly involved in growth and development, but involved in the plant defense mechanism and also absorb UV-rays in plants (Harbone et al., 1986; Hedin et al., 1985; Firmin et al., 1986; Peters et al., 1986; Tomas-Llorent et al., 1990). Many studies have shown that the total flavonoids increase when

plants are exposed to UV-radiation (Les & Sheridan, 1990). As a result, it can be that leaves harvested during the warmer seasons show high levels of total flavonoids, however, in the present study, leaves harvested in summer had the least concentration of total flavonoids as compared to other seasons. Chaves et al. (1993) showed that the concentration of flavonoids was higher in summer and there was a significant difference throughout the seasons. Compared to the present study, a significant difference in the composition of total flavonoids was observed throughout the seasons (Appendix 15). Leaves harvested in autumn resulted in the highest levels of flavonoids compared to other seasons. The concentrations of total flavonoids may vary depending on the age, geographic location and/or season (Chaves et al., 1993). Ghorbani et al. (2012) assessed the total flavonoid content on Flavoparmelia caperata and Physcia dubia, however their comparison did not include the autumn season. Winter showed the highest concentration of total flavonoids in *F. caperata*, followed by spring and then summer. Similarly, our results also show that leaves of *M. oleifera* harvested in spring had high levels of TFC compared with leaves harvested in summer (Figure 12). In *P. dubia*, TFC decreased in spring as compared to both winter and summer (Ghorbani et al., 2012).

These findings indicate that no set response can be used to predict how the biochemical composition may respond to seasonal variations, although environmental stresses are expected to induce the high production of secondary metabolites. Haribal et al. (2001) found that TFC was higher in spring and this was attributed to light exposure and the length of the day. However, the present study shows that TFC in *M. oleifera* leaves was higher in autumn and then followed by spring. From these findings, no correlation is observed between TPC and TFC concerning flavonoids being phenolics, this is a similar observation by Siatka & Kasparova (2010) and this can due to non-phenolic compounds which could influence the TPC in the Folin-Ciocalteu assay. *Moringa oleifera* Lam. may exhibit a physiological response to seasonal variation, because as observed by the differences in the concentration of total phenolics in the leaves. The TFC may be affected by other developmental factors such as extreme temperatures and extreme weather conditions and Shih et al., (2017) found that phenolic content is affected because it decreases as the fruit grows.

3.1.2. Quercetin and kaempferol

3.1.2.1.Individual flavonoids calibration curves

Quercetin and kaempferol calibration curves that were used to quantify the individual flavonoid contents are shown in figure 1.3 and figure 1.4 (shown in appendix 3 and 4,

respectively). Both of these individual flavonoid curves were plotted linearly from 10 mg/L to 50 mg/L and the correlation coefficient was acceptable at 0.99. Before the detection of these individual flavonoids, a blank sample ran 3 times on the UV-HPLC to remove the background noise.

3.1.2.2.Quercetin and kaempferol content of M. oleifera leaves in different seasons

From the standard mixture, using the C18 column, the chromatogram show that quercetin is detected first at a retention time of 6 minutes and kaempferol at 11-12 minutes. These compounds are usually bounded to sugars and can be broken through hydrolysis so that they can be detected (Nuutila et al., 2002). Quercetin appears to be more polar than kaempferol, and it consists of 5 hydroxyl groups than that of kaempferol 4 hydroxyl groups, which could be the reason why Quercetin was detected first (Matshediso et al., 2014).

Table 2: Amount of quercetin and kaempferol.

	Quercetin content (mg/ 100g)	**Kaempferol content (mg/100g)**
Months	**Autumn**	
March	158,03 ± 103,63	23,12 ± 5,59
April	279,69 ± 121,89	10,09 ± 8,50
May	333,71 ± 131,91	24,26 ± 12,15
Mean±SD	**257.142±73.47**	**19.16±7.87**
	Winter	
June	226,89 ± 64,67	7,55 ± 2,51
July	-	-
August	-	-
Mean±SD	**226,89 ± 43.61**	**7,55 ± 2,51**
	Spring	
September	-	-
October	194,64 ± 114,91	10,89 ± 7,03
November	289,10 ± 134,27	20,18 ± 9,49
Mean±SD	**241.87±59.43**	**15.54±7.53**
	Summer	
December	232,31 ± 134,12	10,44 ± 2,10
January	368,05 ± 45,32	10,65 ± 2,54
February	317,98 ± 170,17	13,90 ± 5,56
Mean±SD	**306.12±39.21**	**11.66±1.94**

*- represents the months that the leaves were not harvested.

Autumn

There was an increase in the concentration of quercetin from the start of the season to the end of the season (Table 2), whereas only in the second month of autumn a decrease in the kaempferol concentration was observed (Table 2). Quercetin concentration increased from 158.03 mg/100g to 279.69 mg/100g from March to April and kaempferol concentration decreased from 23.12 mg/100g to 10.09 mg/100g. Quercetin continued to increase 279.69 mg/100g to 333.71 mg/100g from April to May and kaempferol also increased significantly from 10.09 mg/100g to 24.26 mg/100g. May had the highest concentration of both compounds in this season, indicating the significant effects of the environmental changes such decreasing temperature in late autumn reflecting the possibility of significant changes in the environmental conditions such as temperature.

Winter

The concentrations of both compounds dereased significantly from May to June, quercetin dropped from 333.71 mg/100g to 226.14mg/100g and kaempferol from 24.26 mg/100g to 7.55 mg/100g (Table 2). Figures 8B and 8C illustrate the reason why there is no data since the plant shed its leaves in July and in August, the plants were pruned for the new season.

Spring

At the beginning of spring, leaves could not be harvested for analysis because the plant leaves were still immature and since our main objective was to harvest from a full canopy of leaves (Figure 9A). The concentration of quercetin dropped from June to October from 226.89 mg/100g to 194.64 mg/100g and kaempferol increased from 7.55 mg/100g to 10.89 mg/100g. A significant increase in both compounds from October to November was measured from 194.64 mg/100g to 289.10 mg/100g (quercetin) and kaempferol decreased from 10.89 mg/100g to 20.18 mg/100g (Table 2).

Summer

In the first month of summer, the concentrations of both compounds significantly decreased, but quercetin decreased from 289.10 mg/100g to 232.31 mg/100g and kaempferol dropped from 20.18 mg/100g to 10.44 mg/100g from November to December (Table 2). Kaempferol concentration showed a significant variation throughout summer and quercetin showed

fluctuations throughout the summer season. A significant increase in quercetin concentration from 232.31 mg/100g to 368.05 mg/100g was measured from December to January and kaempferol concentration insignificantly increased from 10.44 mg/100g to 10.65 mg/100g. From January to February, quercetin concentration decreased from 368.05 mg/100g to 317.98 mg/100g and kaempferol increased from 10.65 mg/100g to 13.90 mg/100g.

Illustrated in figure 13, minor fluctuations were observed throughout the seasons for both quercetin and kaempferol from the seasonal extracts. Autumn had the highest concentration in both quercetin and kaempferol as compared to all the other seasons. It is important to note that, quercetin concentration was significantly higher than kaempferol in all seasons. From these results, there was no significant effect of seasonal variation on quercetin and kaempferol content of the *M. oleifera* leaf extracts, although they are slight fluctuations, but they are not enough to suggest otherwise (Df= 3, F-value=0.058, Pr(>F)= 0.98, quercetin) (Df= 3, F-value= 2.328, Pr(>F)= 0.151). Quercetin increased insignificantly from autumn to summer. Kaempferol decreased insignificantly from autumn to summer and increased from winter to spring and a slight decrease was observed from spring to summer. The season with the highest level of both quercetin and kaempferol was autumn, followed by spring, summer and the lowest was winter, but for quercetin, summer has the highest concentration and this might be due to the plant being stressed by the increasing summer temperatures. Quercetin content and kaempferol content decreased from 257.14 mg/100g to 226.89 mg/100g (quercetin) and a significant decrease from 19.16 mg/100g to 7.55 mg/100g (kaempferol) from autumn to winter. Quercetin increased from 226.89 mg/100g to 241.87 mg/100g from winter to spring. A significant increase in Kaempferol content from 7.55 mg/100g to 15.54 mg/100g from winter to spring was measured. In summer, an insignificant increase from 241.87 mg/100g to 306.12 mg/100g in the concentration of quercetin was observed from spring to the season (Appendix 17). Kaempferol also illustrated a similar pattern to quercetin, but there was a significant drop in kaempferol concentration from 15.54 mg/100g to 11.66 mg/100g. Depending on the geographic location the influences of seasonal variation may contribute differently to the production of secondary metabolites (Alqahtani et al., 2015). In a study by Alqahtani et al., (2015) they studied Centella asiatica and they found that the highest concentration of kaempferol was observed in summer as compared to winter and quercetin also followed this pattern. Their seasonal pattern started with the highest being summer, autumn, winter, and spring for both compounds. Our study reflects different results because autumn had the highest concentration, followed by spring and winter and the lowest was

summer for both compounds. Chaves et al., (1993) found that in Cistus ladanifer which is a flavonoid-rich plant also showed the highest concentration of kaempferol in summer than in winter. Depending on the amount of light available in the season, the concentration of these compounds may be affected (Winkel-Shirley, 2002; Chalker-Scott, 1999). Exposure to extreme temperatures may influence the concentration of these compounds and as such this might constitute the difference in the trends of how these compounds react to seasonal variation. Observed is that quercetin and kaempferol seem to follow a similar pattern based on the monthly contents of both compounds (Appendix 17 and 19, respectively).

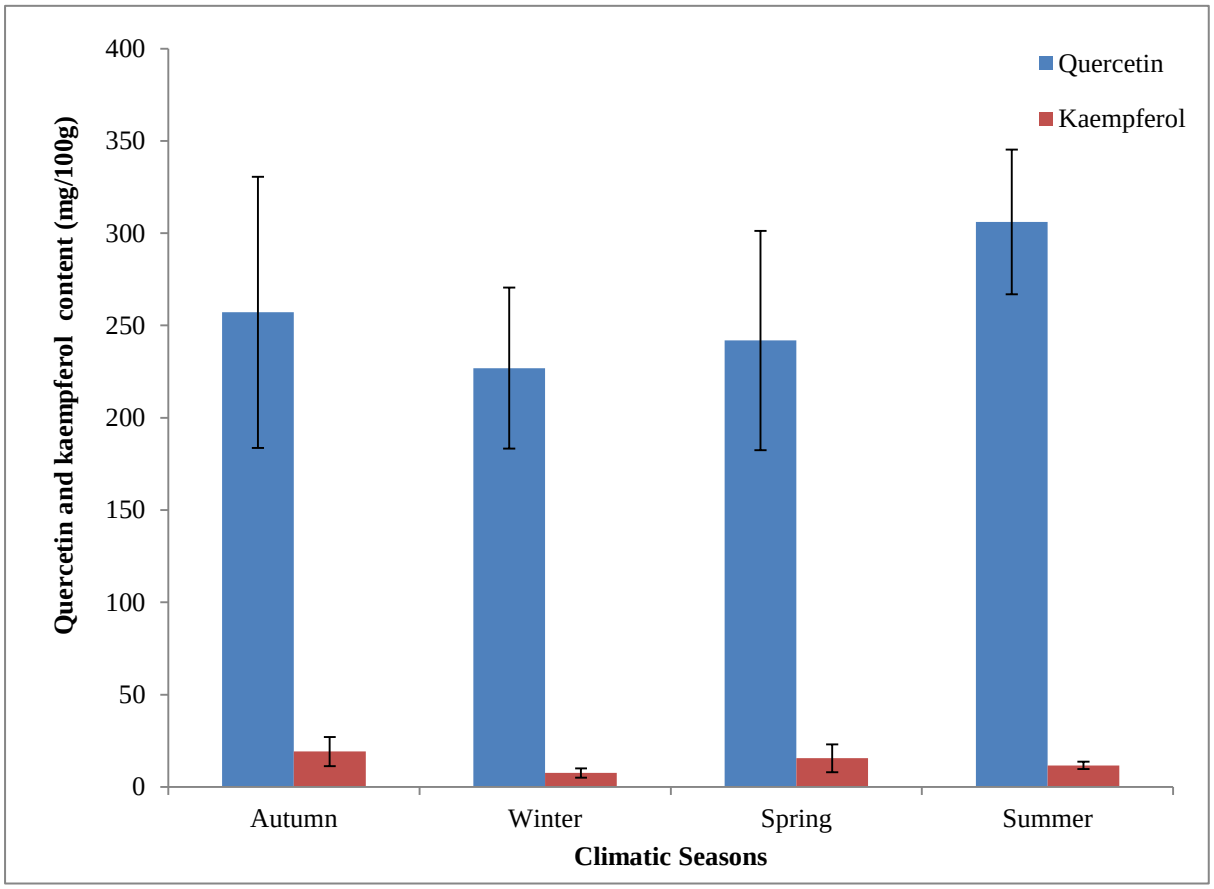


Figure 13: The effect of seasonal on Total Flavonoid content of seasonal variation of *M. oleifera* leaves.

