


An Ethnobotanical, Phytochemical and Metabolomics Investigation of Plants from the Paulshoek Communal Area, Namaqualand

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Chapter 1: General Introduction and Overview of Aims and Objectives

People and plants have a long intertwined history. Before humans could manufacture what they needed, they had to use natural resources to provide the housing, food and clothes necessary for survival. People also relied on nature to provide medicines and more often than not these came in the form of medicinal plants. Early evidence of the use of medicinal plants dates back to 60 000 BCE. Pollen of several plant species was discovered in the grave of a Neanderthal man (species *Homo neanderthalensis*, a close relative of modern humans *H. sapiens*) at an archaeological site in Iraq. Several of the plant species discovered in the grave are still used today as traditional medicine in Iraq (Gurib-Fakim, 2006). More recent records of medicinal plant use date to several thousand years ago. For example, Mesopotamian cuneiform clay tablets documenting the use of medicinal plants such as liquorice and poppy date to 2600 BC (Gurib-Fakim, 2006) and the Ebers Papyrus, which documents the Egyptian use of medicinal plants, dates back to 1500 BC. The use of medicinal plants assumed more significance in some cultures where it took on a deeper meaning beyond the physical healing properties. The Chinese for example, developed a sophisticated holistic medicinal system combining spirituality with the use of herbal mixtures to heal mind and body that dates back to 2700 BC (Wang et. al., 2011). More modern records originate in Europe, where Dioscorides, a Greek physician, recorded the use of medicinal herbs across Europe in the first century AD. His observations resulted in the book "*De Materia Medica*" which was a leading reference book for European healers for almost 1000 years.

For a large part of recorded history, people did not question how the plants worked, but just accepted their use in treating certain illnesses. However, in the early 19th century scientists became interested in investigating components or ingredients responsible for the observed therapeutic activity of plants. At this time chemistry was developing at a rapid pace with newly synthesised paints, dyes and industrial chemicals available for the first time (Rishton, 2008). Medicines however, were still mostly mixtures derived from bark, roots and leaves. Tonics and tinctures made by stewing whole plant parts in alcohol were popular (McChesney et. al., 2007). This led to a scientific investigation of the plants used to make these tonics, utilizing the simple tools available in turn of the century laboratories. Often working in dangerous conditions and using themselves as the test subjects, researchers began discovering compounds. For example, opium and poppy extract (*Papaver somniferum*) had been used to relieve pain for over 7000 years (McCurdy & Scully, 2005) and scientists were quick to isolate morphine as the active compound in poppy juice. A deluge of

newly discovered active compounds followed: strychnine, caffeine, quinine, cocaine, digitoxin and salicylic acid were among the first of these (Cordell, 2011; Butler, 2004; Rishton, 2008).

The "synthetic" or "golden" era of drug discovery and manufacture began after the end of World War 2, when pharmaceutical companies turned their focus from the war time efforts of manufacturing antibiotics and pain medication to the discovery of new drugs. There was a flurry of discovery of plant based compounds to use as new drugs. Screening of plants in order to discover new bioactive compounds for use as leads in drug discovery, and related drug development programs became widespread. However, the screening process was often long and flawed with relatively few hits. For example, most of the plant extracts in a random collection are inactive when tested in a single assay. Of the small percentage that are active, up to 50% yield previously known compounds, rather than the desired novel compounds (Cordell & Colvard, 2005). Automated high throughput screening (HTS) programs capable of testing thousands of compounds daily increased the speed at which testing could be done in an attempt to increase the hit rate on bioactive compounds. However, natural product discovery programs could not meet the demand for large numbers of compounds to feed into HTS programmes (Baker et. al., 2007). In many instances HTS did not deliver the anticipated results and as a result, many pharmaceutical companies, considering the expense versus the potential benefits, downgraded their natural product discovery research in the 1990s and early 2000s.

As an alternative to the unfruitful screening programmes, researchers turned to modern computational methods for discovering compounds to feed into the drug development pipeline. Many structural features common to natural products (e.g., chiral centers, aromatic rings, and degree of molecule saturation) have been shown to be highly relevant to drug discovery efforts (Balunas & Kinghorn, 2005). Several large natural products libraries existed as a result of considerable HTS efforts and these libraries seemed an attractive source of scaffolds for combinatorial synthesis and target-based computer modelling to design new drug compounds (McChesney et. al., 2007). Such efforts have yielded positive results such the discovery of aurantiamide acetate, an inhibitor of the main proteinase of severe acute respiratory syndrome coronavirus, from the Chinese traditional medicinal plant quinghao (*Artemisia annua*) (Barlow et. al., 2012).

Despite the advances of *in-silico* drug discovery there has been a return to natural products isolation and characterisation since the turn of the 21st century. The potential for finding novel, highly active compounds from natural sources is still high, but the hit rate remains low. So the question becomes: *How do we make natural products a relevant and a viable option for drug discovery*? Researchers

need to investigate different ways to improve hit rates and to discover active plant-based compounds faster and more easily. One way of doing this is by incorporating traditional knowledge into screening programmes. Of the currently available plant-derived prescription products 74% are used in a manner which parallels their traditional use (Cordell & Colvard, 2005) suggesting that traditional medicine may be a good starting point for discovering bioactive compounds (Bailly, 2009). However, in most studies the ethnographic information is poorly integrated with laboratory findings and many studies end up as just disjunctive lists of "they use this for that" or "this contains that" (Etkin, 2001).

There are three main ways of integrating ethnographic information into drug discovery efforts. The first is to screen currently used medicinal plants for activity in assays that reflect their traditional use i.e. testing a plant traditionally used to treat diarrhoea for antibacterial activity. A second approach puts currently used medicinal plant species through a random selection of screens not related to their traditional use in the hope of a serendipitous discovery (Cos et. al., 2006). A third, less often used approach, is to mine old herbal texts such as "*De Materia Medica*" for indication of bioactivity in plants that are not necessarily still used traditionally. The first two methods rely on first hand information gained through discussion with people who currently use traditional medicine, with the input of a botanist being valuable in providing a definitive identification of the plant. However, language barriers may lead to misinterpretation of knowledge gained in this way. The third method presents additional problems as the knowledge is gained second or even third hand allowing errors to creep in. Many of these texts were written before the Linnaean system of nomenclature was introduced making plant identification from vague descriptions and poorly executed diagrams even more challenging.

The question of *how we can use traditional knowledge to increase hit rate and create a better overlap of traditional therapeutic objectives and biomedical ones* remains valid*.* Attendant questions are *how chemosensory properties may influence traditional medicinal use and plant choice,* and *how this knowledge can be applied to selection of plant screening targets for developing new pharmaceuticals or botanicals.* A further challenge is to *evaluate how the often disjointed views of the people who use the plants, the anthropologist who records the interviews, the biologist who identifies and harvests the plants, the microbiologist who performs the bioassays, and the chemist who isolates and characterises the active compounds overlap and complement each other*. This challenge assumes further dimensions when one considers recent advances in technologies such as in the field of metabolomics, which permits detailed molecular profiling of plants, and thus provide

an increasingly rich and complex view of plants which can be juxtaposed with an equally rich traditional understanding of plants.

The aim of this thesis is to investigate medicinal plants from different perspectives in an attempt to arrive at a new, integrated and streamlined method for the discovery of bioactive secondary metabolites of plant origin. This will be done through a focused study of the traditionally used medicinal plants of the Paulshoek region of Namaqualand and a demographic study of the people who use them. Trends in traditional medicinal plant choice will be investigated and methods of traditional knowledge acquisition and transfer will be examined. Additional assessment of bioactivity and trends in bioactivity will be conducted and a variety of physico-chemical and computational techniques will be used to determine the major metabolites present in selected plant species. These different approaches to medicinal plants will be brought together in a single holistic method put forward as a possible way of conducting future studies into discovering active metabolites for potential drug development.

Chapter 2: Use of Medicinal Plants by the People of Paulshoek, Namaqualand

1. Introduction

1.1 History of the Cape and early plant use

There has been a long history and tradition of using plants for medicine at the Cape and in Namaqualand. The indigenous San, Nama and Khoekhoen[1] people living in these areas generated their own knowledge of medicinal plants and plant-based remedies through an understanding of the healing effects of the local flora. In addition, European settlers brought with them their knowledge of European medicinal plants, and this, combined with their experience of the local plants and people, developed into the Cape Dutch medicinal traditional pharmacopeia (van Wyk, 2008a). Together, the indigenous knowledge of the San, Nama and Khoekhoen and healing practices of the Cape Dutch settlers provide a rich history of traditional plant use based on the healing properties of unique plants in a unique part of the world. Medicinal plant use is not a recent phenomenon in this area. The greater Cape Floristic Region (extending from the Cape Peninsula inland, towards the Overberg mountains and Little Karoo, and northwards towards the Cedarberg mountains and into Namaqualand) (Born et al., 2007) has been home to groups of pastoralist Khoekhoen and Namaqua people for at least the past 2000 years, as evidenced by archaeological findings of sheep bones at Spoegrivier Cave on the coast of Namaqualand (Webley, 2007; Boonzaier et al., 2000). The unearthed sheep bones provided some of the first evidence of a pastoralist lifestyle in southern Africa. Southern Africa, including Namibia, has been home to groups of hunter-gatherer San/Bushmen for considerably longer, and the area as a whole has been occupied by hominins for at least 1.5 million years (Deacon & Deacon, 1999). There is evidence that hunter-gatherers used medicinal plants to survive and this is where the roots lie for many of the traditional plant use practices still observed in rural parts of the same area today.

Some of the first documents recording the use of plants in the Cape area and in Namaqualand were created by European (mostly Dutch and French) settlers who began occupying the Cape from the mid 1600's. Most of these reports focused on food plants and the functional use of plants as, for example, building materials and leather curing agents (de Wet & Pheiffer, 1979; Forbes, 1986; van

[1] In this thesis the terms "khoikhoi" and "khoekhoe/n" refer to pastoralist groups of indigenous people while the terms "San" and "Bushmen" refer to hunter gather groups. The terms "Namaqua" and "Nama" refer specifically to Nama-speaking groups of pastoralist Khoekhoen and their modern-day descendants.

Wyk & Gericke, 2000; van Wyk, 2002; van Wyk, 2008b). Prior to this there are very few written records of plant use - as medicine or for other purposes - as the indigenous San and Khoekhoen people had an oral tradition of handing down knowledge from one generation to the next. Early scientific observations of the practice of traditional healing (e.g. Laidler, 1928) suggested that it was usual that a healer would take on an apprentice, often his own son, who would live with him and follow him to learn everything he knew. In some cases this meant certain tasks had to be performed to "strengthen" the apprentice to enable him to perform the duties of a healer. For example, a "snake man" or "poison doctor" was a healer who specifically treated snake and scorpion bites. In the course of his work he would collect snake venom to administer to patients in small amounts as an antidote, and would need resistance to snake bites himself. During an apprenticeship, a healer would administer incremental doses of poison to an apprentice so as to build tolerance to venom, as well as to produce antibodies against the venom (which in turn meant that eventually the apprentice's own blood or sweat could be used to treat patients). The system of apprenticeship and oral tradition, which persisted into the early colonial period (Laidler, 1928) meant that that knowledge of medicinal plants remained almost exclusively in the hands of the healer and his apprentice. As a result and except in rare instances (e.g. the Bleek & Lloyd collection of notebooks written between 1870 and 1881, see http://lloydbleekcollection.cs.uct.ac.za/ for a digital version of this rare documentation of |xam and !kung culture) very little of this knowledge was documented.

The establishment of a European colony at the Cape was a turning point after which it became more usual to keep written records. The indigenous knowledge of medicinal plants was recorded in colony "Flora's" e.g. the works of Thunberg, Hermann, Oldenland and van der Stel (Gunn & Codd, 1981). This was not the only effect colonists had on the local pharmacopeia, traditions and people. A supply station was established at present day Cape Town by the Dutch East India Company (Vereenigde Oost-Indische Compagnie (VOC) in Dutch) to provide water and fresh food to passing ships sailing from Holland around Africa en route to the east. Ships also required medical supplies. Initially the VOC had a system whereby medicinal supplies, mainly herbals in the form of raw plants and powders, were sent via sea from Europe to a central distribution centre situated on Java, in the Indian Ocean. However, the plant material did not travel well and often degraded while in transit to the distribution centre, or during the subsequent trip to the intended recipients (Scott & Hewett, 2008). The proposed solution to this supply problem was to establish a "half-way point" at the Cape, bringing plants from Europe and attempting to grow them at the newly established colony. Several European plants were naturalised in this way and were successfully grown in the VOC Company Garden e.g. *Ruta graveolens* (garden rue) (van Wyk, 2008a). Other species did not fare as well, and it

was decided to attempt to seek alternative species from the local indigenous flora (Scott & Hewett, 2008).

At the time, in the late 1600's and early 1700's, it was popular for colonists to travel northwards from the Cape on "journeys of exploration" to find exploitable resources and new areas suitable for farming (Scott & Hewett, 2008). These trips enabled colonists to examine and record plant use by San, Khoekhoen and Namaqua people in order to augment their existing knowledge of plants, particularly medicinal plants. The trips also afforded the opportunity for botanists to seek new or alternative medicinal plants to replace the European ones they were unable to grow at the Cape (Britten, 1920). Substitute plant species were often chosen on the basis of their resemblance to the European plants they were replacing. This method was not without merit as today we know that similar smelling plants may contain similar chemical compounds, and therefore, have a similar pharmacological action (Harborne, 1993; Harborne, 1998). In some instances colonists simply adopted the Khoekhoen and Namaqua use of particular plants (e.g. the use of *Sceletium* species as a calmative agent and mood enhancer (Gericke & Viljoen, 2008), juice from *Aloe* species to treat stomach complaints (Grace et al., 2008) and buchu (*Agathosma* species) to treat a variety of ailments) (Laidler, 1928). In other instances, entirely new concoctions were made from indigenous plants. For example, the oil of *Osmitopsis astericoides* (Asteraceae) was used in the form of a brandy tincture to create a remedy for treating chest troubles and for reducing inflammation and healing wounds when applied directly as a powder (Viljoen et al., 2003). Some of these new remedies and new methods of medicine preparation were adopted by the Khoekhoen and Namaqua people. Results of this back-and-forth intermingling of colonist and indigenous knowledge and practice can be seen today in the wide variety of plants used and the many different methods of treating illness currently employed by the people of Namaqualand.

While the colonists were mounting their trips of exploration and interacting with local people to record traditional knowledge, the 1700's proved to be a challenging time for many Namaqua people. Contact with settler farmers, runaway slaves and more generally with people from the ever-expanding colony brought smallpox, measles, tuberculosis and venereal disease with them. More importantly, these were diseases for which indigenous traditional plant medicine had no remedy (Laidler, 1928). In response to this, new plants were sought and new treatments created from existing plant knowledge, changing the way that Namaqua people viewed medicine and plants (Cocks & Dold, 2000). It is generally thought that there were different types of healers to treat different types of illnesses, in a division similar to that still present in societies reliant on traditional medicine today. A "bossiesdoktor" (plant doctor), for example, used herbal remedies to treat

"natural" illness. Other types of illness were considered spiritual and required a specialist with divining powers or the ability to call on ancestor spirits to assist in diagnosis and treatment (Cocks & Dold, 2000; Richter, 2003; van Wyk, 2008a; Dahlberg & Trygger, 2009). This, and the later 19th century influence of Christian missionaries, settling Dutch farmers and difficult drought years changed the traditional semi-nomadic pastoralist lifestyle. As lifestyles changed, and previously unseen diseases arose, the pharmacopeia changed accordingly to suit people's requirements. Some knowledge was forgotten or lost, while other knowledge was added and plants were discovered to treat the new diseases (Grenier, 1998; Dold & Cocks, 2000, Berkes & Turner, 2006). This is also true of the modern day pharmacopeia which is dynamic and constantly evolving.

While Namaqualand and its people were changing, so too was the structure and composition of society at the Cape. It became important to update and review existing documentation as practices and applications of traditional medicine changed. The old colony Floras were soon no longer sufficient and an attempt at documenting all medicinal plants known in the area was done by Pappe in the mid 1800's (Pappe, 1847; Pappe, 1850). New reviews focusing only on the medicinal tradition of the Namaqua people were published. A particularly good review was done by Laidler (1928), who attempted to record the plant names in the local Namaqua language, along with the method of medicine preparation and the rationale for the choice of plant used. Reviews which focused on the medicinal plants in use at the Cape were collated and updated and botanists began to record more information in more detail. The most comprehensive and often cited general work on medicinal plants in southern and eastern Africa is the 1962 publication of Watt and Breyer-Brandwijk (1962), which provided a good summary of medicinal plant use by the major groups of people in South Africa. Since then there have been several works which document the use of medicinal plants by specific groups of people e.g. Xhosa, Zulu or Sotho in South Africa (Hutchings, 1989; Bhat & Jacobs, 1995; van Wyk et al., 2008a are noteworthy) or which focus on the treatment of a specific category of illness (e.g. wound care) (Grierson & Afolayan, 1999; Archer, 1994).

The late 20th century saw an increase in the interest in medicinal plants and traditional healing by scientists and as well as the general public. This interest was increased by the pharmaceutical industry's investigations into using traditional medicines as starting points for drug development. Some non-scientists became interested for other reasons such as the popular trend in "alternative medicine". With the shift to modern medicine and the supposed apathy of the youth to learning the "old ways" of traditional healing, it was thought that much of the knowledge which was previously passed on orally would be lost if not properly recorded (Dahlberg & Trygger, 2009). This prompted a flurry of activity as scientists, anthropologists and ethnobotanists attempted to record as much of

what is known as possible at various locations around the world. The modern syntheses of authors such as van Wyk et al. (1997) focus on South African plant use and provide historical information as well as information on customs and traditions that are still observed. Books like these are invaluable, but unfortunately are not all-inclusive. The pharmacopeia and traditions of many regions of South Africa, and indeed the world, remain unexplored.

1.2 Namaqualand - the broader context of the study

1.2.1 Location and climate

Namaqualand is an area of about 50 000 km^2 and is located in the upper North West part of South Africa. The area is bounded by the Orange River in the north, the Olifant's river in the South, the Bushmanland plains in the east and the Atlantic Ocean in the west (Desmet, 2007; Cowling et al., 1999; Cowling & Pierce, 1999). This large area encompasses a variety of soil types, vegetation types, altitudes and rainfall regimes, resulting in Namaqualand being divided into seven biophysical regions: Bushmanland, East Gariep, Hardeveld, Knersvlakte, Sandveld, Richtersveld and Kamiesberg (Figure 1) (Desmet, 2007).

The Kamiesberg Mountains form the highest part of Namaqualand, with the highest peak in the range - Rooiberg - standing at 1700 m above sea level. This rugged granitic mountain range is also the wettest part of Namaqualand, receiving up to 400 mm of rain annually, more than double the average annual Namaqualand rainfall of 150 mm (Desmet & Cowling, 1998). The rest of Namaqualand experiences a wide range in rainfall, with a general trend of decreasing annual rainfall amounts the further north (i.e. towards the deserts of Namibia) one travels. The driest parts receive as little as 50 mm of rain per year, which classifies most of Namaqualand as a semi-desert or desert. There is also a distinct difference in rainfall type across the breadth of Namaqualand, with the eastern parts (closer to the Nama-Karoo biome) receiving mostly summer rainfall (November to March), while the more westerly parts experience mostly winter rainfall (May and August) (Desmet, 2007).

