INVESTIGATING THE PRODUCTION OF SECONDARY METABOLITES EFFECTIVE IN LOWERING BLOOD GLUCOSE LEVELS IN *EUCLEA UNDULATA* THUNB. VAR *MYRTINA* (EBENACEAE)

by

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Declaration

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Summary

Euclea undulata Thunb. var *myrtina* is a widely distributed shrub in South Africa. The roots are used by traditional healers for the treatment of diabetes. Research indicates that roots contain epicatechin, lupeol as well α-amyrin-3*O*-β-(5-hydroxy) ferulic acid. It was found that α-amyrin-3*O*-β-(5-hydroxy) ferulic acid inhibits α-glucosidase while epicatechin lowers glucose levels in the blood. Existing literature also indicates the presence of the naphthoquinone 7-methyl-juglone in the roots, although it was not detected in all cases. Due to its cytotoxic nature, 7-methyl-juglone poses a potential threat when *E. undulata* is used as medicinal treatment.

In order to assist the effective and safe use of this plant as a treatment for diabetes, this project aims to determine whether the presence of these metabolites is seasonal. It further aims to contribute to more sustainable harvesting methods by investigating stems and leaves in addition to the roots for the presence of these metabolites.

Key terms:

Euclea undulata; Epicatechin; Lupeol; α-amyrin-3*O*-β-(5-hydroxy) ferulic acid; 7-methyl-juglone; Diabetes mellitus; Secondary metabolites; Statistical analysis; Chemical analysis; Rainy season; Dry season; Roots; Stems; Leaves

List of abbreviations

D_2O	Heavy water,Deuterium oxide, Deuterated water
EtOAc	Ethyl acetate
GPS	Global Positioning System
HPLC	High Performance Liquid Chromatography
KH_2PO_4	Potassium hydrogen phosphate
MeOH	Methanol
NMR	Nuclear Magnetic Resonance
LC-MS	Liquid Chromatography Mass Spectroscopy
OPLS-DA	Orthogonal partial least squares project to latent structures-discriminant analysis
PCA	Principle Component Analysis
TSP	Trimethylsilylpropionic acid sodium salt
WHO	World Health Organisation

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CHAPTER 1

Introduction

1.1 Problem statement

There has been a worldwide increase in popularity in the field of traditional medicine as an alternative form of healthcare. In South Africa traditional medicine is to some extent used by roughly 70% of the population (Weideman, 2005). According to Mander (1998) there are roughly 27 million consumers of medicinal plants in South Africa at present. The interest in medicinal plants within the scientific community is driven by the fact that, according to Gao *et al.* (2001), the treatment of diseases through the use of conventional drugs is threatened by the rate at which pathogenic micro-organisms are currently evolving and developing resistance. Nigro *et al.* (2004) describe the rate at which microbes are developing resistance to treatment as unprecedented, and attributes the current levels of interest in medicinal plants to the attempts to discover and develop new methods of treatment to replace ones that are becoming increasingly ineffective. Gao *et al.* (2001) further strengthen this statement by explaining that plants used by traditional healers and herbalists contain a wide and diverse range of secondary metabolites that have often been applied successfully within the world of health care.

Diabetes mellitus is a collection of diseases that are characterised by the conditions of hyperglycemia and glucose intolerance (Roussel,1998). Motala *et al.* (2008) state that the occurrence of type 2 diabetes (associated with obesity) has increased in Africa over the last two decades and in a report released by Statistics South Africa (2014) on mortality and causes of death in September 2012 it was stated that Diabetes mellitus was the fifth highest cause of natural deaths in South Africa. This report further stated that the majority of natural deaths in the Western Cape could be attributed to Diabetes mellitus. Deutschländer *et al.* (2010) state that this is due to the fact that many African populations become urbanised and adopt Western diets and explain that this results in increasing incidences of obesity.

The Small-leaved guarri, *Euclea undulata* Thunb. var *myrtina*, a member of the Ebenaceae family, is a common as well as widely distributed plant in South Africa (van Wyk *et al*., 2009). Schmidt *et al*. (2002) state that 20 species of the *Euclea* genus occur within South Africa, predominantly in the Western and Eastern Cape, Mpumalanga and Limpopo provinces (Figure 1.1). In addition, there are thirty-five species native to the whole of southern Africa. Although the Ebenaceae family is distributed over the tropical and subtropical parts of the world, it is most abundant in Africa and South-East Asia (Schmidt *et al.,* 2002). According to van Wyk & van Wyk (1997) the two genera of *Diospyros* and *Euclea* are native to southern Africa.

E. undulata grows into a shrub or small tree, reaching an average height of between four and seven meters. It has yellow-green leaves that are characterized by a hard and leathery texture, undulate margins and are arranged opposite (van Wyk *et al.,* 2009 (Figure 1.2). Sexes occur on separate trees (Coats Palgrave & Coats Palgrave, 2002) and it produces small off-white flowers followed by edible (though unpalatable) black fruit of roughly 5 mm in diameter containing a single seed (Figure 1.3) (van Wyk *et al.,* 2009).

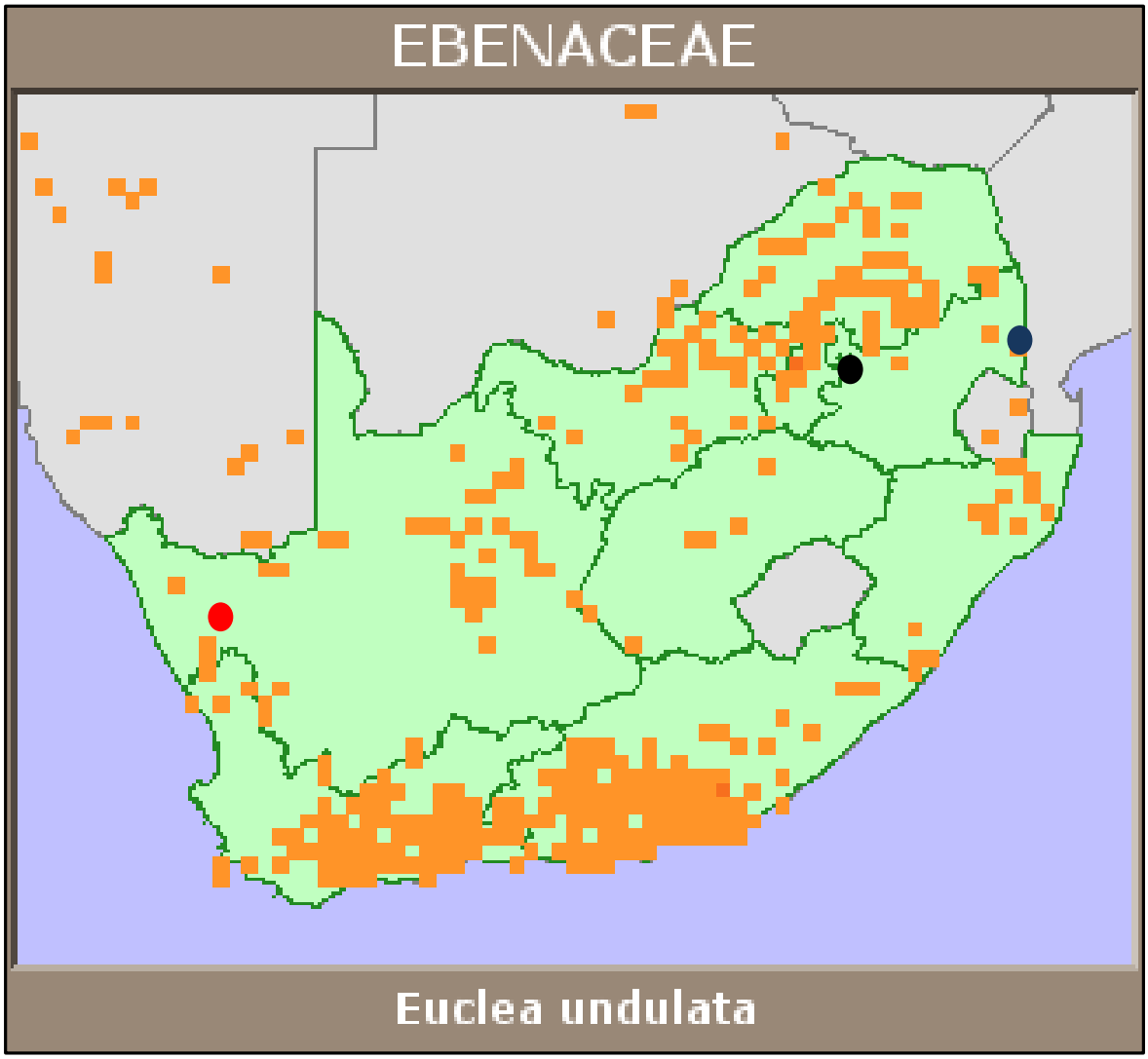


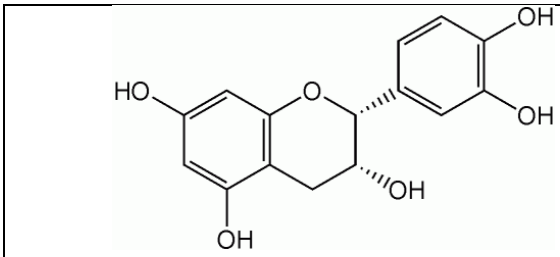
Figure 1.1: Map illustrating the distribution of *E. undulata* in South Africa (Raimondo *et al.*, 2009) as well as the areas where material was harvested for this investigation

Figure 1.2: Undulating leaf margins of *E. undulata* (Coates Palgrave & Coates Palgrave, 2002)

Figure 1.3: Fruit of *E. undulata* (Coates Palgrave & Coates Palgrave, 2002)

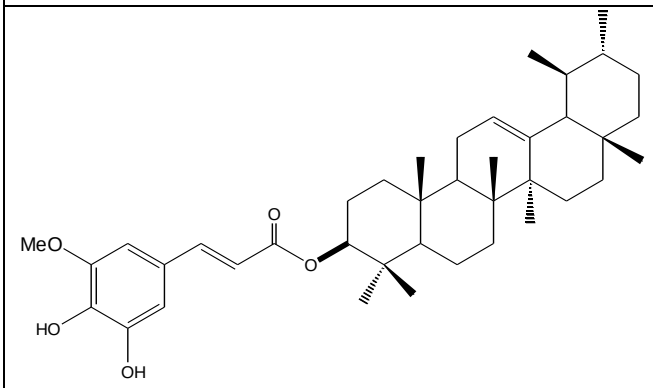
Deutschländer *et al. (*2010) state that herbalists and traditional healers in South Africa use *E. undulata* for the treatment of diabetes. Their research showed that the assay-guided isolation of the crude acetone extract of the root bark of this plant yielded a new triterpene α-amyrin-3*O*-β-(5-hydroxy) ferulic acid in addition to three known compounds betulin, lupeol and epicatechin (Figure 1.4). Of these compounds, α-amyrin-3*O*-β-(5-hydroxy) ferulic acid was found to inhibit α-glucosidase while epicatechin lowers glucose levels in the blood. With roughly 70% of the South African population making use of traditional medicine (Weideman, 2005) and the rise in the occurrence of type 2 diabetes (Motala *et al.,* 2008), an increase in the use of *E. undulata* to treat this condition can be predicted within South Africa.

A potential hindrance in the use of extracts from *E. undulata* for the treatment of diabetes is the possible presence of the cytotoxic naphthoquinone 7-methyl-juglone (Figure1.4). Contradicting findings have been reported in terms of its presence by various sources (van der Vyver & Gerritsma, 1973, 1974; van Wyk *et al.,* 2009; Deutschländer *et al.,* 2010).



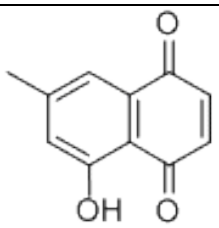
Epicathechin

Vidak *et al.* 2015



α-amyrin-3O-ß-(5-hydroxy)ferulic acid

Deutschländer *et al.* 2010



7-methyl-juglone

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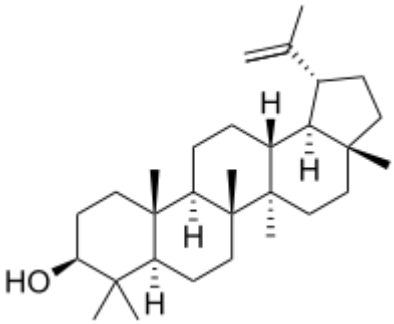

Lupeol

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Figure 1.4: Chemical structures of epicathechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid, 7-methyl-juglone and lupeol

This study aims to investigate the noted discrepancies surrounding the presence of 7-methyl-juglone in *E. undulata* and compare this with the presence of the key metabolites effective in the control of blood glucose levels. During the course of their research, Deutschländer *et al.* (2010) further stated that studies were needed to determine if there is a connection between the production of 7-methyl-juglone and lupeol in *E. undulata* and the other compounds mentioned to have the potential to

control blood glucose levels. The two compounds 7-methyl-juglone and lupeol are not structurally related as lupeol type compounds are derived from squalene (Templeton, 1969) while 7-methyl-juglone derives from acetate units (Thomson, 1971). This study however also aims to investigate the presence of lupeol in relation to the other metabolites.

The fact that the compounds found to be effective in the treatment of diabetes have only successfully been isolated from the roots of *E. undulata* corresponds with the harvesting of this part of the plant by traditional healers (Deutschländer *et al.,* 2010). Root harvesting is less sustainable than the harvesting of aerial parts, and in the interest of the preservation of wild populations it is therefore potentially beneficial to also investigate above-ground structures for the mentioned metabolites. In an investigation conducted by Bapela (2007) on a different member of this genus, *Euclea natalensis* A.DC*.,* it is stated that secondary metabolites are often present in trace amounts and that their presence could be dependent on seasonal environmental change, nutrient status within their environment as well as the particular stage of development of an individual plant.

This study aims to investigate the factors mentioned above by examining root, stem and leaf material harvested from plants from several locations within South Africa during different seasons to represent certain variations in environmental conditions. The samples were air dried and subjected to chemical analysis, the details of which are discussed later in this paper.

1.2 Motivation

In many South African communities, traditional healers and herbalists remain the primary health care providers. Mander *et al.* (2007) state that over 70% South Africans rely on traditional medicine as a form of medical treatment and that it furthermore "is thought to be desirable and necessary for treating a range of health problems that Western medicine does not treat adequately". In addition, the lack of accessibility to modern medicine has contributed to the widespread use of traditional medicines in many poor and rural households in South Africa (Cameron *et al.,* 2008). The table below illustrates the extent to which accessibility of traditional practitioners by South Africans outweighs that of doctors of western medicine (Abdullahi, 2011).

Table 1: Sample ratio of traditional practitioners compared with the ratio of medical doctors to the population

Countries	Ratio Of Traditional Practitioners To Population	Ratio Of Medical Doctors To Population
Kenya, Urban (Mathare) Rural (Kilungu)	1: 833 1: 143–345	1: 987 1: 70 000
Zimbabwe	1: 600	1: 6 250
Swaziland	1: 100	1: 10 000
Nigeria (Benin City) National Average	1: 110 No data	1: 16 400 1: 15 740
South Africa (Venda Area)	1: 700–1 200	1: 17 400
Ghana	1: 200	1: 20 000
Uganda	1: 700	1: 25 000
Tanzania	1: 400	1: 33 000
Mozambique	1: 200	1: 50 000

Considering the importance of the role of traditional forms of treatment in health care provision in South Africa, it becomes evident that traditional medicine is by no means an alternative practice in this country. With the increase in the occurrence of type 2 diabetes discussed earlier (Deutschländer *et al*., 2010), it forms the motivation of this research project to investigate the effectiveness of traditional treatments derived from *E. undulata* as well as the danger of the possible presence of the cytotoxic naphthoquinone 7-methyl-juglone.

Clarity and understanding of the contradictions about the presence of 7-methyl-juglone as described in existing literature (van der Vyver & Gerritsma, 1973, 1974; van Wyk *et al*., 2009; Deutschländer *et al*., 2010) could potentially be used to provide traditional healers with valuable information on the harvesting and utilisation of material from *E. undulata* in a way that improves the safety of their patients. Due to the role of epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in the potential development of diabetes treatments, similar investigations into the presence of these two metabolites were conducted to examine their presence with relation to that of 7-methyl-juglone. Deutschländer *et al.* (2010) stated that studies were needed to determine if there is a link between the production of 7-methyl-juglone and lupeol in *E. undulata*. For this reason, the presence of lupeol was also investigated to aid in potential future research into the possibility of a relationship between the production of 7-methyl-juglone and lupeol.

1.3 Hypothesis

It is hypothesised that the presence of the naphthoquinone 7-methyl-juglone, epicatechin, lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in *E. undulata* might be determined by environmental factors that are either seasonal or non-seasonal.

1.4 Objectives

The following objectives have been identified for this study:

- Determine whether the presence of 7-methyl-juglone, lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and epicatechin in *E. undulata* are subject to the specific seasonal changes of the area.

- Determine the parts of the plant in which 7-methyl-juglone, lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and epicatechin are produced in order to provide guidance for safe, effective and sustainable harvesting.

1.5 References

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CHAPTER 2

Literature review

2.1 The role of plants in medicine as an indication of the relevance of this study

Throughout the ages, plants have played a fundamental role in the treatment of disease as well as the development of traditional medicine systems. (Cechinel-Filho (2012) describes the documented medicinal use of approximately 1000 substances derived from plants in Mesopotamia around 2600 BCE. Similarly, records from Egypt dating back to 1500 BCE describe over 700 forms of medicinal treatments that originated from plants. Cechinel-Filho (2012) further describes similar records in the ancient societies of China, India, Greece and Rome. What makes this information about the role of plants in the past relevant to the world of medicinal science today is the fact that some of the species described within these ancient documents, for example the oils of certain *Cedrus* species, *Commiphora* species and *Papaver somniferum* L.*,* are still used today for the treatment of conditions such as infections, inflammation, coughs and colds. This perhaps reinforces the validity of the statement made by Gao *et al.* (2001) that plants not only contain a wide and diverse range of secondary metabolites that have yielded successful forms of medicinal treatments in the world of today, but also indicates that this has been the case throughout a significant period in history.

As mentioned by Weideman (2005), roughly 70% of the South African population currently makes use of traditional medicine in the treatment of various conditions and ailments. Cechinel-Filho (2012) states that roughly 65% of the world population relies on plant-derived treatments for their primary forms of health care and further states that it also contributes significantly in the remainder of the world population in ‘developed’ countries.

To emphasize the latter, it is useful to consider that of 122 compounds identified in a survey of plant-derived pure compounds used as drugs in countries hosting WHO-Traditional Medicine Centers, 80% were found to be used for the same or related ethnomedicinal purposes, and were derived from only 94 plant species (Cechinel-Filho, 2012).

There are several examples of how ethnomedicine has guided the process of discovery and development of some of the best-known drugs in clinical use today. One example is resperine, an antihypertensive agent isolated from *Rauvolfia serpentine* (L.) Benth. Ex Kurz which is used in Ayurvedic medicine for the treatment of snake bite (Dewick, 2002). Other examples include the isolation of the alkaloid metabolites vinblastine and vincristine from the Madagascar periwinkle *Catharanthus roseus* (L.) G. Don, which are well-known for their clinical use as treatments of cancer (Cechinel-Filho, 2012). When one considers that over ten million new cases of cancer, with over six million deaths, were estimated worldwide in the year 2000 and that there has been a 22% increase in cancer incidence and mortality since 1990 (Parkin *et al.,* 2001), the scale of the contribution made by plants such as these to the world of medicine becomes evident. Salim *et al.* (2008) describe how diosgenin, a steroidal sapogenin obtained from the tubers of various *Dioscorea* species that occur in Mexico and Central America, can be converted chemically into progesterone, which is a hormone that can be used as a female oral contraceptive. Progesterone is furthermore also an important intermediate for the production of cortisone which is an important and widely-used anti-inflammatory drug.

Examples of some of the important contributions plants have made in the past and present spheres of medicine highlight the relevance of the scientific investigation of traditional plant use in the search for new treatment methods for the future. If the findings of this study into the presence of 7-methyl-juglone, lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and epicatechin in *E. undulata* can aid in the safe harvest by traditional healers and herbalists, or indeed assist in the use of this plant to successfully develop new clinical treatment methods for diabetes, it can be concluded that a potentially countless number of individuals could stand to benefit from the results.

2.2 Characteristics of 7-methyl-juglone

Although this study aims to investigate the presence of several metabolites in *E. undulata,* the cytotoxic nature of 7-methyl-juglone combined with the uncertainty over its presence in *E. undulata* and possible extracts used for medicinal purposes warrant the placement of particular emphasis on this compound.

The metabolite 7-methyl-juglone (5-hydroxy-7-methyl-1,4-naphthoquinone) is classified as a naphthaquinone (Mahapatra *et al.,* 2007). Naphthaquinones are organic compounds that are derived from naphthalene. They form a group of secondary metabolites that is widespread in nature and has been identified in several prominent and large plant families including Avicenniaceae, Bignoniaceae, Boraginaceae, Droseraceae, Ebenaceae, Juglandaceae, Nepenthaceae and Plumbaginaceae (Babula *et al.,* 2009). Naphthaquinones usually occur in reduced and glycosidic forms in plants, and have been noted as monomers, dimers or trimers in the *Diospyros* genus of the Ebenaceae family. Naphthoquinones have many physiological roles, for example ubiquinone, plastoquinone and K vitamins are functional constituents of biochemical systems. They are usually yellow or brown in colour and therefore play important roles as dyes in pigmentation (Babula *et al.,* 2009). Babula *et al.* (2009) further state that most naphthoquinones are soluble in alcohol, acetone, chloroform, benzene, DMSO and acetic acid but note that plumbagin and juglone are slightly soluble in hot water.

It is however the cytotoxicity of 7-methyl-juglone that is of interest in this investigation. Evidence of the cytotoxic nature of naphthaquinones can be found in the work of Buch *et al.* (2012) in which the freshly opened flowers of the carnivorous plants of the genus *Nepenthes* were found to contain 7-methyl-juglone and were described as an unsuitable environment for microbial growth as a result. Neuwinger (1994) describes naphthaquinones as 'insecticidal, antibacterial, fungicidal, molluscicidal, termiticidal, antibiotic' as well as 'antitumoral'. Statiauskaite *et al.* (2006) furthermore describe naphthaquinones as capable of eliciting and inducing both apoptotic and necrotic cell death, resulting in this group of secondary metabolites and their derivatives being investigated as possible sources of cancer treatments.

Binneman (2008) states that the quinoids are currently the second largest class of antitumor agents in the world.

If this cytotoxic compound is produced by *E. undulata,* the specific conditions under which it could be present need to be investigated in order to minimize the risk patients are potentially exposed to when this plant is used during the treatment of diabetes.

2.3 Presence of 7-methyl-juglone, lupeol, epicatechin and α-amyrin-3*O*-β-(5-hydroxy) ferulic acid within *Euclea* and related species

One of the aims of this study is to assist in the safe harvest of *E. undulata* by determining the threat posed by the possible presence of 7-methyl-juglone in its use as a treatment for diabetes by traditional healers. Emphasis is placed on a literature review of this compound and on examining existing research into its presence within species related to *E. undulata*. In an attempt to assist in the future development of an effective diabetes treatment, it also aims to investigate the presence of α-amyrin-3*O*-β-(5-hydroxy) ferulic acid and epicatechin in the *Euclea* genus due to their role in the control of blood glucose levels (Deutschländer *et al.,* 2010). Although it is not the aim of this study to determine whether there is a biogenetic reaction between 7-methyl-juglone and lupeol (Deutschländer *et al.,* 2010) the presence of lupeol is investigated in order to possibly assist future research of this nature. The examination of existing literature on the presence of these metabolites in the *Euclea* genus is therefore useful for the purposes of this study. Comparing existing literature on the identification and isolation of these compounds within the Ebenaceae family could provide information with regards to which environmental factors might play a key role, as well as which parts of the plants these compounds are likely to be present.

Van Wyk & van Wyk (1997) mentioned that the genera *Diospyros* and *Euclea* of the Ebenaceae family are native to southern Africa. Examining existing literature on the genus *Euclea*, Van der Vyver & Gerritsma (1973;1974) isolated 7-methyl-juglone and diospyrin from chloroform extracts of the roots of *Euclea crispa* var *crispa* (Thunb.) Gürke and *Euclea undulata* var *myrtina*. Diospyrin and isodiospyrin were isolated from the fruit of *E. crispa* and *E. undulata* respectively. Stem extracts of *E. crispa, E. divinorum* Hiern and *E. schimperi* (A.DC.) Dandy did not show any trace of naphthoquinones and neither did extracts from green fruit of *E. divinorum*.

The findings of this study were confirmed by Neuwinger (1994) who states that 7-methyl-juglone was successfully isolated from chloroform extracts of the roots of the *Euclea* genus, but that naphthaquinones were completely absent in stem extracts. Neuwinger (1994) does however mention that trace amounts were detected in ripe fruit. More recently, Mital *et al*. (2010) successfully isolated 7-methyl-juglone from the roots of *E. natalensis* when conducting an investigation on anti-microbial activity. When examining existing literature on the *Diospyros* genus, several references to 7-methyl-juglone as well as lupeol were found. Sinha *et al.* (2009) also state that 7-methyl-juglone was extracted from stem bark of *Diospyros paniculata* Dalzell. In an investigation conducted by Uddin *et al.* (2014) on root material from *Diospyros lotus* L. both lupeol and 7-methyl-juglone were detected in chloroform extracts. According to a review article done by Mallavadhani *et al.* (1998) plumbagin and 7-methyl-juglone are the most abundant monomeric naphthoquinones in the *Diospyros* genus. The results of their investigation further show that 7-methyl-juglone accumulates in the bark and wood. These findings are similar to those of an investigation done by Gu *et al.* (2004) in which both 7-methyl-juglone and lupeol were extracted from the stem bark of *Diospyros maritima*.

Although the solvent, season and other environmental conditions were not described in all of these cases, the above information gives a clear indication that the species investigated appear to accumulate 7-methyl-juglone in the roots rather than aerial plant parts in most cases.

Perhaps the most insightful information comes from the work done by Bapela *et al.* (2007) who studied the seeds, shoots and roots of *Euclea natalensis* and used chloroform and High Performance Liquid Chromatography (HPLC) to isolate compounds from various plant organs. The results of this study revealed that 7-methyl-juglone was present in very small concentrations in seeds (during dormancy) with concentrations detected at 6.2 and 0 mg/kg – a result that could perhaps be attributed to the fact that, according to Mayer & Poljakoff-Mayber (1982), secondary metabolites generally accumulate in relatively low concentrations in seeds when compared to primary metabolites such as lipids and starch. During the quantitative analysis of chloroform extracts of shoot material, it was concluded that although 7-

methyl-juglone was found to be present (concentrations as high as 1310 mg/kg at one particular stage in development) there was a notable decline as seedlings increased in age (Bapela *et al.,* 2007). When examining root material in the same way it was found that concentrations were generally higher (as high as 3693 mg/kg), but fluctuations were noted when different stages of development were compared.

Two conclusions can be drawn from the results obtained by Bapela *et al.* (2007). Firstly, the results to indicate that 7-methyl-juglone is clearly present in higher concentrations in roots that in aerial structures. When considering that it is the roots of *E. undulata* that are harvested during the treatment of diabetes (Deutschländer *et al.,* 2012), it again highlights the possible threats of this cytotoxic naphthaquinone finding its way into traditional remedies. Secondly, the fact that concentrations of 7-methyl-juglone fluctuates depending on developmental stage could possibly offer explanations for the discrepancies surrounding its isolation from *E. undulata* (Deutschländer *et al.,* 2010).

The role of epicatechin in lowering blood glucose levels is well-documented. Quine & Raghu (2005) found epicatechin to be effective in lowering the glucose levels of the blood of diabetic rats over the period of 35 days. Chakravarthy *et al.* (1982) also mention the ability of epicatechin to lower blood glucose levels to normal levels. The presence of epicatechin in the *Euclea* genus is described by Pretorius *et al.* (2003) who confirm its presence in the leaves of *Euclea crispa* subsp. *crispa,* by describing its successful isolation using ethyl acetate fractions. Hattas & Julkunen-Tiitto (2012) detected epicatechin in the leaves of *E. divinorum* using methanol extractions. Epicatechin was also detected in the *Diospyros* genus. Chen *et al.* (2008) successfully isolated epicatechin from the fruit of *Diospyros kaki* L. cv. Mopan using ethanol as solvent. Choi *et al.* (2015) isolated epicatechin from the dried stem bark and leaves of *Diospyros burmanica* Kurz through methanol extraction. Their research also mentions that plants were collected during the month of February.

It is important to note that, in many of the consulted resources, the environmental and seasonal conditions under which harvesting took place were not described. It does however become evident that epicatechin has been detected in various parts of the plants within the genera studied.

The metabolite α-amyrin-3O-β-(5-hydroxy) ferulic acid was first described by Deutschländer *et al.* (2010) when conducting an investigation into the hypoglycemic properties of the root bark of *E. undulata.* Little additional reference to this compound exists in current literature. Ferulic acid has however been found to increase antioxidant enzyme activity. This neutralises the free radicals that are the primary cause of tissue damage in diabetes (Choi *et al.,* 2011).

Several references were found to the presence of the flavonoid lupeol within the *Euclea* genus. Dagne *et al.* (1993) harvested aerial parts of *Euclea divinorum* in Ethiopia at an altitude of 1800m. Plant material was powdered and an EtOAc extract revealed the presence of lupeol. Sibanda *et al*. (1992) describe the successful isolation of lupeol from the root bark of *E. crispa*. Due to its cytotoxic nature, extensive research has been conducted into the anti-microbial activities of lupeol. Wiegenand *et al.* (2004) state its inhibitory activity against Gram-positive bacterial strains as well as against a drug-sensitive strain of *Mycobacterium tuberculosis* and describe its presence in ethanol extract of the root bark of *E. natalensis*. McGaw *et al.* (2008) describe the antimycobacterial activity of lupeol and 7-methyl-juglone isolated from *E. undulata* and *E. natalensis.* The presence of lupeol has also been documented in the *Diospyros* genus. Adzu *et al.* (2015) describe the successful isolation of lupeol from chloroform extracts of the root bark of *Diospyros mespiliformis* while Prachayasittikul *et al.* (2010) isolated lupeol from dichloromethane extracts from the stems of *Diospyros rubra* Lec.

Sinha & Bansal (2008) successfully isolated both lupeol and 7-methyl-juglone from methanol extracts of the leaves and stem bark of *Diospyros kaki* as well as from ethanol extracts of the stem bark of *Diospyros montana* and *Diospyros melanoxylon.* They further mention that chloroform extracts of the leaves of *D. melanoxylon* also yielded both lupeol and 7–methyl-juglone.

Upon examining existing literature, it becomes evident that lupeol has been detected in various parts of the plants within the *Euclea* and related genera.

2.4 Factors that might influence the presence and successful extraction of secondary metabolites in plants

By examining the existing research on the presence of epicathechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid, 7-methyl-juglone and lupeol in the *Diospyros* and *Euclea* genera it becomes evident that no clear correlation emerges in terms of the plant organs in which each compound has been detected. The consulted resources mention the use of several solvents in their respective extraction and isolation methods and the potential influence of the choice of solvent on the successful extraction of any particular compound should be stressed. To illustrate this Eloff (1998) conducted an investigation in which it was attempted to extract certain secondary metabolites from the leaves of *Anthocleista grandiflora* Gilg and *Combretum erythrophyllum* (Burch.) Sond. using a variety of solvents. Arbitrary values were assigned to each solvent based on how successfully it extracted compounds from plant material. Results indicated that acetone gave the best results with a value of 102 followed by methanol/chloroform/water (81), methylene dichloride (79), methanol (71), ethanol (58) and water (47). Lapornik *et al.* (2005) found that ethanol and methanol extracts made from the fruit of *Ribes rubrum* L. and *Ribes nigrum* L. contained twice more anthocyanins and polyphenols than water extracts. These investigations illustrate that the potential influence of solvent type on the extraction of secondary metabolites from plants.

The effect of environmental stress on the production of secondary metabolites is well documented. The increasing worldwide interest in the production and harvest of secondary metabolites for medicinal purposes has led to a need to better understand the environmental factors that regulate and influence their production in plants (Jafaar *et al.,* 2012). Plants respond to stress in the ecosystem by altering morphology, physiology and biochemistry and Tuteja & Sopory (2008) state that it is an important feature of plant survival to be able to continuously monitor fluctuations in environmental conditions such as light intensity, temperature variations, water and nutrient availability as well as carbon dioxide levels in order to induce physiological defence mechanisms.

Existing literature on the factors that influence the secondary metabolism of plants places particular emphasis on the effect of water stress. Xu *et al*. (2010) define drought stress as 'when water in the soil is reduced to a critical level and atmospheric conditions further contribute to loss of water by the plant' and furthermore describe drought stress as one of the most significant abiotic stresses that influence plant growth and development. Jafaar *et al*., (2012) also name water stress as one of the most important factors in determining the biochemical properties of plants and state that although a lack of water has a negative effect on growth, it can lead to the increased production of certain secondary metabolites. Under conditions of water stress, carbon cannot effectively be translocated to carbon sinks and is accumulated as carbohydrate instead. This results in more carbon being allocated for the use by the plant's secondary metabolism (Jafaar *et al.,* 2012). Soil type can also have a significant impact on the production of secondary metabolites within a plant, since soil type determines soil water capacity (Jafaar *et al.,* 2012).

Gershenzon (1984) stresses the influence of abiotic factors on secondary metabolite production in plants by referring to the environmental factors of light and temperature as having significant influence on the levels of secondary compounds. In the presence of sunlight high levels of oxygen and secondary metabolites are produced during the process of photosynthesis.

According to Ghasemzadeh *et al.* (2010) the presence of sunlight enhances the biosynthesis of phenolic compounds while flavonoid formation is described as completely light dependent. However, they further state that different plants were noted to have different responses to changes in light intensity and that the resulting levels of flavonoid phenolic compound production fluctuated accordingly.

Figure 2.1 provides a generalised overview of the most important environmental factors that could influence the presence and production of secondary metabolites (Mahajan & Tuteja, 2005).