Brossa et al., (2009) studied *Quercus ilex* from two locations and they study two seasons (winter and summer). They found that both quercetin-hexosides and kaempferol-hexosides showed a similar pattern, as the concentration of both compounds were high in winter as compared to summer. In this study, they also established that though these compounds were low, the activity of other flavonoids was found to be higher and it is important to note that the concentration of quercetin was higher than that of kaempferol, which is the same as this the

current study (Brossa et al., 2009). The pattern of this current was the opposite as the concentration of quercetin and kaempferol were insignificantly higher in summer than in winter. Cao et al., (2019) studied the seasonal variation of the *Cyclocarya paliurus* leaves and they observed that quercetin content was high in November, July, August, and May and it was the lowest in June, September and October and kaempferol followed a similar pattern. Assuming the November represents the cooler season and July and August represent the warmer season, we are left with conclusions that the highest concentration of quercetin and kaempferol were observed in winter and followed by winter and the other transitioning seasons. Ifie et al., (2018) studied the effect of seasonal variation on *Hibiscus sabdariffa* (Roselle) and since the seasons in West Africa are split into wet and dry seasons, but typical African seasons are usually autumn, winter, summer, and spring. They found that quercerin3-*O*-sambubioside is higher in the wet season than in the dry season and the wet season commences from March until July, which usually contains autumn and winter seasons and the dry season starts from October till March. These results are quite similar to what we observed because autumn had the highest content of quercetin and summer was low and relatively their dry seasons have high temperatures. The current study winter and summer can serve as dry season as they had a slightly lower concentration of quercetin and kaempferol. Ma et al., (2003) compared the quercetin content of *Apocynum venetum* and *Poacynum hendersonii* and they found that the summer season showed the highest concentration of quercetin and it was followed by spring and then autumn. These results only agree with our study based on the spring season because it was the second-highest whereas summer was the third-highest. It would have interesting to have seen how the season would have compared. The best seasons for harvesting the *M. oleifera* leaves would be late spring, summer, and autumn, but also winter is included because though it was the lowest but the differences were insignificant. Fiordalisi et al., (2019) found that the quercetin content from the propolis extract was higher in summer, followed by autumn, and winter and the lowest concentration was in winter. From all these studies we establish that there is no constant response of quercetin because different seasons introduce different environmental conditions, where some conditions may induce the production of these individual flavonoids and some may not.

3.1.3. Antioxidant activity of M. oleifera leaves in different seasons

The scavenging activity of the *Moringa oleifera* Lam. leaf extracts were collected in four calendar-based seasons and were determined by using the DPPH assay and the monthly findings are shown in Table 3**.** The ability of *M. oleifera* leaves to act as a hydrogen donor in

order to turn it into its reduced form DPPH-H was studied. *M. oleifera* leaf extracts reduced the purple-coloured DPPH to colourless yellow DPPH-H. In autumn, we measured the EC_{50} and there a drop in antioxidant activity from March to April, and then the radical scavenging activity increased in May, highlighting that the plants were stressed (Table 3). This may be the result of transitioning from autumn to the winter season. In winter, only one month was collected, because the leaves had undergone some morphological changes in July and in August the trees were coppiced and pruned to prepare for the next season. June showed a greater radical-scavenging activity than any month in any seasons and this indicates that environmental stresses such as cold temperatures can lead to increasing scavenging activity between May and June. Figure 9A shows that the plants had not sprouted back fully, because leaves were to be harvested from full canopy tree. The EC_{50} reflect the scavenging power of the leaf extract, a lower value indicates a high antioxidant activity, as the extract scavenges the free radicals at a lower concentration and a high EC_{50} value indicates a low antioxidant activity. From winter to spring there was a drop in the radical-scavenging activity of the *M. oleifera* leaf extract. The months of October and November showed a continued drop in the antioxidant activity. In summer, December showed the higher radical-scavenging activity as compared to January and February, and from January the scavenging activity just decreased.

Table 3: Monthly variation on antioxidant activity (EC_{50}) (radical-scavenging activity) of *M. oleifera* leaves (mg/ml).

Months	EC_{50} (mg/ml)
Autumn	
March	2.37 ± 0.14
April	2.97 ± 0.06
May	2.14 ± 0.18
Mean±SD	**2.49±0.43**
Winter	
June	1.61 ± 0.18
July	-
August	-
Mean±SD	**1.61±0.18**
Spring	
September	-
October	3.68 ± 0.66
November	4.38 ± 0.48
Mean±SD	**4.03±0.42**
Summer	
December	1.75 ± 0.13
January	1.92 ± 0.34
February	2.28 ± 0.22
Mean±SD	**1.98±0.27**

*- represents the months that the leaves were not harvested.

The winter and summer *M. oleifera* leaf extract showed a higher scavenging activity of free radicals than the extracts collected in autumn and spring, the average EC_{50} values for each season were 1.61 mg/mL (winter), 1.98 mg/mL (summer), 2.49 mg/mL (autumn), and 4.03 mg/mL (spring) (figure 14). There was a significant difference from a one-way ANOVA in the radical-scavenging activity of the *M. oleifera* leaf extracts from different seasons (Df= 3, F-value= 29.16, Pr(>F)= 0.000117). Hussain et al., (2008) reported that antioxidant activity of *Ocimum basilicum* essential oils showed a greater reduced power in winter, followed by winter, then autumn and summer. These results agree with the present study on the *M. oleifera* leaf extract only on the winter season and the rest of the seasons are different because the trend of the present is winter>summer>autumn>spring. The DPPH assays showed that *M. oleifera* leaf extracts showed a greater ability and capacity to donate the hydrogen atoms in winter and summer, which clearly outlines that these plants might have been under

environmental stresses that are induced by the winter and summer seasons. The stress factors may be longer spells of extreme hot and cold temperatures. Siddhuraju & Becker (2003) the leaf extracts of *M. oleifera* from different agro-climatic origins showed that locations do affect the antioxidants in the leaves of *M. oleifera*.

Sati et al., (2013) reported that DPPH assay showed that the scavenging activity of *Ginko biloba* leaf extracts was at the maximum in autumn than in the rainy and spring season. Shih et al., (2011) reported that winter showed a greater scavenging activity potential as compared to summer, in the leaf extracts of *M. oleifera*. These findings agree with our present findings where the radical-scavenging activity is greater in winter than in summer, although there is a statistically insignificant (Tukey multiple comparisons of means 95% family-wise (winter-summer, P= 0.572). Siddhuraju & Becker (2003) leaf extracts of *M. oleifera* from different agro-climatic origins showed that locations do affect the antioxidants in the leaves of *M. oleifera*. Dalmagra et al., (2018) reported that autumn showed the highest scavenging activity in *Morus nigra* leaf extracts. According to the present study, autumn was the third-highest to exhibit the scavenging activity of free radicals. Ghorbanli et al., (2012) reported the activity of antioxidant enzymes such as catalase, peroxidase, and ascorbate peroxidase were high in winter as compared to summer and spring of the *F. caperata* and *P. dubia* and only polyphenol oxidase were found to have shown higher activity in summer as compared to winter and spring. Sývacý & Sökmen (2004) reported that the stems of *Morus alba* and *M. nigra* showed greater free radical scavenging activity in autumn (October) as compared to other seasons and to support they also found the highest content of total phenolics in autumn (October). Winter showed the lowest scavenging activity in this study, which does not agree with our present study. The possible reason for the higher antioxidant activity may be the limitations to light in winter and also the degradation of the chlorophyll (Sývacý & Sökmen, 2004).

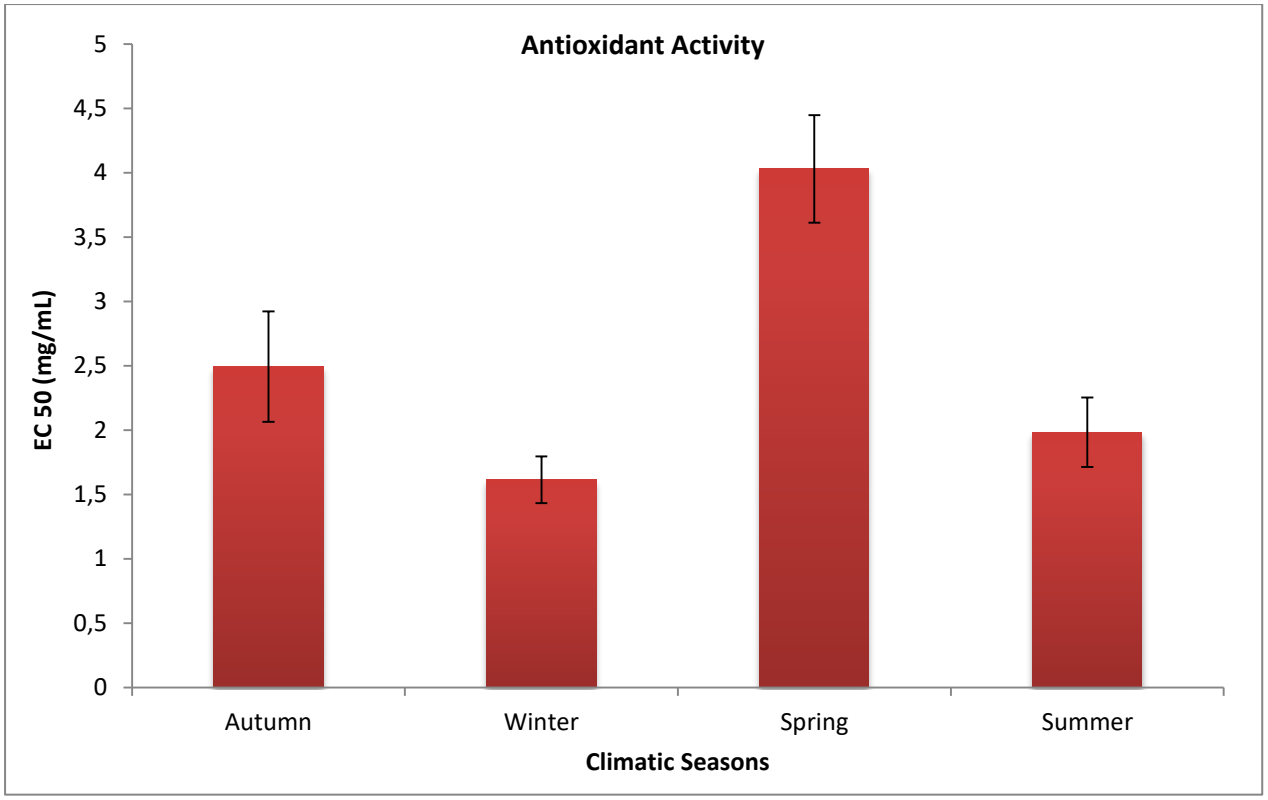


Figure 14: The effect of seasonal variation on Antioxidant Activity of *M. oleifera* leaves.

Ko et al., (2018) reported the antioxidant activity of *Sasa quelpaertensis* to be high in winter as compared to other seasons, which agrees with our present study. Souid et al., (2018) reported that summer season (dry and hot conditions) showed the highest antioxidant activity from the DPPH assay of *Limonium delicatulum*. Iqbal & Bhanger (2006) reported that the free radical scavenging activity of *M. oleifera* was high in winter, except for one location where it was found to be high in spring. These observationsby Iqbal and Bhanger (2006) are similar to the present study, which is winter>summer>autumn>spring. This indicates that antioxidant activity of *M. oleifera* leaves are affected by seasonal variations and from these findings, we observe that seasons and environmental temperature fluctuations show a significant effect on antioxidant activity.

