CHARACTERIZATION OF POLYPHENOLS IN LEAVES OF FOUR DESICCATION TOLERANT PLANT FAMILIES

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Abbreviations

β-	Beta
β-CLA	Beta-carotene linoleic acid
BHT	Butylated hydrotoluene
BSA	Bovine serum albumin
C	Chloroplast
CAT	Catalase
Cu	Copper
CW	Cell wall
CY	Cytoplasm
DAD	Diode array detection
DNA	Deoxyribonucleic acid.
DPPH	1,1 diphenyl –2 picrylhydrazyl
ε	Extinction coefficient
EDTA	Ethylenediaminetetraacetate
ESI	Electro-spray ionisation
ESI-MS	Electrospray ion mass spectrometry
FAO	Food and Agricultural Organisation
Fe^{3+}	Ferric ion
Fe^{2+}	Ferrous ion
$FeCl_3$	Ferric chloride
$FeSO_4.7H_2O$	Ferric sulphate heptahydrate
FRAP	Ferric reducing /antioxidant power
g	gram
GPX	Glutathione-peroxidase
GSH	Glutathione
HCL	Hydrochloric acid
HHDP	2 ,3-α-hexahydroxydiphenyl
HPLC	High-performance liquid chromatography
Hsp	Heat shock proteins
I.A.E.A	International Atomic Energy Agency

IUPAC	International Union of Pure and Applied Chemistry
IOAC	International Organisation for Agriculture and Chemistry
IC_{50}	Concentration required for fifty percent inhibition.
kV	kilovolts
LEA	Late embryonic abundant proteins
L/h	Litres per hour
M	Molar
m^{-2}	per square meter
Me	Metal
mg	Milligram
mg GAE/g DW	Milligram gallic acid equivalents per gram dry weight
mg TAE/g DW	Milligram of tannic acid equivalents per gram dry weight
ml	Millilitre.
mm	Millimetre
Mpa.	Mega Pascal
M_r	Molecular weight
MS	Mass spectrometry
m/z	Mass to charge ratio
N_2	Atmospheric nitrogen
$NaNO_2$	Sodium nitrite
N.D	Not detected
Nm	Nanometer.
NMR	Nuclear magnetic resonance
PAC	Phenol antioxidant coefficient
PARC	Phenol anti-radical coefficient
PP	Polyphenol
PPP	Protein precipitable polyphenols assay
PVPP	Polyvinyl polypyrrolidone
ROS	Reactive oxygen species
S	Starch.
s^{-1}	per second

SDS	Sodium dodecyl sulphate
Se	Selenium
SOD	Superoxide dismutase
T	Thylakoids
TEA	Triethanolamine
TFA	Trifluoroacetic acid
TIC	Total ion chromatograms
TOF	Time of flight.
TPTZ	2,4,6 tripyridyl –s- triazine
µL	micro-litre
µM	Micro molar
UV-Vis	Ultra violet-visible
V	Vacuole.
V	Volts
w/v	Weight to volume ratio.
Zn	Zinc
%	Percent.
^{0}C	Degrees Celsius.
% DW L.E	percent of dry weight as Leucocyanidin equivalents
% EAE DW	percent ellagic acid equivalent of dry weight material

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ABSTRACT

Polyphenols in plants are known to act as antioxidants, antimicrobials, antifungal, photoreceptors, visual attractors and as light screens. In this study polyphenols in angiosperms found in southern Africa and called resurrection (desiccation tolerant) plants were studied. These plants are *Myrothamnus flabellifolius, Xerophyta viscosa, Xerophyta humilis, Xerophyta schlecterii, Xerophyta villosa, Craterostigma wilmsii, Craterostigma plantagineum, Craterostigma pumilum* and *Eragrostis nindensis*. These plants are able to tolerate water stress without undergoing permanent damage. During drying these plants are subjected to different stresses and one such stress is oxidative stress. It has been suggested that polyphenols function as stress protectants in plant cells by scavenging reactive oxygen species (ROS) produced during a period of oxidative stress. In this study the total phenolic content and the related antioxidant capacity of the plants leaf extracts were analysed.

The total polyphenols were measured by the Folin- Ciocalteau assay and tannins by the Folin Ciocalteau/ PVPP (polyvinyl polypyrrolidone) assay. Gallotannins were assayed using both the Rhodanine and the High-Performance Liquid Chromatography (HPLC) methods. Ellagitannins were assayed using the nitrous assay. Determination of condensed tannins (proanthocyanidins) was done using the butanol/HCL reagent. Two protein precipitation methods, protein precipitable phenolics assay and the radial diffusion assay, were used to determine tannins in the resurrection plant extracts. Three separate and complimentary methods, the 1,1 diphenyl –2 picrylhydrazyl (DPPH) assay, the ferric reducing /antioxidant power (FRAP) assay and the β-carotene linoleic acid (β-CLA) assay, were used to determine the antioxidant and radical scavenging capacities of the plants. The phenol antioxidant coefficient (PAC) and the phenol anti-radical coefficient (PARC) were calculated for practical comparison of relative antioxidant potential of phenolics extracted from the selected resurrection plants. The total polyphenolic content of the leaves of the resurrection plants ranges from 10.2 milligram gallic acid equivalents per gram dry weight (mg GAE/ g DW) in *X. villosa* to 247.1 mg GAE/g DW in *M. flabellifolius*. The FRAP ranges from 2.3 mM Fe^{2+} in *X. villosa* to 25.1 mM Fe^{2+} in *M. flabellifolius*. The antioxidant capacities as determined by the DPPH and β-CLA assays

range from 8.4 % to 94.8 %. The PAC and the PARC range from 0.4 to 2.6. *M. flabellifolius* showed the highest polyphenol content, very high FRAP and DPPH inhibition relative to the other resurrection plants. Gallic acid and catechin were used to compare the total antioxidant capacity (FRAP) and the DPPH radical inhibition with polyphenols from *M. flabellifolius*. Studies on the effect of desiccation on the polyphenol content and the antioxidant capacity of the plants showed only minor or no changes.

The major polyphenols in the nine desiccation tolerant plants were tentatively identified by High-Performance Liquid Chromatography (HPLC)-Diode Array Detection (DAD)-electrospray ion mass spectrometry (ESI-MS). The major polyphenols in hydrated *M. flabellifolius* were tentatively identified as di-, tri-, tetra-*O*-galloylglucoses and kaempferol glucuronide. The major polyphenolic compound in hydrated *Craterostigma* species was tentatively identified as acteoside 1 or 2, in hydrated *Xerophyta* species as 5-caffeoylquinic acid with the exception of *X. villosa* where the major polyphenolic compound were identified as ferulic acid and its polymers. In hydrated *E. nindensis* the major polyphenols were naringenin 7-neohesperidoside and rhamnetin (or isorhamnetin). Desiccation resulted in new polyphenols being identified only in *M. flabellifolius* and *X. schlecterii*. The new polyphenols identified are 3,4,5 tri-*O*-galloylquinic acid and a di-caffeoylquinic acid respectively.

The localisation of the polyphenols in the leaves was studied by the use of caffeine and electron microscopy. Leaf sections were stained with uranyl acetate and lead citrate. In *M. flabellifolius* it was observed that polyphenol-caffeine complexes occurred in the vacuoles near cell walls whereas in *X. humilis* the polyphenol-caffeine complexes appear within cell walls. In *X. viscosa, X. villosa, X. schlecterii, C. wilmsii C. plantagineum, C. pumilum* and *E. nindensis* the polyphenol-caffeine complexes could not be visualised.

CHAPTER 1
INTRODUCTION

1.1 Water

Water is a requirement for the existence of life. All biochemical reactions take place in an aqueous environment. Water plays a central role in biochemical processes of all living organisms (Levitt, 1980). Water is involved in the structure and function of biomolecules, the transport of solutes, metabolites and nutrients. Water is incompressible and therefore provides structural support to cells and tissues. In plants, water constitute about 85 % to 95 % of the weight of most plants and therefore water deficit depresses normal metabolic reactions like photosynthesis and respiration, which in turn restrict plant growth and development (Gaff, 1989). There are many factors causing a water deficit in plants, from drought, high salinity and low temperatures (Bonhert et al, 1995). A water deficit occurs when the rate of water uptake is lower than the rate of transpiration and this affects processes like photosynthesis, metabolism and growth are affected. Severe water deficit at the cellular level leads to denaturation of proteins and membranes (Gaff, 1989, 1997; Bray, 1997).

It is estimated that about one third of the earth's land can be classified as arid or semi arid experiencing regular droughts. In arid or semi arid regions of the world crop production is low. In order for plants to survive water stress, which is inevitable in such conditions, they have developed three strategies for survival: these are evasion, avoidance and tolerance (Levvit, 1980). Plants that evade water stress complete their growth and reproductive cycles when sufficient water is available. Those that avoid water stress retard the loss of water or avoid experiencing the stress by increasing their ability to retain water (Bewley and Krochko, 1982). Tolerant plants survive the loss of water and these have been named desiccation tolerant or resurrection plants (Gaff, 1971; Sherwin and Farrant, 1995).

1.2 Desiccation Tolerance in plants

Desiccation tolerance has been defined as the ability of an organism to loose protoplasmic water and be able to revive on rehydration (Bewley, 1979; Proctor and Pence, 2002) after which all metabolic functions are restored (Alpert and Oliver, 2000).

The ability to withstand water deficit is shown by many algae lichens, liverworts, mosses and ferns. Vegetative desiccation tolerance is rare in higher plants (adult flowering plants) but it is a widespread phenomenon in the plant kingdom especially in ferns and their spores and seeds of higher plants (Alpert, 2000; Oliver et al, 2000; Gaff, 1978; Bewley and Krochko, 1982; Crowe et al, 1992, Oliver and Bewley, 1997; Bonhert, 2000). Some angiosperm plants, mostly found in southern Africa, tolerate water stress without undergoing permanent damage, thus they are called desiccation tolerant. These desiccation tolerant plants are found on all continents except the Antarctica (Alpert and Oliver, 2002). These plants can suspend or reduce metabolic activity during dehydration and return to normal metabolism on rehydration. They grow mostly on rocky outcrops where water supply is limited (Gaff, 1971, 1978; Vertucci and Farrant, 1995; Bonhert, 2000, Porembski and Barthlott, 2000). Oliver et al (2000) suggested that desiccation tolerance was a vital prerequisite to land colonisation by aquatic green plants. Between 275 and 375 species of vascular plants are desiccation tolerant whilst the majority occurs in the ferns and monocotyledons (Alpert and Oliver, 2002). *Velloziaceae* is the largest resurrection plant family among the monocotyledons and examples include the angiosperm *X. viscosa*. Understanding the biochemistry of desiccation tolerance, as shown by these plants, is of paramount interest especially in drought prone areas like southern Africa. Development of drought tolerant crops would benefit many people.

1.3 Damage due to Water deficit

There are many ways in which plants respond to desiccation and these responses range from morphological to biochemical changes. Most of the morphological changes or modifications are thought to minimise damage from light in dry tissues. Most experiments to understand desiccation biochemistry and physiology (Farrant, 2000) are done on short term experiments on parts of or whole plants and cells isolated from their natural environments (Yeo, 1998). According to Walters et al (2002) a water deficit causes the loss of cell turgidity. This occurs at water potentials of about –1 to –2 Mpa. As a dehydrating cell loses turgor, tension develops between the collapsing plasma membrane and the rigid cell wall. Once the cell plasma membrane ruptures cell death follows (Iljin, 1957).

Loss of water from a cell results in osmotic imbalance as cell organelles, macromolecules and proteins, dependant on water for their structure, destabilise and concomitantly denature. Additional denaturation of proteins, DNA and the loss of macromolecular integrity can be caused by reactive oxygen species (ROS) (Foyer et al, 1994; Smirnoff, 1993; McAinsh et al 1996). During dehydration cells are vulnerable to oxidative stress due to free radical generation in the form of ROS. The mitochondria and the chloroplasts are the main sources of ROS in the plant cells (Matte Vicre et al, 2003). The uncoupling of the electron transport chain, due to membrane disorder or macromolecule denaturation, results in electrons being transferred to oxygen causing the formation of reactive superoxide radicals. The generated superoxide radicals can react with hydrogen peroxide to form singlet oxygen species and hydroxyl radicals (Halliwell and Gutteridge, 1993). Chloroplast photoinhibition is a process by which molecular oxygen scavenges high-energy electrons from the cascade pathways and generates ROS. It is a universal problem faced by plants (Matte Vicre et al, 2003). During water stress there is decompartmentation of reactions and this could lead to formation of excess ROS and result in cell death.

1.4 Protective Mechanisms

Dehydration of a cell results in generation of mechanical and osmotic stress. Resurrection plants must be able to counter this and maintain the osmotic balance of the cell to prevent disruption of cellular integrity.

It is known that 'orthodox' seeds fill their cell vacuoles with storage proteins, their cytoplasm with lipids and carbohydrates. This helps in preventing mechanical damage as they dry (Farrant, 2000). Some resurrection plants also employ the same strategy.

X. viscosa, X. humilis and *E. nindensis* also fill their vacuoles with these compatible solutes (Farrant, 2000; Vicre et al, 2003; Van der Willigen et al, 2004).

C. wilmsii and *C. plantagenium* undergo extensive cell wall folding so that the plasma membrane remains appressed to the cell wall during dehydration (Vicre et al, 1999, 2004). There is also accumulation of small vesicles associated with desiccated leaf cells of some resurrection plants (Farrant, 2000; Sherwin and Farrant, 1998). The loss of water from cells triggers a cellular signal transduction pathway in which a physical stress is

converted into a biochemical response. Loss of turgidity or change of cell volume could be a signal of water deficit (Bray, 1997).

Compatible solutes such as proline, glycine-betaine, glutamate, manitol, polyols, cyclitols, trehalose, sucrose, fructans and oligosaccharides have been shown to increase in concentration in response to water deficit. Other solutes like citrulline have also been proposed to act as radical scavengers in drought tolerant watermelons plants (Akashi et al, 2001). Induction of proteins like chaperones or heat shock proteins (Hsp), osmotic and late embryonic abundant proteins (LEA) appear to be another mechanism of protecting cellular integrity. LEA proteins accumulate in seeds upon maturation and in vegetative plant tissue in response to heat, cold, osmotic and salt stresses (Ingram and Bartels, 1996; Bray, 1997). Chaperones stabilise and refold denatured proteins as a way of limiting damage caused by water loss (Morimoto et al, 1994; Boston et al, 1996).

Some resurrection plants however dismantle their thyllakoid membranes and degrade their chlorophyll in a controlled manner thereby avoiding or minimising the generation of ROS due to excess light. These plants are called poikillochlorophytes and include *Xerophyta* species *X. viscosa* and *X. humilis* (Gaff, 1977; Hetherington et al, 1982; Sherwin and Farrant, 1998; Tubar et al, 1994, Farrant et al, 2003). Upon rehydration these plants resynthesised their photosynthetic complexes. The homoiochlorophyllous resurrection plants like *M. flabellifolius* and *C. wilmsii* maintain their photosynthetic apparatus and chlorophyll intact during dehydration. However they fold their leaves cell walls in order to hide their photosynthetic complexes from light (Farrant, 2000; Farrant et al 2003). These plants also rely on antioxidants to get rid of ROS (Sherwin and Farrant, 1998; Farrant, 2000).

1.4.1 Antioxidant Systems

In order to avoid cellular disorder, efficient cellular antioxidant mechanisms must be present. Numerous defence mechanisms, both enzymatic and nonenzymic, are believed to protect cells against oxidative injury or stress. The enzymatic antioxidant defences include enzymes capable of removing, neutralising or scavenging free radicals. These enzymes include superoxide dismutase (SOD) and catalase (CAT) whose combined action converts the potentially dangerous superoxide radical and hydrogen peroxide to water and molecular oxygen, thus averting cellular damage (Halliwell, 1987).

Glutathione-peroxidases (GPXs) is a family of enzymes well represented in the cell and play a prominent role in the antioxidant defences. These enzymes however appear ineffective at low water concentrations and it has been suggested tocopherols and ascorbic acid may be more effective under these conditions (McKersie et al, 1988; Pammenter and Berjak, 1999).

The nonenzymic mechanism of the antioxidant defences includes substrates like glutathione (GSH), ascorbate and tocopherols. Glutathione is a substrate of GPX as well as glutathione S-transferase (GST). Selenium also contributes to the antioxidant defences through its involvement in the Se-dependent GPX. The antioxidant defence system requires high amounts of these compounds to be effective.

The presence of water-soluble antioxidants like ascorbate and glutathione is well known. Ascorbate is found in the chloroplasts, cytosol, vacuole and extracellular compartments of the cell. This compound is so prevalent and it is thought to be a major protector of cytosolic and chloroplastic macromolecules. Ascorbate directly scavenges activated oxygen like the superoxide ion.

$$2O_2^{\bullet} + 2H^+ + \text{Ascorbate} \longrightarrow 2H_2O_2 + \text{Dehydroascorbate}$$

Ascorbate also reacts with the hydroxyl radical and it is regenerated from monodehydro- and dehydroascorbate. Glutathione also occurs in all parts of the cell and in most tissues. It directly scavenges free radicals such as singlet oxygen, superoxide and the hydroxyl radical.

$$2\ GSH + \tfrac{1}{2}\ O_2^{\bullet} \longrightarrow GSSG + H_2O$$

Glutathione is regenerated using the enzyme glutathione reductase. Lipid soluble antioxidants include alpha-tocopherol. Alpha-tocopherol is the most prevalent of tocopherols but others like beta (β) and gamma isomers also occur in nature. Alpha-tocopherol is incorporated into membranes especially chloroplasts and it prevents free radical attacks. It is thought to work best at breaking carbon chain reactions from reduced lipids and proteins in membranes.

$ROO^{\bullet}$ + tocopherol ⟶ ROOH + $tocopherol^{\bullet}$

The resulting tocopherol radicals are not reactive and do not propagate the radical reaction, thus it stops chain reactions. Carotenoids act primarily as accessory pigments. They act by directly scavenging singlet oxygen, by reacting with lipid peroxidation products to stop chain reactions and by reacting with excited chlorophyll to prevent the formation of other free radicals. The resultant energy is then dissipated as heat.

β- carotene + $ROO^{\bullet}$ ⟶ β-$carotene^{\bullet}$ + ROOH (inactive product)

In humans the intake of controlled diets rich in fruits and vegetables increased significantly the antioxidant capacity of plasma. This increase could not be explained by the increase in the plasma alpha-tocopherols or carotenoids concentration. Consequently the antioxidant activities of compounds like polyphenols are the focus in many recent studies. (Tsao and Deng, 2004).

The morphological changes or modifications that occur in resurrection plants during dehydration together with the enzymatic defence system of the plant can't explain how these plants survive loss of water during drying. This is because at low water potential the enzymatic defence mechanisms are not effective and protein synthesis is switched off at much higher water potentials during drying. There must be other protectants. Phytochemicals have been implicated as potential protectants. Sugars and polyols are among other substances frequently studied in order to gain insight into mechanisms involved in the adaptation of plants to water stress (Bianchi et al, 1993).

In addition to the antioxidants mentioned above, polyphenols are thought to be part of the nonenzymic antioxidant defence mechanism employed by plants to prevent or minimise the formation of ROS. Numerous studies have conclusively shown that the majority of antioxidant activity of vegetables and other plants may be from compounds such as flavonoids, isoflavonoids, flavones, anthocyanins, catechin and other polyphenols rather than ascorbate, vitamins E and β-carotene (Wang et al, 1996; Kahkonen et al, 1999). These findings have sparked worldwide interest in research on the antioxidant action of polyphenols. One general point must be emphasised however, that polyphenols represent only one possible line of mechanism of protection during drying of many that may be

present in a given resurrection plant. Thus the role of polyphenols in desiccation tolerance has to be considered as only one part of a general series of mechanisms by which resurrection plants avoid cell death during drying.

1.5 Definitions

The terms polyphenols (polyphenolic compounds) and phenolics are used in this study to mean compounds which possess an aromatic ring bearing one or more hydroxyl substituents (Harborne, 1984). Tannins are a subgroup of polyphenols that have the ability to precipitate proteins or react with PVPP (Harborne, 1984; Hagerman, 1998, 2002; Makkar et al, 1995). Tannins are usually polymers of flavanols or gallic acids. These definitions are important as some researchers, for example Haslam (1981), use the term polyphenols to mean tannins and others use molecular weights to differentiate polyphenols or phenolics and tannins. The terms resurrection and desiccation tolerant plants are used in this study to mean plants that loose protoplasmic water and be able to revive on rehydration.

1.6 Plant Polyphenols

Polyphenols are secondary metabolites widely distributed in plants and more than 5000 molecules have been identified. The term polyphenols is a highly inclusive one and include many classes of polyphenols ranging from simple phenolics, flavonols, flavones, flavanols, anthocyanins, isoflavones, tannins and so many others minor classes. (Harborne, 1984; Manach, 2003).

In the past, polyphenols were thought to be waste products, which were deposited by the process of local excretion into the nearest inert compartments for storage. The major compartments where these polyphenols were stored are the vacuoles and the cell walls but these compartments are critical for the functioning of the cell (Hrazdina and Wagner, 1985). Generally plant polyphenols are thought to occur in the vacuoles but there is now evidence that they may also be associated with cell walls. Examples of such polyphenols include the insoluble lignins (Hrazdina and Wagner, 1985; Brett and Waldron, 1996). Vacuoles are now known to be much more dynamic entities of the plant cell than what was originally thought. Plant vacuoles can reversibly and irreversibly store a variety of compounds. There is also evidence of accumulation of flavonoids and anthocyanins in the chloroplasts.

Polyphenols vary in structure from hydroxybenzoic acids and hydroxycinnamic acids having a single ring structure whilst flavonoids can be classified into anthocyanins, flavanols, flavones, and flavonols. Some of the flavonoids can be found as dimers, trimers and polymers. Flavonoids are widely distributed and are found in leaves of most plants. Quinones are also used in primary metabolism whilst anthocyanins are present in flower petals and leaves (Harborne and Simmonds, 1985). Often polyphenols are attached to sugars like glucose and xylose and named glycosides.

The antioxidative properties of polyphenols come from their high reactivity as hydrogen or electron donors and from the ability of the polyphenol-derived radical to stabilise and delocalise the unpaired electron and also from their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans et al, 1997. Flavonoids have been shown to inhibit oxido-reductases. It has been shown recently that polyphenols can be involved in the hydrogen peroxide scavenging cascade in plant cells (Takahana and Oniki, 1997). An example of co-operation between different antioxidant systems is the ascorbate-glutathione (Halliwell-Asada) pathway in the chloroplasts where it provides photoprotection by removing hydrogen peroxide (Noctor and Foyer, 1998). The Halliwell-Asada ascorbic acid: glutathione redox cycle is one of the main anti-oxidative pathways in plants and involves the linking of reducing equivalents in primary metabolic pathways to the antioxidants ascorbic acid and glutathione. Various enzymes are also involved in the Halliwell-Asada pathway (Foyer and Halliwell, 1976).

Redox coupling of plant polyphenols with ascorbate in the hydrogen peroxide – peroxidase system has also been shown (Takahana and Oniki, 1997; Yamasaki and Grace, 1998).

Owing to the presence of these various antioxidant mechanisms, an oxidative stress occurs only when these protective systems are insufficient to cope with the prooxidants that are generated. Many studies have suggested that polyphenols exhibit antiviral, antifungal and antiherbivory activities. However most interest is now being devoted to the antioxidant activity of polyphenols, which is due to their ability to scavenge free radicals.

Plant polyphenols are diverse and therefore are likely to have different chemical and biological properties. It is important therefore, to identify and quantify the individual

polyphenols in the resurrection plants leaves so that their single or synergistic effect can be studied (Soong and Barlow, 2005).

Measurement of the UV absorption spectrum is an extremely useful method of characterising polyphenols. All polyphenols absorb strongly in the ultraviolet region (Harborne, 1964). UV spectra can be used to differentiate between different classes of polyphenols and the positions of the absorption maxima often indicate the number of phenolic hydroxyl groups present.

Different polyphenols are synthesised in cells and their mechanisms of action may also be different. In order to elucidate the role of polyphenols in desiccation tolerance it is necessary to identify the individual polyphenols in the resurrection plants. In this study the quantity, antioxidant capacity and the identity of the polyphenols in the leaves of the resurrection plants are investigated. Polyphenols are a large and a heterogeneous group of compounds and below is a description of some of the major classes.

1.6.1 Polyphenols in the leaves

The leaf is the primary site of plant metabolism and it is not surprising that in the study of polyphenols in plants, the leaves have attracted particular attention. A large number of leaf polyphenols have been isolated and quantified. Polyphenols are known to be unevenly distributed in the leaf. It has been reported that more polyphenols accumulate in older tissues than new tissue. Thus in the study of plant polyphenols the following factors have to be controlled as far as possible:

(1) The age of the plant sample – it has been found that as the plant age, tannins and other polyphenols tend to increase. If it is difficult to get the age of the plant sample then a composite sample must be taken.

(2) The nutritional status of the plant sample- the quantity of polyphenols may depend on the nutritional state of the plant.

(3) The part of the plant (leaves, flowers, stem or bark).

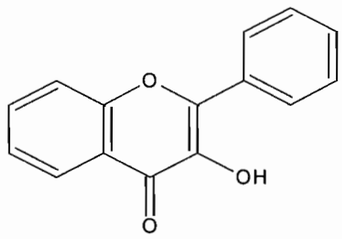
1.7 Flavonoids

More than 4000 molecules exhibiting the typical phenylbenzopyrone structure have been identified. These have been classified as anthocyanidins, flavonols, flavones, isoflavones, flavanones, flavanols and many more minor classes. (Harborne, 1964, 1984). They are

usually glycosylated, and many of them are acylated with aliphatic and aromatic acids. These compounds are widely distributed in nature, albeit not uniformly. Problems encountered in flavonoids analysis are mainly due to solubility and instability. Flavonoids are found in all vascular plants and occur mainly as mixtures. The different classes of flavonoids are described below.

1.7.1 Flavonols

These compounds occur as co-pigments to anthocyanins in petals and also in leaves of higher plants. They are also mostly glycosylated. The three common aglycones of flavonols are kaempferol, quercetin and myricetin. Quercetin can be O-methylated to form the 3-methyl ether isorhamnetin. Rutin is the most common quercetin glycoside. Flavonols are very unstable in basic solution in the presence of oxygen (Harborne, 1973, 1984). These compounds occur frequently in leaves of angiosperm species. They absorb

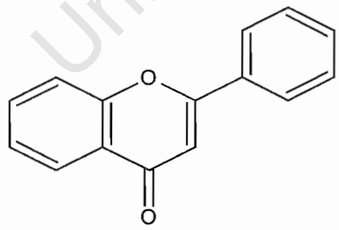


in the 330 -380 nm region but are usually monitored at 360 nm (Manach, 2003).

1.7.2 Flavones

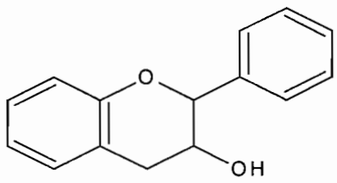
Flavones lack the 3-hydroxyl substitution. The most common flavones are apigenin and luteolin. Bate-Smith (1962) notes that apigenin and luteolin occur sporadically in the leaves of dicotyledons but occur more regularly in flowers. These compounds occur only as glycosides and they also form biflavonyls. The dimeric compounds are formed by carbon to carbon or carbon-oxygen coupling between two flavone units. These compounds absorb in the 320- 350-nm region (Manach, 2003).

Below is the general structure of flavones.



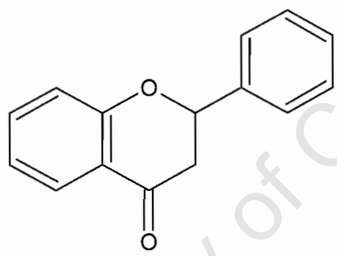
1.7.3 Flavanols

This group includes catechin and isocatechin. Catechin is one of the most widely occurring flavonoids (Harborne, 1964). These compounds can be hydroxylated at the 3-position only (flavan-3-ols) or can be hydroxylated at both 3 and 4 position (flavan-3,4-diols) (Freudenberg and Weinges, 1958; Swain and Bate-Smith, 1956; Manach, 2003).



1.7.4 Flavanones

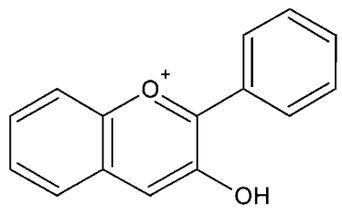
These differ from flavones by having the double bond in the 2,3 position replaced by a single bond. They occur mostly abundantly in a few angiosperm families (Harborne, 1964). Naringenin and eriodictyl are the most common flavanones. Like flavones they can also lack B ring hydroxyl groups or are methylated.



1.7.5 Anthocyanidins

These are mainly based in the petals, leaves and fruits of higher plants. Anthocyanins are all based on the aromatic structure of cyanidin. Basically there are six common anthocyanidins and these are pelargonidin, delphinidin, petunidin, petunidin, malvidin and cyanidin. These can occur combined to various glycosides as anthocyanins. (Harborne, 1984). These anthocyanins are found in all vascular plants.

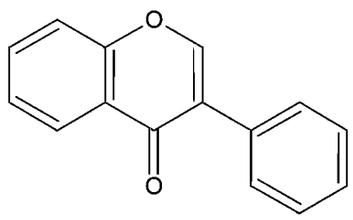
Below is the general structure of anthocyanidins



1.7.6 Isoflavones

They are mainly found in the leguminous family. This group of compounds can have 2-hydroxylation and also 6-hydroxylation.

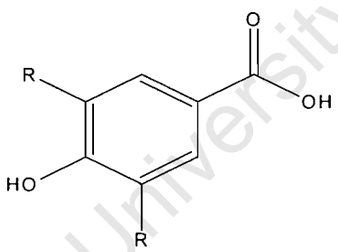
Below is the general structure of isoflavones



1.8 Phenolic Acids

Phenolic acids are usually associated with lignin as ester groups or may be bound to sugars as glycosides. These compounds include hydroxybenzoic acid, protocatechuic acid, vanillic acid and syringic acid. In woody plants gallic and ellagic acid are more commonly found (Harborne, 1984). They are widely distributed in angiosperms. A typical example is benzoic acid and its derivatives and the structure is given below.

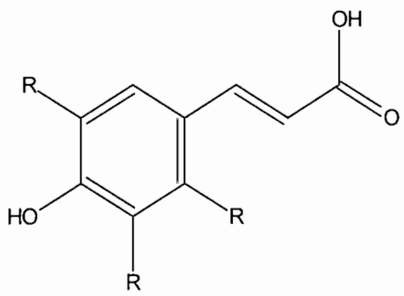
1.8.1 Benzoic Acid Derivatives



1.8.2 Cinnamic acids

These are mainly known as the most widespread group of phenylpropanoids. The most common cinnamic acids are ferulic, sinapic, caffeic and coumaric acids. These compounds are mainly found as conjugates in unprocessed plant material. The most

widespread conjugate of caffeic acid is the quinic acid ester, chlorogenic acid. Chlorogenic acid is now called 5-caffeoylquinic acid (IUPAC, 1976). The term chlorogenic acid is now only used to refer to the family of related quinic acid conjugates (Clifford, 2003).



1.9 Tannins

These compounds have the ability to react with proteins. In the cell tannins are located separately from proteins. These polyphenols are thought to play a key role in the chemical defences of plants. They are basically divided into two groups the hydrolysable and the condensed tannins. Condensed tannins have a flavanol core as a basic structure with carbon – carbon bonds linking one flavan unit to the next by a 4-8 and 6-8 link. The hydrolysable tannins are esters of gallic and ellagic acid.

In this study the word tannin is used to describe those polyphenols that precipitate proteins, gelatine and PVPP. Some researchers like Horvath (1981) defines a tannin as: "any phenolic compound of sufficiently high molecular weight containing sufficient hydroxyls and other suitable groups (i.e. carboxyls) to form effectively strong complexes with proteins and other macromolecules under the particular environmental conditions being studied". This definition based on the molecular weight of the polyphenol is used by some researchers and it means that tannins not only complex proteins but also starch, cellulose and minerals (Giner-Chavez, 1996).

1.9.1 Hydrolysable Tannins

These are synthesised by many plants (Haslam, 1981; Okuda et al, 1990). Pentagalloylglucose is the central compound for hydrolysable tannins. Hydrolysable tannins can further be divided into gallotannins and ellagitannins. Gallotannins consists of a polyol surrounded by gallic acid units and these units can be joined together with

other gallic acid molecules through depside bonds. Ellagitannins are more complex than gallotannins and are formed as a result of oxidative reactions between gallic acid units (Mueller-Harvey, 2001).

Below is a pentagalloylglucose, which is the precursor of gallotannins and ellagitannins.

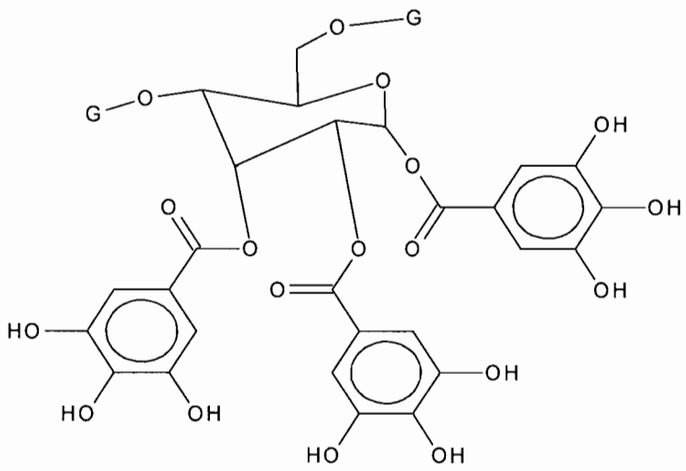


Figure 1.0. Structure of a gallotannin. G- galloyl moiety

The gallotannin shown in Fig 1.0 has five gallic residues. The gallic acid residues can be joined together by a depside bond. The depside bond is more easily hydrolysed than an aliphatic ester bond (Hagerman, 1998, 2002).

Below in Figure 1.1 is an example of a depside bond, which is formed between two gallic acid units.

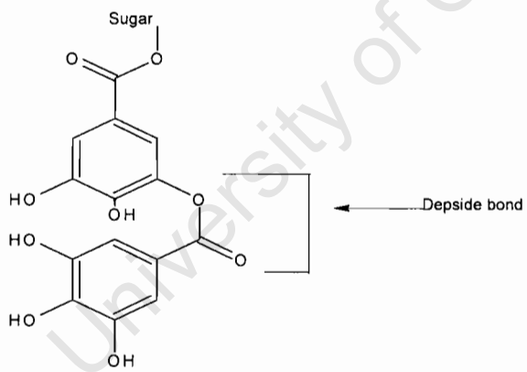


Figure 1.1. A depside bond between gallic acid molecules

1.9.2 Condensed Tannins

These are polyhydroxy flavanol oligomers and polymers linked by carbon-carbon bonds between flavanols subunits. The most widely studied condensed tannins are based on

catechin and epicatechin. The term condensed tannin is generally being replaced by proanthocyanidin as the latter is more descriptive (Hagerman, 1998).
Below is a diagram of a linear polymer based on 4→ 8 linkages. An example is sorghum procyanidin where n = 15 (Hagerman, 1998, 2002). Condensed tannins can be polymers

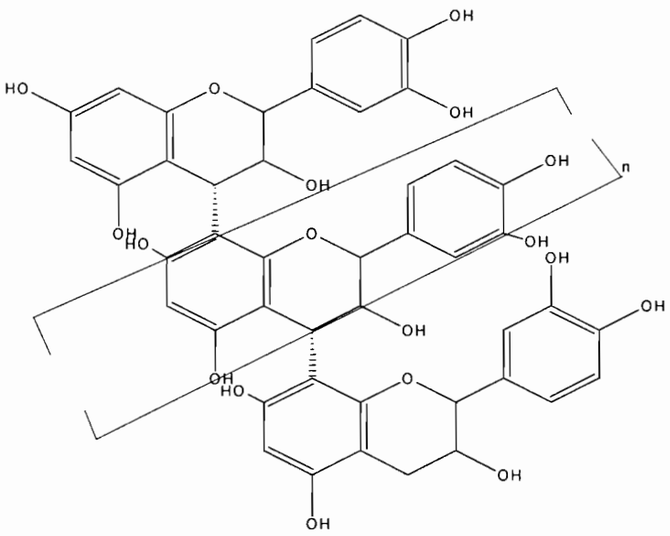


Figure 1.2. Structure of condensed tannins

of 5-deoxy-flavan-3-ols. The 5-deoxy A ring is very reactive and therefore branching is common in polymers made of these monomers. (Hagerman, 1998, 2002).
Plants are sedentary in nature and therefore must develop mechanisms to survive different abiotic stresses. These include drought, UV light, osmotic, heavy metals and oxidative stresses. Polyphenols have been shown to prevent free radical damage in vitro and in vivo biological systems. Therefore they are thought to have a role to play in desiccation tolerance.

1.9.3 Possible Antioxidant mechanism of polyphenols

According to Halliwell and Gutteridge (1993) mechanisms of antioxidant actions are different and can include (1) suppressing reactive oxygen species formation by inhibiting enzymes or by chelating trace elements involved in free radical production (2) scavenging reactive oxygen species and (3) upregulating or protecting antioxidant defences. Most polyphenols fulfil most of the above. Polyphenols efficiently chelate trace metals, which play an important role in oxygen metabolism. Free iron and copper

are potential enhancers of reactive oxygen species formation as shown by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical,

$$H_2O_2 + Fe^{2+} (\text{or } Cu^{+}) \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+} (\text{or } Cu^{2+})$$

or by the copper –mediated low-density lipoprotein oxidation.

$$LH \longrightarrow L^{\bullet} \longrightarrow LOO^{\bullet}$$

where LH represents LDL (Pietta, 2000).

It is nevertheless important to remember that these metals are also essential for many physiological functions and are also cofactors of many enzymes including those involved in the antioxidant defence like Cu, Zn-superoxide dismutase.

The major contribution to metal chelation is due to the catechol moiety as exemplified by the more pronounced bathochromic shift produced by the chelation of copper to flavonoids.

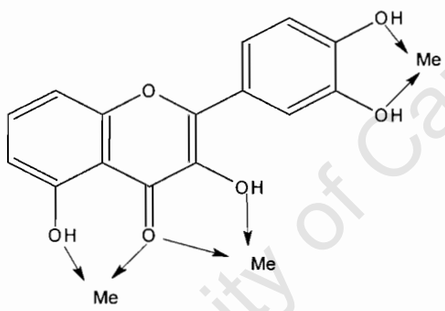


Figure 1.3. Proposed binding sites for trace metals on polyphenols for example flavonoids. (Me- metal)

Most polyphenols have low redox potential and therefore are thermodynamically able to reduce highly oxidising free radicals with redox potentials in the range 2.13 –1.0 V such as the superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen donation (Pietta, 2000).

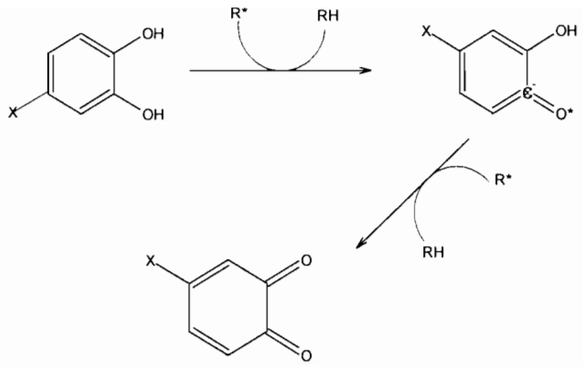


Figure 1.4. Scavenging of ROS by polyphenols. (X could be H, OH, CH_3, aromatic compound etc).

1.10 Quantification of Polyphenols

There are many factors that have to be considered when extracting polyphenols from plant material for quantification and the most important ones are the sample preparation technique, the solvent and storage and consequently has to be optimised. All components of interest must be uniformly enriched in the extract (Robards, Antolovich, Frenzler and Ryan, 2000). Correct extraction must be ensured to avoid chemical modification, which will result in artefacts. The extraction method determines the yield and the chemical properties of the polyphenols extracted (Scalbert, 1992). There are basically three methods of sample preparation and these are air, oven and freeze-drying. Different researchers have shown that the use of these techniques results in different quantities and types of polyphenols (Yu and Dahlgren, 2000; Hagerman et al, 1998, 2002). Variability in plant moisture content makes drying a prerequisite prior to extraction. Oven drying of plant tissue prior to analysis has been reported to decrease polyphenol extractability compared to freeze-drying or the use of fresh plant tissue (Price et al, 1979; Lindroth and Pajutee, 1987; Hagerman, 1988). Makkar (2000) reported that it could be difficult to grind fresh or frozen material so it was suggested that the use of freeze-dried samples might be the solution. It has been shown that the choice of solvent influences polyphenol extractability from plant materials (Yu and Dahlgren, 2000). Polarity of the solvent used also has an impact on the extraction efficiency. The most commonly used solvents are aqueous methanol and acetone. Methanol when used at high temperatures results in methanolysis of depside bonds if the sample contains tannins and digalloyl-compounds.