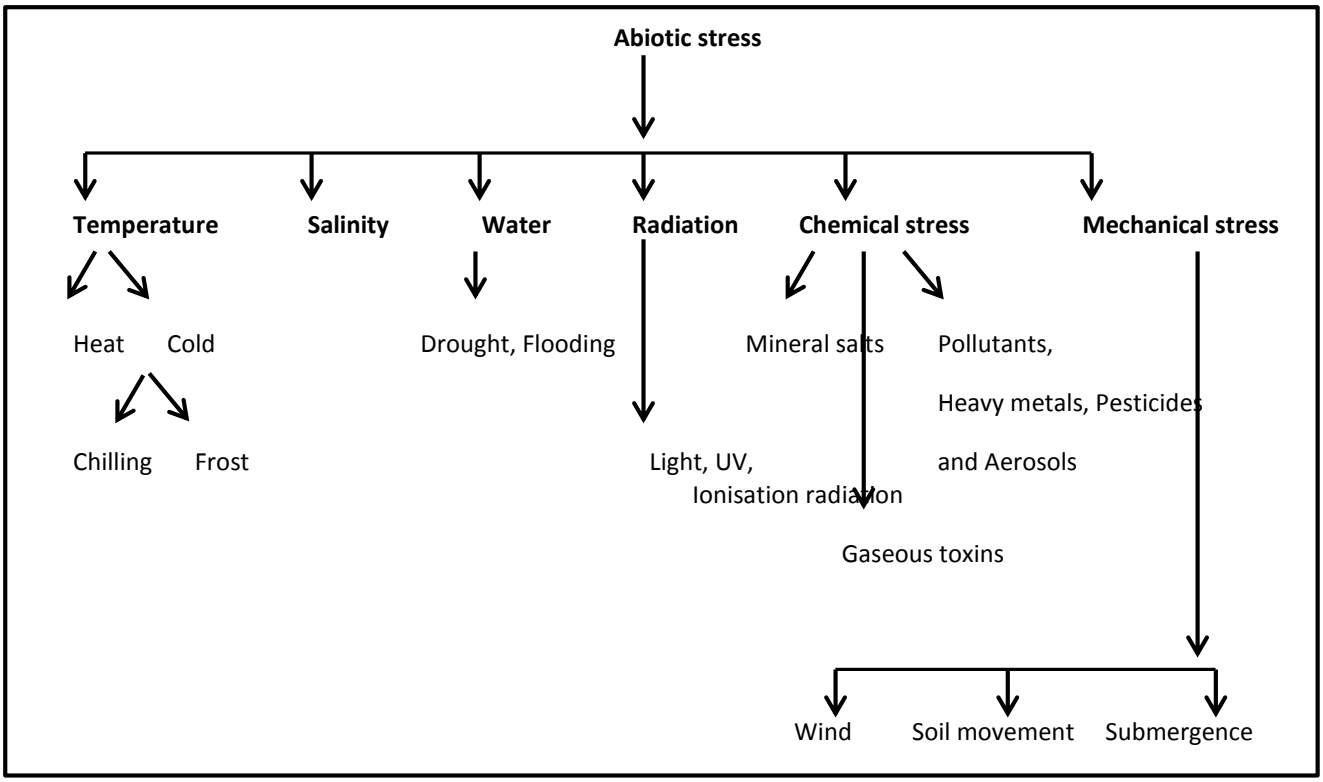

Figure 2.1: Various abiotic stress signals creating stress in plants. (Mahajan & Tuteja, 2005)

A characteristic feature of South African climate patterns is the fact that rainfall is seasonal. The implications are that most plants growing in natural populations in this country are subjected to varying amounts of water availability within a year. If taken into consideration that the country can also be divided into winter and summer rainfall areas it implies that, if the area of natural distribution of a particular plant species is wide enough, certain populations might be exposed to conditions where water is plentiful while temperatures are higher while other populations of the same species could be exposed to the complete opposite.

When keeping in mind the possible effects that abiotic factors could have on secondary metabolite production, it is quite possible that such dramatic variations in environmental conditions could result in the presence of certain compounds in some individuals of the *E. undulata* species while being responsible for the absence in others.

Bapela *et al.* (2007) investigated the possible effect of seasonal changes on the production and concentrations of 7-methyl-juglone in *E. natalensis*. Root material was harvested from natural populations at the end of each season and samples were subjected to the same methods of compound isolation described earlier. Interestingly, the results showed no statistically significant variation in the levels of 7-methyl-juglone over the four seasons. Khan *et al.* (1978) isolated the metabolites 7-methyl-juglone, diospyrin and mamegakinone and later the inactive compound lupeol (Khan, 1985) from root bark of *E. natalensis* using chloroform extracts. In contrast, Khan *et al.* (1978) noted variations in the chemical composition of the roots collected during the rainy and the dry seasons. In the rainy season plants were rich in lupeol and the relative concentration of 7-methyl-juglone was negligible whereas in the dry season the concentrations of the two compounds were reversed. The results of this study are an indication of the potential influence of seasonal environmental change on one of the compounds of interest to this study within the *Euclea g*enus.

2.5 Review of environmental and climatic features of the study area

To investigate the influence of certain seasonal environmental factors on the compounds of interest to this study, plant material was collected during December and July/August in three different localities in the provinces of Gauteng, Mpumalanga and the Northern Cape to encompass both the rainy and dry seasons as well as the differences in winter and summer temperatures in both winter and summer rainfall areas.

2.5.1 Gauteng locality

Material from three individual plants was collected on privately owned land previously used for game farming, within an area around S 25º 28' and E 28º 27'. The area has an elevation of roughly 1320m above sea level. According to Rutherford & Westfall (1994) it forms part of the Savanna Biome and according to Mucina & Rutherford (2006) it falls within the Central Sandy Bushveld bioregion.

It is characterised by sedimentary rock, specifically sandstone, conglomerate and siltstone of the Alma Formation and sandstone, siltstone and shale of the Vaalwater Formation (Mucina & Rutherford, 2006). The soil is described by the Department of Agricultural and Technical Services (1965) as ferruginous lateritic as well as fersiallitic with crystalline acidic rock. In terms of current land use of the area, Biodiversity Geographic Information Systems (BGIS) (2014) describes the area as natural (Figure 2.2).

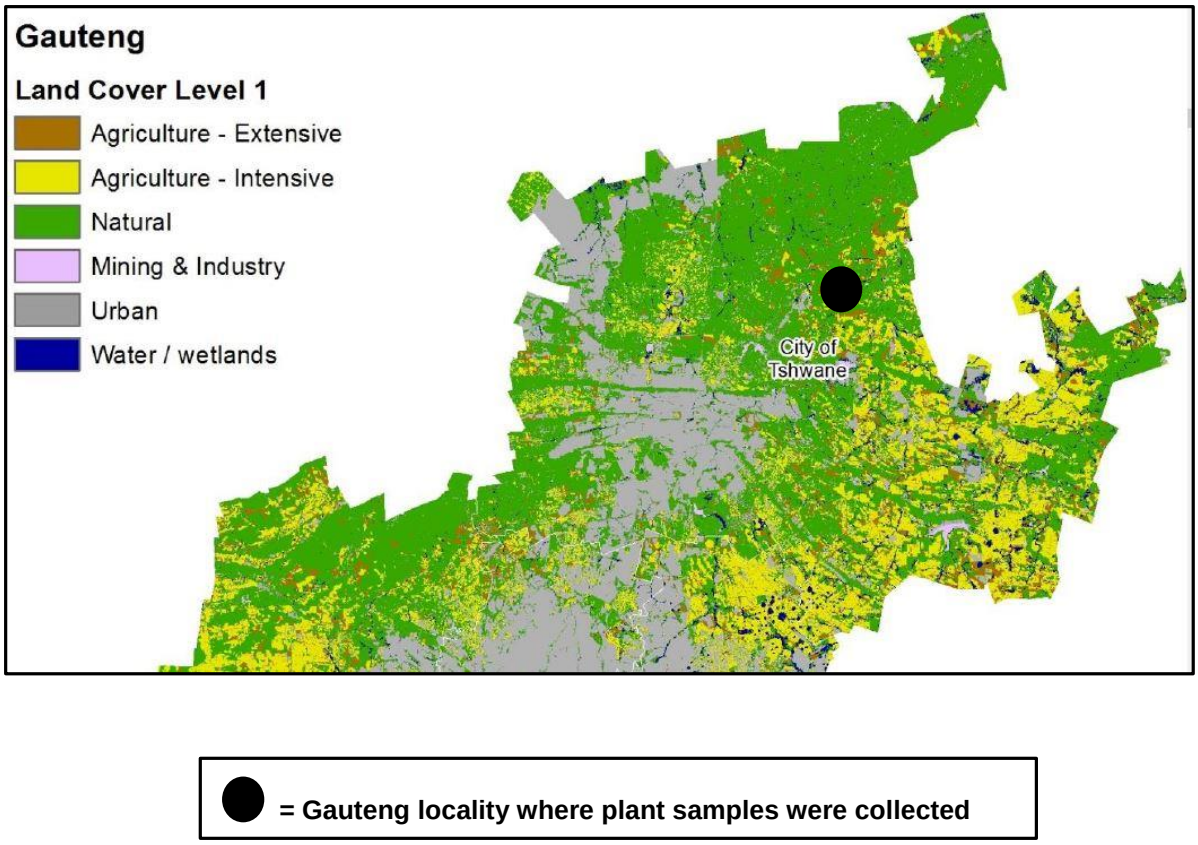

Figure 2.2: Conservation status of Gauteng locality where plant samples were collected (BGIS, 2014)

Data obtained from Wonderboom Airport weather station was used to calculate average monthly temperatures and rainfall between 2010 and 2014 (de Jager, 2015: Personal Communication). This data is summarized in Figure 2.3. and illustrates that the average monthly temperatures for August and December are 15°C and 23°C respectively.

The average minimum temperatures for the months in which plant material was collected (August and December) are 5°C and 16°C respectively. Average maximum temperatures for August and December are 24°C and 29°C respectively. The lowest and highest temperatures recorded in August during this period were 5°C and 25°C respectively.

The lowest and highest temperatures recorded in December during this period were 16°C and 30°C. This indicates that the vegetation in this bioregion will experience great differences between winter and summer temperatures.

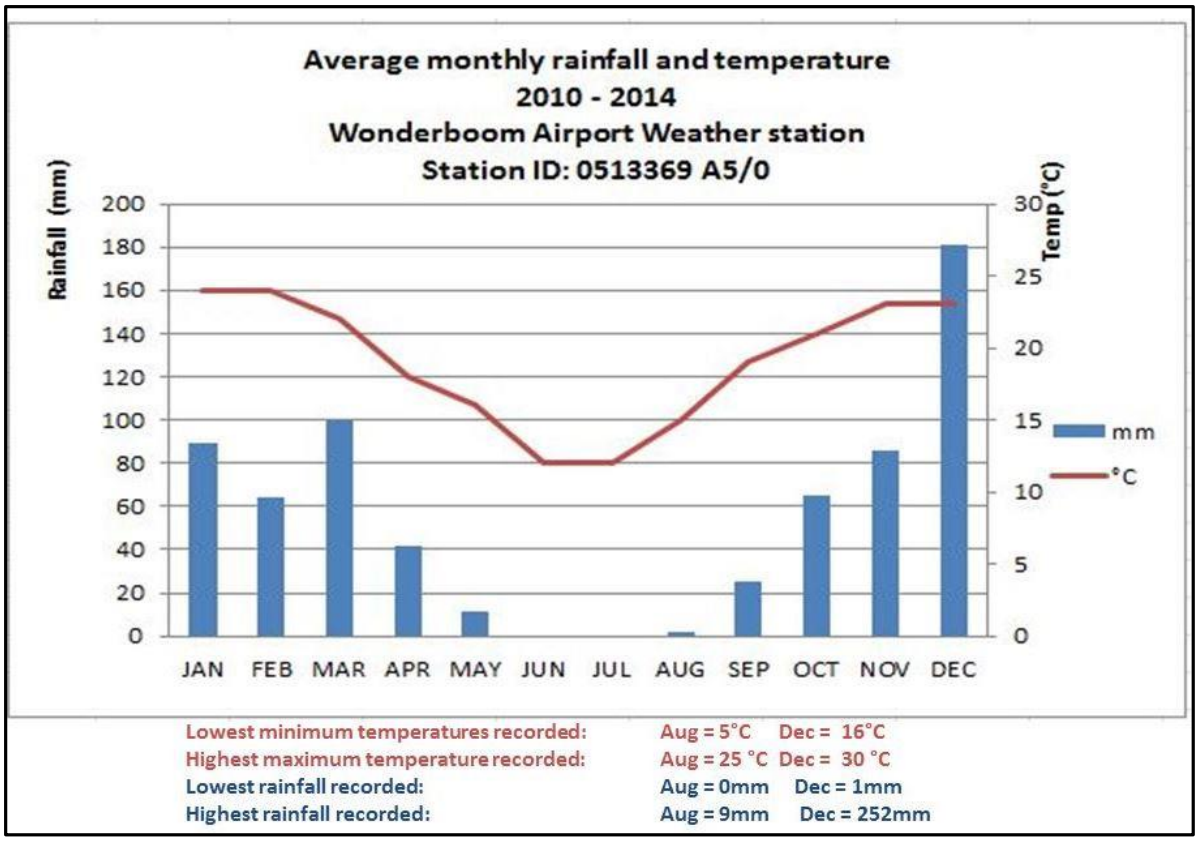

Figure 2.3: Average monthly rainfall and temperature as recorded by the Wonderboom Airport weather station

This Central Sandy Bushveld bioregion falls within the summer rainfall area of South Africa and experiences large differences in rainfall between seasons. According to Lynch (2004) this area receives on average 600mm of rain annually, mostly during the mid- to late summer months of January and February (Schulze, 2008). Mucina and Rutherford (2006) describe winters as very dry. It illustrates that the plants growing in this area would be exposed to dramatic differences in water availability between summer and winter. Data obtained from Wonderboom Airport weather station for 2010 to 2014 (Figure 2.3) indicates that the average monthly rainfall for the months in which plant material was collected (August and December) are 2mm and 181mm respectively (de Jager, 2015: Personal Communication).

2.5.2 Mpumalanga locality

Material from two plants was collected in the Blyde River Nature Reserve, within an area around S 24º 57' and E 30º 77'. It is situated at roughly 1150m above sea level and, according to Mucina & Rutherford (2006), falls within the Northern Escarpment Afromontane Fynbos bioregion of the Grassland Biome. It is characterised by sedimentary rock types (quartzite, shale, and dolomite) of the Transvaal Supergroup (Pretoria Group) according to Viljoen & Reimold (1999). The soil is described by the Department of Agricultural and Technical Services (1965) as red fersiallitic soil. The conservational status of the area is described by Ferrar & Lotter (2007) as well-protected (Figure 2.4).

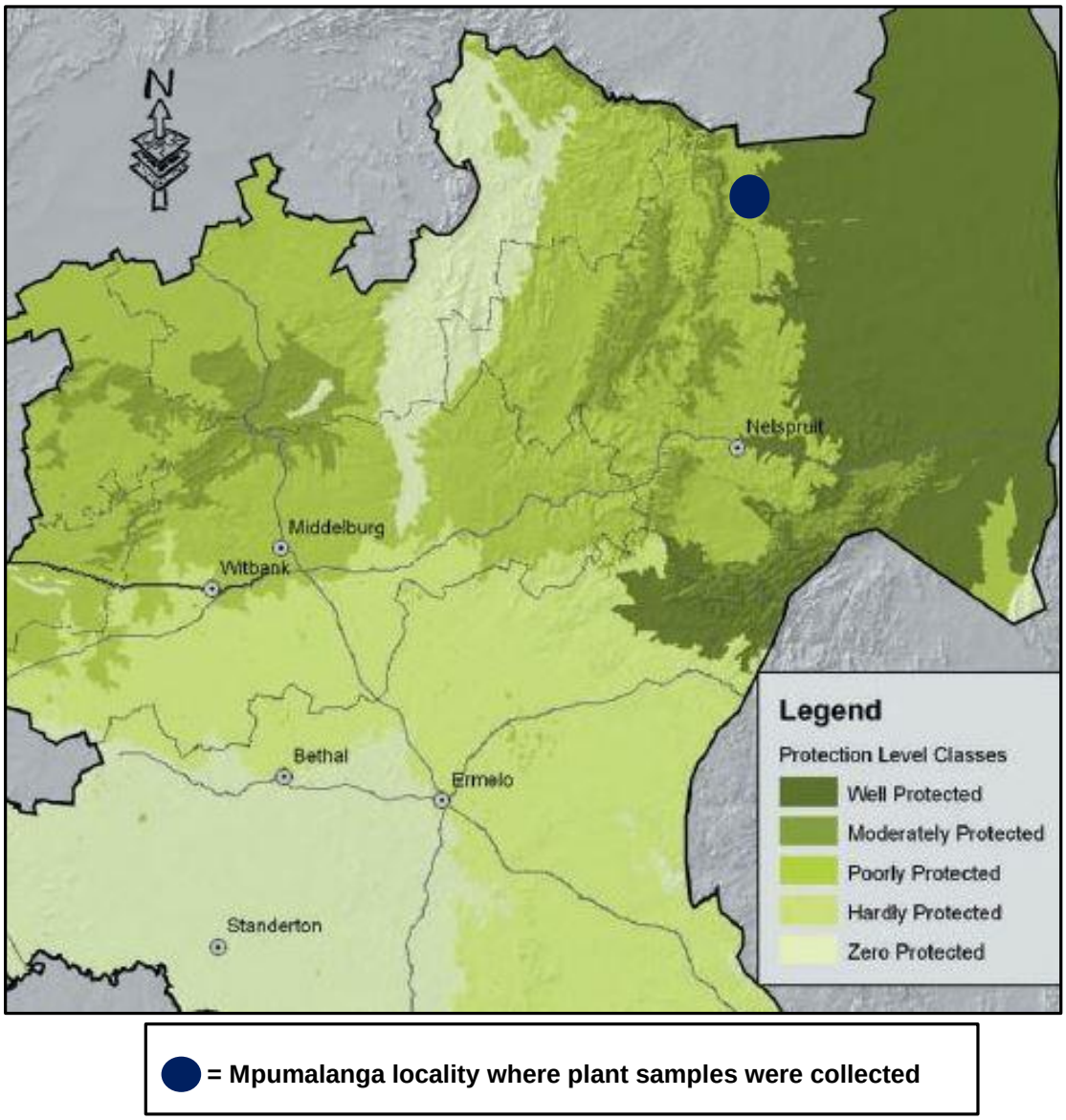

Figure 2.4: Conservation status of Mpumalanga locality where plant samples were collected (Ferrar & Lotter, 2007)

Data obtained from Lydenburg weather station was used to calculate average monthly temperatures and rainfall between 2010 and 2014 (de Jager, 2015: Personal Communication). This data is summarised in Figure 2.5. The average monthly temperatures for August and December are 14°C and 20°C respectively. The average minimum temperatures for the months in which plant material was collected (August and December) are 6°C and 15°C respectively. Average maximum temperatures for August and December are 22°C and 25°C respectively. The lowest and highest temperatures recorded in August during this period were 5°C and 24°C respectively. The lowest and highest temperatures recorded in December during this period were 14°C and 28°C respectively. This indicates that the vegetation in this bioregion will experience great differences between winter and summer temperatures. It is however important to note that Mucina and Rutherford (2006) state that the average temperatures in the study area are cooler than that of the surrounding areas.

Lötter & Beck (2004) state that rainfall in this area varies between 541 mm and 2776 mm per annum, depending on altitude. Mucina and Rutherford (2006) state that the annual summer rainfall is generally greater that 1400mm and is further augmented by mist during large parts of the year. Data obtained from Lydenburg weather station between 2010 and 2014 (Figure 2.5) indicates that the average monthly rainfall for the months in which plant material was collected (August and December) are 3mm and 127mm respectively (de Jager, 2015: Personal communication). Plants growing in this area will be exposed to great differences in water availability between winter and summer months, similar to the Gauteng locality.

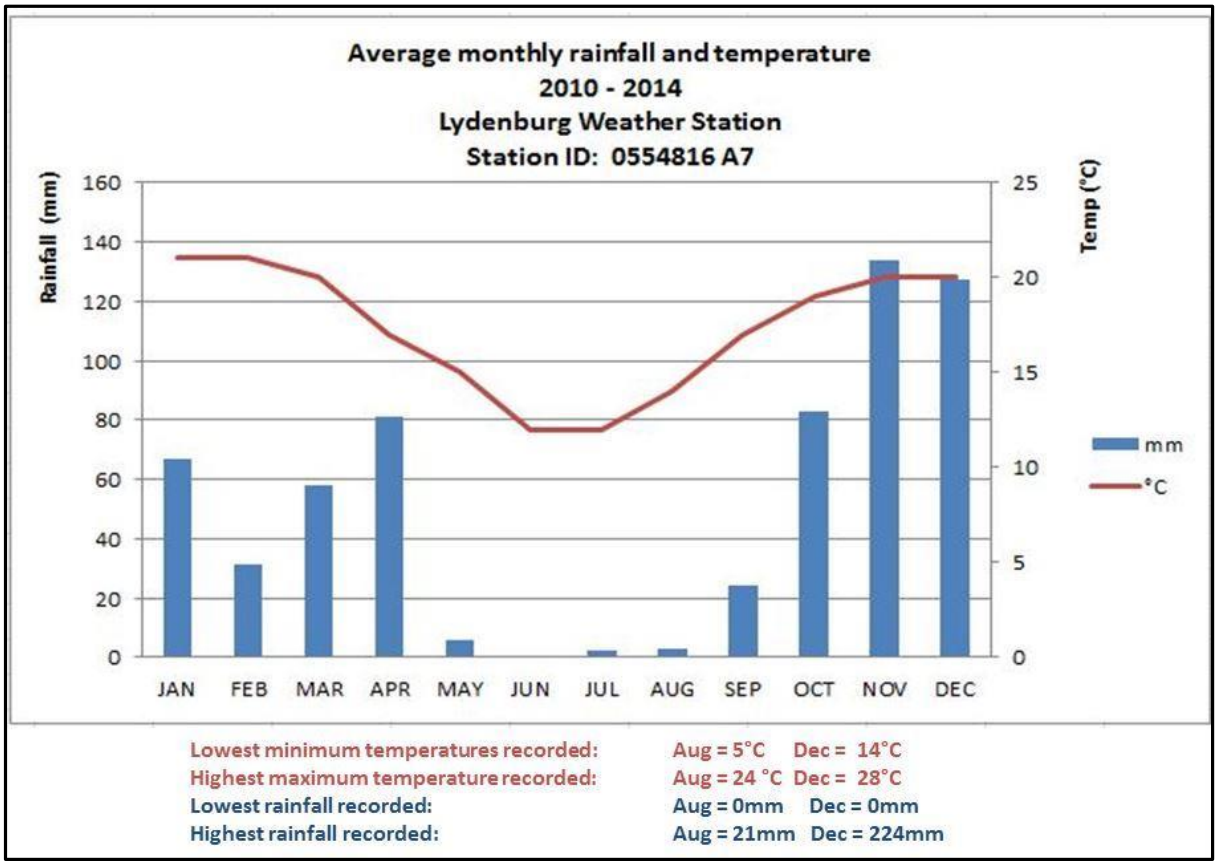

Figure 2.5: Average monthly rainfall and temperature as recorded by the Lydenburg weather station

2.5.3 Northern Cape locality

Material from three plants was collected in the Namaqualand area of the Northern Cape on the road between the settlements of Garies and Leliesfontein, in an area around S 30º 26' and E 18º 03'.

It has an elevation of roughly 770m above sea level and its geology is characterised by quartz-strewn plains and rocky granite outcrops (Olivier & Olivier, 2005). It is characterised by sedimentary rock types (quartzite, shale, and dolomite) of the Transvaal Supergroup (Pretoria Group) according to Viljoen & Reimold (1999). The soil is described by the Department of Agricultural and Technical Services (1965) as litholic soil with crystalline rock.

Natural vegetation is not under threat in the area where plant material was harvested and falls within an area described by BGIS (2007) as least threatened within the region (Figure 2.6). Cowling *et al.* (1999) defines this area as part of the Succulent Karoo biome of southern Africa and Low & Rebelo (1996) refer to the vegetation type as Upland Succulent Karoo.

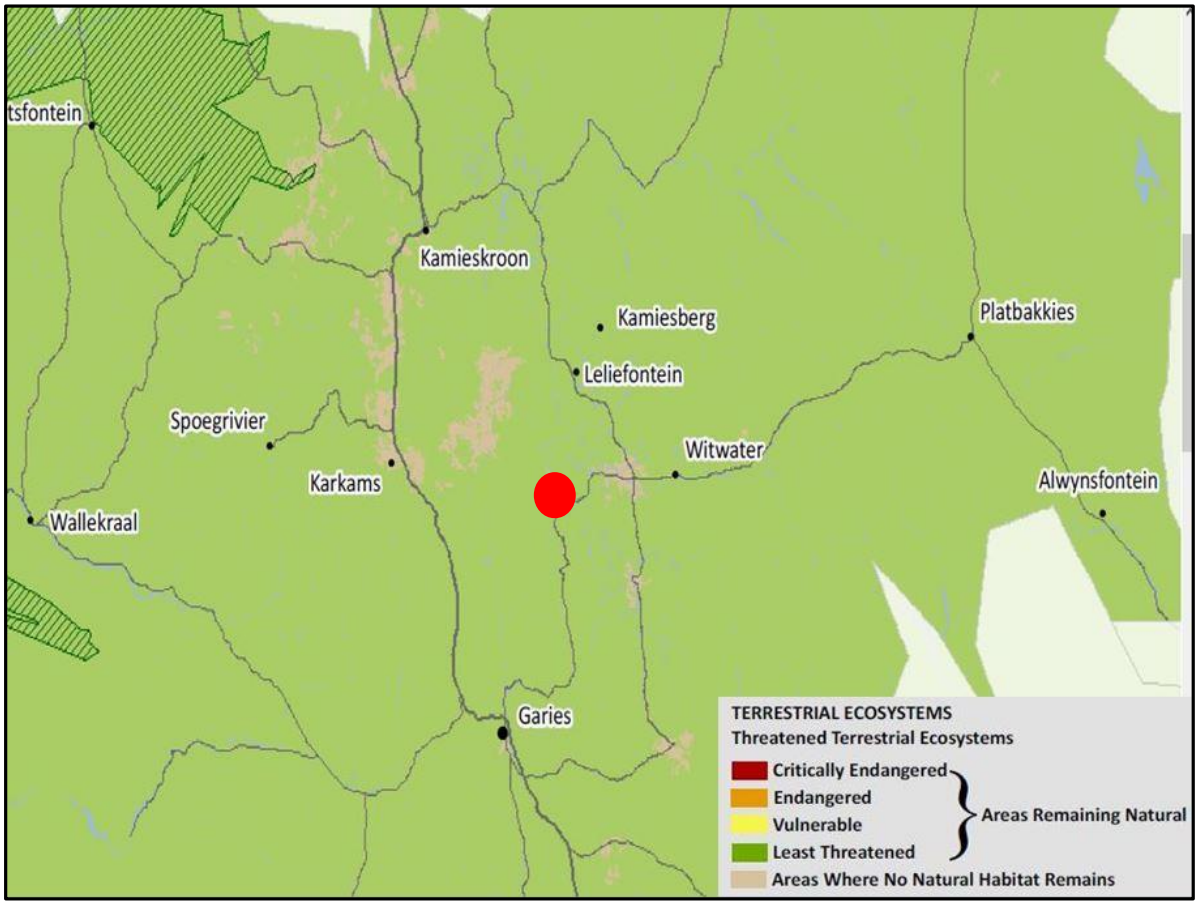

Figure 2.6: Conservation status of Northern Cape locality where plant samples were collected (SANBI (2009)

Mucina & Rutherford (2006) refer to summers as ‘hot’ and state that average maximum and minimum temperatures range between 30°C in January and 5°C in July and further state that frost occurs around 8 days a year. Data obtained from Springbok weather station was used to calculate average monthly temperatures and rainfall between 2010 and 2014 (de Jager, 2015: Personal Communication). This data is summarised in Figure 2.7. The average monthly temperatures for August and December are 12°C and 22°C respectively. The average minimum temperatures for the months in which plant material was collected (August and December) are 7°C and 15°C respectively. Average maximum temperatures for August and December are 17°C and 28°C respectively. The lowest and highest temperatures recorded in August during this period were 5°C and 21°C respectively. The lowest and highest temperatures recorded in December during this period were 12°C and 31°C.

The area is described by Cowling *et al.* (1999) as a winter-rainfall desert. Hoffman & Cowling (1987) state that the Namaqualand area receives an average of 150mm of rain per year and also mention that the rainfall is highly predictable. Mucina & Rutherford (2006) state that the area receives around 160mm of rain per year. They further explain that episodic periods of drought lasting 1-2 years occur in which less than 100mm of rain falls per annum. Dew is however present throughout the winter (Mucina & Rutherford, 2006). Data obtained from Springbok weather station between 2010 and 2014 (Figure 2.7) indicates that the average monthly rainfall for the months in which plant material was collected (August and December) are 38mm and 5mm respectively (de Jager, 2015: Personal communication).

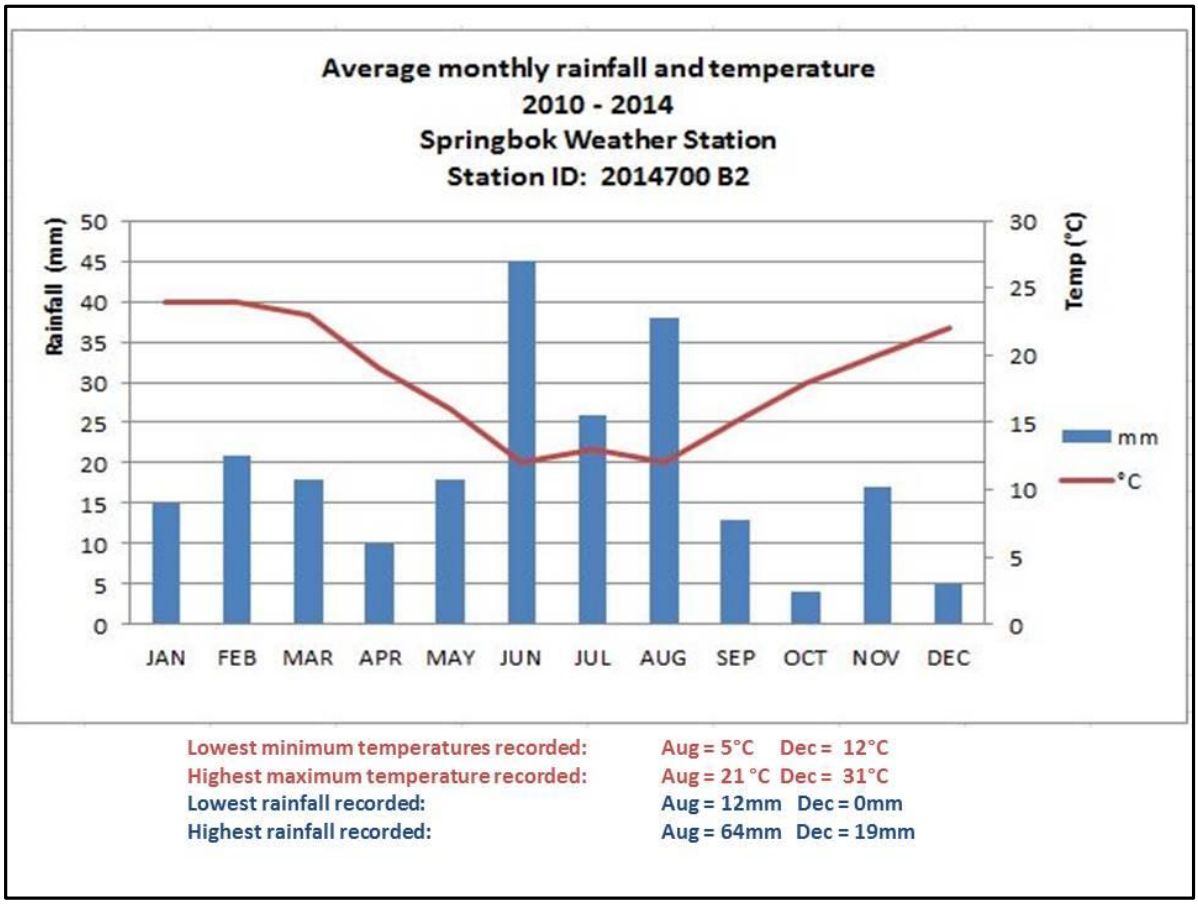

Figure 2.7: Average monthly rainfall and temperature as recorded by Springbok weather station

2.6 Methods of chemical analysis used in this study

2.6.1 Principles of metabolomics

Metabolites are the end product of all cellular processes and activities and are the outcome of enzymatic and protein activity. Metabolomics can be defined as the quantitative measurement of the metabolic response of living systems to pathophysiological stimuli or genetic modification (Worley & Powers, 2013). It can also be described simply as the isolation and identification of small molecules and has become possible with the advances in technology that allow effective compound separation, the determination of the exact mass of substances and the development of high-resolution, high-throughput nuclear magnetic resonance (NMR) spectrometers (Wishart, 2005). Aided by the development of chemometric software to rapidly process spectral or chromatographic patterns, metabolomics has made it

possible to investigate and accurately identify small-molecule metabolites. During this study, NMR spectroscopy was used to investigate the chemical composition of the roots, stems and leaves of *E. undulata* for the secondary metabolites in question.

2.6.2 Principles of NMR spectroscopy

NMR spectroscopy originated from the development of pulsed Fourier transform NMR spectroscopy by Ernst and Anderson. It was initially limited by low sensitivity and by the complexity of the NMR spectra it produced (Cavanagh *et al.,* 2007). The development of more powerful magnets, more sensitive spectrometers as well as the improvement of sample preparation techniques have contributed to NMR spectroscopy becoming a vital tool for studying biological systems, capable of providing detailed information on metabolites (Kim *et al.,* 2011). Advances have been made to enable NMR spectroscopy to accurately determine the three-dimensional structure of molecules at atomic resolution (Cavanagh *et al.,* 2007).

NMR spectroscopy measures the resonances of magnetic nuclei such as ^{1}H, ^{13}C and ^{15}N that interact with an external magnetic field. It provides non-invasive structural analysis of metabolites present in crude extracts, cell suspensions, intact tissues or whole organisms allowing in vivo analysis (Leiss *et al.,* 2011). The NMR gives information about the structure of a compound, by placing a substance in a strong magnetic field that affects the spin of its atomic nuclei.

The nuclei in the atoms in materials are spinning and charged (Chatham & Blackband, 2001) and often form magnetic dipoles which are usually display random orientation (Chatham & Blackband, 2001). When placed in a strong magnetic field the nuclei are reoriented and when the magnetic field is turned off the realignment of the nuclei generates a signal at radio frequencies. These signals are detected by a coil placed around the sample (Chatham & Blackband, 2001).

This pulse of energy released by the nuclei provides data on the molecular structure of the substance (Chatham & Blackband, 2001). The resulting NMR spectra are unique and specific for each single compound and can be used to identify metabolites of biological origin of which no pre-existing knowledge is needed (Leiss *et al.,* 2011).