3.1.4. Comparison of obtained values of phytochemicals with literature

Table 3 below shows the cross-comparison of the current study with other studies regarding the TPC, TFC, and antioxidant activity in *M. oleifera*. Our current study shows that the TPC and TFC were slightly lower than those obtained by Iqbal & Bhanger (2006), however, they were higher than those from Shih et al. (2011). These studies mentioned in table 3 below,

they used methods that are similar to the present study, though some were modified versions of the methods. From this, we can establish that *M. oleifera* grown in different agro-climatic locations will respond differently to seasonal variations. In South Africa, we observed that *M. oleifera* becomes dormant in cold and dry conditions. The antioxidant activity is higher in other locations as compared to our study. This data is essential because it shows how seasonal variations in each location affect the biochemical processes in plants and in particular, the composition of secondary metabolites.

Table 3: Cross comparison with other study seasonal variation on *M. oleifera* leaves.

	Total Phenolic Content	**Total Flavonoid Content**	**DPPH (EC_{50})**
Autumn	8.82±0.34- 12.79±0.29 g/100g (Iqbal &Bhanger, 2006)	6.74±0.27-12.49±0.17 g/100g (Iqbal & Bhanger, 2006)	2.49±0.43 mg/ml (Present study)
	4123.53±1516.97 mg/100g (Present study)	1097.26±32.28 mg/100g (Present study)	
Winter	9.17± 0.45-13.09± 0.38 g/100g (Iqbal & Bhanger, 2006)	7.12± 0.51-12.79± 0.3 g/100g (Iqbal & Bhanger, 2006)	0.2 mg/ml (Shih et al., 2011)
	181.3 mg/100g (Shih et al., 2011)	439,10 ± 12,29 mg/100g (Present study)	1.61±0.18 mg/ml (Present study)
	3467,85 ± 175,11 mg/100g (Present study)		
Spring	8.98±0.30-13.56±0.11 g/100g (Iqbal & Bhanger, 2006)	7.02± 0.24-12.69± 0.21 g/100g (Iqbal & Bhanger, 2006)	5.48- 8.66 mg/ml (Arena & Radice, 2016)
	7.40-10.52 mg/g (Arena & Radice, 2016)		4.03±0.42 mg/ml (Present study)
	1253.59±323.18 mg/100g (Present study)	734.14±148.81 mg/100g (Present study)	
Summer	8.43± 0.41-11.82± 0.31 g/100g (Iqbal & Bhanger, 2006)	6.59± 0.21-12.15± 0.43 g/100g (Iqbal & Bhanger, 2006)	7.49-7.63 mg/ml (Arena & Radice, 2016)
	200 mg/100g (Shih et al., 2011)	322.27±36.46 mg/100g (Present study)	0.387 mg/ml (Shih et al., 2011)
	14.1-14.15 mg/g (Arena & Radice, 2016)		1.98±0.27 mg/ml (Present study)
	3889.61±477.84 mg/100g (Present study)		

CHAPTER FOUR: GENERAL CONCLUSION AND RECOMMENDATIONS

4.1.Concluding remarks

Commercially, *M. oleifera* is considered of economic importance, especially by disadvantaged communities. It is naturalized and cultivated throughout the world because of its medicinal, nutritional and industrial uses. Since it is a drought-resistant species, it is largely favoured because it can adapt easily to various environmental conditions. The responses of plants to seasonal variations are important because they reflects the state at which plants may be and may indicate the season in which it is most suitable to harvest leaves with the highest proportion of secondary metabolites such as TPC, TFC, and Quercetin and kaempferol. The seasonal variation may also reflect how these plants are adapting to the environmental changes that are influenced by climate change. Towards the end of the autumn season, the leaves started to show signs of deterioration as they were changing colour from green to yellow (degradation of chlorophyll and leaf senescence). Leaf senescence continued during winter season, and leaf abscission occurred and this could be due to extreme cold temperatures which are not favourable for the growth and development of *M. oleifera*. As we progressed towards the end of July, the leaves had wilted, dried and died because they were brown, which shows continuous exposure to cold temperature stress, limited light availability, and lack of moisture.

This study demonstrated that *M. oleifera* responds differently to seasonal variations. The total phenolic content and total flavonoid content of *M. oleifera* leaves that were in autumn were higher than all the other seasons. Expected was that concentration of total phenolics to be higher in winter than in summer, but in this study the reverse is true. Literature has shown that *M. oleifera* plants favour the warmer season for growth, and the mature leaves are found in winter, which is why it is expected for the leaves to have a higher concentration of total phenolics in winter than in summer. Interestingly, the concentration of total flavonoids was higher in winter as compared to summer. Literature has shown that there are fluctuations throughout the seasons and that we cannot have set responses or expected to the concentration of total phenolics and flavonoids, because these are affected by geographic locations, different growing conditions, seasonal environmental conditions, fluctuating temperatures, and precipitation patterns (Iqbal & Bhanger, 2006).

Results from the individual flavonoids demonstrates that the fluctuations of the concentrations of quercetin and kaempferol are correlated throughout the season, though

there was a difference in summer where quercetin content was higher than in spring and kampferol content was higher in spring than in summer, however quercetin was higher in concentration as compared to kaempferol. In Autumn, the concentrations were higher than all other seasons. The response pattern was similar to the TFC, however the difference in concentration was between winter and summer. A possible reason for the high concentration of flavonoids can be attributed to some of these compounds contributing to the developmental and growth stages of the leaves because the plants had young *M. oleifera* leaves in spring.

The DPPH assay, the highest antioxidant activity was measured in winter as compared to summer, this was followed by autumn and then spring. Increasing the production of secondary metabolites induces the plant to adapt to the changing environmental conditions, which indirectly affected by climate change.

In conclusion, the results from this study serve as evidence of the responses of *M. oleifera* leaves to seasonal variations. It established that *M. oleifera* leaves show great sensitivity to changing seasonal environmental conditions such as temperature. The evidence suggests that seasonal variation contributes to the quality or quantity of secondary metabolites, due to the fluctuations that are observed throughout the seasons. Spring is the season that the leaves appeared to be less stressed and this might be an indication that plant leaves were still young as such, they had not accumulated. The concentration of total phenolics was higher in autumn, and there was a significant drop in winter and it continued to spring, but from here there was a significant increase. These fluctuations indicate the plant leaves responses to counteracting the seasonal environmental stress such as temperature, light, precipitation, and humidity. The antioxidant activity reflects that plants were stressed more in winter and then summer because the EC_{50} values were low in these seasons. Such evidence serves as a contribution towards the farming industry both subsistence and commercial of *Moringa oleifera* Lam.. This information will contribute towards understanding how seasonal variation affects the production of selected metabolites, and in a nutshell, they are reflective of what happens to the plant metabolites in different seasons. This evidence also reflects on when to harvest the leaves of *M. oleifera* to acquire the highest antioxidant activity and in this study, it is winter and summer.

4.2.Recommendations

We recommend that more antioxidant activity methods should be employed to assess the antioxidant activity of the *M. oleifera* leaves. Future studies on the responses of selected metabolites of *M. oleifera* leaves to seasonal variations should be investigated to add to the existing data that we have to make more climatic projections on how these plants would continue to respond to seasonal changes. Future studies on how seasonal variations may affect the nutritional content such as vitamins and minerals of *M. oleifera* leaves are recommended.