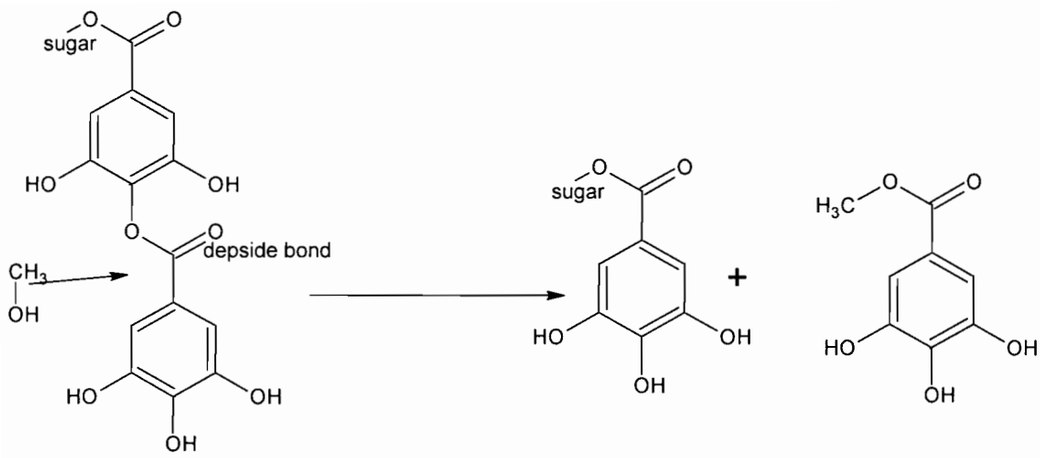


Figure 1.5. Methanolysis of a depside bond.

Other concerns when extracting polyphenols for quantification is the completeness of the extraction and the possible degradation of the extracted polyphenols. It is therefore necessary to optimise extraction parameters for the particular plant materials being used (Yu and Dahlgren, 2000). In order to increase the efficiency of the extraction step, the use of ultrasound was employed (Makkar, 2000; Hagerman, 1998; Hopkins, 1991). It is important to control the temperature of the water bath when using ultrasound, as there is generation of heat. Procedures for quantification of polyphenols should be applicable to all extracts regardless of species or part of the plant (Escarpa and Gonzalez, 2000). Polyphenols are a heterogeneous group of molecules and this makes the analysis of such molecules a problem. Methods for quantifying polyphenols in plant tissues have been reviewed in detail and suitable methods take cognisance of the fact that polyphenols are a large and heterogeneous group of compounds (Mole and Waterman, 1987a, b; Hagerman and Butler, 1989; Scalbert, 1992). Common assays are based on the ability of the polyphenols to form complexes with proteins, formation of depolymerisation products and the reactivity of the phenolic ring (Scalbert, 1992).

The diversity of polyphenols means that whatever reagent is used in the assays and the wavelength used to measure absorbance is going to be a compromise. A single wavelength can only be used when measuring a single class of phenolics or when one class predominates. The wavelength used mostly for general detection is 280 nm (Tsimidou, Papadopoulos and Boskou, 1992; Andersen and Pedersen, 1983).

Results of quantifications are usually expressed as molar equivalents of commonly occurring polyphenols like gallic acid or tannic acid. However, tannic acid is a heterogeneous and variable mixture of galloyl esters and therefore not a good reference standard for polyphenol analysis because of its poorly defined composition (Hagerman, 1998, 2002). Many researchers prefer gallic acid because it is well defined and does not depend on the manufacturer or source (Makkar et al, 1993).

1.11 Localisation of polyphenols in cells of desiccation tolerant plants

X-ray and NMR studies show that caffeine complexes to polyphenols, for example caffeoylquinic acid, by a combination of phenolic hydrogen bonding to the cyclic keto-amide and a hydrophobic interaction (van der Waals attractions and exclusion of water) (Haslam, Lilley; 1985). In the study of polyphenol localisation, the use of caffeine to precipitate polyphenols at their true location is very important in the early stages of tissue preparation. Many studies have been done where lead citrate is used as a staining agent in the study of polyphenols (Reynolds, 1963; Zobel, 1985; Streit and Fengel, 1994, 1995). The use of lead citrate following caffeine precipitation, as a staining agent, permits the investigation of polyphenol localisation and distribution in cells at the high resolution of transmission electron microscopy. This is because it stains polyphenols selectively. When stained with lead citrate, the vacuoles and also the cell walls would contain dark stained phenolic material depending on the concentrations of the phenolic material present. These dark stained material were found to be absent in cells with no polyphenols (Zobel, 1985; Streit, Fengel, 1994, 1995). Streit and Fengel (1995) followed the formation of polyphenols and their distribution in cells of *Schinopsis balansae* Engl. using electron microscopy and proved that the dark stained structures are indeed polyphenols. Localisation of polyphenols in the hydrated and dry leaves was done using caffeine, lead citrate and transmission electron microscopy.

1.12 Studying polyphenols in leaves of desiccation tolerant plants

There are currently two main reasons driving research into plant polyphenols. One is to identify and understand the role of polyphenols as secondary metabolites within plants. Secondly there is interest in the antioxidant properties of the polyphenols. The latter is pertinent to the current work where the amount and possible role of polyphenols in

desiccation tolerance is examined. As plants dry there is bound to be increase in ROS formation. Since these plants survive loss of most of the protoplasmic water they must have an effective antioxidant system to cater for the ROS formed. To date, with the exception of *M. flabellifolius* (Moore et al, 2005) there has been no study done on the amounts and types of polyphenols in the resurrection plants. In addition there hasn't been any study to see how these polyphenols change with drying.

1.13 Aims

The aims of this study are as follows:

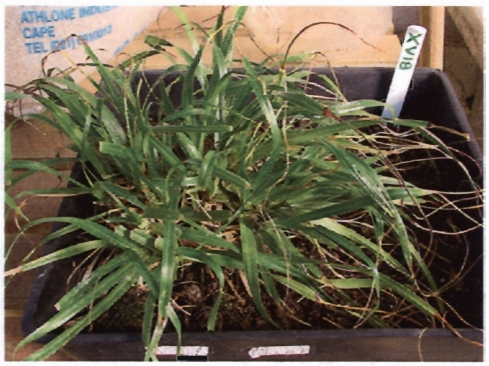
1. To quantitate the total phenol and tannins present in the leaves of desiccation tolerant plants.
2. To determine if there is a correlation in the amount of the total polyphenol and tannins present in the leaves of desiccation tolerant plants.
3. To compare the total phenol and the tannins in the leaves of desiccation tolerant plants with those present in non-desiccation tolerant (desiccation sensitive) plants.
4. To determine the antioxidant capacities of the polyphenols present in the leaves of desiccation tolerant plants.
5. To determine the effect of desiccation on the total polyphenol and antioxidant capacities of the polyphenols present in the leaves of desiccation tolerant plants.
6. To identify the polyphenols present in the leaves of desiccation tolerant plants using HPLC-DAD and mass spectrometry.
7. To determine the effect of desiccation on the polyphenols present in the leaves of desiccation tolerant plants using HPLC-DAD and mass spectrometry.
8. To try to localise the polyphenols using transmission electron microscopy and selective staining.

Desiccation tolerant plants might not be of a direct economic value to the agricultural industry today but they do provide a unique model system to investigate possible mechanisms for improving drought tolerance of crop plants like maize. Understanding how these plants deal with oxidative stress through the nonenzymic mechanism, the only functional mechanism during dehydration, is important.

The quantities of the polyphenols present in the leaves of desiccation tolerant plants are compared with those reported in the literature for a number of other southern African plants. The most important step of optimising the extraction and the method used for quantification was done before any analysis was undertaken. Methodological problems are also reviewed briefly in the discussion.

The desiccation tolerant plants used in this study are *M. flabellifolius, C. wilmsii, C. plantagineum, C. pumilum, X. viscosa, X. humilis, X. schlecterii, X. villosa* and *E. nindensis. M. flabellifolius* belong to the *Myrothamnaceae* family. It is a woody shrub plant often reaching heights of one metre when mature. *C. wilmsii, C. plantagineum* and *C. pumilum* belong to the *Scrophulariaceae* family and most are small herbaceous plants. These plants are all homoiochlorophyllous, as they retain their chlorophyll on drying. *X. viscosa, X. humilis, X. schlecterii* and *X. villosa* belong to the *Velloziaceae* family and are monocotyledonous plants. *E. nindensis* belong to the *Eragrostis* family. These plants are poikilochlorophyllous, as they lose chlorophyll on drying. All these plants are all desiccation tolerant and it was found necessary to use them to investigate the relationship between polyphenols and desiccation tolerance. In addition these desiccation tolerant plants are found locally in South Africa. Two desiccation sensitive plants *Eragrostis capensis* and the rose (*Rosa sp.)* are used for comparative purposes.

Fig 1.6. The hydrated desiccation tolerant plants used in this study



Xerophyta viscosa

Myrothamnus flabellifolius.

Xerophyta schlecterii

Xerophyta humilis

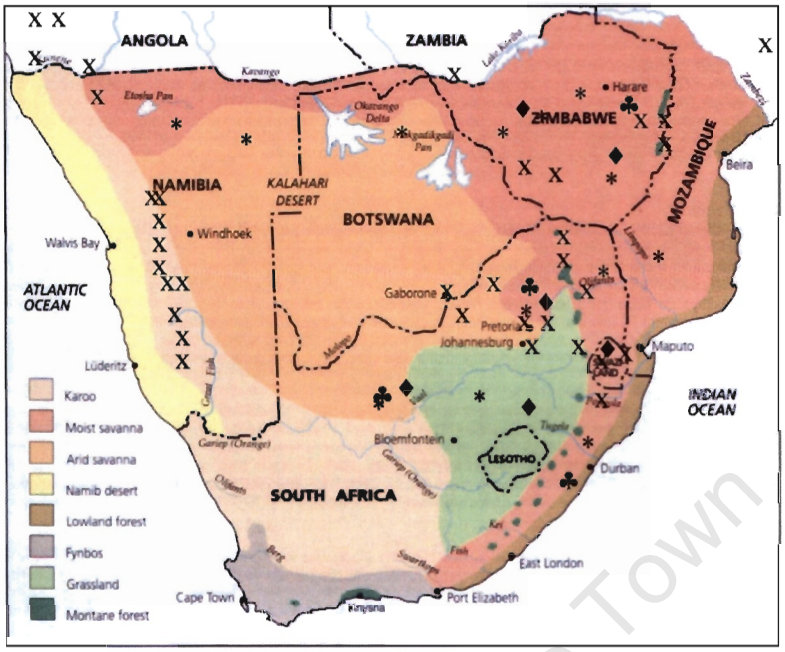
Xerophyta villosa

Craterostigma wilmsii

Craterostigma plantagineum

Craterostigma pumilum

Eragrostis nindensis



HABITAT MAP OF SOUTHERN AFRICA

Figure 1.7. The distribution of desiccation tolerant plants in southern Africa.

x Areas where *M. flabellifolius* is found.

* Areas where *Xerophyta* species are found

♦ Areas where *Craterostigma* species are found.

♣ Areas where *Eragrostis* species are found

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Material

Xerophyta viscosa, Xerophyta humilis, Xerophyta villosa, Xerophyta schlecterii, Craterostigma wilmsii, Craterostigma plantagineum, Craterostigma pumilum, Eragrostis nindensis and *Eragrostis capensis* were obtained from Pilanesberg Nature Reserve in the Northern Province and Buffelskloof Nature Reserve in Mpumalanga Province in South Africa. *Myrothamnus flabellifolius* was obtained from Limpopo Province in South Africa.

The plants were planted in a mixture of peat, river sand and potting soil and maintained in the greenhouse without supplementary lighting. The average temperature of the greenhouse was 28^0C in summer and around 20^0C in winter. The plants were identified taxonomically by Professor J.M. Farrant (Molecular and Cell Biology Department, University of Cape Town). Professor W. Brandt provided the rose plant.

2.1.1 Sample Collection and preparation

Initially, pilot studies were conducted to evaluate the efficiency of various extraction procedures. The results show that the use of freeze-drying and aqueous acetone resulted in a slightly higher yield of phenols than oven drying and methanol (See Appendix 6.1.2) and therefore was used for the preparation of samples and extraction of polyphenols from desiccation tolerant plants.

Fully hydrated leaves were collected from the whole plants randomly and mixed. Sample leaves were then taken from this pool and immediately plunged into liquid N_2. The leaves were freeze-dried in the dark. After 24 hrs the dry sample leaves were ground to a homogenous powder in liquid nitrogen, making sure the sample is always covered in liquid nitrogen, using a mortar and pestle. The resulting sample was sieved through a 0.5 mm screen.

2.1.2 Extraction of polyphenols

Dried plant material (500 mg) was extracted twice with heptane (20 ml) under nitrogen using ultrasound (Bantox Sonorex, TK52H, Bandelin, Berlin, Germany) at 120W for 30

minutes at room temperature. The mixture was centrifuged at 10 286 g for 10 minutes at 4^0C and the supernatant was discarded. This stage removes lipids, chlorophyll and other essential oils. The pellet was dried in vacuo and then treated in the same way using 70 % acetone or 50% methanol as the solvents. The supernatant was collected, stored at –80 ^{0}C and used for the various experiments within two weeks.

2.2 Chemicals

The following chemicals were obtained from the following suppliers:

Sigma: gallic acid, caffeine, ellagic acid, p-coumaric acid, quercetin, remazol brilliant blue R, Kaempferol, tri-fluoroacetic acid, trichloro-acetic acid, iron (11) sulfate heptahydrate.

Fluka: (+)-catechin, chlorogenic acid, o-coumaric acid, iodine, linoleic acid, B-carotene, DPPH, TPTZ, Tween –40, rhodanine.

Merck: Folin Ciocalteau reagent, SDS, Tannic acid, vanillin, ascorbic acid and sodium chloride.

Roche (Boehringer Mannheim): Bovine serum albumin (BSA).

All chemicals and reagents were of analytical grade.

Spectrophotometric Measurements

Spectrophotometric measurements were performed by a UV-Vis spectrophotometer (Ultrospec 1000; Pharmacia, Cambridge, UK).

2.3 Analyses of polyphenolic Compounds

2.3.1 Total Phenolics

Total soluble phenolics in the acetone extracts were determined spectrophotometrically according to the Folin Ciocalteau colorimetric method (Slinkard and Singleton, 1977) using gallic acid as a standard and expressing the results as milligram gallic acid equivalents per g dry weight (mg GAE/ g DW). The total phenolic content in the extracts are not absolute measurements of the amounts of phenolic materials but are in fact based on their chemical reducing capacity relative to an equivalent reducing capacity of gallic acid. The extracts (20µL) were mixed with water (1.58 ml) in a cuvette and 100 µL of the Folin-Ciocalteu reagent (Sigma) was added, and mix well. After 3 minutes 300 µL of

sodium carbonate solution (20 %, w/v) was added and the contents mixed thoroughly. The mixture was allowed to stand for 2 hrs and absorbance was measured at 765 nm against the blank

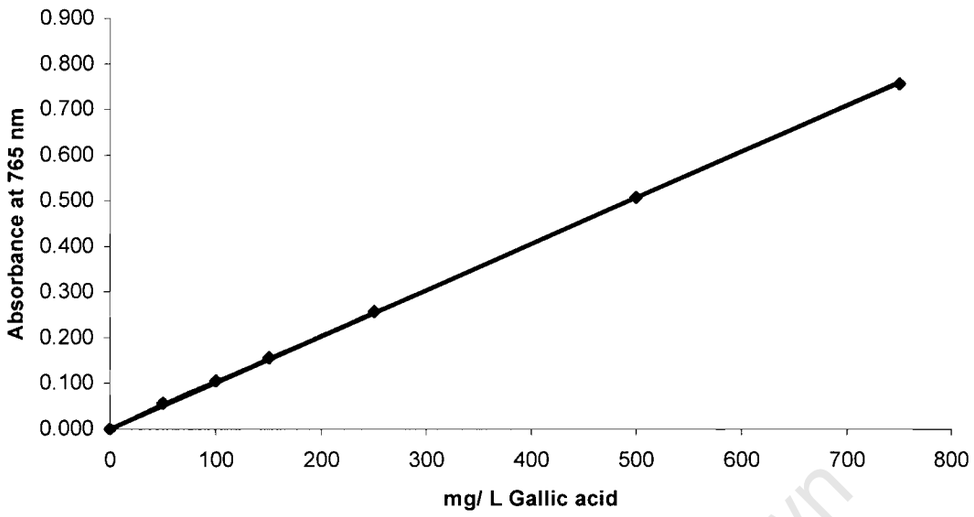


Fig 2.1. The standard curve for the Folin Ciocalteau assay using gallic acid as a standard.

2.3.2 Total Tannins

Tannins were determined using the Folin-Ciocalteau method, using insoluble polyvinyl polypyrrolidone (PVPP) to remove the tannins according to Makkar et al (1993). In short PVPP (100 mg) is mixed with distilled water (1 ml) and the polyphenol containing extract (1 ml) is added. The mixture is vortexed, stored at 4 ^{0}C for 15 minutes and centrifuged at 10 286 g for 10 minutes. The resulting supernatant contains all phenols other than tannins. The phenolic content of the supernatant is determined spectrophotometrically according to the Folin Ciocalteau colorimetric method (Slinkard and Singleton, 1977), calibrating against gallic acid standards and expressing the results as milligram gallic acid equivalents / g dry weight (mg GAE/ g DW) as described for total phenolics. The difference between the total phenolics (above) and the non-tannin phenolics is the amount of tannins in the extract also expressed as milligram gallic acid equivalents per gram dry weight (mg GAE/ g DW).

2.3.3 Gallotannin Determination

2.3.3.1 Rhodanine Assay for Gallotannins

The determination of total gallotannins was done by the quantitative analysis of gallic acid in the hydrolysate of the extracts according to the rhodanine method (Inoue and Hagerman, 1988, Makkar, 2000). In this method gallic acid is determined before and after hydrolysis. Then the sample is hydrolysed so that the gallotannins will release gallic acid, which is also determined together with free gallic acid as total gallic acid. The difference between the total gallic acid and the free gallic acid is proportional to the amount of gallotannins in the sample.

Acid Hydrolysis

The supernatant (3.34-ml) was put into constricted test tubes and acetone was removed by the use of the speedvac. Sulphuric acid (0.1 ml, final concentration was 4 M) was added and the contents frozen. The tubes were vacuum-sealed and heated for 24 hrs at 100 ^{0}C. The contents of the tubes were diluted to 11 ml by adding distilled water. Then 200 µL of this hydrolysed extract was mixed with 300 µL of the rhodanine solution (0.667%, w/v in methanol). The blank was prepared by adding methanol to the hydrolysed extract. After 5 minutes 200 µL of potassium hydroxide (0.5 M) was added. After 3 minutes distilled water (4.3 ml) was added. The absorbance was measured at 520 nm and the rhodanine assay was standardised with gallic acid. The concentration of the gallotannins is therefore expressed as gallic acid equivalents. For the determination of free gallic acid 200 µL of unhydrolysed extract was used in the assay.

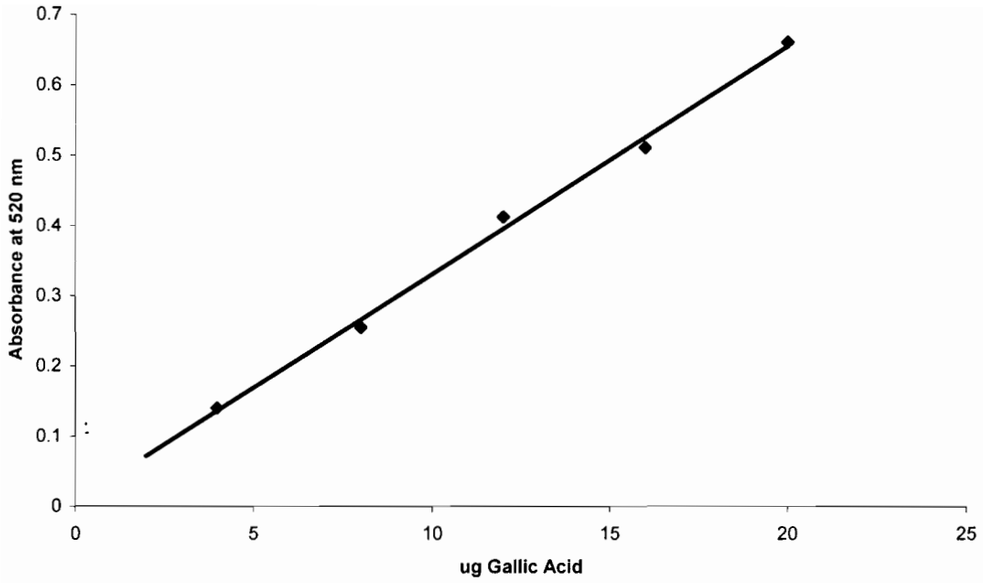


Fig 2.2. Calibration of the Rhodanine assay with gallic acid.

2.3.3.2 HPLC Determination of Gallotannins

Quantification of gallotannins was performed with gallic acid as a standard. Hydrolysed samples were neutralised with a buffer and potassium hydroxide. The amount of gallotannins is obtained by getting the difference between the free gallic acid and the amount of gallic acid obtained after hydrolysis.

Instrumentation and Conditions

Analysis was done using high performance liquid chromatography (HPLC) with a Shimadzu LC- 10 system (Japan, Tokyo) and a Shimadzu SPD-M6A Photodiode array UV-Vis detector (Japan, Tokyo). Separation was achieved in a Jones Reverse Phase C18 column (250 x 4 mm, 5 um, England). The flow rate was 0.7-ml/ min and the volume of sample and standard used was 20 µL. The gallic acid was monitored by UV absorption at 280 nm. Two solvents were used for elution of polyphenols: Solvent: A- Water with 0.1 % TFA; B- Acetonitrile with 0.1 % TFA.

The following gradient programme was used: 0-5 min, 100 % A (isocratic); 5-45 min, 0-100 % B; 45- 55 min, 100 % B (isocratic); 55-70 min, 100-0 % B; 70-73 min, 100 % A.

Gallotannin concentration was calculated as:

(Total gallic acid) minus (free gallic acid) = gallotannins as gallic acid equivalent.

2.3.4 Ellagitannin Determination

Ellagitannins were determined according to the nitrous method (Hagerman and Wilson, 1990). The assay is specific for ellagic acid.

In this method ellagitannins are hydrolysed to ellagic acid which forms a coloured

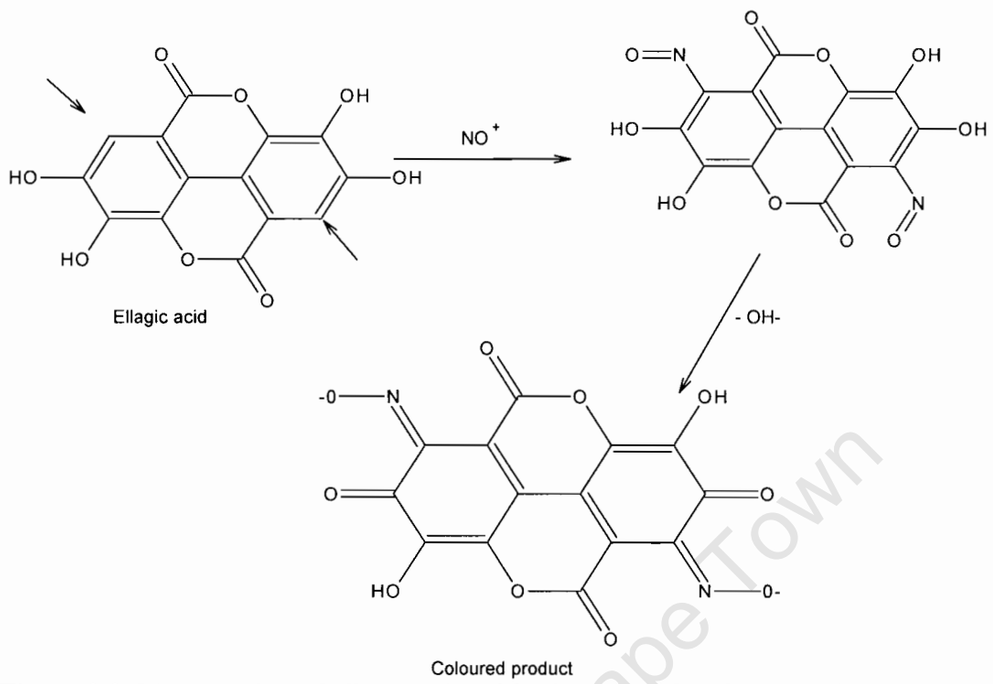


Figure 2.3. The reaction of ellagic acid with nitrous acid to form a coloured complex.

complex with nitrous acid as shown in Fig. 2.3. The method is selective for free ellagic acid only but does not react with a variety of other common plant polyphenols including gallic acid, ellagic acid esters, flavonoids and proanthocyanidins. Extracts (200 µL) were mixed with 1.0 ml of sulphuric acid (4 M) in constricted test tubes and frozen. The tubes were vacuum-sealed and heated for 24 hrs at 100 ^{0}C. The contents were filtered and made up to 10.0 ml with pyridine. Then 1.1 ml of pyridine and 1.0 ml of sample were mixed in a dry test tube. Concentrated HCl (0.1 ml) was added and the sample was brought to 30 ^{0}C to which $NaNO_2$ (0.1 ml; 1 %, w/v) was added with mixing. The absorbance at 538 nm was immediately recorded, and after 36 minutes incubation period at 30^{0}C the absorbance was read again. The difference between the initial absorbance and the absorbance after 36 minutes is proportional to the ellagic acid concentration. The

measured absorbance obeys the relationship: A_{538} = [0.0199 x (mg ellagic acid)] + 0.0237 (Fig 2.4). Ellagic acid was used as a standard and the data were based on experiments done in triplicate. Ellagitannins were calculated according to the formula:
[Total ellagic acid] – [free ellagic acid] = proportional to ellagitannins

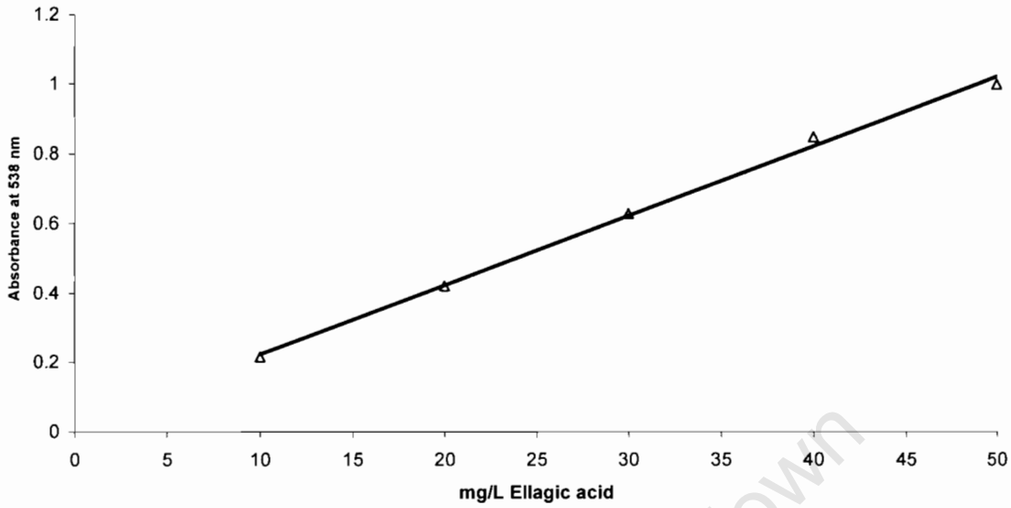


Fig 2.4. Calibration of the nitrite assay with ellagic acid.

2.3.5 Determination of Proanthocyanidins (Condensed Tannins)

Proanthocyanidins were determined using the butanol-HCL method according to Porter et al (1986). Proanthocyanidins yields anthocyanidins upon oxidative cleavage in hot alcohols. This reaction forms unmodified terminal unit and several coloured anthocyanidin ions from the extender units (Hagerman, 1998, 2002).
Plant extracts (500 µL, diluted 10 times with 70 % acetone) were mixed with 3.0 ml of the butanol-HCL reagent and 0.1 ml of the ferric reagent. The tubes are vortexed, capped and heated at 100^0C for 1 hr. The absorbance is read at 550 nm. The blank comprising the unheated mixture is subtracted.

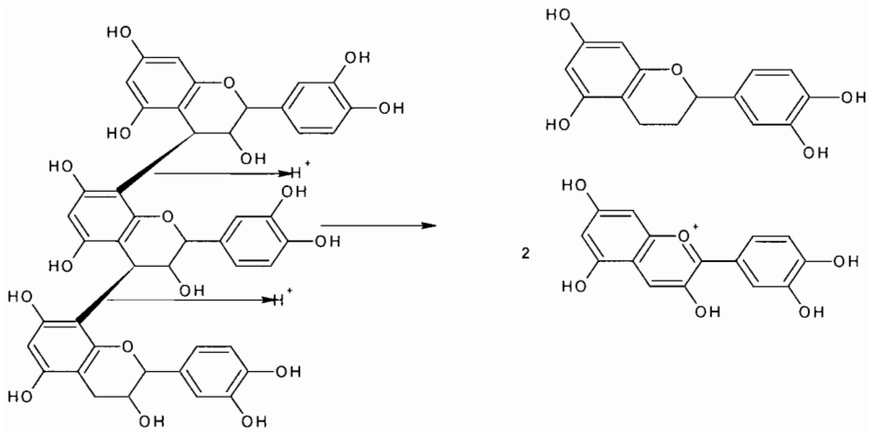


Figure 2.5. The oxidative cleavage of proanthocyanidins in hot alcohol (from Hagerman, 1998, 2002).

Standard

As no suitable anthocyanidin standard was available, the published extinction coefficient of leucocyanidin was used to calculate the anthocyanidin concentration of the extracts (Singh and Singh, 1999; Makkar, 2000).

Leucocyanidin extinction coefficient (ε of 1%, 1 cm, 550nm) = 460 (Porter et al, 1986).

Results were expressed as leucocyanidin equivalents according to the formula:

(A 550 nm x 78.26 x Dilution factor*) / (% Dry Matter)

2.4 Protein Precipitation Methods

2.4.1 Determination of Protein Precipitable Phenolics

The protein precipitable phenolics were determined by a method described by Makkar et al (1988). This method determines the amount of phenolics that precipitate BSA as a percentage of the total phenolics. This method differs from the one described by other researchers as 50 % methanol is used instead of aqueous acetone.

Determination of protein precipitable phenolics (x)

To BSA solution (2 ml) was added increasing amounts of plant extracts topped up to 0.1 ml with 50 % methanol. The mixtures were vortexed and allowed to stand in the

refrigerator overnight. The contents were centrifuged at 3000g for about 10 minutes and the supernatants were removed carefully. SDS (1.5 ml, 1 %) was added to the precipitate and vortexed to dissolve the precipitate.

An aliquot (1.0 ml) of the dissolved complex was taken and a SDS-TEA solution (3 ml) was added. The ferric chloride reagent (1ml) was added and after 20 minutes the absorbance was recorded at 510 nm. The absorbance was converted to tannic acid equivalent using the standard curve. The absorbance values were multiplied by 1.5 to correct for the dilution of the complex. A linear regression of the tannins precipitated as tannic acid equivalent and mg leaf (in the aliquot taken for the assay) was drawn. The slope of the curve (mg tannic acid precipitated / mg leaf; let it be x) represents the protein – precipitable phenolics in the sample.

Determination of Total phenolics in the original plant extracts (y)

Aliquots of the plant extracts were taken and made up to 1 ml with 1% SDS and 3 ml of SDS-TEA solution was added. Ferric chloride reagent (1.0 ml) was added and the absorbance at 510nm was recorded. The absorbance was converted to tannic acid equivalent using the standard curve. A linear regression was constructed between tannic acid equivalent and mg leaf sample (in aliquot taken). The slope of the curve (mg tannic acid equivalent/ mg leaf; let it be y) represents total phenolics.

The percentage of total phenolics which precipitate proteins, called protein precipitable phenolics (tannins) = (x/y) x 100

2.4.2 Radial Diffusion Assay

Tannins or protein precipitable phenolics were also analysed according to Hagerman (1987). Tannin molecules migrate through Agarose gel, which contain bovine serum albumin (BSA). The tannin-protein complex formed in the gel appears as a ring. The diameter of the ring is a measure of the protein precipitation/binding capacity of tannins. Agarose (2.5 g) was dissolved in 250 ml of acetate buffer and heated for 15 minutes with stirring. BSA (250 mg) was added to the Agarose gel maintained at 45^0C and 10.0 ml of the resulting solution was dispensed into Petri plates. The solution was allowed to harden and the plates were covered and sealed with parafilm to prevent drying and cracking. The plates were stored in the fridge at 4^0C.

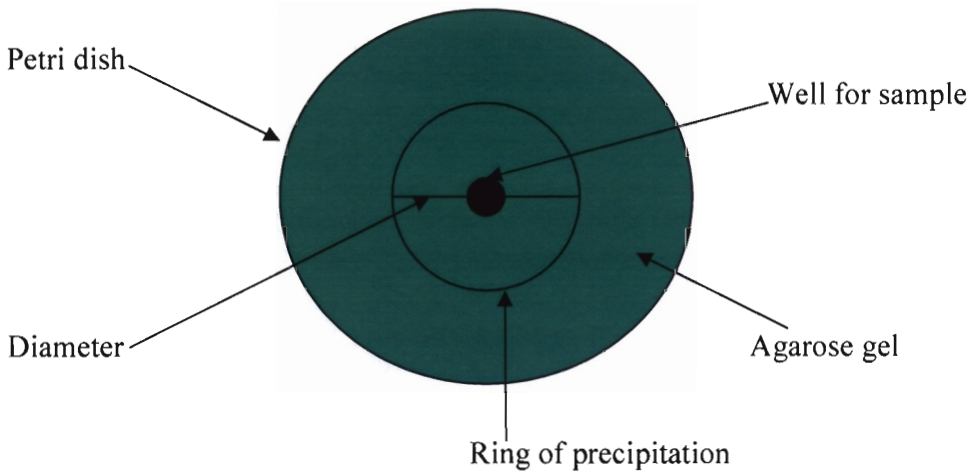


Figure 2.6. The diagrammatic representation of the radial diffusion assay
Wells (4 mm) were punched in the solidified Agarose in the Petri plates and extracts (20 ul) were pipetted into each well. Tannic acid standards (20 µL), of different concentrations, were also pipetted into wells. The Petri dishes were covered, sealed using parafilm and placed in an incubator at 30 ^{0}C. After 96 hrs the diameter of the circle formed was measured. The results were expressed as tannic acid equivalents.

2.5 Antioxidant Capacity Determination

2.5.1 Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing/ antioxidant power (FRAP) assay, developed by Benzie and Strain (1996) and modified by the same authors (1999) was used in this study. The assay is based on the reducing power of a compound or antioxidant. The reduction of Fe^{3+} (ferric ion) to Fe^{2+} (ferrous ion) is taken as unit. The Fe^{2+} ion forms a blue complex with 2,4,6 tripyridyl –s- triazine (TPTZ) which absorbs at 593 nm. High absorbance values at this wavelength indicate higher reducing power of the antioxidant or polyphenol. The FRAP reagent was prepared by mixing one volume 10 mM TPTZ in 40 mM HCl with 10 volumes of 300 mM acetate buffer, pH 3.6 and one volume of 20 mM ferric chloride.

Briefly, FRAP reagent (1.5 ml) was placed into a cuvette and a reagent blank was taken at 593 nm (M1). Samples (50 µL, diluted 10 times) and 150 µL of deionised water was added. Readings were taken immediately and 15 seconds thereafter for 8 minutes. No

further changes in absorbancies occurred after 4 minutes hence the 4-minute reading was used for calculations. A standard curve was prepared by using different concentrations (100- 1000 uM) of $FeS0_4.7H_2O$. All solutions were freshly prepared and catechin and gallic acid were also measured as positive controls and for comparative purposes.

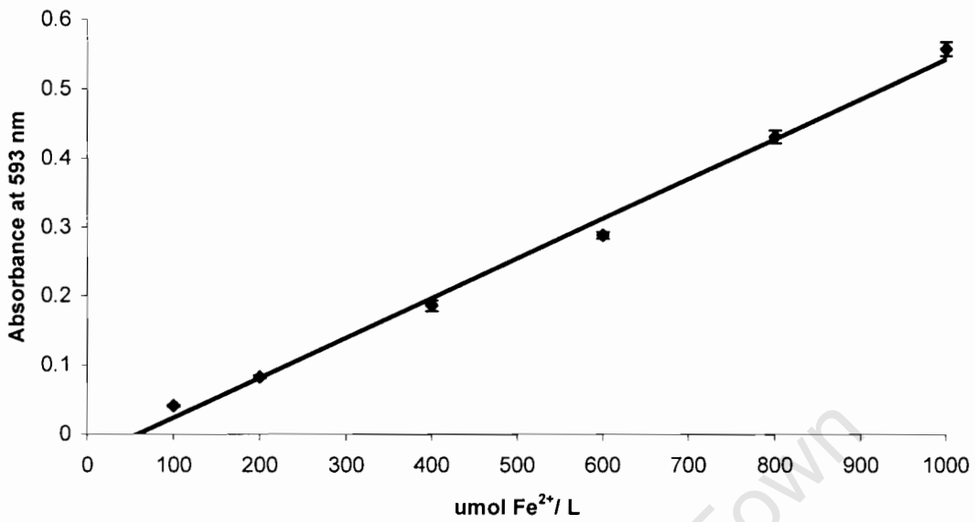


Figure 2.7. The standard curve for the FRAP Assay.

2.5.2 DPPH* Assay

The scavenging activity (electron transferring ability) against the stable DPPH (1, 1 diphenyl-2- picrylhydrazyl) radical of the plant extracts was evaluated according to the method of Brand-Williams et al (1995), with minor modifications. The free radical 1, 1 diphenyl–2-picrylhydrazyl (DPPH) is reduced by the antioxidant that has radical scavenging properties. The radical absorbs at 517 nm and this absorbance disappears upon reduction.

Briefly, aliquots (50 µL) of the leaf extracts and pure compounds were mixed with 2 ml of 6x 10^{-5} M methanolic solution of DPPH radical in a cuvette. Absorbance measurements commenced immediately and the decrease in absorbance at 517 nm was determined after 16 minutes for all samples. The control was the absorbance of the radical without the antioxidant. Catechin and gallic acid solutions in methanol were also tested for comparative purposes. All determinations were done in duplicate and the

percent inhibition of the DPPH radical by the samples was calculated according to the formula of Yen and Duh (1994),

% Inhibition = $[(A_{c(0)} - A_{A(t)}) / A_{c(0)}] \times 100$

Where $A_{c(0)}$ is the absorbance of the control at t = 0 minute and $A_{A(t)}$ is the absorbance of the antioxidant at t = 16 minutes.

2.5.3 Beta-Carotene Linoleic acid Model System

The procedure of Miller et al (1993) with modifications (Wanasundara et al, 1994) was used to measure the antioxidant activity of the extracts. In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation, causing discoloration of β-carotene (Dapkevicius et al, 1998). Readings are taken at 490 nm immediately after mixing the reagents and typically at 15 minutes time intervals for 100 minutes.

In short β-carotene (2 mg) was dissolved in 20 ml of chloroform and a 4-ml aliquot of the solution was added to 40-mg linoleic acid and 400 mg Tween- 40. Chloroform was removed by using the rotary evaporator and oxygenated distilled water (100 ml) was added to the β-carotene emulsion. Aliquots (3 ml) were taken and mixed with 200 µL of the undiluted plant extracts in capped tubes. The tubes were maintained at 50 ^{0}C and oxidation of the β-carotene emulsion was monitored by reading absorbance at 10 minute intervals at 470 nm for 100 minutes. Distilled water was used in place of the plant extracts as a control. Antioxidant activity was expressed as a percent of inhibition relative to control using the equation below.

% Inhibition = [Degradation rate of control- Degradation rate of sample] / Degradation rate of control

Degradation rate = ln (a/b) x 1/t.

Where ln = natural log, a = initial absorbance, b = absorbance at 100 minute interval and t= time (min).