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CHAPTER 3

Materials and methods

3.1 Study areas

To investigate the effect of seasonal changes as well as other environmental conditions on 7-methyl-juglone, epicatechin, lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid production, plant material was collected during the months of December and August in the provinces of Mpumalanga, the Northern Cape and Gauteng. The specific locations within each province are listed with reference to their GPS coordinates in Table 2.

The study areas fall within both the summer and winter rainfall areas of the country. Vegetation within the study area is exposed to dramatic seasonal changes in terms of temperature and rainfall between summer and winter months.

Several non-seasonal differences between the three study areas have also been noted in this study. These include differences in the underlying rock formations, soil type and altitude. Although it is not the aim of this study to identify which non-seasonal factors influence the presence of 7-methyl-juglone, epicatechin, lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in *E. undulata* it is proposed that a comparison between plant materials harvested at different times of the year might provide insight into whether the presence of these metabolites is more strongly influenced by seasonal or non-seasonal environmental conditions.

3.2 Collection of plant materials

Stem, leaf and root bark samples were collected from eight individual plants in the provinces of the Northern Cape, Gauteng and Mpumalanga. These samples were collected during the winter months of July and August 2013 as well as the summer months of December 2013 and October 2015 to represent the dry and rainy seasons within the summer and winter rainfall areas of South Africa respectively. Care was taken to limit damage to the plants and the parts that were cut were treated to prevent fungal infection after harvesting.

Plants were tagged and GPS coordinates and rainfall season were recorded during the collection of materials (Table 2). Voucher specimens were authenticated and deposited at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria.

After collection the samples were dried at room temperature and ground to a homogenous powder.

Table 2: Geographical information and voucher specimens of *Euclea undulata* Thunb. var *myrtina* collected during this study

E. undulata* Thunb. var *myrtina	**GPS location**	**Rainfall season**	**Province**	**Voucher specimen**
Plant 1	S 25º 28' 57.2" E 28º 27' 18.5"	Summer	Gauteng	PRU121023
Plant 2	S 25º 28' 57.1" E 28º 27' 18.3"	Summer	Gauteng	PRU121024.
Plant 3	S 25º 28' 52.4" E 28º 27' 20.3"	Summer	Gauteng	-
Plant 4	S 24º 57' 32.2" E 30º 77' 93.7"	Summer	Mpumalanga	PRU 121691
Plant 5	S 24º 57' 63.2" E 30º 77' 88.9"	Summer	Mpumalanga	PRU 121692
Plant 6	S 30º 26' 06.9" E 18º 03' 38.7"	Winter	Northern Cape	STEYN 2116
Plant 7	S 30º 26' 07.5" E 18º 03' 37.7"	Winter	Northern Cape	STEYN 2117
Plant 8	S 30º 26' 07.7" E 18º 03' 37.5"	Winter	Northern Cape	STEYN 2118

3.3 Extraction of compounds

Metabolomic analysis

The sample preparation, extraction, data acquisition, analysis, data mining and processing were performed by adapting the standard method (Nkomo *et al.,* 2014; Kim & Verpoorte, 2010).

3.3.1 Sample preparation and extraction method

Plant material was harvested in both the summer and winter rainfall areas during August and December 2013/October 2015 and a sample of the leaves, stems and root bark of each plant was prepared by chopping and air drying at room temperature. A total of eight samples of each organ was prepared, each from the Northern Cape area serving as a replicate for the winter rainfall area and each from Gauteng and Mpumalanga serving as a replicate for the summer rainfall area. A powdered sample of 50 mg per treatment was weighed in 2 mL Eppendorff tubes for extraction and analysis. Added to the samples was 0.75 mL of CH_3OH-*d4* (without any standard) and 0.75 mL of potassium dihydrogen phosphate (KH_2PO_4), buffered in deuterium water (D_2O) (pH 6.0) containing 0.1% (w/w) TSP (Trimethylsilylpropionic acid sodium salt). The Eppendorff tubes were vortexed at room temperature for 1 minute, ultrasonicated for 20 minutes at 30°C and then centrifuged for 20 minutes using a microtube centrifugator (13000 rpm, room temperature). The supernatant (more than 1 mL) was transferred to a 1.5 mL Ependorff tube and 800 µL of supernatant was then transferred to a 5mm NMR tube to be subjected to NMR analysis.

3.3.2 Data acquisition and sample analysis

NMR spectral data were obtained using a 600 MHz ^{1}HNMR spectrometer (Varian Inc, California, USA).

The phasing and baseline corrections were conducted using MestReNova software (10.0.2, Mestrelab Research, Spain) with consistent settings for all sample spectra. The chemical shift ranges of methanol (δ 3.17-3.20 ppm) and water (δ 4.4-4.6 ppm) were excluded (Nkomo *et al.,* 2014) and remaining regions between 0.00 and 15.5 ppm were normalized for further analysis.

3.3.3 Data mining and processing

All proton spectra were manually phased, baseline corrected and referenced to TSP (δ 0.00 ppm) using MestReNova software. NMR spectra were bucketed with equal bin width of 0.04 ppm over a region of 0.00 to 15.5 ppm after completion of the phase and baseline corrections. The spectral regions from 4.4 to 4.6 ppm were excluded to eliminate the effects of residual water. Binned data was normalised to the total sum with reference to TSP (δ 0.00 ppm). The ASCII converted data sets were then imported into SIMCA (version 13.0.3; Umetrics) for multivariate data analysis.

Okada *et al.,* (2010) state that multivariate analysis can be used to statistically process large amounts of analytical chemistry data that results from the simultaneous analysis of metabolites. Similar methods of analysis were used in this study to investigate the presence of 7-methyl-juglone, epicatechin, lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in the collected plant material. Multivariate analysis techniques were performed by unsupervised principle component analysis (PCA), orthogonal partial least square discriminatory analysis (OPLS-DA) and hierarchical cluster analysis (HCA) (Mncwangi *et al.,* 2014). This was done using SIMCA-P software (13.0, Umetrics, Sweden) and the Parreto scaling method.

PCA is an unsupervised analysis (Mncwangi *et al.,* 2014) performed to provide an overview of the data and cluster the observations in the form of score scatter plots and loading plots. Scatter score plots from the PC analysis were constructed to identify and evaluate groupings, trends and strong outliers (Mncwangi *et al.,* 2014). The second phase of analysis, the OPLS-DA, is a supervised pattern recognition method (Bylesjö, *et al.,* 2006) of which the main purpose is to separate the systematic variation in the X-matrix into two parts with one part linearly related to the Y-matrix and one that is unrelated to the Y-matrix.

According to Lourenço *et al.* (2013) R2 values of the OPLS-DA model can be considered as measures of goodness of model while Q values indicate its robustness. R2 is the fraction of variance explained by a component. Cross validation of this component provides Q2, which describes the fraction of the total variation predicted by a component. The value of Q2 ranges from 0 to 1 and a Q2 value greater than 0.4 is considered indicative of a good model while those with Q2 values over 0.7 are considered robust (Lourenço *et al*., 2013).

3.3.4 Annotation

Previously published data was used for annotation of compounds that were responsible for separations between treatment samples (Deutschländer *et al*., 2010; Khattar *et al.,* 2015; Wishart *et al.,* 2013; van der Kooy, 2007).

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CHAPTER 4

Results

To investigate the presence of the secondary metabolites epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid, lupeol and 7-methyl-juglone in *E. undulata*, the collection of roots, stems and leaves was done during both the rainy and dry seasons of summer and winter rainfall areas. The results were subjected to statistical analysis in order to determine similarities and differences in terms of chemical composition. Additional chemical analysis of plant material was then performed to determine in which of the harvested plant materials these metabolites were present. Results yielded from the statistical and chemical analyses were then compared to determine whether the production of these metabolites is under seasonal control.

Temperature and rainfall are two of the most prominent aspects of seasonal change. Plants from the summer rainfall area would be exposed to higher levels of rainfall during months in which temperatures are high while experiencing lower levels of rainfall during months when temperatures are low. Conversely, plants from the winter rainfall area will experience higher levels of rainfall during months when temperatures are low and low levels of rainfall during months in which temperatures are high.

In an attempt to investigate the possible effect of temperature and rainfall, the rainy seasons and dry seasons of the two areas were compared respectively. Due to the fact that the rainy and dry seasons of the two regions occur at different times of the year, the plant material in each comparison would therefore have been exposed to similar conditions in terms of water availability while having been exposed to notable differences in temperature. Chemical similarities within rainy or dry seasons might therefore possibly be the result of similar levels of water availability and could indicate that water plays an important role in the production of a specific metabolite.

Differences in chemical composition between rainy and dry seasons might be the result of the differences in temperature experienced by plants within the two regions and could be an indication that temperature has greater influence than water availability on the production of a certain metabolite.

It is important to note that although temperature and rainfall fluctuations represent two of the most prominent seasonal differences between the two regions investigated, there might be other factors that determine whether the production of secondary metabolites is seasonal or not. For this reason, each region was investigated individually by comparing plant material from its rainy and dry seasons. Although these comparisons might not highlight the specific seasonal changes responsible for the production of a particular metabolite, the presence of a specific compound during one season while being absent from the same plant during a different season might indicate that its production is seasonal. Conversely, if a metabolite is found to be present during both seasons it could possibly suggest that its production is not under seasonal control.

The various comparisons made from the results of the statistical and chemical analysis of the harvested plant material can be summarised as follows:

- Comparison of rainy and dry seasons of the summer rainfall area
- Comparison of rainy and dry seasons of the winter rainfall area
- Comparison of rainy seasons of the winter and summer rainfall areas
- Comparison of dry seasons of the winter and summer rainfall areas

The spectral regions for primary metabolites such as glucose and sucrose that are likely to be present in high concentrations were removed from data before statistical and chemical analysis in order to more accurately detect variations in secondary metabolites that were possibly present in smaller concentrations.

4.1 Statistical analysis of plant material

SIMCA software (version 14.0; Umetrics) was used for statistical analysis and comparisons. A principal component analysis (PCA) was done first as unsupervised clustering to identify similarities or differences between sample profiles (Mncwangi *et al.,* 2014). Grouping, trends and outliers were examined from scatter plots generated and cross validation was done by creating hierarchical cluster diagrams (Jung *et al.,* 2011). Orthogonal partial least-squares discriminant analysis (OPLS-DA) was then used to also create score scatter plots to evaluate variations in buckets between groups, followed by cross validation (Jung *et a*l., 2011).

4.1.1 Statistical comparison of rainy and dry seasons of the summer rainfall area

Clustering that was obtained from principal component analysis (R2X = 0.582, Q2 = -0.0601) in Figure 4.1. indicates a split between material harvested during the dry season and that harvested during the rainy season. Within these two seasonal delineations it can be seen that roots, stems and leaves group separately.

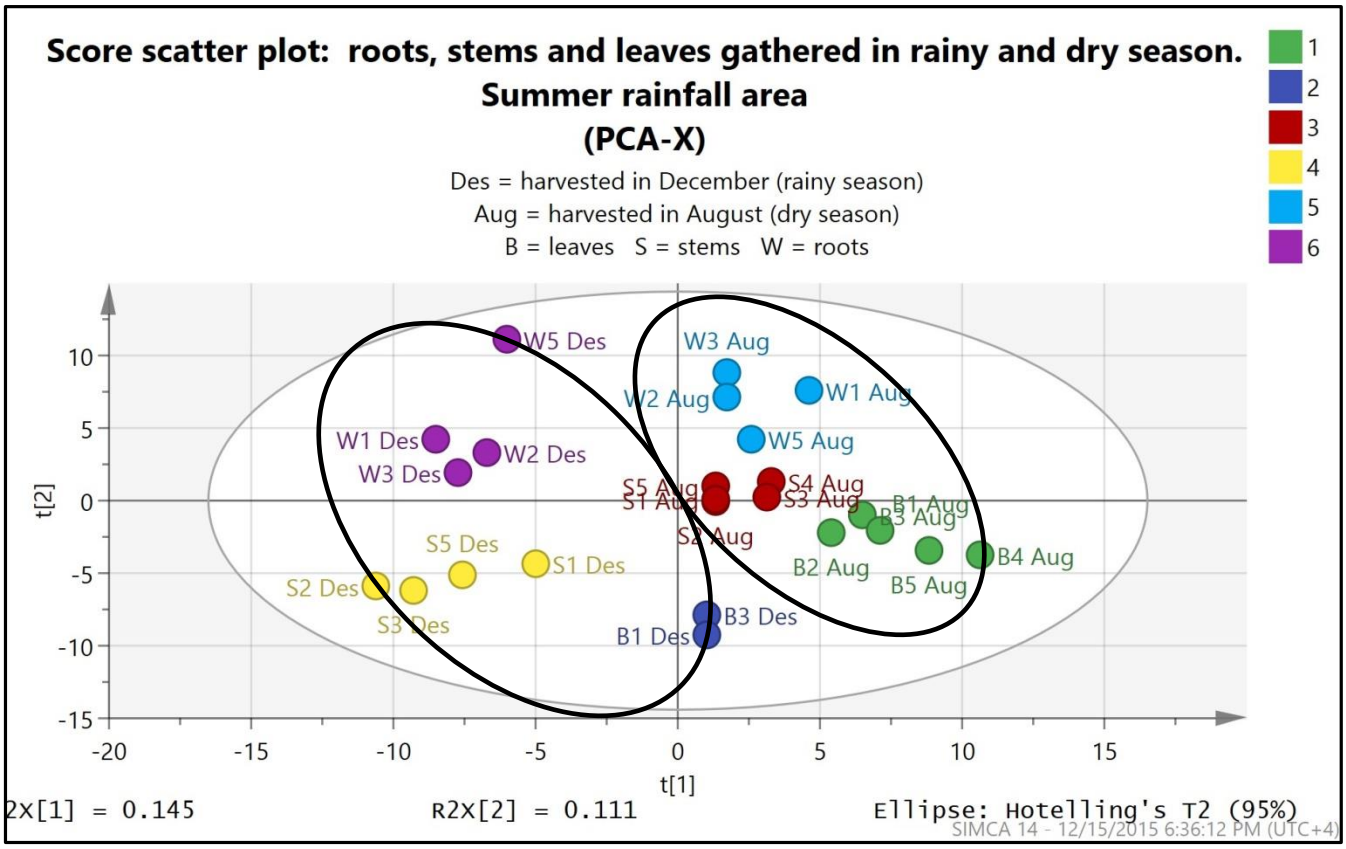


Figure 4.1: Score scatter plot (PCA – X) of metabolites in root, stem and leaf material of *E. undulata* gathered during rainy and dry seasons in summer rainfall area

Further evidence of this can be seen when validating this score scatter plot using a hierarchical clustering diagram (Figure 4.2.) which indicates delineation between the two seasons with the notable exception of the leaves from the rainy season associating more strongly with the material from the dry season than with the other material from the rainy season. It is furthermore evident that there are also delineations between roots, stems and leaves within the respective seasons.

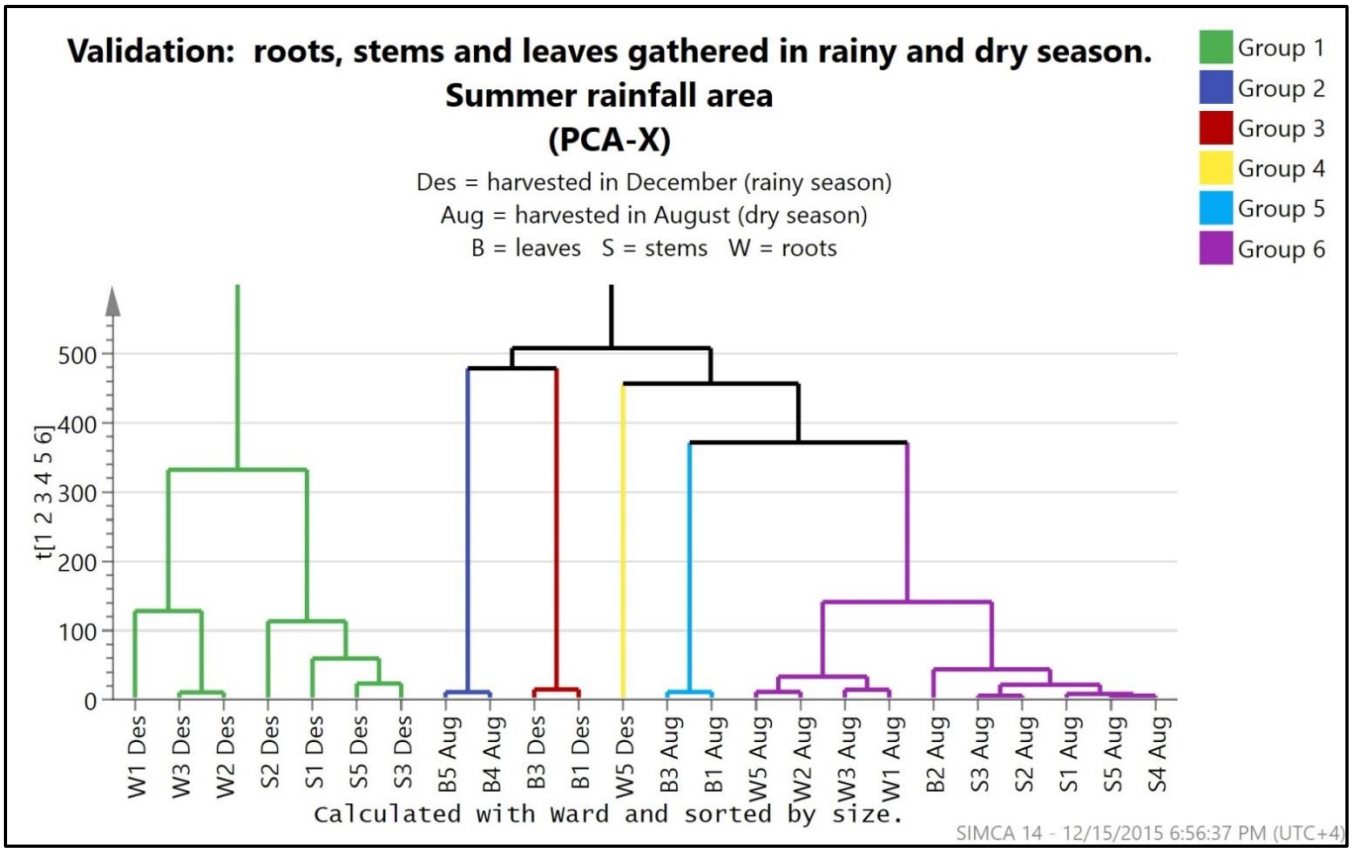


Figure 4.2: Hierarchical cluster diagram for validation of PCA-X model of root, stem and leaf material of *E. undulata* gathered in rainy and dry season of summer rainfall area

The clusters obtained from the score scatter plot of the OPLS-DA model (R2X = 0.513, R2Y = 0.951, Q2 = 0.671) in Figure 4.3 also suggests clear distinction between the plant material harvested during the dry season and material harvested during the rainy season. It is also apparent that leaves from the rainy season forms a cluster relatively closely to the leaves of the dry season.

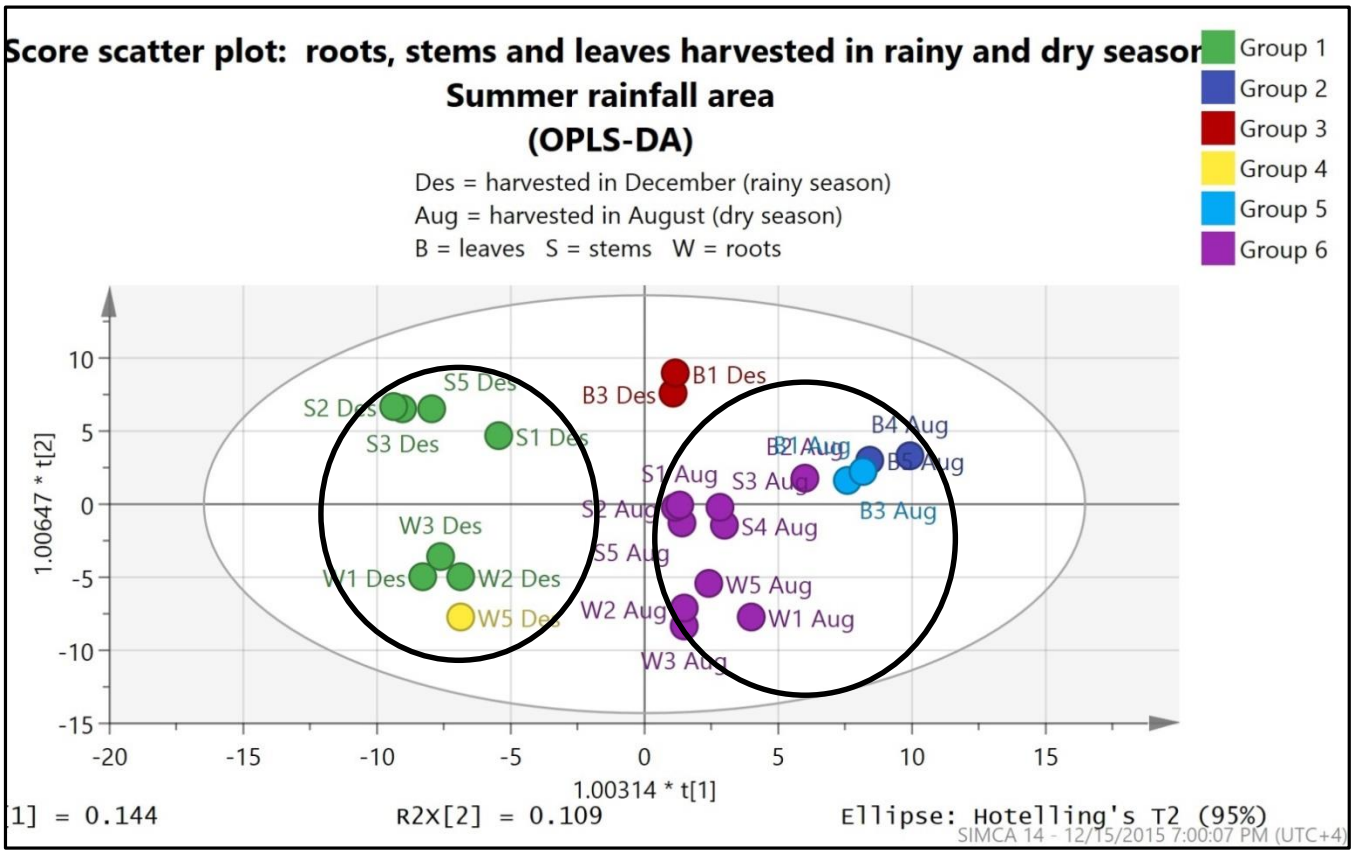


Figure 4.3: Score scatter plot (OPLS-DA) of metabolites in root, stem and leaf material of *E. undulata* gathered during rainy and dry seasons in summer rainfall area

Groupings formed in the hierarchical cluster diagram of the OPLS-DA model (Figure 4.4) also suggest statistically significant differences between the rainy and dry seasons with the notable exception of the leaves from the rainy season associating more strongly with the material from the dry season than with the other material from the rainy season. When examining data for the rainy and dry seasons individually it can be seen that roots, stems and leaves separate into individual clusters. It can also be seen that stems and roots associate more closely with each other than with leaves in material from both seasons. Leaves from the rainy and dry season cluster closely together, suggesting possible chemical similarities.

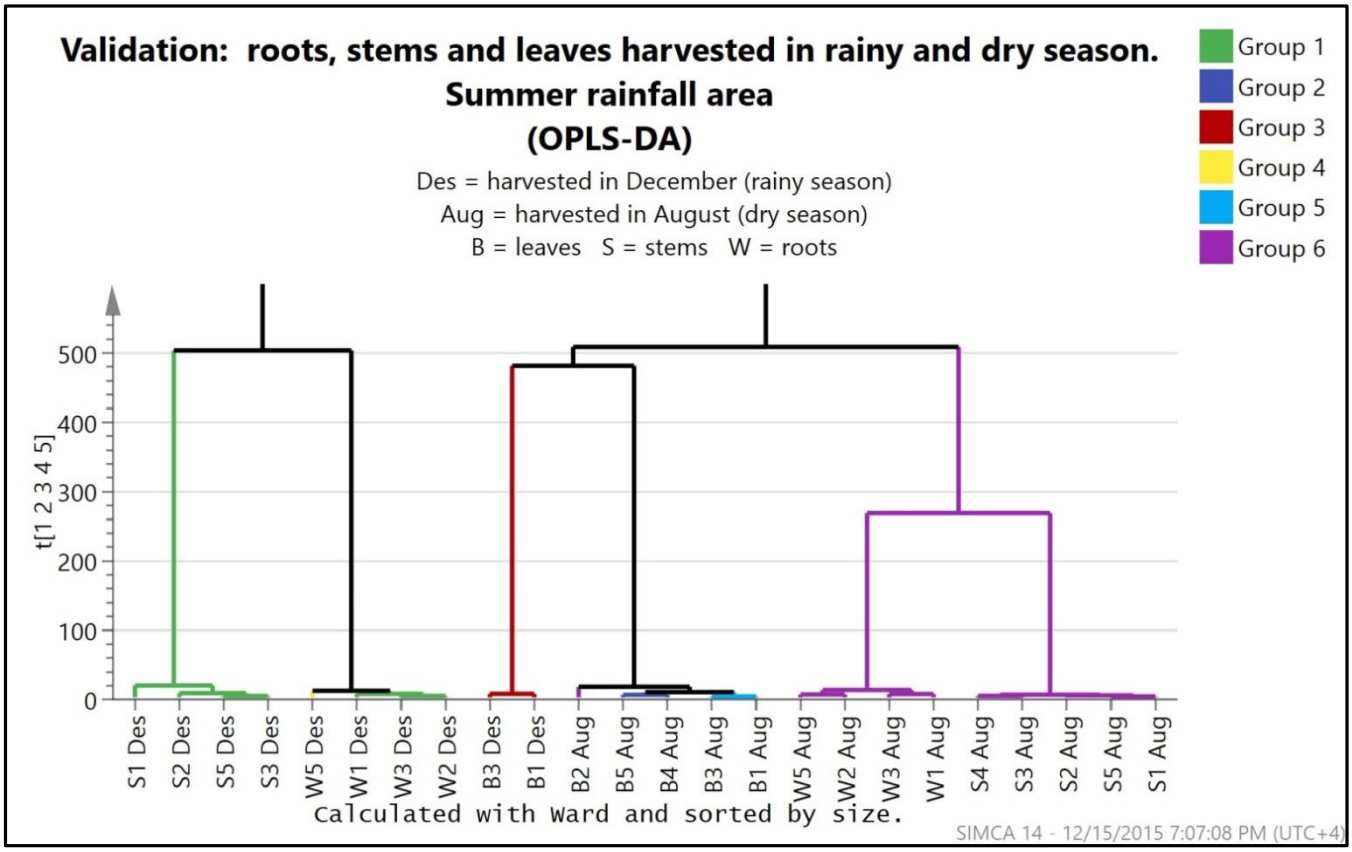


Figure 4.4: Hierarchical cluster diagram for validation of OPLS-DA model of root, stem and leaf material gathered in rainy and dry season of summer rainfall area

4.1.2 Statistical comparison of rainy and dry seasons of the winter rainfall area

Clustering obtained from principal component analysis (R2X = 0.738, Q2 = 0.07) in Figure 4.5. indicates a split between material harvested during the dry season and rainy seasons. Within this seasonal split, material from the dry season groups together closely while material from the rainy season displays separation of leaf material from that of stems and roots.

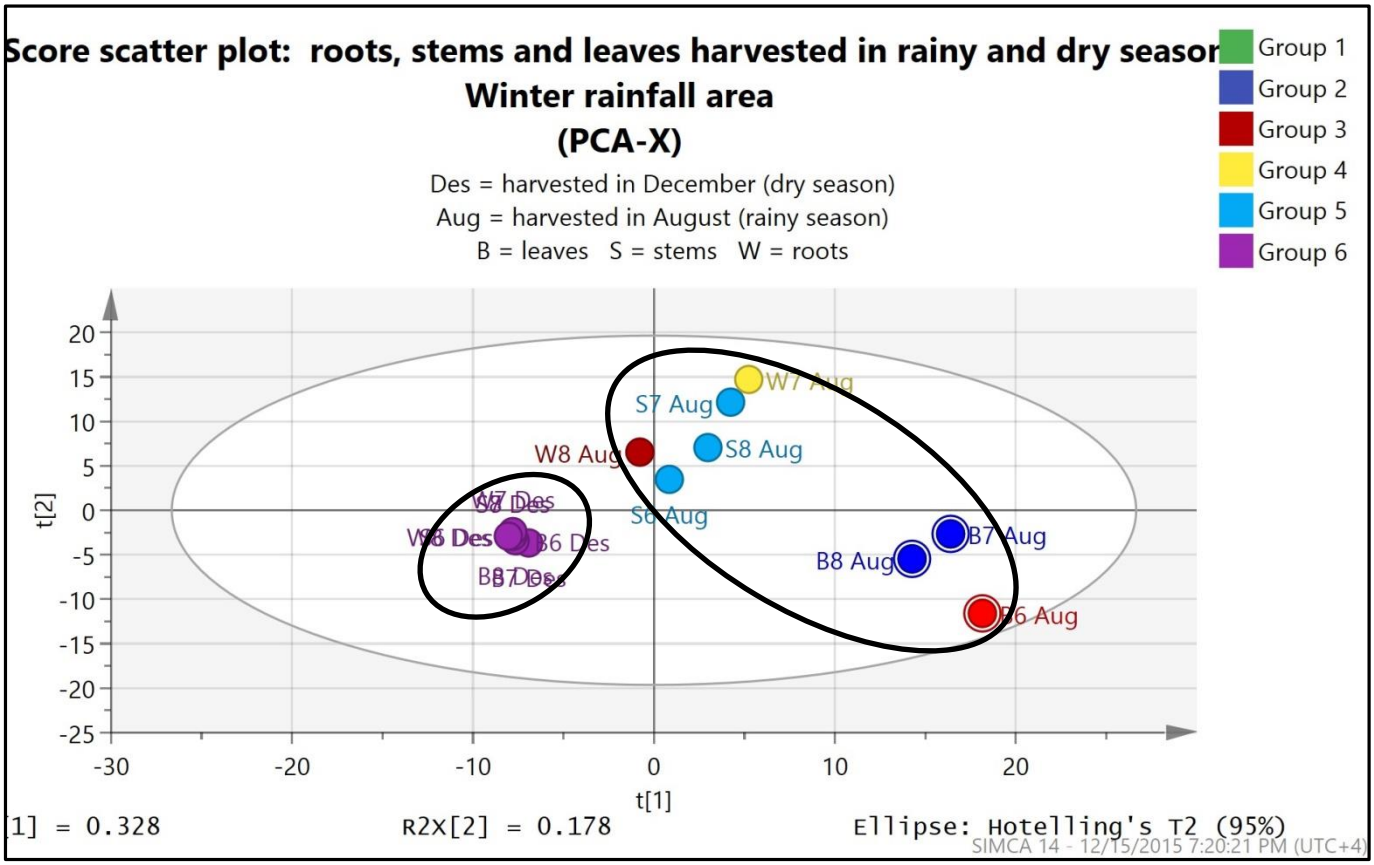


Figure 4.5: Score scatter plot (PCA – X) of metabolites in root, stem and leaf material of *E. undulata* gathered during rainy and dry seasons in winter rainfall area

Further evidence of this can be seen when validating this score scatter plot using a hierarchical clustering diagram (Figure 4.6.) which indicates delineation between the two seasons. Within this seasonal split, slight overlap is visible among groupings of organs from the dry season while organs from the rainy season split into separate groups.

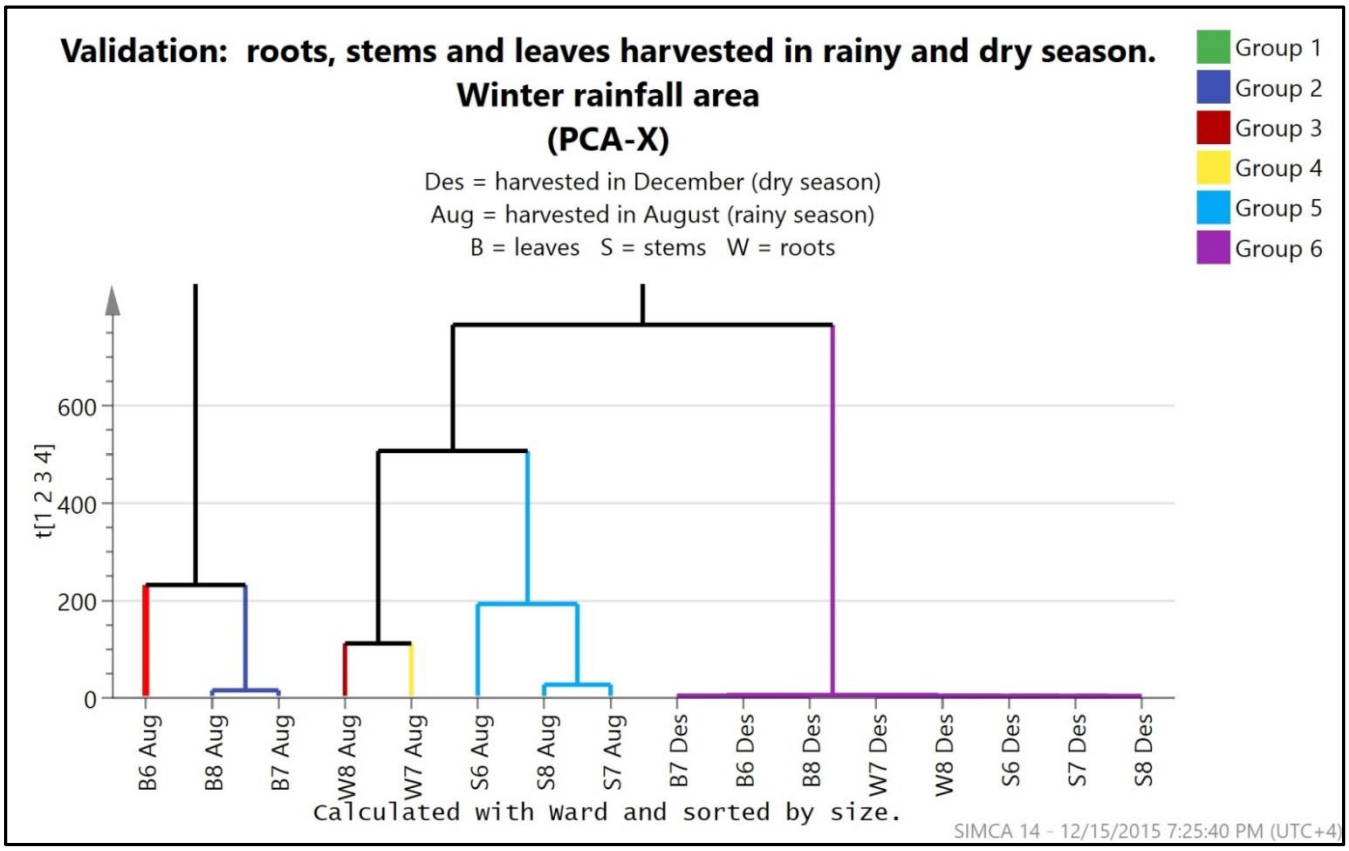


Figure 4.6: Hierarchical cluster diagram for validation of PCA-X model of root, stem and leaf material of *E. undulata* gathered in rainy and dry season of winter rainfall area

The clusters obtained from the score scatter plot of the OPLS-DA model (R2X = 0.709, R2Y = 0.596, Q2 = 0.341) in Figure 4.7 suggests clear distinction between the plant material harvested during the dry and rainy seasons. Within this seasonal split, material from the dry season groups together closely together and forms a cluster relatively close to stem and root material from the rainy season. Stem and root material from the rainy season cluster together while leaf material from the rainy season forms a separate group.