Temperatures are generally mild for the region as a whole, but extremes do occur with peaks of up to 40 °C at the height of summer and occasional frost and snow falling in the Kamiesberg in winter (Cowling et al., 1999).

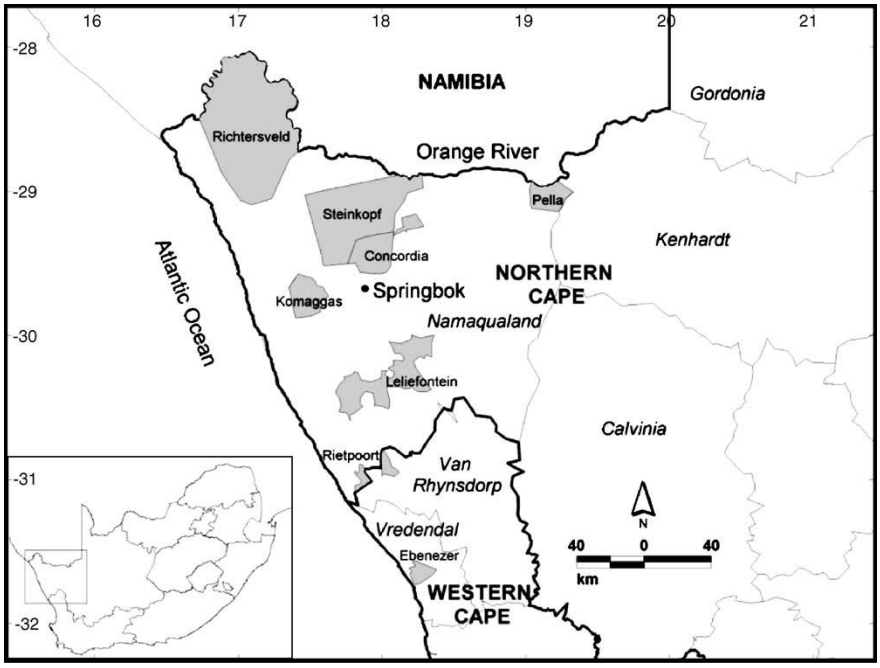


Figure 1: Map of South Africa (insert) and detail of Namaqualand, showing the location of the communal reserves (from Rhode et al., 2006).

1.2.2 Endemism and plant composition in Namaqualand

Namaqualand is part of the Succulent Karoo biome[2] (Milton et al., 1997) and makes up approximately a quarter of the total area covered by this vegetation type (Desmet, 2007). There are an estimated 6.5 endemic plant species per 100 km^2 in the Succulent Karoo. This high level of endemism, combined with a wealth of biodiversity, means that the Succulent Karoo is one of two deserts to be recognised on the list of the top 25 priority "conservation hot spots" globally (Myers et al., 2000). The Succulent Karoo is so speciose that it supports 2.6 times more species than an equivalently sized area of the adjacent Nama-Karoo vegetation type (Cowling et al., 1999).

There are approximately 3500 species in 135 families and 724 genera in Namaqualand, and it is estimated that between 25% and 40% of those are endemic (Desmet, 2007; Desmet & Cowling, 1999). Some experts even suggest greater than 50% endemism within some families (Cowling et al., 1999). For example, there are estimated 850 species of *Mesembryanthemaceae* (the 'mesembs' – a sub-family within the *Aizoaceae*) in 100 genera in Namaqualand (Desmet & Cowling, 1999; Smith et al., 1998). The majority of these species have been proven, on the basis of molecular work, to have evolved relatively recently between the last 3.8 and 8.7 million years (Klak et al., 2004; Ihlenfeldt, 1994). The family is characterized by having hygrochastic fruit capsules which favours short distance

[2] Naming of the biomes e.g. Nama-Karoo follows Mucina & Rutherford, 2006

seed dispersal. Rapid, adaptive allopatric speciation is thought to have been common in the evolutionary history of the family and is one explanation for the high degree of endemism in a relatively small region (Linder & Hardy, 2004; Ellis et al. 2006).

A few families dominate the Namaqualand vegetation, in particular the speciose *Aizoacae*, *Asteraceae* and *Crassulaceae* families (Cowling et al., 1999). The great diversity in these, and several other dominant families, may be explained by looking to past climate conditions and changes in these conditions during the Pleistocene era (ca. 1.8my BP - 100ky BP) (Ridley, 1996; Mckenzie & Barker, 2008). Recent investigations using fossilised pollen from Eksteenfontein Spring Cave in the Northern Cape suggest that the climate shifted from cooler and more humid conditions to drier and warmer conditions resembling the modern day climate of Namaqualand around 12.5ky BP (Scott et al., 2012). Aridity is thought to encourage speciation in general, and it is particularly favourable for CAM adapted species, which many of the Namaqualand species are (Richardson et al., 2001; Landrum, 2002). It has been suggested that even under peak glacial conditions of the Last Glacial Maxim (LGM) (ca. 21 - 18ky BP) the Succulent Karoo biome retained a core bioclimatic range within Namaqualand i.e. there was a stable climatic refuge that didn't vary too much with global trends (Midgley et al., 2005; Midgley & Thuiller, 2007). Thus Namaqualand may have acted as a glacial refuge for succulent species, as did frost free valleys in the Eastern Cape for succulent thicket vegetation during the Pleistocene (Cowling et al., 2009). It is possible that succulent species could have persisted under cooler global temps in the core refugia of the Succulent Karoo and may not have been displaced even under peak glacial conditions (Burgoyne et al., 2005; Ridley, 1996). Thus, Namaqualand provided a stable local climate during times of extremes elsewhere, thereby allowing the region to retain species diversity at a time when other areas were losing theirs (Desmet, 2007). This means regional persistence, fewer extinctions and more speciation once the climate returned to optimum, resulting in the wealth of species diversity in Namaqualand today (Cowling et al., 2009; Jansson, 2003).

Despite this wealth of biodiversity, only a very small percentage of the land area is conserved in formally protected reserve areas. Threats to conservation include mining, poor farm management (specifically overgrazing) and illegal plant harvesting for medicine or black market trade (Lombard et al., 1999). Lombard (1999) proposes a method of choosing new areas for general reserves and many papers deal with restoring degraded rangeland and old mining areas using a variety of ecological restoration techniques (Visser et al., 2004, Carrick & Krüger, 2007; Simons & Allsop, 2007; Botha et al., 2008; Wassenaar et al., 2012), but it is also important to identify priority medicinal plants that may be under threat.

1.2.3 Plant life strategies and other survival adaptations

In addition to high levels of endemism, plants in Namaqualand also exhibit a variety of unique life strategies and growth form adaptations that facilitate these life strategies. Many have specifically evolved to enable continued survival in the semi-desert conditions of the area. While annual rainfall is low for most parts of Namaqualand and individual rainfall events bring only light showers, the rainfall is predictable across the Succulent Karoo (Cowling et al., 1999). Rainfall is considered to be 20% more reliable in Namaqualand than in the neighbouring Nama-Karoo. This is thought to have contributed to the predominance of leaf succulence (as opposed to stem succulence often seen in other semi-desert or desert vegetation types), dwarfism and shallow rooting (Carrick, 2003) as plants need to make the most of moisture when it is available (Figure 2). Together, small, shallow-rooting succulents and geophytes comprise over 50% of the species in Namaqualand, making these the most common growth forms (Desmet, 2007).

Figure 2: Examples of dwarfism on the left (this specimen of *Ornithoglossum vulgare* B. Nord. is approximately 5 cm high) and leaf succulence on the right (the stem of *Cotyledon cuneata* Thunb. is hidden by succulent leaves in this picture). Photographs taken by N. Wheat.

The relatively mild temperatures in autumn and early winter also means that most plants do the major part of their annual growth at these times - a time when light is not at its maximum availability (Esler & Rundel, 1999). Plants have to make the most of light but still be able to minimise the negative effects of heat and short term drought, a feat achieved via leaf succulence and CAM photosynthesis. CAM (Crassulacean Acid Metabolism) photosynthesis is one of the most important adaptations to living in these conditions. As the name suggests, it is commonly found in species of the family *Crassulaceae*, however it is not unique to that family. This method of photosynthesising is also found in mesembs, Aloes and a variety of other families e.g. *Oxalidaceae* and *Orchidaceae* (Rundel et al., 1999). CAM differs from the other methods of photosynthesis, i.e. C3 and C4, in that carbon dioxide is fixed into organic acids at night, with deacidification occurring during the day,

rather than the other way around (Lambers et al., 1998). This means that the stomata are open at night when temperatures are cooler and less moisture is lost – a direct adaptation to the conditions in Namaqualand.

Great endemism, specialised life and survival strategies and recent divergent evolution means that there are many unique plants in Namaqualand, some of which may contain unique secondary metabolites. Theses metabolites may be active in traditional medicines and could be a possible source of templates for future drug development. This study aims to investigate these secondary metabolites in the context of traditional healing systems, with a view to potential drug discovery. The aims of this particular section of the study are covered in more detail in the "Aims and Objectives" section of this chapter.

1.2.4 A brief history of the study area

While plants have evolved different growth forms and life strategies to enable survival in the harsh Namaqualand climate, the people of Namaqualand have also needed to adapt as outside influences forced lifestyle changes upon them. As previously mentioned, Namaqualand has a long history of occupation by small groups of hominins (Deacon & Deacon, 1999). San hunter gatherers lived in Namaqualand prior to groups of Nama-speaking Khoekhoen pastoralists moving into the area approximately 2000 years ago (Webley, 2007). With the advent of colonialism, Namaqualand and its people changed forever, particularly with the creation of the communal reserve areas and the restriction of seasonal transhumance practiced by goat and sheep herding pastoralists. Hoffman and Rhode (2007) suggest that the development of Namaqualand can be divided into three distinct phases - 1) the pastoral ecological revolution: domestic livestock were introduced to the Namaqualand region for the first time and there was a general shift to a pastoral society (approximately 2000 years ago) 2) the colonial ecological revolution: colonists arrived, mission stations were established and cropping was encouraged and 3) the post-agrarian ecological revolution: there was a general decline in agriculture and a shift to post-apartheid transformation. Medicinal plants were used throughout all of these periods to a greater or lesser extent.

Key events in the history of the region are summarized in Table 1. With the influx of people in the 1750's moving into Namaqualand from the Cape to establish "loan farms", came conflict. Land disputes between the indigenous Namaqua people and the settling farmers became more common as the establishment of farms meant that the seasonal movement of pastoralist Namaqua groups was hindered (Rhode et al., 2003). In the late 1800's, the displaced Namaqua people appealed to the church to help them reserve grazing land as more and more farms were being staked out. The church established a mission station at Leliefontein, under the authority of Reverend Barnabas

Shaw, and created six reserve areas - which later became the present day communal reserve areas (May & Lahiff, 2007) (Figure 3). Groups of Namaqua people moved in to the reserve areas with their herds, and were encouraged by missionaries to become more sedentary and give up their semi-nomadic pastoralist lifestyle. At the Leliefontein mission station they were taught to plant and maintain crops such as wheat, oats, barley and rye (Rhode et al., 2003). This agricultural lifestyle was relatively precarious and frequent droughts made cropping difficult. People took their remaining livestock and moved away from the mission station to find pasture, abandoning their crops. In an attempt to retain his congregation during the severe drought of 1820 for example, Rev. Shaw followed them, establishing small mission outposts along the way. The ten outposts thus established were the beginnings of what are the present day villages in the Leliefontein communal reserve area - Klipfontein, Kharkams, Leliefontein, Nourivier, Paulshoek, Tweerivier, Kiess, Rooifontein and Kamassies (Rhode et al., 2003).

In the mid 1800's the role of the church become even more entrenched in Namaqua society as, the church was issued an official "ticket of occupation" by the colonial government at the Cape for the land surrounding the Leliefontein mission station and its outposts. This ticket of occupation conferred a form of "land ownership" to the church. This was the first formal step in the process that eventually led to the displacement of the people of Namaqualand and their segregation into reserve areas under first church, and then state authority (Rhode et al., 2003; May & Lahiff, 2007). It was also around this time that copper mines opened in the area, and many Namaqua people who had no remaining livestock after the droughts of the mid 1820's eventually turned to formal wage earning on the mines and farms surrounding Leliefontein in the mid-1800s, exchanging their pastoralist lifestyle for more a more sedentary one dependant on employers for wages (Scott & Hewett, 2008). As with settling at the mission station in the early 1800's, the change in lifestyle was beneficial, if only for a while. In the late 1800's drought struck again and many people surviving on farm labourers wages found themselves without jobs. Production at the once prosperous copper mines slowed and people who were dependant on mining wages also found themselves without jobs. Widespread destitution followed. Those who were able to do so began farming with small stock as they are easier to keep in times of drought. Goats became the most commonly herded animal in the area and this remains so today (Rhode et al. 2003; Hoffman & Rhode, 2007).

The Mission Station and Communal Reserves Act (enacted in 1913), was the final influence on Namaqua people that cemented the transition from a pastoralist to a sedentary lifestyle. The Act transferred control and governance of communal areas to the state, and in the case of Leliefontein this meant that the church was no longer the local authority. The national government declared

Leliefontein an official “native reserve”, with Afrikaans the main language. Namaqua people could no longer move beyond this area in the traditional ways of transhumance and thus the villages in the reserve started to become more established as people settled down (Rhode et al., 2003; May & Lahiff, 2007). Traditional healers became associated with certain areas, which meant that they used locally available plants and had to share with other healers to obtain plants not found in their immediate vicinity.

Ownership of the land would only begin to be transferred back to the Namaqua people in the late 1990’s, when the post-apartheid government passed The Transformation of Certain Rural Areas Act (TRANCRAA). TRANCRAA allowed the transfer of land held in a trust by the Minister of Agriculture and Land Affairs to either a municipality or a Communal Property Association. The people from Leliefontein elected for their land to be governed by the local municipalities with elected office bearers (May & Lahiff, 2007; Petersen, 2004) rather than by an external national entity.

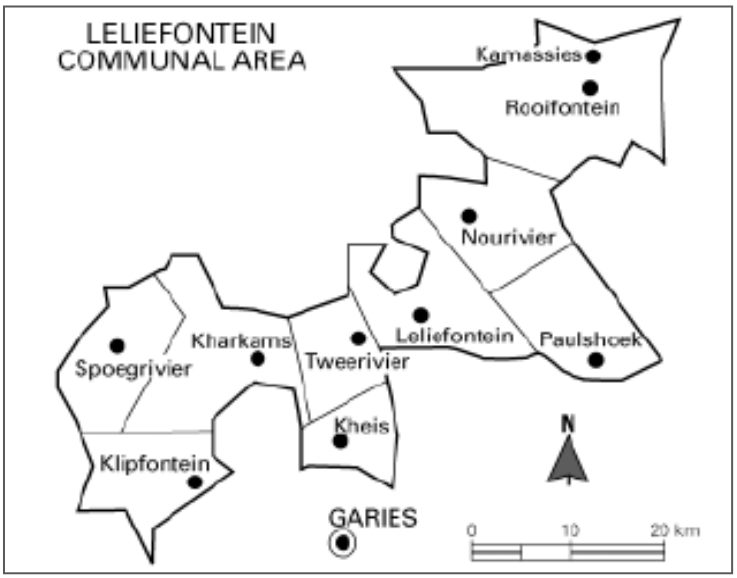


Figure 3: Map of the Leliefontein communal reserve area showing the location of the ten villages. Paulshoek is the most south easterly village (from Rhode et al., 2003)

Table 1: Timeline of major events in the history of Namaqualand and, specifically, the Leliefontein reserve (compiled from data from Scott & Hewett, 2008; Hoffman & Rhode, 2007; Webley, 2007; May & Lahiff, 2007; Rhode et al., 2003; Petersen, 2004; Price, 1976; Lebert & Rhode, 2007; Cousins et al., 2007 and Penn, 1995. Additional specific references given in text).

Date	Event
2100/2000BP	Archaeological evidence at Spoegrivier Cave, on the Namaqualand coast, suggests sheep herding on a small scale. This is the earliest evidence of pastoralism in South Africa
800BP	Archaeological evidence at Bethelsklip (in the modern day Leliefontein communal area) of sheep and goat herding
1600s	European colonists arrive at the Cape and the Dutch East India Company (Vereenigde Oost-Indische Compagnie (VOC) in Dutch) establishes a supply station for ships sailing east from Europe
1661	Colonists mount expeditions of exploration northwards from the Cape and encounter several large groups of Khoekhoen pastoralists whom they call the "Little Namaqua" in the vicinity of the Kamiesberg
1685	Simon van der Stel ventures northward in search of copper and encounters groups of pastoralist "Namaqua" with herds of cattle and sheep. He also encounters a few hunter/gatherer groups of San/Bushmen
1720s	Smallpox, introduced to the colony at the Cape in 1713, spreads northwards and decimates both the European and indigenous populations, continuing to do so for the next 100 years
1750s	The first European colonists move onto "loan farms" in Namaqualand and begin farming
1770s	Conflict arises between indigenous Namaqua people and settling European farmers. Land disputes become more frequent
1779	Namaqua leader "Captain Wildschut" centres his group of pastoralists in the Leliefontein region of Namaqualand for summer grazing
1779	On a trip to Namaqualand, Robert Gordon records the area as inhabited by very few people. He is quoted as saying that "this entire nation consisted of about four hundred men and women, as well as children" (Raper & Boucher, 1988)
late 1780s	A new group of people, the "Bastaards", resulting from the union of loan farmers and Namaqua women, as well as other race groups, arises. Many of these mixed race individuals move to the colony at the Cape to associate themselves with Europeans, in preference to staying in Namaqualand and assuming the "lower status" of the Namaqua people
late 1780s	"Bastaards" allowed to acquire loan farms in Namaqualand
1780	The previous popularity of loan farms wanes and the northward expansion into Namaqualand slows nearly to a halt
1790s	"Bastaards" lose their status in colony society and many are turned into bonded labourers at the Cape. Most return to Namaqualand or settle further afield in Bushmanland
1798	The Kamiesberg has so few people (due to the smallpox epidemic) that it is described by settlers as "formerly inhabited by Namaqua

	Hottentots". A company (presumably the Dutch East India Company) builds a rudimentary road across the Kamiesberg making it easier for "wandering peasantry" to settle in the area
Early 1800s	Pastoralist Namaqua people living in the Leliefontein area, led by Wildschut, appeal to the church to help protect their traditional grazing land from the "wandering peasantry" (settlers, runaway slaves, Bastaards etc.). Missionaries are more than willing to help and the six modern day "coloured reserve areas" (CRA's) are created by formal church protection of land.
1816	Reverend Barnabas Shaw establishes a mission station at Leliefontein. Drought and continuing disease cause the remaining Nama people in the area to seek refuge at the new mission station and Rev. Shaw becomes their leader, governing the area. In an effort to shift the Nama people from a nomadic-pastoralist way of life to a more sedentary one, they are encouraged to plough and plant crops (mostly wheat, oats, barley and rye).
Early – mid 1820s	Namaqua people abandon their crops and leave the Leliefontein mission station with their remaining animals in the wake of severe droughts. Rev. Shaw follows them and establishes mission outposts wherever they go. These outposts eventually became small settlements in their own right and today reflect the locations of some of the main villages in the communal reserve area
1800s	With increasing settlement in the area, the seasonal transhumance of the Namaqua people is limited. Eventually many become sedentary at the mission outposts and by 1874, 268 families (approximately 2000 people) are living in the Leliefontein reserve
1850s	Copper mines are established in Namaqualand, mostly in the vicinity of Springbok
1854	Present day CRA's previously established by the church in the early 1800's are formally acknowledged by the British Cape Colony Government. The Leliefontein mission station is issued a "ticket of occupation" which gives leasehold title to the church. Namaqua people living in the area are only given occupational status.
1855	A large church is built at Leliefontein
1856	A civil commissioner and resident magistrate are appointed at modern day Springbok, further establishing the authority of the colonists and missionaries in the region
1870s	Typhoid breaks out on the copper mines
Late 1870s	Copper production begins to decrease at the mines in Namaqualand
1878	The Cape Government requires the registration of everyone living in Namaqualand in order to collect a new "house duty" tax
Late 1888	The global demand for copper plummets and as a result many mine workers are laid off. This causes widespread poverty in the previously prosperous Leliefontein area
1881 – 1883	A series of severe drought years cause crop failures. Labourers on commercial farms are laid off and previously poor farming families become destitute. Those families able to switch from keeping cattle to herding smaller livestock do so.