2.6 The effect of desiccation on phenolic content and antioxidant capacity

The aim of this part of study was to investigate the effect of natural drying on the quantity of polyphenols and the related antioxidant capacity.

In this study, five selected desiccation tolerant plants, at least one from each family, were dried in a phytotron. The conditions were as follows:

Low light: <1000 umol m^{-2} s^{-1}; Temperature: 18- 25 ^{0}C (day); Temperature: 10 ^{0}C (night); Relative humidity: +/- 70 %; Photoperiod: 12 hrs.

2.6.1 Plants

Plants Used

M. flabellifolius, X. viscosa, X. humilis, C. plantagineum and *E. nindensis*

2.6.2 Treatment

The plants were placed into the phytotron for five days before the start of drying to let them acclimatise to the conditions. In order to determine whether the change of environment has any effect some plants were also placed outside the phytotron. During the acclimatisation period samples were taken to compare the levels of polyphenols for the plants in the phytotron and those outside. There were no major differences observed between these two groups of plants. The plants used were in pots of the same size in order to have almost the same loss from the pots.

After five days of acclimatisation whole plants were dried by withholding water, allowing the plants to dry naturally. At specified number of days sample leaves were taken, polyphenols were extracted as reported before and analysed. Samples leaves were taken from the same three individuals throughout the whole desiccation experiment. Extracts were analysed for total polyphenols and antioxidant capacity using the three different methods mentioned before.

2.6.3 Assays

(1) Total polyphenols- Folin-Ciocalteau assay.

(2) Antioxidant capacity-FRAP Assay, DPPH Assay and the β-CLA Assay.

The assays were done as described above.

Polyphenols in other southern African plant species

The medicinal review of Watt and Breyer-Brandwijk (1962) and Glyphis (PhD Thesis, 1985) were systematically searched to compile a list of total phenolic and tannin levels in a wide sample of southern African plants. Quantitative levels of polyphenols and tannins were obtained for the leaves of the plants.

2.6.4 Statistical Analysis

All assays were repeated at least three times, with duplicates of each sample being used per assay. Correlation analyses of antioxidant activity versus the total phenolic content were carried out using the correlation and regression programme in Windows XP (2005) (Microsoft Software Inc. USA).

2.7 Identification of Polyphenols

2.7.1 Liquid Chromatography Electrospray-Ionisation (ESI) Mass Spectrometry

2.7.1.1 Instrumentation and Conditions

Sample leaves were obtained as described under section 2.1.1 and extraction of polyphenols as described before under section 2.1.2.

Analyses were performed on a Waters Alliance 2690 HPLC system equipped with a quaternary pump, an autosampler and a photo-diode array detector Waters 996 and operating at wavelengths between 200 and 450 nm. The system was coupled to an MS detector Waters API Q (quadruple) -TOF Ultima equipped with an ESI interface. UV and MS data were acquired and processed using operating system MassLynx 4.0.

Compounds were separated on a 250 mm x 4 mm, 5 µm particle size, Jones C18 reverse phase column (England). The mobile phase was a gradient prepared from formic acid in water (solvent A) and formic acid in acetonitrile (solvent B): 0.01 – 5.00 min 100 % A isocratic; 5.01 – 45.00 min, 0-100 % B; 45.01- 55.00, 100 % B isocratic; 55.01 – 65.00 min, 100- 0 % B; 65.01- 70.00 min, 100 % A isocratic.

The last five minutes were used as equilibration time before the next injection. The flow rate used was 700 µl / min and analyses were conducted in the negative mode at room temperature. Aliquots of both extracts and standards (20 µl) were taken into the column by an autosampler. Argon was used as a nebulising and drying gas. The MS acquisition

with the ESI interface was performed under the following conditions: probe high voltage, 3.4 kV; Desolvation gas flow rate, 400 L/ h; Desolvation temperature, 380 ^{0}C; Cone voltage, 35 V; Cone gas flow rate, 50 L/h; MS (full scan) from m/z 100- 1500 with a scan speed of 1s/scan was used to measure the $[M-H]^-$ ions, thus revealing the molecular masses of the components. The elution of the solutes was monitored at 280 nm (the general wavelength for polyphenols) and 330 nm for hydroxycinnamic acids. Total ion chromatograms (TIC) were obtained together with the HPLC chromatogram profiles.

2.8 Identification of Polyphenols in leaves of dry plants

The aim of this part of study was to investigate the effect of natural drying on the types of polyphenols present in the desiccation tolerant plant leaves. The types of polyphenols identified in the dry desiccation tolerant plant leaves were compared to those present in hydrated plant leaves. In this study the whole resurrection plants were dried in the green house for 12 days by withholding water, allowing the plants to dry naturally. Sample leaves were obtained as described under section 2.1.1 and extraction of polyphenols as described before under section 2.1.2.

Different levels of light are known to affect the quantities and types of polyphenols (Sherwin and Farrant, 1998; Farrant et al, 2002, 2003). The plants were dried under the same conditions (greenhouse) as the plants from which we obtained samples for the initial HPLC-DAD ESI MS analysis. It is hoped that this way of drying the plants would elicit the synthesis of polyphenols only associated with the ability to survive desiccation.

2.8.1 Plants Material

M. flabellifolius, X. viscosa, X. humilis, X. villosa, X. schlecterii, C. wilmsii, C. plantagineum, C. pumilum and *E. nindensis*.

2.8.2 ANALYSIS

HPLC-DAD ESI MS (as described above).

2.9 Localisation of Polyphenols in Cells of desiccation tolerant plants

2.9.1 Histochemical Analysis

To determine the subcellular location of the polyphenols within the leaves of the desiccation tolerant plants studied, an ultrastructural investigation was undertaken. The samples are fixed in glutaraldehyde and caffeine. Caffeine is known to precipitate polyphenols at their cellular location (Hayat, 1981). Leaf samples were obtained from at least two different plants of each species. Leaves from fully hydrated plants were cut into about $4mm^2$ pieces and fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 0.5 % caffeine solution. The pieces were washed in 0.1-M phosphate buffer and post fixed in 1-% osmium in phosphate buffer. A series of dehydration steps were undertaken using ethanol and acetone after which the specimens were embedded in epoxy resin (Spur's, 1969) over three days.

The specimens were hardened at 60^0C for at least 16 hrs. Sections were cut using a Reichert Ultracut- S microtome (Vienna, Austria). The sections were stained using 2 % uranyl acetate and 1 % lead citrate (Reynolds, 1963). Viewing and photographs of the sections were taken using a Leo 912 transmission electron microscope (Zeiss, Germany), operating at 120 kV (http://emuo.emu.uct.ac.za/EmforBiologists/lecture2).

Observations of the general cell structure were done in at least four different sections from the leaves prepared.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 The total phenol and tannin content of the resurrection plants leaf extracts

Preliminary studies showed that freeze-drying and the use of 70 % acetone as the solvent together with ultrasound at room temperature was superior to oven drying and the use of 80 % methanol (Appendix. 7.1.2), giving the highest yield. Therefore acetone extraction of freeze-dried material was used for a detailed study of the polyphenols present in the selected nine resurrection plants. In the same preliminary studies it was also shown that there was no difference in the use of tannic acid or gallic acid as a standard in the Folin Ciocalteau method.

The results obtained in the determination of total phenol and tannin are shown in Table 3.1. The extracted total phenols ranged from 10.2 to 247.1 mg GAE/g DW (milligram gallic acid equivalent per gram dry weight) according to the Folin Ciocalteau method. *M. flabellifolius* exhibited the highest total soluble phenol content of 247.1 mg GAE/g DW whilst *X. villosa* and *E. nindensis* showed the lowest total soluble phenol contents of 10.2 mg GAE/g DW and 10.5 mg GAE/g DW respectively. *C. wilmsii, C. plantagineum, C. pumilum, X. schlecterii, X. humilis* and *X. viscosa* gave total soluble phenol contents in the range 38.0 to 48 mg GAE/g DW. The resurrection plants can be divided into three groups in terms of total soluble phenolic content: (1) very high total soluble phenols (> 200 mg GAE/g DW) n = 1; (2) low total soluble phenols (20- 50 mg GAE/g DW), n = 6; (3) very low total soluble phenols (0 – 12 mg GAE/g DW), n = 2. *X. villosa* has lower total soluble phenols compared to the other *Xerophyta* species.

The tannin content ranged from 0 to 208.7 mg GAE/g DW. *M. flabellifolius* contained the highest amount of tannins of 208.7 mg GAE/g DW and tannins contribute about 85 % of the total soluble phenols in this plant. *C. wilmsii, C. plantagineum, X. schlecterii, X. humilis* and *X. viscosa* gave tannin contents in the range 1.7 – 8.9 mg GAE/g DW whilst *C. pumilum, X. villosa* and *E. nindensis* seem to contain no tannins. The nine resurrection plants could be grouped into three groups again in terms of tannin contents as (1) very

high total tannin (> 200 mg GAE/g DW), n = 1; (2) very low total tannin (1.7 – 8.9 mg GAE/g DW), n = 5; and (3) no tannins (< 0.01 mg GAE/g DW), n = 3.

Table 3.1. Total soluble phenolic content of leaves of resurrection plant.

Resurrection plant Name	Total phenolics[a] (mg GAE/g DW)	Non tannin phenolics[b] (mg GAE/g DW)	Total Tannins[c] (mg GAE/g DW)
Myrothamnus flabellifolius	247.1 (15.8)	39.4 (13.6)	208.7 (7.2)
Craterostigma wilmsii	47.9 (1.3)	41.7 (1.7)	6.2 (0.6)
Xerophyta schlecterii	45.8 (5.1)	36.9 (4.7)	8.9 (0.4)
Craterostigma plantagineum	43.4 (1.6)	41.7 (2.3)	1.7 (1.0)
Craterostigma pumilum	41.5 (2.3)	41.8 (1.9)	N.D
Xerophyta humilis	38.9 (0.6)	35.6 (1.2)	3.2 (0.6)
Xerophyta viscosa	39.6 (1.5)	37.2 (1.9)	2.3 (0.5)
Eragrostis nindensis	10.5 (1.1)	10.5 (1.0)	N.D
Xerophyta villosa	10.2 (0.7)	10.3 (0.9)	N.D

[a] mg GAE/g DW -milligram gallic acid equivalent per gram dry weight material as calculated by the Folin-Ciocalteau method .
[b] mg GAE/g DW -milligram gallic acid equivalent per gram dry weight material as calculated by the Folin-Ciocalteau/ PVPP method
[c] mg GAE/g DW -milligram gallic acid equivalent per gram dry weight material as calculated by the Folin-Ciocalteau/ PVPP method. Obtained as the difference between the total phenolics and the non-tannin phenolics.
(x) is the standard deviation.
N.D- not detected.

There is a large variation in total phenol (total polyphenol) and tannin contents in the leaves of desiccation tolerant plants and the contents compare well with reported values for non-desiccation tolerant plants. Hoffman (2001) found that the leaves of the grass *Andropogon spec.* has total phenols of 16 mg tannic acid equivalents (TAE) /g DW and tannin concentration of 6 mg TAE/g DW as determined by the Folin Ciocalteau and Folin Ciocalteau/ PVPP assays respectively using tannic acid as the standard. Koukoura and Nostis (2000) found the total phenol content in *Medicago sativa* as 18,3 mg TAE/g DW as determined by the Folin Ciocalteau method.

The quantities of total soluble phenols and tannins in *E. capensis* and the rose plant are shown in Table 3.2 below. These two plants are desiccation sensitive and are used in this study for comparison purposes with the desiccation tolerant plants. *E. capensis* gave total phenol concentration of 9.9 mg GAE/g DW and no tannins were detected in the plant. This correlates well with the quantities shown by *E. nindensis* and *X. villosa*. The rose plant (Rosa *sp.*) gave total phenol concentration of 162.0 mg GAE/g DW and tannin concentration of 72 mg GAE/g DW. The rose plant is woody and showed very high total polyphenol and tannins compared to all desiccation tolerant plants used in this study except *M. flabellifolius*.

From the results it is apparent that the ability to tolerate desiccation does not seem to be conferred by the quantity of polyphenols or tannins present in the leaves. This does not mean polyphenols are not involved in desiccation tolerance, as they could be part of an array of compounds involved in desiccation tolerance.

Table 3.2. Total phenol and tannins in two desiccation sensitive plants, *Eragrostis capensis* and the rose plant.

Plant	Total phenol[a]	Total tannin[b]
Eragrostis capensis	9.9	ND
Rose	162.0	72.5

[a] mg GAE/g DW -milligram gallic acid equivalent per gram dry weight material as calculated by the Folin-Ciocalteau method .
[b] mg GAE/g DW -milligram gallic acid equivalent per gram dry weight material as calculated by the Folin-Ciocalteau/ PVPP method
N.D- not detected.

The quantities of total soluble polyphenols and condensed tannins in the leaves of some South African shrubland plant species are shown in Table 3.3. The concentrations of the total polyphenols reported for the plants are comparable to most of the nine-desiccation tolerant plants used in this study except *M. flabellifolius.*

The listed total polyphenol concentrations from Glyphis (1985) were obtained for studies done in summer. The plants given in the table are all desiccation sensitive plants. *Thesium aggregatum* and *Willdenowia striata* gave total phenolic value of 42.0 mg TAE/g DW and 38.0 mg TAE/g DW. These concentrations of total phenols are comparable to those of *C. wilmsii, C. plantagineum, C. pumilum, X. schlecterii, X. humilis, and X. viscosa.* The method used in this study, the Folin Ciocalteau assay, is the same method used by Glyphis (1985). It is clear from Table 3.1, Table 3.2 and Table 3.3 that there is no distinction between desiccation tolerant and desiccation sensitive plants in terms of total phenol and tannin content, with the exception of *Myrothamnus flabellifolius.*

Table 3.3. Total soluble polyphenols (mg TAE/g DW) and total condensed tannins (mg QE/g DW) in leaf material of some South African shrubland plant species.

Plant	Total Phenolics[a]	Total Condensed tannins[b]
Colpoon compressum	173.0	140.0
Chrysanthemoides incana	68.3	0.0
Eriocephalus africanus	65.0	0.0
Nylandtia spinosa	84.0	0.0
Olea exasperata	35.0	0.0
Pterocelastrus tricuspidatus	86.0	15.5
Salvia aurea	90.0	0.0
Thesium aggregatum	42.0	0.0
Willdenowia striata	38.8	24.5

[a] – Folin Ciocalteau assay as tannic acid equivalents /g D.W.

[b] – Butanol –HCl assay as quebracho equivalents /g DW.

(Table 3.2 – values from Glyphis, 1985).

Harborne (1993) pointed out that polyphenols are widely distributed in the plant kingdom and sometimes present in high concentrations. Larson (1988) and Oliver (Oliver et al, 1998) noted that phenolic acids and flavonoids are abundantly present in dry seeds, pollen and resurrection plants (Larson, 1988; Oliver et al, 1998). Caution should be

exercised when making comparisons of polyphenol quantities in different plant genera and/or species (Swain and Hillis, 1959).

According to White (1957) tannins are divided into hydrolysable and condensed tannins. Hydrolysable tannins have been further divided into other classes including gallotannins (gallic acid and glucose as the core) and ellagitannins (ellagic acid and glucose as the core) (Haslam, 1966). Condensed tannins are much more heterogeneous and are generally of larger molecular size than hydrolysable tannins. Tannins can form strong complexes with proteins, gelatine and PVPP. It has been found that the number and arrangement of phenolic groups around the glucose or quinic acid core affects the protein binding capacity of hydrolysable tannins (Haslam, 1974).

3.1.1 Hydrolysable Tannins in resurrection plants leaf extracts

To further investigate the possible involvement of polyphenols in desiccation tolerance the nature of the polyphenols was analysed. The quantities of hydrolysable tannins and condensed tannins in the nine resurrection plants were determined and are given below. Results on the hydrolysable tannins as gallotannins and ellagitannins are summarised in Table 3.4. Gallotannins were detected in *M. flabellifolius, C. wilmsii* and *X. schlecterii* using the rhodanine assay. However the content of gallotannins in *C. wilmsii* and *X. schlecterii* was 10^4 times lower compared to the content of gallotannins in *M. flabellifolius*. The content of gallotannins in *M. flabellifolius* using the rhodanine assay was found to be 146 mg GAE/g DW (milligram gallic acid equivalents per gram dry weight) whilst *C. wilmsii* and *X. schlecterii* contain 0.014 mg GAE/g DW and 0.013 mg GAE/g DW respectively.

C. plantagineum, C. pumilum, X. viscosa, X. humilis, X. villosa and *E. nindensis* gave no response in the rhodanine assay according to the results obtained. The content of gallotannins in *M. flabellifolius* using the HPLC method was found to be 104 mg GAE/g DW. *C. wilmsii, C. plantagineum, C. pumilum, X. schlecterii, X. viscosa, X. humilis, X. villosa* and *E. nindensis* appear to contain no gallotannins according to the HPLC method.

Table 3.4. Total soluble Hydrolysable tannins content of leaves of resurrection plant.

Resurrection plant Name	Gallotannins (mg GAE/g DW)[a]	Gallotannins (mg GAE/g DW)[b]	Ellagitannins[c] (mg EAE/g DW)
Myrothamnus flabellifolius	146.0 (2)	104.0 (24.0)	32.0 (0.4)
Craterostigma wilmsii	0.014 (0.001)	ND	0.015 (0.004)
Xerophyta schlecterii	0.013 (0)	ND	0.014 (0.001)
Craterostigma plantagineum	N.D	N.D	0.0064 (0)
Craterostigma pumilum	N.D	N.D	N.D
Xerophyta humilis	N.D	N.D	N.D
Xerophyta viscosa	N.D	N.D	N.D
Eragrostis nindensis	N.D	N.D	N.D
Xerophyta villosa	N.D	N.D	N.D

[a] mg GAE/g DW –milligram gallic acid equivalent per gram dry weight material as determined by the Rhodanine Assay (Hagerman, 1988).
[b] mg GAE/g DW –milligram gallic acid equivalent per gram dry weight material as determined by the HPLC method.
[c] mg EAE/g DW–milligram ellagic acid equivalent per gram dry weight material as determined by the Nitrous Assay (Wilson and Hagerman, 1990).
N.D- not detected.
(x) is the standard deviation.

In the determination of ellagitannins, using the nitrous assay, *M. flabellifolius, C. wilmsii, C. plantagineum* and *X. schlecterii* were the only plants that gave positive results. *M. flabellifolius* gave 32 mg EAE/g DW, *C. wilmsii* gave 0.015 mg EAE/g DW, *C. plantagineum* gave 0.0064 mg EAE/g DW and *X. schlecterii* gave 0.014 mg EAE/g DW of gallotannins as ellagic acid equivalents. *M. flabellifolius* contains mainly gallotannins and relatively low quantities of ellagitannins. *M. flabellifolius* stands out as having more gallotannins and ellagitannins than any other resurrection plant studied.

The differentiation of between gallotannins and ellagitannins is based on assays only as in reality gallotannins could also be bound to ellagic acid and ellagitannins could be bound to gallic acid (Mueller-Harvey, 2001). *C. wilmsii* and *X. schlecterii* appear to

contain the same amounts of gallotannins and ellagitannins. Ossipov et al (1997) found that in the leaves of young birch trees the total content of gallotannins varied from 3 to 30 mg GAE/g DW as gallic acid equivalents. This is higher than the quantities found for the other desiccation tolerant plants except *M. flabellifolius*. Birches are generally known to contain high levels of polyphenols in leaves often exceeding 100 mg GAE/g DW.
Again there is no discernible pattern in the nine resurrection plants in terms of the levels of hydrolysable tannins, with only *M. flabellifolius* giving positive responses in all the assays used. *C. wilmsii* and *X. schlecterii* have same levels of gallotannins and ellagitannins according to the rhodanine and nitrous assays.
Most studies done on plants tend to concentrate on condensed tannins as these are analytically more accessible via the simple butanol assay. Condensed tannins form strong bonds with proteins and the complexes formed are not easily digested compared to those formed between hydrolysable tannins and proteins.

3.1.2 Total condensed tannins in the leaves of resurrection plants

The reactivity of condensed tannins with molecules of biological significance has important physiological consequences. Analysis of condensed tannins is difficult due to the structural complexity of these compounds. Commonly used methods include the use of the butanol-HCl reagent. This reagent was used in the determination of condensed tannins in the nine selected resurrection plants.
M. flabellifolius gave a positive response in the assay yielding 29 mg LE/g DW (milligram leucocyanidin equivalents per gram dry weight). *C. wilmsii, C. plantagineum, C. pumilum, X. schlecterii, X. viscosa, X. humilis, X. villosa* and *E. nindensis* all gave no response in the butanol-HCL assay. Condensed tannins are reported to interact with proteins forming insoluble protein-tannin complexes through hydrogen bonding and hydrophobic interactions. The quantities of condensed tannins are always low in plant leaves. Condensed tannins are known to be synthesised in response to many factors and among them is herbivory, thus their low content in all plants is expected as the plants were kept in the greenhouse prior to analysis. It has also been shown that aqueous acetone enhances the butanol/ HCl reaction with condensed tannins and increases colour intensity (Yu and Dahlgren, 2000).

Table 3.5. Total soluble condensed tannins content of leaves of resurrection plant.

Resurrection plant Name	Condensed Tannins[a] (mg LE/g DW)
Myrothamnus flabellifolius	29.0 (0.4)
Craterostigma wilmsii	ND
Xerophyta schlecterii	ND
Craterostigma plantagineum	ND
Craterostigma pumilum	ND
Xerophyta humilis	ND
Xerophyta viscosa	ND
Eragrostis nindensis	ND
Xerophyta villosa	ND

[a] mg LE/g DW –milligram leucocyanidin equivalents per dry weight material as determined by the Butanol-HCL method (Porter et al, 1986).
N.D- Not detected.
(x) is the standard deviation.

Purification of internal standards, for use in the determination of condensed tannins, is time consuming and a complex procedure especially in this study where multiple species are used. Even with internal standards available, polyphenols and tannins in plants are a large heterogeneous group of compounds exhibiting different reactivity to the various analytical reagents.

The amount of condensed tannins in the leaves of *Andropogon spec.* were found to be 0.3 mg LE/g DW by the butanol-HCL method (Hoffman, 2001). Thus *M. flabellifolius* leaves contain relatively high amounts of condensed tannins. Table 3.3 summarises analyses done by Glyphis (1985) on different South African shrubland plants. *Colpoon compressum* contains 140 mg QE/g DW (milligram quebracho equivalents per gram dry weight), *Pterocelastrus tricuspidatus* gave 15.0 mg QE/g DW, *Willdenowia striata* gave 24.0 mg QE/g DW condensed tannins as quebracho equivalents. The standard used by

Glyphis (1985) is quebracho whilst in this study the results are reported as leucocyanidin equivalents. Both results show variation in condensed tannins in the different plants. Other plants studied by Glyphis (1985) appear to contain no condensed tannins. This is consistent with the results obtained in this study where, except *M. flabellifolius*, the rest of the plants show no presence of condensed tannins.

Bate-Smith (1962) also pointed out the correlation of proanthocyanidins (condensed tannins) occurrence in plants with a woody habit of growth. The same is observed in this study where of the nine desiccation tolerant plants only *M. flabellifolius* is woody and it is the only one containing condensed tannins. There appears to be no correlation in the amount of both hydrolysable and condensed tannins in the desiccation tolerant plants. There is no distinction again in the amount of hydrolysable and condensed between desiccation tolerant and desiccation sensitive plants.

3.1.3 Tannin determination by protein precipitation

The ability to precipitate proteins differentiates tannins from other polyphenols compounds. The radial diffusion assay permits the simultaneous analysis of many samples and is used to measure total tannin levels in the leaf extracts. The results are summarised in Table 3.6.

The radial diffusion assay results for *M. flabellifolius* show the amount of tannins as 195.0 mg TAE/g DW (milligram tannic acid equivalents per gram dry weight). *C. wilmsii, C. plantagineum, C. pumilum, X. schlecterii, X. viscosa, X. humilis, X. villosa* and *E. nindensis* extracts did not precipitate BSA which means that no tannins are present or are present in low amounts. The results obtained for *M. flabellifolius* in the radial diffusion assay agree with results obtained in the Folin Ciocalteau/PVPP assay. The formation of the precipitation ring in the radial diffusion assay is influenced among other factors by the stoichiometry of the reaction between the tannins present in the extracts and the BSA.

Using the protein precipitable phenolic (PPP) assay the protein precipitable phenolics in *M. flabellifolius* were found to be 148.0 mg TAE/g DW of leaf material. *C. wilmsii, C. plantagineum, C. pumilum, X. schlecterii, X. viscosa, X. humilis, X. villosa* and

E. nindensis extracts gave no response in this assay. Comparing these results to those obtained by the Folin Ciocalteau/PVPP method it can be seen that the Folin Ciocalteau/PVPP method gives a high value (208.7 mg GAE/g DW) to the results obtained by this protein precipitation (PPP) method (148.0 mg TAE/g DW) for *M. flabellifolius. C. wilmsii, C. plantagineum, X. schlecterii, X. humilis* and *X. viscosa* also showed the presence of tannins in the Folin Ciocalteau/ PVPP assay but in the radial and the PPP assay they appear to contain no tannins. Reasons for the differences could be due to the fact that insoluble PVPP used in the Folin Ciocalteau / PVPP assay is more efficient in precipitating polyphenol than BSA used in the PPP and radial diffusion assays. PVPP and BSA may have different binding characteristics and pH optima. The protein precipitable phenolics (PPP) assay also takes into accounts only the slope of the linear regression and ignores the y-intercept. Makkar and Becker (1994) suggested the addition of different quantities of protein to a fixed amount of tannin or tannin-containing extract and the determination of the maximal protein bound by the tannin. The ferric chloride method (used in the PPP assay to determine tannins) is known to underestimate or give values that are lower to those of the Folin Ciocalteau method (used in the Folin Ciocalteau /PVPP assay). Hoffman (2001) found that the results of the Folin Ciocalteau method exceeded those of the ferric chloride assay up to an order of magnitude. The PPP assay also uses methanol to extract the phenolics as acetone would inhibit the interaction between the protein and the tannins. Methanol has been shown to extract less polyphenols compared to acetone (see Appendix A section 7.1.2).

The results for the radial diffusion and the Folin Ciocalteau/PVPP assays are almost the same for *M. flabellifolius* hence they can be taken as the more accurate values. Hoffman (2001) found that in the leaves of the plants he studied the Folin Ciocalteau/ PVPP method always gave higher values than the protein precipitable phenolic method in terms of the total phenolics and tannins. The *Guira senegalensis* leaves gave the total tannins as 96.0 mg TAE/g DW by the Folin/ PVPP method and as 17.0 mg TAE/g DW by the protein precipitable phenolic assay (Hoffman, 2001). The results obtained in this study illustrate that the quantification of polyphenols and tannins using a single method can be misleading as different methods yields different quantities. The same large variation is also observed in desiccation sensitive plants as shown by Table 3.2 and Table 3.3.

Table 3.6. Summary of tannin analysis using three separate and complimentary assays

Plant	Folin/PVPP[a]	Radial Assay[b]	PPP Assay[c]
Myrothamnus flabellifolius	208.7	195.0	148.0
Craterostigma wilmsii	6.2	ND	ND
Xerophyta schlecterii	8.9	ND	ND
Craterostigma plantagineum	1.7	ND	ND
Craterostigma pumilum	ND	ND	ND
Xerophyta humilis	3.2	ND	ND
Xerophyta viscosa	2.3	ND	ND
Eragrostis nindensis	ND	ND	ND
Xerophyta villosa	ND	ND	ND

[a] milligram gallic acid equivalents per dry weight.
[b] milligram tannic acid equivalents per dry weight.
[c] milligram tannic acid equivalents per dry weight as determined by the ferric chloride/BSA method
N.D – not detected.

3.1.4 CONCLUSION

There is a large variation in total polyphenol and tannin contents in the plants used in the study. Of the nine resurrection plants studied only *M. flabellifolius* leaves contains unusually high amount of polyphenols and tannins. *C. wilmsii, C. plantagineum, C. pumilum, X. schlecterii, X. viscosa, X. humilis, X. villosa* and *E. nindensis* leaves have amounts that are comparable to other desiccation sensitive plants and grasses. The amount of polyphenols and tannins do not seem to be directly correlated to the ability to tolerate desiccation. The role played by the polyphenols in *M. flabellifolius,* that appear to be higher than normal, is uncertain at this moment.

M. flabellifolius contains relatively high amounts of gallotannins. *C. wilmsii, C. plantagineum, X. viscosa, X. humilis* and *X. schlecterii* seem to contain relatively low or no tannins. As stated before polyphenols and tannins could be synthesised in response to herbivory and diseases (Harborne, 1964). Condensed tannins are usually found in woody plants. Polyphenols might have many other roles they play in plants that are

related to desiccation tolerance. Using the results obtained so far, polyphenols do not seem to be directly involved in desiccation tolerance. However, quantity only does not define function. The levels of the polyphenols in the hydrated state might not show that polyphenols are directly involved in desiccation tolerance hence the plants were dried under controlled conditions and the levels of polyphenols monitored prior to drying and during drying over a period of time. One of the roles that has been assigned to polyphenols is being antioxidants. Polyphenols are good candidates as antioxidants because of their favourable redox potentials and the relative stability of the aryloxy radical (Hagerman, 1998, 2002). The antioxidant capacities of the extracted phenolics were also analysed.

3.2 Antioxidant Capacity

During dehydration, plant cells are subjected to a number of stresses one of which is oxidative stress. Therefore an efficient cellular antioxidant mechanism must be present in desiccation tolerant plants for them to avoid cellular disorders. The enzymatic defence mechanism is not effective at low water potential, therefore the plants must use the nonenzymic antioxidant defence mechanism to prevent or minimise the formation of reactive oxygen species. The antioxidant capacity of polyphenolic extracts of the nine desiccation tolerant plants used in this study, as determined by the FRAP assay are shown in Table 3.7.

3.2.1 Total Antioxidant Capacity (FRAP) of leaf extracts of the desiccation tolerant plants

Table 3.7. Total antioxidant capacity determined as FRAP and the related total phenolic content of the desiccation tolerant plant extracts.

Resurrection plant Name	FRAP[a] (mmol Fe^{2+}/L)	Total phenolics[b] (mg GAE/ g DW)	Total phenolics[c] (mmol GAE/ L)	PAC[d]
Myrothamnus flabellifolius	25.1 (0.8)	247.1 (15.9)	36.3	0.7
Craterostigma wilmsii	11.5 (0.4)	47.9 (1.3)	7.1	1.6
Xerophyta schlecterii	8.7 (0)	45.8 (5.1)	6.7	1.3
Craterostigma plantagineum	10.9 (0.4)	43.4 (1.6)	6.4	1.7
Craterostigma pumilum	7.8 (0.2)	41.5 (2.3)	6.1	1.3
Xerophyta humilis	7.7 (0)	38.9 (0.6)	5.7	1.4
Xerophyta viscosa	8.0 (0.3)	39.6 (1.5)	5.8	1.4
Eragrostis nindensis	3.4 (0.1)	10.5 (1.1)	1.5	2.3
Xerophyta villosa	2.3 (0.1)	10.2 (0.7)	1.5	1.5

[a] FRAP - Ferric reducing / antioxidant power.
[b] mg GAE/ g DW - milligram gallic acid equivalent per gram dry weight material
[c] mmol GAE/ L- millimole gallic acid equivalent per litre of extract.
[d] PAC -Phenol antioxidant coefficient, calculated as FRAP (mmol Fe^{2+}/L)/Total phenolics (mmol GAE/ L).
(x) Standard deviation in brackets, n = 3.

The total antioxidant capacity (FRAP) of the leaf extracts determined by the ferric reducing antioxidant power (FRAP) assay are summarised in Table 3.7. These ranged from 2.3 to 25.1 mmol Fe^{2+}/L of extract. *M. flabellifolius* has the highest FRAP value of 25.1 mmol Fe^{2+}/L of extract whilst *X. villosa* has the least FRAP value of 2.3 mmol Fe^{2+}/L of extract. Using the FRAP values the nine desiccation tolerant plants can be divided into three groups: (1) low FRAP (1.0- 4.0 mmol Fe^{2+}/L), n = 2; (2) good FRAP (5.0- 12.0 mmol Fe^{2+}/ L), n = 6; (3) very high FRAP (>20.0 mmol Fe^{2+}/L), n = 1. The antioxidant capacities when measured with the FRAP assay ranged: *M. flabellifolius* >>> *C. wilmsii* > *X. schlecterii* > *C. plantagineum* > *C. pumilum* >*X. humilis* > *X. viscosa* > *E. nindensis* and *X. villosa.*

The phenol antioxidant coefficient (PAC) was calculated, as the ratio between FRAP (mmol/L) and total phenol (converted to mmol GAE/L). The PAC is used for comparison of antioxidant efficiency of total phenolics from different plant extracts (Katalinic et al, 2005). The calculated PAC ranged from 0.7 to 2.3. The PAC of *M. flabellifolius* was the lowest (0.7) yet it contained the highest amounts of phenolics and the PAC of *E. nindensis* (2.3) was the highest yet it contained among the least amount of phenolics. Wang et al (1996) and Kahkonen et al (1999) concluded that most of the antioxidant activity of vegetables and some other plants might be from flavonoids, isoflavones, flavones, anthocyanins, catechin and isocatechin. The high antioxidant efficiency of *E. nindensis* extracts could be explained on the basis of the high antioxidant activity of some individual phenolic units, which may act as efficient antioxidants rather than contributing to high total phenolics (Kaur and Kapoor, 2002). Thus the low PAC value for *M. flabellifolius* might mean that the polyphenols in *M. flabellifolius* are not very efficient antioxidants and therefore have other roles. The FRAP value for *X. villosa* (2.3 mmol Fe^{2+}/L) is different from *X. schlecterii* (8.7 mmol Fe^{2+}/L), *X. humilis* (7.7 mmol Fe^{2+}/L) and *X. viscosa* (8.0 mmol Fe^{2+}/L). However *X. villosa* has almost the same PAC value (1.5) as *X. schlecterii* (1.3), *X. humilis* (1.4) and *X. viscosa* (1.4) plants.

Cao and Prior (1998) noted that there are many methods that can be used to measure the antioxidant capacity of compounds and the FRAP method is one of those methods. The FRAP method has a wide range of applications from being used on fruits, wines, animal tissues and pure compounds (Ghiselli, Nardini, Baldi and Scaccini, 1998; Modun et al,

2003). The antioxidant capacities of the phenolics in these resurrection plants were also analysed using two other methods, namely the β-carotene linoleic acid assay and the DPPH assay. Different phenolics have different antioxidant capacities therefore it was found necessary to characterise the individual polyphenols in the leaf extracts (under HPLC-DAD- ESI MS section 3.4).

This is the first time the antioxidant capacities of these resurrection plants have been compared to the amount of phenols present in the resurrection plants extracts. FRAP was used as it is reproducible and linearly related to the molar concentration of the antioxidants present (Benzie, Wai and Strain, 1999). Katalinic et al (2005) used the same method and found that the medicinal plant *Mellisa officianalis L.* has very high FRAP (25.2 mmol Fe^{2+}/L) values and PAC (>3). Interestingly *M. flabellifolius* is used by some people as a medicinal plant to heal wounds and abrasions.

Results from Table 3.1 show that *M. flabellifolius* contains more tannins than other phenolics. Other researchers have postulated that the high antioxidant activity of vegetables is mainly due to flavonoids, flavones, anthocyanins, catechin and isocatechin rather than from vitamin C, E and β-carotene. The same researchers also noted that non-tannin phenolics are more efficient as antioxidants than tannin phenolics (Wang et al, 1996; Kahkonen et al; 1999). *M. flabellifolius* contains less non-tannin phenolics and could be the reason why its PAC is low compared to the other plant extracts. However, the differences in the PAC values could be due to the use of crude extracts that are not pure phenolic. *M. flabellifolius* contains high levels of phenolics and has a high FRAP value. The leaf extract of *M. flabellifolius* was selected for further analysis.

3.2.1.1 Comparison of antioxidant capacity of *M. flabellifolius* leaf extract with gallic acid and catechin

M. flabellifolius leaf extract contains high quantities of polyphenols and it has a high FRAP value hence it was used for comparison purposes to gallic acid and catechin. The dose response of *M. flabellifolius* extract compared with that of pure antioxidants, catechin and gallic acid is shown in Figure 3.1. Gallic acid is a strong natural antioxidant (Heinonen, Lehtonen and Hopia, 1998; Zheng and Wang, 2001). Gallic acid is known to decrease the peroxidation of ox-brain phospholipids (Milic, Djilas and Canadovic-

Brunnet, 1998). *M. flabellifolius* phenolics reducing equivalent was about 1.6 times higher when compared to Fe^{2+}. The reducing capacity of pure catechin and gallic acid was more than twice (2.2) and nearly quadruple (3.9) that of Fe^{2+} respectively. These results confirm the results obtained by Benzie who found that the relative antioxidant efficiency of catechin to Fe^{2+} was 2 on average (Benzie et al, 1999; Katalinic et al, 2004). The responses given by the plant extracts depend on the composition of the extracts.

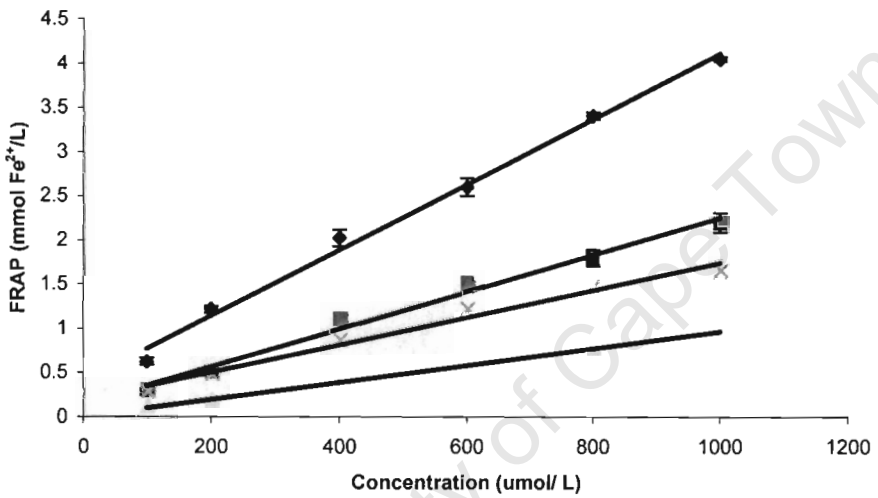


Figure 3.1. Dose response of (x) *M. flabellifolius* phenolics, (■) catechin, (♦) gallic acid and (▲) Fe^{2+} over the concentration range 100 – 1000 uM in the FRAP assay for reducing (antioxidant) activity. *M. flabellifolius* concentration was calculated based on the Folin Ciocalteau results as gallic acid equivalents. Bars: standard deviation, n=3.

The results obtained in this study show that *M. flabellifolius* leaf extract has high antioxidant capacity as the crude extract gave high relative antioxidant response (1,6 times the reducing capacity of Fe^{2+}). This is close to the relative antioxidant response given by pure catechin and is almost half the response of pure gallic acid. Thus the purified extract from *M. flabellifolius* can be considered to be rich in antioxidants. *M. flabellifolius* is known to survive desiccation for long periods of time (Kranner et al, 2002). This could be due to the presence of high amounts of polyphenols that are able to remove reactive oxygen species efficiently. Kranner et al (2002) demonstrated that the

ability of resurrection plants to 'resurrect' correlates with the capacity to maintain a number of antioxidant systems on rehydration. It was also suggested that the whole array of antioxidants and pigments are required to provide protection against desiccation-induced free radical attack.

3.2.2 Antioxidant Capacity determined by DPPH

The DPPH assay was used to measure the radical scavenging abilities of the plant extracts. The assay is used in the determination of antioxidant activity of pure compounds as well as plant extracts (Brand-Williams, Cuvelier and Berset, 1995; Kulisic, Radonic, Katalinic and Milos, 2004; Yen and Duh, 1994).

Table 3.8. Radical scavenging ability of leaf extracts of the resurrection plant (diluted 10 x)

Plant	% Inhibition of DPPH	Total phenolics (mg GAE/g DW)	Phenol anti-radical coefficient (PARC)[d]
Myrothamnus flabellifolius	94.8 (0.4)	247.1	0.4
Craterostigma wilmsii	47.7 (1.0)	47.9	1.0
Xerophyta schlecterii	36.3 (1.5)	45.8	0.8
Craterostigma plantagineum	54.3 (1.3)	43.4	1.2
Craterostigma pumilum	40.0 (1.4)	41.5	1.0
Xerophyta humilis	31.7 (2.4)	38.8	0.8
Xerophyta viscosa	36.1 (0.6)	39.6	0.9
Eragrostis nindensis	24.0 (2.6)	10.5	2.3
Xerophyta villosa	8.4 (2.1)	10.2	0.8

[d]- Phenol anti-radical coefficient, calculated as percent inhibition of DPPH radical / Total phenol (mg GAE/ g D.W), (x)- is the standard deviation, n = 3.