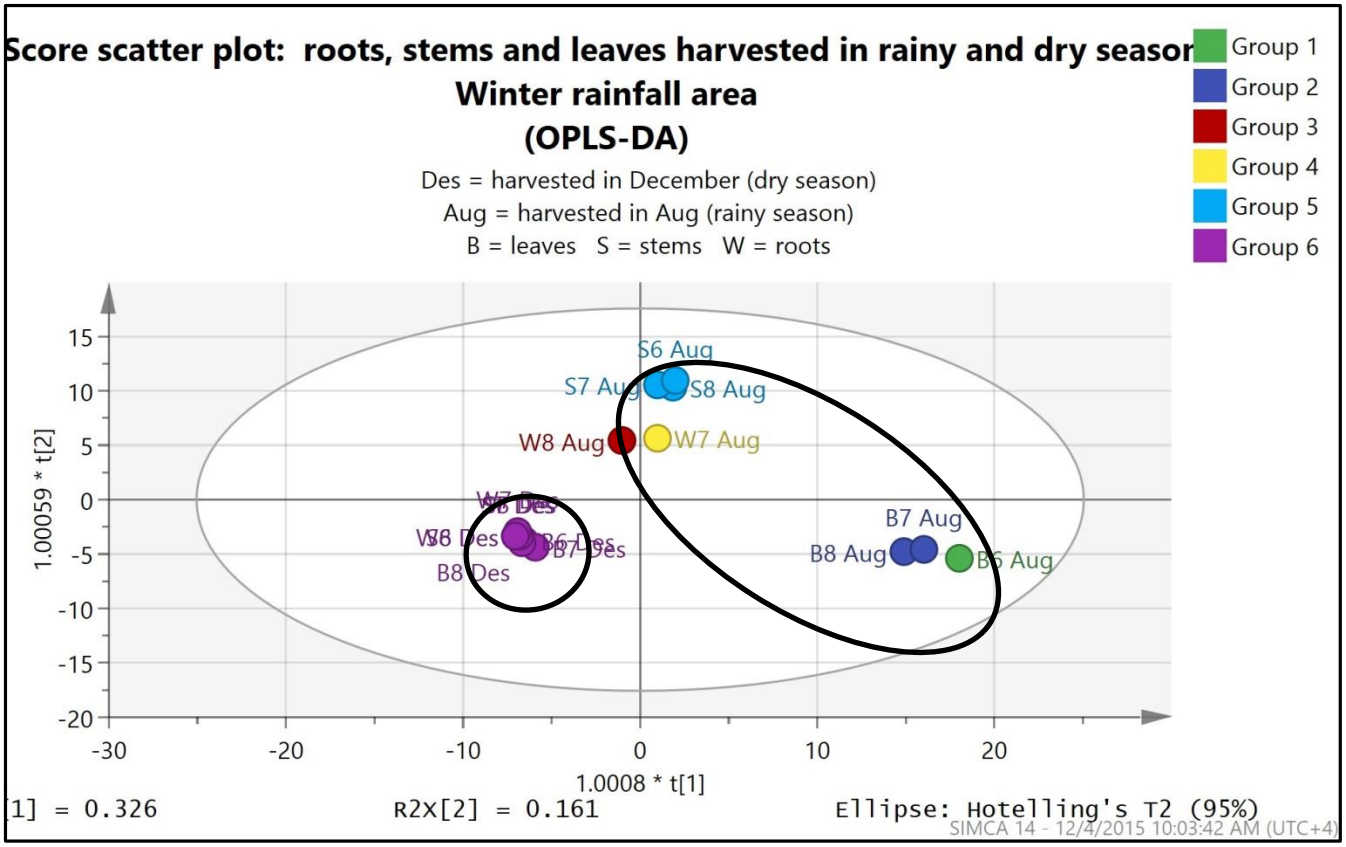


Figure 4.7: Score scatter plot (OPLS-DA) of metabolites in root, stem and leaf material of *E. undulata* gathered during rainy and dry seasons in winter rainfall area

Groupings formed in the hierarchical cluster diagram of the OPLS-DA model (Figure 4.8) also suggest statistically significant differences between the rainy and dry seasons. Within this seasonal split, groupings that separate organs from each other are also evident. Roots and stems from the rainy seasons affiliate more closely to material from the dry season than to leaves from the rainy season.

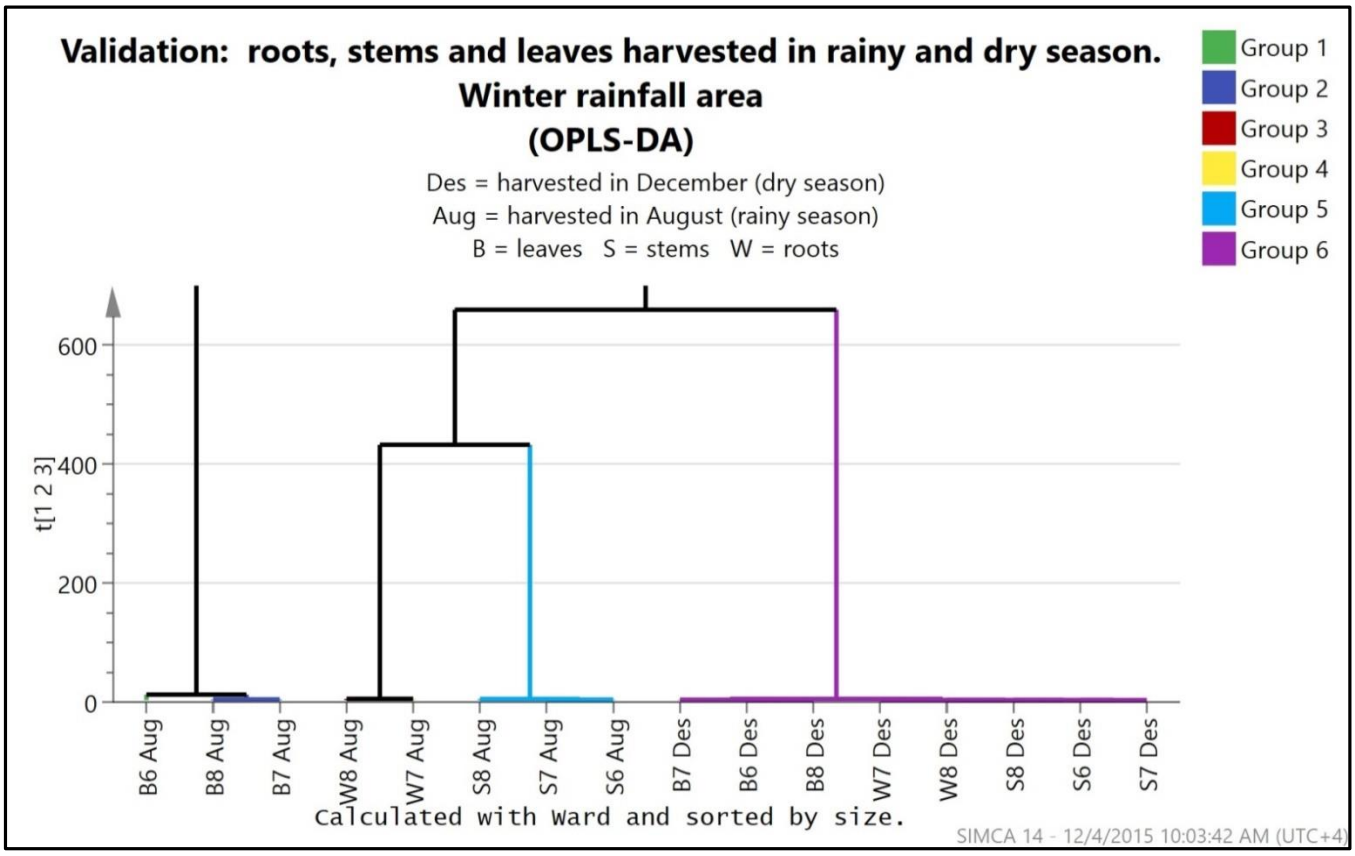


Figure 4.8: Hierarchical cluster diagram for validation of OPLS-DA model of root, stem and leaf material gathered in rainy and dry season of winter rainfall area

4.1.3 Statistical comparison of rainy seasons of the winter and summer rainfall areas

In spite of a minor degree of overlapping among certain clusters obtained from the principal component analysis (R2X = 0.461, Q2 = 0.0791) in Figure 4.9 three clear groupings of roots, stem and leaf materials are evident. A seasonal split between plant material from the two rainfall seasons is also visible.

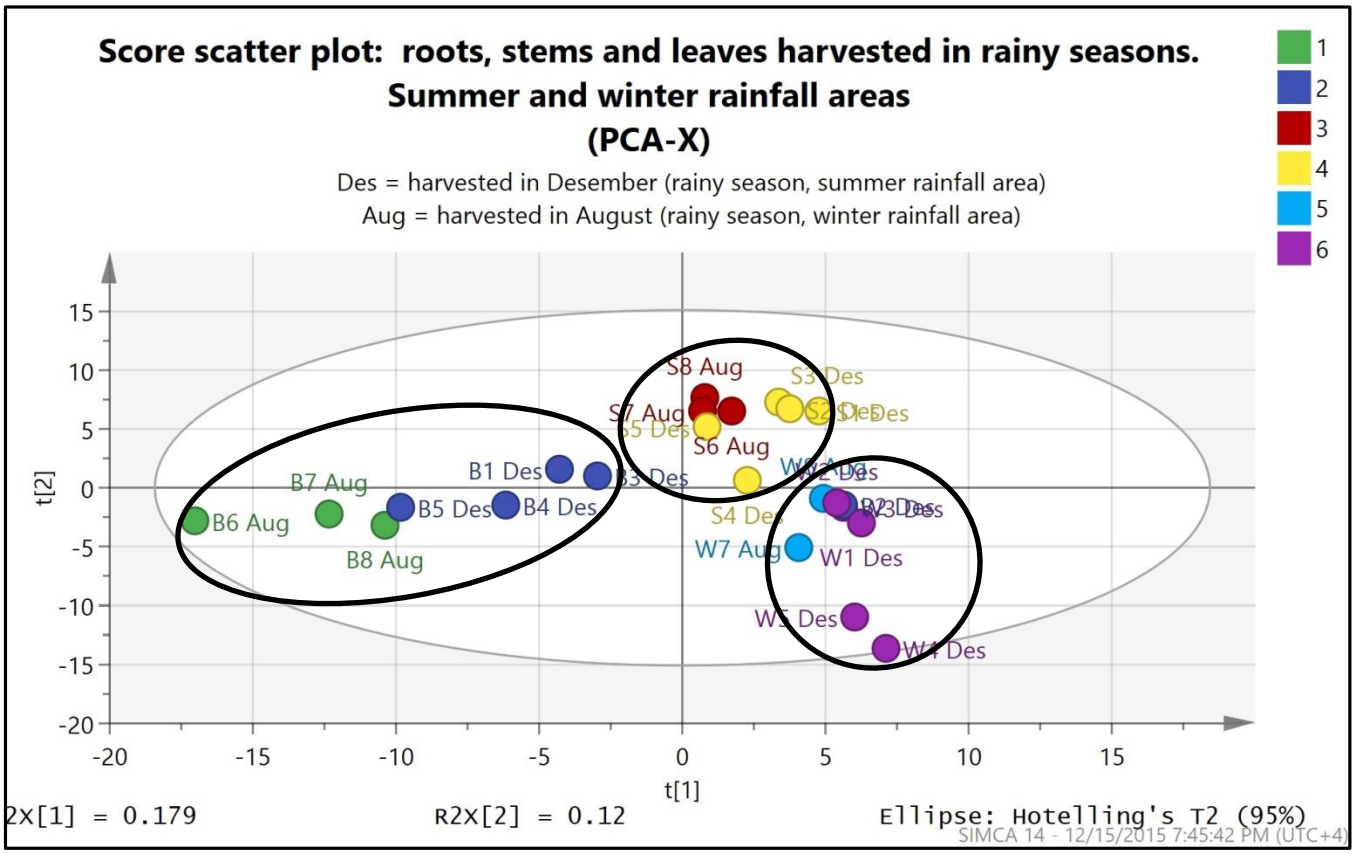


Figure 4.9: Score scatter plot (PCA – X) of metabolites in root, stem and leaf material of *E. undulata* gathered during rainy seasons of summer and winter rainfall areas

Although the hierarchical clustering for this model in Figure 4.10 indicates slight discrepancies and overlap among groupings, delineations suggest a clear clustering of roots, stems and leaves into three groups. Within these groups, the clusters indicate notable delineation between summer and winter rainfall areas.

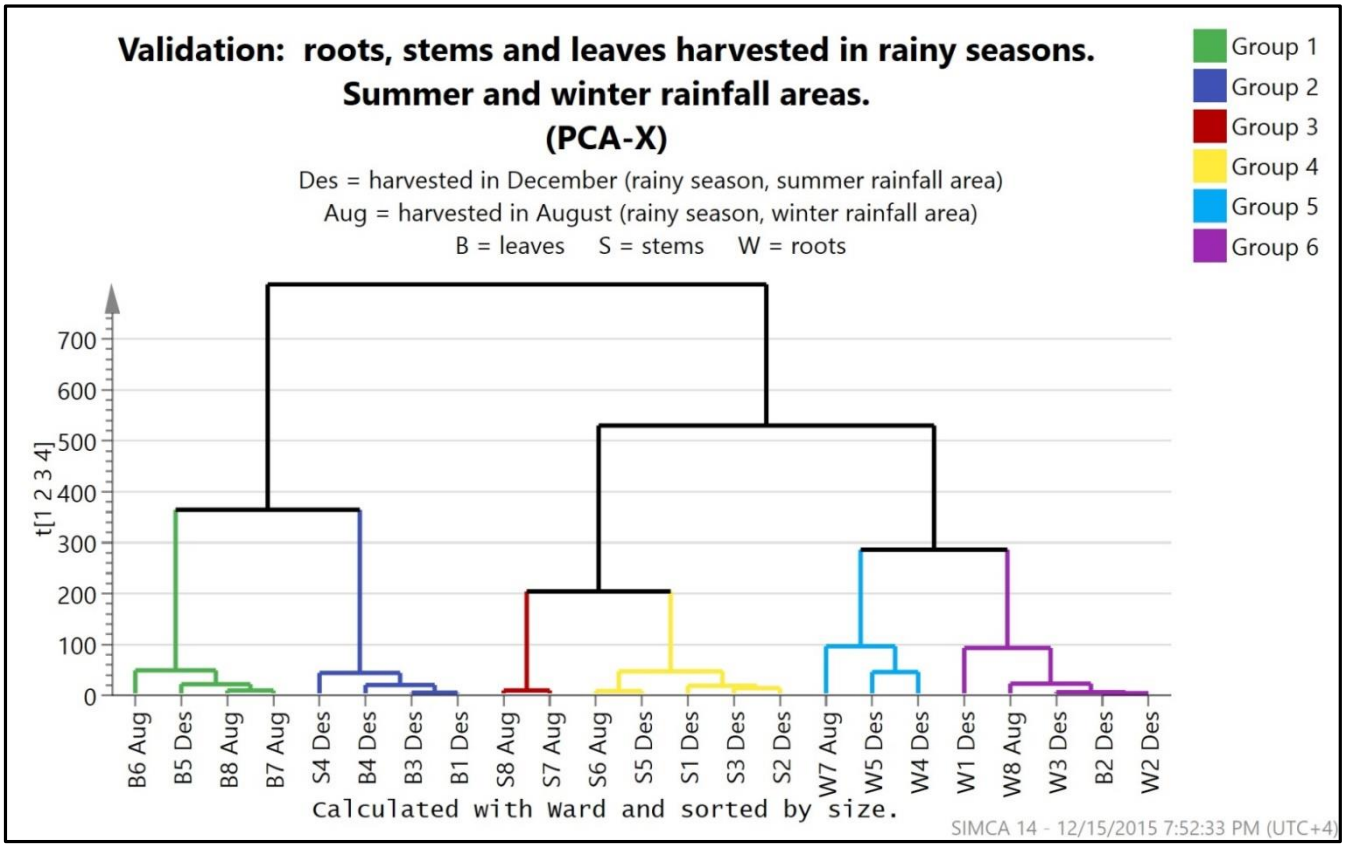


Figure 4.10: Hierarchical cluster diagram for validation of PCA-X model of root, stem and leaf material of *E. undulata* gathered in rainy seasons of summer and winter rainfall areas

Orthogonal partial least-squares discriminant analysis (OPLS-DA) was used to create a score scatter plot to evaluate variations in buckets between groups, followed by cross validation (Jung *et al*., 2011). The groupings obtained from the score scatter plot of the OPLS-DA model (R2X = 0.492, R2Y = 0.909, Q2= 0.439) in Figure 4.11 indicate three clear delineations in terms of root, stem and leaf material.

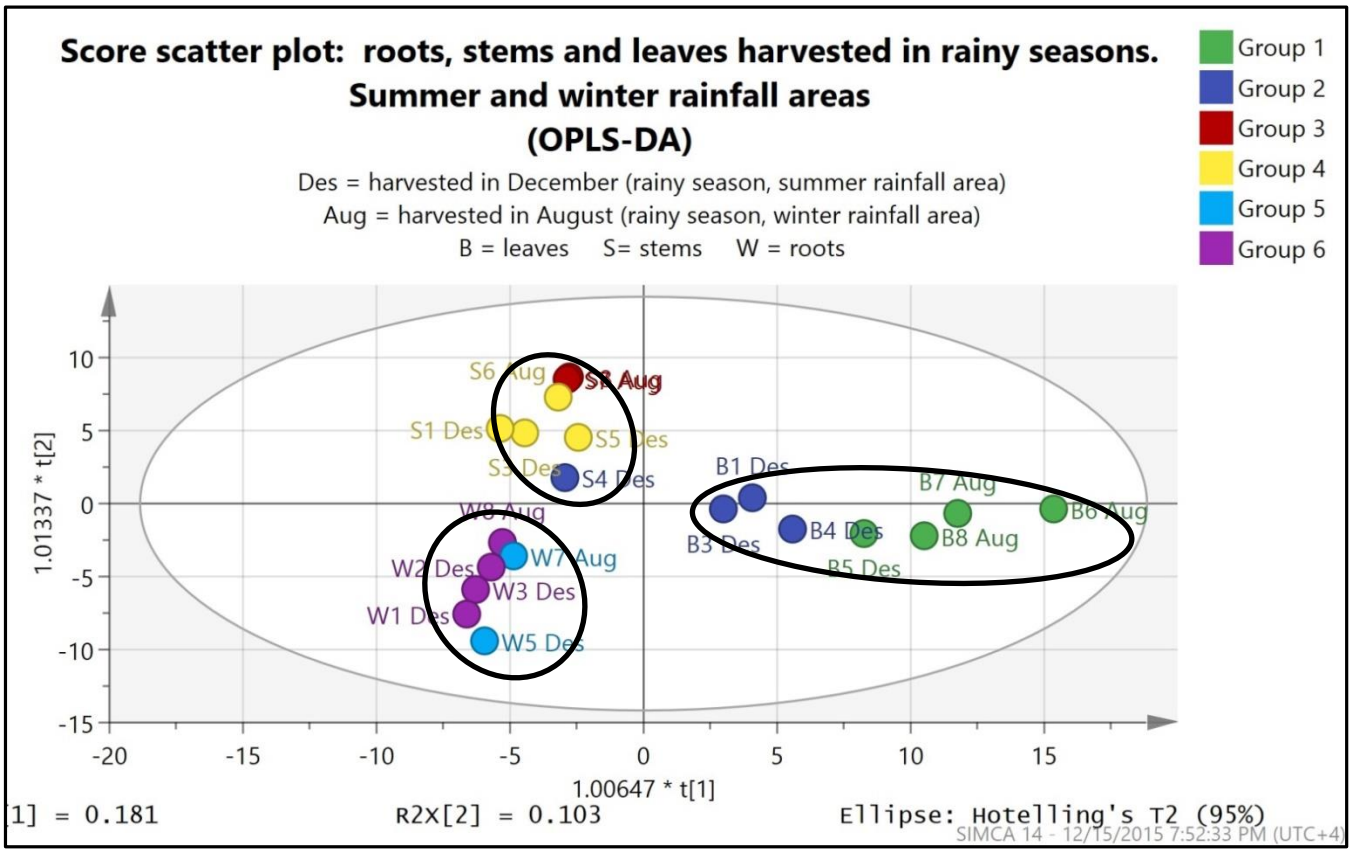


Figure 4.11: Score scatter plot (OPLS-DA) of metabolites in root, stem and leaf material of *E. undulata* gathered during rainy seasons of winter and summer rainfall areas

Clusters created by the hierarchical validation (Figure 4.12) also indicate separation of root, stem and leaf material into three clear groups. Within these three groups, it can be seen that material from the winter and summer rainfall areas also form separate groupings.

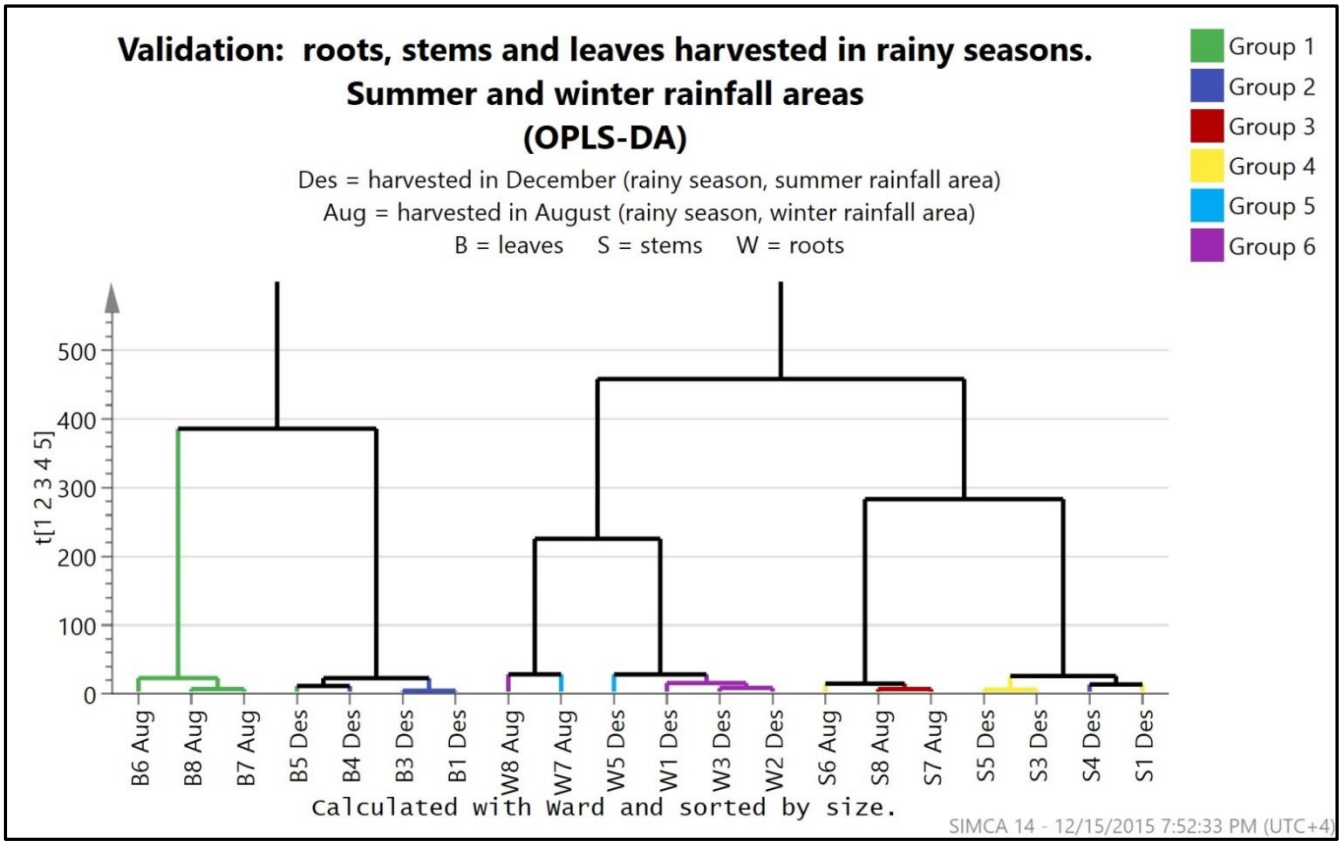


Figure 4.12: Hierarchical cluster diagram for validation of OPLS-DA model of root, stem and leaf material of *E. undulata* gathered in rainy seasons of winter and summer rainfall areas

4.1.4 Statistical comparison of dry seasons of the winter and summer rainfall areas

The clusters that were obtained from the principal component analysis (R2X = 0.777, Q2 = -0.0332) in Figure 4.13 indicate separation between material from the two rainfall areas. Material from the dry season of the winter rainfall area groups together very closely while that of the summer rainfall area separates loosely into groups based on plant organ. It can also be seen that roots and particularly stems from the summer rainfall area lie closer to the cluster for the winter rainfall area than the leaves do.

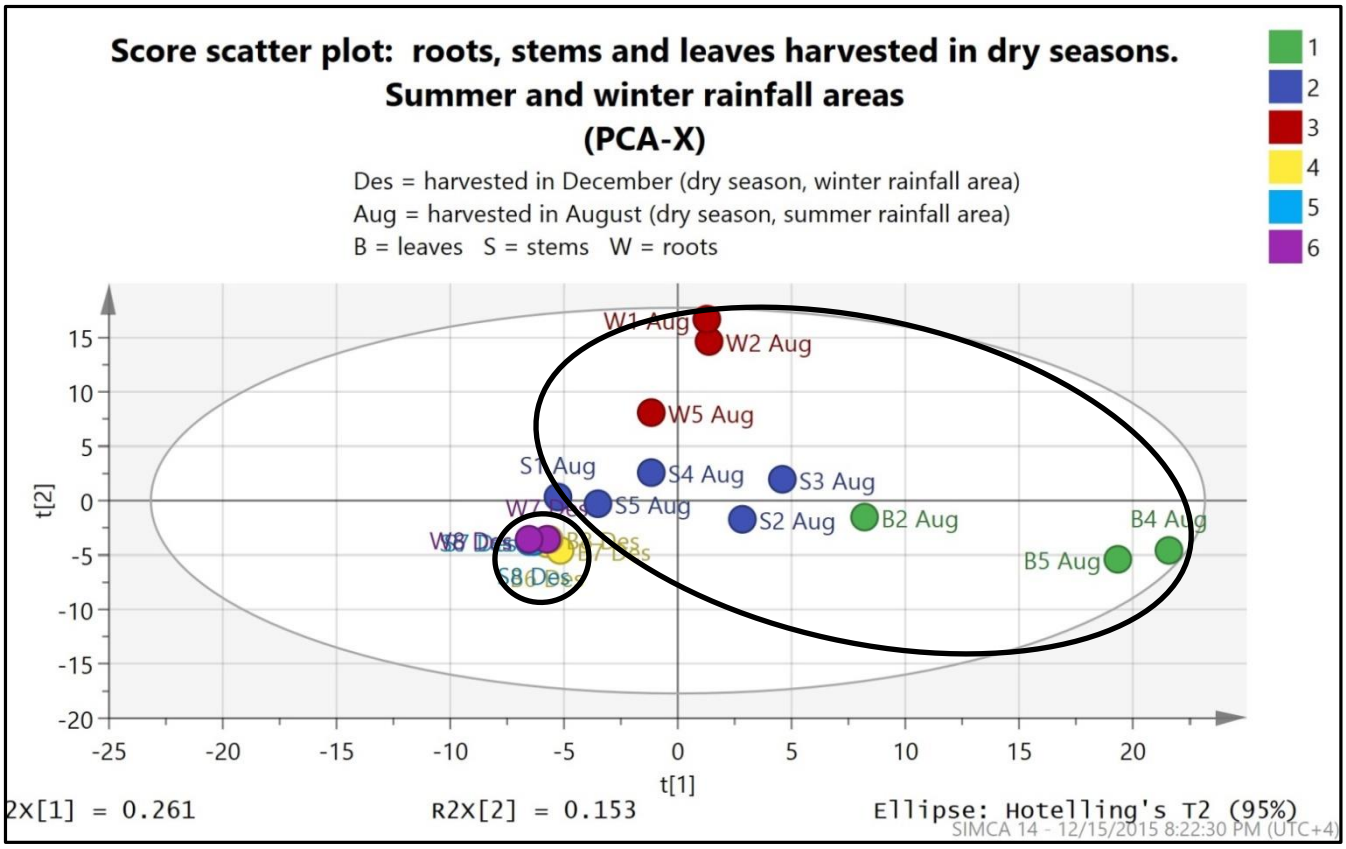


Figure 4.13: Score scatter plot (PCA – X) of metabolites in root, stem and leaf material of *E. undulata* gathered during dry seasons of summer and winter rainfall areas

Hierarchical clustering for this model in Figure 4.14 indicates delineation between summer and winter rainfall areas, although it can be seen that stems and roots from the summer rainfall area might be more chemically similar to plant material from the winter rainfall area than to leaves from the summer rainfall area. Slight discrepancies and overlap among groupings from material harvested in the winter rainfall area are evident and suggests possible chemical similarity between plant organs. Delineations in material from the summer rainfall area suggest a clear clustering of roots, stems and leaves into three groups.

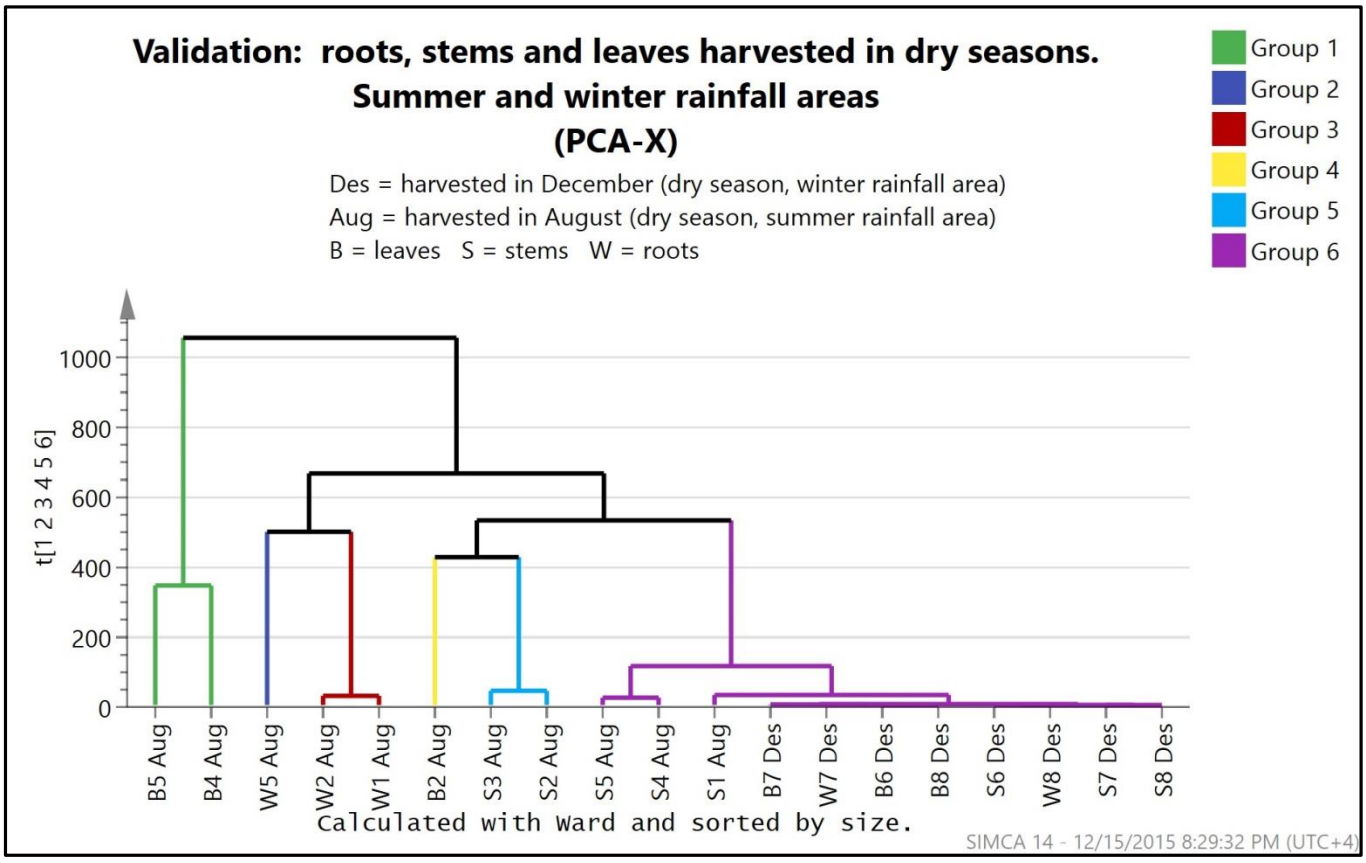


Figure 4.14: Hierarchical cluster diagram for validation of PCA-X model of root, stem and leaf material of *E. undulata* gathered in dry seasons of summer and winter rainfall areas

Orthogonal partial least-squares discriminant analysis (OPLS-DA) was used to create a score scatter plot to evaluate variations in buckets between groups, followed by cross validation (Jung *et al.,* 2011). The groupings obtained from the score scatter plot of the OPLS-DA model (R2X = 0.529, R2Y = 0.645, Q2 = 0.244) in Figure 4.15 indicate separation between material from the two rainfall areas. Material from the dry season of the winter rainfall area groups together very prominently while that of the summer rainfall area separates into groups based on plant organ. It can be seen material from the summer rainfall area forms clusters based on organ. It is also evident that roots and especially stems from the summer rainfall area group closer to material from the winter rainfall area than the leaves of the summer rainfall area do.