1895	More drought hits the already stricken area
1909	The Mission Station and Communal Reserves Act (enacted in 1913) means that church control and mission station governance of the Leliefontein reserve are replaced by direct state authority
1913	Leliefontein becomes an official "native reserve" under control of the state. Afrikaans becomes the *lingua franca,* although older people still speak Nama. Most people still refer to themselves as "Namaqua"
1919	After struggling for years, the Cape Copper Company shuts down and closes all its' mines in Namaqualand. Many miners lose their jobs.
Early 1920s	More farmers, many of them Namaqua, settle in the reserve and start farming with cattle and goats and planting crops. Due to the hardship of this way of life, many reserve settlers are convinced at this time to sell all of their possessions and move to Steilhoogte (a farm on the southern side of the Olifants River) for a better life, only to return destitute
1920s	Leliefontein (the settlement) becomes overpopulated and people begin leaving as land pressures increase. Some people move to the mission station outpost of Paulshoek (which was used at the time to keep rams separate from the rest of the herd in the run up to the breeding season)
1925	Returning residents left destitute after the Steilhoogte debacle work on the Garies/Platbakkies road for basic food rations
1920s	Global economic depression
1930s	Severe drought hits again forcing even more families to move from outside into the reserve and from the overcrowded Leliefontein village to Paulshoek
1920 – 1960	The influx of farmers to the area means widespread ploughing and a rapid expansion of croplands
1940	The Land Settlement Act gives grazing rights inside the reserve to white farmers. These rights could be converted to ownership rights
1950s	Many of the former "Bastaards" and their descendents leave the Leliefontein reserve to settle further north in the Richtersveld
1950s	Many recently created croplands are abandoned as the younger generation move away to find more profitable work on commercial farms and diamond mines
1950s	The Group Areas Act confines coloured people (i.e. Namaqua descendants and people of mixed heritage) to the CRA's and forces white farmers living in the area to move outside the reserve. Many people who had moved away returned to settle in the reserve. Commercial farms (owned by white famers) bordering on the communal area are fenced with the assistance of government subsidies, restricting communal grazing.
1960s	Villagers become fully sedentary for the first time in several of the villages associated with communal areas. Most people are dependent on state grants, income generated from farm labour, or money sent home by family members working at the mines or in

	the major urban centres of Namaqualand
1963	The Coloured Rural Areas act allows the Minister of Coloured Affairs to review land use and tenure in the CRA's, dividing up the land into "economic units" and allocating these units to selected individual farmers (this act was subsequently amended and then replaced by the Rural Areas Act of 1979)
1970s and 1980s	More people settle in the towns associated with the communal areas as they are seen as places where one can survive on a very tight budget, thus making it more affordable for the unemployed and poor to live there
1979	The Rural Areas Act is passed by government which formalised the notion of "economic units" of land (first mentioned in the Coloured Rural Areas Act of 1963) which would be privately leased to farmers. This is first implemented in Leliefontein in 1984
1984	Leliefontein reserve is divided into 47 farming units for private lease. Many people lose access to grazing land and angry residents opposing the new policy and force would-be occupants off the new units
1988	The Rural Areas Act is overturned by the Cape Supreme Court. The policy of "economic units" is set aside and communal land is restored to communal farmers
1990s	Land reform policies and the RDP (Reconstruction and Development Programme) bring infrastructure improvements to the village such as RDP housing. A land reform programme is stated to increase the commonage area.
1993	The Land Titles Adjustment Act allows for the updating of land title deeds that had not been maintained (e.g. through non-registration of change of ownership upon inheritance of land) and allows investigation by a commissioner into the rightful owners where the legal ownership was unclear.
1994	Transition to democracy in South Africa
1996	The constitution of South Africa is established, which obliges the government to implement a programme of land reform, restoring historical land and redistributing land and resources
1995	Communities in Namaqualand request a formal district planning project to manage land reform in their areas
1998	The Transformation of Certain Rural Areas Act (TRANCRAA) allows the transfer of land held in a trust by the Minister of Agriculture and Land Affairs to either a local Municipality or a Communal Property Association
1999	The District Planning and Management Project requested by residents in 1995 is completed and provides a framework for future resolution of conflicting land claims as well as an action plan for the acquisition of new communal land
2000	The local municipalities of Richtersveld, Khai Ma, Kamiesberg and Nama Khoi are established within the greater "Namakwa District". Leliefontein reserve falls under the Kamiesberg municipality and is currently administered by a Commonage Committee

1.3 Paulshoek - the study area

1.3.1 Climate and vegetation

There are six communal reserve areas in Namaqualand which were previously known as "Coloured Reserve Areas" (CRA's): Richtersveld, Komaggas, Steinkopf, Leliefontein, Concordia and Pella (Figure 3). These are the ultimate result of church protection of land during the 1850's (Table 1). As of 2007, they make up 22.6% of the land area in Namaqualand (May & Lahiff, 2007). Specifically, this study was done in the village of Paulshoek (30°25'S; 18°15'E) in the communal reserve of Leliefontein. The Leliefontein reserve covers approximately 190 000 ha of land in the Kamiesberg region of Namaqualand. A post-apartheid land distribution programme known as the Municipal Commonage Programme acquired an additional 33 000 ha between 1998 and 2000, bringing the total reserve area to 223 000 ha (Lebert & Rhode, 2007).

Paulshoek is the most easterly of ten villages in the reserve and is relatively isolated, being situated 27 km away from the nearest village and about 50 km along a gravel road from the town of Garies. The village lies approximately 1400 m above sea level and receives an average of 180 mm of rain annually (unpublished data). This is a slightly higher than the average annual rainfall for Namaqualand but not nearly as much precipitation received by some of the wetter parts of the Kamiesberg. There are no permanent rivers in the village and people rely on borehole water for their main water supply which is augmented by household water storage tanks which collect run-off water from roofs. A small dam fed by precipitation run-off from a large granite outcrop behind the village supplements the water supply, but water shortages are a common feature of the village life (Petersen, 2004). The climate is generally mild, but temperatures can reach up to 37°C in summer, and in winter there may be frost and light snow due to the altitude. Temperature extremes may occur in a single 24 hour period, from near freezing at night to upper 30's during the day, especially during October and November (Petersen, 2004).

The vegetation is typical of the Kamiesberg region and is dominated by species belonging to the families *Mesembryanthemaceae*, *Aizoaceae*, and *Asteraceae*. Succulence is common, as are geophytes. Larger shrubs and a few trees grow along seasonal river courses and at the base of granite outcrops, where runoff accumulates (Le Roux, 2005). The communal grazing land surrounding the village is degraded in places. Like other disturbed areas in Namaqualand it has become dominated by *Galenia africana*, which is generally unpalatable to livestock (Simons & Allsop, 2007). Studies suggest that under prolonged heavy grazing, the perennial-dominated shrubland becomes replaced by annuals and geophytes and that this trend is not easily reversed once grazing pressure has been reduced (Anderson & Hoffman,

2007; Todd & Hoffman, 1999). This may be one of the contributing factors to the noticeable difference between the quality and composition of the vegetation on the communal rangelands and the adjacent commercial farms. Stocking rates on communal lands tend to be higher than the rates recommended by the South African Department of Agriculture and in some cases twice the number of recommended animals grazes a particular area of the commons (Hoffman & Ashwell, 2001). The impact of this on vegetation is most noticeable along fence lines, where glaring differences between communal and commercial land often exist (Todd & Hoffman 1999, Todd & Hoffman, 2009). Heavy or overgrazing is one of the main causes for vegetation degradation and loss of biodiversity, with an estimated 30% reduction in species numbers in the areas immediately surrounding a stock post (Hendricks et al. 2005; Haarmeyer et al., 2010). Recent studies suggest that under a heavy grazing regime woody perennial shrubs, many of which are medicinal plants, decrease and fast growing annuals and herbaceous species increase (Anderson & Hoffman, 2011). Moderate grazing is thought to have only a moderate effect on vegetation (Haarmeyer et al., 2010). The study of Goldberg (1998) suggested that there was no significant impact on medicinal plants from either grazing or harvesting, but the true effect of grazing on medicinal plants is not well documented and deserves further study.

1.3.2 Creation of the modern village and its infrastructure

Leliefontein mission station was established in the early 1800's (Table 1). Later, satellite mission outposts were established and these became the ten villages in the reserve, of which Paulshoek is one (May & Lahiff, 2007; Petersen, 2004). The reserve was acknowledged by the government at the Cape in the mid 1850's and the Leliefontein reserve was officially established as a "coloured reserve" in 1913, but it wasn't until the 1960's that the first permanent houses were built in Paulshoek. It is said that Paulshoek got its name because rams being kept separate from the rest of the herd prior to breeding season were penned in the area by a man named Paul Joseph - hence "Paul se hoek" ("Paul's corner") (Petersen, 2004). Prior to this, there were few people living in Paulshoek mostly herding goats or planting crops. Most of them lived in traditional mat houses ("maatjieshuise") and built outdoor shelters for cooking, but some had more permanent structures. With the advent of the Group Areas Act in the 1950's, more people moved into the reserve as a whole. Paulshoek experienced an influx of people as it offered a small school, a church and the possibility of grazing land, and was therefore attractive to those seeking security in uncertain times (Rhode et al., 2003). This trend continued and there was a fivefold increase in people in Paulshoek between 1960 and 1997 (Rhode et al., 2003).

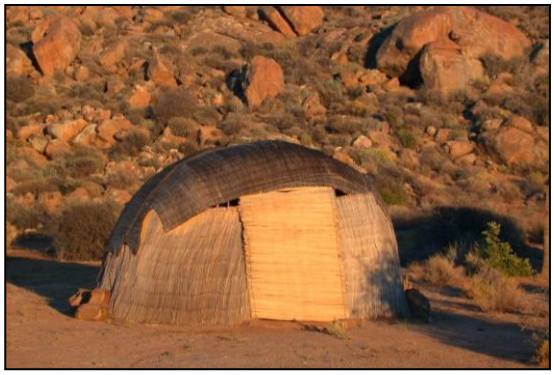


Figure 4: Traditional reed mat house "matjieshuis" (left) and typical modern houses (right), with outhouse on the premises (photos taken by Timm Hoffman (left) and N. Wheat (right)).

Today there are approximately 100 houses in Paulshoek, housing between 400 and 450 people (99 houses and 405 individuals were recorded in a survey of the people living in the village in 2010 (Duijnstee, 2011).

The number of inhabitants fluctuates as people return to stay with family when out of work, leave to search for work or seek opportunities in another village in the reserve or in the nearest large centres e.g. Vredendal. Some of the houses are simple Reconstruction and Development Programme (RDP) style houses built by the government as part of the post-apartheid upliftment project aimed at improving the quality of life of the poorest South Africans (May & Lahiff, 2007; Petersen, 2004; Rhode et al., 2003). However, many houses consist of more than two rooms and some still have a traditional cooking shelter built from dried bushes and branches (referred to as a "kookskerm") in the yard (Figure 4).

In recent years, since the establishment of a local municipality for the Kamiesberg region and the implementation of TRANCRAA, there have been significant improvements. Most houses now have electricity (running on a pre-paid meterage system), telephones (also on a pre-paid meterage system) and piped water to a tap on the premises. There is still no indoor plumbing. There is at least one household with dial-up internet access and several small shops (run by village inhabitants and a neighbouring commercial trader) that sell basic foodstuffs, toiletries and household goods. Despite the improvements in living conditions, many people in Paulshoek continue to live in poverty. In an assessment of the village conducted in 2003, approximately 30% of people were living on less than $1 a day, the United Nations measure for the least developed and poorest countries (Rhode, et al., 2003). In a more recent study, it was shown that, due to a large increase in inflation, the mean average total income from employment has only increased moderately since then and almost 85% of village

inhabitants are unemployed (Duijnstee, 2011). Only 20% of households in the greater Leliefontein reserve have a regular income (Berzborn, 2007) and many people rely on state grants. In Paulshoek, 50% of the entire village income is from state support, with the most common forms being elderly, child and disability grants (Duijnstee, 2011).

Education levels in the village tend to be low, with the average adult obtaining a total of six years of education. Education has been steadily improving over the past years, with more people being educated, but the average level has not increased much above grade 6 (Rhode, et al., 2003; Duijnstee, 2011). This is most likely a result of there being a nursery school as well as a permanent primary school in the village, providing education up to grade 7. There is no secondary school in the village and learners must attend boarding school in one of the towns outside the village to obtain education beyond grade 7. For many families this expense is too great. However, many people now realise the value of education and an increasing number of learners are completing their schooling despite the difficulty involved for them and their families.

1.3.3 Healthcare and medicinal plant use in Paulshoek

Overall, there has been a general decrease in agriculture (fewer crop lands), and an increased dependence on state subsidies and wages since the village started expanding in the 1960's (Hoffman & Rhode, 2007). Pastoralism is still important but it is not the main source of income for most households, as most practice economic diversification with multiple livelihood strategies (Berzborn, 2007). Keeping herds of small livestock such as goats and sheep is often practised as an insurance policy against unemployment or to provide meat and milk for family needs (Rhode et al., 2003). A recent study of household incomes in Paulshoek indicated that income generated from selling livestock accounts for only 5% of the average household income (Duijstee, 2011).

Many people are entirely or largely dependent on state pensions and grants for their survival, which means that there is often very little money to cover basic health care expenses. Although there is no permanent medical facility in the village, a mobile clinic with a doctor and a nurse visits once a month. In addition to the clinic there is a recently appointed Community Health Worker, whose purpose is to educate people in aspects of healthcare, to facilitate access to healthcare and occasionally to treat minor illnesses or wounds (Duijstee, 2011). The mobile clinic service provides checkups and vaccinations for infants as well as basic medication to those living with HIV or tuberculosis (TB). It also provides chronic medication to those with illnesses such as high blood pressure. In a study done on health in Paulshoek by the Medical Research Council (MRC), it was found that the most common ailments

recorded at the clinic were high blood pressure, and respiratory diseases such as TB and asthma. The incidence of these diseases seems elevated due to the majority of adults being regular smokers (Rhode et al., 2003). Most services are free of charge, but occasionally a small fee is incurred. Despite the minimal cost involved, many people cannot afford to pay either the clinic or the traditional "bossiesdoctor" for treatment and thus the most accessible form of health care available to them is treatment with basic home remedies. While specific knowledge of how to treat serious illnesses such as cancer, strokes and mental illnesses is kept confidential and is only known to specialist healers, many lay people have a working knowledge of the plants used to treat everyday illnesses such as colds and stomach upsets (Dahlberg & Trygger, 2009; Dold & Cocks, 2000). Even though many people use home remedies out of necessity and the inability to make use of other available facilities, many people prefer to treat themselves and their families with home remedies as a first line of treatment (Cocks & Dold, 2000). If home remedies don't work, then the help of a Western doctor or the services of a traditional healer may be sought.

Traditional veterinary knowledge has also arisen out of necessity. Plants are responsible for a large number of animal deaths in South Africa, and herders living on remote stock posts are unable to gain the assistance of a Western veterinary doctor, and in many cases cannot afford the service if it is available (Kellerman et al., 1998). Thus, most herders have some knowledge of plant remedies to aid animal illnesses such as diarrhoea, gallsickness, heartwater, coughing and helminth infestation. It has been estimated that 75% of livestock herders in the Eastern Cape use plant-based treatments for animal illnesses (Masika et al., 2000; McGaw & Eloff, 2008) and the same may be true for herders in Namaqualand. Most herders also have some knowledge of the plants that their animals graze on and can prevent unnecessary poisoning, paralysis or liver damage due to ingestion of toxic plants (Bath et al., 2005). While this is vital knowledge for rural farmers, the topic falls beyond the scope of this study.

1.4 The importance of this study and its relevance

Medicinal plants play an integral role in the lives of modern Namaqualand inhabitants, and have done so for hundreds of years. Investigating trends in medicinal plant use may provide insight into how knowledge systems are created and how traditional use is maintained and may be continued in a modern society. It is also important to document traditional plant use so that this knowledge can be retained for future generations, particularly now that all of the traditional healers formally recognised in the village by the community passed away during the course of the study, leaving the village without a traditional healer.

Investigating the status of plant use in the community is also important in order to compare it with plant use in other parts of South Africa as well as other areas globally. This information may assist government in creating policy for traditional health care systems, for those that practice as healers and for traditional medicines themselves. Recording and detailing the actual plants used to create remedies may also provide a starting point for further investigations into biological activity or the nature of secondary metabolites present in those plants.

1.5 Aims/questions and objectives of this section

The main aim of this part of the study was to get a general overview of medicinal plant use in the village of Paulshoek and the demographics of medicinal plant users. A second aim was to compare this information with data from 14 years ago, in an attempt to highlight trends or changes in medicinal plant use over time (Goldberg, 1998). Plant choice was also investigated in order to establish if particular families are preferred for creating remedies for particular types of illnesses. In addition, methods of knowledge acquisition and commonly held perceptions of medicinal plants and their uses were investigated. In summary, the aims of this section of research are to:

1) Investigate the extent of knowledge of medicinal plant use within the village population
    a. Record all plants that are used for medicinal purposes
    b. Investigate the demographics of people who use home remedies and compare this with results from 14 years ago
2) Examine trends in plant choice and establish whether certain families are preferred for treating particular types of illnesses
3) Investigate how medicinal plant knowledge is acquired and transferred between users
4) Investigate the role of medicinal plants in the community and examine commonly-held perceptions of them and their use
5) Contrast this with the views and practices of specialist healers in the village

2. Methods

Ethical clearance for this research was obtained from the Faculty of Science Research Ethics Committee (based at the University of Cape Town), approval number SFREC 032_2011. Participants were informed of the nature of the project and no one was obliged to participate in the study. Personal information was kept confidential, with hard copies of interview transcripts stored in a locked desk drawer and electronic copies stored in a database on a password protected computer.