APPENDICES

Appendix 1

1.1 Gallic acid standard

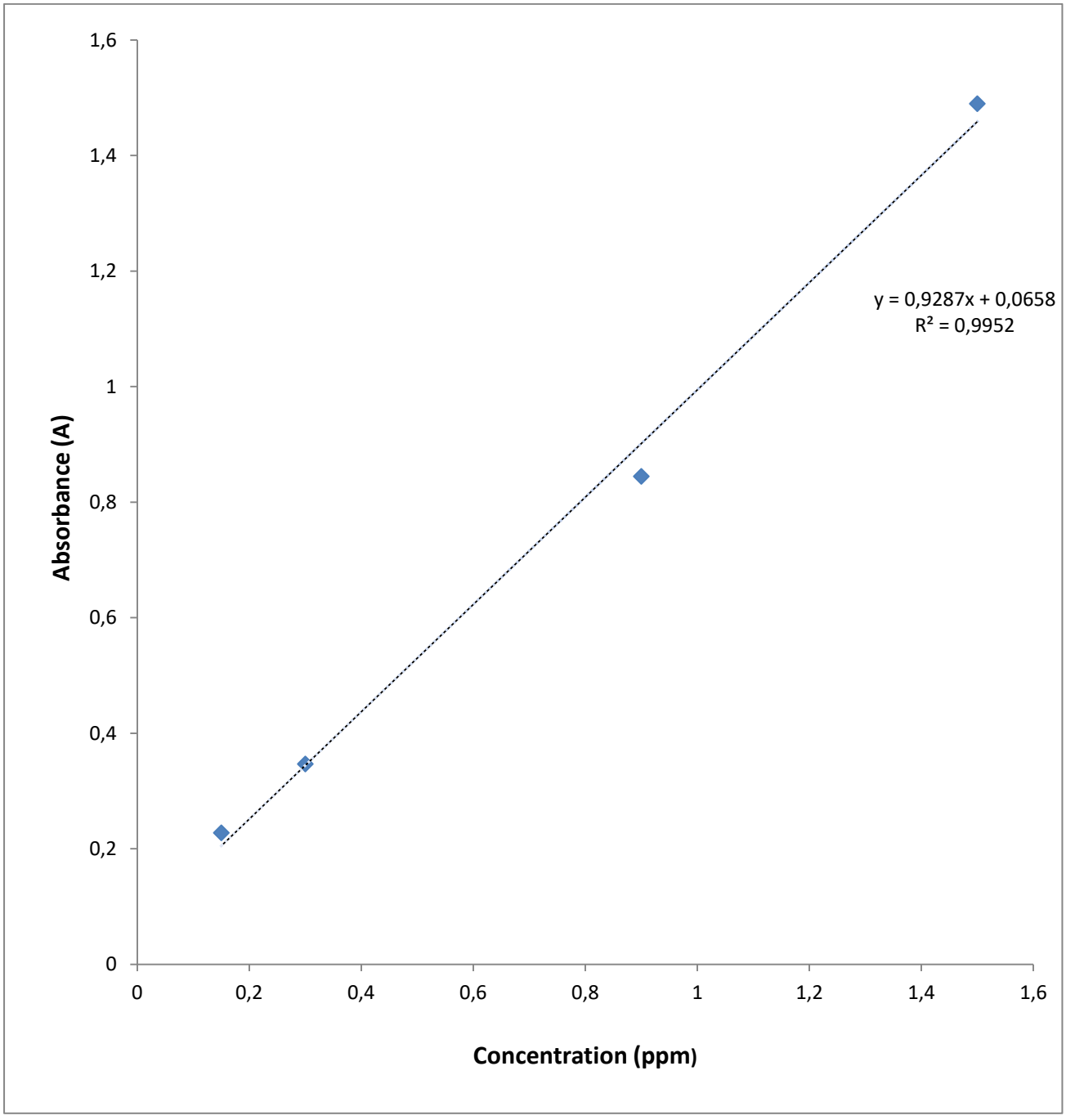


Figure 1.1: Gallic acid standard calibration curve

Appendix 2

1.2 Quercetin standard

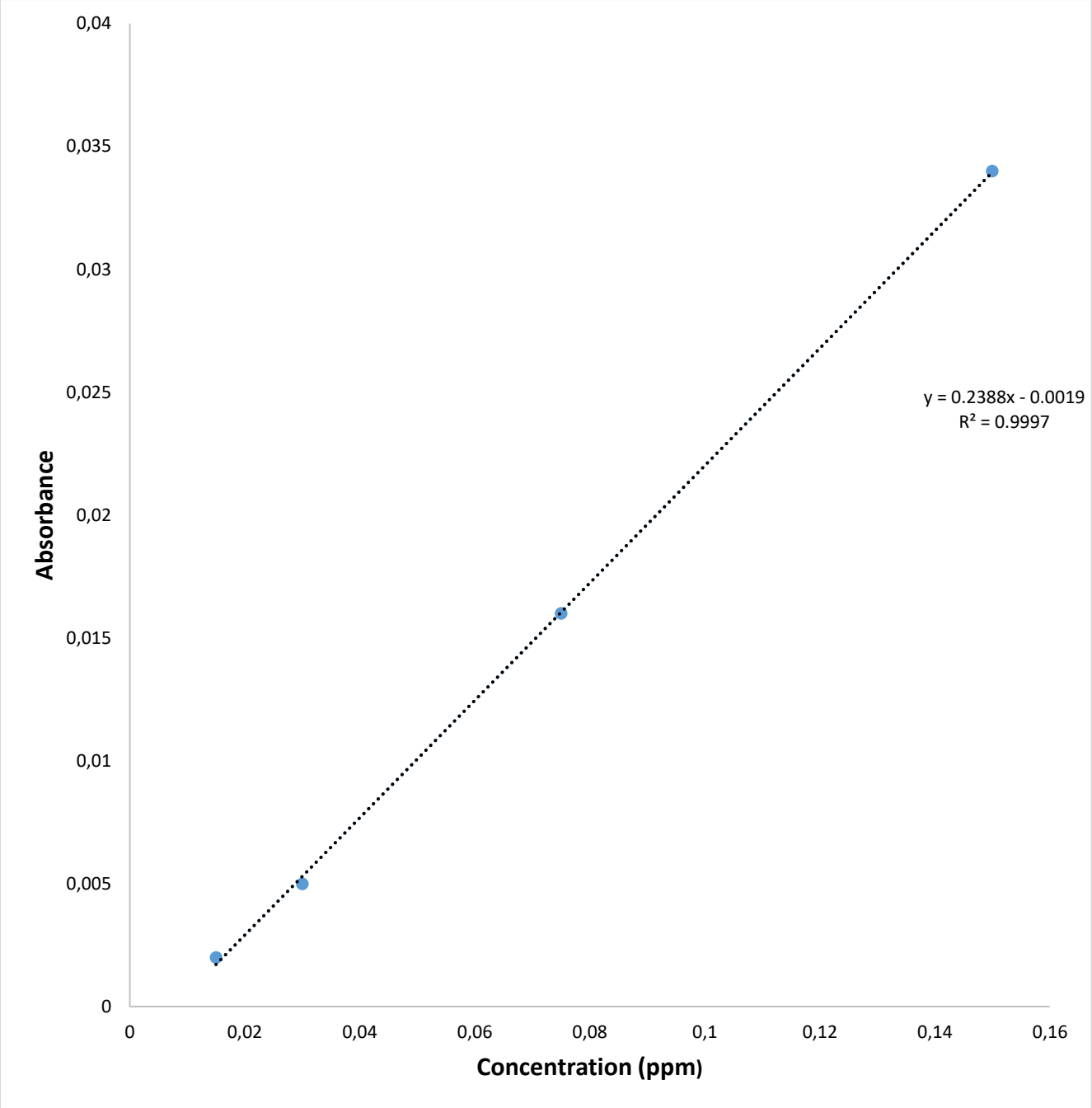


Figure 1.2: Quercetin standard calibration curve

Appendix 3

1.3 Quercetin standard

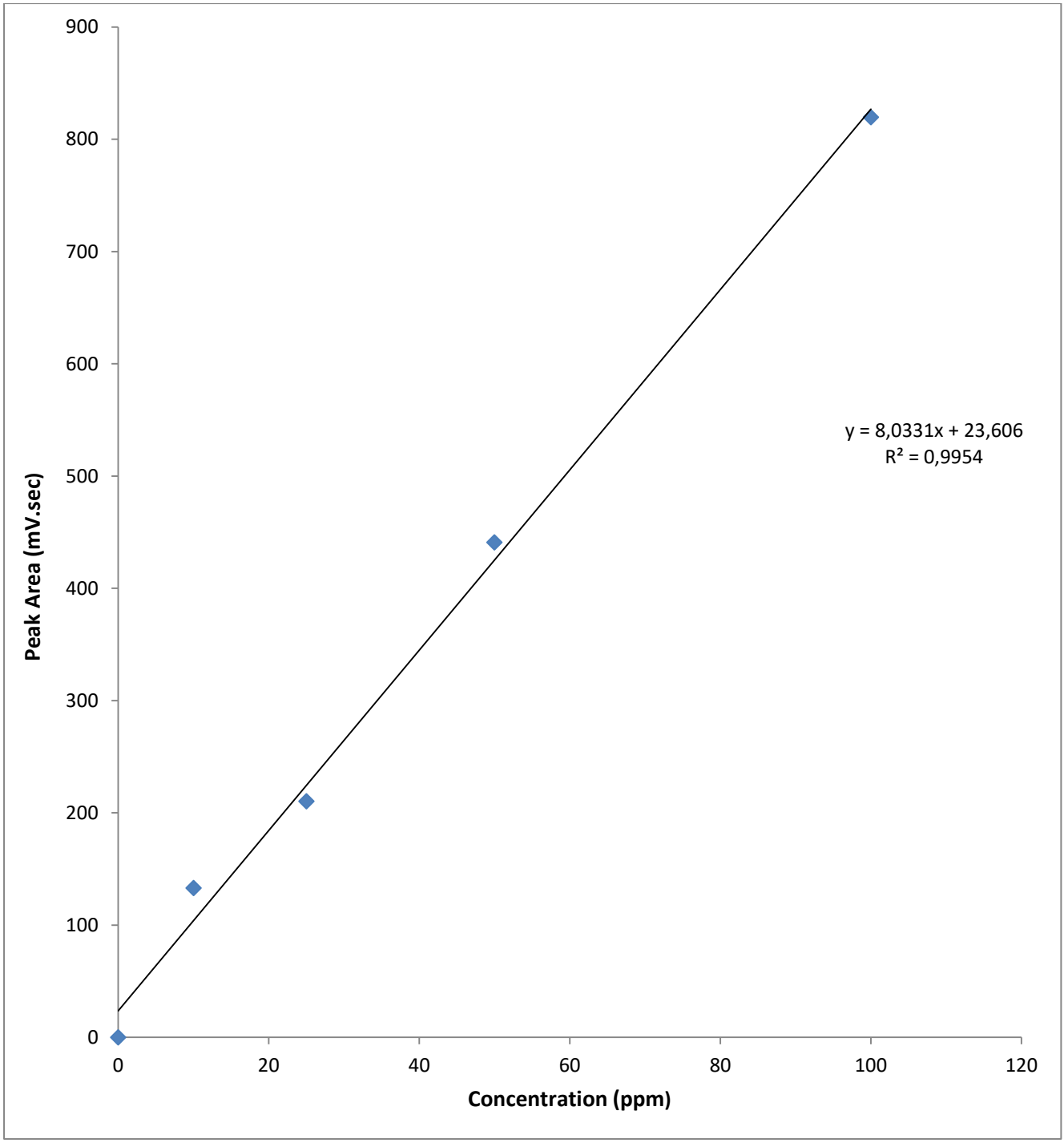


Figure 1.3: Quercetin standard calibration curve

Appendix 4

1.4. Kaempferol standard

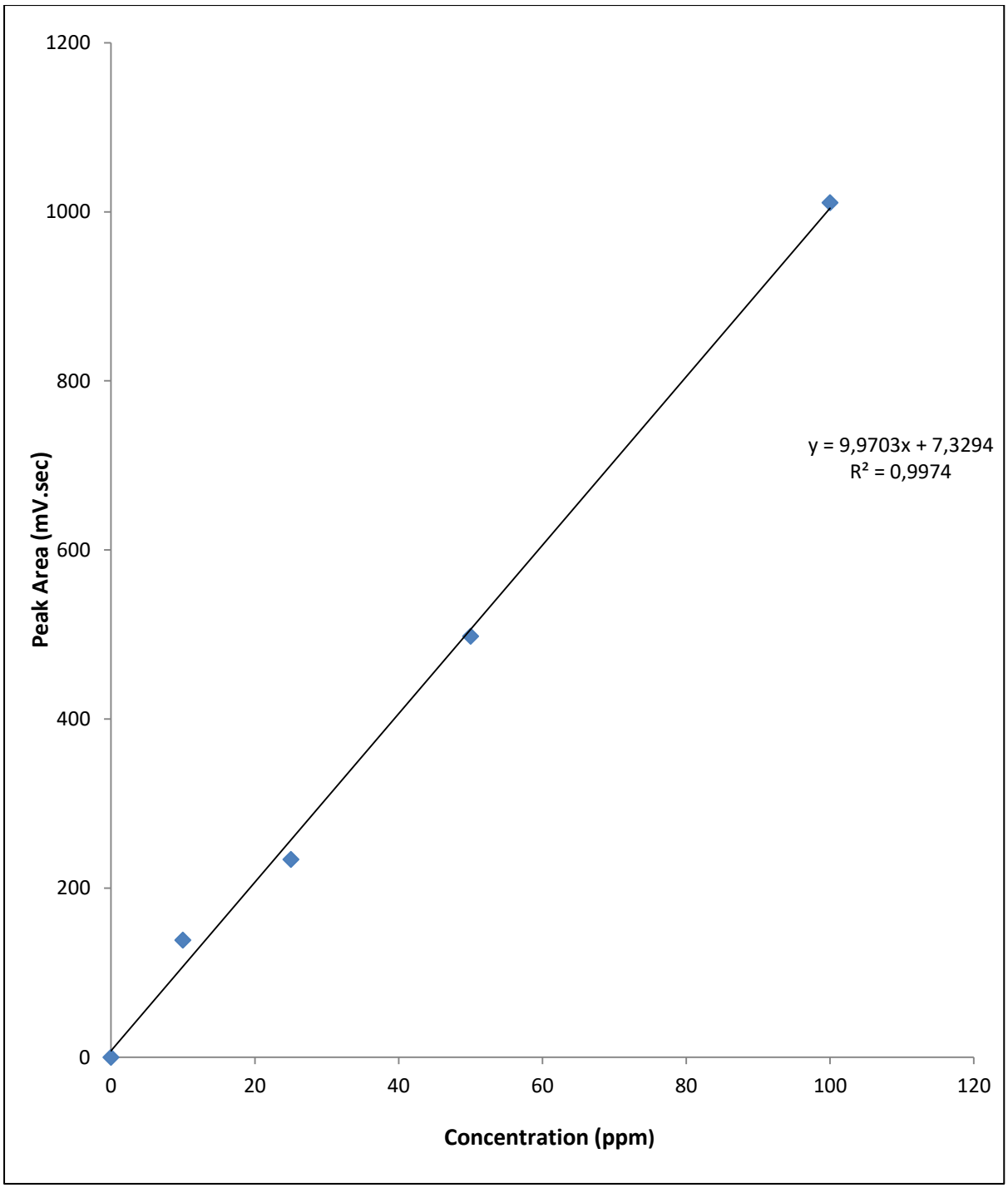