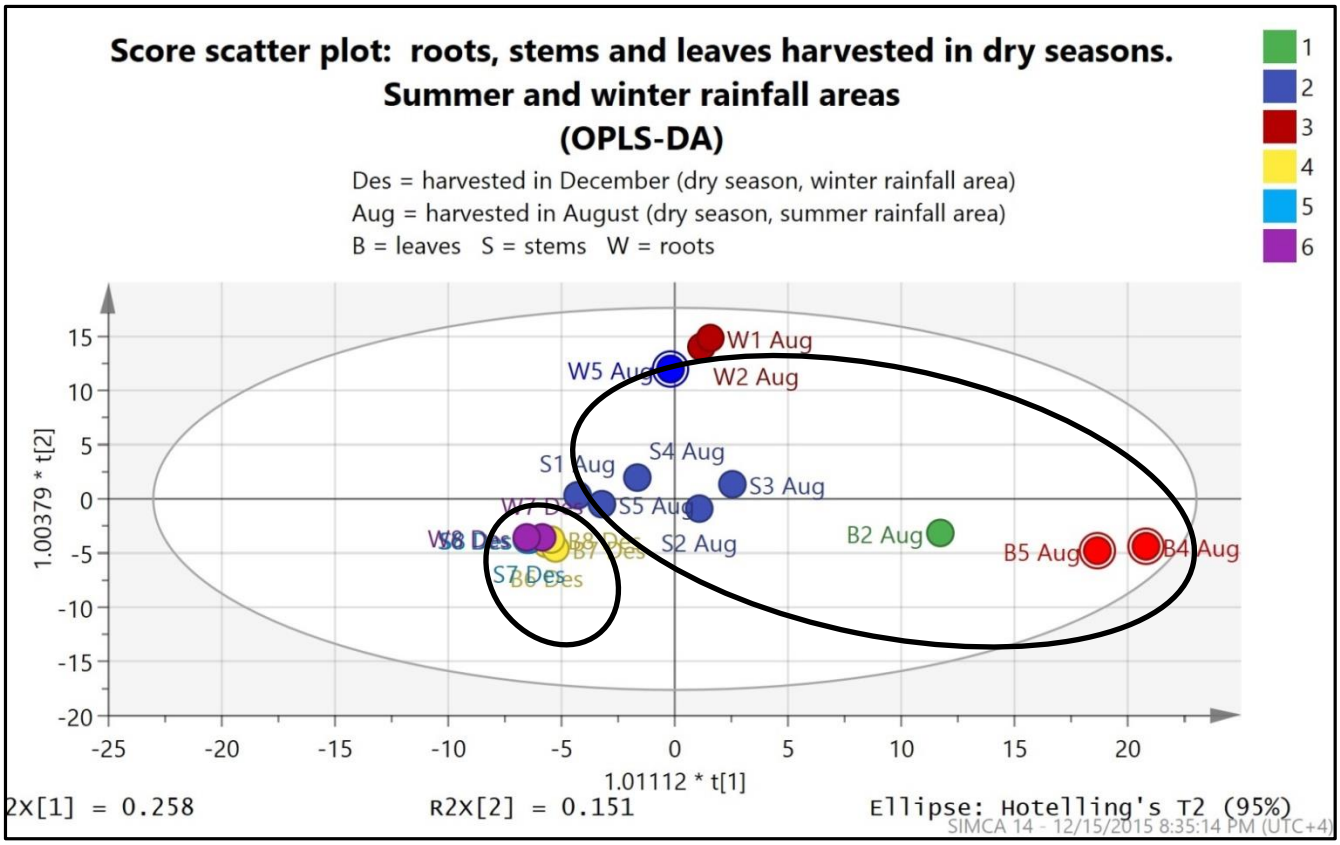


Figure 4.15: Score scatter plot (OPLS-DA) of metabolites in root, stem and leaf material of *E. undulata* gathered during dry seasons of winter and summer rainfall areas

Clusters created by the hierarchical validation (Figure 4.16) indicate separation between rainfall areas but most prominently indicate that stems and roots from the summer rainfall area might be more chemically similar to plant material from the winter rainfall area than to leaves from the summer rainfall area. The close clustering of material from the winter rainfall area suggests considerable chemical similarity between plant organs. Delineations in material from the summer rainfall area suggest a clear clustering of roots, stems and leaves into three groups.

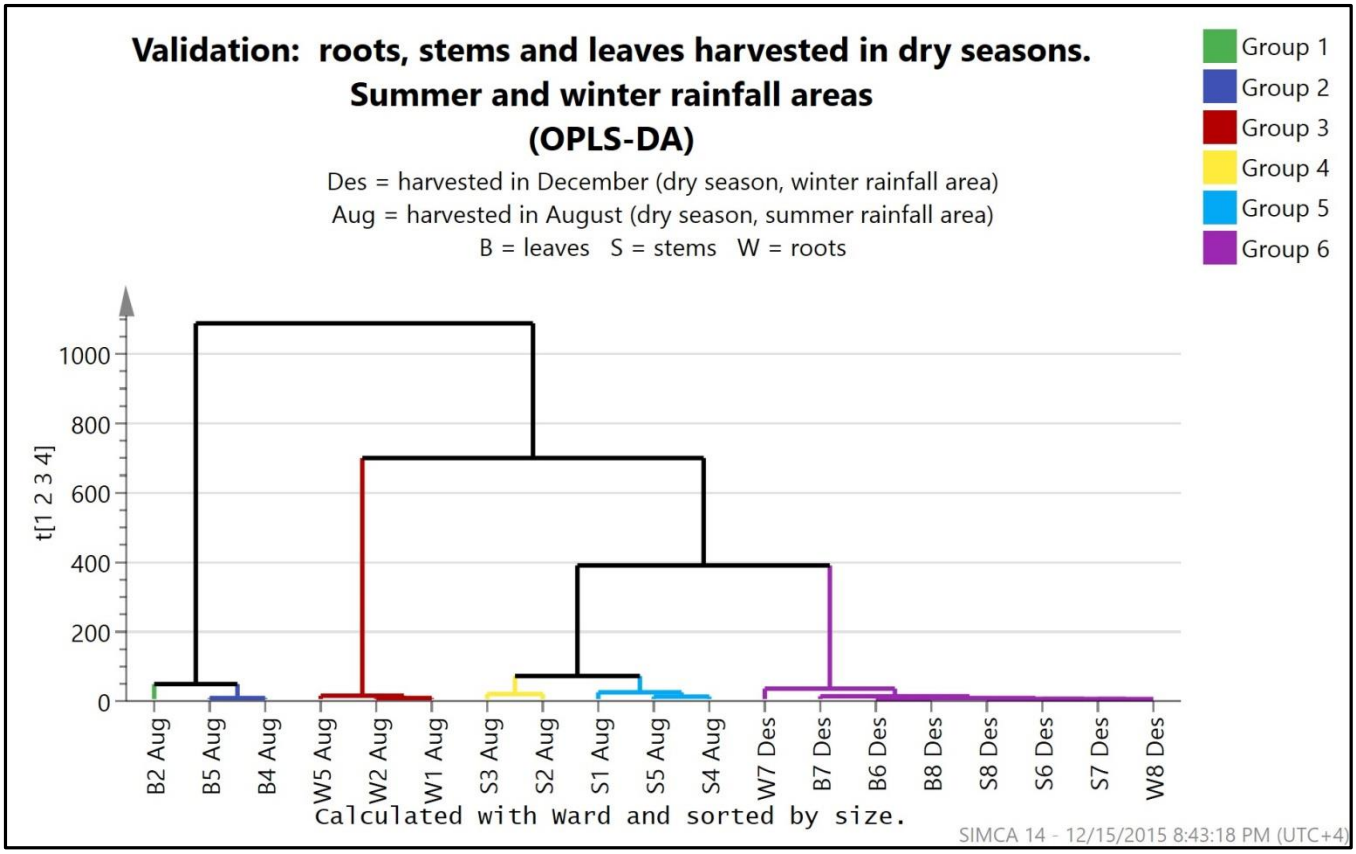


Figure 4.16: Hierarchical cluster diagram for validation of OPLS-DA model of root, stem and leaf material of *E. undulata* gathered in dry seasons of winter and summer rainfall areas

4.2 Chemical analysis of plant material

In order to determine in which plant material the metabolites lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid, epicatechin and 7-methyl-juglone were present, the OPLS-DA data was used to create score contribution plots for roots, stems and leaves harvested during both rainy and dry seasons of the summer and winter rainfall areas respectively. These score contribution plots were investigated individually to determine in which plant samples these metabolites were present.

Values below 0 on the y-axis of the score contribution plots indicate negative association and suggest that metabolites corresponding to these Primary ID (bin) values are likely to be absent. Values above 0 on the y-axis on the score contribution plots indicate positive association and suggest that metabolites corresponding to these Primary ID values are likely to be present in the plant sample.

The bin values associated with lupeol (Khattar *et al.,* 2015), α -amyrin-3O-ß-(5-hydroxy) ferulic acid (Deutschländer *et al*., 2010) epicatechin (Wishart *et al.,* 2013) and 7-methyl-juglone (van der Kooy, 2007) are listed in Table 3 in ppm (600 MHz in H_2O). The bin values for a metabolite that was found to associate positively with the Primary ID (bin) values on the contribution plots, suggested the presence of that metabolite within the plant material.

Table 3: Specific NMR regions of epicatechin, 7-methyl-juglone, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol used to determine the presence or absence of these metabolites in plant samples in OPLS-DA contribution plots

Compound	Corresponding peaks in ppm on ^{1}H NMR spectra (600 MHz in H_2O)	Primary ID value on OPLS-DA contribution plot (bin value)
lupeol	0.76	17/18
	0.81	19
	0.92	22
	1.02	24
	1.67	41
	3.16	80
	4.76	120
α -amyrin-3O-ß-(5-hydroxy) ferulic acid	0.84	20
	1.16	41
	3.85	97/98
	4.61	117
	5.10	129
	6.32	159
	6.72	169
	7.46	187/188
	7.56	190
epicatechin	4.16	105/106
	4.60	115/116
	6.72	169
	6.84	172
	7.16	181/182
7-methyl-juglone	2.37/40	60
	6.88	173
	7.06	177/178
	7.42	186/187

In order to further investigate plant material for the presence of these secondary metabolites, ^{1}H NMR spectra of collected samples were compared to ^{1}H NMR spectra of lupeol as seen in Figure 4.17 (Khattar *et al.,* 2015), epicatechin in Figure 4.18 (Wishart *et al.,* 2013), 7-methyl-juglone in Figure 4.19 (van der Kooy, 2007) and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in Figure 4.20 (Deutschländer *et al*., 2010). Values found to be present on ^{1}H NMR spectra of harvested material indicate the presence of the associated metabolites within that plant sample.

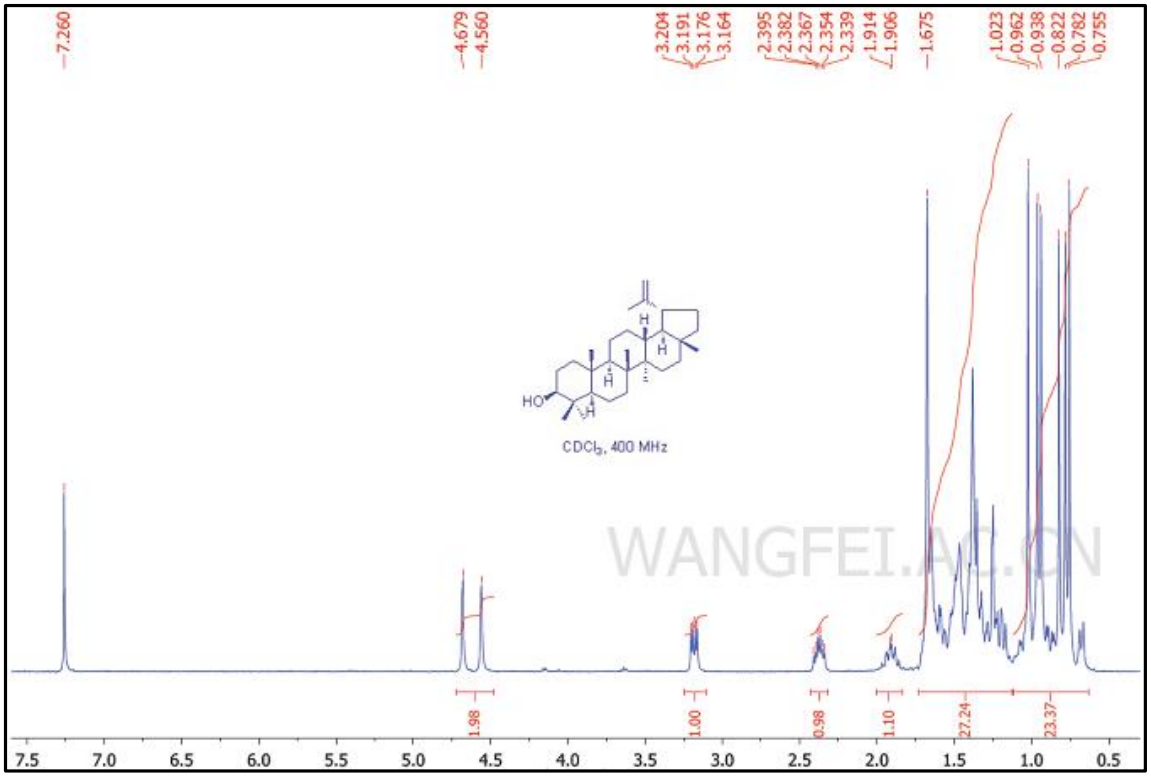


Figure 4.17: ^{1}H NMR spectrum for lupeol (500MHz in H_2O) (Khattar et al., 2015). The ^{1}H NMR data (in ppm) for lupeol used in this study are: δ = 0.76, 0.81, 0.92, 1.02, 1.67, 3.16, 4.76

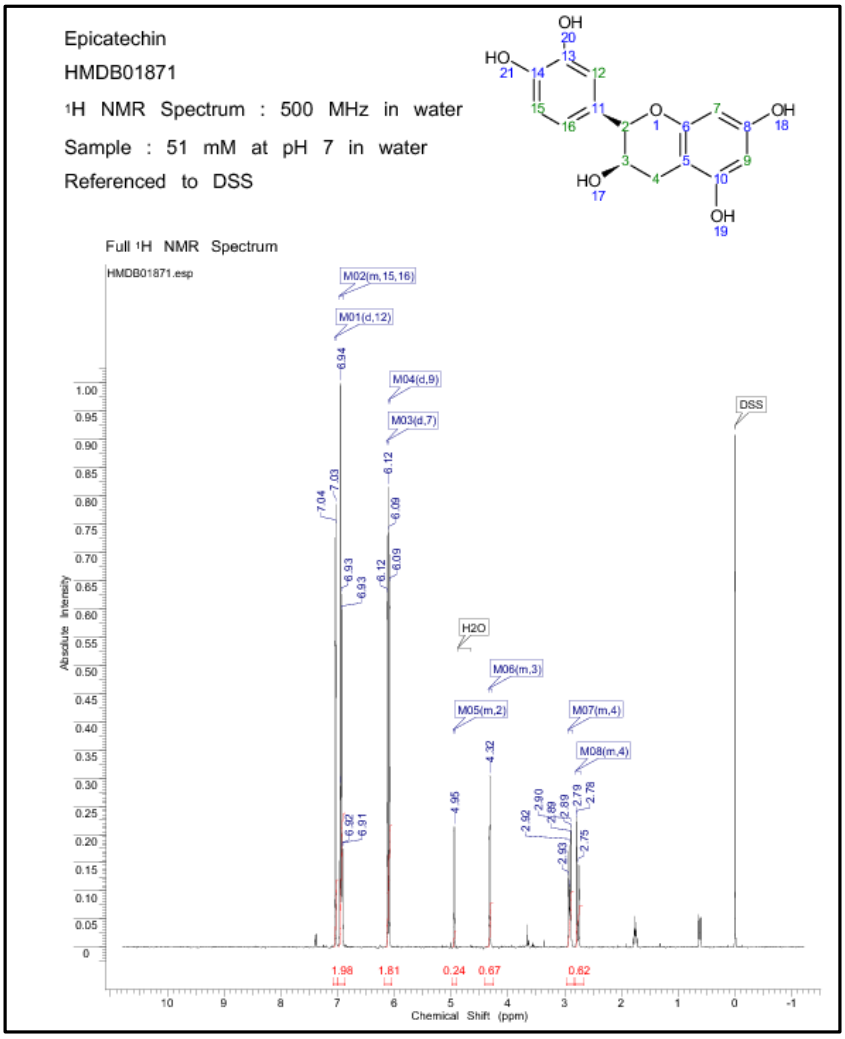


Figure 4.18: ^{1}H NMR spectrum for epicatechin (500MHz in H_2O) (Wishart et al., 2013). The ^{1}H NMR data (in ppm) for epicatechin used in this study are: δ = 4.16, 4.60, 6.72, 6.84, 7.16

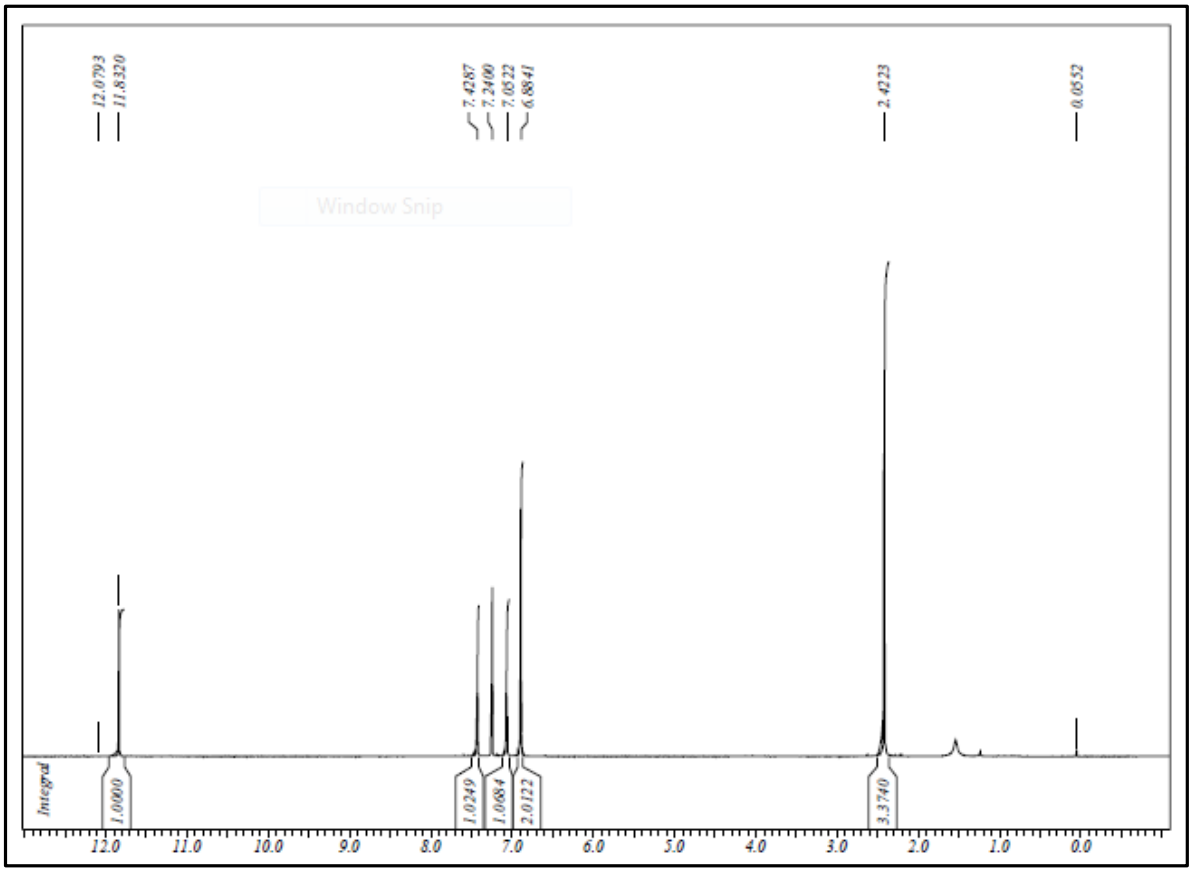


Figure 4.19: ^{1}H NMR spectrum for 7-methyl-juglone (500MHz in H_2O) (van der Kooy, 2007). The ^{1}H NMR data (in ppm) for 7-methyl-juglone used in this study are: δ = 2.40, 6.88, 7.06, 7.42

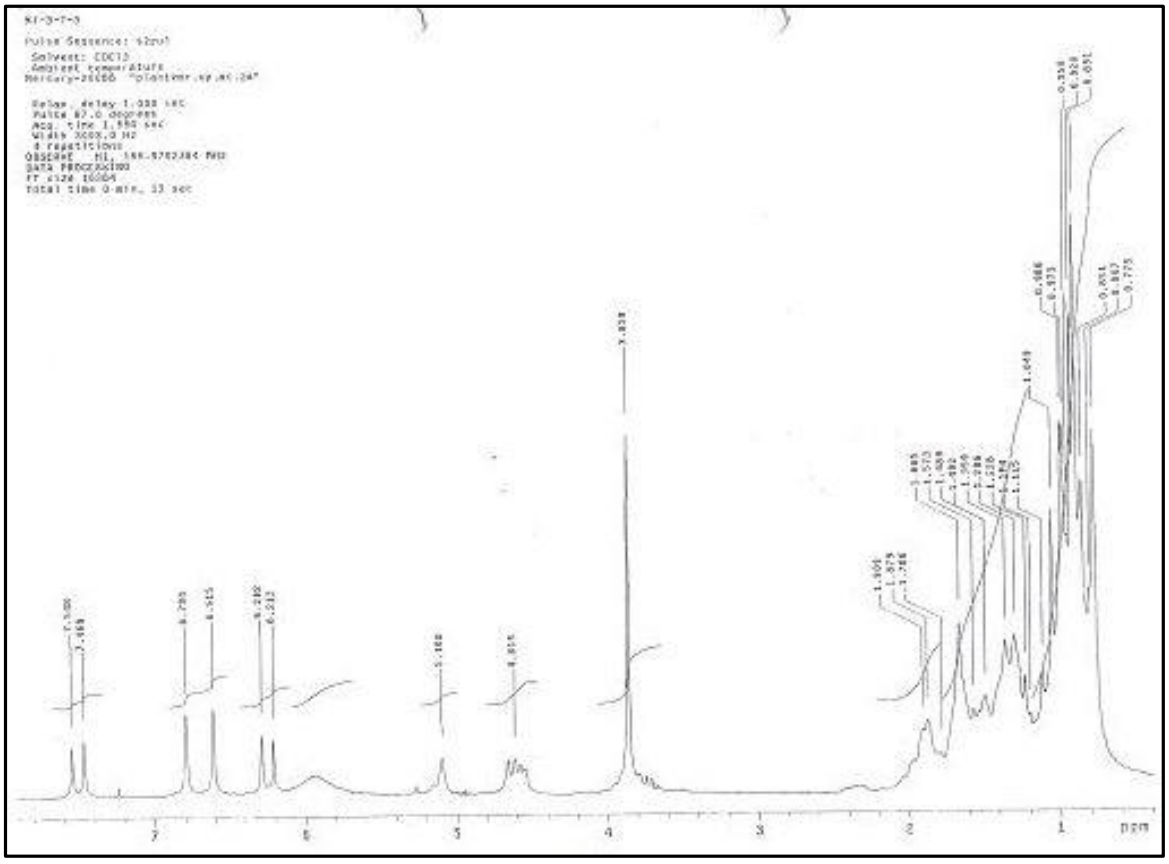
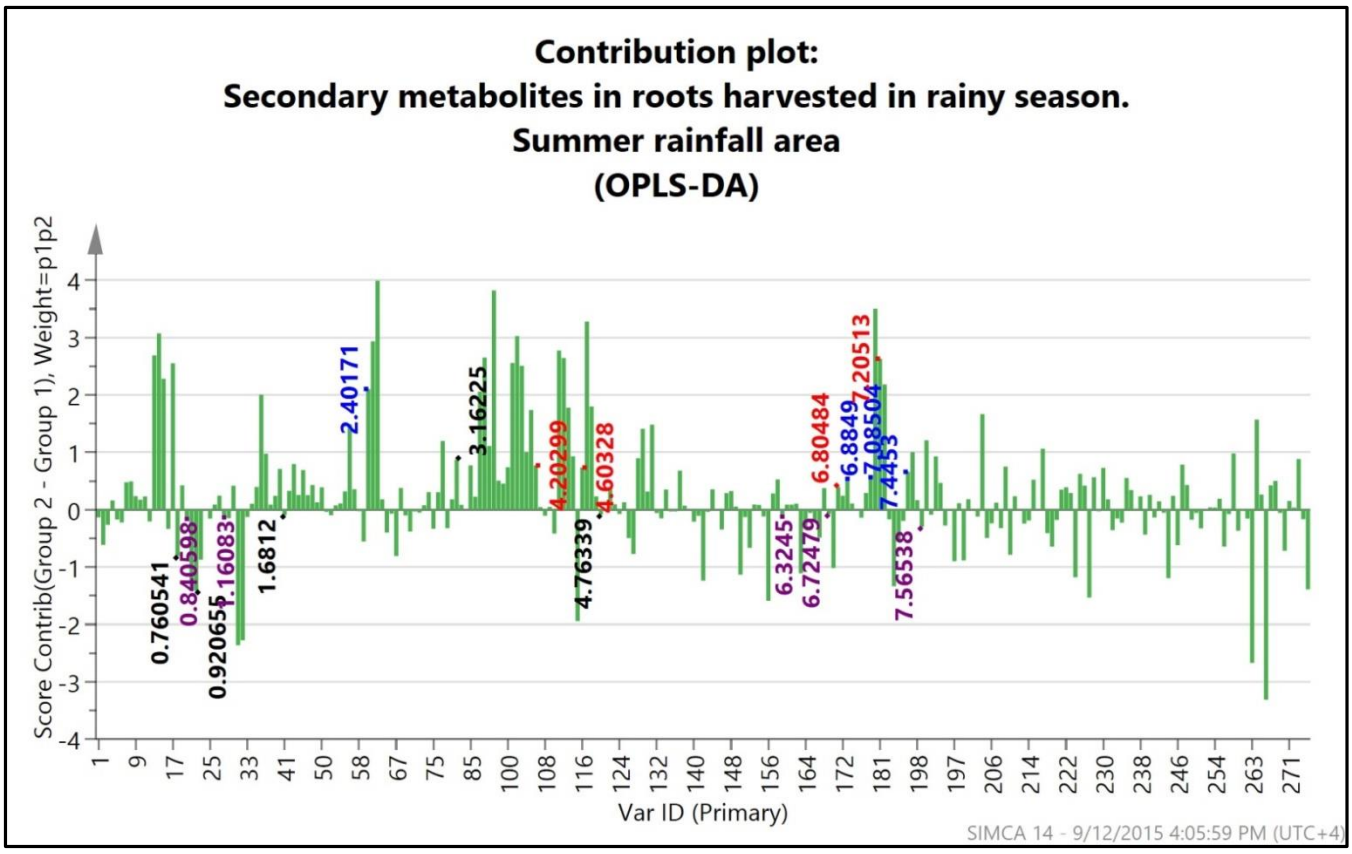


Figure 4.20: ^{1}H NMR spectrum for α -amyrin-3O-ß-(5-hydroxy) ferulic acid (500MHz in H_2O) (Deutschländer 2010). The ^{1}H NMR data (in ppm) for α -amyrin-3O-ß-(5-hydroxy) ferulic acid used in this study are: δ = 0.84, 1.16, 3.85, 4.61, 5.10, 6.32, 6.72, 7.46, 7.56

4.2.1 Chemical analysis of the rainy season of the summer rainfall area

4.2.1(i) Roots harvested in the rainy season of the summer rainfall area

The contribution plot for roots gathered during the rainy season shown in Figure 4.21 shows that the bin values and ^{1}H NMR spectra peak values for 7-methyl-juglone and epicatechin associate positively with this plant material and suggests that these metabolites are present in the roots during the rainy season. The values associated with lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid mostly associate negatively, suggesting that these metabolites are either absent or present in concentrations too low for detection.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values for α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.21: Score contribution plot of root material gathered during rainy season of summer rainfall area

The ^{1}H NMR peak values for epicatechin and 7-methyl-juglone have been identified and labelled on the spectra for root material harvested during the rainy season of the summer rainfall area, while the peak values associated with lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid could not be identified (Figure 4.22). This suggests that epicatechin and 7-methyl-juglone are possibly the only two of the four investigated metabolites present in concentrations high enough to be detected and corresponds to the data on the score contribution plot in Figure 4.21.

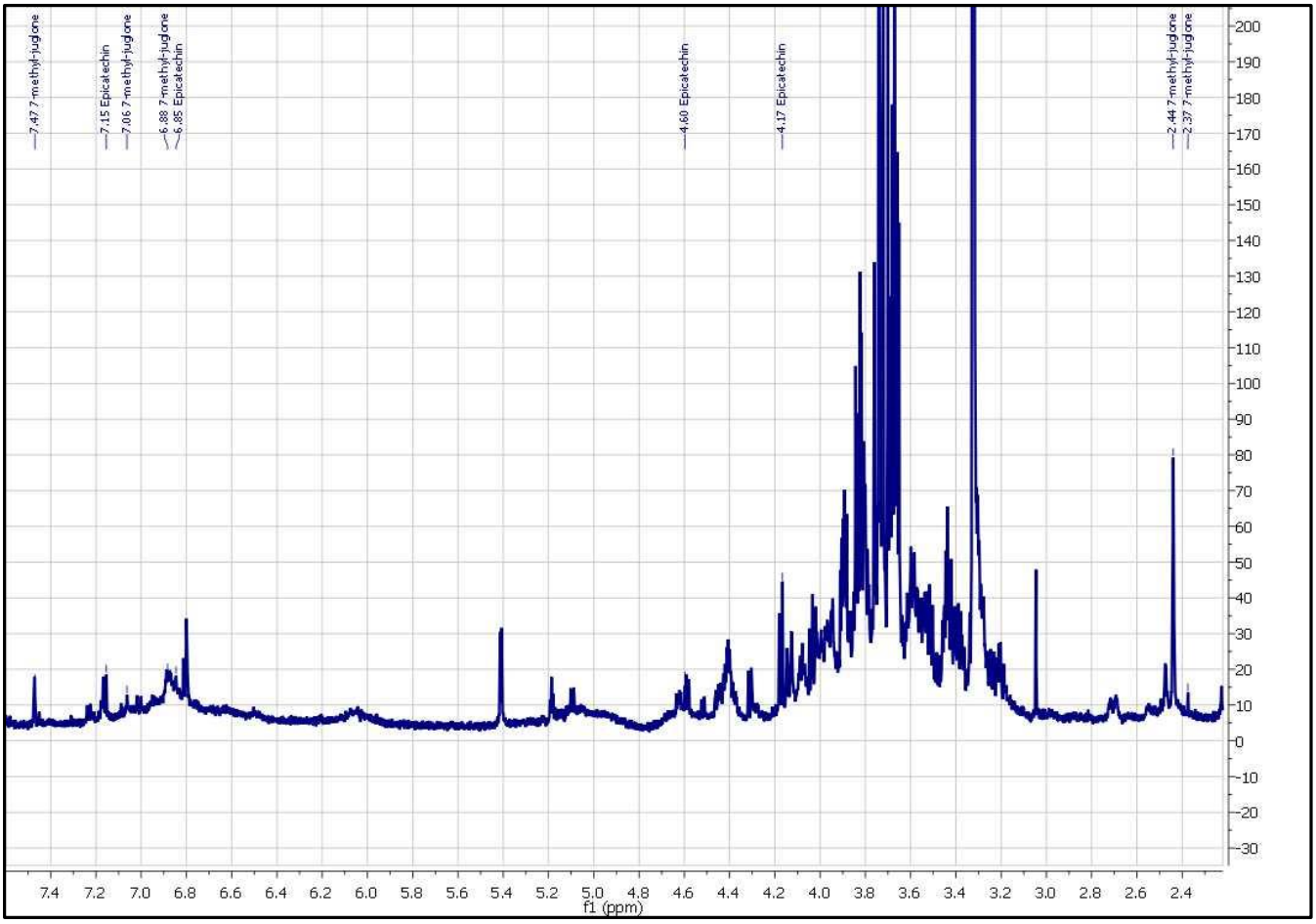
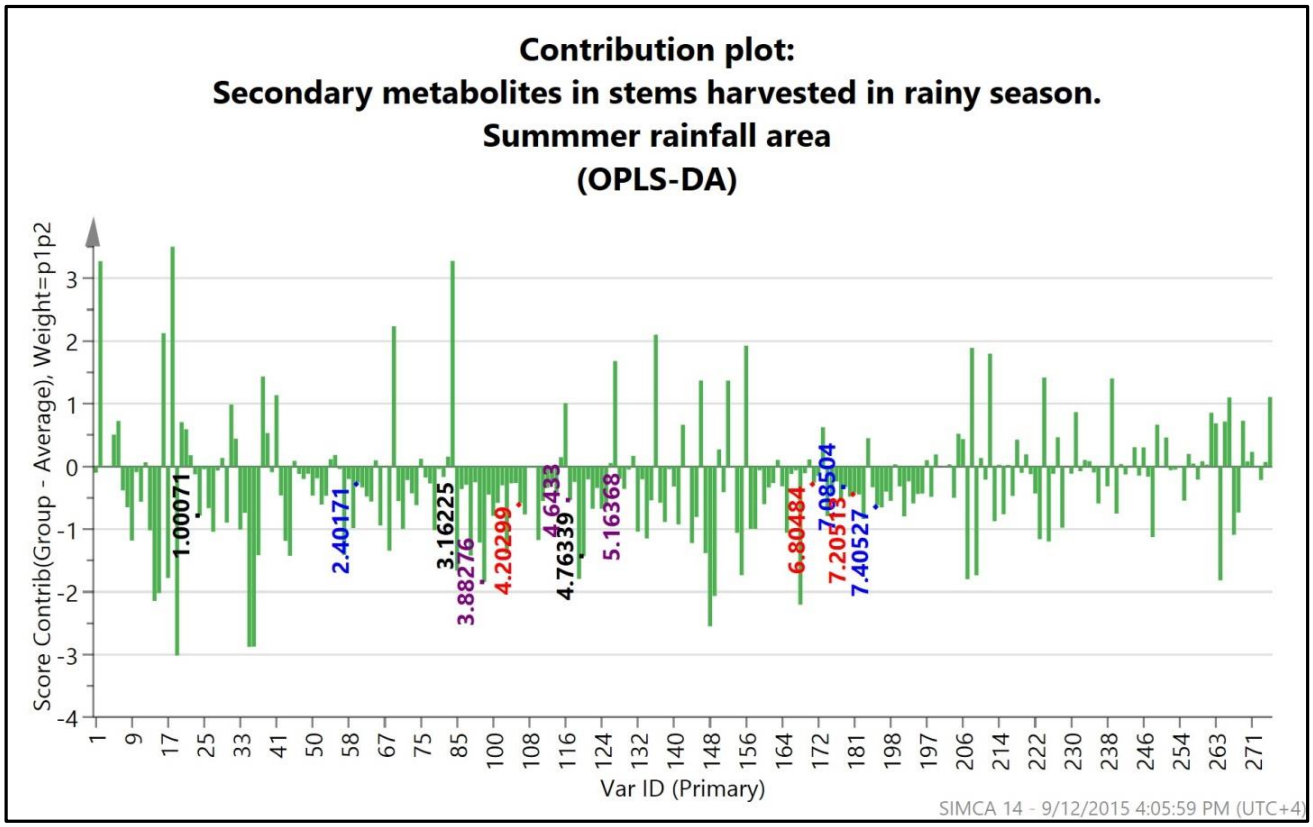