2.1 Interviews with villagers

Interviews with villagers took place between September 2010 and September 2011. An initial questionnaire was created in order to investigate the extent of use of medicinal plants by individuals not practicing as traditional healers, and to gain a basic overview of which species were commonly used in the preparation of home remedies (See Appendix A: "Question Set One"). Households that responded positively to this initial questionnaire were subsequently revisited and asked a series of follow up questions designed to investigate the demographics of those respondents in more detail (See Appendix A: "Question Set Two"). The second questionnaire also investigated general perceptions of medicinal plants and their use in Paulshoek, and allowed interviewees to comment freely on this and to give their opinions regarding remedy efficacy in relation to 'Western' medicine as well as historical anecdotes and anything else they felt was relevant and should be recorded.

Information gathered during interviews was compiled using Microsoft® Office Excel® 2007 and compared to data gathered in Paulshoek in a similar fashion previously (Goldberg, 1998), as well as to ethnobotanical data previously gathered for the greater Richtersveld area (Archer, 1994).

2.2 Interviews with healers

Interviews with two of the village healers, Oom Jacobus 'Kooitjie' Corjeus and Oom Gert 'Julk' Dirkse, now both deceased, took place in July 2009. These brief interviews, along with additional recorded interviews conducted over the past five years with the same people (M.T. Hoffman, unpublished recordings and transcripts), allowed comparison of opinions and perceptions of medicinal plants and their use between non-specialist villagers and formal traditional healers ("bossiesdoktors" or "kruiedoktors"). Interviews also allowed comparison of practices between non-specialist villagers preparing home remedies and the more formal system of diagnosis and treatment employed by traditional healers.

2.3 Plant collection and identification

The questionnaire surveys provided a basic list of the most commonly used plants in the preparation of home remedies. Walks were taken through the veld with a local field assistant in order to create a more comprehensive list of medicinal plants from the Paulshoek region, which included historical use as well as applications that have fallen out of common use. This is a modified version of the "active participant observation" method (Martin, 1995). Instead of observing someone who is involved in collecting plants for their own medicinal needs (as is done with "active participant observation"), guided walks were taken with a field assistant. The field assistant was someone who would usually collect medicinal plant, but was not actively involved in the activity at that time. The field assistant, (Ms. Marianna Lot) lives in Paulshoek and her father was a traditional healer in the area before his death. Even though she didn't officially train as a *kruiedoktor* she picked up knowledge of medicinal plants, their common names and uses from her father and is very knowledgeable in this area. She regularly makes use of plant-based home remedies and has an extensive knowledge of the methods used for preparing remedies. She has also been trained as a para-ecologist by BIOTA Southern Africa (see http://www.biota-africa.org/reg_south_paraecol_ba.php) and has worked on several other research programmes in the surrounding areas, making her a capable field researcher and valuable guide for the area. Walks were taken in several different areas around the village to encompass a variety of soil and vegetation types and to maximise the likelihood of encountering the widest selection of medicinal plants possible. Ms. Lot would identify plants of interest and provide common names and uses for each. Samples were taken from each plant identified in this manner for the purposes of formal identification and as herbarium samples. Species were identified by the author (M.Sc. Botany, UCT) and by Prof. M.T. Hoffman (Director of the Plant Conservation Unit at the University of Cape Town), who has extensive experience working in Namaqualand, specifically in the Leliefontein Reserve.

Herbarium samples were placed in a plant press and rotated with fresh paper weekly until they were adequately dry. This was done in accordance with standard pressing techniques (Victor et al., 2004). Spirit collections were made of succulents and spiky plants not suitable for pressing, according to the method outlined in Victor et. al (2004). Spirit collections were preserved in a standard Kew Solution containing 5% glycerine, 70% EtOH (70%), and 25% distilled water (www.kew.org). Additional photographs were taken of dried herbarium material for reference purposes and inclusion in data profiles (See Appendix B). Herbarium samples were submitted to the Bolus Herbarium at the University of Cape Town. An exhaustive literature search was performed on all plants collected in order to uncover

any additional uses, historical and present, in other areas or by different cultural groups. Additional information was included in the resulting plant profiles submitted with this thesis.

In addition to the system of informal guided walks, a typical south-facing rocky slope with sandy soil was selected as a collection site for additional exhaustive species sampling of the area. Paired sample squares (quadrats) measuring 2 m x 2 m were marked out along a north-south transect running up the slope. All new species were collected from quadrat one. All new species not already collected on veld walks or in quadrat one were collected from quadrat two, and so on along the transect, until a negligible number of new species was being collected per additional quadrat surveyed. This is in accordance with the law of diminishing returns, which says there is a point at which surveying another quadrat or interviewing another person will not add any significant or new information to the study and it becomes inefficient to continue collecting data (Martin, 1995).

A total of 10 sample quadrats were investigated in this fashion. Herbarium samples were made for each species and additional small samples of leaves, fruit or flowers were collected and placed in bags to be taken back to the University of Cape Town for analysis (See Chapter 3). Species collected in this way were identified by the author (M.Sc. Botany, UCT) and by Prof. M.T. Hoffman (Director of the Plant Conservation Unit at the University of Cape Town). This sampling strategy was a mixture of two commonly accepted sampling methods, namely *random sampling* and *ethnopharmacological sampling*. Random sampling involves collecting any plant in a given area that can be collected in a sufficient amount of a given quality for a given purpose. Here the main purpose was to collect non-damaged (through insect activity or grazing etc.) species for analysis (see chapter 3). This gives a good indication of the plants with potential medicinal application in an area. Random sampling is also the best method if any statistical analysis is to be performed on the data (Cunningham, 2001). Ethnopharmacological sampling involves sampling only plants that are used medicinally and is most likely to produce positive bioactive hits (Martin, 1995).

2.4 Investigating trends in plant choice

In order to determine which families are most often used for which types of illness, a series of regression analyses were performed at the family level. The number of species used medicinally per family (MSPE) was the independent variable, and the number of uses per family for a particular category of treatment was the dependant variable (Moerman, 1991). The rationale behind this is that it is a way of highlighting which families are favoured (“hot”) for treating particular types of illness. In essence, the approach uses least-squares regression analysis to establish an equation that describes the relationship

between the two variables under investigation. Assuming the relationship is linear, the equation will have the form:

$$y = A + Bx$$

where y is the dependant variable, x the independent variable, B is a coefficient representing the slope of the line and A is a constant representing the y axis intercept. The equation can be used to predict the expected number of uses per family for a certain category of illness. These predicted values are then compared to the actual values per use category. Subtracting the predicted value from the actual count gives the residual value for that data category.

Residuals can be calculated for each family. Families with positive residuals are the ones used more often than regression would predict, while those with negative residuals are used less often than would be expected. Therefore, families are designated either “hot” or “cold” and the choice to use (or avoid) species from certain families to treat certain illnesses is not random. Extreme outliers are of interest as they represent families that are either particularly favoured, or particularly avoided for medicinal use (Douwes et al. 2008; Moerman, 1991; Treyvaud Amiguet et al., 2006; Molander et al., 2012). This method was followed for the medicinal species mentioned in interviews with Paulshoek residents.

3. Results

3.1 Extent of use of medicinal plants

A few people declined to be interviewed, or were not available when the interviews were being conducted and were not included in this study. A total of 71 households in Paulshoek, out of approximately 99 were interviewed in order to investigate the extent of knowledge of medicinal plants and the use of traditional home remedies by villagers. Of the participating households, it was found that 70.4% had used plant based remedies in the previous year, compared with 70.0% recorded in a similar survey of medicinal plant use in Paulshoek in 1998 (Goldberg, 1998). Thus, the number of people using medicinal plants had not changed significantly in the time between the two studies, suggesting that plant use in the village is not in decline. However, while the percentage of people using plant medicine has not changed, the demographics of the group may have.

Children (minors under the age of 18) made up 27.7% of people using medicinal plants, 57.6% of which were under the age of 10. Thus, children under 10 years of age comprised 15.9% of the total population

using medicinal plants. Adults over the age of 18 made up 72.3% of the people who used medicinal plants. Goldberg (1998) reported that more than 50.0% of people using medicinal plants were children, which suggests that there has been a decline in the number of children using plants in the 14 years between the two surveys and an overall increase in the number of adults using medicinal plants. Since the percentage of the population using medicinal plants has not varied significantly over the past 14 years, and the total population size has not varied significantly, it must be assumed that there has been a general shift in the demographics of people using medicinal plants. A few of the older people recorded in Goldberg's study (1998) have passed away in the interim years, and many of those recorded as minors in the previous study will now be adults. This resulted in an overall increase in the number of people in the adult group, and a decrease in percentage of children using plants. However, while some of the observed drop in the percentage of minors using medicinal plants can be explained by previously recorded children growing up, the rest of the decline implies fewer children are being born in the village and that a large number of new minors born in the interim years between studies are *not* using medicinal plants. These indicators of an ageing and declining population were also recorded in a 2010 survey of the village (Duijnstee, 2011), which indicated that the number of children in the village had dropped since 1995. This trend was explained as result of a combination of fewer children being born in the village and that mothers with children were more likely to move away from Paulshoek in search of work. Both of these events have led to a decrease in the number of children using medicinal plants in the village, and will eventually result in declining numbers of adults using medicinal plants in coming years. As recorded in this study, Duijnstee (2011) also showed that the number of elderly people had increased.

Each household consisted of an average of four people, ranging between one and eight occupants per house, as compared with the average of six people per household, ranging between one and twelve occupants per house, reported in a 2003 study of Paulshoek (Rhode et al., 2003). The number of houses has not changed significantly, so this does not indicate that people just prefer to live in smaller groups, but rather it suggests that there has been an overall decline in the number of people living in Paulshoek permanently, as was previously suggested by other recent studies (Duijnstee, 2011).

Of the 29.6% of respondents that said they did not use medicinal plants, 18.2% had used plant based home remedies in the past, but no longer did so. There were various reasons given for the cessation the most common being that the respondent was old and unable to collect plants, make medicine or remember which plants to use. One respondent said they no longer used medicinal plants as the plants

they preferred had died out, and, most interestingly, one person was under the impression that it was illegal to collect plants and so did not make home remedies anymore. The remaining 11.4% of respondents currently do not use medicinal plants, and have not done so in the past.

3.2 What is used and for what purpose?

The aim of this section was to investigate the extent of knowledge of medicinal plants of non-specialist villagers in order to identify which species are used and for what purposes as well to establish if there are preferences in plant use among non-specialists.

3.2.1 How many species are used?

A total of 50 plants in 27 families were described during interviews with villagers and talks with Ms Lot, 18 of which were mentioned by her alone. The common names and uses of these plants were recorded and they were identified to species level (Table 2). In addition to the information about medicinal plants, common names and applications of other locally-used, non-medicinal plants were also recorded. Most of these non-medicinal species mentioned were used for grazing or firewood, while some had other functional uses such as traditional building materials, insect repellents or for food (Table 3).

In the previous study of Goldberg (1998), it was recorded that 22 plants were used by traditional healers, but only 15 were being used regularly for home remedies by villagers in Paulshoek. Other studies reported that 45 species were known in the greater Richtersveld area, but only 20 of those were being used regularly (Archer, 1994). Thus, the number of plants used in the creation of home remedies by village residents in Paulshoek appears to have trebled, but this may be due to a more rigorous sampling approach adopted in this study. Comparing these numbers to results from similar studies in other regions of South Africa indicates that they are not significantly different. For example, Bhat & Jacobs (1995) reported 26 medicinal plants in 21 families used by people in rural Transkei; van Wyk et al. (2008) reported the medicinal use of 86 plant species (72 indigenous) in 39 families in a study of the South Eastern Karoo; Dahberg & Trygger (2009) reported 49 species in 29 families used in rural KwaZulu-Natal; and Thring & Weitz (2006) reported 36 species in 19 families in use in the Overberg region of the Western Cape. This gives a mean number of 23 families used per region. Paulshoek, with its 27 reported families, is comparable with these other South African studies.

An average of 7 medicinal plants was known per person interviewed, with the majority of people naming between 6 and 10 medicinal plants (Figure 5). Less than 5.0% of respondents in the general survey could name and give uses for more than 15 species, and a similar number only named a single

Table 2: A list of the plants used in Paulshoek for medicinal purposes and a brief description of the uses for which they are intended

Scientific name	Local name	Medicinal use
***Aloe microstigma* Salm-Dyck subsp. *microstigma* (Asphodelaceae)**	Vuurpylalwyn	In the past, the leaves were boiled and the liquid reduced to a thick yellow syrup which would be given to livestock to treat gall sickness.
***Antizoma miersiana* Harv. (Menispermaceae)**	Bloubos, Dawids wortel (David's root), inkbos	A decoction of the root is taken as an emetic and for fever and stomach complaints. Dried plant material has veterinary application for the treatment of horses with bot fly, a parasitic fly of the family *Oestridae* that uses horses as a larval host. This use is not common in Paulshoek currently, but was popular in the past.
***Aptosimum spinescens* (Thunb.) Weber (Scrophulariaceae)**	Ambeibos	A cooled decoction of the leaves is used to treat an unidentified illness "ambei". Old literature (Laidler, 1928) refers to the "ambiaer bush" which was used to treat haemorrhoids. It seems that the "ambei" mentioned in this instance also refers to haemorrhoids.
***Asparagus aethiopicus* L. (Asparagaceae)**	Hakiesdoringbos	A decoction made from the roots of *A. aethiopicus* and an unidentified plant, "wildekanniedood", is used to treat tuberculosis.
***Asparagus rubicundus* P.J. Bergius (Asparagaceae)**	Wag-'n-bietjie bos	The lower stem and roots are dried and ground into a powder which is used a snuff to treat headaches.
***Ballota africana* (L.) Benth. (Lamiaceae)**	Kattekruie	A hot or cold poultice is prepared from fresh leaves and placed on burns or wounds to reduce inflammation. A similar poultice can be applied to relieve pain.
***Carpobrotus edulis* (L.) L. Bolus (Aizoaceae)**	Hotnotsvye	The juice of the leaf is used to treat fever blisters.
***Chrysocoma ciliata* L. (Asteraceae)**	Metjiesbos, ouwerfbos	A decoction of the leaves is taken to treat stomach pain.
***Colpias mollis* E.Mey. ex Benth. (Scrophulariaceae)**	Klipblom	Ground up then chewed to help heal sores.
***Cotyledon orbiculata* L. *var. orbiculata* (Crassulaceae)**	Pêpêbos, skaapiesbos	The outer leaf surface is removed and placed directly on the skin to treat cracked lips. The leaf juice is used to treat fevers and sores in the mouth.
***Crassula atropurpurea* (Haw.) Dietr. var. *watermeyeri* (Compton)**	Rooirantjie	The outer cuticle of "rooirantjie" is placed on sore lips and fever blisters to help speed the healing process.

Tölken (Crassulaceae)		
***Crassula muscosa* L. *var. muscosa* (Crassulaceae)**	Hoendervoet, volstruistoon	The leaves are stripped off the stems and boiled in water. The resulting decoction is used to treat a range of general children's illnesses.
***Cynanchum africanum* (L.) Hoffmanns (Apocynaceae)**	Bobbejaantou, jakkalsgaring, jakkalsbos	A decoction is taken by pregnant women to help expel the placenta and as a general tonic after giving birth. A decoction is also drunk for general body pains. Crushed fresh or dried leaves are mixed with animal fat or petroleum jelly to make an ointment or plaster to treat burns and sores. The plant can also be put into water used to wash hair and this is said to strengthen brittle hair.
***Dicerothamnus rhinocerotis* (L.f.) Koekemoer (Asteraceae)**	Renosterbos/xhou (although Bergrenoster is found in the mountains and the Witrenoster is found in rivers, they are the same taxonomic species)	A cooled decoction of "witrenoster" leaves is used to soak burning, itchy feet. The buds of the "witrenoster" are dried and crushed and the powder put into wounds to prevent infection. A decoction of "bergrenoster" is used to clean the kidneys, urinary tract and help expel phlegm from airways and placenta after giving birth. To treat spiritual illness or "vuil winde" (lit. "foul winds" meant to describe an evil spirit or an evil spell or curse), a decoction of "bergrenoster", "kalkoentjiebos" and "balerja" is made
***Diospyros austro-africana* De Winter var*. austro-africana* (Ebenaceae)**	Kraaibos	The juice of the unripe berry is swallowed to treat stomach pain and flatulence. A decoction of the leaves is drunk as an emetic and to treat intestinal problems.
***Dodonaea viscosa* Jacq. var. *angustifolia* (L.f) Benth, (Sapindaceae)**	Xhoubie, toubee, wilde olino	A cooled decoction made from the leaves of "wilde olino", "balerja", "taaibos", "salie" and "koorsbos" is taken every few hours to treat colds and flu. A decoction of the plant by itself is used to break fever.
***Eriocephalus brevifolius* (DC.) (Asteraceae)**	Roosmaryn	A decoction is used to wash skin to treat rashes and pimples.
***Eucalyptus camuldensis* Dehnh (Myrtaceae)**	Bloekomblare/blue gum	General home remedy. Placed on sore areas to relieve pain. Also used specifically to treat back pain and headaches. The leaves are boiled and placed on the areas that are sore.
***Euryops multifidus* (Thunb.) DC. (Asteraceae), *Othonna cylindrica* (Lam.) DC (Asteraceae) and others**	Rapuis (there are five types of rapuisbos recognised: kallataaibosrapuis, bruinrapuis, stinkrepuis/bergrapuis/geelrapuis, gemsbok/sandrapuis/swartrapuis)	The resin of any "rapuisbos" can be dried, crushed and mixed with fat to make an ointment which is used to remove thorns or splinters. "Kallataaibosrapuis" leaves can be chewed to treat colds.
***Galenia africana* L. (Aizoaceae)**	Kraalbos, geelbos	Leaves may be used dry or fresh. When powdered they are used for toothache. A decoction is used to treat several ailments: sores on the head, aching legs,

		bladder problems (when mixed with ostrich egg shell).
***Gethyllis afra* L. (Amaryllidaceae)**	Koekemakrank	The plant juice is used to treat bald patches as well as spots or blemishes on the skin.
***Hermannia amoena* Dinter ex. Friedr.-Holzh. (Malvaceae)**	Jeukbos	The leaves are boiled and strained and the decoction drunk for nausea. This liquid is also used to treat weakness resulting from long illnesses, especially diabetes. The leaves may also be crushed and mixed with petroleum jelly to make an ointment for insect stings. Used in treating "vuil winde" (lit. "foul winds" but meant to describe an evil spirit or an evil spell or curse).
Hermannia cuneifolia* Jacq. var*. cuneifolia (*Malvaceae*)	Agtdaegeneesbossie	A decoction of the plant is used to help build strength in the body (presumably after illness) and as a tonic for general body pains and sores.
***Leonotis leonurus* (L.) R.Br. (Lamiaceae)**	Wilde dagga	Inhaled as smoke or snuff for respiratory problems. A tea may also be made from the plant to treat asthma.
***Manochlamys albicans* (Aiton) Aell. (Chenopodiaceae)**	Seepbos	A decoction of the leaves of the "seepbos" combined with "ambeibos" and an unidentified plant, "xhoro", is used to treat flatulence and stomach pain.
***Melianthus pectinatus* Harv. subsp. *pectinatus* (Melianthaceae)**	Kruidtjie-roer-my-nie	Dried plant material is ground with animal fat or petroleum jelly to make an ointment which is used to treat pain.
***Mentha longifolia* (L.) Huds.**	Kruistement, ballerja	A tea made from the leaves is drunk for colds and flu. This plant is also mentioned as in ingredient in several other combination medicines for a variety of illnesses.
***Microloma sagittatum* (L.) R. Br. (Apocynaceae)**	Bokhoringbos, bokhoutbos	The root is ground to powder and used in treating "vuil winde" (lit. "foul winds" but meant to describe an evil spirit or an evil spell or curse).
***Monoculus hyoseriodes* (DC.) B. Nord. (Asteraceae)**	Dassiepisbos/Dassiegousblom	General home remedy and tonic, illness unspecified
***Montinia caryophyllacea* Thunb. (Montinaceae)**	Peperbos	None officially, but the leaves apparently taste peppery, hence the common name. Some people believe the sharp taste indicates a good and "healthy" plant.
***Nicotiana glauca* Graham (Solanaceae)**	Twaksboom	The leaves are mixed with petroleum jelly or castor oil to make an ointment which is applied to treat pain. A bandage is wrapped over the ointment, and the ointment is reapplied until the pain subsides.
***Oncosiphon suffruticosum* (L.)**	Stink kruid	A decoction of "stink kruid" is taken for headaches, stomach problems and nausea.