The results obtained show that for the desiccation tolerant plants used (diluted 10 x, v/v), there is a large variation in DPPH inhibition ranging from 8.4 % to 94.8 %.
M. flabellifolius showed the highest percent inhibition of the DPPH radical of 94.8 % whilst the least was for *X. villosa* with its percent inhibition of the DPPH radical at 8.4 %.

Overall these nine resurrection plants can be grouped into three groups again: (1) very low antioxidant activity (0- 30 % inhibition), n = 2; (2) low antioxidant activity (31- 55 % inhibition), n = 6; (3) high antioxidant activity (80- 100 % inhibition), n = 1. In the DPPH radical scavenging method, the absorbance decreases as a result of a colour change from purple to yellow as the radical is scavenged by the antioxidant through donation of hydrogen to form the stable DPPH-H (Katalinic et al, 2005). The phenol anti-radical coefficient (PARC) was also calculated as the ratio between percent inhibition (scavenging) of the DPPH radical and total phenols and is used to compare the anti-radical efficiency of the total phenolics. The calculated PARC ranged from 0.4 for *M. flabellifolius* to 2.3 for *E. nindensis*. The trend observed here for the PARC is the same observed with the PAC in the FRAP assay. The ratios show that polyphenols in *E. nindensis* are more effective radical scavengers per unit mass compared to those in *M. flabellifolius*.

The homoiochlorophyllous plants, *M. flabellifolius (94.8 %), C. wilmsii, C. plantagineum* and *C. pumilum* (40 – 54 %) appear to have higher percent inhibition of the DPPH radical relative to poikilochlorophyllous plants (*Xerophyta* species and *E. nindensis,* 8.4- 36.3%). Kranner et al (2002) suggested that homoiochlorophyllous plants might need better protection against free radical attack as compared to the poikilochlorophyllous plants. Poikilochlorophyllous plants dismantle their photosynthetic apparatus during desiccation and this strategy avoids free radical formation caused by energy transfer from excited chlorophyll to ground state oxygen (Kranner et al, 2002).

It has been found that the scavenging action of polyphenols is closely related to their structure. Katalinic et al (2005) reported the percent inhibition of the synthetic antioxidant, BHT, as determined by the DPPH assay as 49.2 %. *C. wilmsii, C. plantagineum, C. pumilum* and *X. schlecterii* extracts (diluted 10 x, v/v) also have percent inhibition in the same range.

Chu et al (2000) found that although white cabbage and crown daisy have low phenolic content they have moderate anti-oxidant activity. This was attributed to the presence of other phytochemicals such as ascorbic acid, tocopherol and pigments, which can also make a contribution to the total antioxidant activity (Chu et al, 2000). In this study

pigments and possibly carotenoids were removed by the use of heptane before the phenolics were extracted using 70 % acetone.

It is recommended that the antioxidant activity of extracts be evaluated by different methods rather than depending on the results of a single method. The percent inhibition of DPPH radical for *X. villosa,* like the total phenol and FRAP value, is substantially lower (8.4 %) compared to the other *Xerophyta* species (31- 36 %) and this may indicate an incorrect classification. *X.villosa* is morphologically different from the other *Xerophyta* species as shown by Figure1.6.

3.2.2.1 Comparison of the free radical scavenging ability of *M. flabellifolius* leaf extract with that of catechin and gallic acid

The DPPH radical scavenging ability of *M. flabellifolius* phenolics, catechin and gallic acid at different concentrations are shown in Fig 3.2 below. The same trend observed in

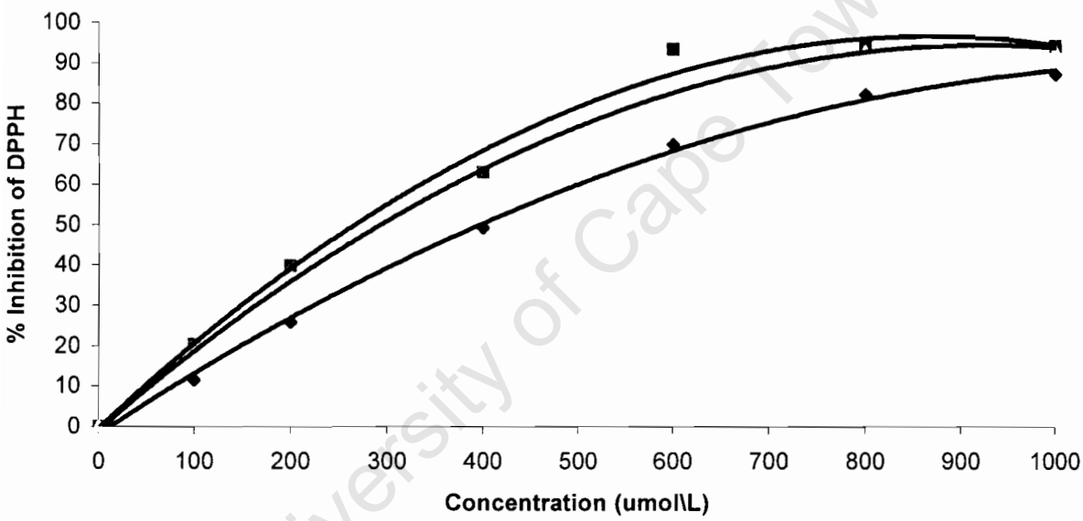


Figure 3.2. The percentage inhibition of the free DPPH radical in the presence of different concentrations of antioxidants (♦) *M. flabellifolius* phenolics, (▲) catechin, (■) gallic acid. *M. flabellifolius* concentration was calculated based on the Folin Ciocalteau results as gallic acid equivalents.

the FRAP assay is observed here where catechin and gallic acid have free radical abilities that are high compared to that of *M. flabellifolius* extract. *M. flabellifolius* extracts gave lower inhibitions compared to gallic acid and catechin. However, in this study the results show that *M. flabellifolius* extracts are good at inhibiting free radicals in solutions since the extract is crude but give good inhibitions compared to these two pure compounds. The IC_{50} (concentration required to give 50 % inhibition) for gallic acid, catechin and *M. flabellifolius* polyphenols was obtained from the Figure 3.2. Gallic acid gave an IC_{50} of 280 µmol/ L, catechin gave 295 µmol/ L and *M. flabellifolius* polyphenols gave an IC_{50} of 390 µmol/L.

3.2.3 Antioxidant capacity determined by the β-carotene linoleic acid method

The percent inhibition of linoleic acid oxidation by the resurrection plant extracts ranged from 25.9 % to 93.6 % as shown in Table 3.9. *M. flabellifolius* polyphenols had the highest antioxidant capacity with inhibition at 93.6 % and *E. nindensis* polyphenols had the least antioxidant capacity with inhibition at 25.9 % inhibition. Again there is a large variation in the percent inhibition as in the DPPH assay. The resurrection plants can be grouped into three categories: (1) very low antioxidant activity (0- 30 % inhibition), n = 2; (2) low antioxidant activity (31- 55 % inhibition), n = 6; (3) high antioxidant activity (80- 100 % inhibition), n = 1.

Table 3.9 Antioxidant activity of the resurrection plant extracts in the β-carotene linoleic acid assay

Plant	% Inhibition of β-carotene Bleaching	Total phenolics (mg GAE/ g DW)	Phenol Antioxidant Coefficient (PAC)[a]
Myrothamnus flabellifolius	93.6 (2.1)	247.1	0.4
Craterostigma wilmsii	54.7 (1.2)	47.9	1.1
Xerophyta schlecterii	50.1 (0.9)	45.8	1.1
Craterostigma plantagineum	53.6 (1.5)	43.4	1.2
Craterostigma pumilum	35.8 (1.0)	41.5	0.9
Xerophyta humilis	31.7 (2.0)	38.9	0.8
Xerophyta viscosa	40.1 (2.5)	39.6	1.0
E. nindensis	25.9 (0.7)	10.5	2.5
Xerophyta villosa	26.6 (0.8)	10.2	2.6

[a]-Phenol antioxidant coefficient (PAC), calculated as the ratio between percent inhibition of β-carotene bleaching / total phenol (mg GAE/ g DW).

(x)- standard deviation, n = 3.

X. villosa is the only plant that showed a different percent inhibition in the DPPH assay (8.4 %) and the β-carotene linoleic acid assay (26.6 %). Sokmen et al (2005) reported that *Geranium sanguineum L* exhibited a strong antioxidant capacity in the β-carotene linoleic

acid assay, achieving 88 % inhibition. This plant is a medicinal plant, rich in phenolics (Sokmen et al, 2005). The percent inhibition shown by this plant is lower than that of *M. flabellifolius* (93.6 %) but higher than the other resurrection plants (25- 55 %).

The calculated PAC ranged from 0.4 for *M. flabellifolius* to 2.6 for *X. villosa*. Many researchers report that non-tannin phenolics like flavonoids, anthocyanins, catechin and flavones are more efficient antioxidants than tannin phenolics.

The same trend that was observed in the FRAP and the DPPH assays is observed in the β-carotene linoleic acid assay. *M. flabellifolius* extract (93.6 %) stands out of the rest as having the highest ability to inhibit the oxidation of linoleic acid.

A summary of the antioxidant activity of *M. flabellifolius* extract in comparison with pure compounds, BHT, gallic acid, catechin and vitamin C is given below.

Table 3.10. Antioxidant activity of *M. flabellifolius* polyphenols in comparison with pure antioxidants determined by three methods (FRAP, DPPH, β-CLA).

Compound or plant extract	FRAP (relative to Fe^{2+})[a]	DPPH (IC_{50}, umol/L)[b]	β-Carotene-Linoleic (% Inhibition)[c]
Gallic acid	3.9	280	ND
Catechin	2.2	295	ND
M.flabellifolius	1.6	390	93.6
Vitamin C	1.9- 2.1[*]	-	-
BHT	-	-	88-89 %[#]

[a] Relative activities of individual antioxidants to the reaction of Fe^{2+} (representing one-electron exchange reaction and taken as unity).

[b]Determined by the DPPH radical scavenging method.

[c]Determined by the β-carotene Linoleic acid assay.

[*]Vitamin C results – according to Benzie and Strain (1996).

[#]BHT results – according to Sokmen et al (2005)

N.D - not determined.

M. flabellifolius polyphenols (93.6 %) were more powerful at inhibiting the bleaching of β-carotene than reported results of pure BHT (88-89 %) for the same volume used in the

assay. Catechin gave a relative reducing capacity of 2.2 to Fe^{2+}. Other researchers found similar results, with Katalinic et al (2005) getting the relative reducing capacity as average 2.0 (1.9-2.1). Gallic acid and catechin are used in this study for comparison purposes and also as positive controls.

M. flabellifolius extracts are crude whilst gallic acid and catechin are pure, therefore *M. flabellifolius* polyphenols appear to be good antioxidants. These three separate and complimentary methods (FRAP, DPPH, and β-CLA) were used to study changes in antioxidant activity during desiccation (section 3.3).

3.2.4 Correlation between the antioxidant capacities determined by the FRAP, DPPH and β-CLA Assays

The antioxidant capacities of the polyphenols present in the leaves of the desiccation tolerant plants were compared.

Table 3.11. Comparison of the correlation coefficients between the antioxidant capacities of the polyphenols extracted from the leaves as determined with three different methods (FRAP, DPPH and β-CLA)

	r		
	FRAP	DPPH	β-CLA
FRAP	1	0.98	0.97
DPPH	0.98	1	0.95
β-CLA	0.97	0.95	1

The magnitude of correlation between variables was quantified by the correlation coefficient *r*. A correlation coefficient above 0.9 was considered significant, n = 3.

The correlations between the antioxidant capacities of the extracted polyphenols as determined by the three assays were found to be significant (Table 3.11). There is a significant correlation between FRAP and the DPPH scavenging ability (0.98), between FRAP and the ability to inhibit the bleaching of β-carotene (0.98) and between the DPPH

scavenging ability and the ability to inhibit the bleaching of β- carotene (0.95). This means that any one of the three methods can be used for the evaluation of antioxidant capacity of the resurrection plant extracts.

3.2.5 CONCLUSION

The large variation in antioxidant capacities shown by the desiccation tolerant plants, as determined by the three methods, has been reported before in desiccation sensitive plants (Cao et al, 1996; Wang et al, 1996; Gazzani et al, 1998). This implies that phenolics are not the main factor determining desiccation tolerance. There is also a large variation in the phenol antioxidant coefficient, showing that the polyphenols present in the leaves of these plants are of different efficiency. Using the PAC and the PARC obtained there is a general trend that the higher the amount of tannins the less efficient are the phenols as antioxidants. This is in contrast with what some researchers suggest. Hagerman (1998, 2002) suggests that condensed and hydrolysable tannins are more effective radical scavengers than small phenolics. However, that could be as a result of the use of different methods in the determination of the antioxidant capacities. The antioxidant capacities of the polyphenols in the hydrated leaves are unlikely to be directly responsible for desiccation tolerance in resurrection plants. At this stage, however, it is not possible to rule out that polyphenols play an important role in water stress tolerance especially in the case of *Myrothamnus flabellifolius*, which contains high amounts of polyphenols. The involvement of polyphenols in desiccation tolerance was further investigated by analysing polyphenol concentration and antioxidant capacities of the polyphenols in response to desiccation.

3.3 The effect of desiccation on polyphenol content and the antioxidant capacities of the leaves of desiccation tolerant plants

In order to understand the role of polyphenols in desiccation tolerance it was decided to study the polyphenol contents and the related antioxidant capacities of the leaf extracts during desiccation in order to elucidate the possible role of phenolics in desiccation tolerance. It is known that protein synthesis and enzyme activity is switched off at high relative water contents. Therefore any synthesis and change in polyphenol content can only occur at high relative water contents.

Water contents of the drying leaves were expressed as relative water content (RWC) i.e. the water content of the drying leaves as a percentage of the water content of the leaves when fully hydrated. The water content was calculated according to the following formula: **[Starting weight – Dry weight] / Starting weight x 100.**

Starting weight being the weight of the leaves at different drying stages and dry weight being the weight of freeze dried leaves taken at different drying stages.

The water content of fully hydrated leaves was taken as 100 % relative water content.

Table 3.12.The relative water contents (RWC, %) of the leaves at different drying stages.

Plant	0 DAYS	2 DAYS	4 DAYS	6 DAYS	8 DAYS
M. flabellifolius	100	95	72	45	25
C. plantagineum	100	80	55	22	18
X. humilis	100	88	60	38	20
X. viscosa	100	90	64	34	16
E. nindensis	100	86	54	29	17

RWC –expressed as percentages of the fully hydrated water leaves.

3.3.1 Change in total polyphenol content with drying

Table 3.13. The total phenolic concentrations in the plant extracts at different drying times.

Plant	0 DAYS	2 DAYS	4 DAYS	6 DAYS	8 DAYS
M. flabellifolius	244.7 (1.7)	277.1 (1.0)	267.3 (0.0)	298.9 (0.1)	314.1 (2.8)
C. plantagineum	42.8 (0.8)	50.4 (1.8)	54.4 (2.8)	58.0 (1.8)	59.1 (0.9)
X. humilis	37.3 (1.4)	33.3 (0.3)	39.1 (0.8)	38.6 (0.5)	40.6 (2.8)
X. viscosa	35.9 (1.9)	43.0 (4.5)	40.4 (1.8)	43.1 (1.8)	43.2 (2.0)
E. nindensis	11.1 (0.7)	8.0 (0.6)	10.0 (0)	10.2 (2.1)	9.5 (1.8)

Total phenolics as mg GAE/ g DW

(x) is the standard deviation, n = 3.

There was a general increase in total polyphenols in *M. flabellifolius* from 244.7 mg GAE/g DW to 314.1 mg GAE/ g DW over the 8 days of drying. This represents approximately 28 % increase in phenolic concentration over the 8 days. The same trend observed in *M. flabellifolius* is observed in *C. plantagineum* where phenolics increased from 42.8 mg GAE/ g DW to 59.1 mg GAE/ g DW over the 8 days and this is a 38 % increase in the concentration of phenolics. In both *M. flabellifolius* and *C. wilmsii* there was also an observed leaf colour change to purple possibly due to anthocyanins. The increase in total phenol concentration could be due to de novo synthesis, modification or relocation of phenolics from other parts of the plant. In *X. humilis, X. viscosa* and *E. nindensis* there was generally no major change in the concentration of total phenolics over the 8 days used in the study. The resurrection plants used in this study were dried naturally by withholding water over the days of the study.

Overall there is a differential response to desiccation in the plants used in the study. In *M. flabellifolius* and *C. plantagineum* plants there appear to be a slight increase in the quantities of phenolics over time while in *E. nindensis, X. viscosa* and *X. humilis* there appear to be no change in total phenol concentration.

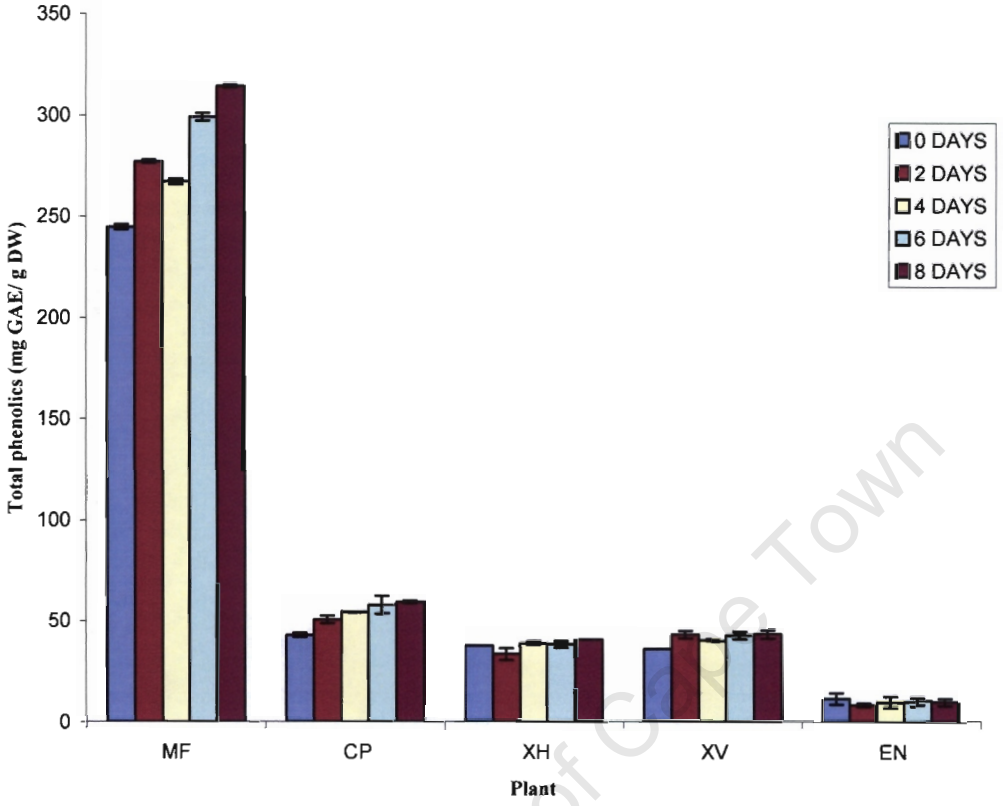


Figure 3.3. Change in phenolic content with drying in the resurrection plant extracts. Bars - standard deviation, n = 3. Abbreviations used: MF-*M. flabellifolius*, CP- *C. plantagineum*, XH-*X. humilis*, XV-*X. viscosa*, EN-*E. nindensis*.

The high amount of phenolics in hydrated *M. flabellifolius* however might mean that the antioxidant –based defence systems are constitutively expressed protection mechanisms (Kranner et al, 2002). This would mean the phenolics are present at all times and not synthesised in response to desiccation.

3.3.2 Change in Antioxidant Activity (FRAP) with drying

Since there was a strong correlation between the content of polyphenols and the ferric reducing capacity, it was imperative to see if any change in phenolic content during drying would result in a change in the ferric reducing abilities of the resurrection plant extracts.

Table 3.14. Change in FRAP (mmol Fe^{2+}/ L) of plant extracts at different drying times.

Plant	0 DAYS	2 DAYS	4 DAYS	6 DAYS	8 DAYS
M.flabellifolius	25.1 (0.8)	27.5 (0.2)	27.5 (0)	28.7 (8.3)	27.9 (1.0)
C. plantagineum	10.9 (0.4)	11.2 (0.2)	10.7 (0.2)	10.8 (0.3)	10.9 (0.5)
X. humilis	7.7 (0)	7.3 (0.1)	7.5 (0.1)	7.4 (0.3)	7.5 (0.1)
X. viscosa	8.0 (0.3)	8.2 (0.1)	8.2 (0.3)	8.5 (0.2)	8.2 (1.4)
E. nindensis	3.4 (0.1)	2.9 (0.1)	3.2 (0.9)	3.2 (0.1)	3.2 (0.5)

FRAP – mmol Fe^{2+} / L

(x) – Standard deviation, n = 3.

In *M. flabellifolius* there was a slight increase in the FRAP value for the 8 days of drying from 25.1 mmol Fe^{2+}/ L to 27.9 mmol Fe^{2+}/ L. This is an increase of about 10 % over the period of the study. The change in antioxidant capacity appear to be related with the change in phenolic quantity in this plant as there was also a general increase in polyphenol content over the same drying period. Desiccation appears to have little or no effect on the antioxidant capacity of *X. viscosa*, *X. humilis*, *C. plantagineum* and *E. nindensis*. Overall desiccation appears to have no effect on the antioxidant capacity, as measured by the FRAP assay. Kranner et al (2002) found that ∝-tocopherol, β-carotene, chlorophyll a + b and ascorbic acid decreased when *M. flabellifolius* was dried over 12 days. However, glutathione and zeaxanthin increased over the same period (Kranner et al, 2002).

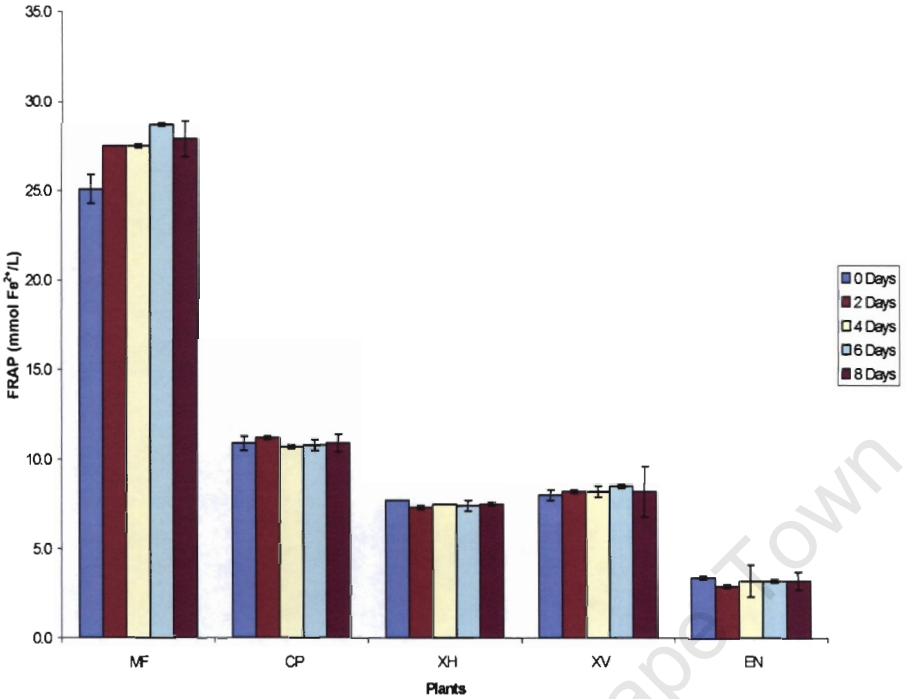


Figure 3.4. Change in FRAP of plants leaf extracts at different drying times.

Bars – standard deviation, n = 3. Abbreviations used: MF-*M. flabellifolius*, CP- *C. plantagineum*, XH-*X. humilis*, XV-*X. viscosa*, EN-*E. nindensis*.

3.3.3 Change in DPPH radical Inhibition with drying

Table 3.15. Change in percent DPPH radical inhibition with drying

Plant	0 DAYS	2 DAYS	4 DAYS	6 DAYS	8 DAYS
M. flabellifolius	94.8 (2.4)	96.3 (0.4)	95.8 (0.0)	96.9 (0.1)	98.2 (0.0)
C. plantagineum	54.3 (1.4)	65.9 (1.3)	71.8 (2.1)	71.9 (0.5)	68.9 (0.5)
X. humilis	31.7 (0.8)	35.5 (7.3)	29.9 (0.3)	30.0 (0.5)	32.6 (3.2)
X. viscosa	36.1 (0.8)	60.2 (0.9)	57.9 (0.5)	55.3 (0.7)	56.2 (1.6)
E. nindensis	24.0 (0.6)	16.9 (3.1)	13.1 (0.4)	15.2 (0.1)	14.2 (5.1)

Radical scavenging ability- % DPPH scavenging capability

(x) – Standard deviation, n = 3.

In *M. flabellifolius* there was no major change in radical scavenging ability. In *E. nindensis* there was a general decrease in the radical scavenging ability of around 40 % over the 8 days of the study. In *C. plantagineum* there was a general increment in radical scavenging ability of about 27 % over the same period whilst in *X. viscosa* there is an increase of about 55 % over the 8 days of the study. In *X. humilis* there is no change in the radical scavenging ability over the 8 days.

Desiccation appears to cause a differential response in the antioxidant capacities of the leaves of the desiccation tolerant plants.

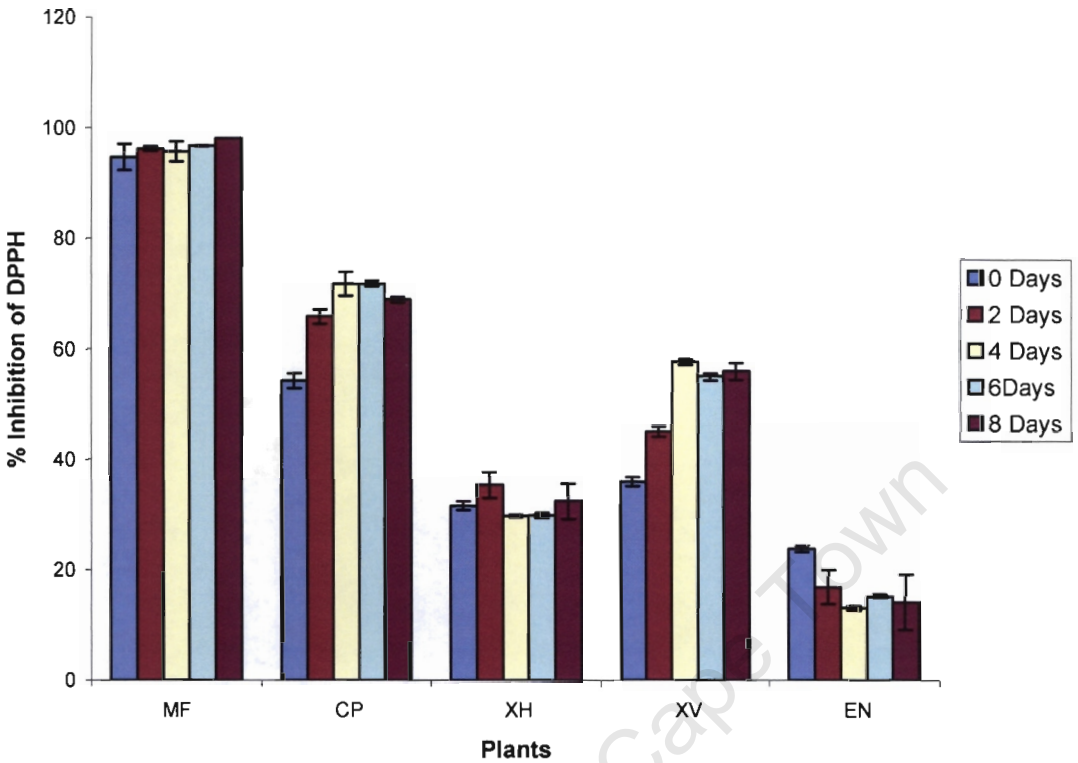


Figure 3.5. Change in percent DPPH inhibition at different drying times.

Bars – standard deviation, n = 3. Abbreviations used: MF-*M. flabellifolius*, CP- *C. plantagineum*, XH-*X. humilis*, XV-*X. viscosa*, EN-*E. nindensis*.

3.3.4 Change in β-Carotene bleaching Inhibition with drying

Table 3.16. Change in percent inhibition of β-carotene bleaching at different drying times

Plant	0 DAYS	2 DAYS	4 DAYS	6 DAYS	8 DAYS
M. flabellifolius	93.6 (3.4)	94.5 (1.5)	96.8 (2.5)	98.1 (1.6)	97.8 (2.4)
C. plantagineum	48.2 (1.1)	50.0 (2.5)	57.3 (1.8)	55.4 (0.9)	59.0 (1.7)
X. humilis	33.8 (1.0)	34.2 (0.8)	37.1 (2.1)	35.3 (1.5)	38.4 (2.9)
X. viscosa	48.5 (2.9)	55.8 (3.2)	62.7 (1.4)	60.2 (3.0)	57.3 (2.5)
E. nindensis	24.6 (1.7)	23.9 (2.3)	20.7 (1.1)	19.0 (0.5)	16.8 (1.3)

Percent Inhibition of β-carotene bleaching

(x) – Standard deviation, n = 3.

The resurrection plants gave different responses to desiccation in terms of inhibition of β-carotene bleaching. There was generally no change in the percent inhibition of β-carotene bleaching in *M. flabellifolius*. In *C. plantagineum* and *X. humilis* there were increases in the percent inhibition of β-carotene bleaching. In *X. viscosa* there was an overall increase in percent inhibition of β-carotene bleaching of around 18 %. In *E. nindensis* extracts there was a general decrease (31 %) over the drying period.

The slight differences observed in the three methods used in this study might be due to differences in the types of phenolics present in the plants. The differences could also be due to changes in the nature of phenolics present in the plants as the plants dry.

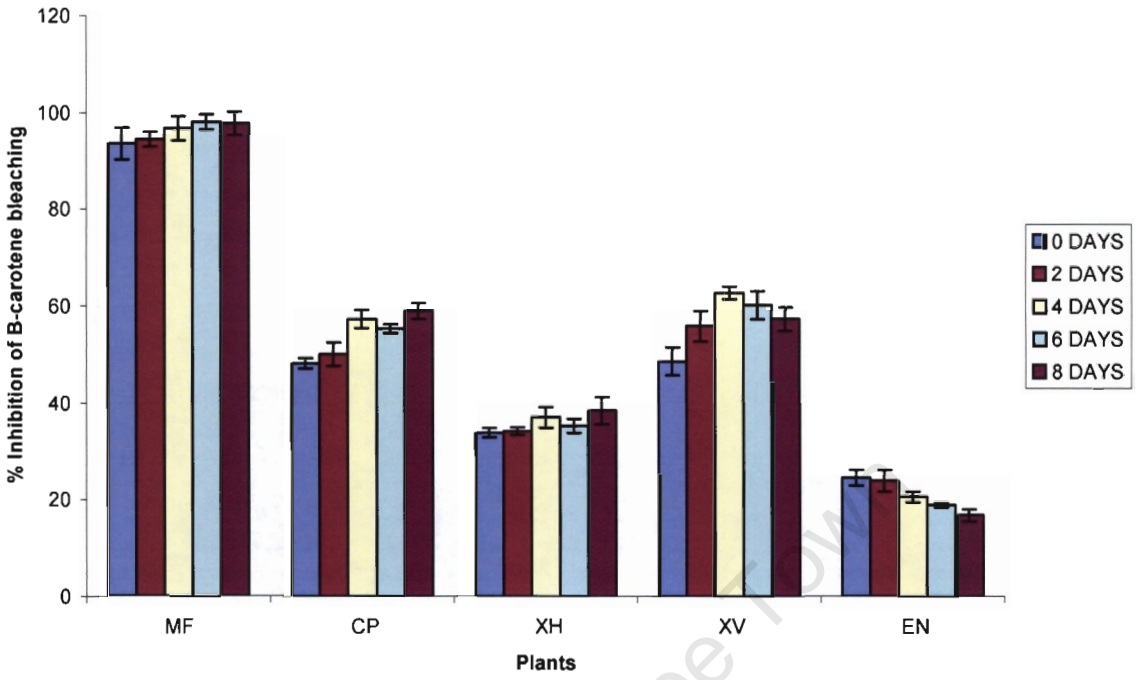


Figure 3.6. Change in percent inhibition of β-carotene bleaching at different drying times. Bars – standard deviation, n = 3. Abbreviations used: MF-*M. flabellifolius*, CP- *C. plantagineum*, XH-*X. humilis*, XV-*X. viscosa*, EN-*E. nindensis*.

3.3.5 CONCLUSION

At least one plant from each family was chosen for the study. Desiccation produced differential responses in the amount of polyphenols and the antioxidant capacities of the leaves of the desiccation tolerant plants. From the results it appears that polyphenols are not directly involved in desiccation tolerance and the antioxidant activities of the polyphenols are not the main mechanism by which desiccation tolerant plants survive desiccation.

The differences shown by the three methods used to determine the antioxidant capacities could be a result of the desiccation tolerant plants having different polyphenols. It should be noted that antioxidant activity in plants is not limited to polyphenols and therefore polyphenols could actually be part of an array of antioxidants present in plants.

3.4 Identification of polyphenols in leaves of the desiccation tolerant plants

The leaf extracts from the nine desiccation tolerant plants were subjected to a fairly detailed polyphenol analysis using HPLC, diode array and mass spectrometry to specifically identify the compounds present. Rouseff et al (1990) indicated that chromatographic gradient systems composed of acetonitrile or formic acid and water in a C-18 column sharpen peak shapes and improve analytical sensitivity and resolution for the HPLC analysis of polyphenolic compounds. Therefore formic acid and water were used as the mobile phases in this study. According to UV spectra obtained with diode array detection, the maximum absorption of hydroxycinnamic acids occur around 325 nm and those of hydroxybenzoic acids near 280 nm. The optimal conditions used for the HPLC-DAD ESI MS analysis were determined as described in section 2.7.1.2 and Appendix 7.3.1. The molecular ions of individual polyphenols and their UV-Vis spectra are valuable confirmatory data when compared with the authentic standards or reference data.

Electro-spray ionisation (ESI) is one of the most often used soft ionisation techniques in mass spectrometry. ESI eliminates the need for neutral molecule volatilisation prior to ionisation. In the negative mode, ESI spectra of the phenolics show a predominant molecular ion $[M-H]^-$ with minimum fragmentation. It is known that the negative ion mode generally provides improved detection limits (Robards et al, 2003). Standards solutions of several phenolic compounds were analysed (see Appendix C section 7.3.1). The peaks of the polyphenols were identified by:

- The retention time obtained for the extract in comparison with standards.
- The UV-Vis spectrum of the eluted solute
- The ESI-MS data of the compounds/ peaks.

A tentative identification of the polyphenols in the desiccation tolerant plant leaf extracts is done based on the three criteria above. According to our knowledge this is the first time these polyphenol compounds are identified in these desiccation tolerant plants, except the compound 3,4,5 tri-*O*-galloylquinic acid (Moore et al, 2005).

HPLC-DAD ESI-MS RESULTS

3.4.1 *Myrothamnus flabellifolius*

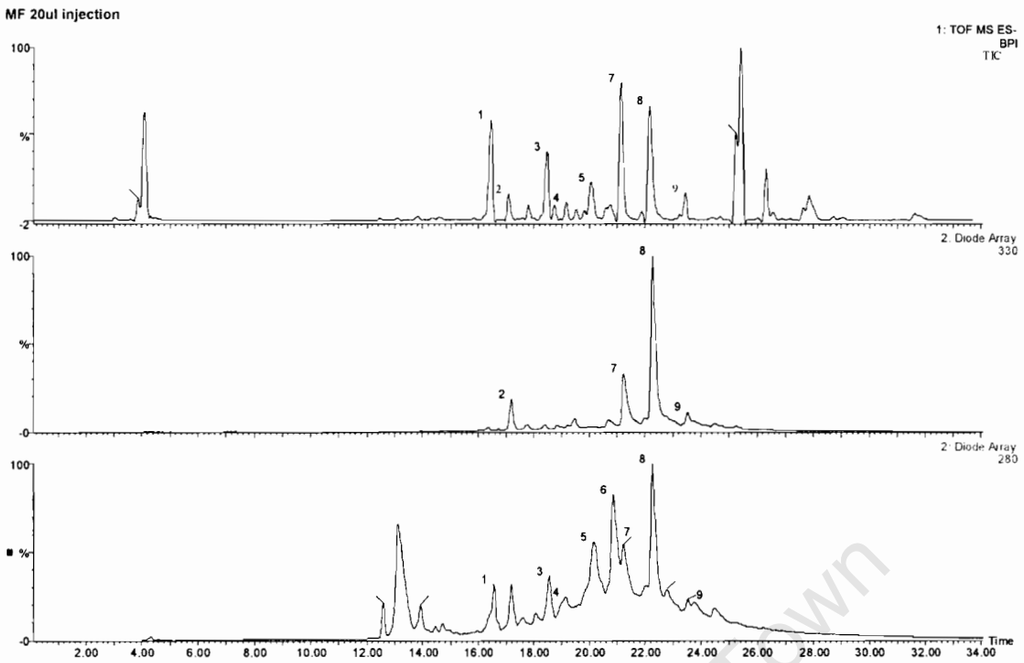


Figure 3.7. HPLC elution profiles of acetone extracts from hydrated *Myrothamnus flabellifolius* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

The major polyphenols in *Myrothamnus flabellifolius* were identified as:

Peak 3: m/z 575 -was identified as a procyanidin A-type dimer. This is confirmed by the λmax at 277nm. This ion would give other ions like 423 as seen in peak 1. (Le Roux et al, 1997; Soong and Barlow, 2005).

Peak 5: m/z 787 -was tentatively identified as 1,2,3,6-tetra-*O*-galloyl-β-D-glucose. Again the UV absorbance confirms the presence of gallic acid residues.

Peak 6: m/z 615, 482, 481 -It is a identified as a mixture of 1,6-di-*O*-galloylglucose, 1,2,6-tri-*O*-galloyl-glucose and 2,3-∝-hexahydroxydiphenyl (HHDP) (α/β)-glucose. (Owen et al, 2003; Salminen et al, 1999; Hussein et al, 2003).

Peak 7: m/z 955,477 - was tentatively identified as a Quercetin glucuronide. UV absorbance is confirmed at 264 nm and 354 nm. $[2M-H]^-$ = 955; $[M-H]^-$ = 477. (Seeram et al, 2005).

Peak 8: m/z 923,462,461- was identified as a Kaempferol glucuronide $[M-H]^-$ =461; $[2M-H]^-$ = 923; $[2M-CH_2]^-$ = 909; [M] neutral ion = 462.

Table 3.17. Analysis of the HPLC and MS chromatograms for *Myrothamnus flabellifolius* extract

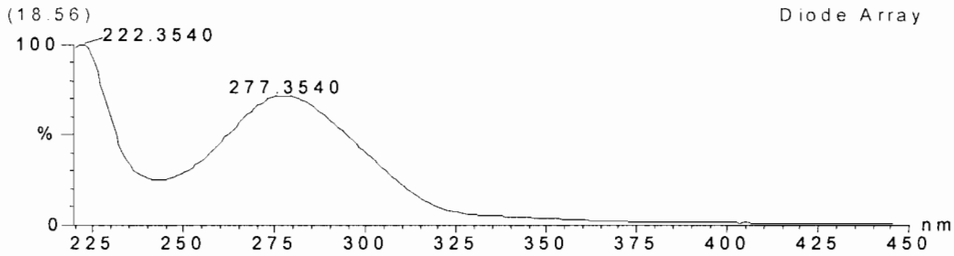
Peak	R.T(min)	$[M-H]^-$ (m/z)	Fragment ion (m/z) at -35 V	λ_{max} (nm)	Tentative ID (Theoretical Molecular weight)
1	16.56	575	423	277	Procyanidin type-A dimer (576)
2	17.20	447	423, 391, 337	310	Flavone glycoside-several (448)
3	18.56	575	525, 479	277	Procyanidin type –A (576)
4	19.15	786	729, 537, 431	276	1,2,3,6 tetra-*O*-galloyl-β-D-glucose(788) ?
5	**20.16**	**787**	**349**	**278**	**1,2,3,6 tetra-*O*-galloyl- β -D-glucose(788)**
6	**20.86**	**615**	**482, 481**	**278**	**1,2,6-tri-*O*-galloylglucose**
(Mixture)		**2,3-α-hexahydroxydiphenyl (α/β)-glucose (482) + 1,6-di-*O*-galloylglucose**			
7	21.23	477	633,955	264, 354	Quercetin glucuronide (478)
8	**22.26**	**461**	**909, 462,**	**265, 347**	**Kaempferol glucuronide (462)**
9	22.78	811	431, 183	265	Possibly Ellagic acid pentose Conjugate

NB- major peaks are shown in bold.