Figure 1.4: Kaempferol standard calibration curve

Appendix 5

1.5. Quercetin and kaempferol raw data from the MEOH extracts

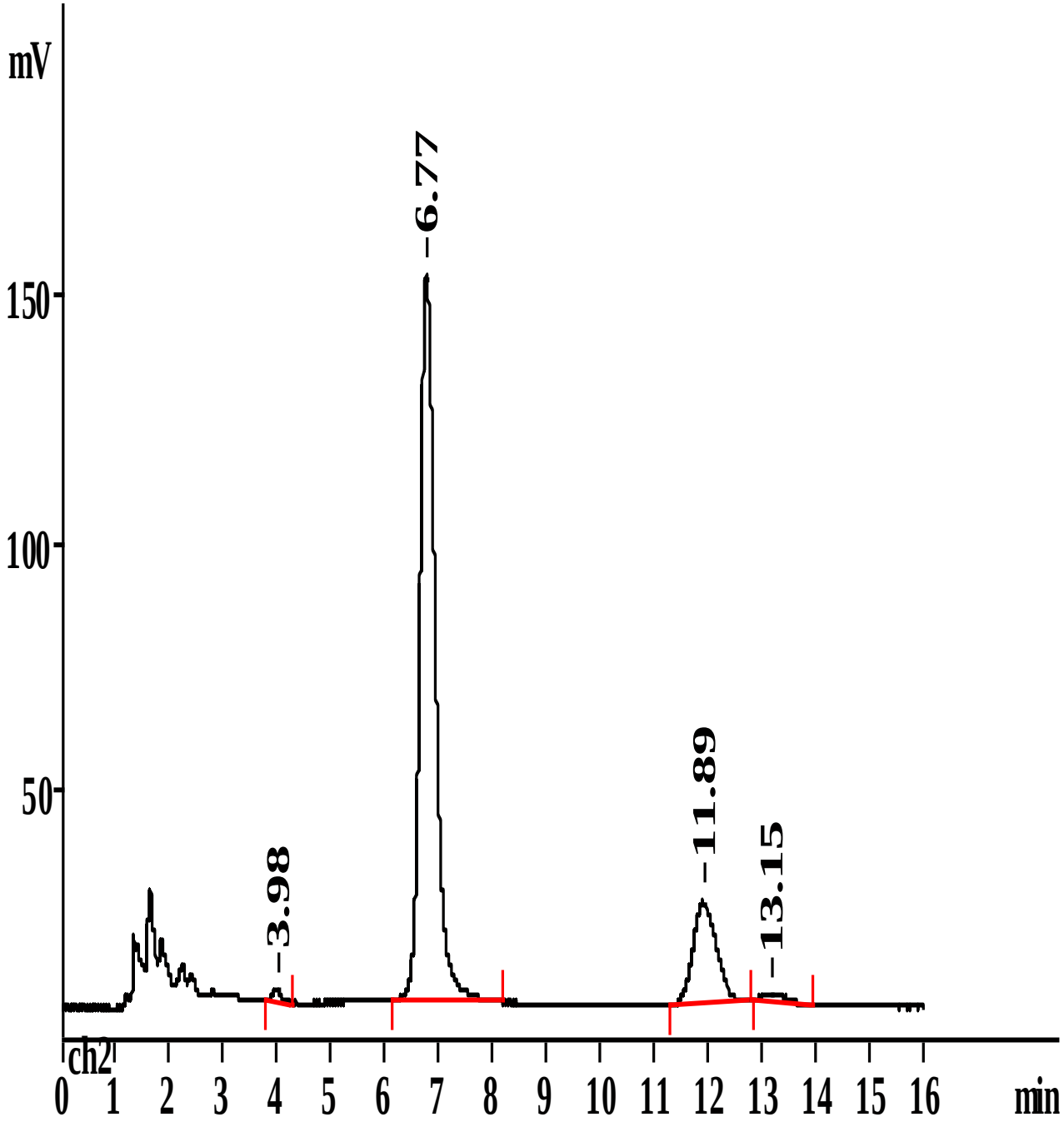


Figure 1.5: Concentrations of 6.77-quercetin and 11.89-kampferol in March

Appendix 6

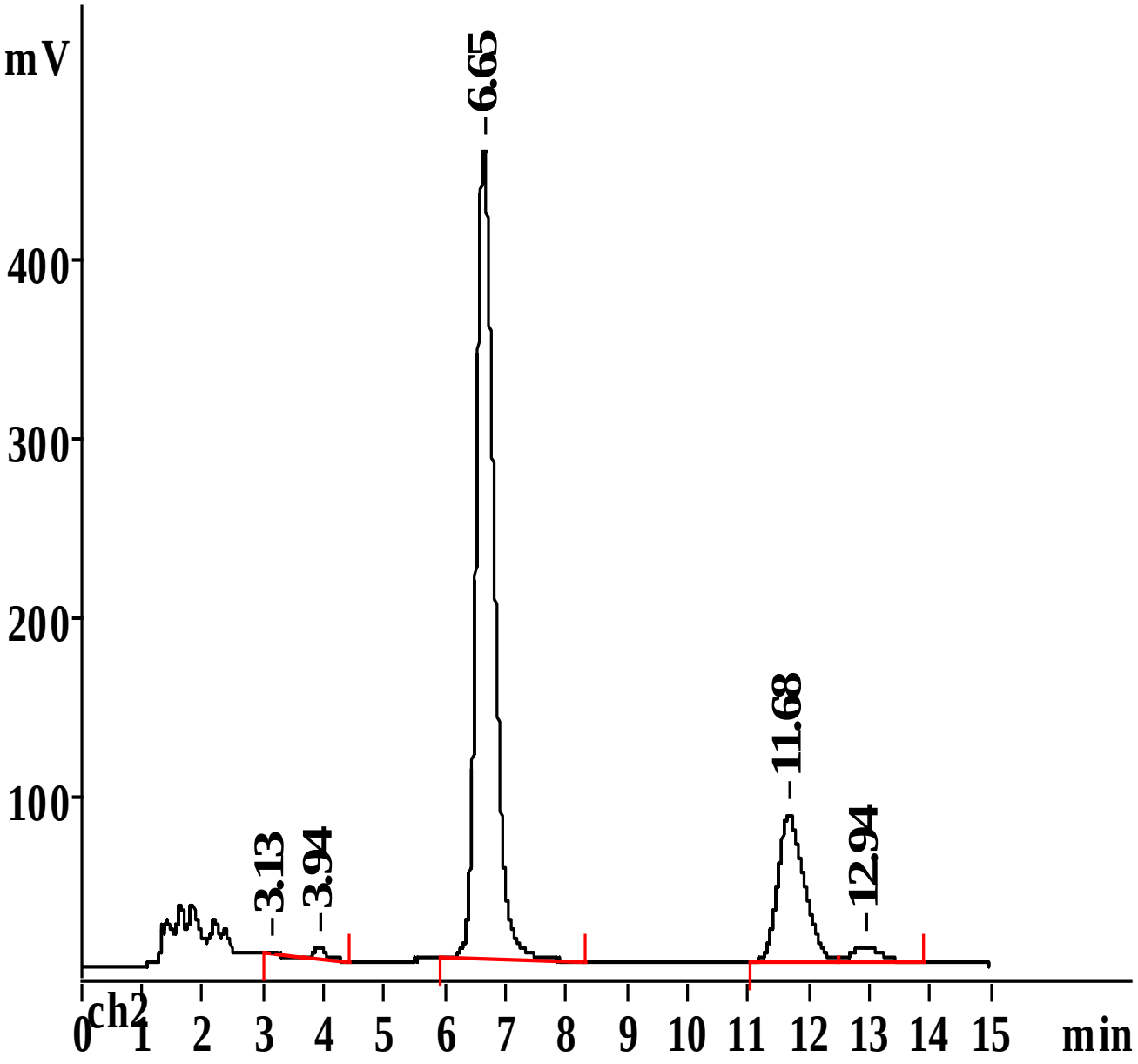


Figure 1.6: Concentrations of 6.65-quercetin and 11.68-Kaempferol in April

Appendix 7

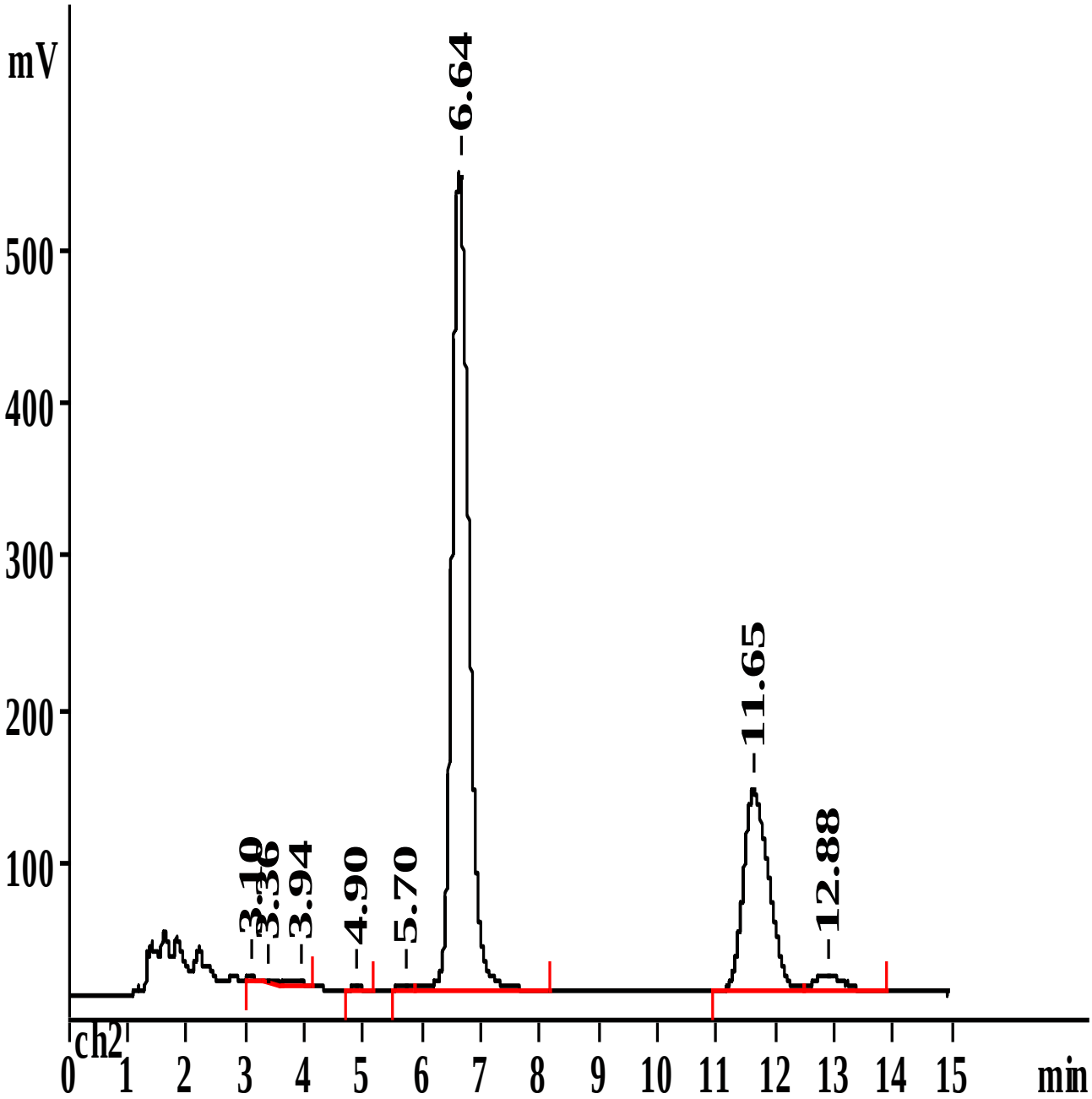


Figure 1.7: Concentrations of 6.64-quercetin and 11.65-kaempferol in May