		Old literature sates that “stink kruid” was used to treat stomach pains and as a plaster/poultice for scorpion stings, however this use does not seem prevalent in Paulshoek.
***Pelargonium alternans* J.C. Wendl. (Geraniaceae)**	Skaapbos	In the past, people would crush up the leaves with petroleum jelly or animal fat to make an ointment which would be applied to sores.
***Pentzia incana* (Thunb.) Kuntze (Asteraceae)**	Wilde als	A decoction of the leaves is used to treat high blood pressure.
***Polymita albiflora* (L.Bol.) L.Bol. (Aizoaceae)**	Muisoor	The leaf juice or a decoction of the leaves is swallowed to relieve stomach pain.
***Ruschia fredericii* (L.Bolus) L. Bolus (Aizoaceae)**	Vygiebos	The leaves are chewed and the juice is swallowed to help relieve stomach ache.
Ruta graveolens L. (Rutaceae)	Wynruik	General home remedy and tonic with multiple uses.
***Salsola kali* L. (Amaranthaceae)**	Khakiebos	A decoction of the plant is used to treat diabetes.
***Salvia dentata* Aiton (Lamiaceae)**	Salie, bergsalie	The leaves are chewed to treat influenza. For a stronger medicine for colds and flu, the leaves of “salie”, “kalkoentjiebos”, “balerja”, “taaibos” and “koorsbos” are made into a decoction to be taken at 4 hour intervals.
***Sceletium tortuosum* (L.) N.E.Br. (Mesembryanthmaceae)**	Kougoed	Chewed to relieve hunger and to lift moods. Also a bit of the leaf juice is placed on a rag and given to babies to suck on to calm them down, help them to sleep and to relieve winds (indigestion).
***Searsia undulata* (Jacq.) T.S.Yi, A.J.Mill. & J.Wen (Anacardiaceae)**	Taaibos	Tea made from the leaves, sometimes in combination with leaves of “salie”, “balerja” and “koorsbos”, is drunk for colds and flu. Crushed leaves mixed with kidney fat are used to make an ointment or plaster for boils, ringworm and abscesses.
***Senecio cinerascens* Aiton (Asteraceae)**	Vuurhoulap, handjiesbos	The leaves are used to make a warm poultice which is placed on burns and wounds. The poultice is also placed on painful areas to reduce the ache.
***Seriphium plumosum* L. (Asteraceae)**	Hotnoskooigoed	General home remedy, mood enhancer, calmative and recreational material. Also used to calm down restless babies and children and for constipation.
***Stachys rugosa* Aiton (Lamiaceae)**	Koorsbos, TB bos, Hen-met-kuikens	A cooled decoction of the leaves is drunk to break fevers. It is also combined with “balerja”, “salie”, “xhoubie” and “taaibos” in a decoction used to treat colds and flu. This plant is also used to help women to fall pregnant (treat infertility) and as a general health tonic during pregnancy.

***Sutherlandia frutescens* (L.) R.Br. (Fabaceae)**	Kalkoentjie, kankerbos, (klein) Jantjie Bêrend	A cooled, strained decoction of the root, stalk and leaves is used to treat diabetes. A decoction of "kalkoentjie", "salie" and "balerja" is taken for asthma, while a decoction of equal amounts of "kalkoentjie" and "balerja" is taken for high blood pressure. In the past, people would use the bush to treat cancer but details of the treatment are not known (or were not provided during this investigation).
***Termitomyces schimperi* (Pat.) R. Heim (Lyophyllaceae)**	Ajoos ("termite mushroom")	The spores of this fungus are mixed with lard to make an ointment which is used to treat allergic reactions and rashes.
***Tetragonia fruticosa* L. (Aizoaceae)**	Roosmaryn	The leaves are mixed with other plants in a range of different medicinal preparations.
***Trichodesma africanum* (L.) Lehm. (Boraginaceae)**	Brandnetel	General home remedy and tonic, illness unspecified
***Tulbaghia violacea* Harv. (Alliaceae)**	Wilde knoffel	Made into a tea for colds and flu. The bulbs can be eaten "as is" for the same purpose.
***Viscum capense* L.f. (Viscaceae)**	Voëlent	Cooled tea made from the entire dried plant is drunk as a general health tonic and a decoction of the plant is used to treat diabetes. The same decoction is given to sheep and goats to help expel placenta after giving birth.

Table 3: Additional plants used in Paulshoek for non-medicinal purposes

Scientific name	Local Paulshoek name	Non-medicinal use
***Albuca* spp.**	Slymstok	The stalk and bulb are eaten by humans and livestock.
***Aloe dichotoma* Mass. (Asphodelaceae)**	Kokerboom	The hollowed out stem is covered with a lid and used to store food. It keeps things cool as a fridge would do.
***Antizoma miersiana* Harv. (Menispermaceae)**	Bloubos, Dawids wortel (David's root)	Firewood and grazing.
***Antizoma miersiana* Harv. (Menispermaceae)**	Inkbos	The green leaves are grazed and it is used as kindling.
***Aridaria noctiflora* (L.) Schwantes (Mesembryanthemaceae)**	Vleisbos	Grazing.
***Aslcepias fruticosa* L.**	Tontelbos	The fluffy fibres associated with the seeds are used to get fires going

(Apocynaceae)		
***Asparagus aethiopicus* L. (Asparagaceae)**	Hakiesdoringbos	Grazing.
***Asparagus rubicundus* P.J. Bergius (Asparagaceae)**	Wag-'n-bietjie bos	Grazing for goats.
***Ballota africana* (L.) Benth. (Lamiaceae)**	Kattekruie	Grazing.
***Carpobrotus edulis* (L.) L. Bolus (Aizoaceae)**	Hotnotsvye	The fruit is edible and is used to make traditional jam.
***Cheiridopsis denticulata* (Haw.) N.E.Br. (Aizoaceae)**	Xhotsiama	Grazing.
***Cheiridopsis namaquensis* (Sond.) Hartmann (Aizoaceae)**	Xhotsiama, type noordepool	Grazing.
***Chrysocoma ciliata* L. (Asteraceae)**	Metjiesbos, ouwerfbos	In the past, the entire dried bush was used as a broom to sweep hearths. People would also use it as cushioning when carrying firewood on their backs.
***Cotyledon orbiculata* L. *var. orbiculata* (Crassulaceae)**	Pêpêbos, skaapiesbos	In the past people would blow through the broken off flower stems to make a noise to draw animals out of hiding in order to hunt them.
***Cynanchum africanum* (L.) Hoffmanns (Apocynaceae)**	Jakkalsbos, jakkalsgaring, bobbejaantou	Grazing. It is pointed out that the leaves are believed to be toxic to livestock when they are covered with morning dew.
***Cyphia crenata* (Thunb.) C.Presl. (Lobeliaceae)**	Bouroe/berg bouroe	In the past the root/corm was eaten as a source of nutrition and hydration when out in the veld.
Dicerothamnus rhinocerotis* (L.f.) Koekemoer *(*previously *Elytropappus rhinocerotus)	Renosterbos/xhou (Bergrenoster is found in the mountains and the Witrenoster is found in rivers, but are presumably the same species)	The leaves are put into smelly shoes to remove foot odours.
***Diospyros austro-africana* De Winter var*. austro-africana* (Ebenaceae)**	Kraaibos	Branches are used to make brooms for domestic use.
***Dodonaea viscosa* Jacq. var. *angustifolia* (L.f) Benth, (Sapindaceae)**	Xhoubie, wilde olino	Firewood and grazing. Farmers at stockposts also use the branches to make shelters or screens for themselves and their animals.
***Euphorbia decussata* E. Mey.**	Soetmelkbos	Grazing. Provides a sticky "glue" that was used as bubblegum in the past.

ex Boiss. (Euphorbiaceae)		
***Euphorbia mauritanica* L. (Euphorbiaceae)**	Bittermelkbos	Building shelters and kraals.
***Fockea edulis* (Thunb.) K.Schum (Apocynaceae)**	Kambroo (two different species are called kambroo – berg kambroo and stink kambroo/bitter kambroo)	"Berg kambroo" has an edible tuber. In the past, the tuber of the "bitter kambroo" was used to make a type of honey beer.
***Galenia africana* L. (Aizoaceae)**	Kraalbos, geelbos	Firewood, building of cooking shelters and grazing (only when green, otherwise the leaves cause illness in livestock).
***Gethyllis afra* L. (Amaryllidaceae)**	Koekemakrank	The fruit is eaten by humans and animals.
***Grielum humifusum* Thunb. (Neuradaceae)**	Pietsnot/duikerwortel	The root is eaten by humans and animals.
***Hermannia cuneifolia* Jacq. (Malvaceae)**	Vet-en-brood bos	Grazing.
***Hirpicium alienatum* (Thunb.) Druce (Asteraceae); *Pentzia incana* (Thunb.) Kuntze (Asteraceae); *Pteronia incana* (Burm.) DC. (Asteraceae); *Eriocephalus microphyllus* DC. (Asteraceae) and others**	Xhibbie/krakrakie (There are ± 7 species that are all called "xhibbie")	Grazing. Cushions were made from the "wool" of xhibbie (presumably *E. microphyllus*).
***Hoodia gordonii* (Mass.) Sweet ex Decne (Apocynaceae)**	Bossielêr	In the past, "bossielêr" was used as a veld food and to stop hunger pains
***Hypertelis salsoloides* (Burch.) Adamson (Molluginaceae)**	Haas suuring	Grazing. People can eat it too, but it has a sour taste.
***Larryleachia cactiformis* (Hook.) Plowes (Apocynaceae)**	Bobbejaanseep	Used as soap for cleaning hands when out in the veld.
***Lebeckia sericea* Thunb. (Fabaceae)**	Fluitjiesbos	Firewood and grazing.
***Leipoldtia schultzei* (Schltr. & Diels) Friedr. (Aizoaceae)**	Female Xhouroe	Firewood and building cooking shelters.

***Lycium ferocissimum* Miers./ *Lycium cinereum* Thunb. (Solanaceae)**	Large/small kirriedoring bush	Firewood and grazing. Berries are eaten by humans.
***Manochlamys albicans* (Aiton) Aell. (Chenopodiaceae)**	Seepbos	Firewood and grazing.
***Melianthus pectinatus* Harv.**	Kruidjie-roer-my-nie	Grazing for mountain goats.
***Mesembryanthemum guerichianum* Pax. (Aizoaceae)**	Soutslaai	Grazing. In the past, people would use the plant to remove hair from animal skins in the process of making leather. The leaves combined with sand were used to scour clean pots and pans.
***Microloma sagittatum* (L.) R. Br. (Apocynaceae)**	Bokhoringbos, bokhoutbos	The flowers and pods are eaten by animals and humans.
***Monoculus hyoseriodes* (DC.) B. Nord (Asteraceae)**	Dassiegousblom/dassiepisbos	Grazing.
***Montinia caryophyllacea* Thunb. (Montinaceae)**	Pepperbos	In the past, the branches were used to make "canes" for administering corporal punishment to children.
***Nicotiana glauca* Graham (Solanaceae)**	Twaksboom	NOTE: leaves of *N. glauca* are used as a substitute for tobacco in many parts of South Africa due to their high content of anabasine, a nicotine-like compound. However this use does not seem to be prevalent in Paulshoek.
***Ornithoglossum vulgare* B. Nord. (Colchicaceae)**	Raap	The bulb is fried and eaten.
***Pelargonium alternans* J.C. Wendl. (Geraniaceae)**	Skaapbos	Grazing.
***Polymita albiflora* (L.Bol.) L.Bol. (Aizoaceae)**	Muisoor	Firewood and building shelters (specifically cooking shelters).
***Pteronia incana* (Burm.) DC. (Asteraceae)**	Asbos	In the past, people would use the ash made by burning the leaves and branches in the process of soap making. The ash was also considered a good whitener for clothing (i.e. it was used as bleach).
***Quaqua mammillaris* (L.) Bruyns (Apocynaceae)**	Ouroena/green ouroena/aroena	Eaten in the past as a source of nutrition and hydration when out in the veld.
***Ruschia fredericii* (L.Bolus) L. Bolus (Aizoaceae)**	Vygiebos	Grazing. Also as a source of water when out in the veld for extended periods.
***Ruschia robusta* L.Bol.**	Long legged Xhouroe	Firewood and building cooking shelters.

(Aizoaceae)		
***Salsola kali* L. (Amaranthaceae)**	Rolbos, donkiebos	Grazing for donkeys.
***Salvia dentata* Aiton (Lamiaceae)**	Salie, bergsalie	Grazing.
***Sarcocaulon salmoniflorum* Moffet (Geraniaceae)**	Kersbos	Firewood.
***Scirpus nodosus* Rottb. (Cyperaceae)**	Matjies	The reeds were cut down, bundled together and sew into mats. The mats were used to make the traditional round houses - "matjiehuise" – of the area
Searsia erosa (Anacardiaceae)Unidentified (Restionaceae)	Besembos (two types are recognised: "regular besembos" and "jagbesembos")	Grazing. Bundles of sticks from the "besembos" are used to make brooms and implements for threshing oats, wheat or rye.
***Searsia undulata* (Jacq.) T.S.Yi, A.J.Mill. & J.Wen (Anacardiaceae)**	Taaibos	Firewood and grazing. In the past, hot water was poured on crushed bark and the mixture was used to dye animal skins.
***Termitomyces schimperi* (Lyophyllaceae)**	Ajoos ("termite mushroom")	In the past, the ointment was also used as sunscreen and as a substitute for makeup.
***Tetragonia fruticosa* L. (Aizoaceae)**	Roosmaryn	Grazing.
***Thesium lineatum* L. f. (Santalaceae)**	Pennetjiesbos, witstorm	Firewood. Sharpened shoots were used to pin out animal skins during tanning.
Ubiquitous daisy plants e.g. *Arctotis* spp	Gousblom	Grazing.
Unidentified bulbous spp. e.g. *Babiana*	Uintjies (There are five types of uintjies: klipuintjies, bobbejaanuintjies, swartuintjies, tol uintjies and perdeklou uintjies)	The bulbs of the different uintjies can be fried or boiled in goat milk and eaten.
Unidentified but presumably some sort of *Gazania* spp.	Botterblom	Grazing.
Unidentified *Salsola* spp.	Ganabos	Firewood.
Various Oxalis spp. (Oxalidaceae)	Suuring (There are four types of suurings: bobbejaan suuring,	Livestock may eat "suuring" and people sometimes boil the bulbs in goat milk to make a type of porridge.

	haassuuring, langbeen suuring, and skilpad suuring)	
***Zygophyllum microphyllum* L.f. / *Zygophyllum foetidum* Schrad. & J.C.Wendl. (Zygophyllaceae)**	Perdebos	Limited grazing.

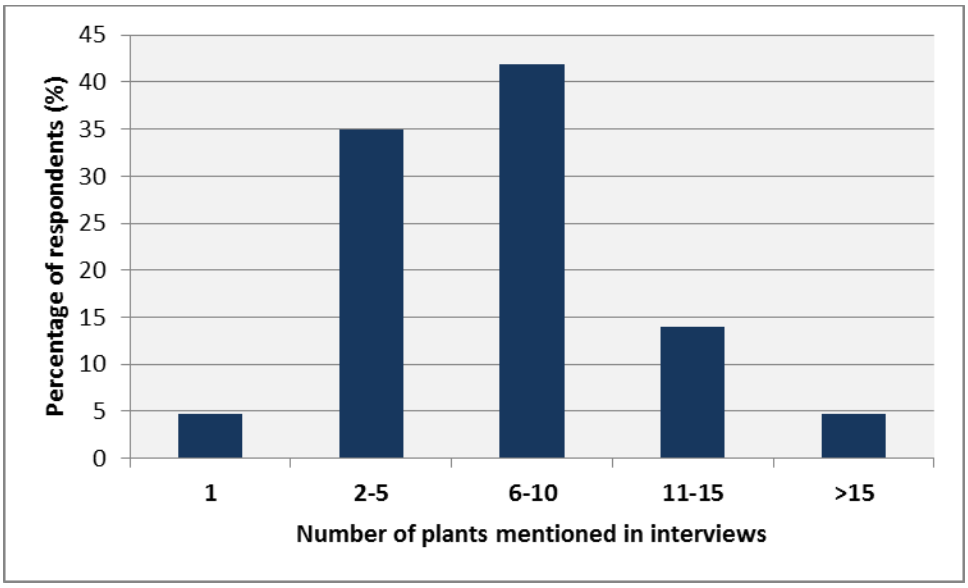


Figure 5: Average number of plants known to respondents

plant. The exception was Ms. Lot who, during walks and conversations, could name and give uses for 32 species. This is comparable to similar studies done, for example, in rural KwaZulu-Natal, where the average number of medicinal plants known to non-specialists was 8, ranging between 1 and 59 (Dahlberg & Trygger, 2009). This indicates that the majority of people know a few plants and remedies while there are a handful of more knowledgeable non-specialists, most of whom had had some familial relationship to a healer e.g. daughter (as in Ms. Lot's case) or spouse (in the KwaZulu-Natal example).

Some people mentioned that plants could be used in combination, for example to make a better treatment for colds and flu (see entry "salie" in Table 2) and some plants had multiple applications e.g. "jeukbos" - *Hermannia amoena* - which is used to treat nausea, weakness and insect stings. This sentiment was echoed by the healers who said that lots of different plants are combined to make their medicines. The majority of people regard mixing plants like this to be more specialised knowledge, held by more experienced, often older, members of the community and in particular by the healers.