Figure 4.22: Presence of epicatechin and 7-methyl-juglone on ^{1}H NMR spectra (600 MHz in H_2O) of roots harvested during the rainy season of summer rainfall area

4.2.1(ii) Stems harvested in the rainy season of the summer rainfall area

The contribution plot for stems gathered during the rainy season of the summer rainfall area (Figure 4.23) indicates that the bin values and ^{1}H NMR spectra peak values for lupeol, epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and 7-methyl-juglone associate negatively with the plant material in these samples. This either suggests that none of these three metabolites are present in the stems of *E. undulata* during the rainy season of the summer rainfall area or indicates that their concentrations are too low to be detected chemically.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

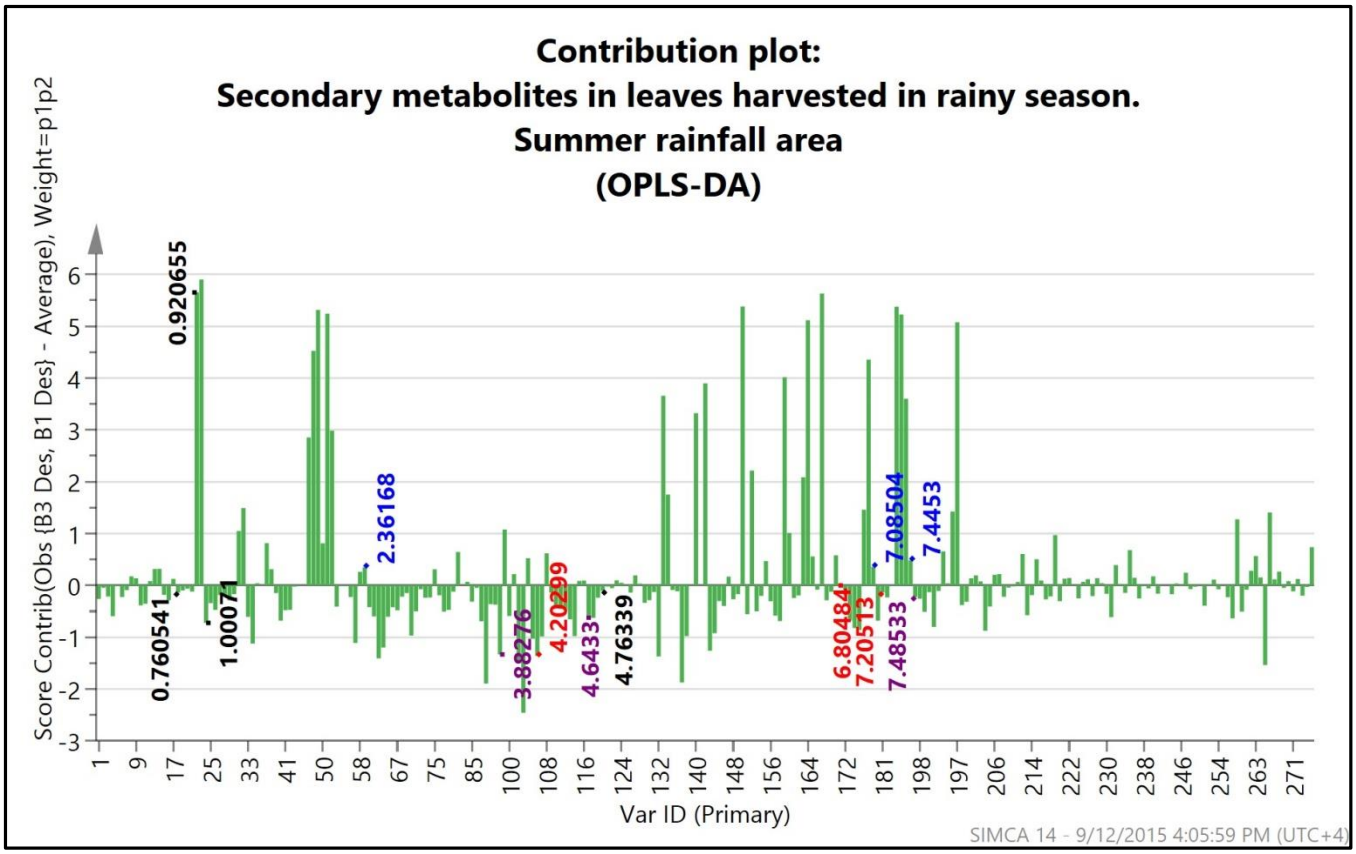
Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values for α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.23: Score contribution plot of stems gathered during rainy season of summer rainfall area

4.2.1(iii) Leaves harvested in the rainy season of the summer rainfall area

The contribution plot for leaves gathered during the rainy season (Figure 4.24) indicates that the bin values and ^{1}H NMR spectra peak values for lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and epicatechin associate negatively with the plant material while those for 7-methyl-juglone associated positively. This suggests that 7-methyl-juglone is present in leaves during the rainy season while the other three metabolites are either absent or not present in concentrations high enough for chemical detection.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.24: Score contribution plot of leaf material gathered during rainy season of summer rainfall area

The ^{1}H NMR peak values for 7-methyl-juglone were identified on the spectrum of leaves gathered in the rainy season of the summer rainfall area, while the values associated with epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol could not (Figure 4.25). This corresponds to the data from the contribution plot in Figure 4.24 that suggested that 7-methyl-juglone is likely to be the only one of these metabolites present in these leaves in high enough concentrations to be detected.

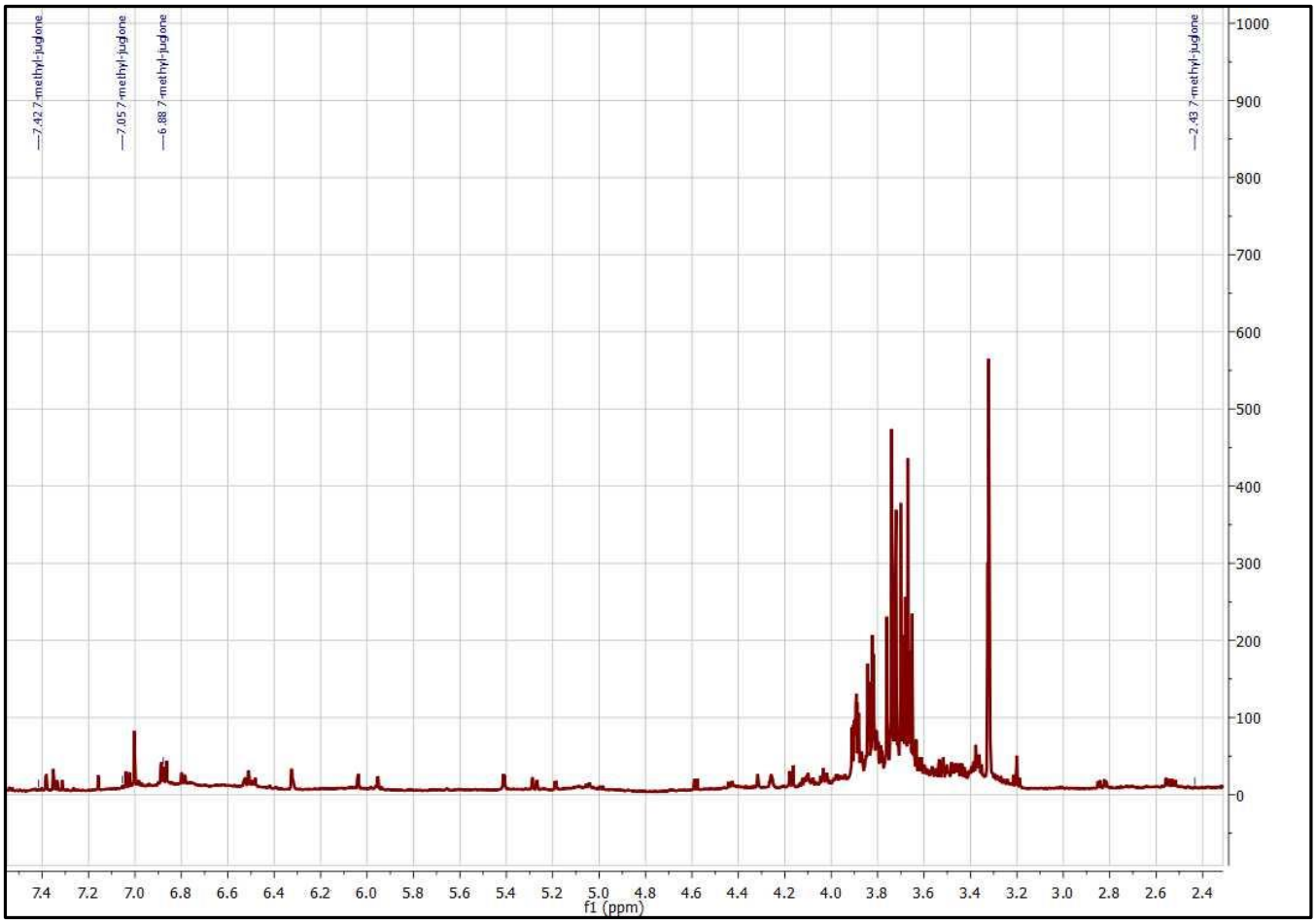
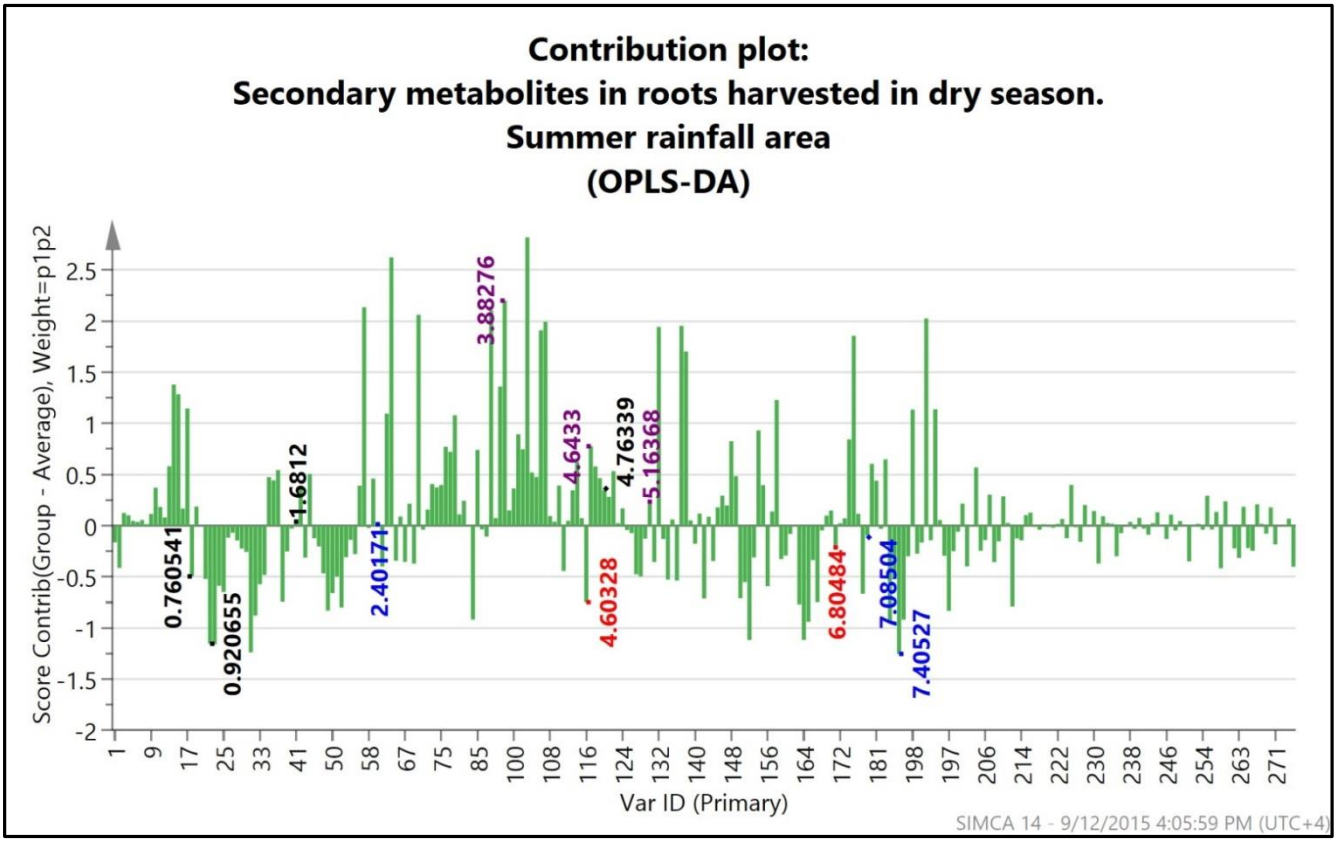


Figure 4.25: Presence of 7-methyl-juglone ^{1}H NMR spectra (600 MHz in H_2O) of leaves gathered in rainy season of summer rainfall area

4.2.2 Chemical analysis of the dry season of the summer rainfall area

4.2.2(i) Roots harvested in the dry season of the summer rainfall area

The contribution plot for roots gathered during the dry season shown in Figure 4.26 indicates that the bin values and ^{1}H NMR spectra peak values for epicatechin and 7-methyl-juglone associate negatively with this plant material while those for α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol mostly associate positively. This suggests that while lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid appear to be present, the other metabolites are either absent or present in concentrations too low to be detected.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.26: Score contribution plot of root material gathered during dry season of summer rainfall area

The ^{1}H NMR peak values for α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol were identified on the spectra for root material gathered in the dry season of the summer rainfall area while those for 7-methyl-juglone and epicatechin could not (Figure 4.27). This corresponds to the data from the contribution plot in Figure 4.26 that suggested that while α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol appear to be present, the other two metabolites are likely to be either absent or present in concentrations too low to be detected.

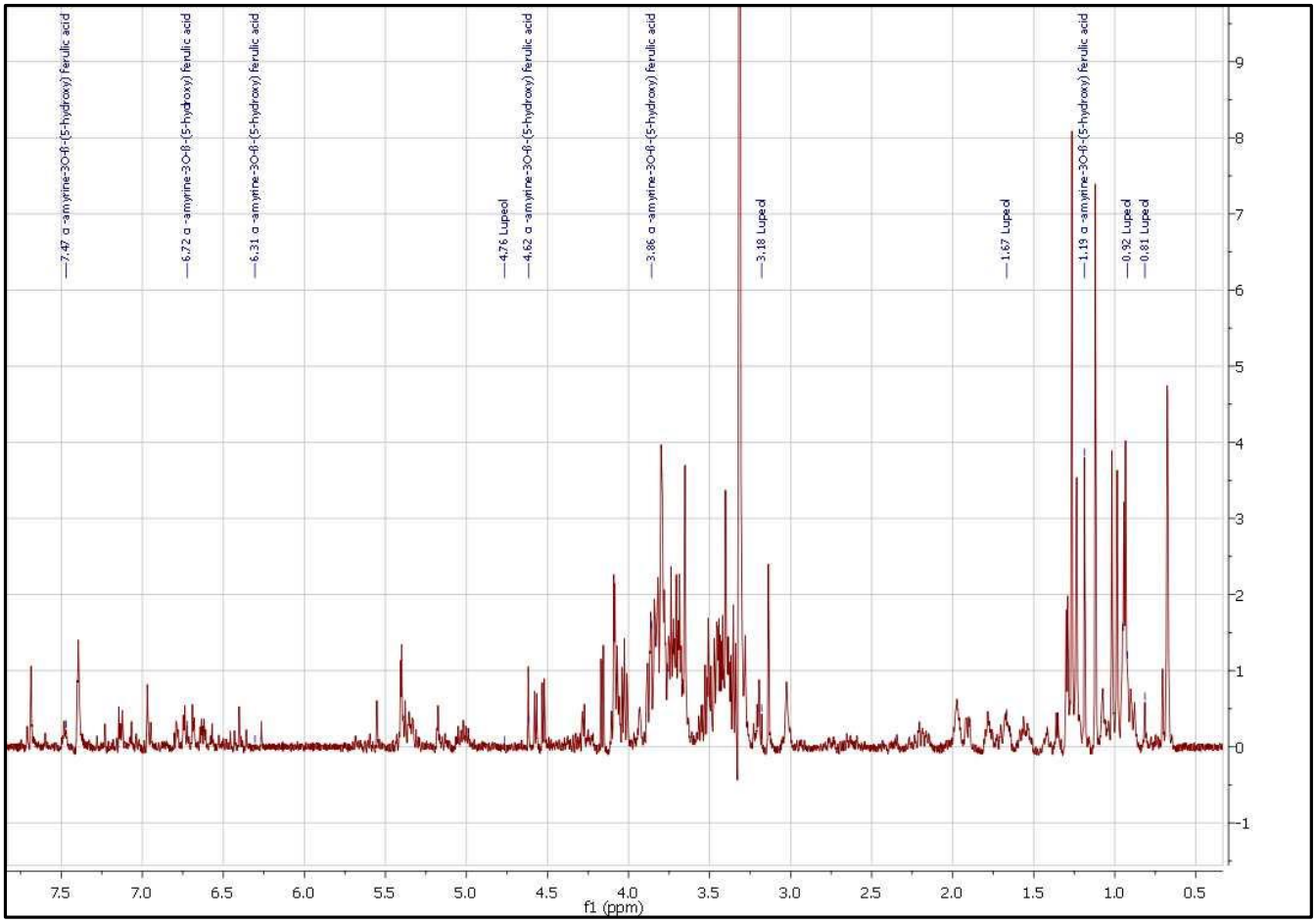
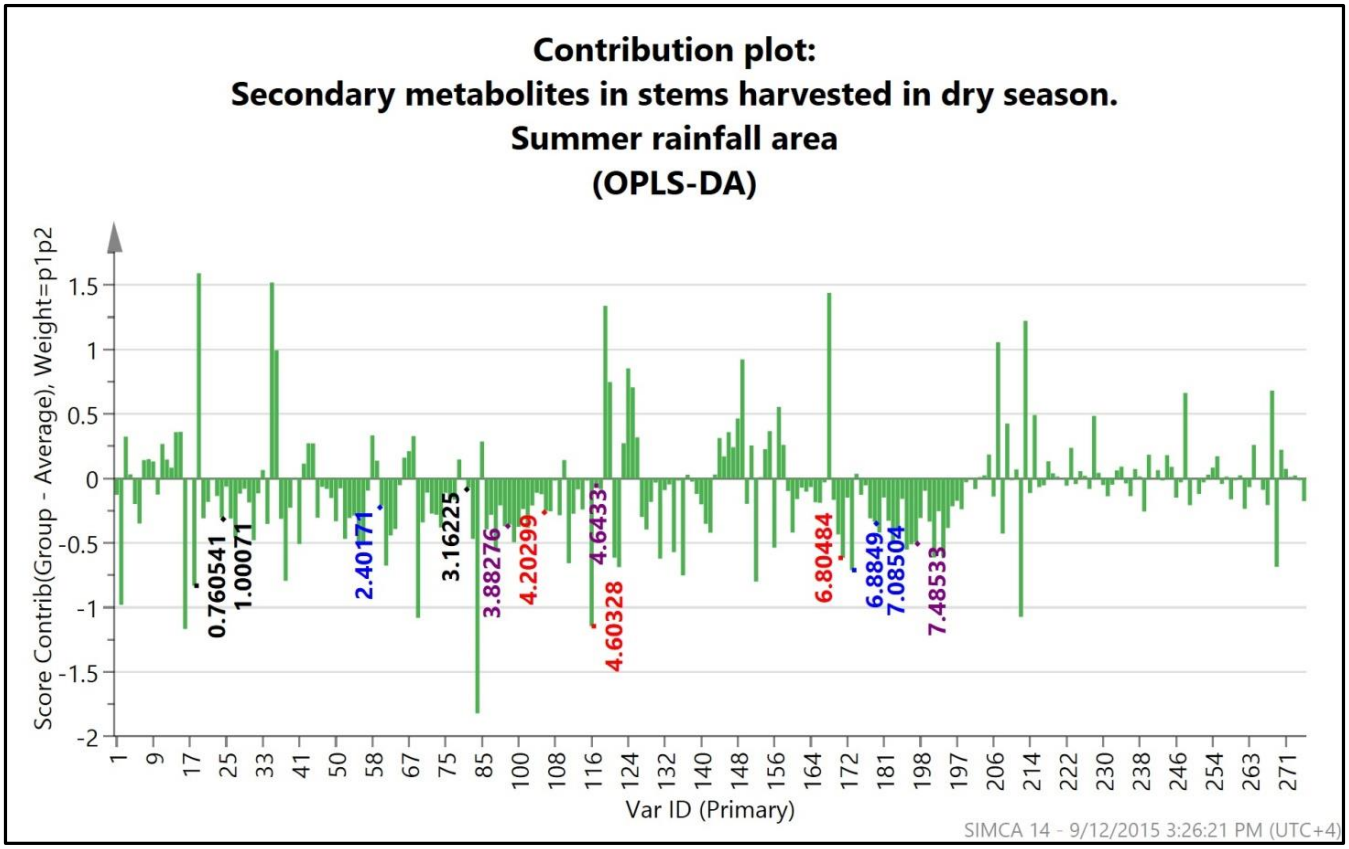


Figure 4.27: Presence of α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol on ^{1}H NMR spectra (600 MHz in H_2O) of roots gathered in dry season of summer rainfall area

4.2.2(ii) Stems harvested in the dry season of the summer rainfall area

Score contribution plots of stem material harvested in the dry season of the summer rainfall area were created from the OPLS-DA score scatter plot data to investigate the presence of epicatechin, lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and 7-methyl-juglone. This contribution plot (Figure 4.28) indicates that the bin values and ^{1}H NMR spectra peak values for all four metabolites associate negatively with the plant material in these samples and suggests that these metabolites absent from the stems of *E. undulata* during the dry season or were present in concentrations too low to be detected.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

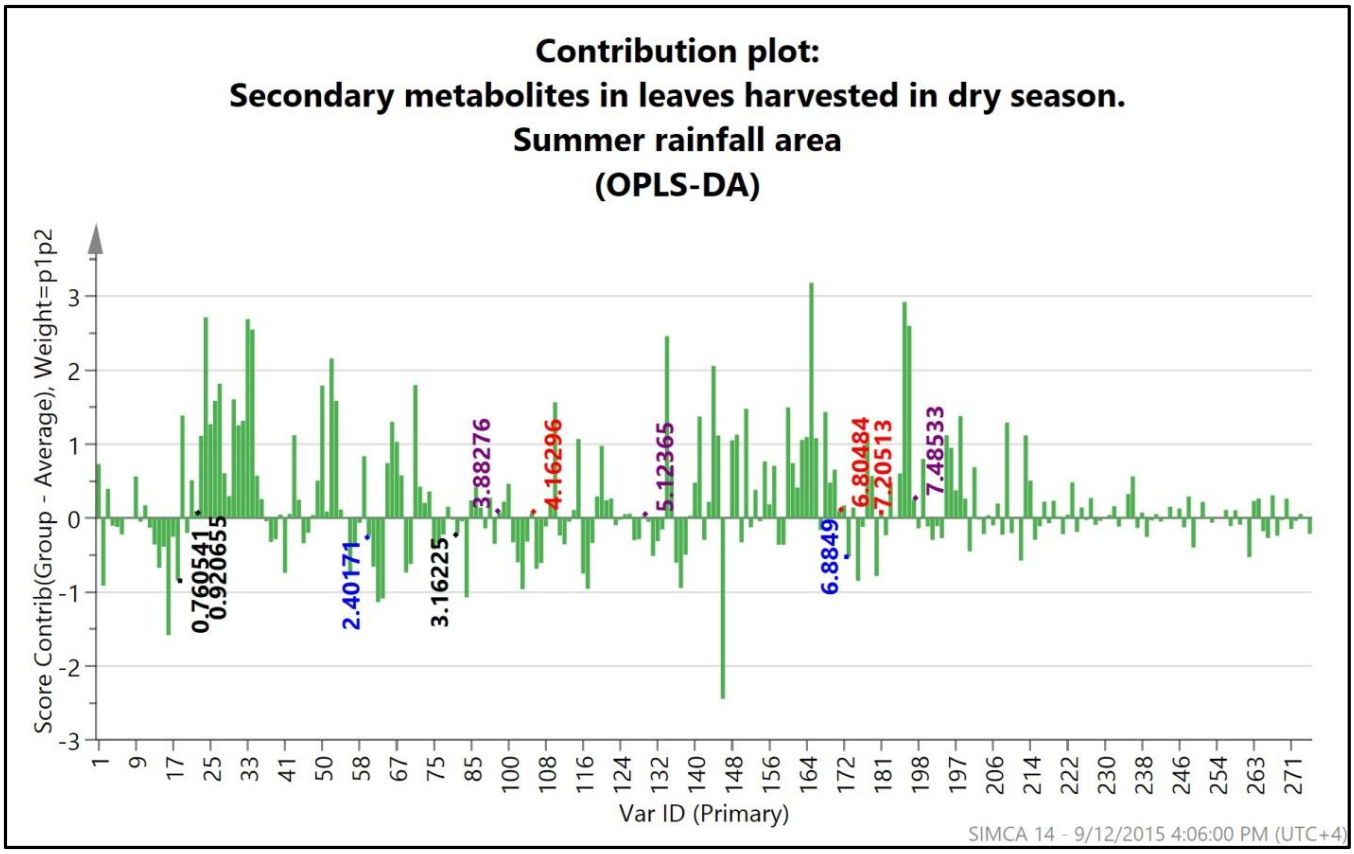
Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.28: Score contribution plot of stems gathered during dry season of summer rainfall area

4.2.2(iii) Leaves harvested in the dry season of the summer rainfall area

The contribution plot for leaves gathered during the dry season of the summer rainfall area (Figure 4.29) indicates that the bin values and ^{1}H NMR spectra peak values for epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid associate positively with the plant material in these samples while those for lupeol and 7-methyl-juglone associate negatively. This suggests that epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid are likely to be present in the leaves during the dry season, while lupeol and 7-methyl-juglone are either absent or present in concentrations too low to be detected.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.29: Score contribution plot of leaf material gathered during dry season of summer rainfall area

The ^{1}H NMR peak values for epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid have been identified and labelled on the spectra for leaf material harvested during the dry season of the summer rainfall area while those for 7-methyl-juglone and lupeol could not (Figure 4.30). This suggests that epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid are present in this leaf material and corresponds to the data on the OPLS-DA contribution plot in Figure 4.29 that suggests that epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid are the only two of these metabolites that could be detected.

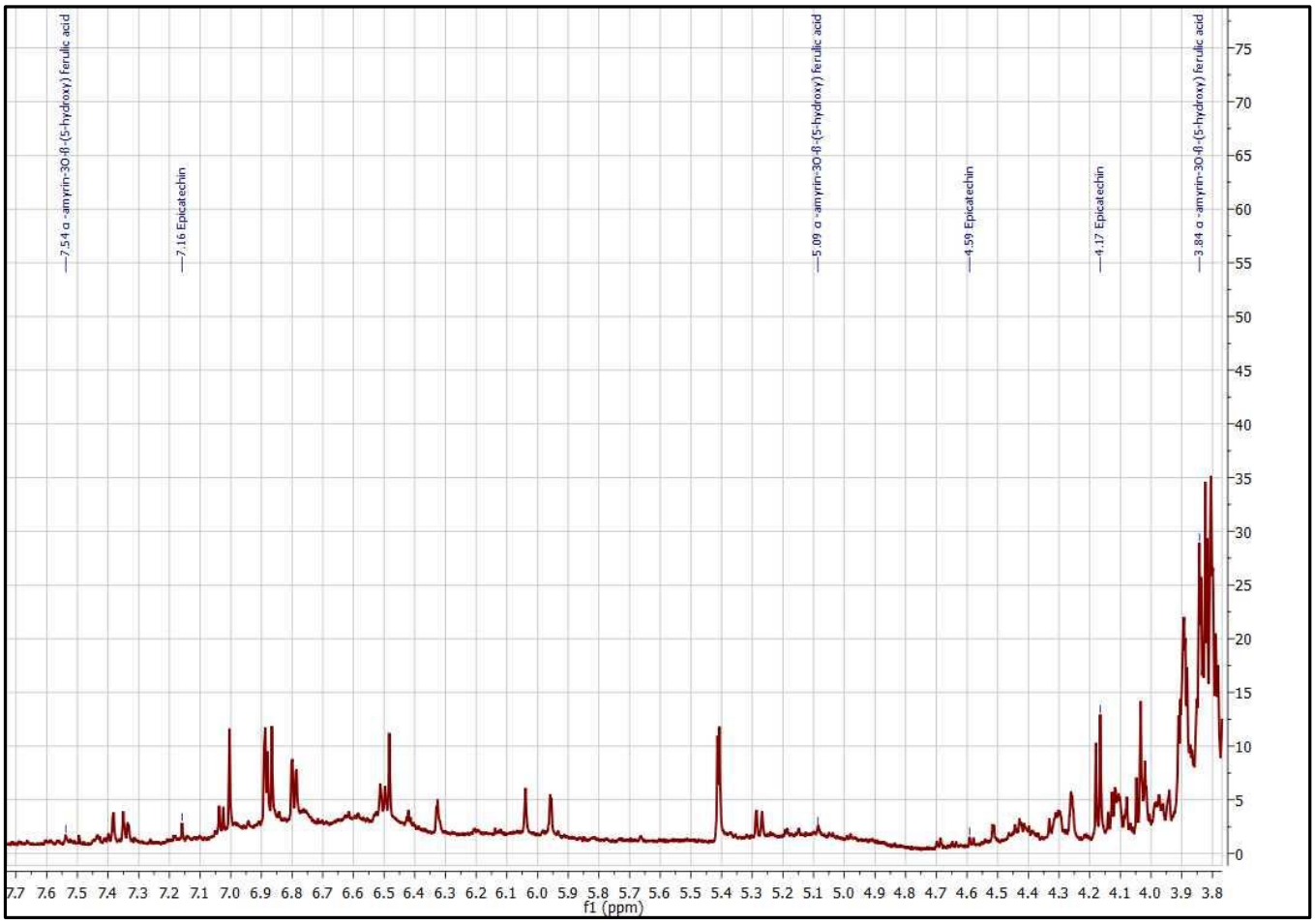
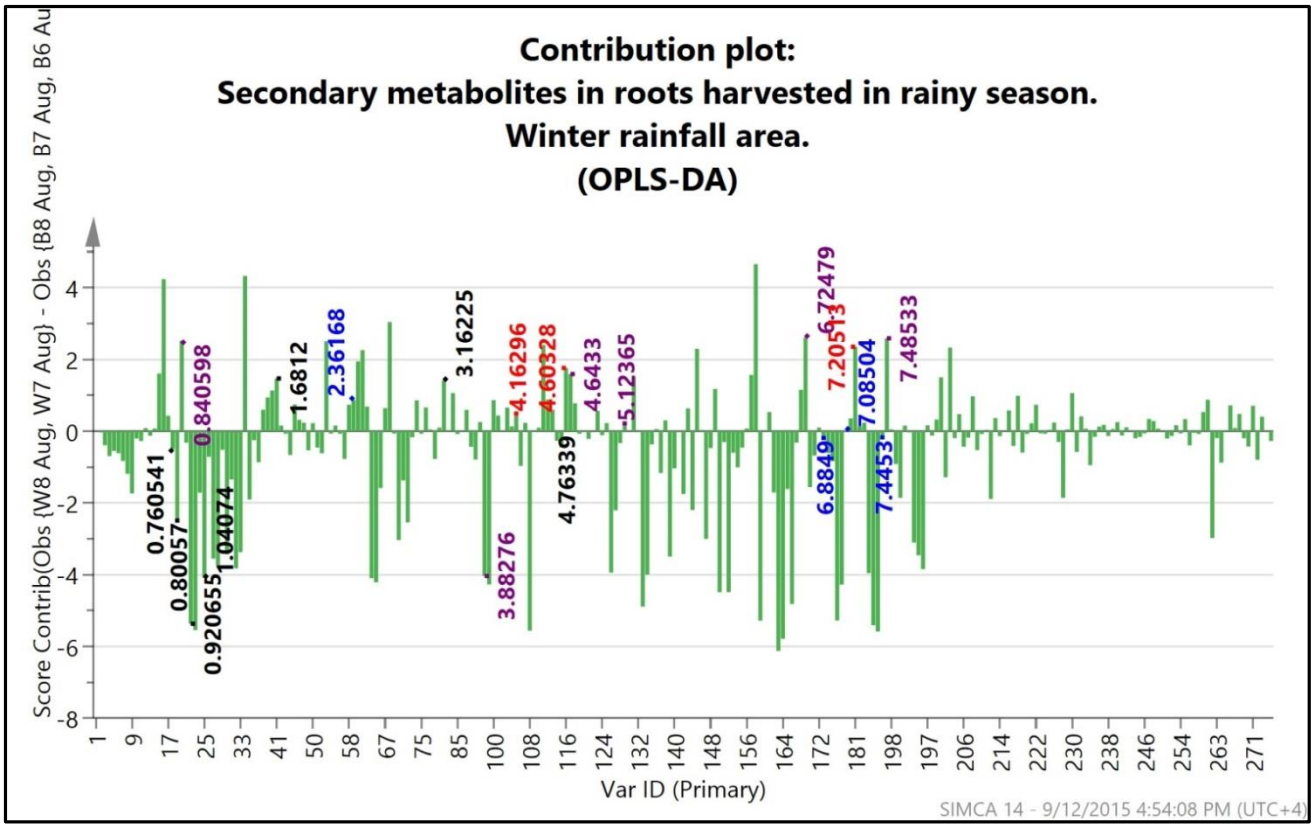


Figure 4.30: Presence of epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid on ^{1}H NMR spectra (600 MHz in H_2O) of leaves harvested during dry season of summer rainfall area

4.2.3 Chemical analysis of the rainy season of the winter rainfall area

4.2.3(i) Roots harvested in the rainy season of the winter rainfall area

The contribution plot for root material gathered in the winter rainfall area shown in Figure 4.31 indicates that the bin values and ^{1}H NMR spectra peak values for epicatechin, 7-methyl-juglone and α -amyrin-3O-ß-(5-hydroxy) ferulic acid generally associate positively with the plant material in these samples while those for lupeol mostly associate negatively. This suggests that epicatechin, 7-methyl-juglone and α -amyrin-3O-ß-(5-hydroxy) ferulic acid might be present in the roots during the rainy season of the winter rainfall area. Lupeol is possibly absent or present in concentrations too low to be detected.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.31: Score contribution plot of root material gathered during rainy season of winter rainfall area

The ^{1}H NMR spectra for root material harvested in the rainy season of the winter rainfall show that peak values associated with epicatechin, 7-methyl-juglone and α -amyrin-3O-ß-(5-hydroxy) ferulic acid are present while those for lupeol are not (Figure 4.32). This suggests that lupeol is the only one of these metabolites absent from this root material (or present in concentrations too low for chemical detection) and corresponds to the data on the contribution plot in Figure 4.31 that suggested that epicatechin, 7-methyl-juglone and α -amyrin-3O-ß-(5-hydroxy) ferulic acid are possibly the only three of these metabolites present in the roots in the rainy season of the winter rainfall area.