Appendix 8

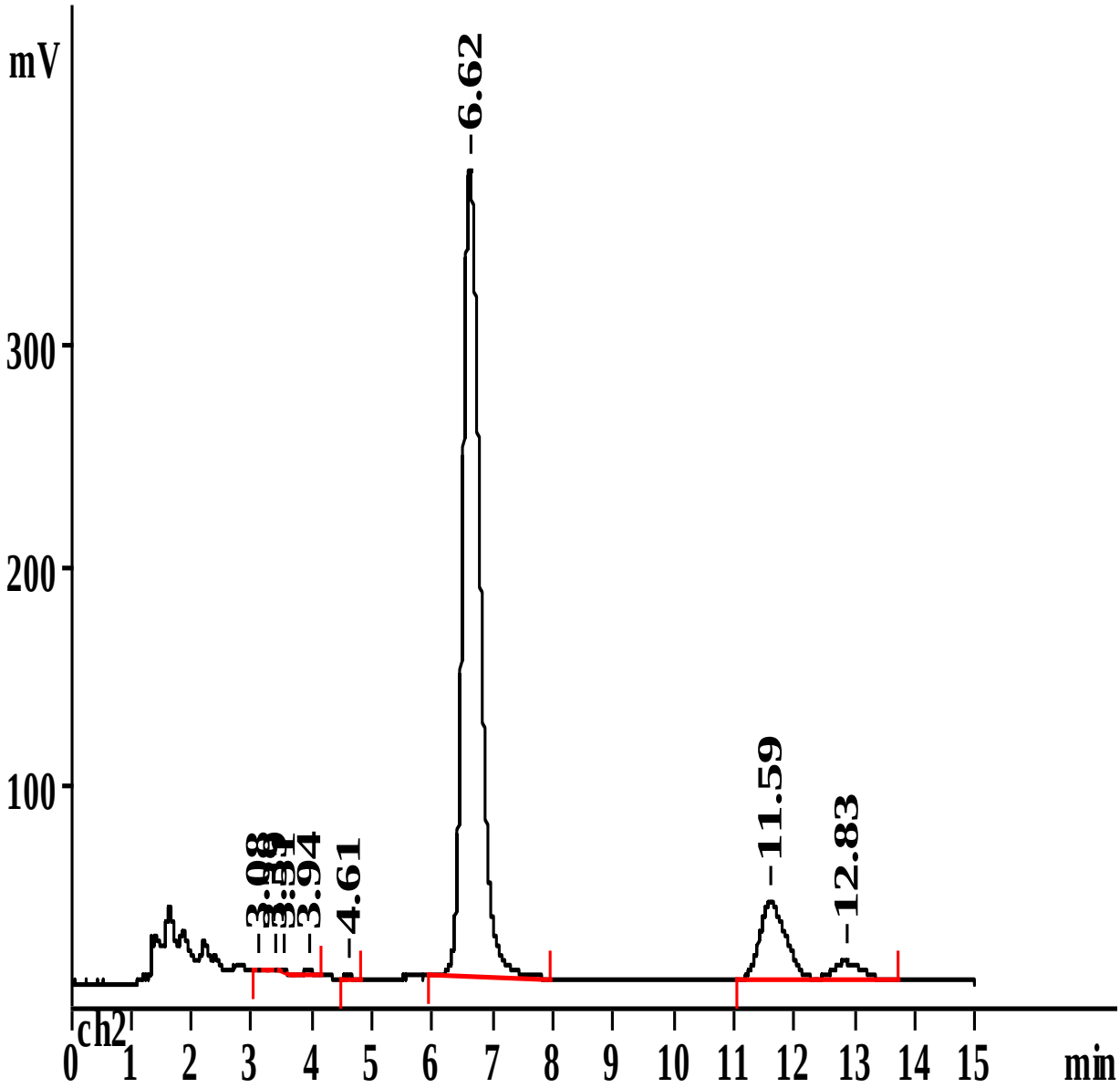


Figure 1.8: Concentrations of 6.62-quercetin and 11.59-kaempferol in June

Appendix 9

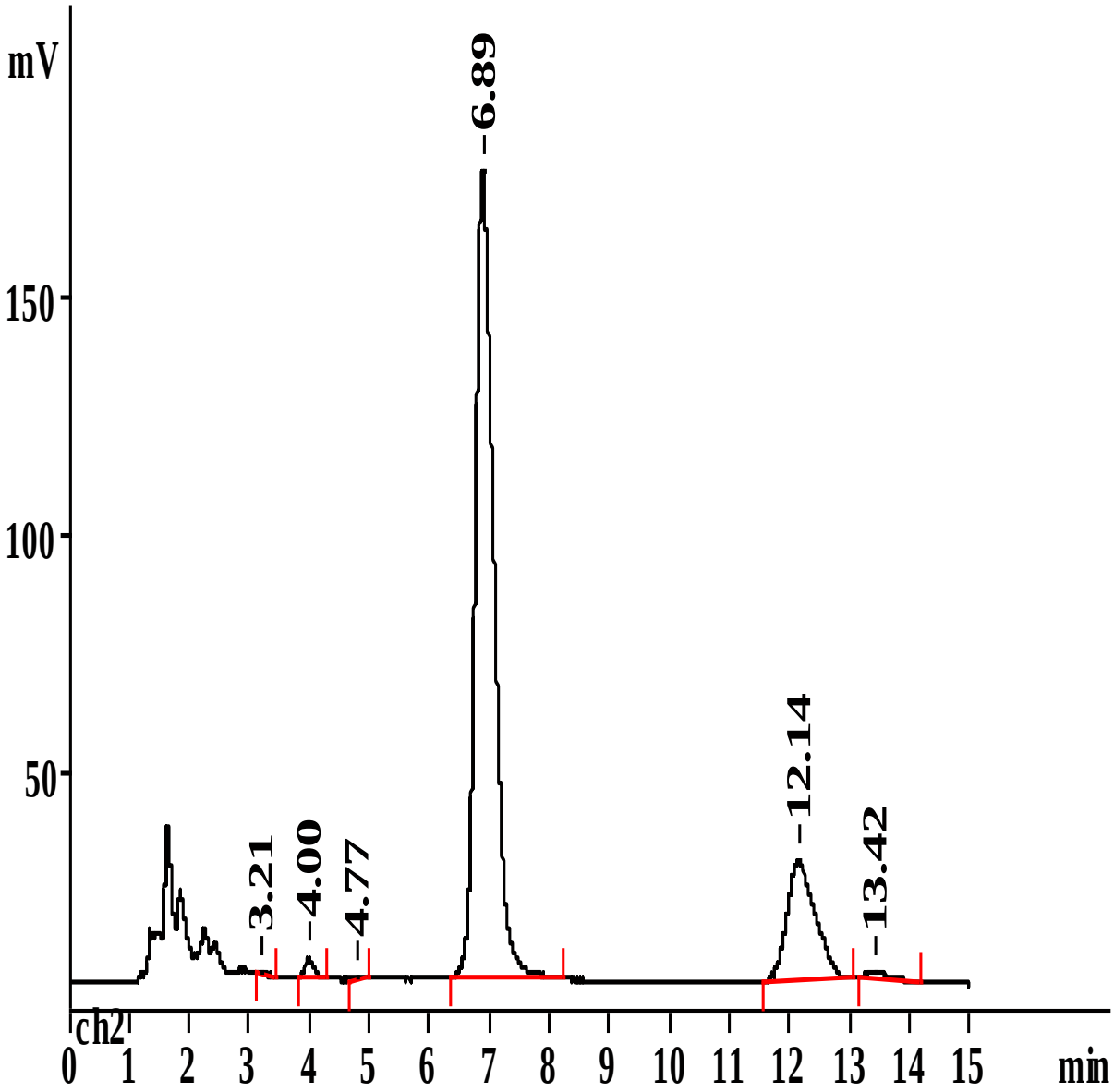


Figure 1.9: Concentrations of 6.89-quercetin and 12.14-kaempferol in October

Appendix 10

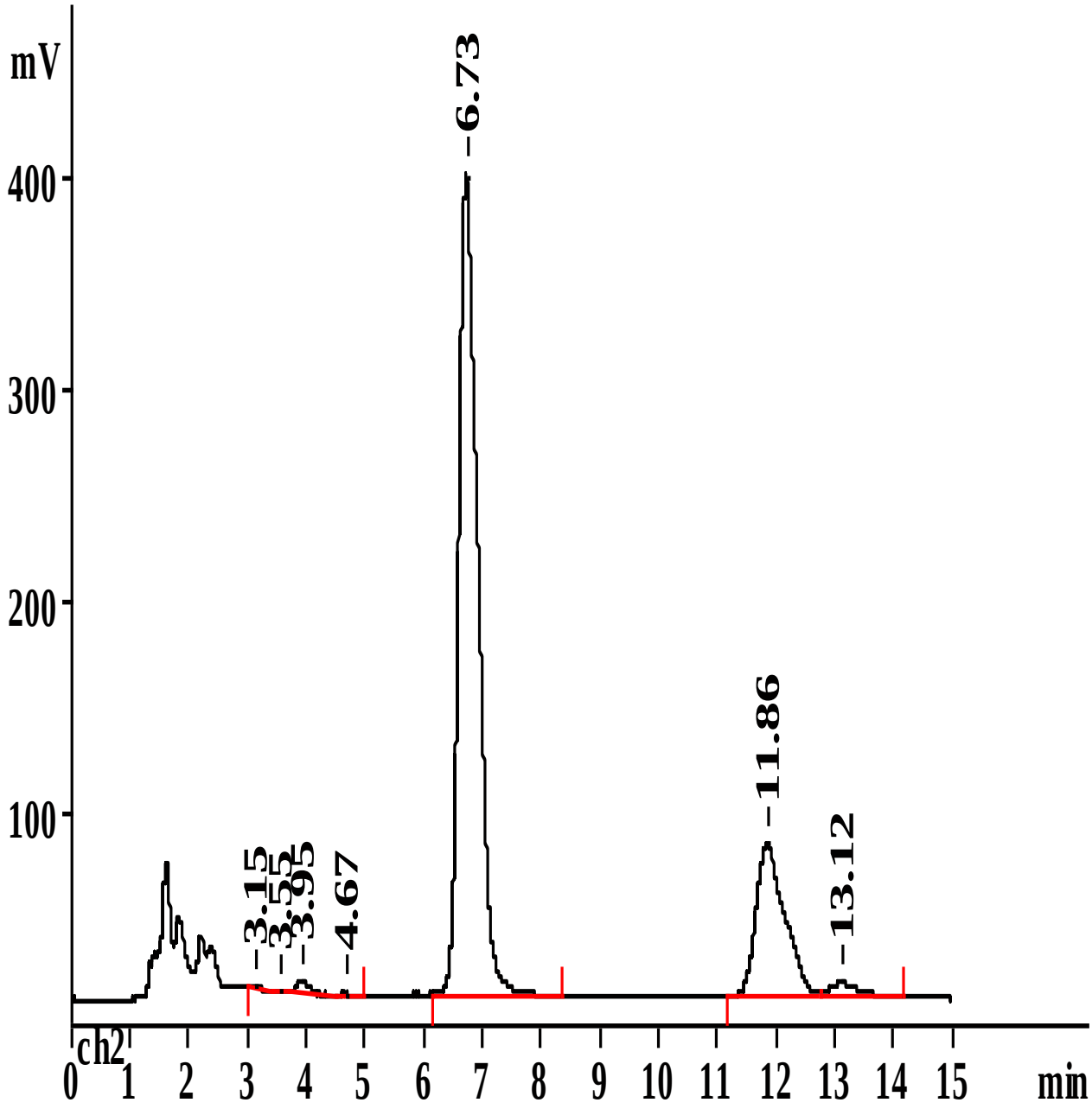


Figure 1.10: Concentrations of 6.73-quercetin and 11.86-kaempferol in November

Appendix 11

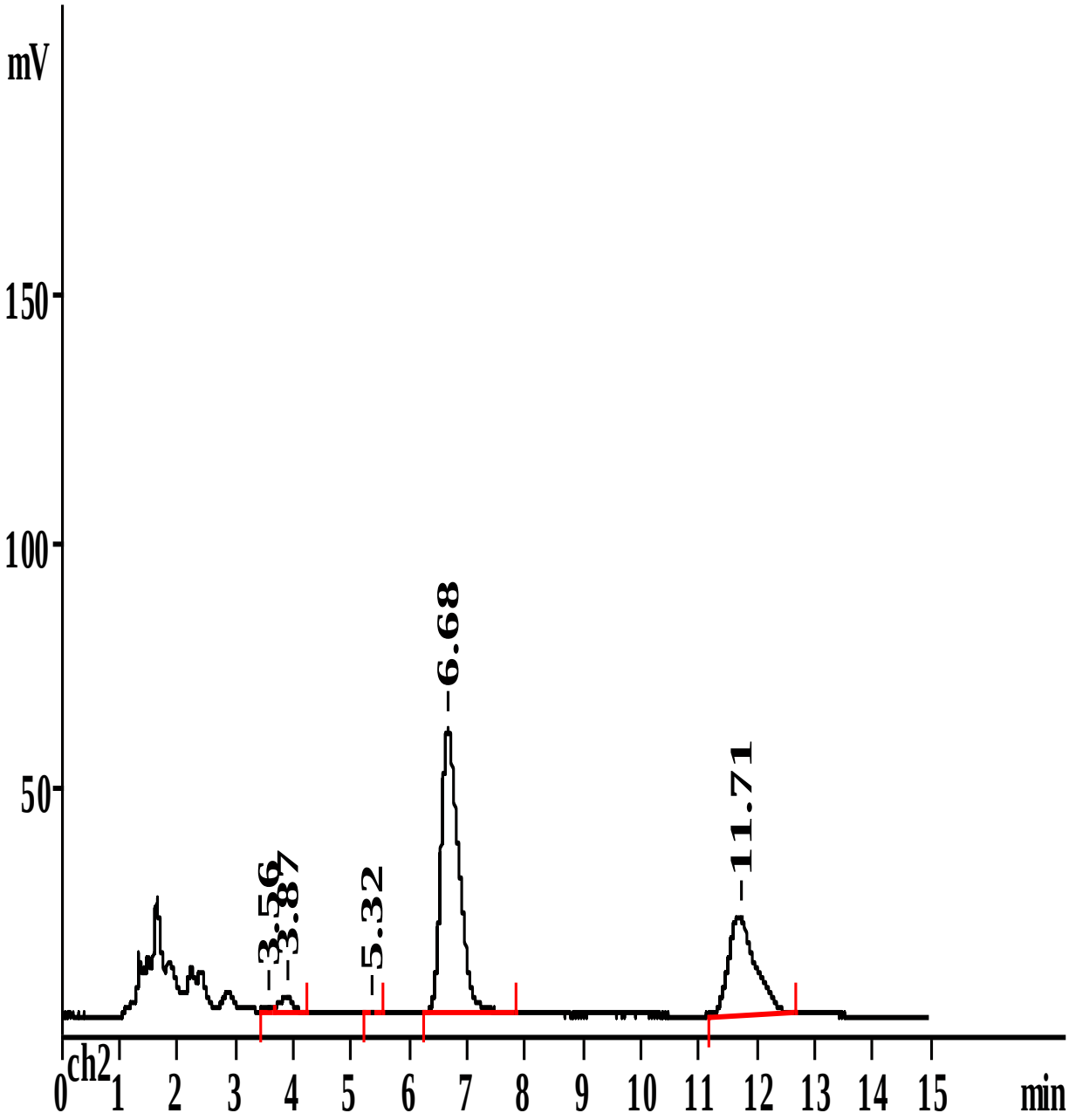