3.2.2 Most common illnesses treated with plant medicines

Participants in this study mentioned 27 illnesses that are commonly treated with home remedies. These could be grouped into 7 broad categories: 1) "colds and flu" 2) "digestive/bowel problems" (e.g. constipation, diarrhoea, excessive flatulence) 3) "aches and pains" (e.g. headache, body ache or toothache) 4) "respiratory problems" (e.g. asthma) 5) "women's problems" (including healthcare during pregnancy, childbirth and urinary tract infections) 6) "wound care and sores" and 7) "other". This last

category included a variety of non-specific minor ailments, which could be symptomatic of other illnesses, such as itchy feet, insomnia and bald spots. There were also a variety of ailments that only a few, or in some cases a single person, treat with medicinal plant home remedies. These include ailments such as skin problems and kidney problems, among others. Figure 6 gives a breakdown of all 27 illnesses mentioned during interviews. The size of pie slices is representative of the relative frequency with which that ailment was mentioned.

The most common category of illness that people treat with home remedies is “colds and flu”, followed by “stomach problems”, and “aches and pains” of different types. Together, these constituted 83.1% of all illnesses mentioned. This is consistent with the results of Archer (1994), who found that the most common illnesses treated with medicinal plants in the greater Richtersveld area could be grouped into “colds and flu”, “aches and pains”, and “stomach ailments”. Other studies of different rural areas of South Africa highlight a similar trend. For example, Bhat & Jacobs (1995) found that in rural Transkei the most commonly treated illnesses could be grouped into “coughs, colds and fevers”, “stomach complaints” (including stomach ache, diarrhoea, and constipation), “wounds”, and other minor ailments similar to those mentioned in the Paulshoek survey (i.e. earache, toothache and skin problems). Dahberg & Trygger (2009) in rural KwaZulu-Natal also recorded the most common groups of illnesses treated with home remedies as “aches and pains”, “coughs and colds”, “stomach problems” and others miscellaneous ailments such as skin problems, kidney problems, wounds and asthma.

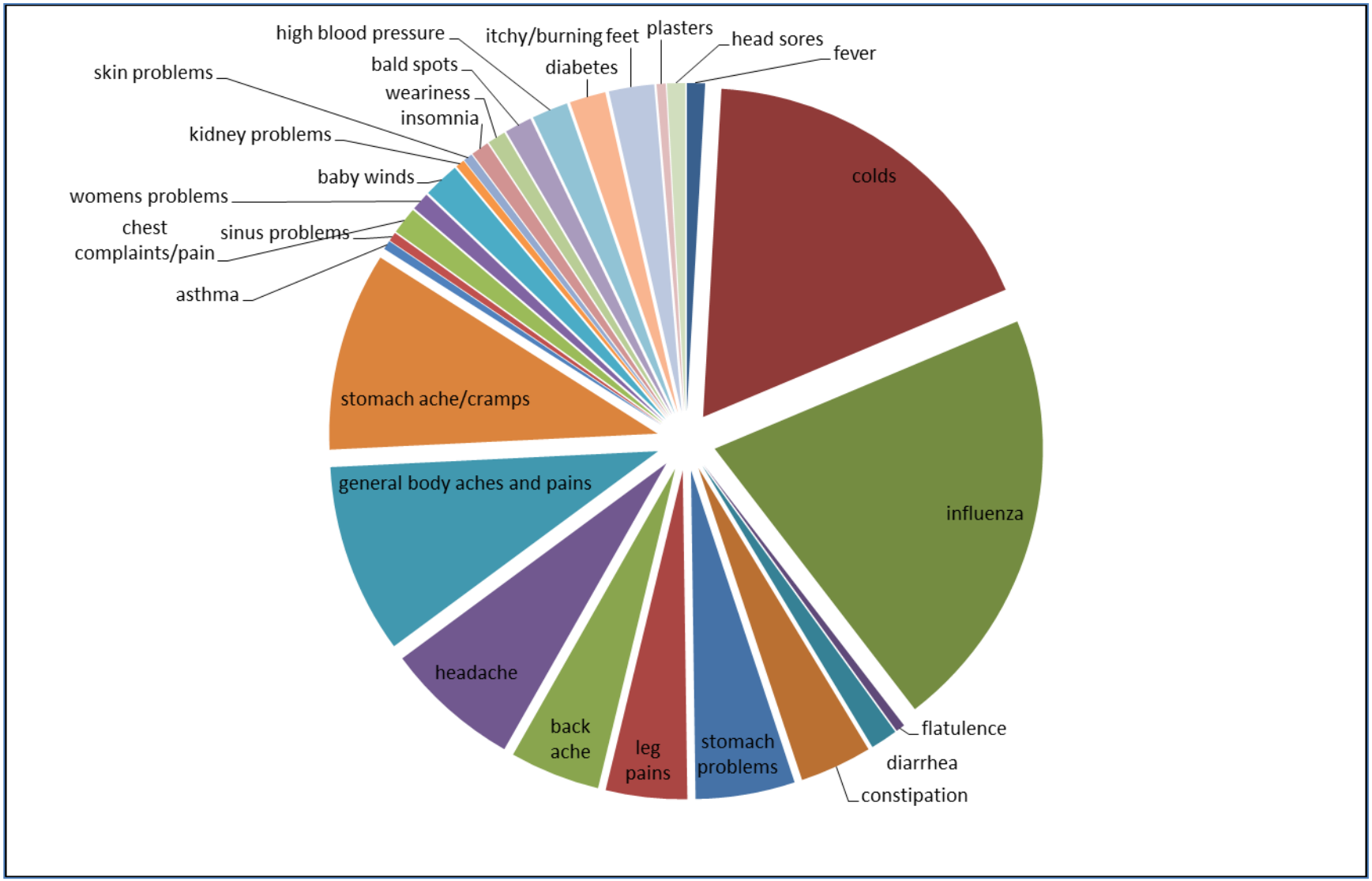


Figure 6: Breakdown of all 27 illnesses mentioned during interviews. Size of pie slices are representative of how often the particular illness was mentioned.

However, in other areas of South Africa, illnesses falling into the "other" category in Namaqualand, which were the least frequently mentioned illnesses, were some of the most often treated complaints. For example, van Wyk et al. (2008) found that problems of the bladder, kidneys, stomach and back were the most commonly treated ailments in the south eastern Karoo region. Colds and "minor" ailments (similar to those mentioned in Paulshoek - skin problems, insomnia, diarrhoea, fever, earache etc.) were also treated with home remedies, but the majority of respondents mentioned those four areas (bladder, kidneys, stomach and back) as being the most commonly treated at home. Thring & Weitz (2006) found the most common illnesses treated with home remedies in the Overberg region of the Western Cape were arthritis, bladder and kidney problems, diabetes, fever and stomach problems. Perhaps people in these areas are more prone to these types of illnesses, for whatever reason, or perhaps there is simply a higher occurrence of these illnesses in this area because of some environmental or external influence not experienced in Namaqualand.

Overall, it seems that the majority of people in Paulshoek, indeed in many rural South African areas, make simple home remedies to treat common illnesses that are self-evident and require no specialist knowledge to diagnose e.g. diarrhoea. Diagnosis and treatment of more serious or obscure illnesses and those with indistinct symptoms remains the domain of the traditional healer. Oom 'Kootjie' Corjeus, one of the healers living in Paulshoek, described his method of diagnosis via consultation with the patient, listening to their description of the problem and sometimes involving a spiritual aspect such as using a pendulum to divine the diagnosis using his "God-given talent". Once diagnosis is complete, he knows which plants to use to make a medicine to treat the problem and makes it by combining plants from the stocks he has on hand. In his experience, the most commonly treated illnesses were headaches, stomach problems, general pains, diabetes and more general treatment such as making plasters for wounds. He also assists with birthing and treats sick animals and pets. The late Oom Gert 'Julk' Dirkse, a second healer living in Paulshoek at the time of this study, dealt more with illnesses resulting from spiritual causes. He, and many inhabitants of Paulshoek, believed that spiritual problems manifest as physical illnesses. His diagnosis was carried out through divining using specific items in an almost ritualistic fashion, and he claimed to be able to treat people over a distance, without needing to be near them. His method of diagnosis sometimes showed that the patient has a "regular" illness, not caused by spiritual means and, if this was the case, he would advise the patient to seek treatment from the clinic. Such illnesses include high blood pressure, diabetes, rheumatic pain, colds and sinusitis.

In general, surveys and interviews indicated most people would rather make their own medicine at home as the first course of treatment. Then, if the condition persists, visit a conventional doctor or the clinic. If the doctor or clinic is unable to cure the malady, the herbalist/traditional healer is the final resort. This is the same view that was expressed during Goldberg's investigation (Goldberg, 1998), but differs from the other areas in which traditional and Western health care systems coexist. For example, in a study performed in KwaZulu-Natal, villagers preferred home remedies as the first line of treatment, followed by visiting the traditional healer if home remedies didn't work and then finally the Western clinic (Dahberg & Trygger, 2009). This sequence of treatment is also more common in areas that have limited access to Western health care e.g. rural Mozambique and other parts of Africa (Bruschi et al., 2011; Agyare et al., 2009), but may not represent preference for traditional healers over Western doctors as all options are not always available.

3.2.3 Which species are used?

The 50 medicinal plants mentioned by people in Paulshoek in the course of this study fell into 27 different families. However, nearly half of the medicinal plants mentioned during the survey fell into four main families. These were (in descending order): *Asteraceae*, *Lamiaceae, Mesembryanthemaceae*, and *Crassulaceae*. These families are also some of the most speciose in Namaqualand in general.

The trend of a few families dominating the list of medicinal plants is not limited to this instance in Namaqualand and it has been observed in other regions of South Africa as well. For example, in studies conducted in the Karoo, Transkei and Overberg regions, the majority of medicinal plants fell into the family *Asteraceae* (van Wyk et al., 2008; Bhat & Jacobs, 1995; Thring & Weitz, 2006), which is also the most frequently-cited family in this study in Namaqualand. While *Asteraceae* is the most commonly cited family in all of these areas, the actual genera and species used differ from region to region. Another example of a dominant medicinal plant family is *Lamiaceae,* which is one of the most commonly used families for medicinal purposes across several different regions of South Africa (van Wyk et al., 2008; Thring & Weitz, 2006). Some species are used across the country e.g. *Leonotis leonuris* as they have wide distributions, while other species, and sometimes families, are region specific such as species of *Solanaceae* and *Apocynaceae* resulting in heavy weighting of these families in particular areas. Despite regional biases, *Asteraceae* and *Lamiaceae* seem to be among the most commonly used families for medicinal purposes generally across all regions.

In Paulshoek, at first glance the particular species that are used seems to have changed over time suggesting a general shift in the pharmacopeia. Of the species mentioned in Archer's study (1994), Goldberg's study (1998) and this study, the only medicinally used species common to all lists were

Ballota africana, *Sutherlandia frutescens*, *Mentha longifolia* and *Salvia dentata*. If one only considers the study done by Goldberg (as this was done in Paulshoek and is more specific to the area than the broader study conducted by Archer) almost all plants were common to both lists. However, on closer examination it becomes apparent that in some instances the same genera were mentioned in both studies, but different species of those genera were listed e.g. *Pentzia*, *Pteronia*, *Rhus, Chrysocoma* and *Sceletium*. This may indicate that species were not correctly identified during the previous study (some species are very similar in appearance and can't be easily told apart when not in flower or without a microscopic investigation of certain features), or that very closely related species are interchangeable in home remedies. This is the practice in other areas e.g. KwaZulu-Natal where people said they had other species they could substitute into remedies if one was not available (Dahlberg & Trygger, 2009). The most popular plants recorded during this study, i.e. those mentioned most frequently in interviews, were *Sutherlandia frutescens*, *Dodonaea viscosa* var. *angustifolia*, *Searsia undulata* and *Salvia dentata*. Interestingly, *S. frutescens* is not common around the village and grows predominantly in the mountain fynbos region of the Kamiesberg, and neither is *D. viscosa*. This suggests that people go out of their way to acquire these plants and share them amongst themselves.

In contrast to local villagers, the healers possessed large dedicated sheds or huts to store lots of plant material sourced from all over the countryside, particularly from the fynbos region of the Kamiesberg. People often bring plants from other areas, and healers will share what they have amongst themselves. Most non-specialist villagers, however, do not stockpile in this fashion and collect only when needed.

3.2.4 Are certain families preferred for making home remedies?

The null hypothesis, H_0, was that plants from different families have the same probability to be chosen to treat a particular illness. Illnesses mentioned in interviews varied broadly and for the purposes of this analysis, recorded medical conditions were classified into 11 broad categories termed "use categories". A similar approach has been adopted in previous studies and these were used to guide the selection of use categories in an attempt to standardise the process (Salis-Lagoudakis et al. 2011; Moerman & Estabrook, 2003; Douwes et al., 2008). The 11 categories were: cardiovascular and blood related disorders e.g. high blood pressure (CAR), dermatological conditions (DER), gastro-intestinal conditions (GIT), General (including remedies described as "health tonics") (GEN), gynaecological conditions and fertility treatment (GYN), neurological conditions (NEU), musculo-skeletal conditions (MUS), Oto-rhino-laryngology (ear nose and throat, including mouth ailments) (ENT), respiratory and pulmonary ailments (PUL), urinary, bladder and kidney conditions (URI), other e.g. diabetes (OTH). Table 4 lists the types of illnesses encompassed by each

Table 4: Indications and therapeutic categories used in regression analysis

Use category	Abbreviation	Medical conditions included in this category
Cardiovascular and blood related conditions	CAR	Hypotension
Dermatological conditions	DER	Burn ointment, allergy treatment (dermatological antihistamine), sooth rashes, insect stings, boils, abscesses, wound and "sores" treatment, ringworm cure, spots and pimple cream, fever blisters, treat bald patches, itchy feet
Oto-rhino-laryngology conditions	ENT	Toothache, oral sores
General conditions, not specifically identified	GEN	General tonic, building strength after illness, tonic for children, general post partum tonic
Gastro-intestinal conditions	GIT	Emetic, sore or aching stomach, nausea (antiemetic), hunger pains, flatulence, diarrhoea, constipation (laxative)
Gynaecological conditions and fertility treatment	GYN	Help expel placenta after giving birth, infertility, tonic during pregnancy, alleviate excessive menstrual bleeding
Musculo-skeletal conditions	MUS	Body pains and aches, back ache, general muscular pain relief
neurological conditions	NEU	Mood enhancer, fever (febrifuge), headaches (analgesic), sedative, narcotic
Other, miscellaneous conditions not fitting the other categories	OTH	Hair treatment, diabetes, infant calmative agent, splinter removal, veterinary application, treatment of spiritual illness, cancer, haemorrhoids
Respiratory and pulmonary ailments	PUL	TB, colds and flu, asthma, expectorant
Urinary, bladder and kidney conditions	URI	Bladder problems, kidney and urinary tract infections

category. It stands to reason that there is a causal correlation between the medical application and the bioactivity (as determined by underlying phytochemistry) which should be revealed by this analysis (Weckerle et al., 2011). As medicinal plant species were allocated to 11 use categories, regression analysis was performed for each of the 11 categories. For the sake of brevity, one case example is described in full and a summary of the remaining 10 is presented.

3.2.4.1 Case example: use category "respiratory and pulmonary ailments" (PUL)

A regression analysis for the use category "respiratory and pulmonary ailments", which includes TB, asthma and respiratory infections, was performed at the family level. The number of species used for this illness category was plotted against the total number of medicinal uses per family (Figure 7). From this we can see that the data point referring to the family "*Lamiaceae*" is an outlier, far off the regression line described by the equation $y = 0.2993x - 0.1209$. This indicates that species from this

family are utilised much more often to treat respiratory ailments than if species were selected at random for this purpose. This suggests that *Lamiaceae* is a family of interest for respiratory and pulmonary illnesses and species in this family may yield compounds related to the treatment of these types of illnesses.

Residuals were calculated for all data points plotted in regression analysis (Figure 8). This confirms what was visually apparent from the regression plot - that *Lamiaceae* has a much larger residual value for this category of illness than any other family. Residual values also indicate that four families are tied for second position - *Sapindaceae*, *Fabaceae*, *Anacardiaceae* and *Alliaceae*. These families have slightly lower positive residuals than *Lamiaceae* and do not lie as far off the regression line. Hence, they are used marginally more than if plant use was random, but are not as preferred as species in the family *Lamiaceae*. *Mesembryanthemaceae* has the largest negative residual, indicating that species in this family are avoided when selecting plants to treat respiratory ailments.

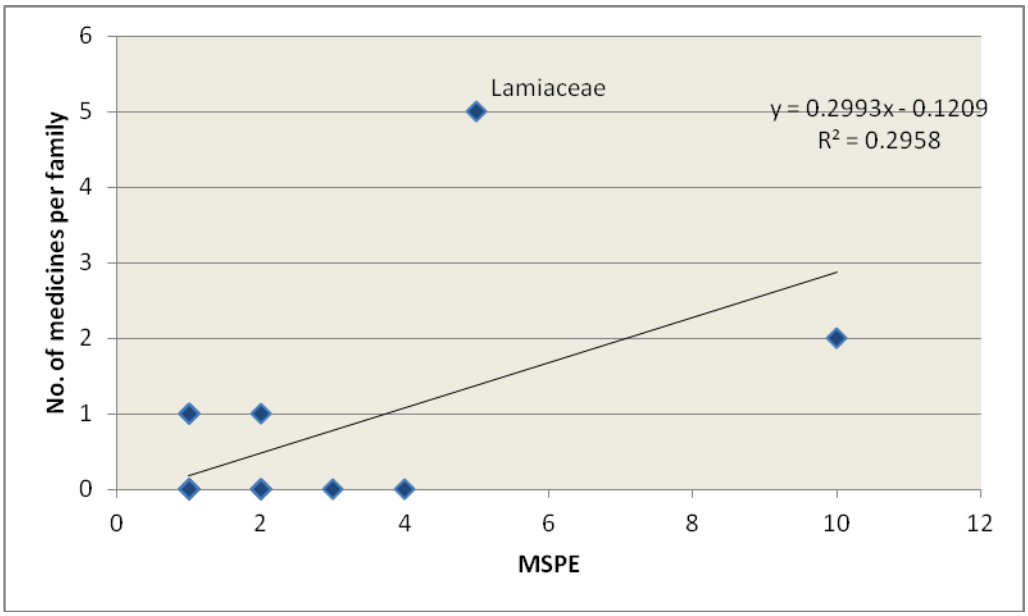


Figure 7: Regression analysis for Pulmonary medicines: the number of medicinally used species (MUSPE) vs. the number of uses for this type of treatment per family.