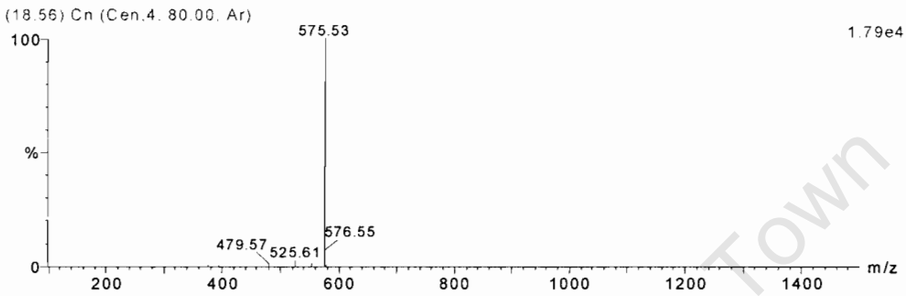
The polyphenols in *M. flabellifolius* occur mainly as tannins. The major polyphenols identified in *M. flabellifolius* leaves are 1,6-di-*O*-galloylglucose, 1,2,6 tri-*O*-galloylglucose, 1,2,3,6 tetra-*O*-galloylglucose and kaempferol glucuronide. In the system used, additional galloyl groups appear to make the molecules more polar probably due the increase in the number of hydroxyl groups present (Santos-Buelga et al, 2003). This is the first time these compounds have been identified in this plant and no studies have been done so far to see if they could possibly be involved in desiccation tolerance. Condensed tannins are present as shown by the presents of procyanidin type –A dimers. Condensed tannins are found mainly in woody plants of which *M. flabellifolius* is one. Non tannin polyphenols are present in the form of quercetin glucuronide, kaempferol glucuronide

and flavone glycosides. These results agree with those obtained by the Folin Ciocalteau/PVPP method, which show the predominance of tannins in *M. flabellifolius.* The UV spectrum and the MS spectrum of some of the peaks are shown below.

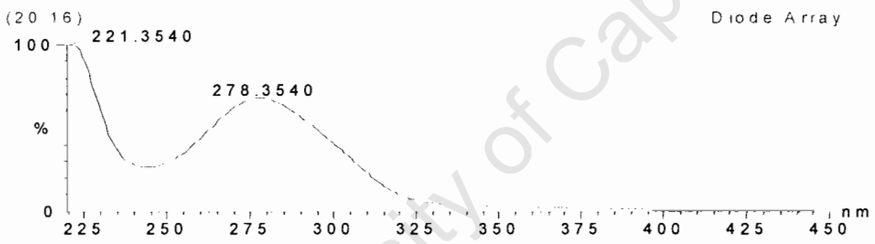
Peak 3 UV Spectrum



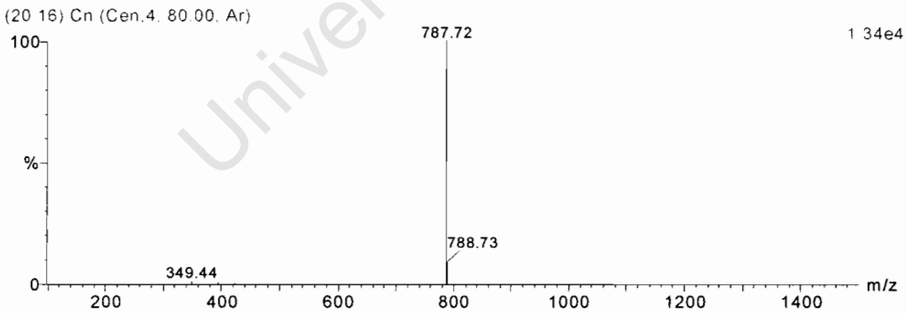
Peak 3 ESI MS spectrum



Peak 5 UV Spectrum



Peak 5 ESI MS spectrum



3.4.2 *Xerophyta viscosa*

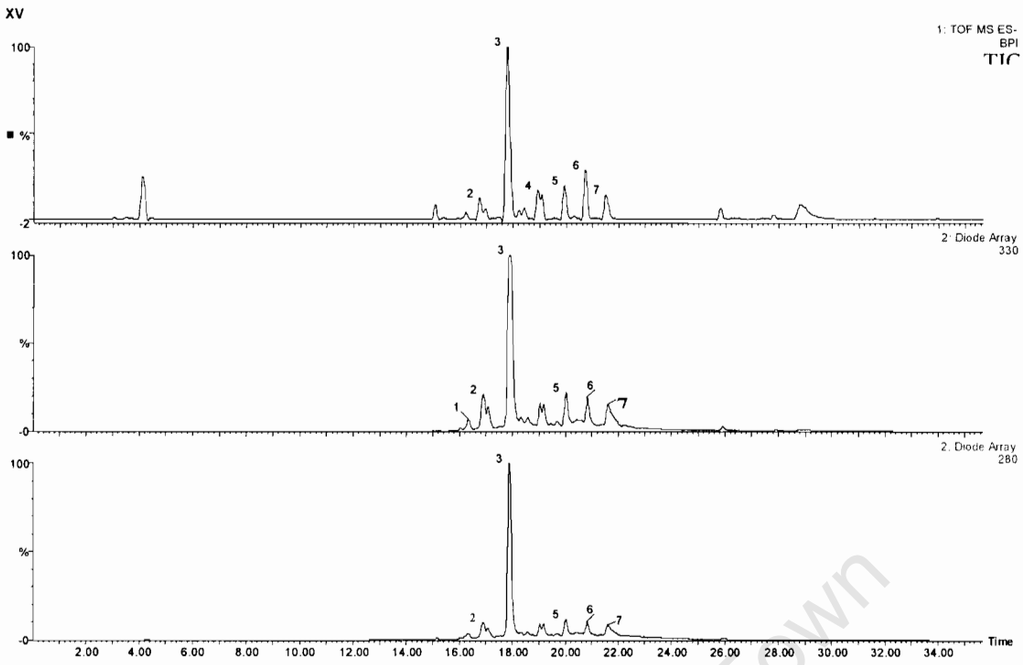


Figure 3.8. HPLC elution profiles of acetone extracts from hydrated *Xerophyta viscosa* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

The major polyphenols in *X. viscosa* were identified as:

Peak 3: m/z 707 and 353- this is the dimer and the monomer of caffeoylquinic acid. On the basis of the retention time the polyphenol has been assigned as 5-caffeoylquinic acid (Sanchez-Rabaneda et al, 2002). .

Peak 6: m/z 609 and 1220- this is identified as rutin (Seeram et al, 2005; La Torre et al, 2005).

Peak 7: m/z 787- this was identified as 1,2,3,6 tetra-*O*-galloylglucose.(Salminen et al,1999;Owen et al, 2003).

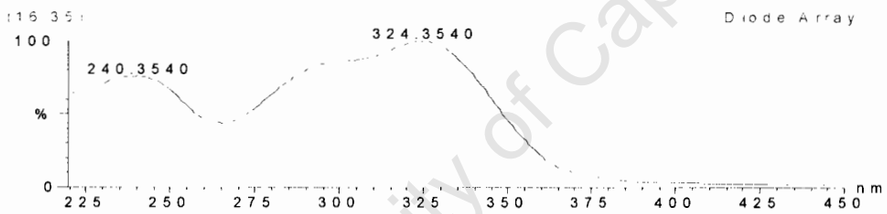
Table 3.18. Analysis of the HPLC and MS chromatograms of *Xerophyta viscosa* extract.

Peak	R.T(min)	$[M-H]^-$ (m/z)	Fragment ion (m/z) at 35 eV	λ_{max} (nm)	Tentative ID (Theoretical molecular weight)
1	16.34	353	-	240, 324	3-caffeoylquinic acid(354)
2	16.91	501	-	241, 326	Flavone glycoside, Hydroxycinnamic acid
3	**17.89**	**707**	**353**	**241, 326**	**Dimer of 5-caffeoylquinic acid(708).**
4	19.25	651	-	254, 342	Not known
5	20.02	625	933, 1252	254, 347	Not known
6	20.82	609	1220	254, 348	Rutin and Dimer (610/1220)
7	21.61	787	-	252, 330	tetra-*O*-galloyl-glucose (788)

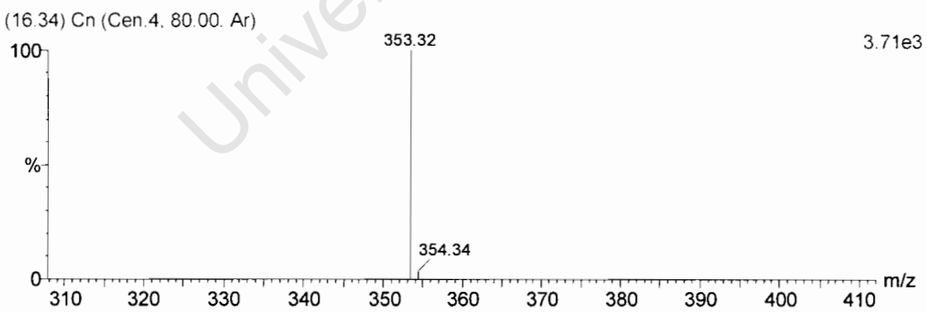
NB –major peaks shown in bold.

The major phenolic compound in *X. viscosa* is the dimer of 5-caffeoylquinic acid (m/z 707, 353). This has been proven correct by comparing the ESI MS data of the standard and the major peak. Most polyphenols are non-tannin in agreement with the results of the Folin-Ciocalteau/PVPP assay, which showed less tannin in the leaves of this plant. Peak 7 however shows the presence of tannins though in small amounts.

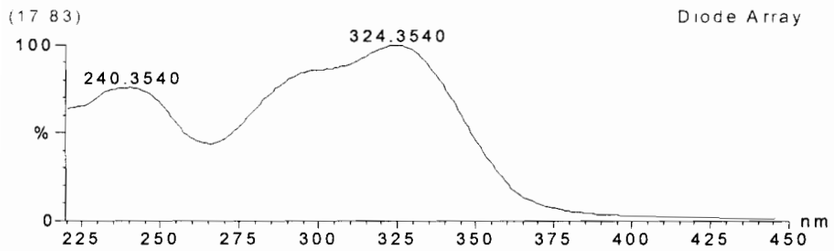
Peak 1 UV Spectrum



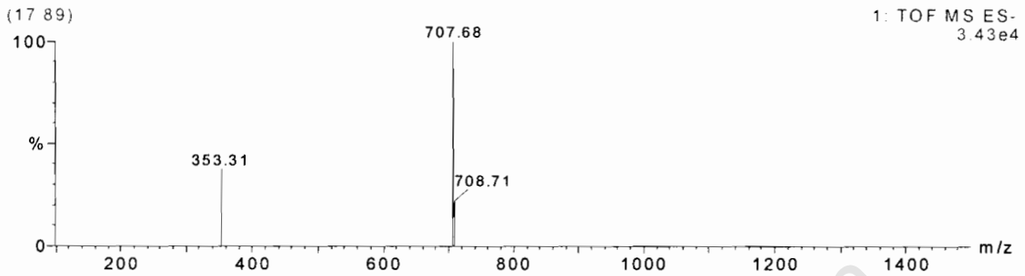
Peak 1 MS spectrum



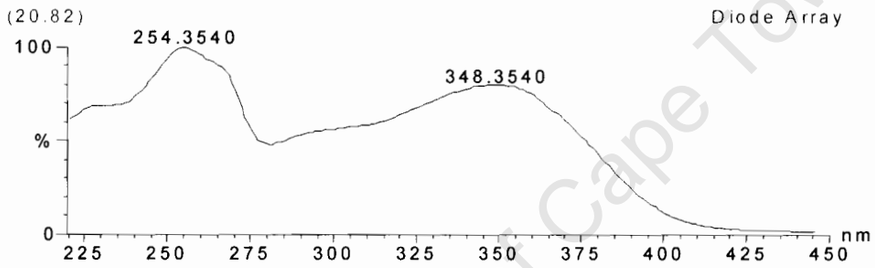
Peak 3 Spectrum



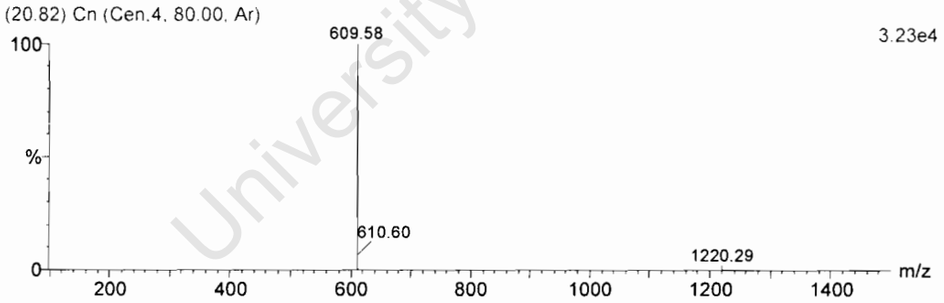
Peak 3 MS spectrum



Peak 6 Spectrum



Peak 6 MS spectrum



3.4.3 *Xerophyta humilis*

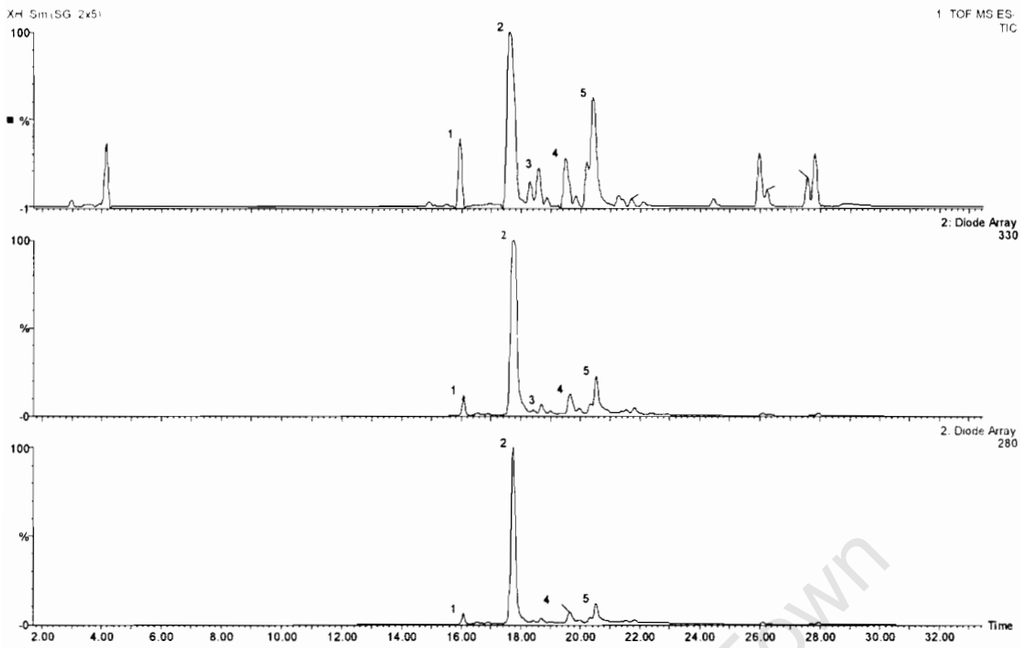


Figure 3.9. HPLC elution profiles of acetone extracts from hydrated *Xerophyta humilis* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Some of the major polyphenols in *X. humilis* leaves were identified as:

Peak 1: m/z 353. This was tentatively identified as an isomer of 3-caffeoylquinic acid. The UV spectrum also confirms this. (Sanchez-Rabaneda et al, 2002; Clifford, 2003).

Peak 2: m/z 191 and 353. This is 5-caffeoylquinic acid. Showed the deprotonated molecule [M-H]- (m/z 353) and the m/z corresponding to the deprotonated quinic acid (m/z 191). The presence of 5-caffeoylquinic acid was also confirmed by the UV spectrum (Sanchez-Rabaneda et al, 2002; Clifford, 2003).

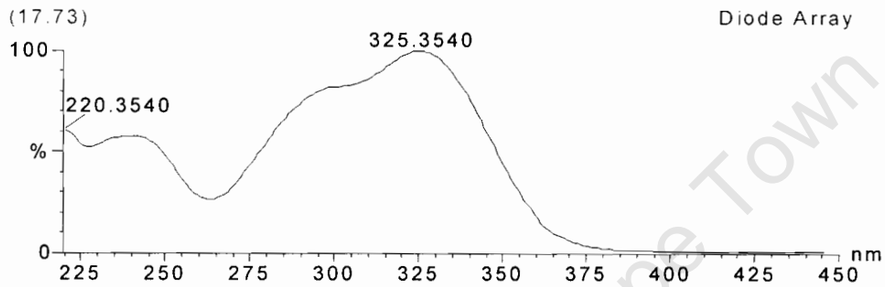
Peak 5: m/z 787. This was identified as 1,2,3,6 tetra-*O*-galloylglucose.(Salminen et al,1999;Owen et al, 2003).

Table 3.19. Analysis of the HPLC and MS chromatograms for *Xerophyta humilis* extract

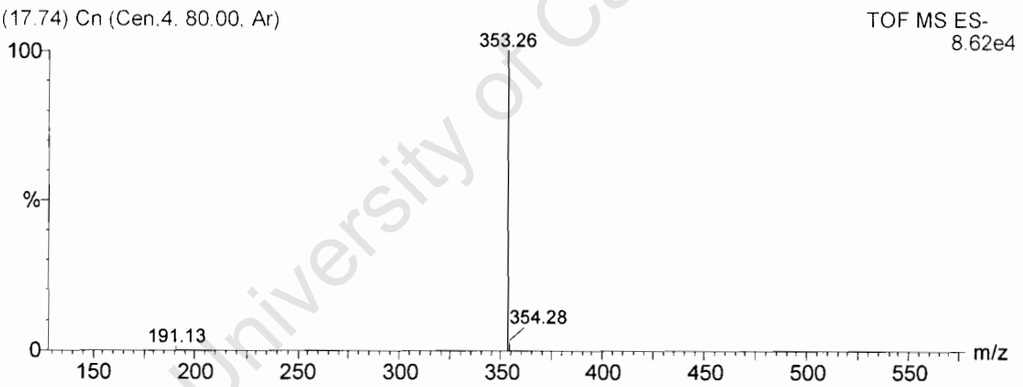
Peak	R.T(min)	$[M-H]^-$ (m/z)	Fragment ion (m/z) at 35 eV	λ_{max} (nm)	Tentative ID (Theoretical molecular weight)
1	16.07	353	-	240, 325	3-caffeoylquinic acid (354).
2	**17.74**	**353**	**191**	**326**	**5-caffeoylquinic acid (354).**
3	18.69	757	353	350	Tetramer of ferulic acid (758).
4	19.65	1252	919, 625	348	Not known.
5	20.54	787	-	278	Tetra-*O*-galloylglucose (788).

NB- major peaks shown in bold.

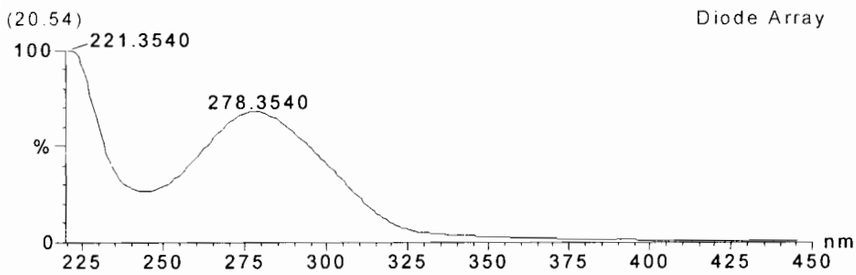
Peak 2 UV Spectrum



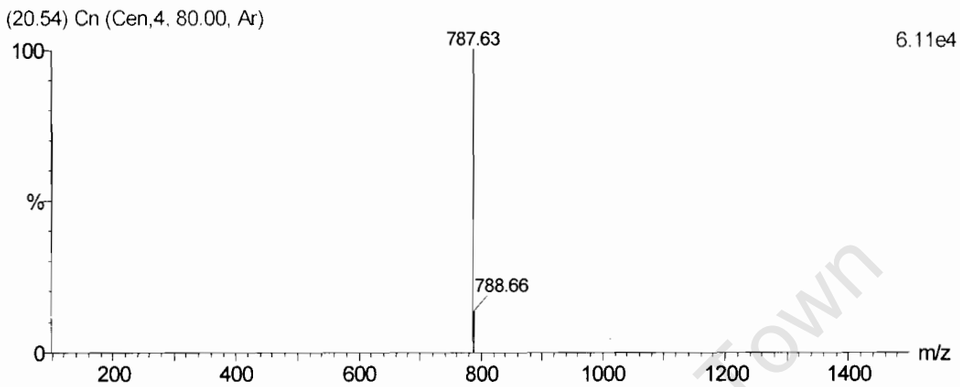
Peak 2 MS spectrum



Peak 5 UV Spectrum



Peak 5 ESI MS spectrum



The major polyphenolic compound in *X. humilis* was tentatively identified as 5-caffeoylquinic acid. There was also another compound identified as 3-caffeoylquinic acid (peak 1). This compound is also identified in *X. viscosa*. Most polyphenols are non-tannin in agreement with the results of the Folin-Ciocalteau assay, which showed less tannin in the extract. Rutin, which was detected *in X. viscosa* leaves, is not detected in this plant. Most of the polyphenols appear to be the same in both *X. viscosa* and *X. humilis*.

3.4.4 *Xerophyta schlecterii*

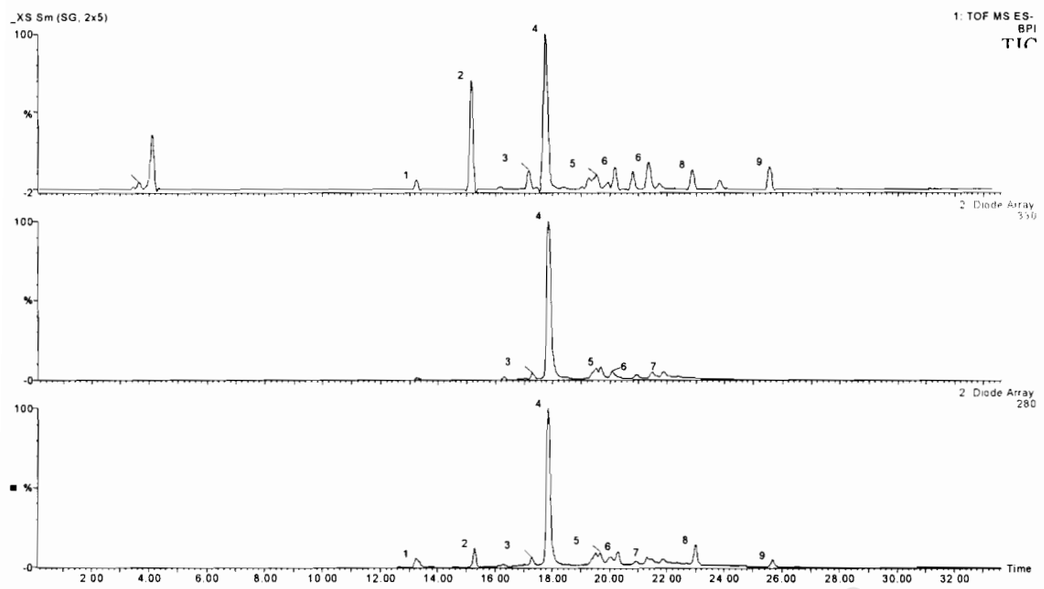


Figure 3.10. HPLC elution profiles of acetone extracts from hydrated *Xerophyta schlecterii* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Some of the compounds in *X. schlecterii* were identified as:

Peak 2: m/z 631, 315. It was tentatively identified as a galloyl-hexahydroxydiphenyl (HHDP)- glucose moiety and this is confirmed by the UV spectrum. (Salminen et al, 1999).

Peak 4: m/z 353, 707. Tentatively assigned to the dimer of 5-caffeoylquinic acid. Even the UV spectrum confirms this. (Sanchez-Rabaneda et al, 2002; Clifford, 2003).

Peak 7: m/z 463, 927. Tentatively identified as myricetin rhamnoside and the dimer. $[M-H]^-$ = 463, $[2M-H]^-$ = 927.(Owen et al, 2003).

Peak 8: m/z 579, 813. Tentatively identified as catechin dimer, confirmed by the UV spectrum

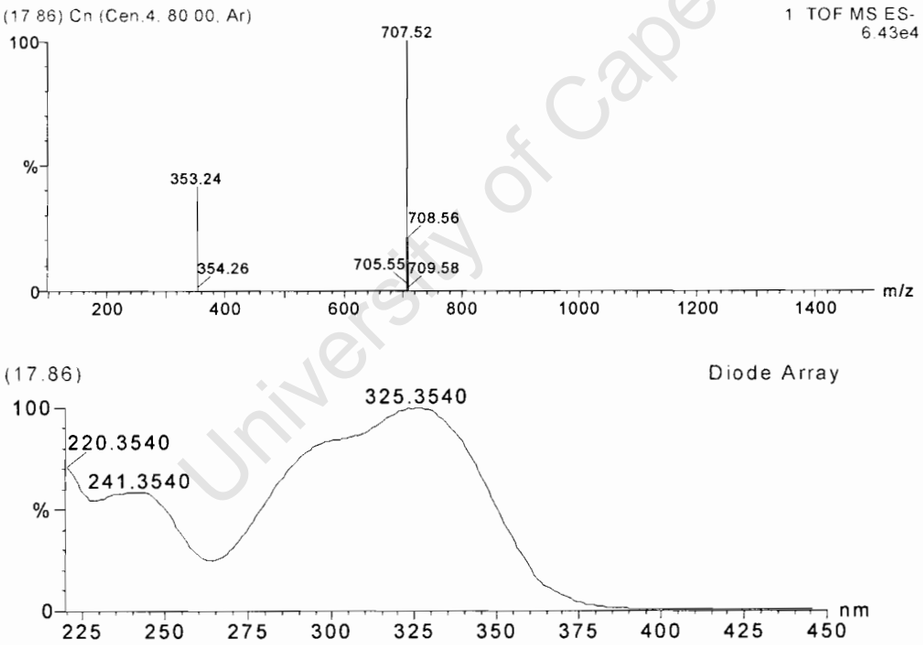
Table 3.20. Analysis of the HPLC and MS chromatograms for *Xerophyta schlecterii* extract.

Peak	R.T(min)	$[M-H]^-$ (m/z)	Fragment ion (m/z) at 35 eV	λ_{max} (nm)	Tentative ID (theoretical molecular weight)
1	13.24	345	691, 692	299	Unknown
2	15.29	631	315	279	galloyl-HHDP-glucose(632)
3	17.29	829	523, 507, 477	280-345	Isorhamnetin glucoside.
4	**17.86**	**707**	**353**	**325**	**Dimer of 5-caffeoylquinic acid(708)**
5	19.68	625	529, 433	255.340	Ellagic acid conjugate (626).
6	20.29	641	449	293	Unknown.
7	21.33	927	463	352	Dimer of myricetin rhamnoside (928)
8	23.01	579	813	278	Epicatechin dimer (579).
9	25.68	621	-	277	Unknown

NB – major peaks shown in bold.

The major polyphenol identified is 5-caffeoylquinic acid. This is the same as in *X. viscosa* and *X. humilis*. Tannins are also present as shown by the detection of galloyl-HHDP-glucoses.

Peak 4 ESI MS and UV spectra.



3.4.5 *Xerophyta villosa*

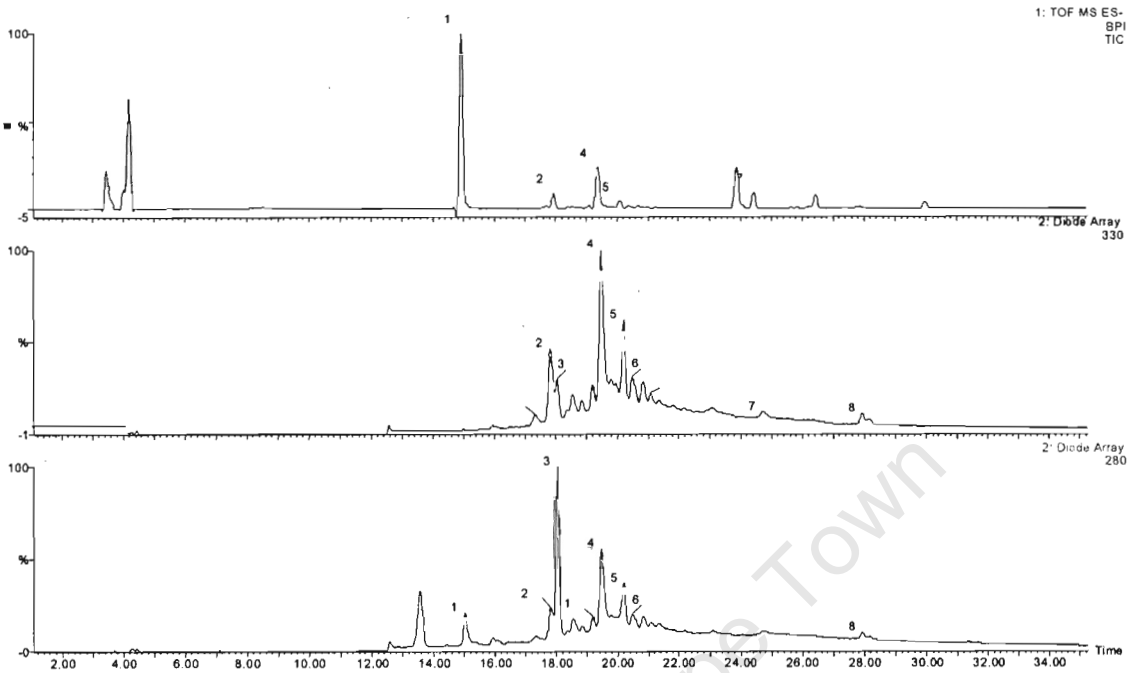


Figure 3.11. HPLC elution profiles of acetone extracts from hydrated *Xerophyta villosa* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

The compounds in *X. villosa* were identified as:

Peak 2: m/z 191, 353, 707. Tentatively identified as 5-caffeoylquinic acid and its dimer. Well supported by the UV spectrum. The compound gives the deprotonated quinic acid ion (m/z 191) (Sanchez-Rabaneda et al, 2002; Clifford, 2003).

Peak 3: m/z 287, 387, 741, 775, 776. Identified as a dimer of ferulic acid and the tetramer. $[2M-H]^-$ = 387, $[4M-H]^-$ = 775, $[4M]^-$ = 776. (Owen et al, 2003)

Peak 4: m/z 563, 755 Tentatively identified as a trimer of ferulic acid. $[3M-H_2O]^-$ = 563, $[4M-H_2O]^-$ = 755.

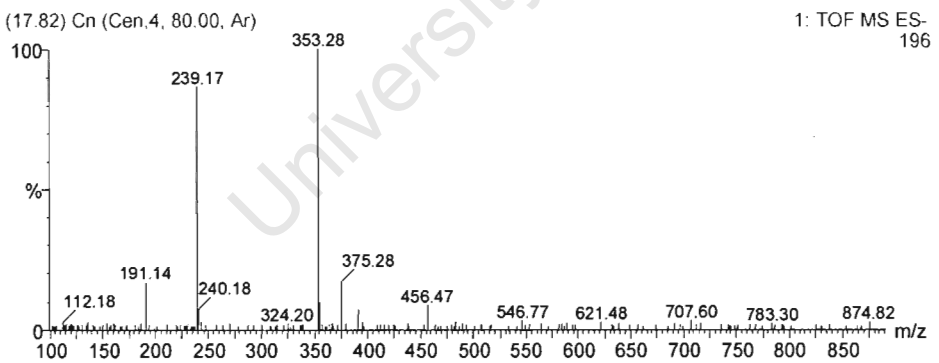
The major polyphenols in *X. villosa* appear to be ferulic acid and its polymers. 5-caffeoylquinic acid (peak 2) is also present but in small amounts based on the peaks. The results obtained for *X. villosa* have been different from all the *Xerophyta* species. Most polyphenols detected are non-tannin agreeing with the results of the Folin-Ciocalteau/PVPP assay, which showed no tannin in the extract.

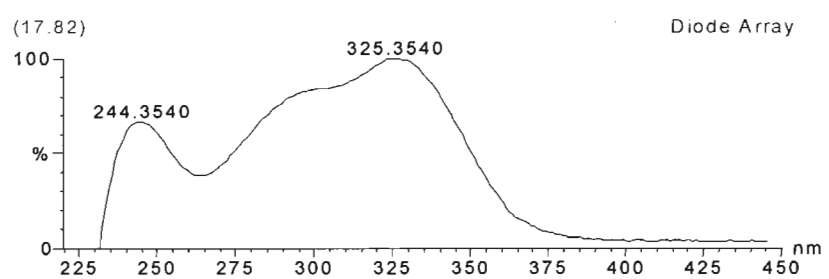
Table 3.21. Analysis of the HPLC and MS chromatograms for *Xerophyta villosa* extract.

Peak	R.T(min)	$[M-H]^-$ (m/z)	Fragment ion (m/z) at 35 eV	λ_{max} (nm)	Tentative ID (Theoretical molecular weight)
1	15.03	255	-	225,274	Unknown.
2	17.82	353	239, 191/707	244, 325	5-caffeoylquinic acid (354) Plus dimer (708)
3	**18.05**	**387**	**776, 741, 755, 287**	**234, 283**	**Dimer of ferulic acid (388) Tetramer (777) [Tetramer-H_20-H]$^-$=755**
4	**19.48**	**563**	**755**	**238, 271, 332**	**Trimer of ferulic acid [Trimer-H_20]$^-$=563 [Tetramer–H_20]$^-$=756-1=755**
5	20.22	709	623, 415, 253	271, 332	Acteoside 1 or 2 (624)?
6	20.48	563	533, 417, 198	270, 332	Unknown
7	24.72	1108	1109	265	Unknown
8	27.93	413	283, 172	243	Unknown

NB – major peaks shown in bold.

Peak 2 ESI MS and UV spectra.





3.4.6 *Craterostigma wilmsii*

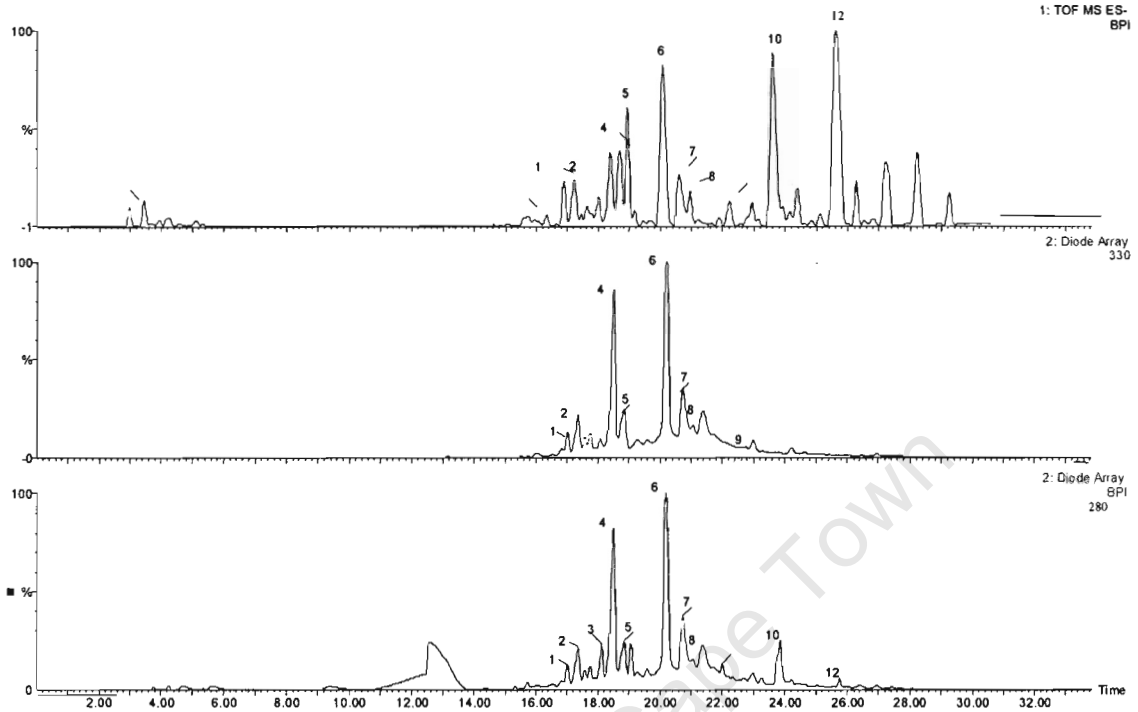


Figure 3.12. HPLC elution profiles of acetone extracts from hydrated *Craterostigma wilmsii* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Some of the polyphenols in *Craterostigma wilmsii* were identified as:

Peak 3: m/z 281, 341, 385, 635, 771. Tentatively identified as a tri-*O*-galloylglucose.

Peak 4: m/z 443, 785, 1036. Tentatively identified as digalloyl-HHDP-glucose (Salminen et al, 1999).

Peak 6: m/z 623, 1248. Tentatively identified as Acteoside 1 or 2 dimer. There are two possibilities of compounds giving the $[M-H]^-$ with m/z 623, the major peak in this plant. (1) Firstly acteoside 1or 2, contains a caffeoyl, rhamnosyl and glucosyl moieties; will give $[M-H]^-$ m/z 623, UV absorption is at 284 nm and 326 nm; Loss of deoxyhexosyl residue (146 amu) will give a fragment of m/z 477.(2) The other possibility is isorhamnetin 3-*O*-rhamnoglucoside, contains rhamnosyl and glucosyl moieties and will also give $[M-H]^-$ with m/z 623, with UV absorption at 256 nm and 356nm; Loss of

deoxyhexosyl residue (146 amu) will also give a fragment of m/z 477. The peak observed was found to absorb maximally at 254 nm and 326 nm. This indicates the presence of a caffeoyl moiety (326 nm) and therefore the polyphenol was identified as acteoside 1 or 2 (Owen et al, 2003). NMR studies would be necessary to know the isomer present.

Peak 7: m/z 593, 1188. Could not distinguish between Kaempferol 3-*O*-rutinoside and Kaempferol 7-*O*-neohesperidoside. The corresponding dimer is also there (Sanchez-Rabaneda et al, 2002).

Peak 8: m/z 623. Tentatively identified as Acteoside 1 or 2. (Owen et al, 2003).

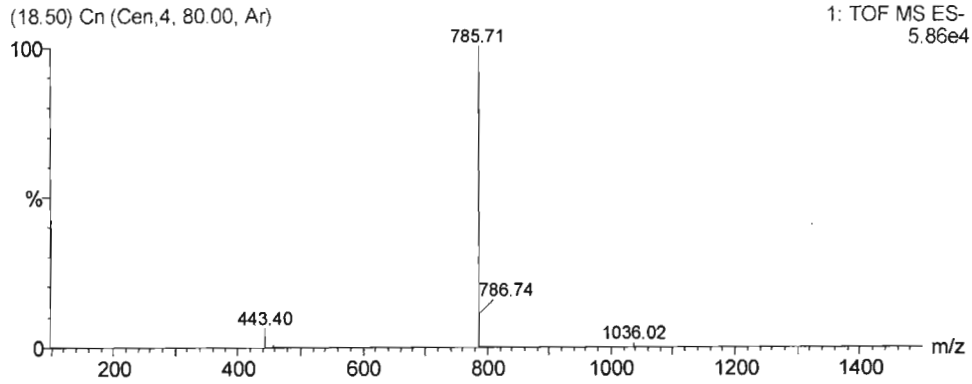
Table 3.22. Analysis of the HPLC and MS chromatograms for *Craterostigma wilmsii* extract.