Figure 1.11: Concentrations of 6.68-quercetin and 11.71-kaempferol in December

Appendix 12

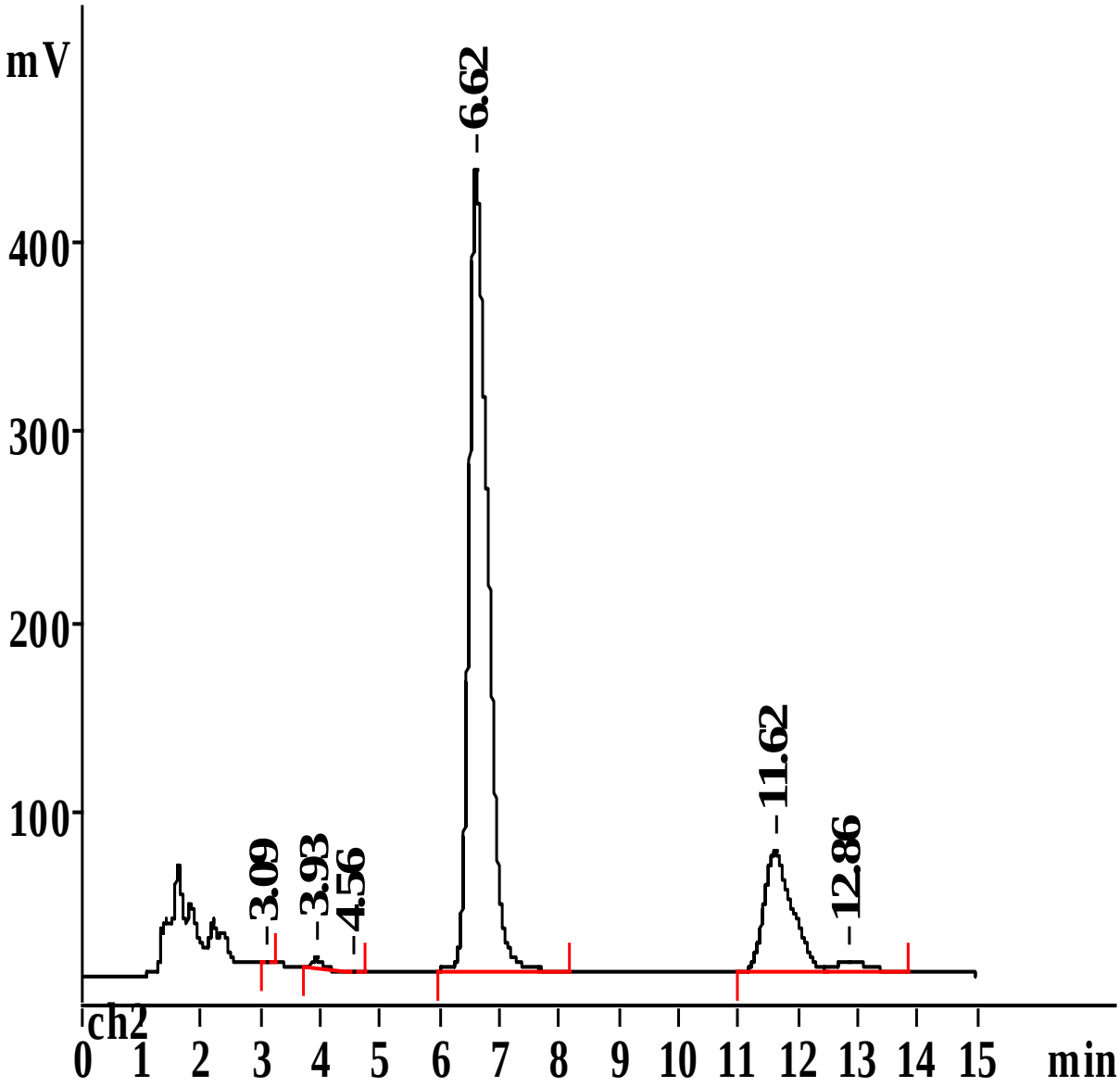


Figure 1.12: Concentrations of 6.62-quercetin and 11.62-kaempferol in January

Appendix 13

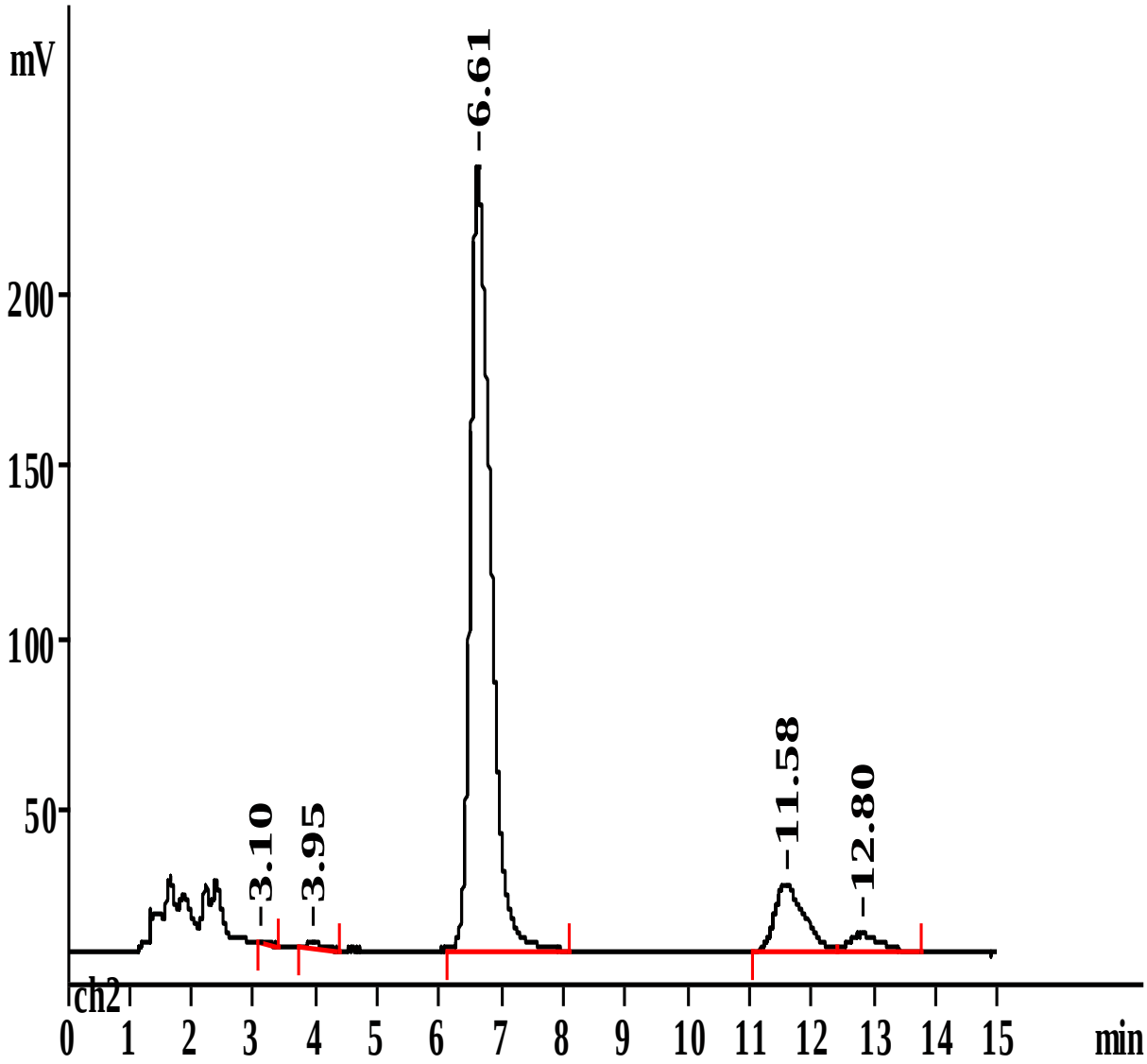


Figure 1.13: Concentrations of 6.61-quercetin and 11.58-kaempferol in February

Appendix 14

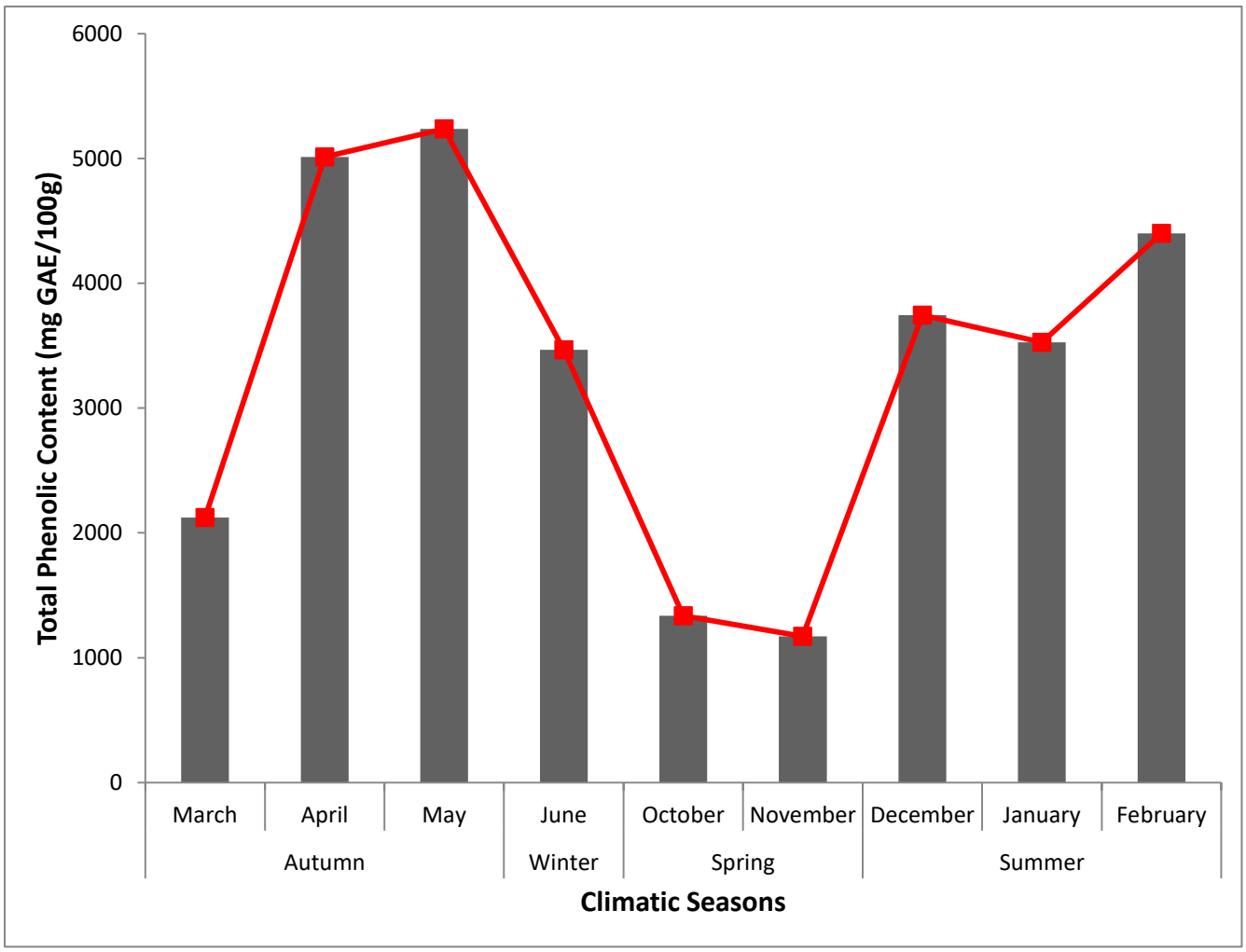


Figure 1.14: Total phenolic content of monthly *M. oleifera* leaf collection.