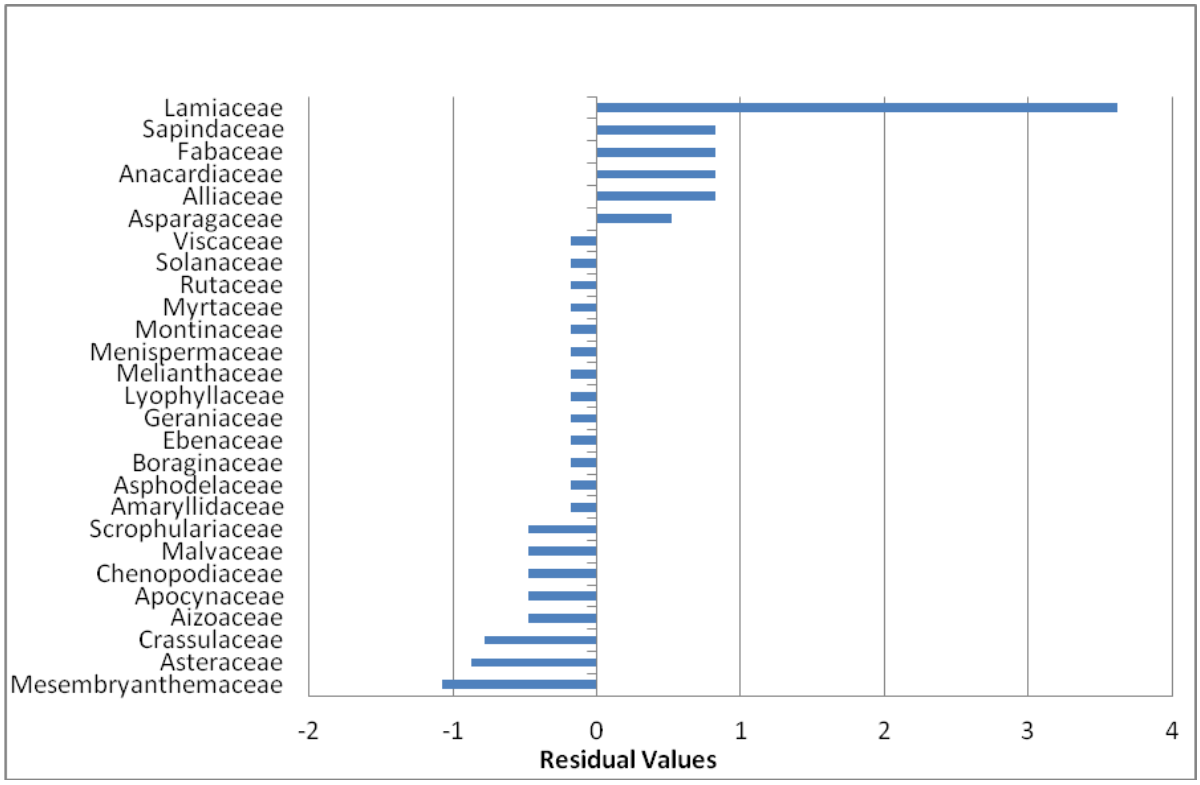


Figure 8: Residual values for the category PUL indicating that species of *Lamiaceae* are most preferred in this category.

A summary of the largest positive and largest negative residual values is given for each of the use categories investigated (Table 5). From this summary we can see that species in the family *Mesembryanthemaceae* are consistently avoided, having the largest negative residual for four of the 11 use categories. Families that have the highest positive residual in more than one use category are *Fabaceae*, *Aizoaceae, Lamiaceae* and *Myrtaceae*. This implies that species in these families are most often selected for the use categories in question. When the second largest positive residual is also considered other families also appear to be popular. *Asteraceae* and *Viscaceae* are also selected more than once for different use categories. However, a seemingly contradictory pattern also emerges, with *Lamiaceae* being one of the most avoided families as well as one of the most selected. This may mean that species in this family are particularly active, and that that activity is suitable for treating certain illnesses but not others. The activity may in fact be contraindicative in certain illnesses and thus the same species favoured for one illness are avoided for others.

Table 5: Summary of the major results for residual analysis performed on each use category. In some instances, two (or more) families were equally favoured for a single application.

Use category	Highest +ve residual	2nd highest +ve residual	Lowest -ve residual
CAR	Fabaceae	Asteraceae	Lamiaceae
DER	Amaryllidaceae Anacardiaceae	-	Lamiaceae
ENT	Aizoaceae	Crassulaceae	Asteraceae
GEN	Malvaceae	Boraginaceae Myrtaceae Montinaceae Rutaceae Viscaceae	Mesembryanthemaceae
GIT	Ebenaceae	Menispermaceae	Lamiaceae
GYN	Lamiaceae	Viscaceae	Mesembryanthemaceae
MUS	Melianthaceae Solanaceae Myrtaceae	-	Mesembryanthemaceae
NEU	Menispermaceae Myrtaceae Sapindaceae	-	Scrophulariaceae
OTH	Fabaceae Viscaceae	-	Lamiaceae
PUL	Lamiaceae	Fabaceae Sapindaceae Anacardiacae Alliaceae	Mesembryanthemaceae
URI	Aizoaceae	Asteraceae	Lamiaceae

Table 6: Comparison of growth types of medicinal plants (1998 vs. present study)

Growth form	Goldberg (1998) (%)	This study (%)
Large tree or shrub	26.7	28.0
Small shrub	26.7	38.0
Perennial herb	26.7	14.0
Annual herb	6.6	4.0
Succulents	13.3	12.0
Geophytes	0	4.0

3.2.5 Which growth forms are most used?

A wide variety of plant functional types or growth forms is used, as indicated by the diverse array of families recorded (Table 2). People do not limit themselves to using only shrubs or only succulents, for example, as a source of raw materials for making medicines. Despite the fact that the majority of plants fell into one of four main families (see section 3.2.3), there was still a diversity of growth forms within these families. Of the 50 medicinal plant species identified during this study, small shrubs were the most utilised growth form (38.0), followed by large trees and large shrubs (28.0%) (Table 6). This is consistent with other studies in other rural areas of South Africa. For example, Dahlberg & Trygger (2009) reported that trees and shrubs were the most commonly used growth forms in rural KwaZulu-Natal, with 34.0% of the plants used by local people and 47.0% of plants used by healers falling into this group.

In Goldberg's study (1998), it was reported that large trees, large and small shrubs and perennial herbs were equally the most frequently utilised for plant medicines in Paulshoek. While large trees, large and small shrubs and perennial herbs remained the most frequently used categories of growth form, there appears to have been a general shift to using more trees and shrubs and fewer perennial herbs. Perennial herbs dropped from 26.7% of the plants used (1998) to 14.0% (current study), while the use of both large trees and shrubs and small shrubs increased slightly (from 26.7% (1998) to 28.0% (current study) for large trees and shrubs and from 26.7% (1998) to 38.0% (current study) for small shrubs). A decline in use was also evident in annual herbs, which dropped from 6.6% (1998) to 4.0% (this study). Succulents made up approximately the same percentage of plants used in both studies (13.3% in 1998 vs. 12.0% current study). No geophyte use was recorded in 1998, but this study shows that 4.0% of the total number of medicinally used plants was comprised of geophytes.

3.3 Plant use in practice

During the survey, interviewees were asked about methods of plant collection and remedy creation in order to get a better idea of medicinal plant uses and practices. Of the people that said they use plant based home remedies, 30.3% said they only use medicine when it is prepared by someone other than themselves (friends or family, rather than a formal traditional healer). Therefore, 69.7% of people responding positively in the survey actually collect plants (are "collectors") and know how to make medicine (are "preparers"). This is an increase from 14 years ago when Goldberg (1998) reported that 70.0% of people in Paulshoek used plant medicine, but only 36.0% of people actually collected plants and made home remedies. Thus, over the past 14 years, there has been a

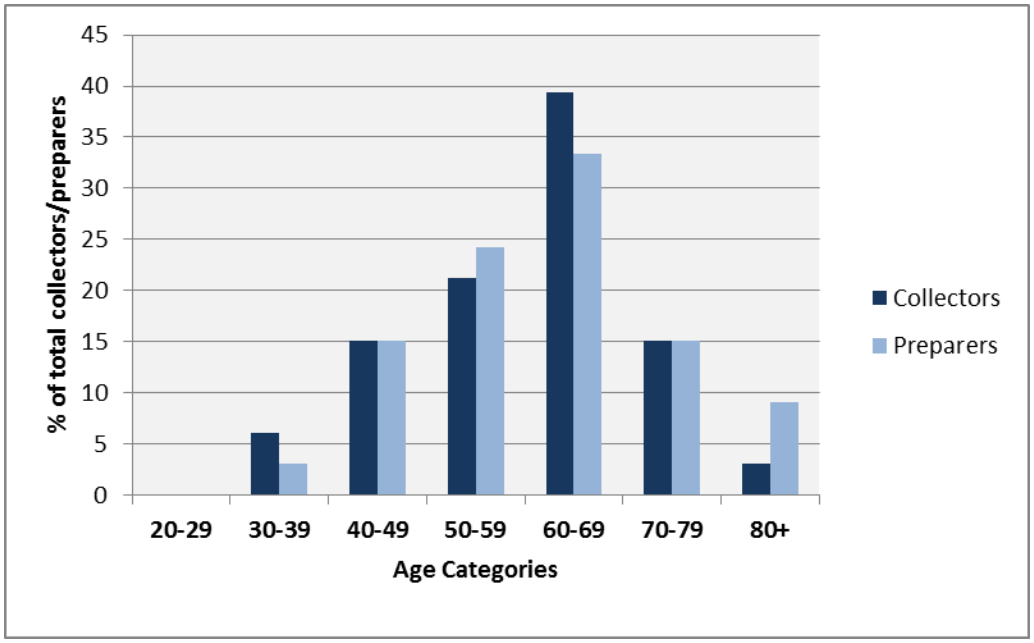


Figure 9: Trends in ages of people collecting plants and preparing medicine, by age group

substantial increase in the number of people who can identify plants, collect them and make medicine from them, suggesting that knowledge is being shared and new people are being shown how to collect and prepare medicine.

The average age of collectors was 60 years (ranging between 35 and 80 years), while the average age of preparers was 62 years (ranging between 39 and 96 years). Goldberg (1998) reported that more than 80.0% of collectors were over 50 years old, and none were younger than 40 years old. Of the people in the current study that collected and prepared medicine, 78.8% of collectors and 81.8% of preparers were over the age of 50 (Figure 9). This does not represent a significant difference from the previously reported number.

The majority of people that collect and prepare medicine fall into the 60-69 year old category. While the percentage of people over 50 is virtually unchanged since Goldberg, there has been a dynamic shift in the composition of this group i.e. the group does not only consist of the same individuals from 14 years ago. Even the youngest person recorded in the Goldberg survey would now be at least 64 (assuming the youngest to be 40 years old in 1998) and the majority of the respondents from that study would now fall into the 60-69 year old and the 70-79 year old categories.

There are still a substantial number of people in the 70-79 year old and 80+ age categories that prepare medicine (24.2% of the total), and it is often the case that a single older person makes medicine for more than one house. There are still some people (18.2%) in the 70-79 year old category that collect plants, but the number drops to only 3.0% for over 80's, suggesting that people

are just physically unable to do it any longer. This feeling of being "too old" to collect plants was echoed by the healers. Gert Dirkse stated that he felt a bit too old to climb mountains to collect plants, and had enlisted the help of a younger man to do the job for him. Oom Kootjie also said that he was too old and unwell to collect plants anymore, and that his personal health and inability to go into the veld had affected the number of people who wanted to be treated by him.

The youngest person to be recorded in the previous study would now be at least 63. Thus, the fact that during this survey collectors and preparers were recorded in the 30-39 and the 40-49 age brackets means that younger people are starting to collect medicinal plants and to prepare the medicine themselves (rather than relying on the older generations). Even though there are only a very few younger people learning to collect and/or prepare plant remedies (6.1% of collectors and 3.0% of preparers in this survey were under 40), it is a positive sign as there were no collectors or preparers in this category 12 years ago (Figure 9). Despite this positive trend, there were still no people in the 20-29 year old category that either collected or prepared medicine.

3.4 How is knowledge acquired and transferred?

In order to investigate how knowledge regarding medicinal plant use is acquired and transferred, people were asked where they had learnt about medicinal plants and, if they had learnt from another person, the gender of that person. We already know that there has been an increase in the number of people with knowledge about medicinal plants over the past decade (see part 3 of the Results section of this chapter), and this section investigates how these people acquired their knowledge.

3.4.1 How did they learn?

Three quarters of respondents (75.6%) had acquired their knowledge from family members (mostly a parent or grandparent, rather than from a member of the extended family), 17.8% had learnt from other knowledgeable (non-family) members of the community and only 6.6% had learnt what they know from a traditional healer. One respondent said he was self-taught, through trial and error, although for lay people this was the exception rather than the rule and the majority of people were taught what they know.

The opposite seems true for healers though, with both healers saying that they tasted plants and experimented on themselves to learn about plant properties. While Oom 'Kootjie' Corjeus said that he had been taught the basics by his father-in-law, he had expanded his knowledge through tasting plants from the veld and selecting them according to certain criteria e.g. bitter tasting plants could be used for stomach ailments and "harsh" tasting plants were good for treating headaches. As mentioned before, traditional healing involves a spiritual side, and this is evident as even though he

tastes and tests plants, he attributes the ability to correctly identify new plants as a "God-given talent". Oom Gert 'Julk' Dirkse says that he taught himself but was also guided by God as he mainly treats illnesses with a spiritual cause. His method of selecting new plants for making medicine is, appropriately, more abstract. Once again, he says if he notices a new plant, he will taste the plant himself, and then he can tell if the plant will be good for medicine by drying it, crushing it to a powder and using a pendulum to divine its potential. Independent verification of such approaches to medicinal plant identification has not as yet been undertaken.

Some people who use medicinal plants have no knowledge of them and this may be quite common (or an emerging trend). 30.3% of people only take medicine that other people make, and can't identify plants or make medicines.

3.4.2 Gender roles

Gender does not appear to be as big a determining factor in the level or type of medicinal plant knowledge in Namaqualand as it does in other areas of South Africa. In Paulshoek, 28.9% of respondents learnt what they know about medicinal plants exclusively from men, and 17.8% learnt exclusively from women. The majority of people, 53.3%, gained knowledge from multiple sources, both male and female, suggesting that there is no gender bias one way or another when it comes to knowledge of plant-based, home remedies. This is in contrast to results from similar studies in other rural areas where women (mothers and grandmothers) were found to hold the majority of home remedy knowledge (Thring & Weitz, 2006; Gedif & Hahn, 2003; Hernández et al., 2003, Karunamoorthi & Tsehaye, 2012). While this was the case for knowledge of home remedies, formal traditional healers tended in most instances to be men (Dahlberg & Trygger, 2009; Dold & Cocks, 2000).

3.5 Perceptions of plant use and their role in the community

An important aspect of this study was to investigate the role of medicinal plants in the community, and to evaluate commonly-held perceptions of them and their use. To this end, interviewees were asked a few set questions and then asked to comment freely on any aspect of medicinal plant use, healers, and "Western" medicine and to add anything else they felt was important.

3.5.1 Clinic vs. veld - what is the relative role of each in healthcare?

Many people perceive plants as a better treatment than medicine obtained from the clinic. 22.0% of people said plants were better medicine and it is "healthier" to use plants, mostly due to the belief that they don't leave side effects. There is also the idea that plants collected from the veld provide the "best" (i.e. most effective) medicine. Despite this belief, many people are planting plants they use the most often in their gardens. 61.4% of people using medicinal plants collect the plants they

use from both the veld and from a cultivated garden on the homestead. 29.6% of people only collect plants from the veld, and a small amount (4.6%) only use plants grown in the garden (Figure 10).

The traditional healers mostly use plants collected from the veld, and are aware of using this resource sustainably. Oom Gert 'Julk' Dirkse, for example, stressed that one should only break off pieces to use and not pull the whole plant out of the ground when collecting. He has also planted a few species in his garden, but the vast majority of the plants he uses are collected from far afield.

Despite the prevalence of using plant medicine, 77.2% of people had also visited the clinic in the previous year, with the main reasons cited as "for the baby", "for chronic medication", "for diabetes", or "for high blood pressure". There appears to be a large number of people being treated for high blood pressure and diabetes in the village. No one in the survey had visited a traditional healer in the previous year. 22.8% of people had not visited the clinic either and relied entirely on home remedies for their health care. These people tended to be older people.

100.0% of the people using medicinal plants said they would be interested in a workshop or learning more, especially about what other people had to say. Many also thought that it would be a good opportunity to get the youth interested.

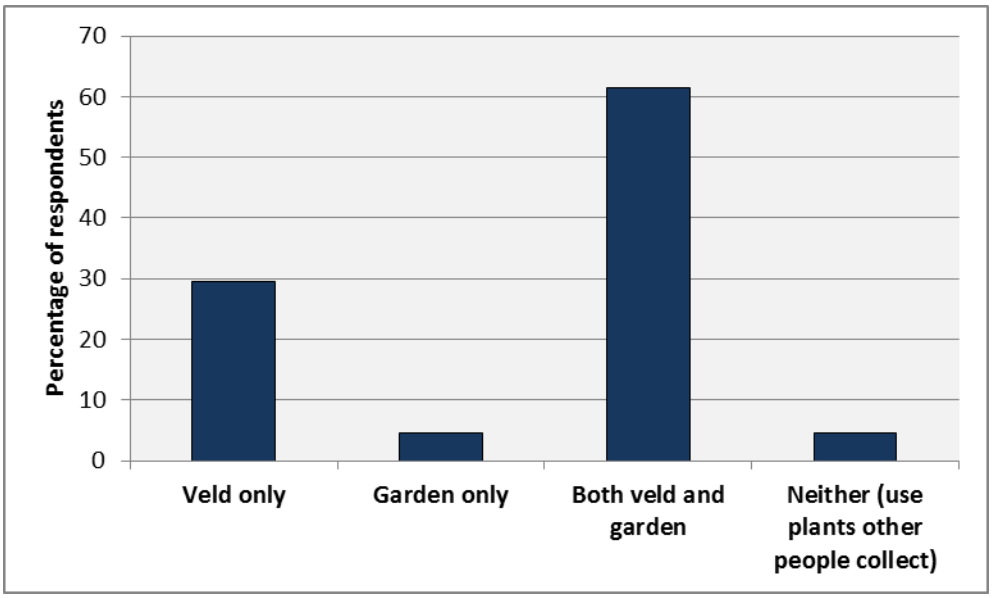


Figure 10: The percentage of interviewees that collect plants from each source

3.5.2 Widely held perceptions of medicine and medicinal plants

Other comments made during the interviews could be grouped into four broad categories:

3.5.2.1 Concern with the loss of traditional knowledge

Many people were concerned that there has been a loss of knowledge about plants and traditional plant medicine as the most knowledgeable people have died and not passed on what they know. It was felt that "the researchers" came too late to preserve most of what was known, and now only a fraction of what was known in the past has been recorded. There was a general feeling that more was known in the past by grandparents and that the most knowledgeable people still alive are the older generations, but they often can't remember everything as the memory is not too good as age progresses, and so their knowledge has also been lost.