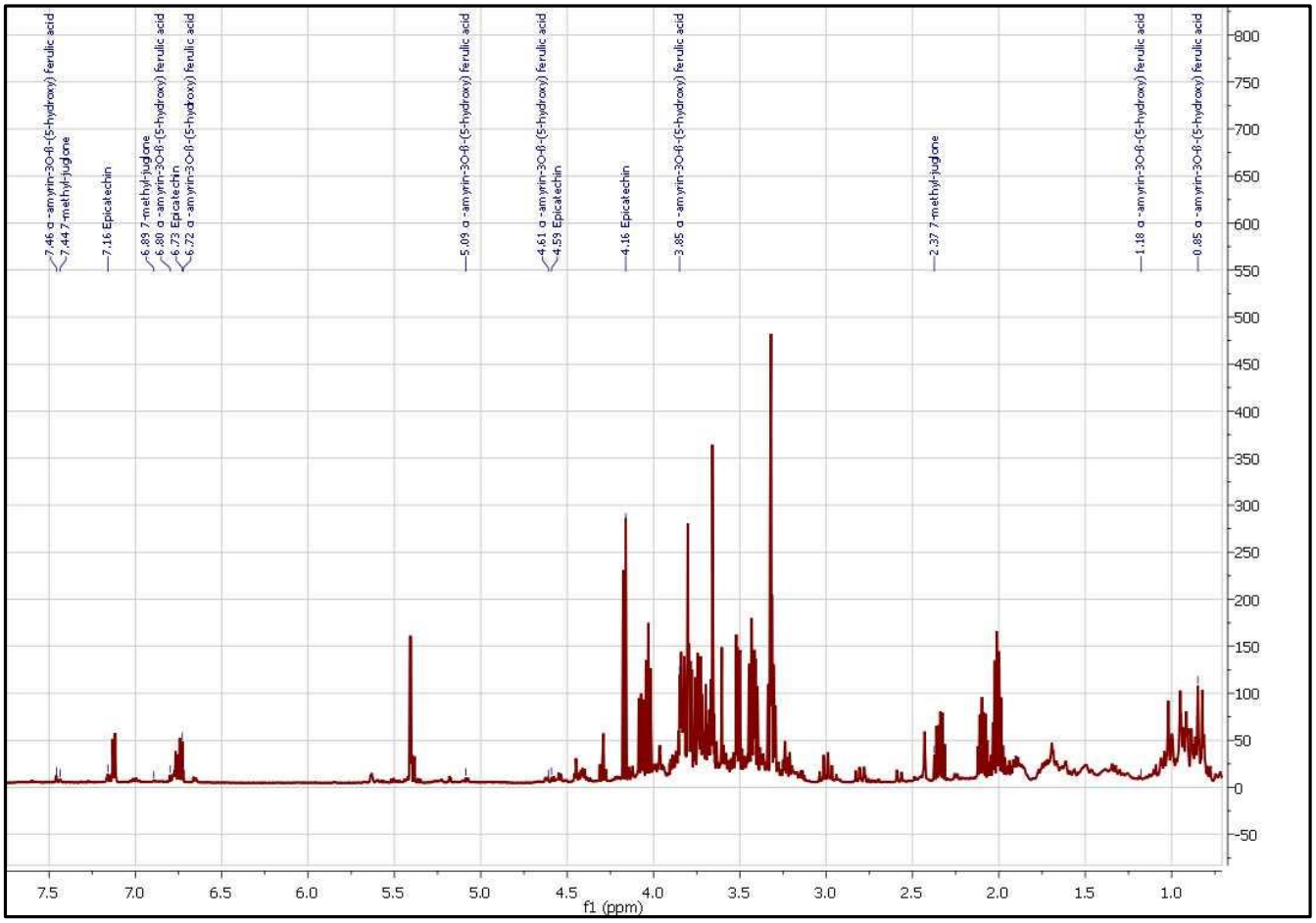
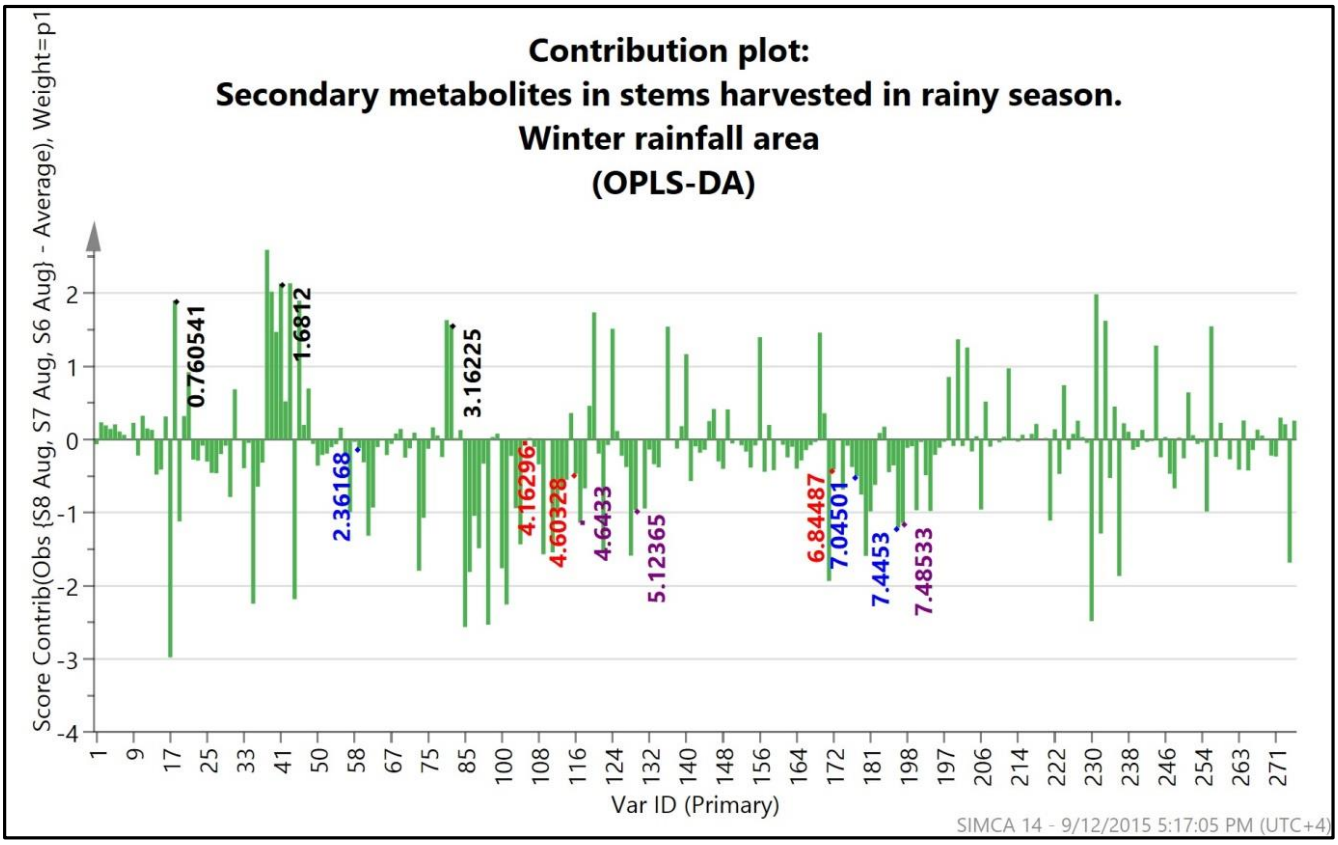


Figure 4.32: Presence of epicatechin, 7-methyl-juglone and α -amyrin-3O-ß-(5-hydroxy) ferulic acid on ^{1}H NMR spectra (600 MHz in H_2O) of roots harvested during the rainy season of winter rainfall area

4.2.3(ii) Stems harvested in the rainy season of the winter rainfall area

The contribution plot for stems gathered in the rainy season of the winter rainfall area (Figure 4.33) shows that bin values and ^{1}H NMR spectra peak values for epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and 7-methyl-juglone associate negatively with the plant material in these samples while those for lupeol associate positively. This indicates that lupeol is likely to be present in the stems during the rainy season while suggesting that the other metabolites are either absent or present in concentrations too low for chemical detection.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.33: Score contribution plot of stems gathered during rainy season of winter rainfall area

The ^{1}H NMR spectrum for stem material gathered during the rainy season of the winter rainfall area (Figure 4.34) shows the peak values (ppm) that indicate the presence of lupeol. The ^{1}H NMR values for epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and 7-methyl-juglone were not present.

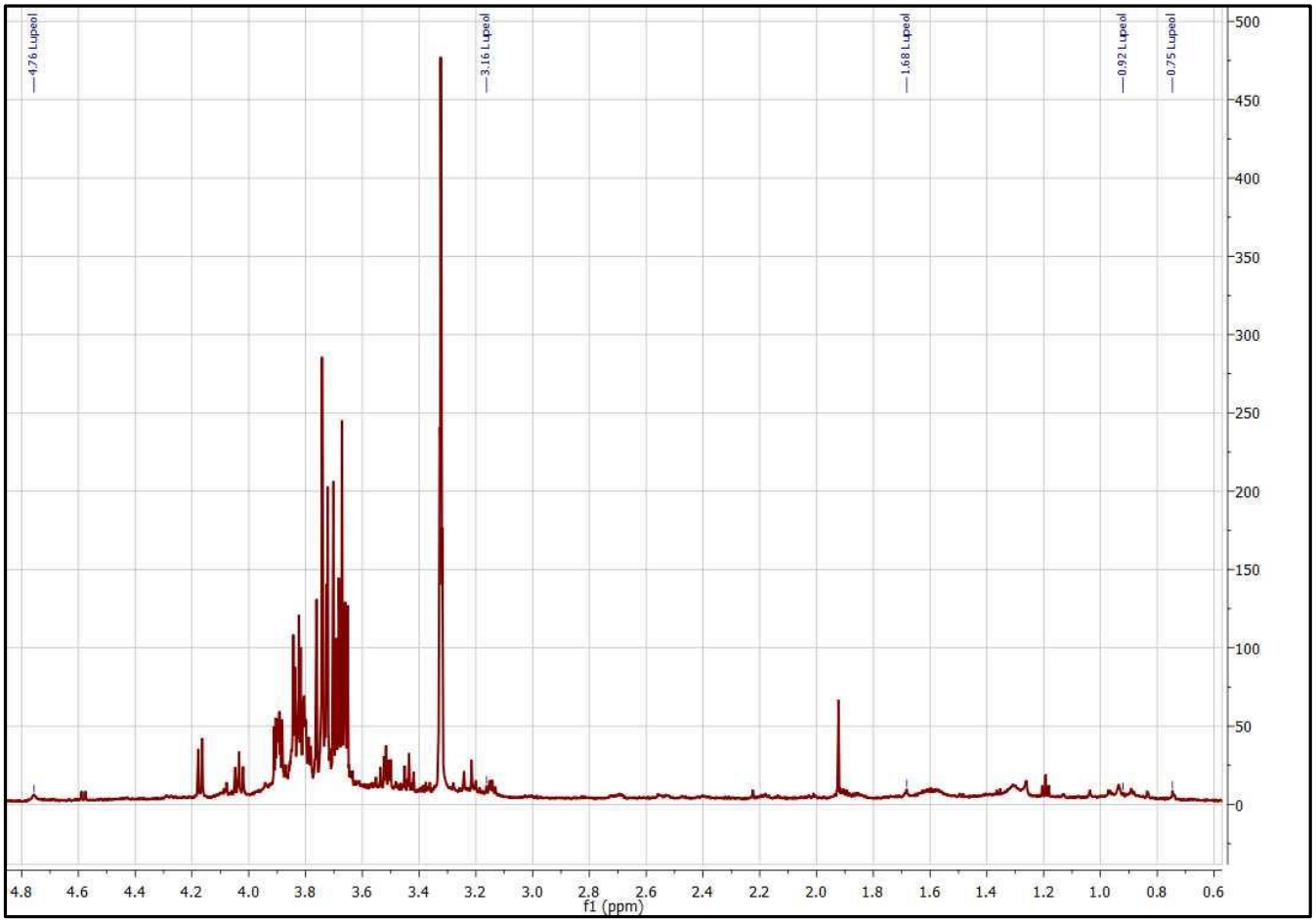
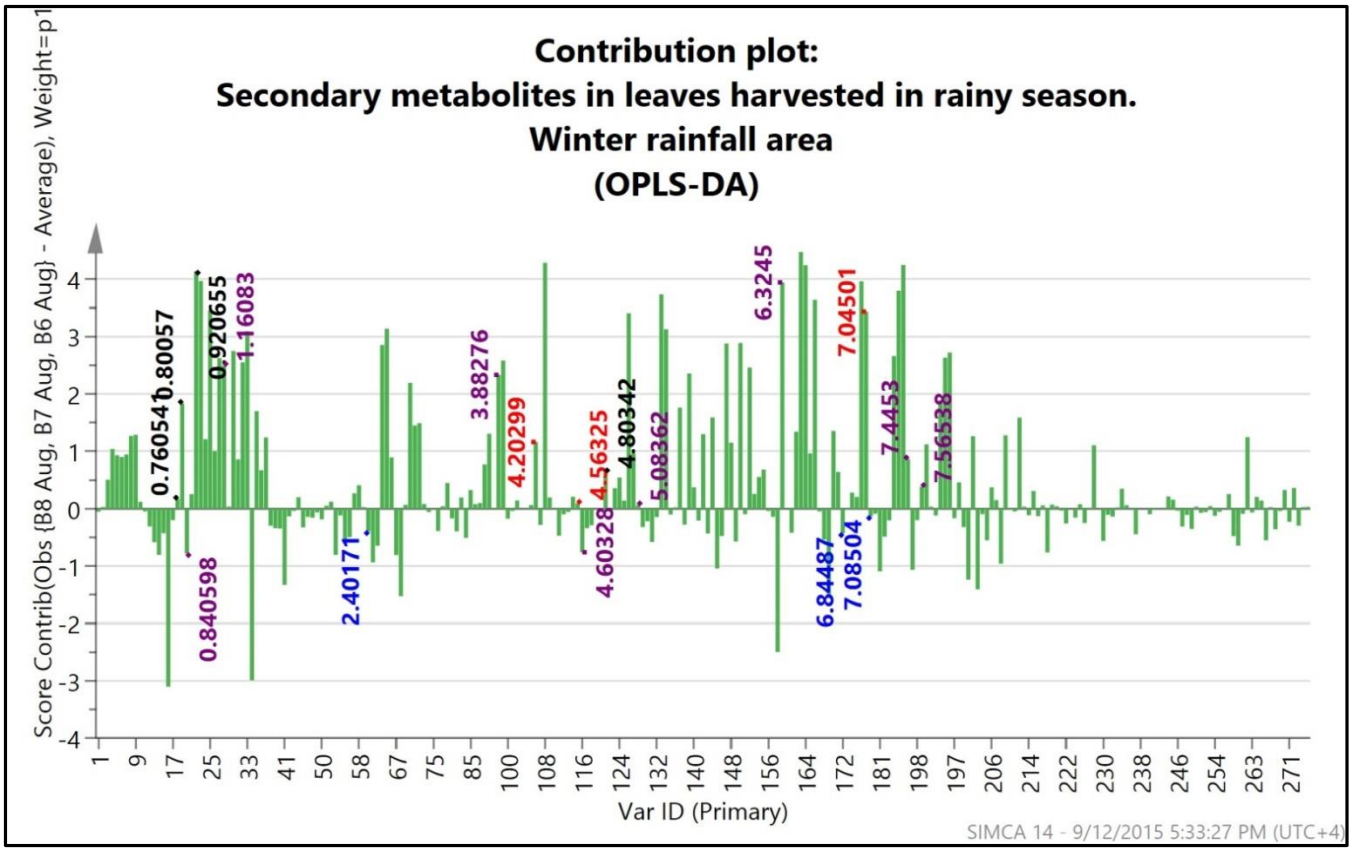


Figure 4.34: Presence of lupeol on ^{1}H NMR spectra (600 MHz in H_2O) of stems gathered during rainy season of the winter rainfall area

4.2.3(iii) Leaves harvested in the rainy season of the winter rainfall area

The contribution plot for leaves gathered in the rainy season of the winter rainfall area (Figure 4.35) illustrates that the bin values and ^{1}H NMR spectra peak values for epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol mostly associate positively with the plant material in these samples while those for 7-methyl-juglone largely associate negatively. This suggests that epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol are possibly present while 7-methyl-juglone might either be absent or present in concentrations too low to be detected chemically.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.35: Score contribution plot of leaf material gathered during the rainy season of winter rainfall area

The ^{1}H NMR spectrum for leaf material from the rainy season of the winter rainfall area (Figure 4.36) shows the peak values that indicate the presence of epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol. Values associated with 7-methyl-juglone were not present on the spectra. This corresponds to the data in the contribution plot in Figure 4.35 that suggests that epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol are possibly present in leaves during the rainy season of the winter rainfall area while 7-methyl-juglone is likely to be absent or present in concentrations too low for chemical detection.

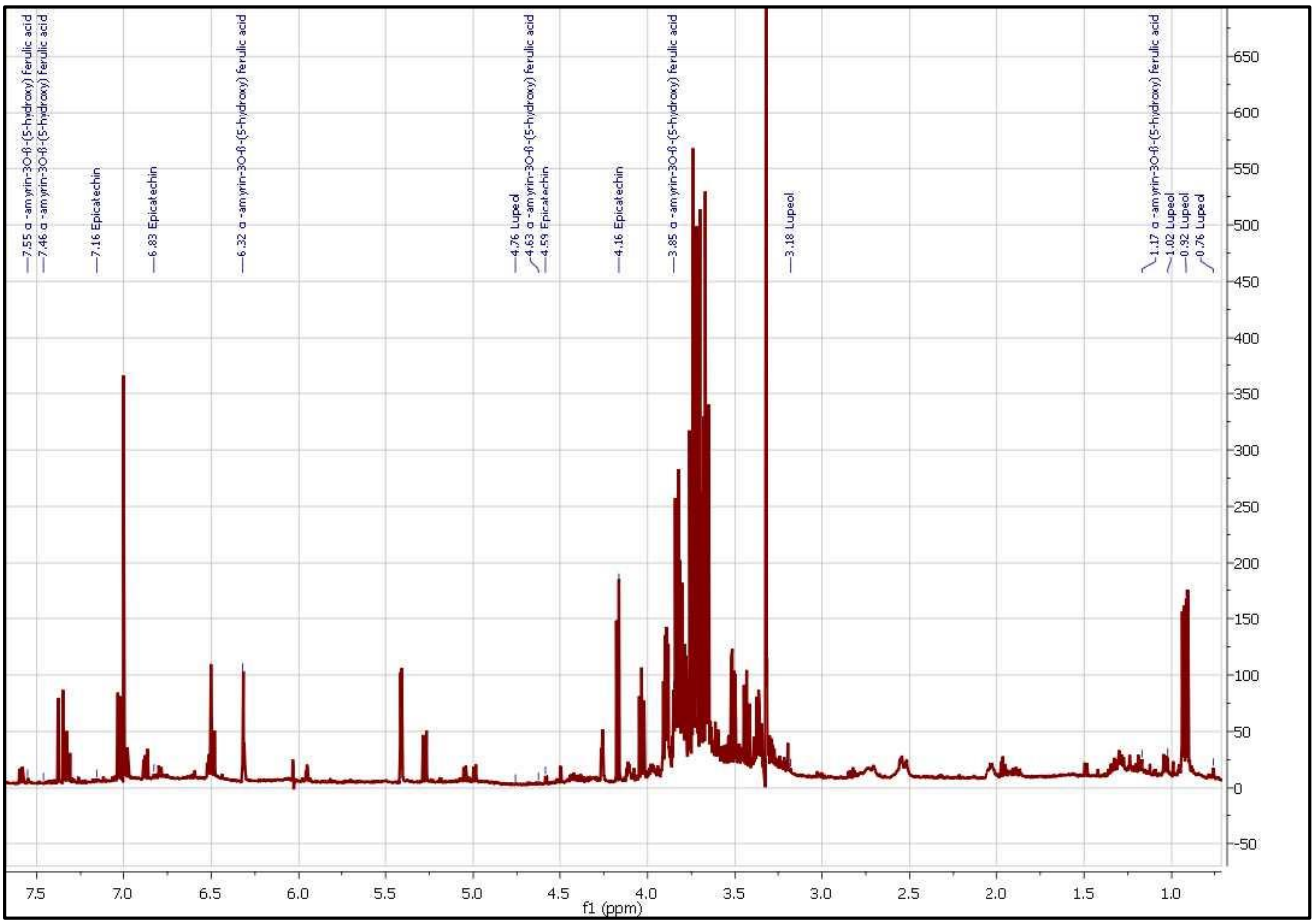
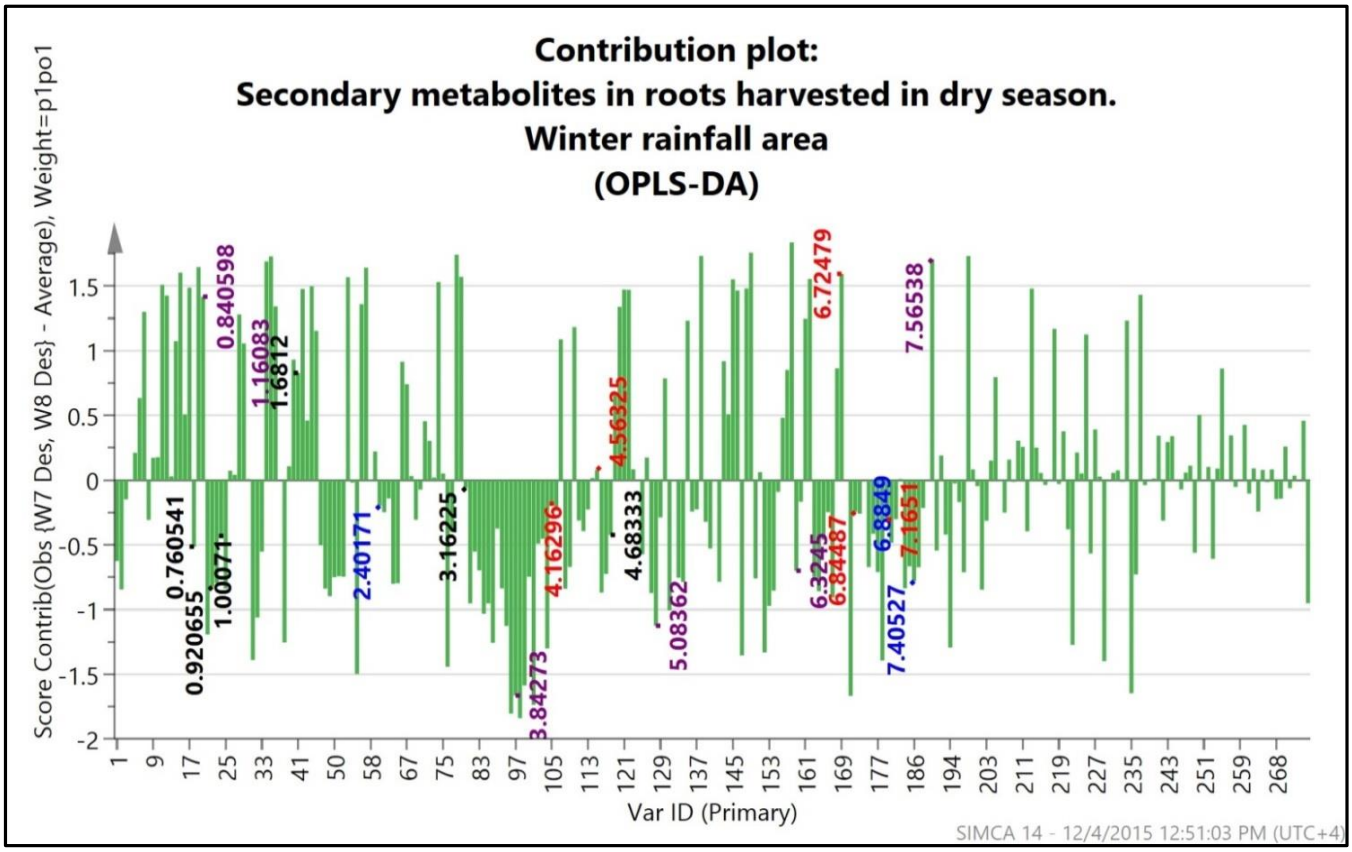


Figure 4.36: Presence of lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and epicatechin on ^{1}H NMR spectra (600 MHz in H_2O) of leaves harvested during rainy season of winter rainfall area

4.2.4 Chemical analysis of the dry season of the winter rainfall area

4.2.4(i) Roots harvested in the dry season of the winter rainfall area

The contribution plot for root material gathered in the dry season of the winter rainfall area shown in Figure 4.37 indicates that many of the bin values and ^{1}H NMR spectra peak values for epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid associate positively with the plant material in these samples while those for lupeol and 7-methyl-juglone generally associate negatively. This suggests that epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid might be the only of these metabolites present in the roots during the dry season of the winter rainfall area. Lupeol and 7-methyl-juglone are possibly absent or present in concentrations too low to be detected through chemical analysis.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.37: Score contribution plot of root material gathered during dry season of winter rainfall area

The ^{1}H NMR spectra for root material harvested in the dry season of the winter rainfall area show that peak values associated with epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid are present while those for lupeol and 7-methyl-juglone are not (Figure 4.38). This suggests that α -amyrin-3O-ß-(5-hydroxy) ferulic acid and epicatechin might be the only of these metabolites present while lupeol and 7-methyl-juglone are possibly absent or present in concentrations too low for chemical detection. This corresponds to the data on the contribution plot in Figure 4.37 that suggested that epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid are possibly the only two of these metabolites present in detectable amounts in the roots during the dry season of the winter rainfall area.

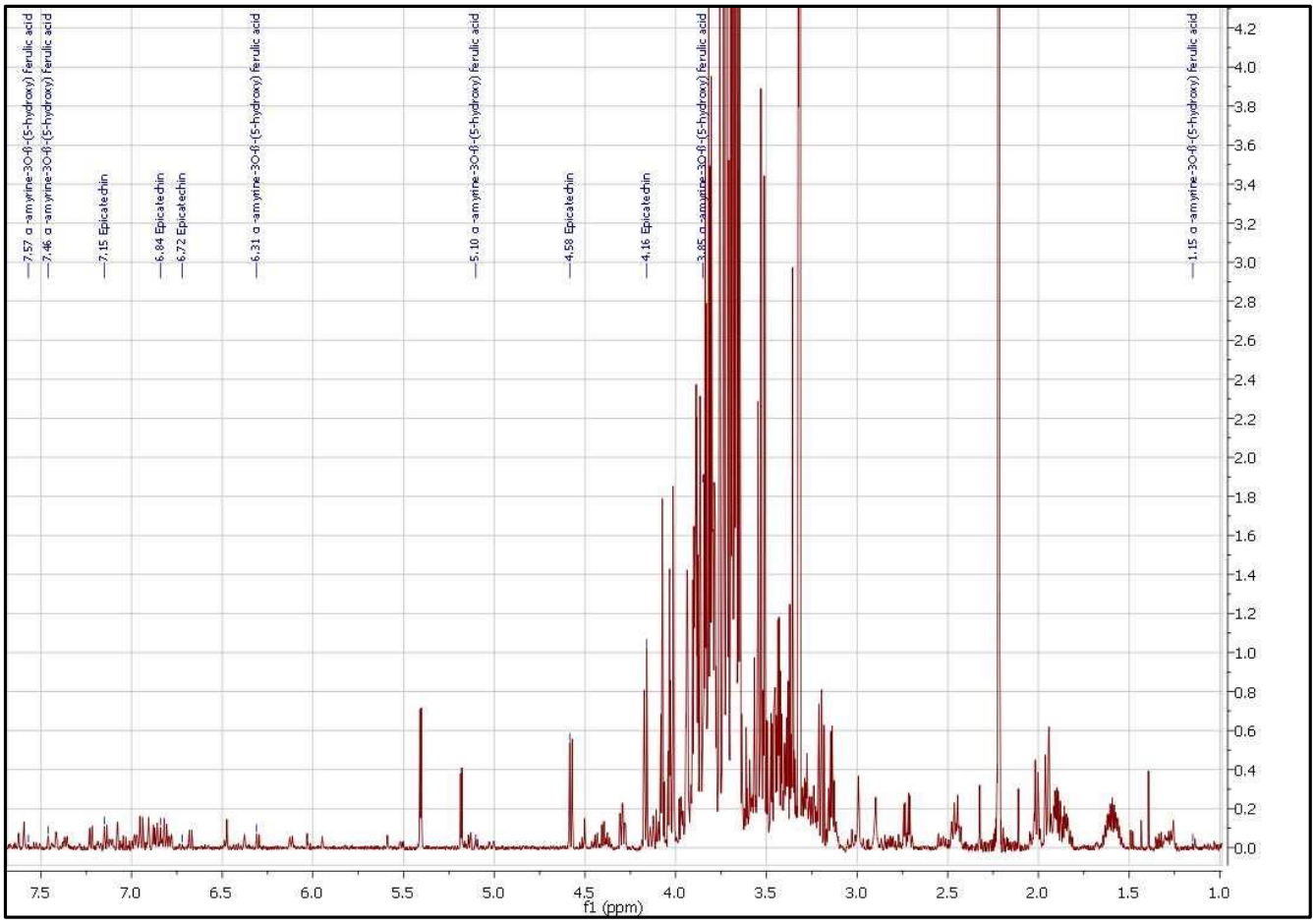
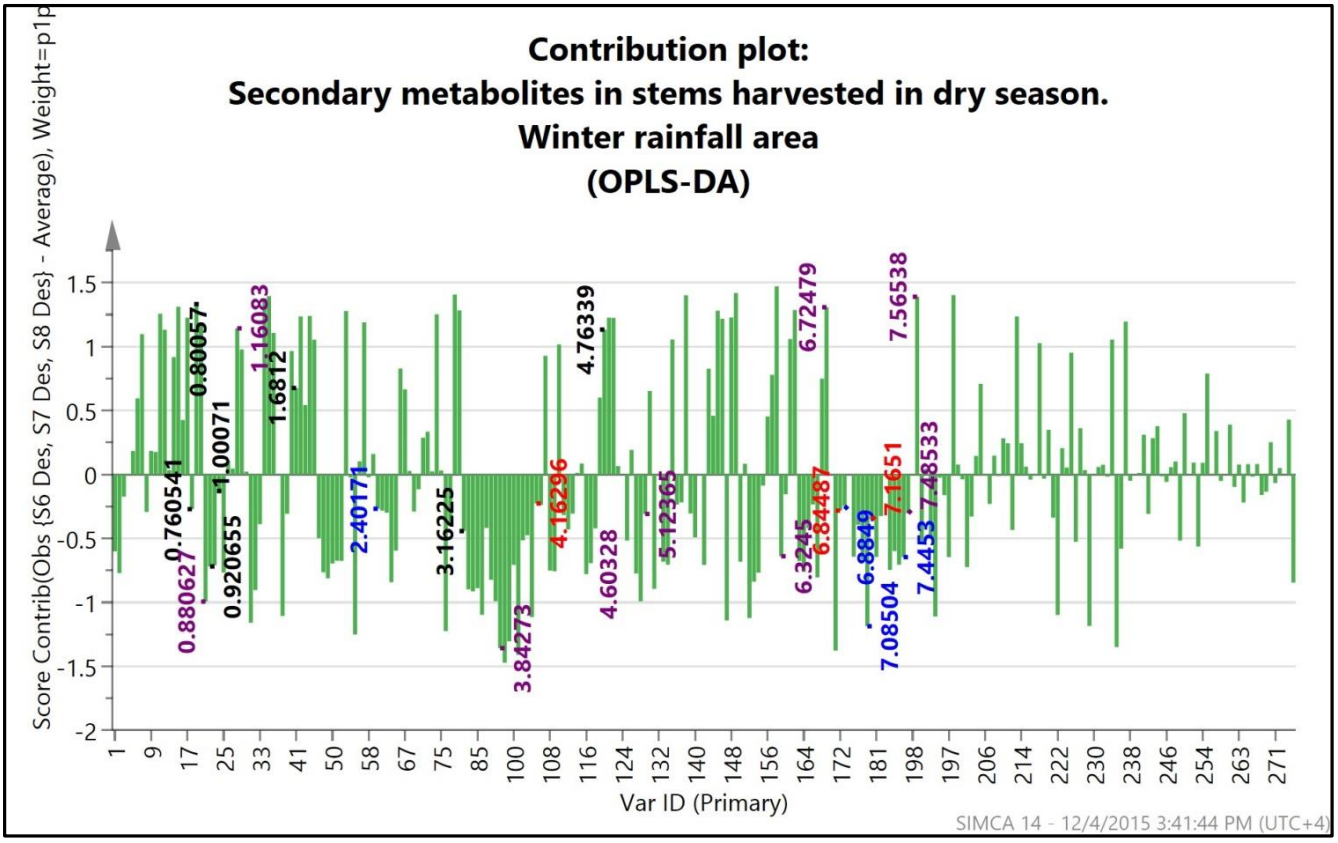


Figure 4.38: Presence of epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid on ^{1}H NMR spectra (600 MHz in H_2O) of roots harvested during the dry season of winter rainfall area

4.2.4(ii) Stems harvested in the dry season of the winter rainfall area

The contribution plot for stems gathered in the dry season of the winter rainfall area (Figure 4.39) shows that most of the bin values and ^{1}H NMR spectra peak values for lupeol associate positively with the plant material. Values for 7-methyl-juglone and epicatechin associate negatively and so do most for α -amyrin-3O-ß-(5-hydroxy) ferulic acid. This suggests that lupeol is likely to be present in the stems during the dry season while 7-methyl-juglone, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and epicatechin are likely to either be absent or present in concentrations too low for chemical detection.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.39: Score contribution plot of stems gathered during dry season of winter rainfall area

The ^{1}H NMR spectrum for stem material gathered during the dry season of the winter rainfall area (Figure 4.40) shows the peak values (ppm) that indicate the presence of lupeol. The ^{1}H NMR values for epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and 7-methyl-juglone were not present. This corresponds to the data on the contribution plot in Figure 4.39 that suggests that lupeol might be the only one of these metabolites present in detectable amounts in these samples.