Peak	R.T(min)	$[M-H]^-$ (m/z)	Fragment ion (m/z) at 35 eV	λ_{max} (nm)	Tentative ID (Theoretical molecular weight)
1	17.02	475	951	242 , 276	Unknown
2	17.35	801	387, 341	254, 265	Unknown
3	18.12	771	635, 385, 341	268	tri-*O*-galloyl-glucose (772).
4	**18.50**	**785**	**1036, 443**	**279**	**Digalloyl-HHDP-glucose (787).**
5	18.85	743	371	---	Unknown.
6	**20.20**	**623**	**1248**	**222, 330**	**Acteoside 1 or 2 (624)**
7	20.73	593	1188	---	Kaempferol 3-*O*-rutinoside (594) Kaempferol 7-*O*-neohesperidoside (594)
8	21.40	623	-	222, 330	Acteoside1 or 2 (624)
9	22.98	709	651	---	Unknown.
10	23.85	897	707	---	Quercetin rhamnoside(2M=898)
11	24.138	867	821/693	245,354	Quercetin arabinoside? Naringenin 7-*O*-hesperidoside?.
12	25.75	705	707/ 938	----	Unknown

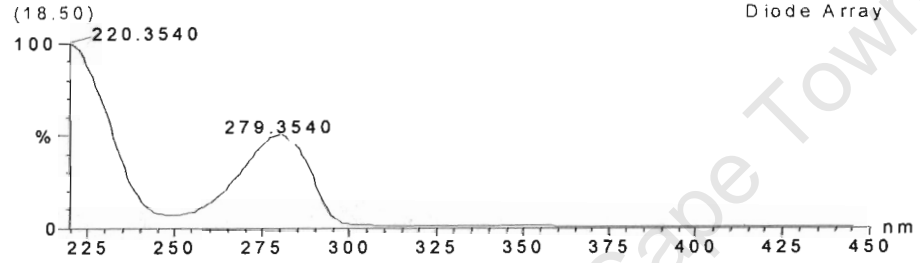
NB – major peaks shown in bold.

The major polyphenol in *C. wilmsii* was identified as acteoside 1 or 2. The *Craterostigma* species have different major polyphenols compared to the *Xerophyta* species. These polyphenols are likely to have different chemical and biological properties and therefore contribute differently to desiccation tolerance if they are involved.

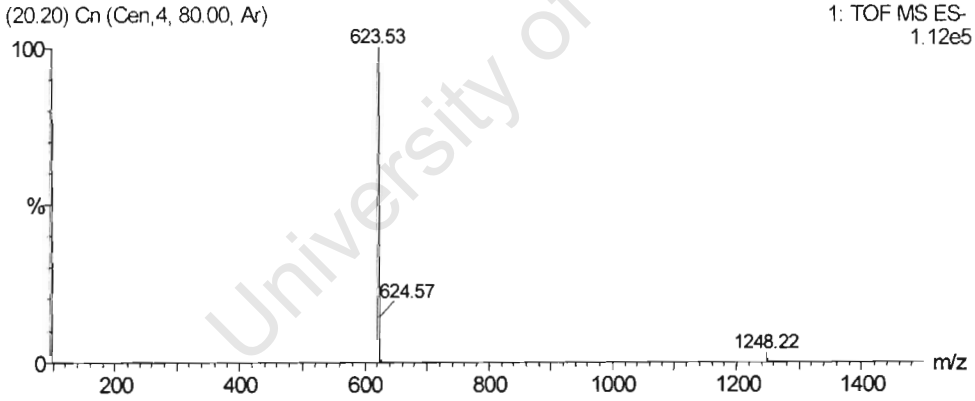
Peak 4 ESI MS spectrum



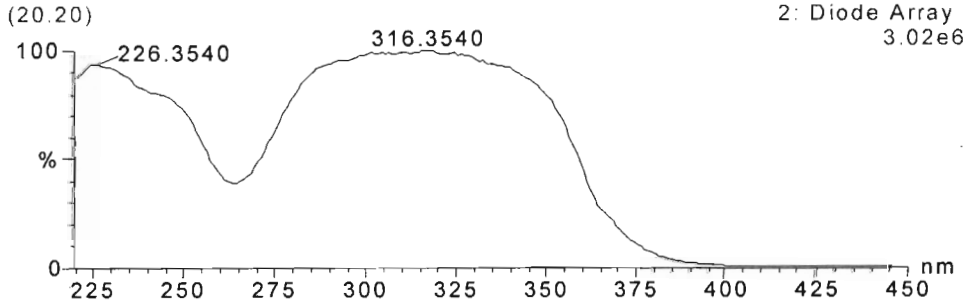
Peak 4 UV spectrum



Peak 6 ESI MS spectrum



Peak 6 spectrum



3.4.7 *Craterostigma pumilum*

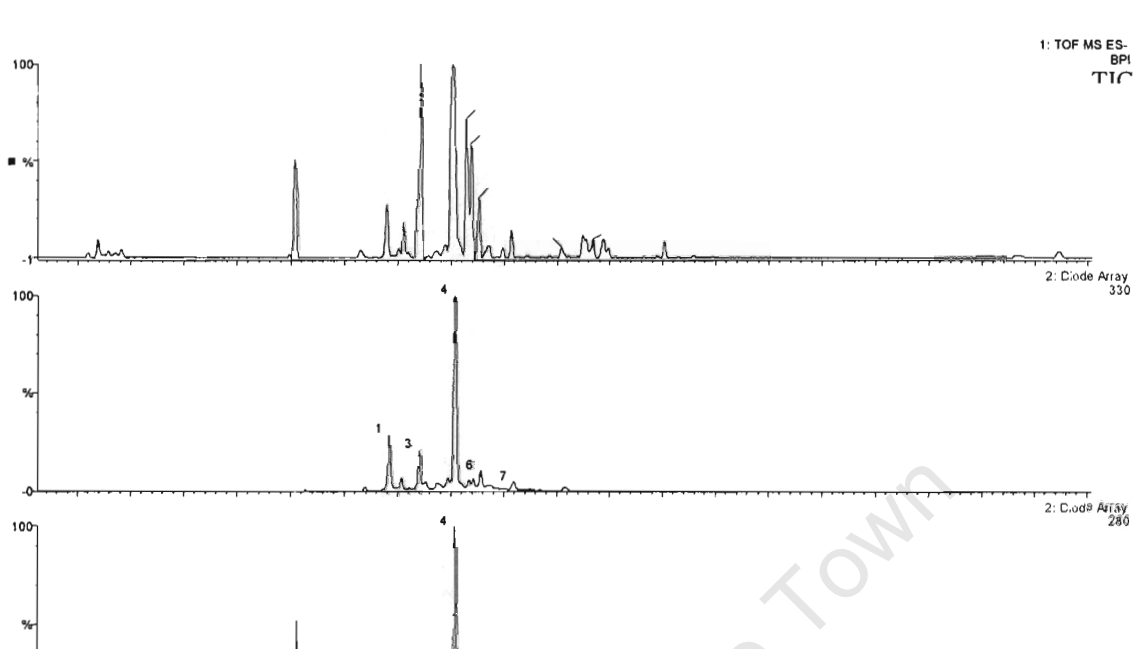


Figure 3.13. HPLC elution profiles of acetone extracts from hydrated *Craterostigma pumilum* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Some of the polyphenols in *C. pumilum* were identified as:

Peak 1: m/z 401, 803. Unknown.

Peak 2: m/z 351. Unknown.

Peak 3: m/z 443, 639,887. Unknown.

Peak 4: m/z 623, 1248. Tentatively identified as Acteoside 1 or 2. (Owen et al, 2003).

Peak 6: m/z 637, 1276. Unknown. This compound exists also as a dimer.

Peak 7: m/z 651, 679, 855. Unknown.

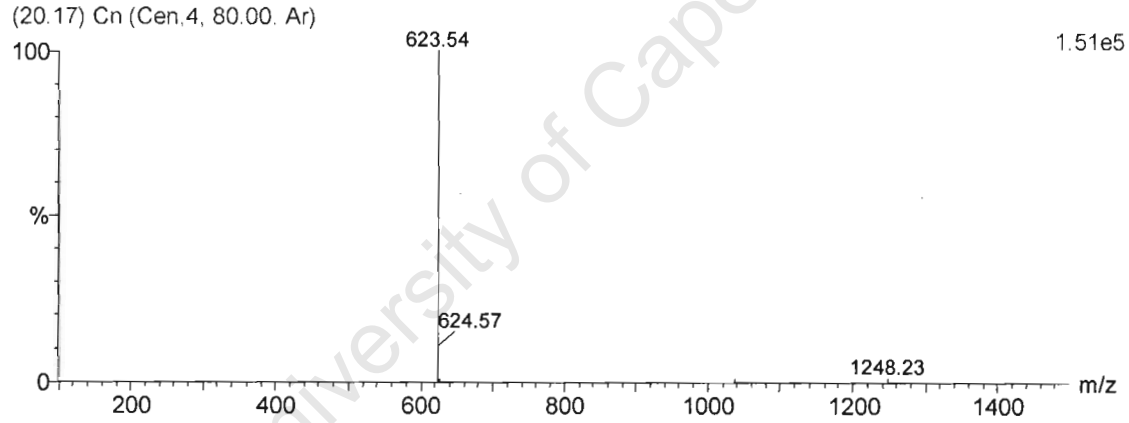
Both *C. wilmsii* and *C. pumilum* have Acteoside 1 or 2 as the major polyphenol in the leaves. However, *C. wilmsii* also contain digalloyl-HHDP- glucose as a major component.

Table 3.23. Analysis of the HPLC and MS chromatograms for *Craterostigma pumilum* extract.

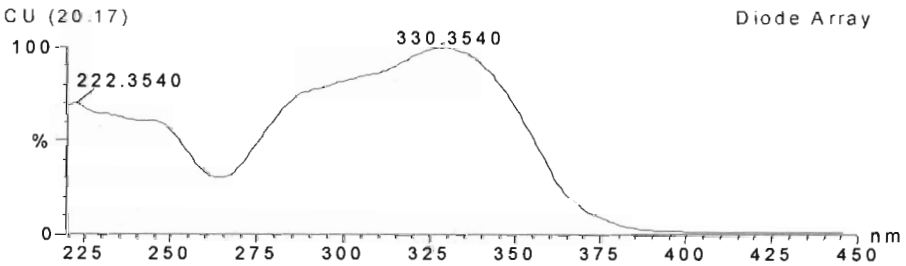
Peak	R.T(min)	$[M-H]^-$ (m/z)	Fragment ion (m/z) at 35 eV	λ_{max} (nm)	Tentative ID (Theoretical molecular weight)
1	17.09	803	311, 401	244, 325	Unknown
2	17.67	351	----	ND	Unknown
3	18.51	443	639, 887	244, 328	Unknown
4	**20.17**	**623**	**-**	**222, 330**	**Acteoside 1or 2 (624)**
5	21.06	623	-	ND	Acteoside 1 or 2 (624)?
6	21.41	637	1276	250, 328	Unknown
7	22.97	651	679, 855	247, 329	Unknown

NB – major peaks shown in bold.

Peak 4 ESI MS spectra



Peak 4 spectrum



3.4.8 *Craterostigma plantagineum*

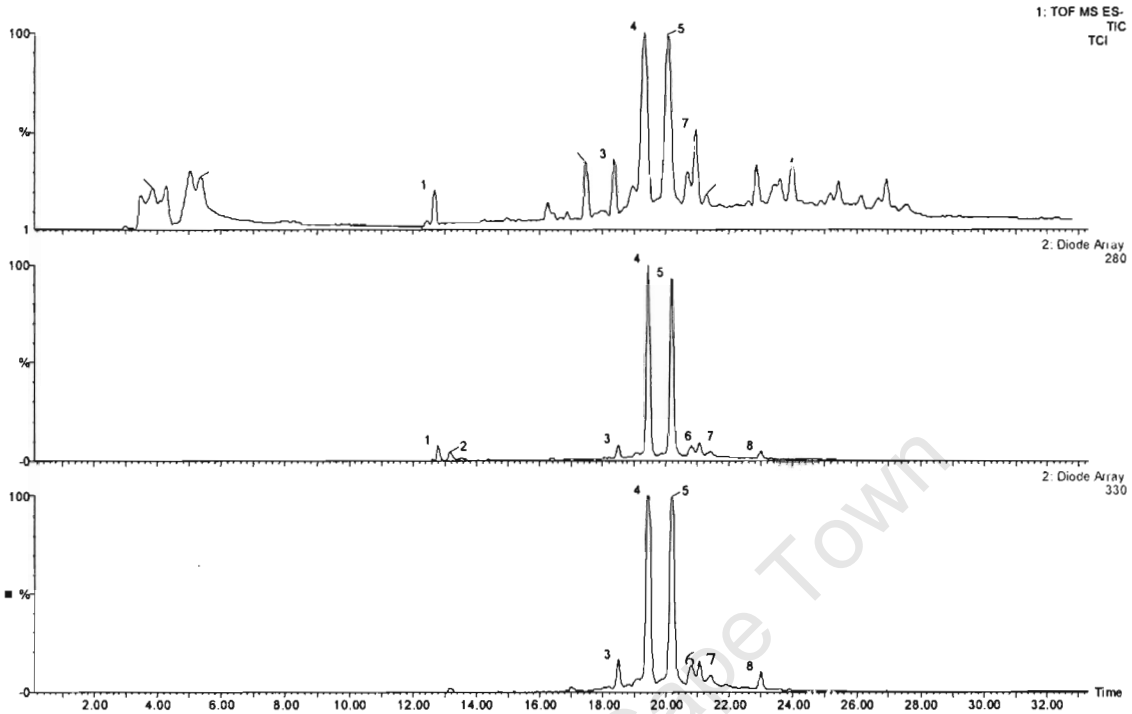


Figure 3.14. HPLC elution profiles of acetone extracts from hydrated *Craterostigma plantagineum* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 280nm (middle) and HPLC profile at 330 nm (bottom) absorptivity.

The major polyphenols in *C. plantagineum* were identified as:

Peak 3: m/z 399, 489, 785. Tentatively identified as either digalloyl-HHDP-glucopyranose or 1,2,3,6-tetra-*O*-galloylglucopyranose.

Peak 4: m/z 755. Tentatively identified as a tetramer of ferulic acid [4M-H_20-H]- = 756-1= 755.

Peak 5: m/z 623,1248. Tentatively identified as Acteoside 1 or 2 and the corresponding dimer (Owen et al, 2003).

Peak 6: m/z 579, 1160. Tentatively identified as Naringenin- 7-*O*- neohesperidoside and the dimer. (Sanchez-Rabaneda, 2002).

C. plantagineum leaves contain acteoside 1 or 2 as a major polyphenol together with tetra-*O*-galloylglucose.

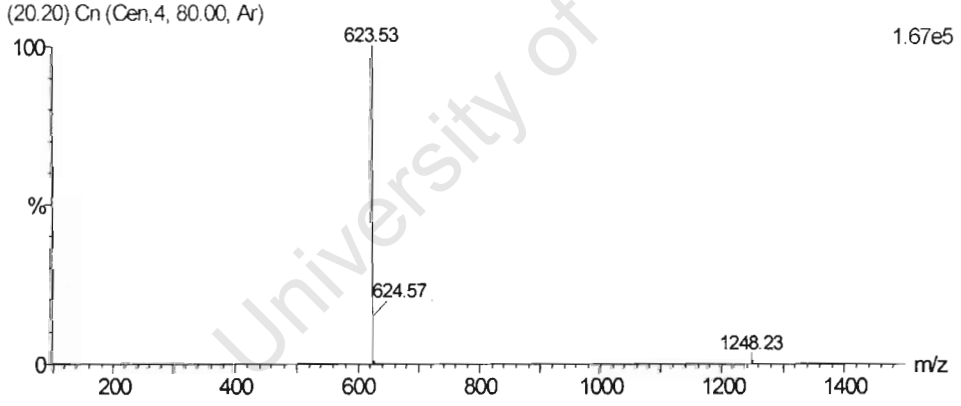
Table 3.24. Analysis of the HPLC and MS chromatograms for *Craterostigma plantagineum* extract.

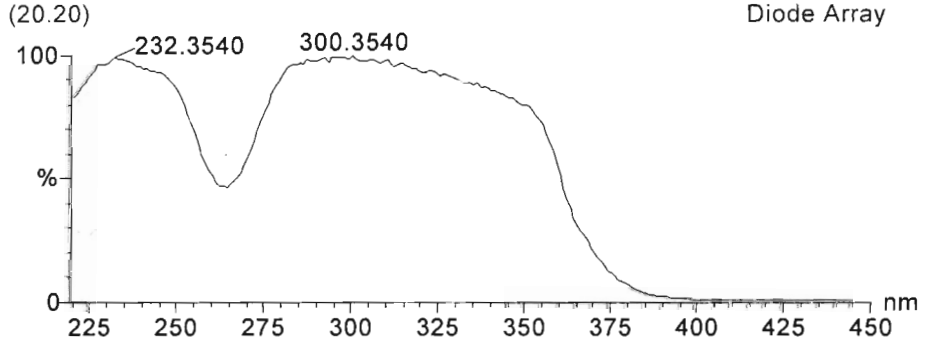
Peak	R.T(min)	M-H]⁻ (m/z)	Fragment ion (m/z) at 35 eV	λ_{max} (nm)	Tentative ID (Theoretical molecular weight)
1	12.80	689	727, 516	220, 263	Unknown
2	13.18	ND	ND	ND	ND
3	18.50	785	489, 399	221, 329	galloy-HHDP-glucopyranose(788) Tetra-*O*-galloylglucopyranose
4	**19.43**	**755**	-	**231, 280-360**	**Tetramer of ferulic acid (756)**
5	**20.20**	**623**	**1248**	**232, 280-360**	**Acteoside 1or 2 (624)**
6	20.70	579	1160	252, 343	Naringenin-7-*O*-neohesperidoside(580) + Dimer
7	21.07	637	1276, 1086	221, 329	Digalloyl-bis-HHDP-glucopyranose-1087
8	23.00	651	-	234, 329	Unknown

NB- major peaks shown in bold.

N.D - not detected.

Peak 5 ESI MS and UV spectra.





3.4.9 *Eragrostis nindensis*

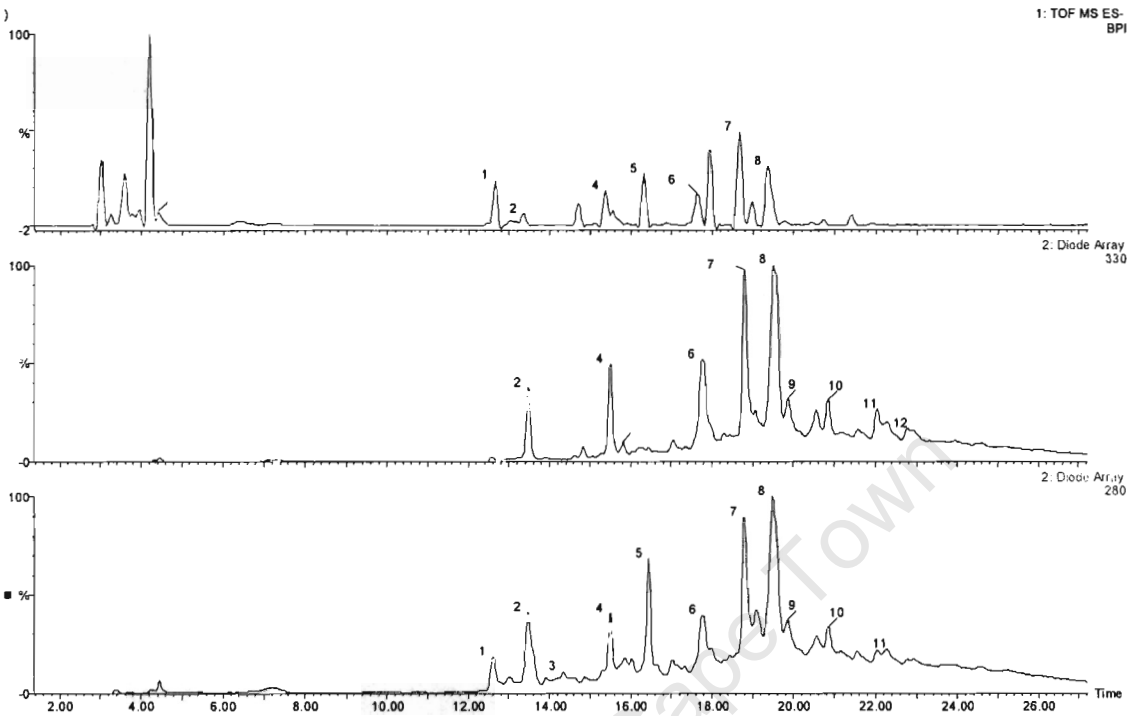


Figure 3.15. HPLC elution profiles of acetone extracts from hydrated *Eragrostis nindensis* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

The compounds in *E. nindensis* were identified as:

Peak 4: m/z 315, 631. Tentatively identified as Rhamnetin or isorhamnetin and its corresponding dimer. (Sanchez-Rabaneda et al, 2002).

Peak 5: m/z 315. Tentatively identified as Rhamnetin and isorhamnetin. (Sanchez-Rabaneda et al, 2002).

Peak 6: m/z 359. Tentatively identified as Ophiopogonanone E.(Min Ye and Dean Guo, 2004).

Peak 7: m/z 429. Unknown. The UV spectrum shows the compound as a hydroxycinnamic acid

Peak 8: m/z 579. Tentatively identified as Naringenin 7-*O*- neohesperidoside. (Sanchez-Rabaneda et al, 2002).

Peak 10: m/z 495, 607, 608, 609. This is tentatively identified as Rutin (Sanchez-Rabaneda, 2002).

Table 3.25. Analysis of the HPLC and MS chromatograms for *Eragrostis nindensis* extract

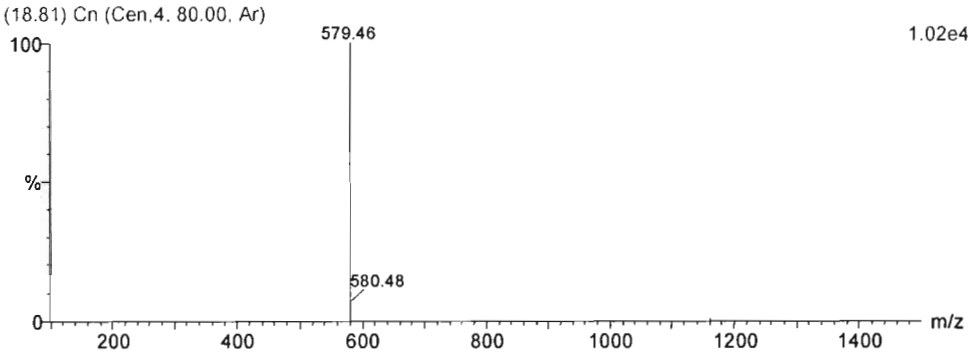
Peak	R.T(min)	$[M-H]^-$ (m/z)	Fragment ion (m/z) at 35 eV	λ_{max} (nm)	Tentative ID (Theoretical molecular weight)
1	12.61	333	-	ND	Unknown
2	13.47	245	-	ND	Unknown
3	14.34	341	487, 387	ND	Dimer of ferulic acid-387.
4	**14.84**	**315**	**315, 631**	**240, 323**	**Isorhamnetin, Rhamnetin (316)**
5	**15.51**	**315**	**-**	**240, 323, 300**	**Isorhamnetin, Rhamnetin (316)**
6	16.45	359	-	224, 282	Ophiopogonanone E (360).
7	**17.77**	**429**	**385**	**240, 324**	**Hydroxycinnamic acid**
8	**18.81**	**579**	**-**	**271, 343**	**Naringenin-7-*O*-neohesperidoside (580)**
9	19.51	593	563	270, 339	Unknown
10	20.84	607	608, 609, 495	245, 270, 343	Rutin (610)
11	22.04	491	204, 319, 368	243, 340	Unknown

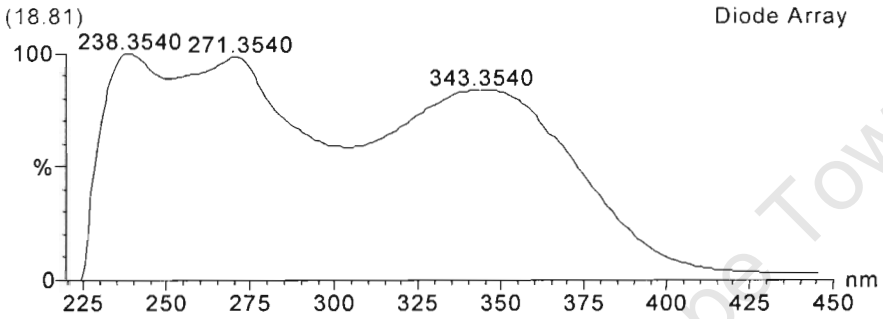
NB – major peaks shown in bold.

N.D-Not detected.

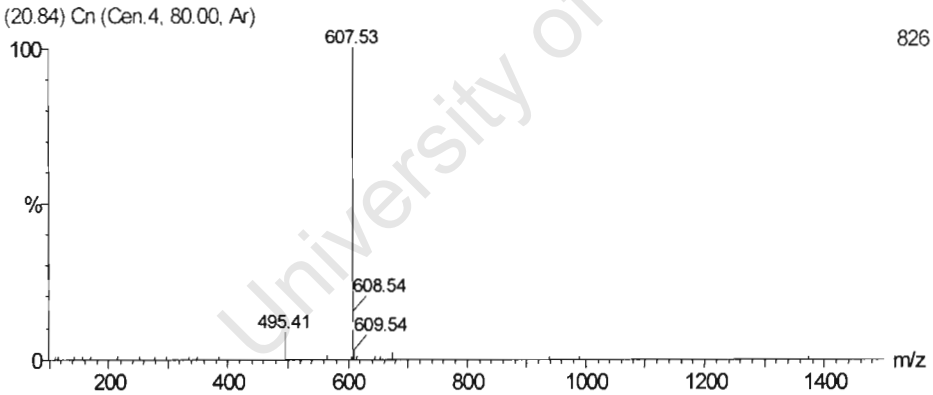
This plant is very different from the other desiccation tolerant plants. There are no tannins in the extracts of this plant and this agrees with the results from the chemical assays. *E. nindensis* and *X. viscosa* leaves contain rutin. The major polyphenols in *E. nindensis* appear to be isorhamnetin and naringenin 7-*O*-neohesperidoside.

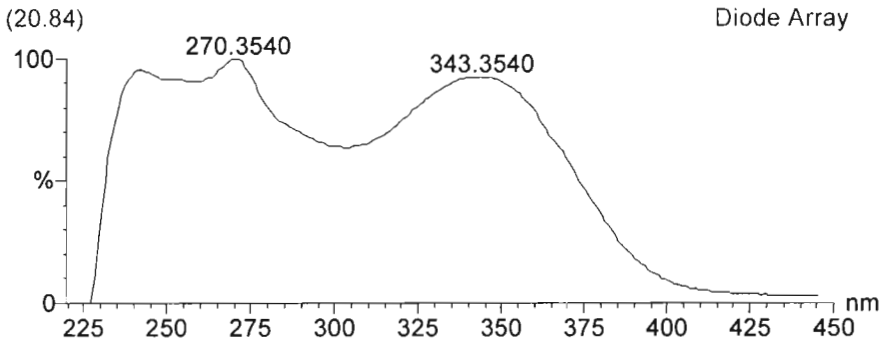
Peak 8 ESI MS and UV spectra.





Peak 10 ESI MS and UV spectra





3.4.10 CONCLUSION

The leaves of the nine desiccation tolerant plants contain a wide range of polyphenols. This appears to be the first attempt to identify the polyphenols in these plants, with the exception of *Myrothamnus flabellifolius*. The major polyphenols in *Myrothamnus flabellifolius* were identified as multi-galloylated glucoses and kaempferol glucuronide. In the *X. schlecterii, X. viscosa* and *X. humilis* the major polyphenol present was tentatively identified as 5-caffeoylquinic acid and/or its dimer. In *X. villosa* however the major phenolic is ferulic acid and its polymers. The compound 5-caffeoylquinic acid is also present in *X. villosa* but as one of the minor polyphenols in the leaf extract. The major polyphenols in *C. wilmsii, C. plantagineum* and *C. pumilum* is acteoside 1 or 2 (acteoside or isoacteoside). The major polyphenols in *E. nindensis* were identified as naringenin 7-*O*-neohesperidoside, rhamnetin, isorhamnetin and also Ophiopogonanone E. One exception for these *Xerophyta* species is the presence of rutin (quercetin 3-*O*-rutinoside) in *X. viscosa* only. Minor constituents were identified as rhamnetin glucoside and other flavones glycosides in the leaves of *X. viscosa* and *X. humilis*. Williams et al (1993) found that *Xerophyta retinervis,* a desiccation sensitive plant from South Africa contains quercetin 3-methyl ether, chrysoeriol 7-*O*-glucoside and flavone mono- and di-*C*-glycosides. The *Xerophyta* species studied (desiccation tolerant) therefore appear to contain closely related polyphenols as in desiccation sensitive plants.

There appears to be no common polyphenols in all the desiccation tolerant plants. The results obtained show how closely related the desiccation tolerant plants are to each other. Plants of the same family do contain the same major polyphenol with the exception of *X. villosa* which contain ferulic acid and its polymers whilst the rest of the *Xerophyta* species contain 5-caffeoylquinic acid. The polyphenols identified differ widely and therefore their contribution, if any, to desiccation tolerance is bound to be different.

3.5 Comparison of polyphenols in hydrated and dry leaves of desiccation tolerant plants

Most of the predominant polyphenols present in the resurrection plants were identified for the first time using mass spectrometry. There appears to be no discernible similarities in the types of polyphenols in the different plants except those of the same family. In order to understand how the types of polyphenols change with drying, the plants were dried and mass spectrometry was used to identify the polyphenols present in the dried plants. Understanding the changes in the polyphenols present in the desiccation tolerant plants may lead to an understanding of how these compounds contribute towards desiccation tolerance. The polyphenolic compounds in the dry plants were compared to those present in hydrated plants.

Water contents of the drying leaves were expressed as relative water content (RWC) i.e. the water content of the drying leaves as a percentage of the water content of the leaves when fully hydrated. The water content of fully hydrated leaves was taken as 100 % relative water content.

Table 3.26 The relative water contents (RWC, %) of the leaves after 12 days of drying.

Plant	RWC (%).
M. flabellifolius	10
C. wilmsii	8
C. pumilum	5
C. plantagineum	6
X. humilis	12
X. viscosa	10
X. schlecterii	12
X. villosa	8
E. nindensis	5

RWC –expressed as percentages of the fully hydrated water leaves.

The chromatograms of hydrated and dry plants (drawn to the same scale) are shown here to show the similarities and differences that occurred, in terms of polyphenols detected, after 12 days of drying.

3.5.1 Myrothamnus flabellifolius

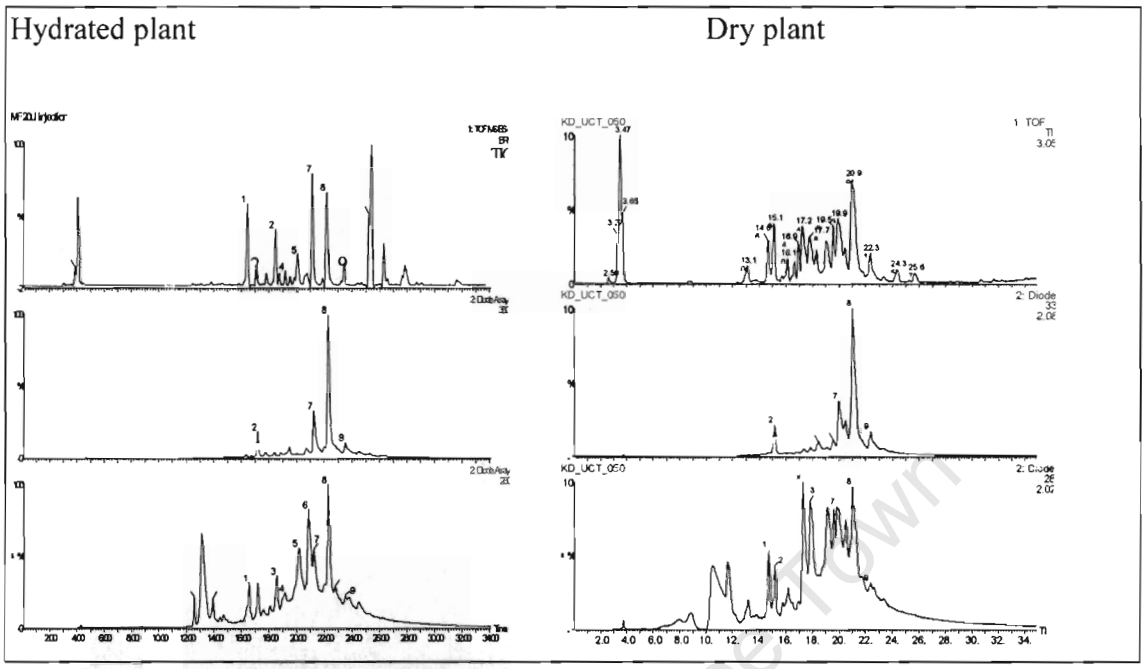


Figure 3.16. HPLC elution profiles of acetone extracts from hydrated and desiccated *Myrothamnus flabellifolius* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

The elution profiles determined at 330 nm for both hydrated and desiccated plants are generally similar. However, using the elution profiles determined at 280 nm the following observations were made:

Similarities

General similarities include the presence of 1,2,3,6- tetra-*O*-galloylglucose (m/z 787) , quercetin 3-*O*-rhamnoside (m/z 447), Quercetin glucuronide (m/z 477), Kaempferol glucuronide (m/z 461) and also procyanidin A-type dimers (m/z 423/ 431/479) in both hydrated and dry plants.

Differences

One major difference between the hydrated and dry plants is the presence of 3,4,5 tri-*O*-galloylquinic acid (peak x, m/z 647) in large quantities in the dry plant. This compound was tentatively identified before (Moore et al, 2005). The compounds 1,2,3,6- tetra-*O*-galloylglucose (m/z 787) and Quercetin glucuronide (m/z 478/447) are present in large quantities in the dry plant compared to the hydrated plant.

3.5.2 *Xerophyta viscosa*

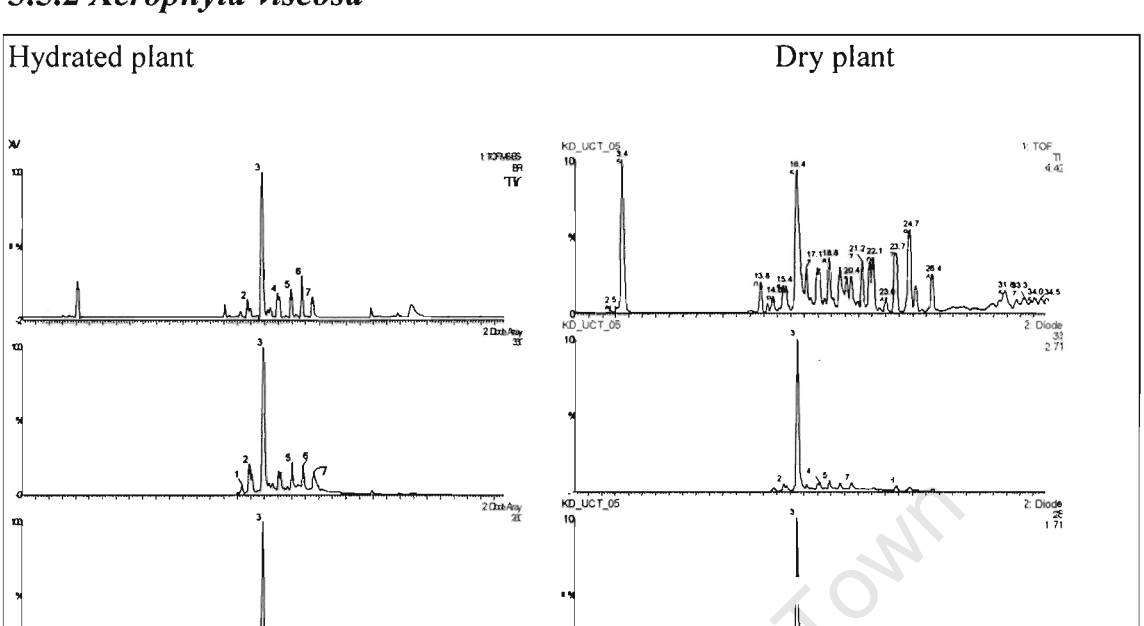


Figure 3.17. HPLC elution profiles of acetone extracts from hydrated and desiccated *Xerophyta viscosa* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Similarities

There appear to be no major differences however between the hydrated and dry plants in terms of polyphenols present. The dimer of 5-caffeoylquinic acid (formerly chlorogenic acid) (m/z 707, 353) is still the major phenolic detected in both the hydrated and dry plants. The presence of rutin (m/z 609) in both hydrated and dry plants is also observed. The compound 1,2,3,6 tetra-*O*-galloylglucose (m/z 787) is detected in both plants though as one of the small peaks.

Differences.

The small peaks (peaks 1,2,4,5,6,7) present in the hydrated plant appear to be depleted in the dry plant. This could be as a result of degradation, oxidation or rearrangement of the polyphenols during desiccation. There is also the presence of high molecular weight compounds as shown by the total ion chromatogram (TIC-top) of the dry plant. These compounds appear to be higher galloylated compounds of the compound 1,2,3,6 tetra-*O*-galloylglucose (m/z 787, 803, 805, 1126, 1140, 1168).

3.5.3 *Xerophyta humilis*

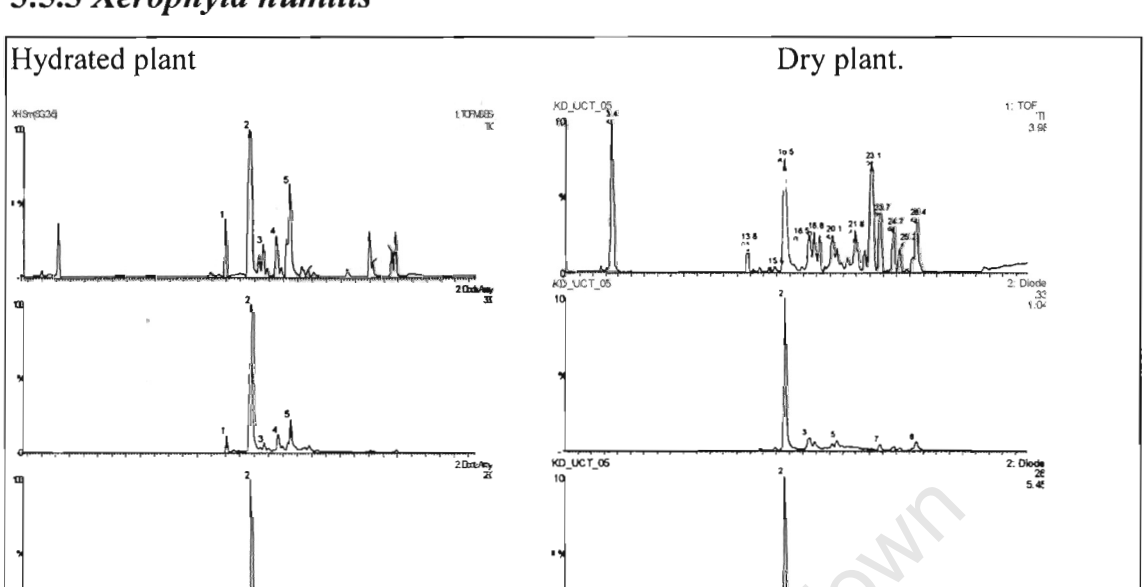


Figure 3.18. HPLC elution profiles of acetone extracts from hydrated and desiccated *Xerophyta humilis* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Similarities

5-caffeoylquinic acid (peak 2, m/z 353) is the major polyphenol detected in both hydrated and dry plants. The tannin 1,2,3,6 tetra-*O*-galloylglucose (m/z 787) is detected in both plants.

Differences.

The small peaks (4,5) are depleted in the dry plant and new more hydrophobic compounds of high molecular weight (m/z 803, 805, 1240, 1272) appear. These compounds are assigned to higher galloylated compounds of the tannin 1,2,3,6 tetra-*O*-galloylglucose (m/z 787).