Appendix 15

Table 4: Tukey multiple comparisons of means 95% family-wise confidence level of total phenolics and flavonoid content

Seasons	TPC (P_{value})	TFC (P_{value})
Winter-Autumn	0.0000158	0.0000001
Spring-Autumn	0.0000001	0.0000001
Summer-Autumn	0.0918112	0.0000001
Spring-Winter	0.0000001	0.0000001
Summer-Winter	0.0041413	0.0000444
Summer-Spring	0.0000001	0.0000001

Appendix 16

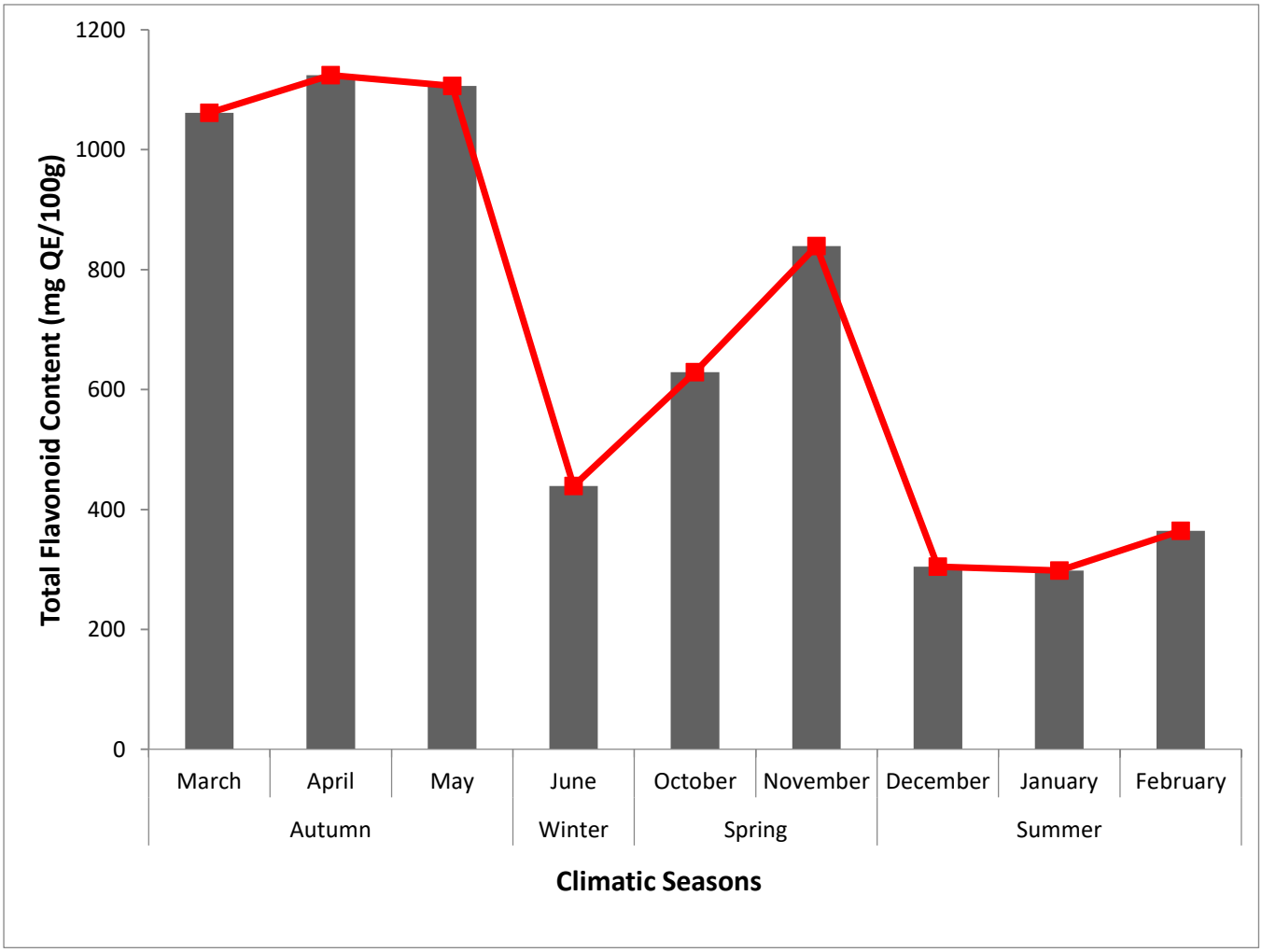


Figure 1.15: Total flavonoids content of monthly *M. oleifera* leaf collection.

Appendix 17

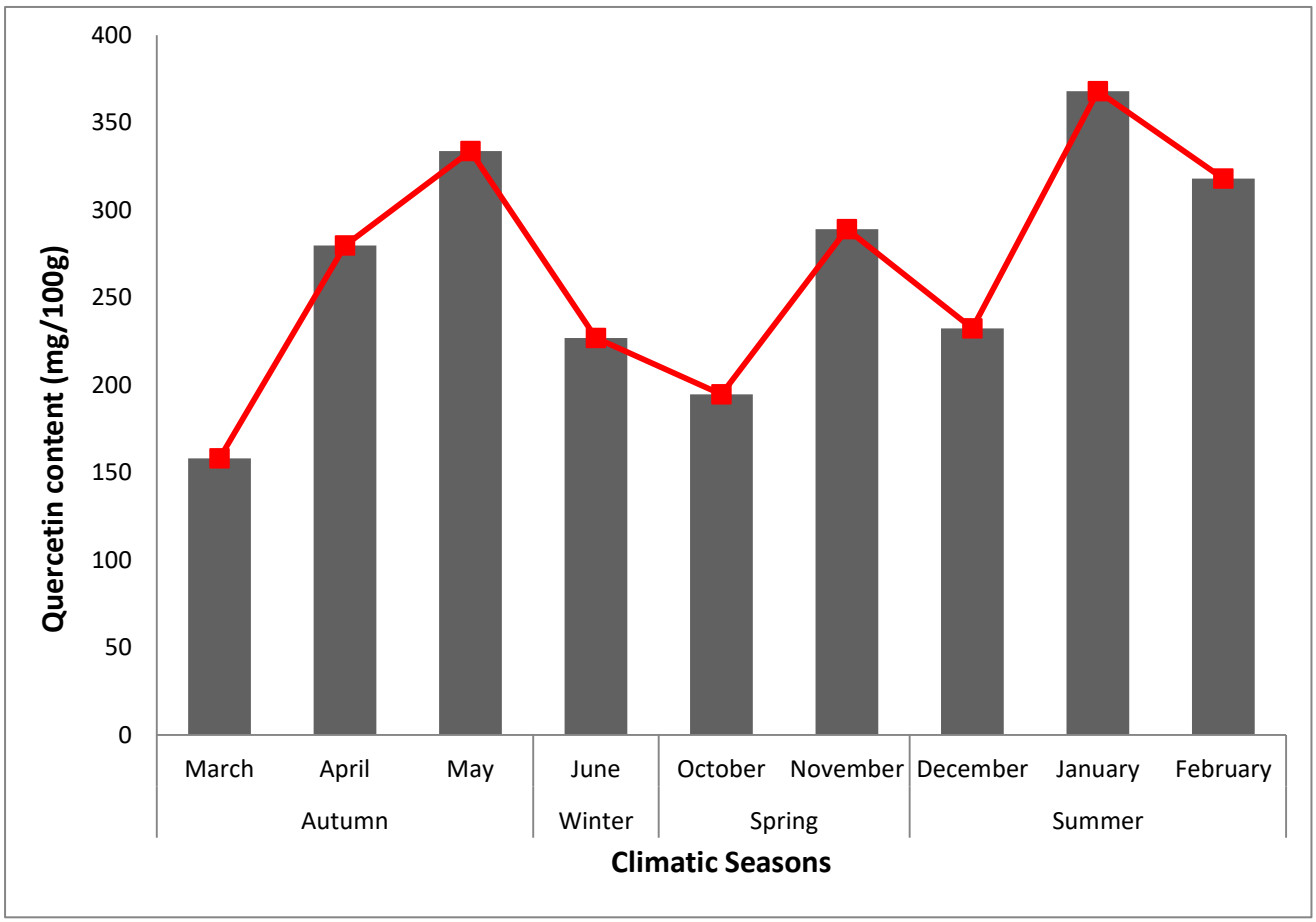


Figure 1.16: Quercetin content of monthly *M. oleifera* leaf collection.

Appendix 18

Table 5: Tukey multiple comparisons of means 95% family-wise confidence level of Kaempferol and quercetin

Seasons	Kaempferol (P_{value})	Quercetin (P_{value})
Winter-Autumn	0.1333553	0.9744449
Spring-Autumn	0.8607205	0.9964924
Summer-Autumn	0.4212003	0.9946946
Spring-Winter	0.3723055	0.9966889
Summer-Winter	0.8115061	0.9979803
Summer-Spring	0.8360291	0.9999877

Appendix 19

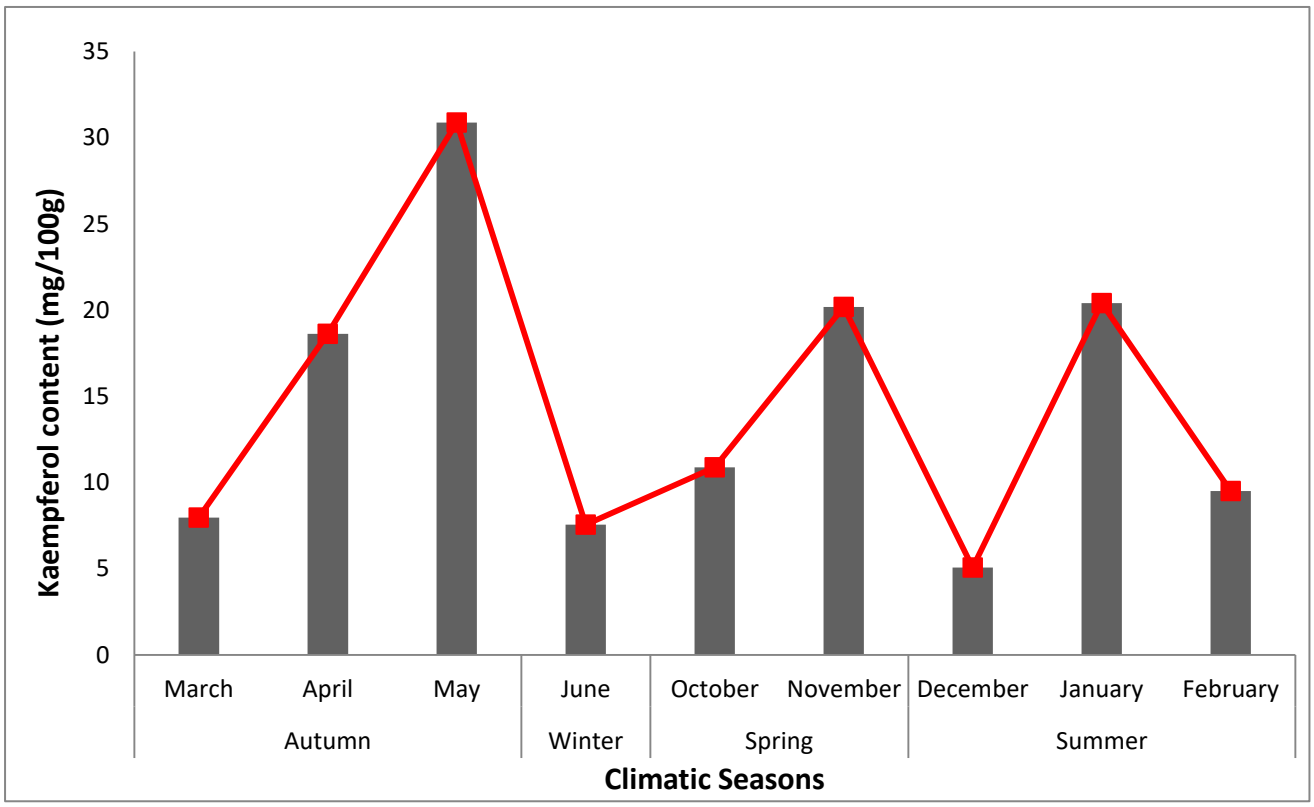


Figure 1.17: Kaempferol content of monthly *M. oleifera* leaf collection.

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