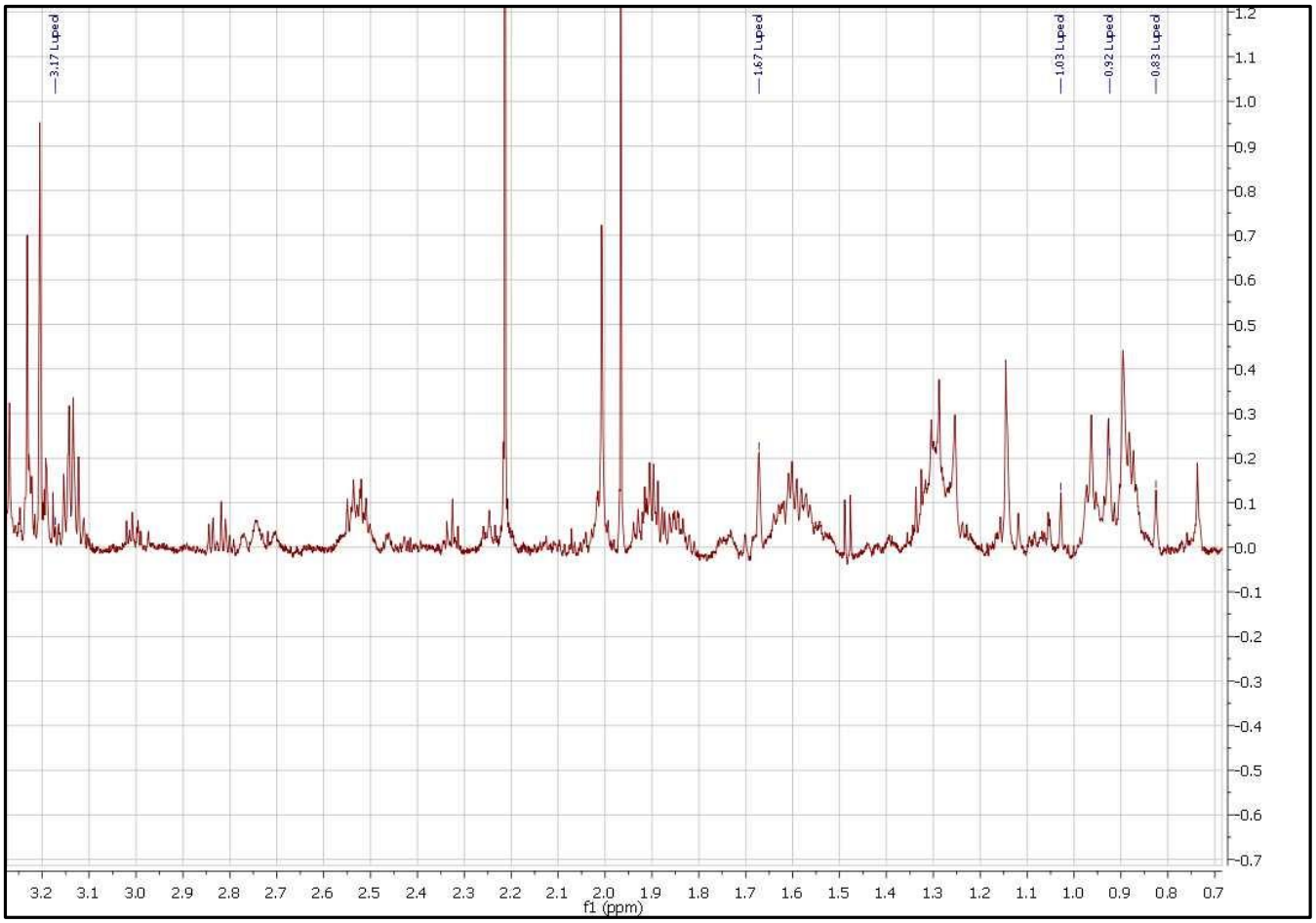
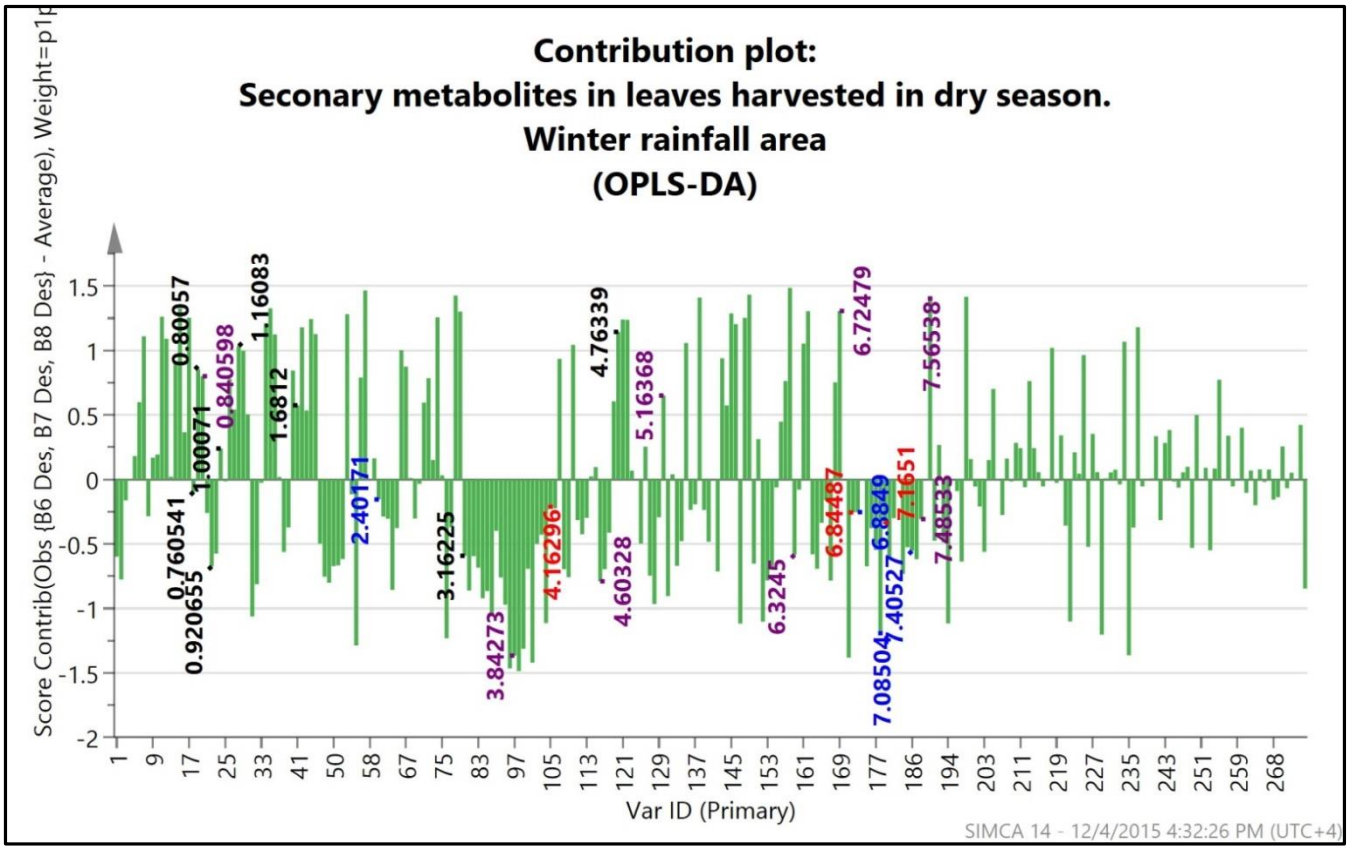


Figure 4.40: Presence of lupeol on ^{1}H NMR spectra (600 MHz in H_2O) of stems gathered during dry season of the winter rainfall area

4.2.4(iii) Leaves harvested in the dry season of the winter rainfall area

The contribution plot for leaves gathered in the dry season of the winter rainfall area (Figure 4.41) illustrates that a large number of bin values and ^{1}H NMR spectra peak values for α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol associate positively with the plant material in these samples while those for 7-methyl-juglone and epicatechin associate negatively. This suggests that α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol are possibly present while 7-methyl-juglone and epicatechin are likely to be absent or present in concentrations too low to be detected chemically.



Blue = ^{1}H NMR peak values associated with 7-methyl-juglone

Red = ^{1}H NMR peak values associated with epicatechin

Black = ^{1}H NMR peak values associated with lupeol

Purple = ^{1}H NMR peak values associated with α -amyrin-3O-ß-(5-hydroxy) ferulic acid

Figure 4.41: Score contribution plot of leaf material gathered during the dry season of winter rainfall area

The ^{1}H NMR spectrum for leaf material from the dry season of the winter rainfall area (Figure 4.42) shows the peak values that indicate the presence of lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid. Values associated with 7-methyl-juglone and lupeol were not present on the spectra. This corresponds to the data in the contribution plot in Figure 4.41 that suggests that α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol are possibly the only of these metabolites present in concentrations high enough for chemical detection.

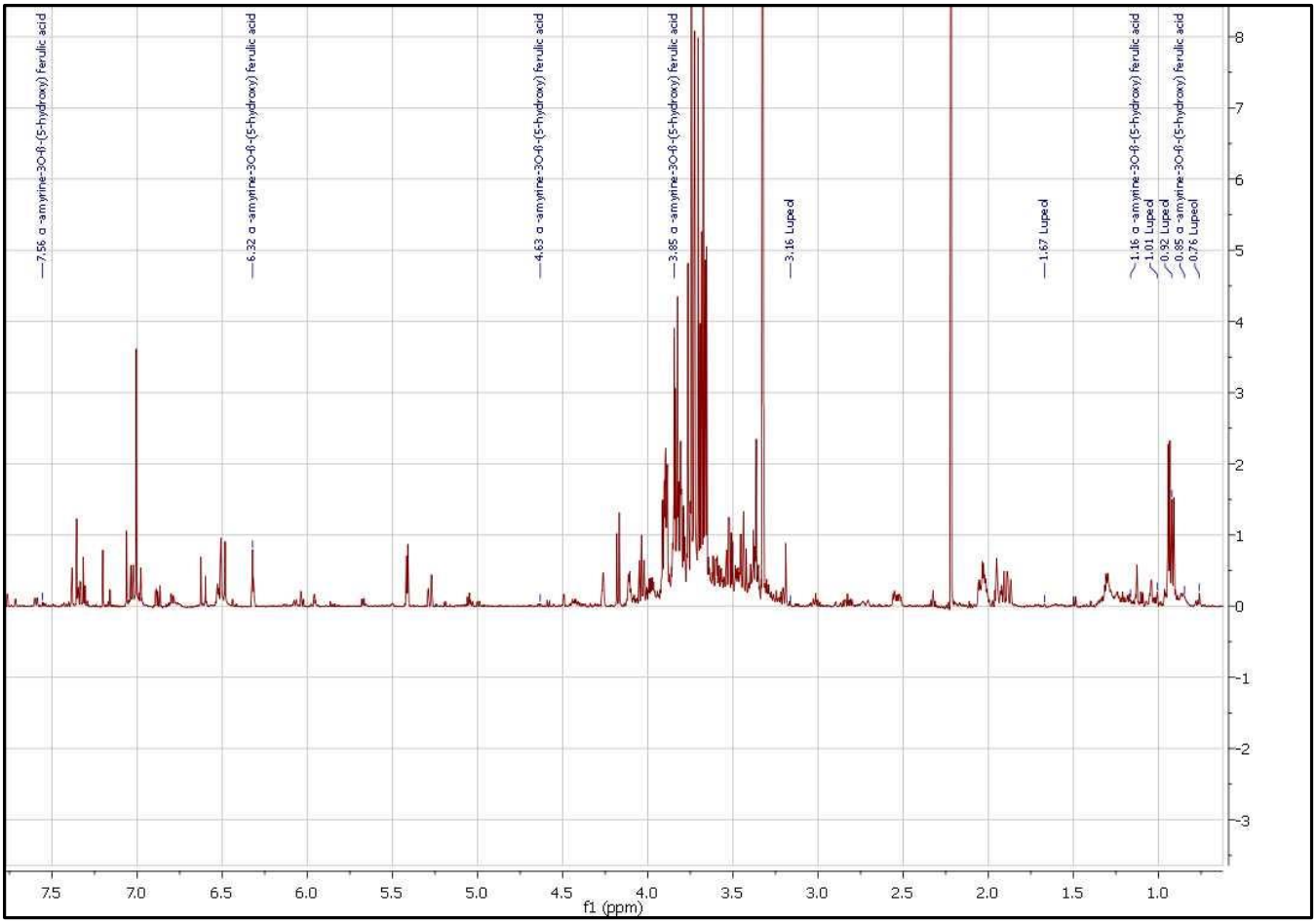


Figure 4.42: Presence of lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid on ^{1}H NMR spectra (600 MHz in H_2O) of leaves harvested during dry season of winter rainfall area

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CHAPTER 5

5.1 Discussion

In an attempt to identify possible correlations between the presence of these metabolites and the potential influence of certain seasonal environmental factors, this study used the average temperature and rainfall values to compare plant samples in which these metabolites were detected. These comparative results of chemical analysis are summarised in Table 4.

Table 4: Presence of 7-methyl-juglone, α -amyrin-3O-ß-(5-hydroxy) ferulic acid, epicatechin and lupeol in detectable concentrations in roots, stems and leaves of *E. undulata* during rainy and dry seasons of summer and winter rainfall areas

Rainfall area	Season	Organ	Metabolite			
			Lupeol	Epicatechin	7 methyl-juglone	α -amyrin-3O-ß-(5-hydroxy) ferulic acid
Summer rainfall area	Rainy season (Dec) Ave. temp: 22°C Ave. rainfall: 154mm	Roots	x	✓	✓	x
		Stems	x	x	x	x
		Leaves	x	x	✓	x
	Dry season (Aug) Ave. temp: 14°C Ave. rainfall: 2mm	Roots	✓	x	x	✓
		Stems	x	x	x	x
		Leaves	x	✓	x	✓
Winter rainfall area	Rainy season (Aug) Ave. temp:12°C Ave. rainfall: 38mm	Roots	x	✓	✓	✓
		Stems	✓	x	x	x
		Leaves	✓	✓	x	✓
	Dry season (Dec) Ave. temp: 22°C Ave. rainfall: 5mm	Roots	x	✓	x	✓
		Stems	✓	x	x	x
		Leaves	✓	x	x	✓

To investigate the presence of epicatechin, α -amyrin-3O-ß-(5-hydroxy) ferulic acid, lupeol and 7-methyl-juglone the following statistical comparisons of roots, stems and leaves were made using SIMCA software (version 14.0; Umetrics)

- Comparison of rainy and dry seasons of the summer rainfall area.
- Comparison of rainy and dry seasons of the winter rainfall area.
- Comparison of rainy seasons of the winter and summer rainfall areas.
- Comparisons of dry seasons of the winter and summer rainfall areas.

An evaluation of this statistical analysis was then conducted by comparing data yielded from the chemical analysis of plant material to that of the statistical data.

When examining the statistical data it is notable that the Q2 values of the OPLS-DA models obtained fall between the values of 0.4 and 1, which is considered an empirically inferred acceptable range for biological models (Worley & Powers, 2013). The statistical comparisons of the rainy and dry seasons of the summer rainfall area as well as the statistical analysis of the dry seasons of the two rainfall areas however reveal in their PCA models Q2 values that are lower than 0.4. Although these values have been directed in the case of this study and are attributed to natural variations within the plant samples, they are a possible indication that these models lack robustness (Lourenço *et al*., 2013).

Worley & Powers (2013) state that Q2 values have no standard of comparison or critical value for inferring significance and explain that unsupervised and unbiased methods such as PCA analysis provide only an informative initial observation of a dataset. They further suggest that it should ideally be used to formulate an initial biological conclusion which an OPLS-DA analysis can thereafter test in more detail.

5.1.1 Comparing rainy and dry seasons of the summer rainfall area

Data obtained from OPLS-DA Hierarchical clustering of root, stem and leaf material from the summer rainfall area suggested possible chemical similarities between stems and roots in the rainy and dry seasons respectively (Figure 4.4).

Stems and roots from the rainy season appear to have the absence of lupeol and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in common (Table 4). This similarity might contribute to the affiliations between stems and leaves observed in the OPLS-DA groupings in Figure 4.4 while the differences in terms of 7-methyl-juglone and epicatechin might contribute to the fact that stem and leaf material still separates into different groups in the OPLS-DA validations.

When examining the data for the dry season, roots and stems have the absence of epicatechin and 7-methyl-juglone in detectable concentrations in common (Table 4). These similarities might possibly contribute to the affiliations between stems and leaves observed in the OPLS-DA groupings in Figure 4.4 while the apparent presence of α -amyrin-3O-ß-(5-hydroxy) ferulic acid and lupeol in roots only might contribute to the fact that stem and leaf material still fall in separate groups in the OPLS-DA validations.

Grouping of leaf material in the OPLS-DA hierarchical clustering diagram suggested possible chemical similarities between leaves from the two seasons (Figure 4.4). Chemical analysis reveals the likely absence of lupeol to be the only similarity (Table 4). This similarity might contribute to the affiliations observed in the OPLS-DA clusters in Figure 4.4 while the differences observed in the results might contribute to the fact that leaf material from the two seasons still fall in separate groups in the OPLS-DA hierarchical cluster diagram. Results from the OPLS-DA data could also be attributed to chemical differences and similarities that fall outside the scope of this study.

Statistical analysis obtained from OPLS-DA score scatter plots (Figure 4.3) indicated separation between material from the rainy and dry seasons. The apparent absence of all four metabolites from all the stem material as well as that of lupeol from all

leaves were the only similarities observed between the rainy and dry seasons while various differences were detected in terms of the presence of all four investigated metabolites in root and leaf material from the two seasons (Table 4). These chemical differences might contribute to the delineation between the rainy and dry seasons observed in score scatter plots. The fact that certain of the metabolites investigated appear to be present in some plants at certain times of the year while absent at other times could be a possible indication that their presence might be influenced by seasonal change.

It is important to note that the results observed in the OPLS-DA data could also be attributed to chemical differences and similarities that fall outside the scope of this study.

5.1.2 Comparing rainy and dry seasons of the winter rainfall area

The clustering together of root and stem material from the rainy season on OPLS-DA scatter plots suggested possible chemical similarities between these organs (Figure 4.7).

When examining the chemical analysis from the rainy season it becomes evident that stems and roots share no similarities in terms of the metabolites studied (Table 4). These differences might contribute to the fact that stem and root material still delineate into separate groups in the OPLS-DA Hierarchical cluster diagram (Figure 4.8) in spite of the fact that they cluster together in close proximity. Results also suggest that any similarities in terms of chemical composition might be the result of compounds not included in this investigation.

Statistical data obtained from OPLS-DA score scatter plots of material from the winter rainfall area (Figure 4.7) indicated close clustering of root, stem and leaf material from the dry season indicating chemical similarities amongst these organs. Chemical analysis revealed the likely absence of 7-methyl-juglone to be the only similarity shared by all three organs (Table 4). This might contribute to the affiliations observed in OPLS-DA data, although chemical similarity could possibly also be the result of shared metabolites that fall outside the scope of this investigation.

The differences in terms of α -amyrin-3O-ß-(5-hydroxy) ferulic acid, lupeol and 7-methyl-juglone concentrations might contribute to the fact that the roots, stems and leaves cluster in separate groups in the hierarchical cluster diagram (Figure 4.8).

Statistical analysis obtained from OPLS-DA data (Figure 4.7) indicated that material from the dry season clustered together closely while indicating more prominent delineation between roots, stems and leaves from the rainy season into separate clusters. This suggested more chemical similarity amongst organs from the dry season and less chemical similarity amongst organs from the rainy season. Chemical analysis indicates the apparent absence of 7-methyl-juglone to be the only similarity shared by all three organs during the dry season. This might contribute to the affiliations observed amongst the organs in the OPLS-DA data although metabolites outside the field of this investigation could also have contributed.

Chemical analysis also reveals notable similarities between organs from the two seasons, with the difference in the presence of 7-methyl-juglone in roots and epicatechin in leaves being the only exceptions. These chemical similarities might possibly contribute to clusters observed in OPLS-DA validations (Figure 4.8) that suggested possible chemical affiliations between material from the two seasons. The fact that 7-methyl-juglone and epicatechin appear to be present in some organs at certain times of the year while absent at other times could be a possible indication that their presence might be influenced by seasonal change.

It is important to reiterate that the results observed in the OPLS-DA data could also be attributed to chemical differences and similarities that fall outside the scope of this study.

5.1.3 Comparing rainy seasons of the winter and summer rainfall areas

Data obtained from OPLS-DA hierarchical clustering of root, stem and leaf material from the rainy seasons of the summer and winter rainfall areas suggested possible chemical similarities between root material from the two rainfall areas (Figure 4.12). Chemical analysis reveals that roots from the different rainfall areas appear to have the absence of lupeol as well as the presence of epicatechin and 7-methyl-juglone in common (Table 4). These similarities could possibly contribute to the affiliations observed on the OPLS-DA Hierarchical cluster diagram while the difference in terms of α -amyrin-3O-ß-(5-hydroxy) ferulic acid could contribute to the fact that root material from the different rainfall areas still fall in separate groups in the OPLS-DA validations. It is also possible that groupings in the Hierarchical cluster diagrams are the result of chemical differences or similarities that fall outside the scope of this study.

Data obtained from OPLS-DA Hierarchical clustering also suggested possible chemical similarities between stems from the two rainfall areas (Figure 4.12). Chemical analysis reveals that stems from the different rainfall areas have the absence of 7-methyl-juglone, epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in common (Table 4). These similarities could possibly contribute to the affiliations observed on the OPLS-DA Hierarchical cluster diagram while the difference in terms of lupeol could possibly contribute to the fact that stem material from the different rainfall areas still fall in separate groups in the OPLS-DA data. It is also possible that data groupings in the Hierarchical cluster diagrams are the result of chemical differences or similarities that fall outside the scope of this study.

The OPLS-DA hierarchical further suggested possible chemical similarities between leaves from the two rainfall areas (Figure 4.12). Chemical analysis reveals no detectible similarities in terms of the metabolites investigated (Table 4). The chemical data suggests that the affiliations observed in the OPLS-DA data are likely to be the result of chemical similarities not investigated in this study. The differences in chemical composition observed in the results could possibly contribute to the fact that leaf material from the different rainfall areas fall in separate groups in the OPLS-DA data.

Chemical analysis reveals notable similarity between roots and stems, with the presence of α -amyrin-3O-ß-(5-hydroxy) ferulic acid in roots and lupeol in stems being the only detectable difference with regards to the investigated metabolites. As the rainy seasons fall within opposite seasons, these results might possibly suggest that availability of water contributes significantly to the presence and production of lupeol, epicatechin and 7-methyl-juglone in stems and roots of *E. undulata* and that chemical similarities might possibly arise under certain conditions in plants in different locations. No similarities were detected in terms of the presence of any of these metabolites in the leaves.

5.1.4 Comparing dry seasons of the winter and summer rainfall areas

The most prominent delineations obtained from OPLS-DA Hierarchical clustering of root, stem and leaf material from the dry seasons of the summer and winter rainfall areas indicate that organs from the winter rainfall area cluster together very closely (Figure 4.15) and a degree of overlap is visible on validation diagrams of this material (Figure 4.16). Chemical analysis reveals the absence of 7-methyl-juglone to be the only similarity in terms of the metabolites investigated. Stems and leaves furthermore appear to have the presence of lupeol as well as the absence of epicatechin in common (Table 4). These similarities might contribute to the delineations observed in the statistical analysis, although statistical similarities might also be the result of metabolites that fall outside the scope of this investigation.

Chemical analysis reveals notable similarity between stems, with lupeol detected in the winter rainfall area being the single difference with regards to the investigated metabolites. Also notable is the absence of 7-methyl-juglone from all organs during the dry seasons of both areas as well as the presence of α -amyrin-3O-ß-(5-hydroxy) ferulic acid in leaves and roots from both areas.

5.1.5 Comparing the results of this study to existing literature on related plant species

When examining the root material harvested in this study, it is particularly the results of the summer rainfall area that clearly indicate seasonal variations in the presence of the four metabolites investigated (Table 4). Existing literature shows that Van der Vyver & Gerritsma (1973;1974) isolated 7-methyl-juglone from chloroform extracts of the roots of the *Euclea* genus while Deutschländer *et al.* (2010) could not. Bapela *et al.* (2007) successfully identified 7-methyl-juglone in the roots of *E. natalensis* using chloroform extracts, as did Mital *et al*. (2010). The seasonal fluctuations in detectable concentrations of 7-methyl-juglone noticed in this investigation might explain why Deutschländer *et al.* (2010) were unable to locate 7-methyl-juglone during their investigation even though other researchers managed to detect this metabolite.

The seasonal presence of α-amyrin-3*O*-β-(5-hydroxy) ferulic acid, lupeol and epicatechin in the investigated root material (Table 4) corresponds to the work of Deutschländer *et al.* (2010) who successfully identified the presence of these metabolites in the roots of *E. undulata* using acetone extracts. Adzu *et al.* (2015) describe the successful isolation of lupeol from chloroform extracts of the root bark of the related species *D. mespiliformis, and* Sibanda *et al*. (1992) describe the successful isolation of lupeol from the root bark of *E. crispa*. These findings further correlate to the results for the dry season of the summer rainfall area of this investigation (Table 4). The seasonal fluctuations observed in this study however suggest that the successful identification of these metabolites in root material of *E. undulata* and related species might depend upon the season of harvesting.

Results of the stem material indicate the complete absence of the investigated metabolites from all harvested material with the exception of the presence of lupeol in the winter rainfall area (Table 4). This correlates to the research of Gu *et al.* (2004) which describes the presence of lupeol in chloroform extracts of the stem bark of *D. maritima.* Existing literature also shows that the absence of 7-methyl-juglone from all harvested stems in this investigation (Table 4) are similar to the results of a study

conducted by Neuwinger (1994) in which it was found that naphthaquinones were completely absent in chloroform stem extracts. The results of this study indicate that detectible concentrations of lupeol appear to be either present or absent in certain areas, regardless of seasonal change. This might suggest that environmental factors outside the scope of this study determine its presence and offers a possible explanation for the fact that existing literature indicates the successful identification of lupeol in only some of the investigated members of the *Euclea* and *Diospyros* genera.

Results of the leaf material analysed in this investigation indicate that chemically detectible concentrations of all four metabolites appear to be influenced by either seasonal or geographical change. Epicatechin was detected in the leaves of the dry season of the summer rainfall area as well as the rainy season of the winter rainfall area (Table 4). This correlates to the findings of Pretorius *et al.* (2003) who confirmed its presence in ethyl acetate fractions of the leaves of *E. crispa* subsp. *crispa*. Existing literature also shows that Hattas & Julkunen-Tiitto (2012) detected epicatechin in the leaves of *E. divinorum* using methanol extractions.

The results of this investigation indicate that 7-methyl-juglone was present in the leaves from the rainy season of the summer rainfall area and that lupeol was detected in all leaf material harvested in the winter rainfall area, regardless of season (Table 4). Existing literature also indicates the presence of these two metabolites in related species. Sinha & Bansal (2008) identified lupeol and 7-methyl-juglone in methanol extracts of the leaves of *D. kaki* as well as chloroform extracts of the leaves of *D. melanoxylon.*

Although existing literature does not always indicate seasonal and environmental conditions under which research was done, it confirms the presence of lupeol, epicatechin and 7-methyl-juglone in the leaves of the *Diospyros* and *Euclea* genera and correlates to the presence of these metabolites in at least some of the samples analised in this investigation.

5.2 Conclusion

5.2.1 Possible influence of seasonal environmental factors on the presence of 7-methyl-juglone, lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid

One of the objectives identified in this study was to investigate the possible influence of seasonal change on the presence of these metabolites in order to assist the potential development of a treatment for diabetes from *E. undulata.*

The results of the summer rainfall area indicate various seasonal fluctuations in the levels of 7-methyl-juglone, lupeol, α -amyrin-3O-ß-(5-hydroxy) ferulic acid and epicatechin in leaves and roots. It is interesting to note that stems appear to be devoid of all these metabolites during both the rainy and dry seasons.

The results from the winter rainfall area indicate far less seasonal fluctuation. Most metabolites appear to be either present or absent in the respective organs regardless of season. Fluctuations were however detected in epicatechin levels in leaves and 7-methyl-juglone levels in roots.

The influence of environmental factors on the secondary metabolism of plants has been studied extensively and is well-described in existing literature. Xu *et al.* (2010) describe drought stress as one of the most significant abiotic forms of stress to affect plant growth and development. Jafaar *et al*. (2012) also state that water stress is one of the most important factors in determining the biochemical properties of plants. This is reflected in the findings of this study where varying levels of rainfall between seasons coincided with fluctuations in the presence of certain metabolites. This suggests that the drastic differences in rainfall experienced between the summer and winter months of the areas where plants were harvested could play a significant role in the presence of 7-methyl-juglone, lupeol, epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in *E. undulata.*

It is the conclusion of this study that it is indeed possible that the presence of these metabolites could be controlled by seasonal environmental factors. This could possibly provide a potential explanation for the contradictions in existing literature on the presence of 7-methyl-juglone in *E. undulata* (van der Vyver & Gerritsma, 1973, 1974; van Wyk *et al.,* 2009; Deutschländer *et al.,* 2010).

5.2.2 Recommendations for future development of successful, safe and sustainable treatments for diabetes from *E. undulata*

Another objective of this study was to investigate the possible viability of the development of a treatment for diabetes from *E. undulata.* This would rely upon the presence of epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid as the active compounds (Deutschländer *et al.,* 2010) coinciding with the absence of 7-methyl-juglone as a cytotoxic naphthaquinone (Statiauskaite *et al.,* 2006).

The results from the summer rainfall area only found epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid to be present at the same time in the leaves of the dry season. Results also indicate the absence of 7-methyl-juglone from leaves during the dry season. This suggests that the leaves from the dry season of this area are a possible sustainable source of plant material for research into the development of a diabetes treatment.

Similarly, results from the winter rainfall area found leaves of the dry season to contain epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in the absence of 7-methyl-juglone. This again highlights the potential of these leaves from dry seasons as a sustainable source for the potential investigation for treatment development. The presence of epicatechin and α -amyrin-3O-ß-(5-hydroxy) ferulic acid was also detected in roots from both seasons, although 7-methyl-juglone was found in samples from the dry season. This suggests that, although it is less sustainable than the harvest of leaves would be, the roots from the rainy season of this area are also a possible source for the development of a diabetes treatment.

It is also suggested that the safe and effective use of *E. undulata* by traditional healers is further aided by the employment of high performance liquid chromatography (HPLC) or liquid chromatography mass spectroscopy (LC-MC) in future research into the seasonal presence of the metabolites in question. These methods of chemical analysis could identify compounds present in relatively high concentrations in harvested material, thereby guiding the practice of seasonal use in all regions of the country.

5.3 Possible future research

There are many environmental factors outside the scope of this study that could play a potential role in the presence of the metabolites investigated in *E. undulata.* Research has shown a wide range of environmental conditions to have a possible influence on the production of secondary compounds. Tuteja & Sopory (2008) state that plants alter their metabolite production in response to changes and seasonal fluctuations in light conditions, carbon dioxide levels, water and nutrient availability as well as temperature variations. Gershenzon (1984) describes factors such as low levels of light and temperature, fungal infections and herbivory as having notable influence on the production of secondary metabolites in plants. Ianson (2005) describes the production of secondary metabolites in response to herbivory as a mechanism that results in avoidance of the plants as a food source.

All plants investigated in this study were harvested in protected areas and would therefore be exposed to natural fluctuations in these environmental factors. The recommendation can therefore be made to investigate the influence of factors such as these on the presence of epicatechin, lupeol, 7-methyl-juglone and α -amyrin-3O-ß-(5-hydroxy) ferulic acid in *E. undulala* in future research into diabetes development.

Several non-environmental factors are also known to influence the presence and production of secondary metabolites. The role of genetics in the production of secondary metabolites is well-documented. Bibb (2005) describes the role of genes in the production of secondary metabolites, explaining how the production of various secondary metabolites is controlled by the production of proteins by gene expression. Bouvier *et al.* (2003) describe the role of genetics in secondary metabolite production

in their research to identify a gene that controls the production of certain carotenoid derivative secondary metabolites within the *Crocus* genus. The possible influence of genetic factors was not investigated in this study and a further recommendation could therefore be made to include this in potential future research into secondary metabolite production in *E. undulata* when developing a potential diabetes treatment. Existing literature indicates gender as another example of a non-environmental factor that influences secondary metabolite production. Strauss *et al.* (2004) describe the 'optimal defence theory' that predicts that tissues that are the most valuable to the plant are expected to be defended the best. This includes reproductive structures and might suggest notable chemical differences between the two separate sexes of *E. undulata* as described by Coats Palgrave & Coats Palgrave (2002). Massei *et al.* (2006) describe certain gender-related differences in concentration of secondary metabolites in dioecious plants and explain that male plants generally grow faster since female plants allocate more resources to reproduction and chemical defences. When investigating *Juniperus oxycedrus macrocarpa* L. it was found that in addition to growing faster males also had higher concentrations of both phenolic and terpenoid secondary metabolites (Massei *et al.* 2006). When investigating *Salix rigida* Muhl. Elmqvist *et al.* (1991) found that female plants displayed higher levels of tannins and describe a notable reduction in secondary metabolite levels during fruiting. The influence of gender was not investigated in this study, and the recommendation could therefore be made that this be included in future investigations into the development if diabetes treatments from *E. undulata.*

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