3.5.4 *Xerophyta schlecterii*

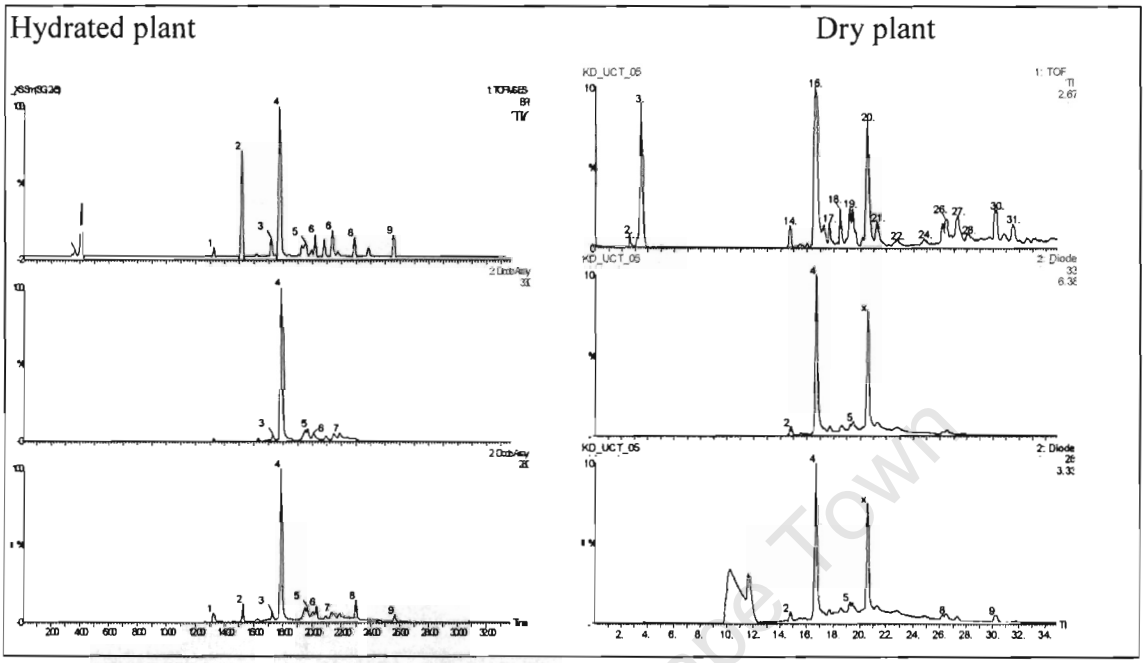


Figure 3.19. HPLC elution profiles of acetone extracts from hydrated and desiccated *Xerophyta schlecterii* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Similarities

5-caffeoylquinic acid (peak 4, m/z 353) is the major polyphenol detected in both hydrated and dry plants. The ion m/z 625 is still detected in both and is unknown. Peak 2 (m/z 315, 631) was tentatively identified as galloyl-HHDP-glucose is present in both hydrated and dry plants.

Differences

There is another major peak in the dry plant (peak x, m/z 515) and which is tentatively identified as a di-caffeoylquinic acid (MW- 516, $[M-H]^- = 515$]) (Clifford, 2003). This compound is not detected in the hydrated plant. The compound 1,2,3,6 tetra-*O*-galloylglucose (m/z 787) is detected in the dry plant only whilst in the hydrated plant epicatechin dimers, galloyl-HHDP-glucose and ellagic acid pentose conjugates appear to constitute the tannins.

3.5.5 *Xerophyta villosa*

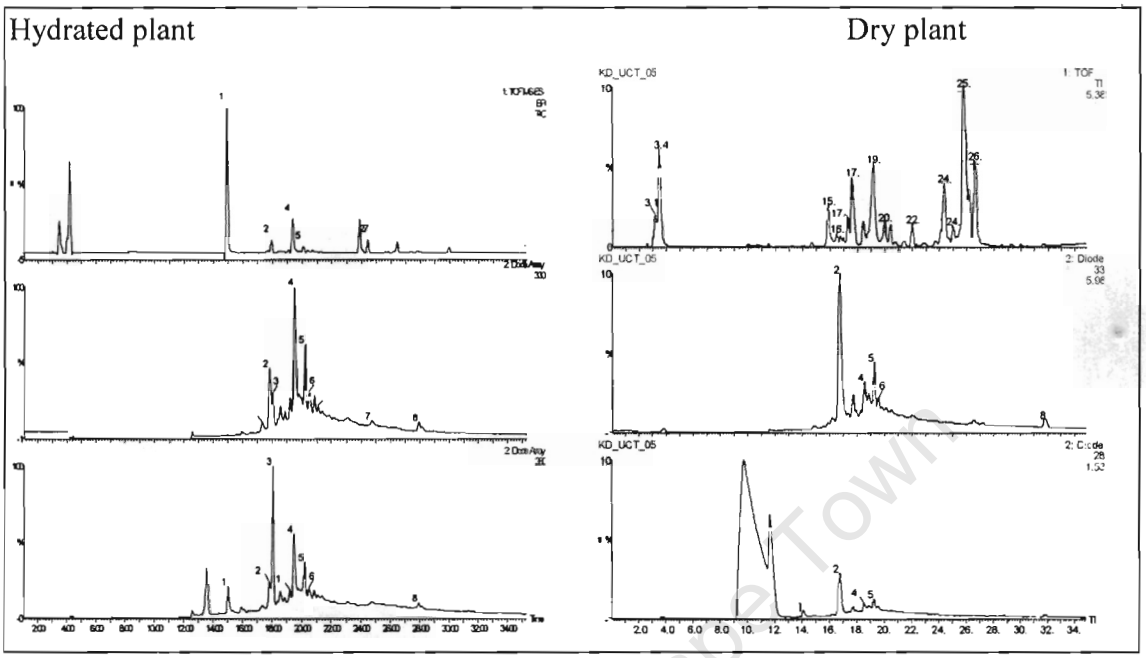


Figure 3.20. HPLC elution profiles of acetone extracts from hydrated and desiccated *Xerophyta villosa* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330 nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Using the 330 nm HPLC profile (middle) the following observations were made:

Similarities

5-caffeoylquinic acid (peak 2, m/z 353) is still present in both hydrated and dry plants.

The deprotonated compound (m/z 623) is detected in both hydrated and dry plants, and is assigned to be Acteoside 1 or 2.

Differences

The major peaks in the hydrated plant appear to be ferulic acid and its polymers (peak 3,4, m/z 387, 563, 741, 755, 775, 776).

The major peak in the dry plant has been tentatively identified as 5-caffeoyquinic acid (peak 2, m/z 353) and it appears as a minor peak in the hydrated plant. The presence of procyanidin A-type dimers or catechin (m/z 579) in the dry plant only

Multi-galloylated tannins (m/z 785) are also detected in the dry plant only.

The solvent caused the large peak observed at 280nm for the dry plants (no mass).

3.5.6 *Craterostigma wilmsii*

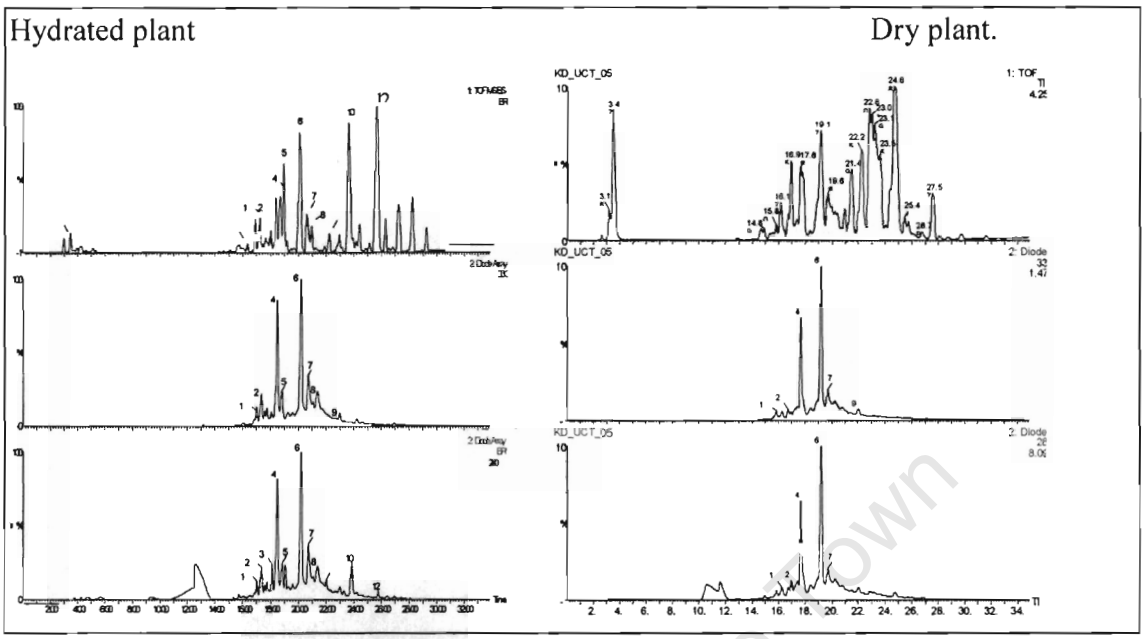


Figure 3.21. HPLC elution profiles of acetone extracts from hydrated and desiccated *Craterostigma wilmsii* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

The following observations were observed.

Similarities

The two major peaks in both hydrated and dry plants appear to be digalloyl-HHDP-glucose (m/z 785) and Acteoside 1 or 2 (m/z 623). There appear to be trimers of catechin (m/z 839) in both hydrated and dry plants.

The compounds Kaempferol neohesperidoside (m/z 593,1188) and quercetin rhamnoside (m/z 895) are detected in both hydrated and dry plants.

The tannin 1,2,3,6- tetra-*O*-galloylglucose is detected in both the hydrated and dry plants.

Differences

There appear to be depletion of small peaks (1,2,5,7,8, 10) in the dry plant.

3.5.7 Craterostigma pumilum

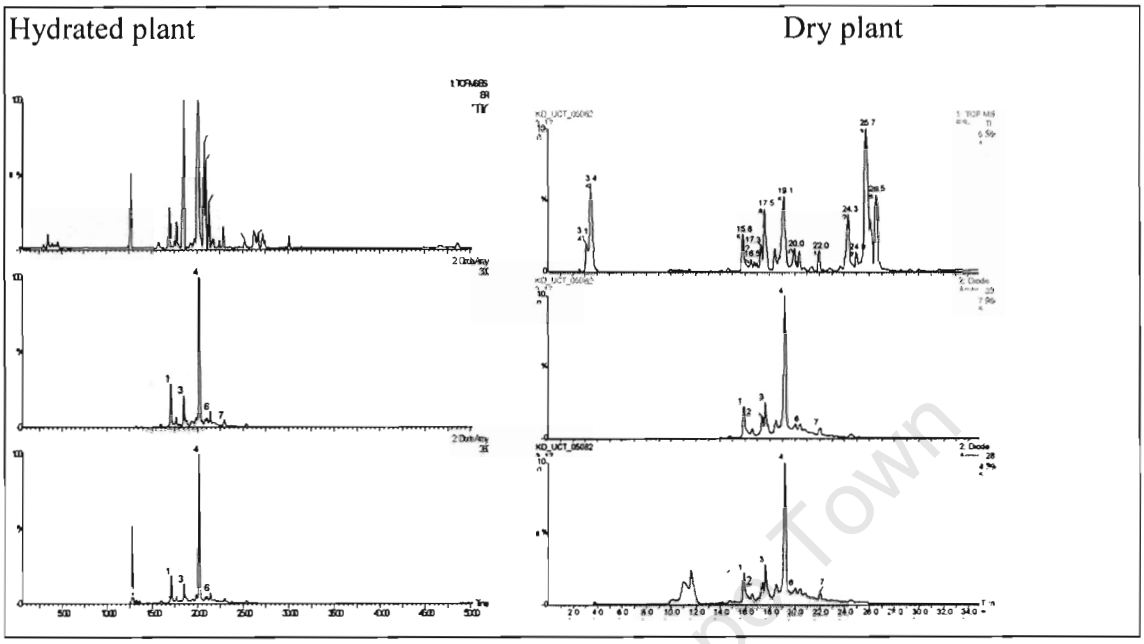


Figure 3.22. HPLC elution profiles of acetone extracts from hydrated and desiccated *Craterostigma pumilum* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

Similarities

The major peak in both hydrated and dry plants was tentatively identified as Acteoside 1 or 2 (m/z 623).

Differences

There is the presence of unidentified high molecular weight compounds (m/z 794, 1002, 1004, 1164, 1166) in the dry plant. These compounds are not detected in the hydrated plants.

3.5.8 Craterostigma plantagineum

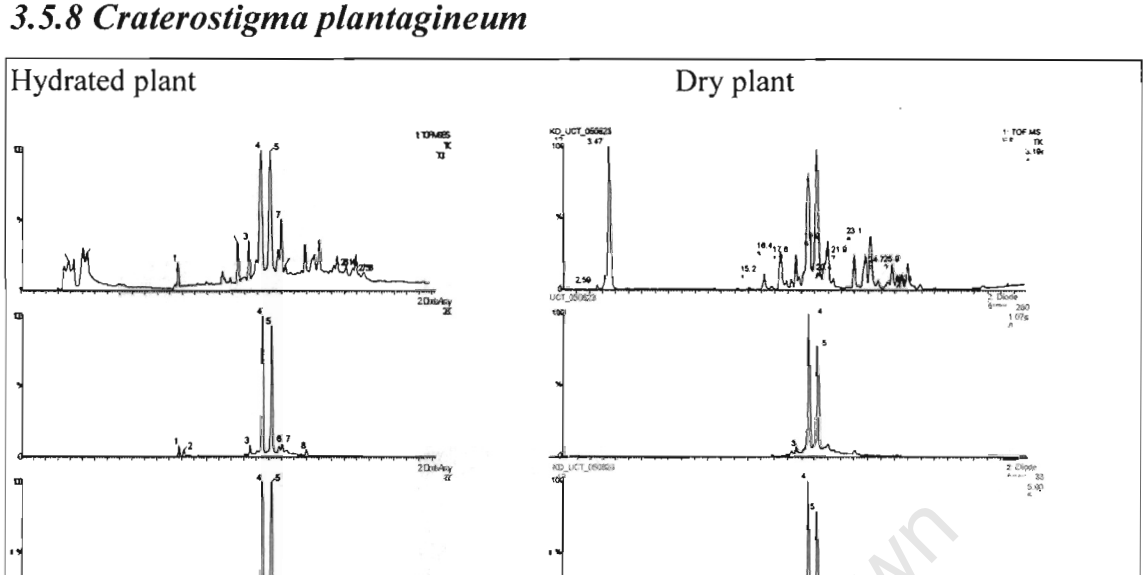


Figure 3.23. HPLC elution profiles of acetone extracts from hydrated and desiccated *Craterostigma plantagineum* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 280 nm (middle) and HPLC profile at 330 nm (bottom) absorptivity.

Similarities

The major peaks in both plants appear to be Acteoside 1 or 2 (m/z 623) (peak 4) and tetramer of ferulic acid (m/z 755) (peak 5). The compound 1,2,3,6-tetra-*O*-galloyglucose was detected in both hydrated and dry plants.

Differences

The tetramer of ferulic acid (peak 5) is partially depleted in the dry plant.

No major differences between the hydrated and dry plants

3.5.9 Eragrostis nindensis

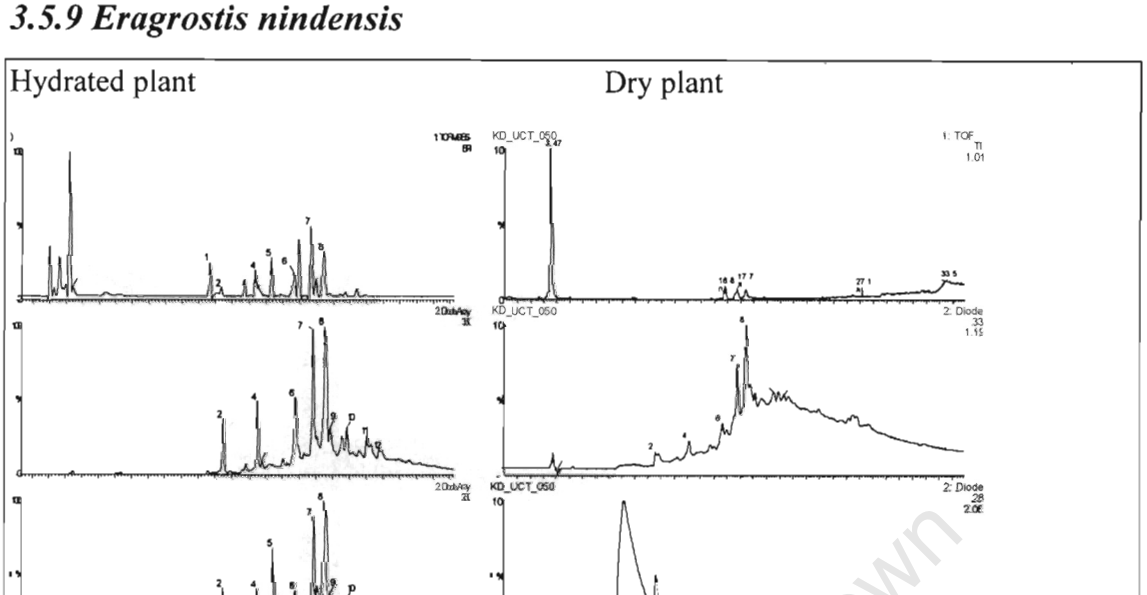


Figure 3.24. HPLC elution profiles of acetone extracts from hydrated and desiccated *Eragrostis nindensis* leaves. The profiles were recorded as MS total ion chromatogram (top), HPLC profile at 330 nm (middle) and HPLC profile at 280 nm (bottom) absorptivity.

The following observations were made using the 330 nm HPLC profile and mainly restricted to the types of polyphenols present.

Similarities

The presence of Naringenin 7-*O*-neohesperidoside in both plants

The presence of the compound giving m/z 563 in both plants though it is unidentified.

There appear to be no tannins in both hydrated and dry plants.

The presence of either rhamnetin or isorhamnetin in the hydrated plant only.

Differences

There is the apparent presence of rutin in the hydrated plant. The presence of the ion m/z 359 which was tentatively identified as Ophiopogonanone in the hydrated plant only.

A dimer of ferulic acid (m/z 387) is present in the hydrated plant.

The presence of Kaempferol rhamnoside in the dry plant.

The large peak observed at 280 nm for the dry plant is caused by the solvent.

3.5.10 CONCLUSION

The compounds identified in both the hydrated and dry plants were compared. Any change in the types of polyphenols as the plants dry, might give clues to the polyphenolic compounds that are used by the plants to survive in the absence of water.

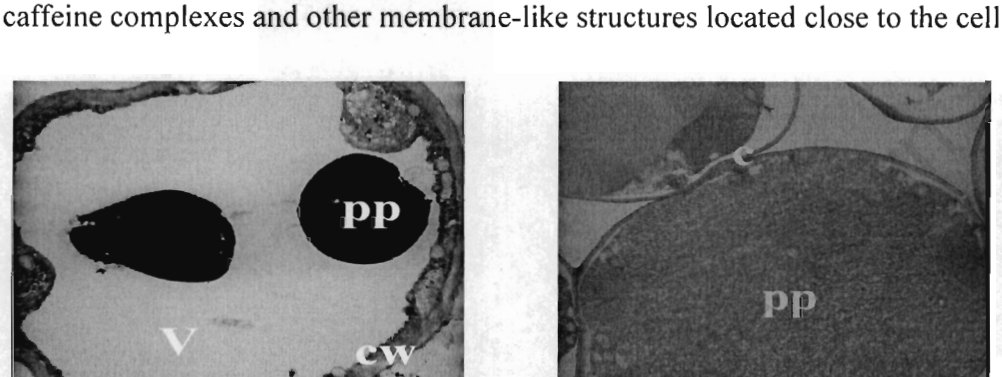
One major difference in *M. flabellifolius* between the hydrated and the dry plants is the detection of 3,4,5 tri-*O*-galloylquinic acid (m/z 647) in the dry plant. This compound was detected before in dry *M. flabellifolius* plants (Moore et al, 2005). There is also the presence of large quantities of 1,2,3,6 tetra-*O*-galloylglucose (m/z 787) and quercetin 3-*O*-rhamnoside (m/z 447). The compound 3,4,5 tri-*O*-galloylquinic acid has been shown to stabilise an artificial membrane system, liposomes, against desiccation provided the phenolic concentration was between 1 and 2 µg/µg phospholipid (Moore et al, 2005). There are large quantities of 1,2,3,6, tetra-*O*-galloylglucose present in the dry *M. flabellifolius* plant than in the hydrated plant. It is possible that the two compounds, 1,2,3,6 tetra-*O*-galloylglucose and 3,4,5 tri-*O*-galloylquinic acid, might play a role in how *M. flabellifolius* tolerates desiccation. In desiccated *X. schlecterii* a new compound was tentatively identified as a di-caffeoylquinic acid. Since this compound was not present in the hydrated plant it might be involved in desiccation tolerance. No major changes were observed between hydrated and desiccated plants in *X. viscosa, X. humilis, C. wilmsii, C. plantagineum* and *C. pumilum*. In desiccated *E. nindensis* kaempferol rhamnoside was detected. There appear to be degradation of polyphenols in desiccated *E. nindensis* as the HPLC profile at 280-nm show fewer peaks than the hydrated plant. It is known that polyphenols are easily oxidised.

Generally desiccation did not result in major changes in the nature of the polyphenols in the plants studied. Oxidation and dimerisation of existing polyphenols may account for minor differences observed. It is possible that the plants synthesise the compounds they require in advance to tolerate desiccation and not in response to desiccation. The other possibility is that polyphenols are not directly involved in desiccation tolerance. The new compounds identified in *M. flabellifolius* and *X. schlecterii* might be necessary for desiccation tolerance, so further analyses like the use of liposomes as model membranes can be done to see if these compounds protect the membranes against desiccation.

3.6 Localisation of polyphenols in the cells of desiccation tolerant plants

This study was undertaken to characterise leaf ultrastructure and to localise polyphenols in the cells of hydrated and dry leaves of desiccation tolerant plants. Two plants *Myrothamnus flabellifolius* and *Xerophyta humilis* showed the presence of polyphenols in the hydrated leaves.

In hydrated *M. flabellifolius* leaf cells, polyphenols appear to be associated with the vacuoles (Fig 3.25 (A)). A typical cell contained polyphenols as large polyphenol-caffeine complexes and other membrane-like structures located close to the cell walls but



(A) (B)

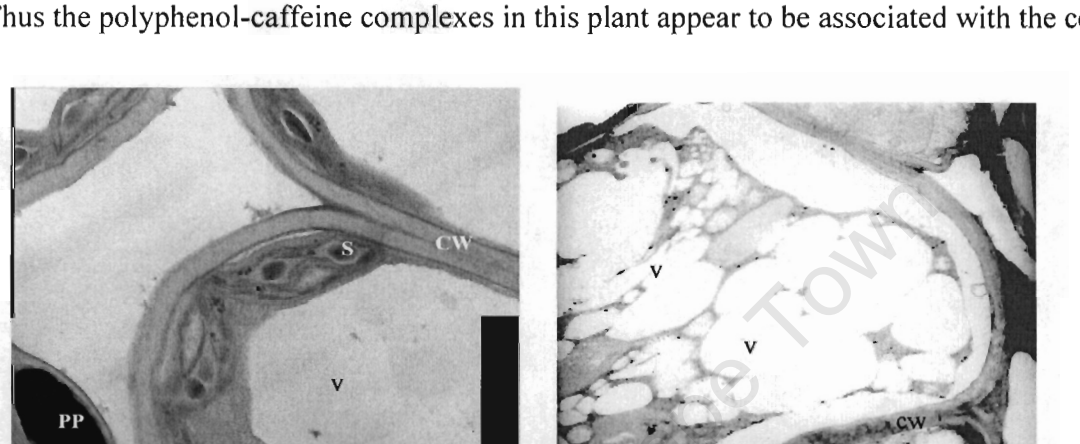
Figure 3.25. Subcellular organisation and polyphenolic deposition in hydrated (A) and dry (B) mesophyll cells from leaves of *Myrothamnus flabellifolius*.
CW-cell wall, S- starch, PP- polyphenol, V-vacuole.

in the vacuoles. Most of the polyphenols appear to be in mesophyll cells. Subcellular organisation of mesophyll cells of hydrated *M. flabellifolius* shows chloroplasts and starch granules (Fig 3.25 (A)) along the cell membranes, but otherwise no unusual features. Dehydrated leaf mesophyll cells of *M.flabellifolius* display diffuse precipitate complexes occupying and filling the vacuolar space (Fig 3.25 (B).

The results obtained in this study for *M. flabellifolius* correlates well with what Moore et al (2005) and Streit and Fengel (1995) found in terms of location of polyphenols. The vacuoles also have other electron dense material as reported by Sherwin and Farrant (1996). The location of the polyphenols in the vacuole could also indicate a function in mechanical stabilisation upon dehydration. Polyphenols are potent antioxidants and their

vacuolar location, adjacent to the cytoplasm may indicate a role in alleviation of oxidative stress during desiccation.

The subcellular organisation of hydrated cells of *X. humilis* shows the presence of well defined thyllakoids, starch and also plastoglobuli as small electron dense material in the chloroplasts. Mesophyll cells that appear to have polyphenol-caffeine complexes in the cell walls can be found occasionally adjacent to cells without the complexes (Fig 3.26 A). Thus the polyphenol-caffeine complexes in this plant appear to be associated with the cell

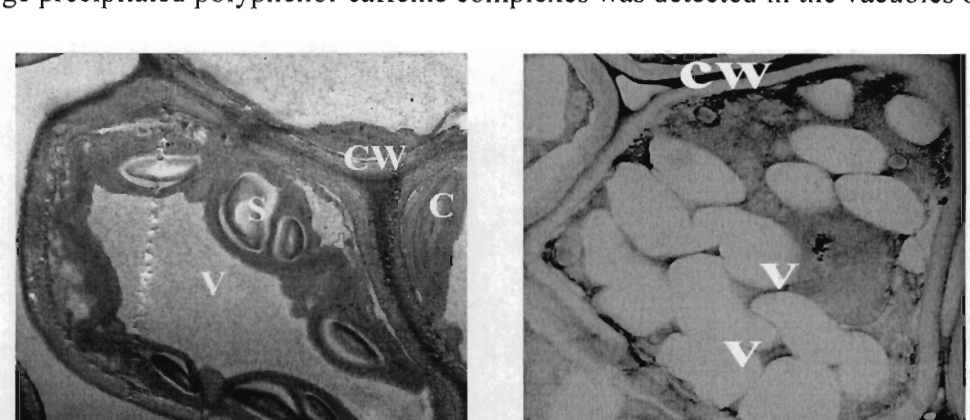


(A) (B)

Figure 3.26. Subcellular organisation of hydrated and dry mesophyll cells from leaves of *Xerophyta humilis*. CW-cell wall; PP-phenolic-caffeine complexes. V- vacuole; S- starch granule.

walls. There is the presence of many vacuoles in the desiccated cells of *X. humilis* (Fig 3.26 B). No polyphenol-caffeine complexes were visualised in the desiccated cells. *Xerophyta* species are known to fill vacuoles with compatible solutes on drying as an antidote to mechanical stress (Farrant, 2000; Vicre et al, 2003; Van der Willigen et al, 2004).

In *X. viscosa* there is diffuse electron dense material in the vacuole (Fig 3.27 A). Whether this is a result of polyphenol-caffeine interaction could not be verified. No formation of large precipitated polyphenol-caffeine complexes was detected in the vacuoles or cell



(A) (B)

Figure 3.27. Subcellular organisation of hydrated (A) and dry (B) mesophyll cells from leaves of *Xerophyta viscosa*. CW-cell wall; S- starch; C-chloroplast, V- vacuole.

walls of cells of this plant. This could be attributed to the low levels of polyphenols present in this plant. Most of the starch was present within the chloroplasts and most cells. Organelles were appressed against the cell membrane as is reported for other hydrated plant material. In the desiccated cells there is the presence of many vacuoles (Fig 3.27 B) like in *X. humulis*.

Similarly there were no polyphenol-caffeine complexes found in the hydrated (A) and dry (B) mesophyll cells from leaves of *C. wilmsii* (Fig 3.28). There is extensive cell wall folding (arrows) in the desiccated cells (B). *Craterostigma* species are known to undergo extensive cell wall folding so that the plasma membrane remains appressed to the cell wall during drying to minimise mechanical stress (Vicre et al, 2004).

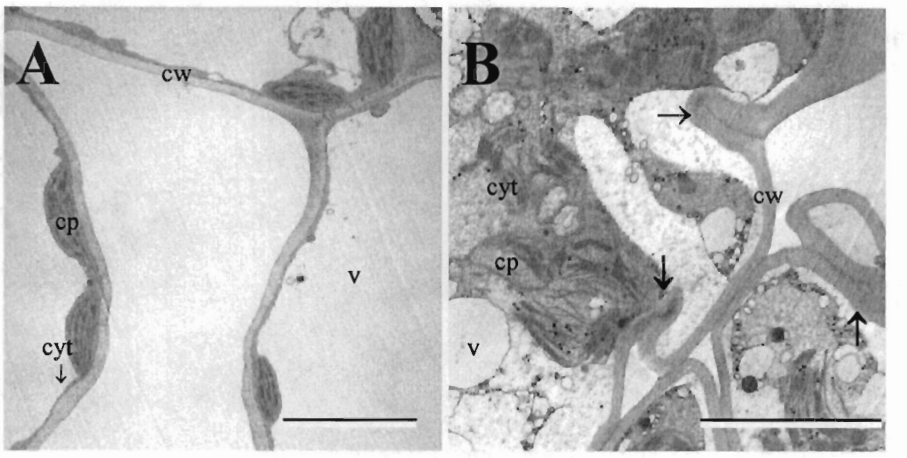


Figure 3.28. Subcellular organisation of hydrated (A) and dry (B) cells of *Craterostigma wilmsii*. C-chloroplast; S-starch; V- vacuole; Cyt-cytoplasm.

In *E. nindensis* cells there were no caffeine-polyphenol complexes observed (Fig 3.29). The subcellular organisation of mesophyll cells of this species shows a large central vacuole and peripheral cytoplasm in which well formed chloroplasts were evident.

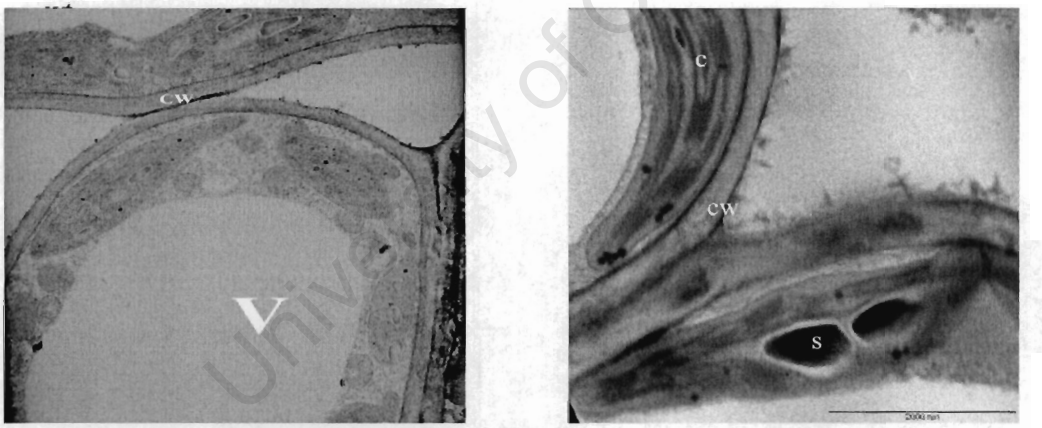


Figure 3.29. Subcellular organisation of hydrated mesophyll cells from leaves of *E. nindensis*. CW-cell, S-starch, C-chloroplast.

Polyphenol-caffeine complexes were not visualised in both hydrated and desiccated cells and only hydrated cells are shown in this study.

In the rest of the plants used in this study i.e. *X. schlecterii, X. villosa, C. wilmsii, C. pumilum* and *C. plantagineum* the caffeine-polyphenol complexes could not be visualised in both hydrated and desiccated cells. In a microscopic study on the origin of polyphenols in the wood rays of *Abier alba*, Parameswaran and Bauch (1975) observed the formation

of phenolic substances in vacuoles arising from the rough endoplasmic reticulum. According to Stafford (1989) polymerisation of phenolic monomers occurs at the membranes of vacuoles and most polyphenols are subsequently deposited in the lumina of the vacuoles. However, Streit and Fengel (1946) and Hillis (1971) showed that some polyphenols are also deposited in the cell walls.

3.6.1 Conclusion

Histochemical analysis revealed differences in localisation of polyphenols among the resurrection plants studied. In *M. flabellifolius* polyphenols appear to be associated with the vacuoles, though close to cell walls, whereas in *X. humilis* polyphenol-caffeine complexes appear to be associated with the cell wall. In *X. viscosa, X. schlecterii, X. villosa*, *C. wilmsii, C. plantagineum, C. pumilum* and *E. nindensis* no polyphenol-caffeine complexes were visualised. The data obtained here compares well with the quantification of polyphenols as determined by the Folin-Ciocalteau assay (section 3.1). Low concentrations of polyphenols in *X. viscosa, X. schlecterii, X. villosa*, *C. wilmsii, C. plantagineum, C. pumilum* and *E. nindensis* would mean that it is not possible to visualise polyphenol-caffeine complexes. In *X. humilis* the polyphenol-caffeine complexes appear to be associated with the cell walls. In both *X. humilis* and *X. viscosa* dry mesophyll cells contain vacuoles and it was found that these vacuoles are filled with compatible solutes (Farrant, 2000; Vicre et al, 2003). In *Craterostigma wilmsii* the dry cells undergo extensive cell wall folding as reported before (Vicre et al, 1999, 2004).
In *M.flabellifolius* the diffuse precipitate complexes occupying the vacuolar space in dehydrated cells could indicate a function in membrane and mechanical stabilisation and also involvement in alleviation of oxidative stress during desiccation. Polyphenols complex proteins and they are likely therefore to be found in vacuoles and possibly cell walls. The presence of polyphenols in the cytoplasm might be detrimental to the cell as polyphenols might interfere with enzymatic reactions.

CHAPTER 4

GENERAL DISCUSSION

Analysis of polyphenols is complicated by the diversity of structures found within this group of compounds. Many analytical methods have been used to quantify polyphenols in plant materials. Commonly used methods include the Folin Ciocalteau assay, the ferric chloride assay and the Prussian blue assay. These methods entail oxidation-reduction reactions in which the phenolate ion is oxidised. These assays are not specific for particular groups of polyphenols but serve to quantify the total concentration of polyphenolic hydroxyl groups in the plant extract of interest (Hagerman and Butler, 1994; Waterman and Mole, 1994). The Folin Ciocalteau assay is an improved version of the Folin-Denis method. The Folin Ciocalteau reagent is designed to avoid formation of precipitates which interfere with spectrophotometric measurements (Singleton, 1990) and it was used in this study to quantify the total phenol concentrations in the leaf extracts of the desiccation tolerant plants. The initial drying of the plant material and the extraction methods have significant effects on polyphenol analyses. The investigations done in this study show that the optimal protocol for drying and extraction of polyphenols from the plant materials studied was freeze-drying and the use of aqueous acetone respectively. Freeze-dried plant material and aqueous acetone (70%) gave superior recovery to the use of oven –dried plant material and aqueous methanol (Appendix A, section 7.1.2).

One of the aims of this study was to obtain data on the antioxidant and radical scavenging potential of the polyphenolic extracts isolated from the leaves of the desiccation tolerant plants. This information may aid in the understanding of how desiccation tolerant plants survive an inevitable increase in reactive oxygen species as they dry. Three methods, FRAP, DPPH, and the β-carotene linoleic acid assays were used to establish the antioxidant capacities of the polyphenols extracted from the leaves of the desiccation tolerant plants. There are several other methods used to characterise the antioxidant capabilities of polyphenols and these include the deoxyribose method and the metmyoglobin (Randox) method.

Chromatographic gradient systems composed of formic acid and water in a C-18 column are known to sharpen peaks and improve analytical sensitivity and resolution for HPLC

analysis of polyphenols (Rouseff et al, 1990). Therefore formic acid and water were used as the mobile phases in this study.

The investigations done in this study show that polyphenol content in the leaves of the desiccation tolerant plants show a large variation from as low as 10.2 mg GAE/g DW in *X. villosa* to 247.1 mg GAE/g DW in *M. flabellifolius*. These levels of total polyphenols are comparable to other non-desiccation tolerant plants as shown in Table 3.2 and Table 3.3. The ability to tolerate desiccation does not seem to be conferred by the quantity of polyphenols or any subclasses of polyphenols present in the leaves of the plants. *M. flabellifolius* contains mainly gallotannins, which constitutes about 80 % of the total phenols. *C. wilmsii, C. plantagineum, C. pumilum, X. viscosa, X. humilis, X. schlecterii, X. villosa* and *E. nindensis* contain mostly non-tannin polyphenols.

There is a large variation in the antioxidant capacities of the polyphenols present in the leaves of the desiccation tolerant plants. The FRAP ranged from 2.3 to 25.1 mmol Fe^{2+}/ L and the DPPH scavenging ability ranged from as low as 8.4 % in *X. villosa* to as high as 94.8 % in *M. flabellifolius*. According to the β-carotene linoleic acid assay the antioxidant activity ranged from 25.9 % in *E. nindensis* to 93.6 % in *M. flabellifolius*. Many researchers have shown that non-desiccation tolerant plants have the same antioxidant capacities. How desiccation tolerant plants survive the onslaught of reactive oxygen species as they dry cannot be explained by the antioxidant capacity of polyphenols only. However, polyphenols were responsible for the antioxidant capacity observed as shown by the significant correlation between polyphenolic concentration and the FRAP, DPPH inhibition and the β-carotene bleaching inhibition. It is proposed that the antioxidant protection for desiccation tolerant plants may possibly be attributed to a combination of more than one group of compounds. At the same time polyphenols of several different types and other compounds in the desiccation tolerant plants might form a synergistic multilevel defence system.

This study showed that *M. flabellifolius* extracts possess a significant reducing power and free radical scavenging ability. There was a variable response to desiccation in terms of total phenols and antioxidant capacities. An increase in total phenol on drying in *M. flabellifolius* and *C. plantagineum* resulted in a general increase in antioxidant activity. The results obtained in this study indicate that desiccation tolerance must be the outcome

of the interplay of more than one (and probably many) mechanisms or processes (Blackman, 1991, 1992). Javanmardi, Stushnoff, Locke and Vivanco (2003) found that the antioxidant activity of plant extracts is not limited to polyphenols. The activity may also come from other antioxidant secondary metabolites such as carotenoids and vitamins. Kranner et al (2002) also suggests that protection against desiccation-induced free radical might require the whole array of antioxidants and pigments and not a single antioxidant. This would require techniques like genetic engineering to target a set of characters and not a single one. Kranner et al (2002) noted that it is already known that there are cases where genetically increasing the concentration of a single antioxidant had little or no effect on stress tolerance. Well known morphological changes take place when these resurrection plants dry and it is possible that together with the help of polyphenols they make the plant desiccation tolerant.

In the present study HPLC-ESI-MS analysis revealed the wide array of polyphenols present in the resurrection plants extracts. These are listed in Tables 3.17 to Table 3.25. Desiccation produced variable responses in the types of polyphenol compounds identified in the plants. New compounds were identified in dry *M. flabellifolius* and *X. schlecterii*. These compounds are 3,4,5 tri-*O*-galloylquinic acid and a di-caffeoylquinic acid respectively. Minor changes were observed in *X. viscosa, X. humilis, X. villosa, C. wilmsii, C. plantagineum* and *C. pumilum.* The resurrection plants might synthesised the compounds they require to survive desiccation when still hydrated and not in response to desiccation. Compounds like LEA proteins and other polyols have been shown to be involved in desiccation tolerance. It is known that polyphenols are useful as chemotaxonomic markers (Ducrey, Wolfender, Marston and Hostettmann, 1995). The results obtained in this study might mean *X. villosa* may have been classified wrongly as it has total polyphenol content, antioxidant capacity and types of polyphenol that are different from the other *Xerophyta* species.

Cells of *M. flabellifolius* revealed electron dense phenolic-caffeine precipitates within the vacuoles of the leaf mesophyll cells. It is also known that polyphenols oxidise and crosslink in the presence of osmium tetroxide into hard complexes. In *X. humilis* these dark stained structures (phenolic-caffeine complexes) appear to be associated with the cell walls. In *X. viscosa, X. villosa, X. schlecterii, C. wilmsii, C. plantagineum,*

C. pumilum and *E. nindensis* plants there was no direct indication of localisation of polyphenols in vacuoles or cell walls of the cells. In dry plants *Xerophyta* species show mesophyll cells with lots of vacuoles whilst *Craterostigma wilmsii* show mesophyll cells with extensive cell wall folding as reported before. In dry *Myrothamnus flabellifolius* the mesophyll cells contain a central vacuole filled with diffuse polyphenol-caffeine complexes. The polyphenols in the resurrection plants are likely to be localised in the vacuoles of the cells, as they were easily extractable. We suppose that polyphenols are diffusely localised in the vacuoles and act mostly as antioxidants and /or incorporated into primary walls as cross-linked fragments or lignin-like compounds.

CHAPTER 5

FINAL CONCLUSION

In this study we investigated the quantities, nature and the possible role of polyphenols in desiccation tolerance. Chemical analyses show that there is a large variation in total phenol and tannin contents and total antioxidant capacities in the leaves of the nine desiccation tolerant plants. There is no distinction between desiccation tolerant plants and non-desiccation tolerant plants in terms of quantity and the nature of polyphenols. There is significant linear correlation between polyphenols concentration and the antioxidant capacity of the extracts and this show that polyphenols contribute to the antioxidant defence systems of the desiccation tolerant plants. However, the antioxidant capacities are not different from other non-desiccation tolerant plants. Comparative studies at family level show that *Xerophyta* species have comparable total polyphenols and antioxidant capacities, with the exception of *X. villosa*. *Craterostigma* species also do have comparable levels of polyphenols and antioxidant capacities. Overall these results indicate that polyphenols are not directly linked to desiccation tolerance.

The HPLC ESI-MS analytical method used in this study allowed the positive identification of polyphenols in the extracts without the need to purify the extracts. The resurrection plants contain a wide range of polyphenols and are reported in this study for the first time, except the compound 3,4,5 tri-*O*-galloylquinic acid.

M. flabellifolius contains mainly tannins and the major polyphenols were identified as multi-galloylated glucoses and kaempferol glucuronide. *Xerophyta* species contain 5-caffeoylquinic acid as the major phenolic with the exception of *X. villosa*, which has ferulic acid and its polymers, as the major polyphenols and *Craterostigma* species contain acteoside1 or 2 (verbascoside or isoverbascoside) as the major polyphenolic compound. Further analyses like nuclear magnetic resonance (NMR) are required to determine the isomer present. Desiccation has a variable effect on the polyphenols identified in the desiccation tolerant plants. In *M. flabellifolius* and *X. schlecterii* there was the appearance of new compounds, 3,4,5 tri-*O*-galloylquinic acid and a di-caffeoylquinic acid, when these plants were subjected to desiccation. These compounds might have a role in desiccation tolerance.

Histochemical detection shows large polyphenol-caffeine complexes in the vacuoles of *M. flabellifolius.* This localisation of polyphenols in the vacuole could indicate a function in membrane stabilisation and in alleviation of oxidative stress during desiccation. In *X. humilis* the polyphenol-caffeine complexes were visualised in the cell walls. No polyphenol-caffeine complexes were visualised in *E. nindensis, X. villosa, X. viscosa, X. schlecterii, C. pumilum, C. wilmsii and C. plantagineum*. This could be due to the low concentrations of polyphenols detected.

It is understood that desiccation tolerance is a complex phenomenon and it is hoped that this study will add to the knowledge already there about this phenomenon. From the results obtained herein it is uncertain whether polyphenols play any role, if at all, in desiccation tolerance. The role played by the large quantities of polyphenols in *M. flabellifolius* and the new compounds, detected in *M. flabellifolius* and *X. schlecterii* after desiccation, is still unclear at this stage. More work is still to be done to conclude on the possible role of polyphenols in desiccation tolerance.

5.1 Recommendations

Future studies should focus on individual phenolic compounds, as identified by mass spectrometry, and follow the changes of these compounds as the plants dry. Polyphenols could contribute to desiccation tolerance in many other ways. Polyphenols are known to be antibacterial, antiviral and antifungal. Therefore purified compounds from these resurrection plants can be studied to see if they can act against bacteria, viruses and fungi. Polyphenols are also UV absorbing so could have a role in light stress. Polyphenols could be used in the synthesis of lignins by the plant after periods of desiccation. As Zucker (1983) noted, individual polyphenols warrants closer attention as important chemicals, especially in desiccation tolerance, than they have received to date.

Targeting antioxidant metabolism and polyphenols in genetic engineering can help in producing drought resistance plants, but it should be borne in mind that the whole array of antioxidants and other pigments is required for the protection of plants against free radical attack. As Kranner et al (2002) noted our knowledge of protection mechanisms against desiccation-induced oxidative stress is still incomplete and a lot still needs to be done.

CHAPTER 6

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CHAPTER 7

7.1 Appendix A

7.1.0 Establishment of optimum conditions for extraction of polyphenols from leaves of desiccation tolerant plants

Polyphenols can be extracted by a range of methods. Correct extraction should be done to prevent chemical modification, which will result in artefacts. The conditions used during extraction should be as mild as possible to avoid oxidation, thermal degradation and other biochemical changes to the compounds in the sample. Extraction of polyphenols from plant material pose a great challenge as not only does the extraction method determines the yield but it also determines the nature of the polyphenols extracted (Scalbert, 1992). Typical solvents are aqueous methanol and acetone in an inert atmosphere. Other concerns when extracting polyphenols from plant material is the completeness of the extraction and degradation of the extracted polyphenols. It is therefore necessary to optimise extraction parameters for the particular plant materials in use (Yu and Dahlgren, 2000). An attempt was made to optimise some of the factors that affect the extraction of polyphenols in the plant materials in this study.

Therefore the following factors were investigated and compared:

1.0 Sample preparation techniques: oven drying and freeze-drying.

2.0 Aqueous methanol and aqueous acetone as extracting solvent.

3.0 The reference standard to use in the Folin Ciocalteau assay-gallic acid and tannic acid

4.0 Sonicating time required for maximum extraction of polyphenols.

7.1.1 Extraction of polyphenols

Fully hydrated leaves were collected from the whole plants randomly and mixed. Sample leaves were then taken from this pool and immediately plunged into liquid N_2. The leaves were freeze-dried or oven dried (at 40 ^{0}C) in the dark. After grinding the samples were extracted using the following solvents

1.0 70 % aqueous acetone.

2.0 80 % aqueous methanol.

The polyphenols were extracted as described in section 2.1.2 under nitrogen and total polyphenols determination was done as described in section 2.3.1.

7.1.2 Optimum conditions for extraction of polyphenols

From Figure 7.1 it was concluded that freeze-drying and the use of acetone as the solvent appears to be the best way to prepare samples and extract the polyphenols in the plants

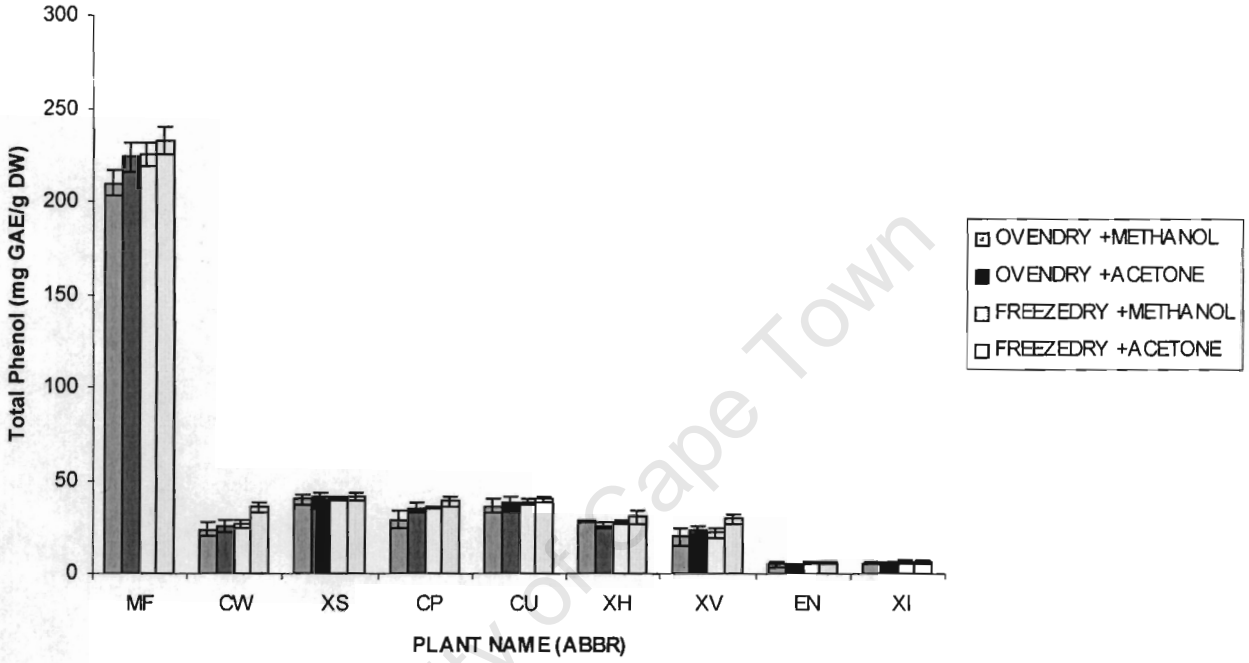


Fig 7.1.The influence of drying technique and solvent on the extraction of polyphenols from desiccation tolerant plants using the Folin Ciocalteau method. Bars-standard deviation, n = 3. Abbreviations used: MF-*M. flabellifolius*, CW-*C. wilmsii*, CU-*C. pumilum*, CP- *C. plantagineum*, XH-*X. humilis*, XV-*X. viscosa*, XS-X. *schlecterii*, XI-*X. villosa*, EN-*E. nindensis*.

investigated. From the results summarised in Figure 7.1 it is evident that the four methods used give fairly similar results. In the case of *M. flabellifolius, C. wilmsii, C. plantagineum* and *X. viscosa* freeze drying followed by the use of 70 % acetone, as the solvent appears to be slightly superior to the other methods. Consequently it was decided that freeze drying and 70 % acetone be the used in the study. Furthermore freeze-drying is milder than oven drying.

7.1.4 Sonicating Time

To increase the efficiency of the extraction step the use of ultrasound is usually employed (Hopkins, 1991). These disrupt cells and they work by generating intense sonic pressure waves in liquid media. The pressure waves cause the formation of microbubbles that grow and collapse violently. This process is called cavitation and the implosion of the bubbles generates a shock wave with sufficient energy to break cell membranes and also some covalent bonds (Hopkins, 1991). The sonicator used had a thermostat to keep the temperature low. To create an inert atmosphere it is important to purge the extract with gaseous nitrogen. Below we determined the time to sonicate in order to extract the polyphenols from the desiccation tolerant plants efficiently.

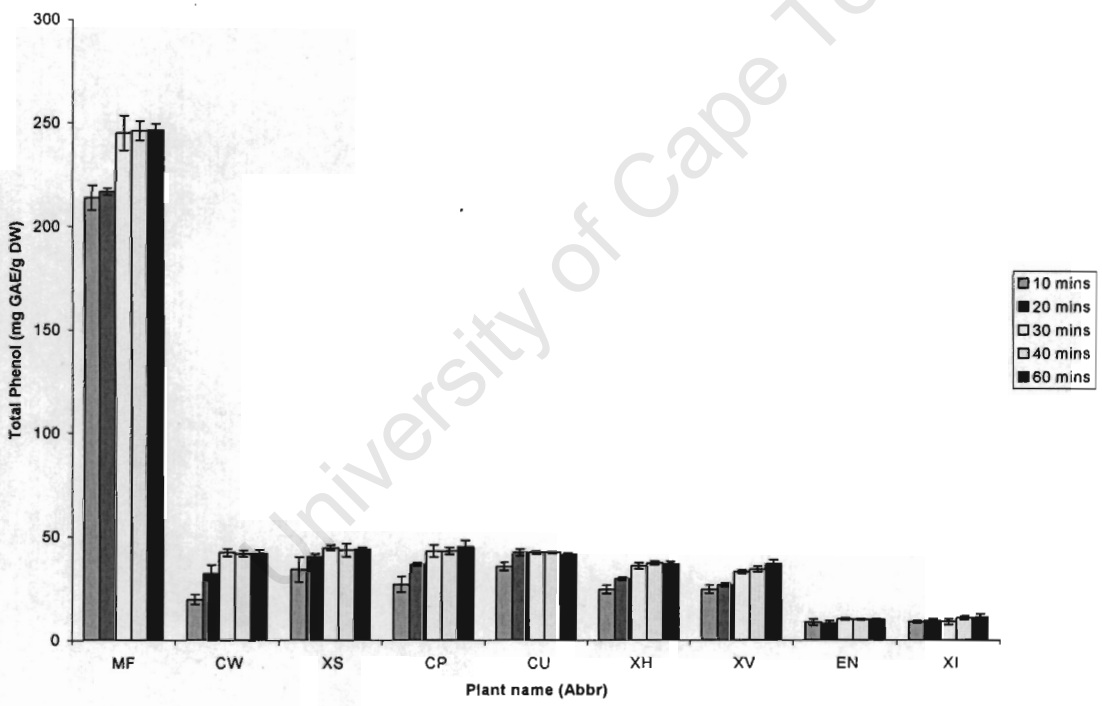


Figure 7.2. The effect of sonicating time on total polyphenols extracted. Bars-standard deviation, n = 3. Abbreviations used: MF-*M. flabellifolius*, CW-*C. wilmsii*, CU-*C. pumilum*, CP- *C. plantagineum*, XH-*X. humilis*, XV-*X. viscosa*, XS-X. *schlecterii*, XI-X. *villosa*, EN-*E. nindensis*.

Folin Ciocalteau method was used to quantify the polyphenols extracted at each different time and gallic acid was used as the standard. From the results it is evident that

polyphenol yields increase as a function of time reaching a maximum at 30 minutes. Consequently 30 minutes was chosen as the sonicating time since it was enough time to extract most polyphenols and also it saves time.

7.1.3 Choosing the best Standard for the Folin Ciocalteau assay

Gallic acid and tannic acid are both standards used in the Folin Ciocalteau assay.

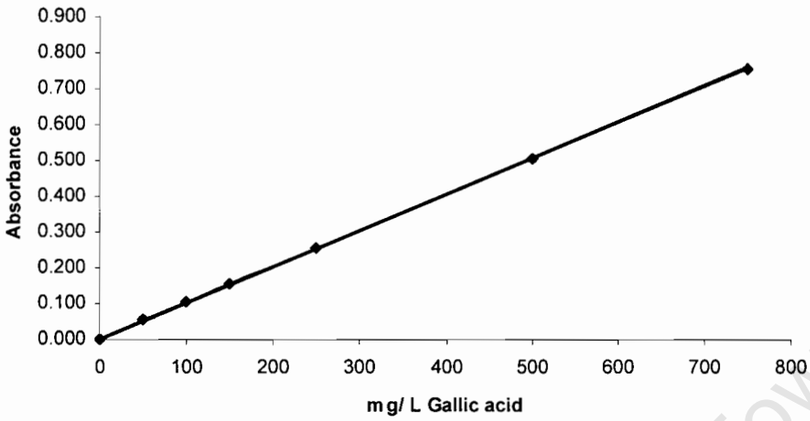


Figure 7.3. The standard curve for the Folin Ciocalteau assay using gallic acid as a standard.

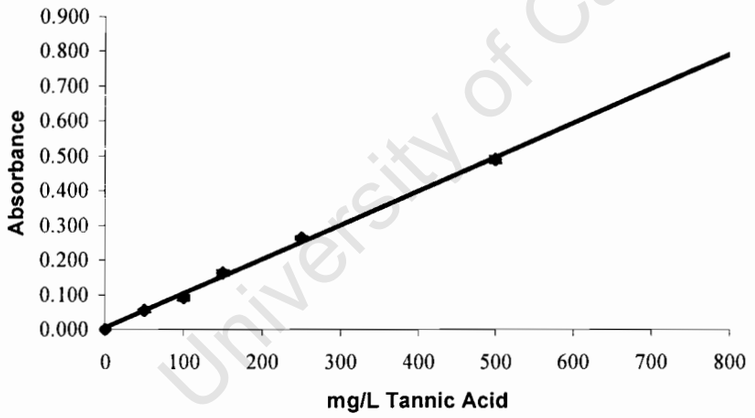


Figure 7.4. The standard curve for the Folin Ciocalteau assay using tannic acid as a standard.

Polyphenols comprises a large heterogeneous set of chemicals. Consequently the choice of a reference standard is extremely important. The standard curves obtained using the Folin Ciocalteau method for both gallic acid and tannic acid are shown in Figure 7.3 and Figure 7.4. From the results it is evident that there is a very similar and linear relationship between absorbance and concentration in the range 0-800 mg /L of gallic acid or tannic acid. Many researchers use gallic acid in the Folin Ciocalteau assay (Hagerman, 1998, 2002). Others however use tannic acid. It was decided that it is better to use gallic acid as it is well defined unlike tannic acid, which can vary from supplier to supplier.

7.3 Appendix C

7.3.1 Optimisation of the polyphenols of the polyphenols separation and identification using HPLC-DAD ESI MS

Table 7.1 below shows a list of standard compounds used in this study. A mixture of these standards was run on the system to optimise the chromatographic conditions. The optimum conditions achieved are those reported before in this section.

Formic acid was used to improve peak purity and shape. The concentration of formic acid required to improve the MS spectrometer sensitivity was found to be 0.1 %. Repetitive analysis of the standards shows that the average standard deviation of the retention times was 0.8 min.

Table 7.1. Retention times, absorbance maxima and main ions observed for the standards. M.W –the theoretical molecular weight of the standards.

Compound	Retention time (min)	λ_{max} (nm)	M.W	Main ion (at 35 V) (m/z)
Gallic acid	12.29	271	170	169
Ellagic acid	22.59	367	302	301
Ascorbic acid	3.73	242	176	175/351
Quercetin	17.95	293/369	302	301/603
p-Coumaric acid	19.62	287-340	164	163
o-Coumaric acid	21.44	273/324	164	163
(+)-Catechin	16.47	278	290	289/579
5-caffeoylquinic acid	16.76	326	354	353/707
Vanillin	19.44	279/309	152	151

Some of the HPLC and MS spectra observed for the standards used in the study are shown below.

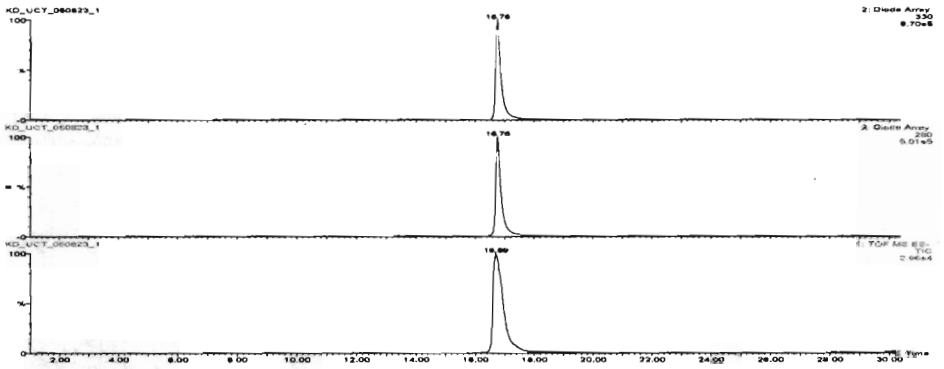


Figure 7.5. HPLC elution profiles of pure 5-Caffeoylquinic acid recorded as HPLC profile at 330 nm (top), at 280 nm (middle) and MS total ion chromatogram (bottom).

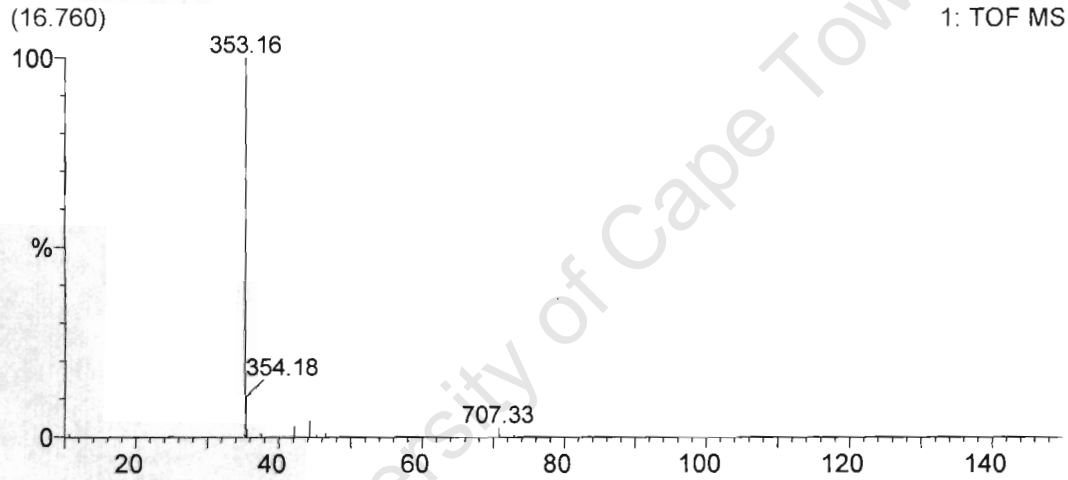


Figure 7.6. Mass spectrum of 5-Caffeoylquinic acid.

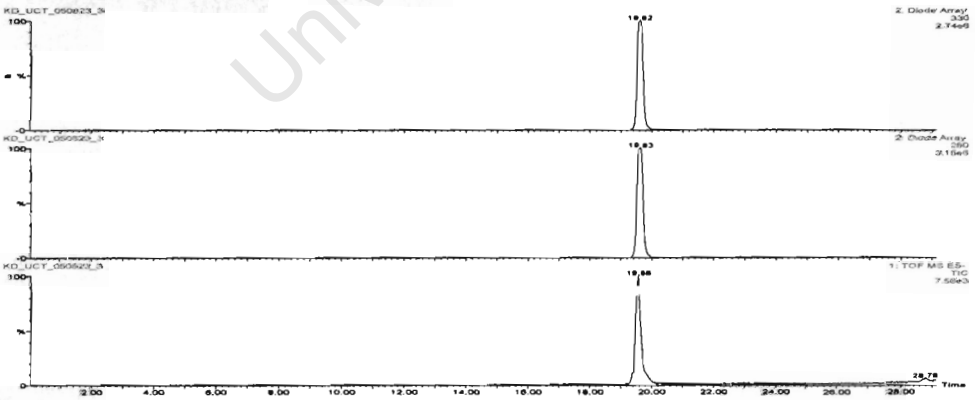


Figure 7.7. HPLC elution profiles of pure p-coumaric acid recorded as HPLC profile at 330 nm (top), at 280 nm (middle) and MS total ion chromatogram (bottom).

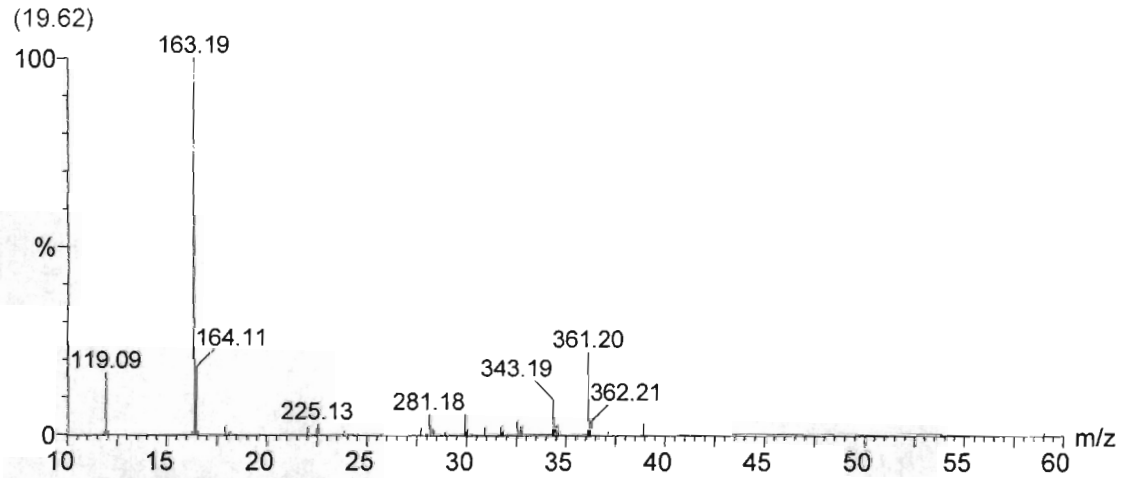


Figure 7.8. Mass spectrum of p-Coumaric acid.

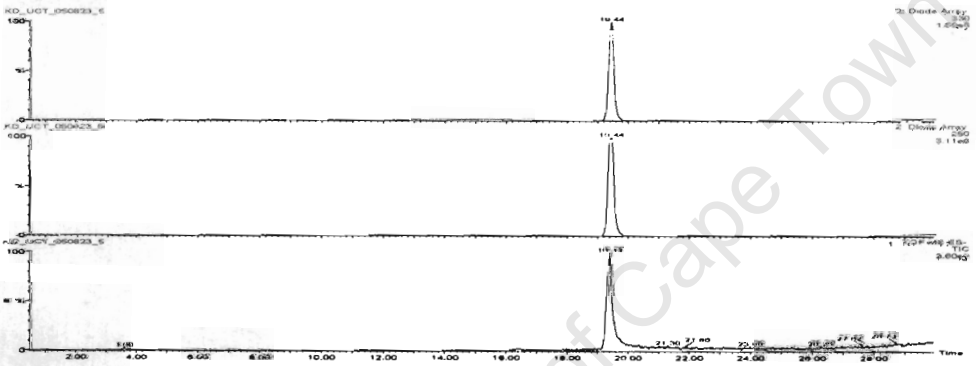


Figure 7.9. HPLC elution profiles of pure vanillin recorded as HPLC profile at 330 nm (top), at 280 nm (middle) and MS total ion chromatogram (bottom).

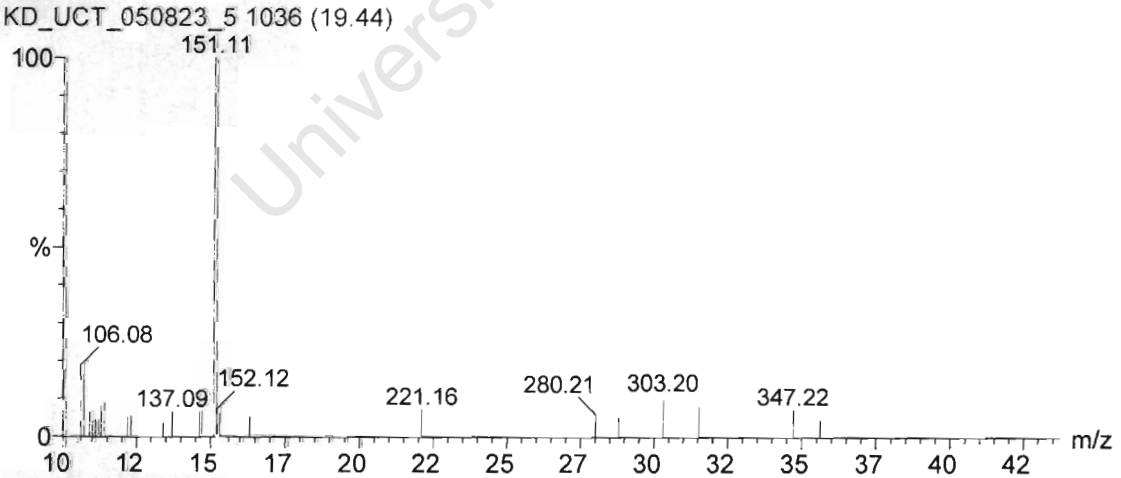


Figure 7.10. Mass spectrum of Vanillin

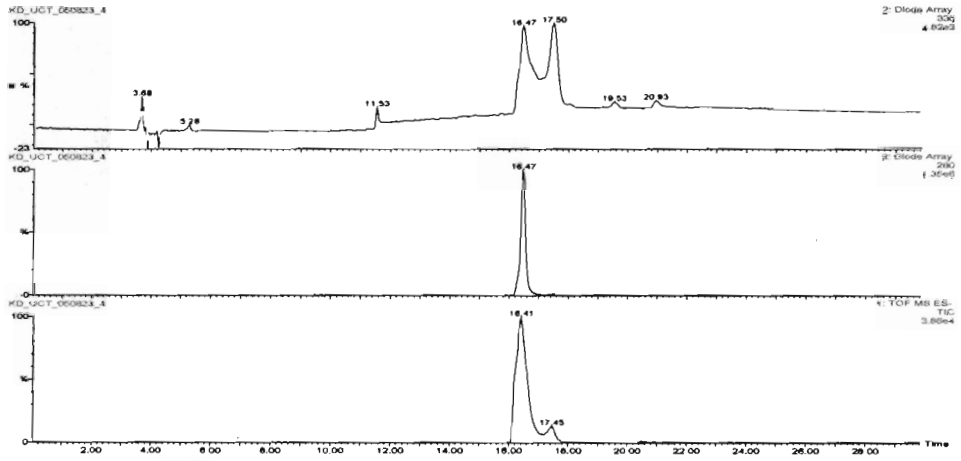


Figure 7.11. HPLC elution profiles of pure catechin recorded as HPLC profile at 330 nm (top), at 280 nm (middle) and MS total ion chromatogram (bottom).

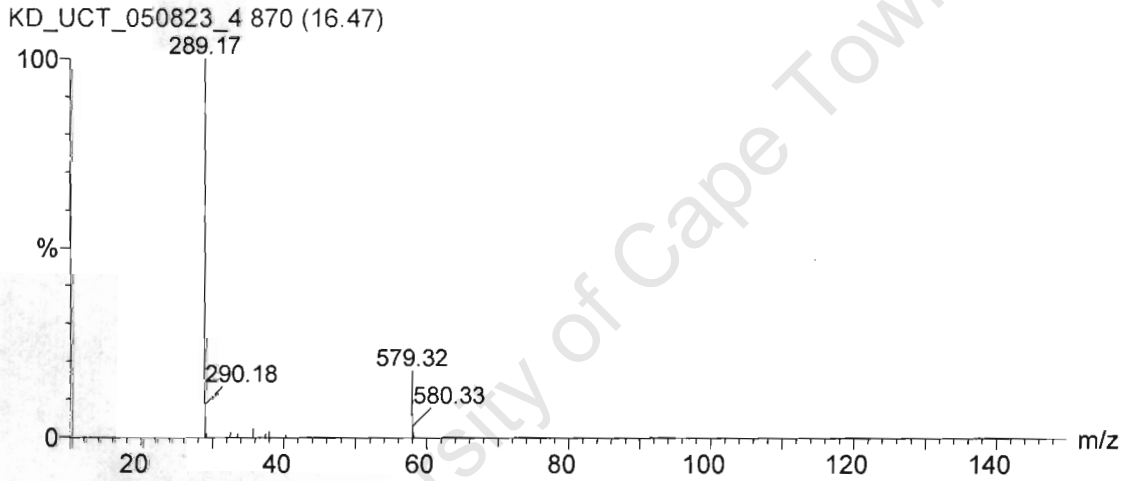


Figure 7.12. Mass spectrum of Catechin.

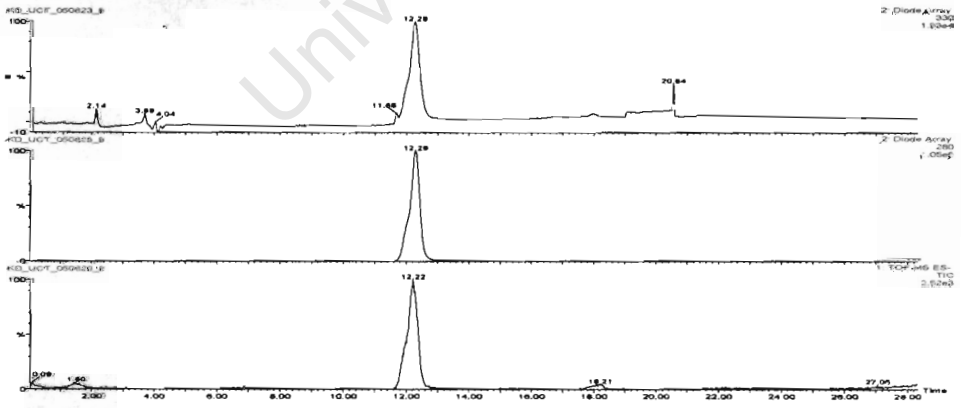


Figure 7.13. HPLC elution profiles of pure gallic acid recorded as HPLC profile at 330 nm (top), at 280 nm (middle) and MS total ion chromatogram (bottom).

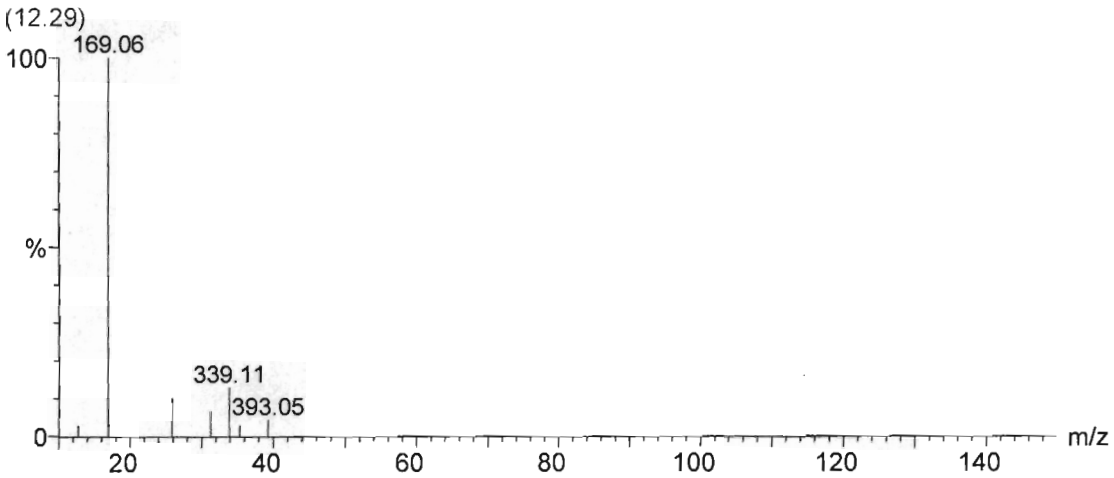
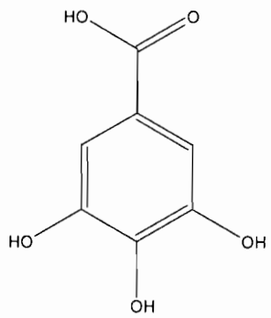


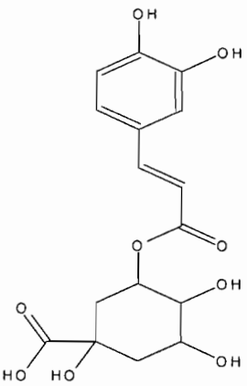
Figure 7.14. Mass spectrum for gallic acid

Most UV spectral data in literature are referred to measurements done in methanol but in the case of online identification the solvent is different hence the spectral characteristics obtained from online analysis might be slightly different. (Santos-Buelga et al, 2003). UV-Vis spectra recorded online with HPLC-DAD may differ slightly from those recorded for methanol solutions of the isolated compounds but they provide very useful information for the identification of the compounds.

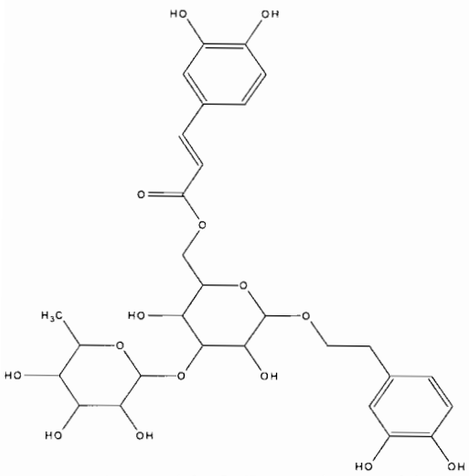
Structures of polyphenolic compounds identified in leaves of the desiccation tolerant plants



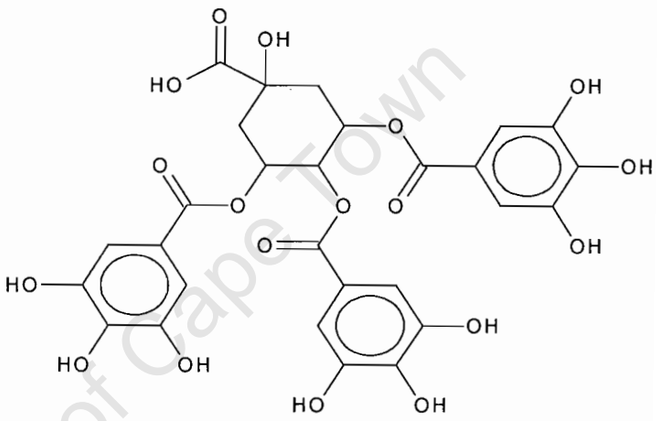
Gallic acid



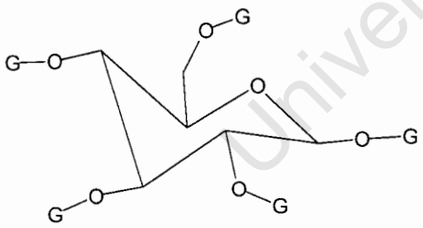
5-caffeoylquinic acid.



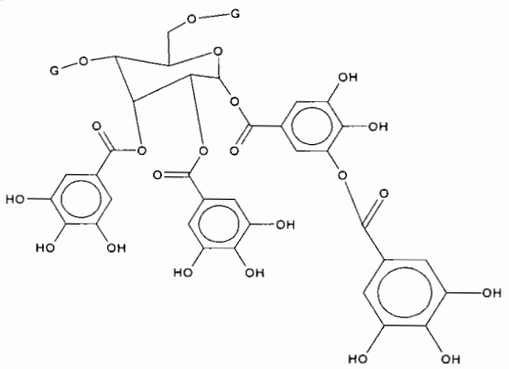
Acteoside 2 (Isoverbascoside).



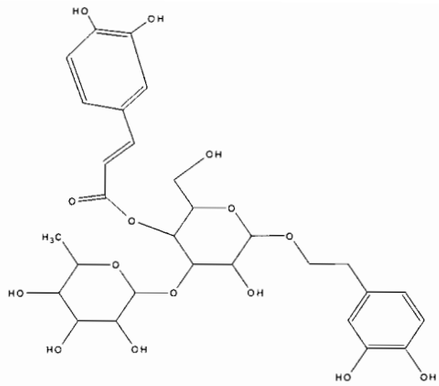
3,4,5 tri-*O*-galloylquinic acid.



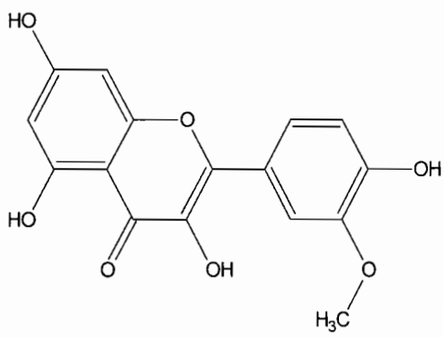
Penta-galloylglucose.



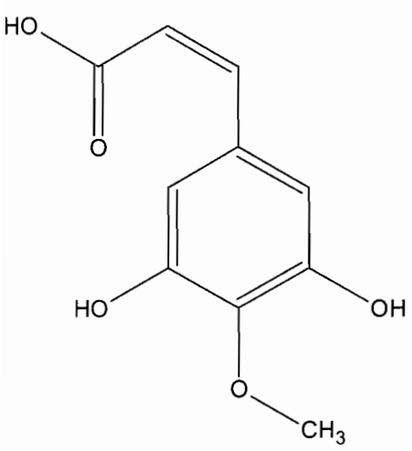
A galloyl-glucose tannin.



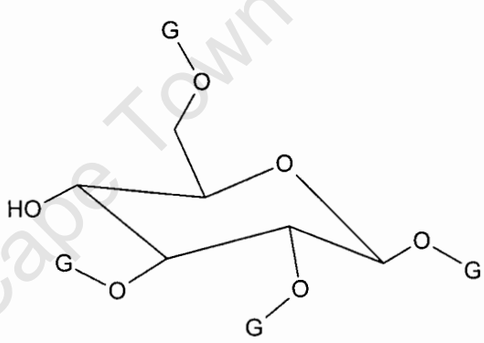
Acteoside 1 (verbascoside)



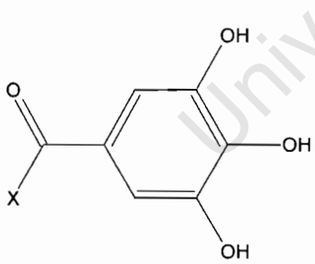
Isorhamnetin



Ferulic acid



1,2,3,6 tetra-*O*-galloyl-glucose.
G- galloyl moiety



Galloyl moiety