3.5.2.2 Concern that the traditional ways are no longer relevant in the modern day

It was suggested that the tradition of using medicinal plants is dying out and just isn't as important in modern life as they were to people in the past. One person said that in the past they used plants because they didn't know about germs, disease and vaccinations. Now that more people have at least some understanding of basic healthcare (due to an improvement in education) there is little need for people to use plants when there is the clinic. There was also the perception that it is more difficult to work with plants nowadays as people think you need to register to work with plants and it's just easier to avoid medicinal plants altogether. This belief is incorrect - while there may be a drive to formalise and regulate traditional medicine practice in South Africa, there is no such rule governing the use of home remedies using your own garden plants.

3.5.2.3 Perceptions that plant medicine is better than Western medicine

Several people felt that local plants make better medicine than that prescribe by the "Western" doctor. There was a variety of reasons given to support this idea - mostly that they are "safer", cheaper and have no side effects. Different people said that plants are the secret to a long life; and plants work better than the medication from the clinic for illnesses like diabetes and high blood pressure. One person said that the doctor's pills "calcify" his body, but plant medicine does not do this.

3.5.2.4 Perception that plants and plant knowledge is valuable - should it be shared?

Many of those people that use medicinal plants felt that other people don't know the value of plants and the knowledge of how to make medicine. Most people were of the opinion

that the knowledge should be shared, particularly that "the researchers" need to share what information has been collated with everyone in the village, especially the youth. They were interested in learning what their neighbours use plants for and possibly discovering new uses for plants. People were, for the most part, happy that research is being done on the topic, but there was one exception. One person refused to share any information on the topic and insisted on being paid. He said it was his secret. However, people were more than willing to share and get involved as they see it as an opportunity for the youth and themselves to learn something about their traditions which they feel are dying out with the older generation.

3.5.3 Will the youth learn traditional ways?

People were divided on this topic. Half of the respondents felt that the youth will learn and continue the tradition while half suggested that they would not and that they have no interest. 100.0% of respondents said they felt there was benefit to having a workshop about medicinal plants and most were of the opinion that youth might use plants if they were told how to use them and were given more information at such a workshop. It seems that over the years more people have started thinking that the youth won't learn, as the general perception during the Goldberg study in 1998 was that knowledge would be passed on and the youth would be keen to learn. This belief may stem from the fact that both traditional healers have passed away recently, neither having trained an apprentice.

4. Discussion

4.1. The role and importance of plants in the lives of Paulshoek residents

Plants play an integral role in the lives of Paulshoek residents, particularly for the purpose of healthcare. Many people view plants and knowledge of plant use as valuable, and wish to preserve their traditional healthcare system. So much so that a few residents were unwilling to share their knowledge of medicinal plants and believe that it should be kept secret from "outsiders". This view however seems to be the exception rather than the rule in Paulshoek, but is often encountered in other rural areas. For example, in the Transkei it is believed that herbal preparations will lose their potency if the secret of how to make them is revealed to anyone from "outside the village" (Bhat & Jacobs, 1995). Plant use is so widespread in the Paulshoek community that 70.0% of residents use medicinal plants, and 22.8% of those use plant-based home remedies exclusively. This is in line with the World Health Organisation's estimate that between 60 and 75% of the global population relies primarily on plants to fulfil their healthcare needs (World Health Organization, 2002), and slightly

more than 70% of South Africans living in rural areas do the same (Van Staden, 2008). Despite there being virtually no change in the percentage of people in Paulshoek using medicinal plants since Goldberg's (1998) survey, subtle changes in the demographics of medicinal plant users have occurred over time. These changes hint at an underlying shift in attitude to, and use of, traditional methods of healthcare. For example, 18.2% of people interviewed in this study had used plant-based home remedies in the past, but no longer did so, suggesting that there are other forces at work influencing the use of medicinal plants in Paulshoek and it is a more complicated and delicate health care system than appears on first glance.

While many people who use plant-based remedies view them as being "better than Western medicine", there are a number of people who feel that plant medicine is old fashioned and outdated. This disparity in attitudes may be linked to age, with older generations preferring the "traditional ways", and the younger generations, who have had more exposure to a Western lifestyle and culture, rejecting what they perceive as "the old ways" (Gedif & Hahn, 2003; Satimia et al. 1998; Karunamoorthi & Tsehaye, 2012).

The average age of collectors and preparers recorded in this study was 60 and 62 years respectively. Also the majority of people collecting and preparing were over the age of 50, and none were in their 20's. This creates the impression that it is only the older generations involved in medicinal plant use. This, in turn, may be the reason that many children are not using medicinal plants as their young parents do not have the knowledge required to gather plants and prepare medicine. They are left with the situation where a few older people prepare medicine for several others. In fact, this study shows that 30% of people using plant medicine are only able to access medicine via a third party i.e. when it is made by someone else. It would seem, at least on first investigation, that the majority of plant knowledge *is* held by older people.

Despite this, there are a handful of younger people learning to collect and/or prepare plant remedies. Once again there are some interesting dynamics at work. While not many young people (defined here as being under the age of 30) are learning about the medicinal value of plants, there has been a 100% increase in the number of people who can identify plants, collect them and make medicine from them since the 1998 study by Goldberg. This indicates that the older generations who hold the knowledge are sharing and teaching others, but mostly with other people who are at least 50 years old, rather than with the younger generation in their 20's. The reason for this may be that only those older than 50 are seen as responsible and mature enough to be taught the ways of plant use. Or it may just be that as people get older, health becomes more of a concern and this prompts them to learn how to make medicine for themselves, or at the very least how to treat a nagging

personal pain or illness (Dalhberg & Trygger, 2009). This demographic pattern of plant use seems to be common in some areas (Gedif & Hahn, 2003; Satimia et al. 1998; Karunamoorthi & Tsehaye, 2012), while in other rural areas the situation is reversed. In some areas it is the "middle aged" people (30 – 50 years) who know the most about medicinal plants and their uses, while the older generations report that they have forgotten most of what they once knew (Dalhberg & Trygger, 2009). There are also villages, for example in Kenya, where children pick up plant knowledge and treat their own ailments without adult supervision (Geissler et al., 2000). Neither of these situations seemed to be the case in Paulshoek. In future, the demographics of plant users in Paulshoek may shift to either one of these models, but there are currently no indicators that this is imminent.

Gender does not seem to play as a big a role in the traditional medicinal systems present in Paulshoek as it does in other areas of South Africa, and globally. Both men and women are involved in plant collection and the preparation of medicines, which is different to other areas where it is mainly the women involved in preparing home remedies while the role of formal healer is often reserved for a man (Thring & Weitz, 2006; Gedif & Hahn, 2003; Hernández et. al, 2003**;** Karunamoorthi & Tsehaye, 2012). Dahlberg & Trygger (2000) suggest the reason for this distinction could be due to the traditional roles assigned to young adults, particularly in rural environments. Daughters tend to stay at home with their older female relatives to help with cooking, household duties and looking after the younger children. Male children are sent to tend fields or livestock. Therefore, women have a more immediate need to treat ill children at home and have the opportunity to learn home remedies while spending time with female relatives who pass on their personal experiences and knowledge. Tending livestock and fields tends to be a more individual activity, where the males would not have the opportunity to learn about the medicinal value of plants, unless they are apprenticed to a formal traditional healer.

In the past in Namaqualand, women seemed to have had a better economic standing than they did in the late 20th century, particularly with regard to stock farming and land ownership. But the loss of land and changes to society that occurred in the 19th and early 20th centuries lead to increasing marginalisation of women to reproductive and domestic roles (Archer & Meer, 1997). In theory, all community members residing in the communal areas are entitled to residential and arable land plots if they wish to farm. In practice it was not that simple. Research undertaken between 2003 and 2005 suggested that women who wanted to farm faced difficulties in obtaining land as land was traditionally passed along family lines from father to son or to another suitable male family member. There was a general reluctance to bequeath land to daughters. Commonly held opinions given in different villages in Namaqualand were that "*fathers focus on their sons when it comes to farming*

with the understanding that they will become farmers and household heads one day" and "*a woman's function is to support the husband, take care of the house, rear the children and do the washing and cook while livestock remain the men's domain*" (Kleinbooi & Lahiff, 2007). This echoes the findings of Dahlberg & Trygger (2000) and suggests that we should expect to see a similar pattern of female dominance in plant collection and medicine preparation, which is not what was observed.

Despite these commonly held perceptions of gender roles, a study conducted in the communal areas of Namaqualand, including Leliefontein, indicated that many women played a vital role in the functioning of farms (Kleinbooi & Lahiff, 2007). Some women interviewed during the study consider them and their husbands as partners in a joint farming enterprise, despite the fact that the land was registered in the husbands' name. A minority of women had been able to get land in their own names and engaged in small individual farming activities such as vegetable production. It may be this longstanding blurring of the "traditional roles" that lead to the observed pattern of relative equality between the sexes (at least in regard to medicinal plant knowledge) in Paulshoek.

Over the last 15 years in Paulshoek there has been an increase in the number of female-headed households from 30% in 1995 to 44% in 2010 (Duijnstee, 2011). Since the head of a household is also the property owner, 44% of the property in 2010 was in the hands of women. In 1995 this situation wasn't as prevalent. This may be because strong social convention dictated that land ownership was transferred through inheritance along male familial lines. Today, women have rights to land and it is easier for them to obtain land ownership. More women are informed of their rights to own land in the communal reserves and do so without having to rely on male family members. Women can apply directly to the municipality for access to land on which to farm. Even though this was legally allowed in the past, social conventions and tradition dictated that it was nearly always a man who acquired land in his name. Even if a wife acted as part owner, sharing all duties and responsibilities it was still the husband whose name was on the title deed (Kleinbooi & Lahiff, 2007). They also participate in community meetings and are involved in decisions that affect the village. Despite the fact that many women do accept the traditional role of home maker and child rearer, they are not resigned to these roles, and they have not been enforced. Not all men believe in the patriarchal system that, until recently, has dominated communal societies and this may have resulted in the observed pattern of gender in plant use (Kleinbooi & Lahiff, 2007).

Paulshoek residents had different attitudes towards traditional healers and the use of home remedies which are viewed as very different practices despite both being part of the "traditional healthcare system". Residents are confident in preparing simple plant remedies for day to day

ailments, but healers are considered as having specialist knowledge that the average person does not possess. This is most likely a reflection of the spiritual link that healers use in diagnosis and patient treatment. Still, home remedies are the first choice for most people, despite there being reasonably priced alternative healthcare options available. This preference is seen in other areas of South Africa, as well as globally. Healers are visited as a last resort, or for obscure ailments, and sometimes as the third option after Western medicine has been considered. (Dahlberg & Trygger, 2009; Giovannini et. al, 2011). With home remedies, people have the power to manage their own healthcare, which is important to a society which has been historically displaced, dispossessed and excluded from decision-making processes regarding their own future.

4.2 The influence of ethnobotany, education, and government policy on the use of medicinal plants

Many people in Paulshoek are concerned about the effect that "modernisation" and "the West" are having on traditional methods of healthcare. Now that there are widespread campaigns aimed at educating people about health and hygiene and which promote Western medicine and healthcare services, people may be less inclined to use traditional plant medicines. It was mentioned during interviews that people had little knowledge about vaccinations and the ways in which various diseases are transferred between people before health education came along, and now that they do, they take their babies to the clinic and are more aware of unsanitary practices. In some parts of Africa there has been confusion once a village receives health education, particularly those areas where the belief in witchcraft persists. For example, in a study performed in the Kilombero District of south eastern Tanzania it was found that information given to the community regarding mosquitoes, malaria prevention and treatment had merged with local knowledge. Some people were under the impression that a witch could cause the symptoms of malaria without the person actually contracting the disease. So people did not always go to the clinic for treatment, despite the extensive education they received regarding malaria (Meula et al., 2002). This type of confusion does not seem to have happened in Paulshoek. People have assimilated health education and not misinterpreted the information. For example, people have been told about diabetes and what it is. The local name for the illness is "suiker siekte" (lit. "sugar sickness") indicating an understanding of the disease and its causes.

While the majority of parents with infants and young children do take their children to the clinic for vaccinations, it does not mean that they neglect the traditional plant medicines in favour of the clinic. While there is a definite and noticeable effect of health education and modernisation on the inhabitants of the village, it is not necessarily a negative one. It also does not necessarily mean that traditional ways will be abandoned. The introduction of pharmaceuticals does not have to displace

traditional healthcare systems and a complementary system may evolve. This was the case in rural India where Western medical information and treatment have been incorporated alongside traditional Indian plant medicine (Lambert, 1996; Minoncha, 1980). Sometimes the preference for traditional medicines is so strong that it survives long distance migration. For example, Bolivian and Peruvian immigrants in London purchased traditional Andean plant medicines from "Latino shops" that import dried plants from South America. Roughly 50% of the traditional medicines used in Andean countries is available in the UK through these shops. People still prefer the remedies of their home villages over the free Western healthcare available through the National Health Service (Ceuterick et al., 2011). Similarly Chinese migrants in the UK also prefer traditional Chinese medicines and visit the increasing number of traditional Chinese herbalists, acupuncturists and doctors practicing in Britain (Green et al., 2006). In South Africa, an increase in the use of traditional medicines, particularly dried plant materials, has been documented in some urban centres. Pietermaritzburg in KwaZulu-Natal is one such city where there is enough demand for medicinal plant materials to necessitate a large informal market (Ndhlala et al., 2010). The use of traditional medicine is not restricted to rural areas, and studies suggest that rural residents who migrate to urban centres continue to use traditional remedies (Marsland, 2007). However, the incidence of this trend in migrants from rural Namaqualand to urban centres in the Western Cape has not been investigated.

In many instances, as in other areas of the world where Western medicine and traditional healthcare systems co-exist, people use a mixture of home remedies and Western pharmaceuticals (Etkin et al., 1990; Meula et al., 2002; Giovannini et al., 2011; Giovannini & Heinrich, 2009; Calvet-Mir et al., 2008; Scrimshaw & Cosminsky, 1980). In Paulshoek, 75% of people had visited the clinic for treatment in the year preceding this study, indicating that the majority of residents practice medical pluralism[3].

The extent of medical pluralism in South Africa is not well understood, but in other African countries, such as Kenya, studies suggest that it is a widespread practice (Nagata et al., 2011; Janzen, 1978). This is a cause for concern amongst medical practitioners as there may be unknown pharmacokinetic and pharmacodynamic interactions between medicines dispensed from the clinic and traditional plant remedies (Langlois-Klassen et al., 2008). In some cases, patients do not disclose their use of plant medicines and the interactions between pharmaceuticals, particularly antiretroviral agents (ARVs), and plants are of particular concern. Of the commercially available plant medicines, "African potato" (*Hypoxis* spp.) and "Cancer bush" (*Sutherlandia frutescens)* have been tested and in some

[3] Medical pluralism refers to the practice of drawing on both traditional and Western health care systems for personal wellbeing and treatment of illness

instances appear to inhibit ARVs (Mills et al., 2005; Nyika, 2007). In the case of nevirapine these traditional treatments cause increased uptake of the drug into the blood, resulting in incorrect dosage and possible toxicity (Brown et al., 2008). Most of the plants being used in Namaqualand, and in South Africa, have not been tested for drug interactions or safety (Nagata et al., 2011; Street et al., 2008). In Paulshoek in particular, there are a number of people who mentioned that they receive chronic medication for high blood pressure and diabetes, among other illnesses. The interactions between medicinal plants and these drugs have not been studied and could be a cause for concern as the village relies increasingly on pharmaceutical products delivered by the mobile clinic (Ernst, 2000). The danger of plant-drug interaction is not limited to rural villages. The use of Complementary and Alternative Medicines (CAM, which covers a range of treatments includes aromatherapy, herbal medicines etc.) is on the rise in developed nations such as the UK. Products remain largely unregulated, non-standardised and untested for interactions with each other or with pharmaceuticals. Such products are available to the public without supervision or assistance from a health care professional and people similarly do not disclose their use (Wahlberg, 2007). In some areas where medical pluralism exists, for example, in Thailand, traditional treatments have been adopted by Western practitioners. Hospitals offer treatment with traditional remedies, and a combination of traditional and "modern" hospital staff provide treatment. This combination of the two healthcare systems supposedly allows the "safer" concomitant administration of herbal and pharmaceutical preparations. At the very least it ensures that doctors are aware of the exact treatment a patient has received (Chotchoungchatchai et al., 2012). A similar system might be successful in South Africa, but would take many resources, much careful management and careful integration to achieve. There is still the view that the isolated active compounds from traditionally used plants have a weaker ability to heal than extracts created from whole plants, or plant parts (Rodriguez-Fragoso et al., 2008). So, perhaps this is not feasible considering the current state of South Africa's public health system infrastructure, but it is something that might be an appropriate goal.

Another effect of increasing Western influence on traditional medicinal plant use was investigated in a previously isolated village in rural Mexico (Giovannini et al., 2011). The study determined that there is a negative association between increasing levels of schooling and the use of medicinal plants. A similar trend has also been observed in African villages (Satimia et al 1998; Gedif & Hahn, 2003; Karunamoorthi & Tsehaye, 2012). This may also be true in Paulshoek. Education levels rise as education facilities become incorporated into the national system. Young adults in Paulshoek are the most educated (having on average twice the number of years of schooling that their parents have),

and are also the age group least involved in collecting, preparing and using plant medicine. The long-term of effects of this remain to be seen.

In addition to increased levels of education, is an increased level in the standard of living, as a result of government projects to uplift the poorest communities by building houses and roads and introducing services such as sanitation and electricity. Better sanitation and clean water means that fewer water-borne diseases such as diarrhoea and bacterial infections are likely to occur. This means that there may be less of a demand for home remedies to treat these ailments, which in turn may, in coming years, lead to a decreased reliance on medicinal plants and a loss of traditional knowledge of these treatments. In addition, now that many houses have electricity people can use electrical or gas-powered cookers instead of having to rely solely on wood-fuelled fires in outdoor cooking shelters. This means that there is less smoke inhalation and possibly in the future we will see fewer incidences of asthma and other respiratory illnesses. Many people still use fires, and the damage caused by years of smoke inhalation cannot be undone in a few years. Perhaps the younger generations will see a decline in respiratory problems in the coming years.

The presence of the clinic, increased levels of education and better amenities are not the only influences that may affect traditional healthcare systems. Government policy is also a factor. The government has proposed the regulation of medicinal plants, healers and the trade in medicinal plants mostly in response to concerns regarding the safety of traditional medicines and the overexploitation of raw resources. Many cases of poisoning are caused by the incorrect use or preparation of plant medicines, and usually involve self-administration or administration to children. Between 1991 and 1995, 43% of traditional medicine poisoning cases treated in Johannesburg hospitals were caused by plants (Stewart et al., 1999). Similar trends are observed elsewhere and are not limited to developing or rural areas. For example, poison control centres in the United States of America reported that for the period 2000 – 2007 “dietary supplements, *herbs* and homeopathic products” was the highest group of products associated with poisoning requiring hospitalisation (Vassilev et al., 2009).

Other concerns regarding traditional medicines which may influence government policy and the use of plants are incorrect plant identification, heavy metal contamination and microbial contamination. In a test of dried plants sold in various African markets for the purpose of treating various illnesses with herbal teas, it was found that many of them contained large microbial concentrations at levels that would be unacceptable to commercial food supplement manufacturers (Street et al., 2008). In the USA, herbal preparations and traditional medicines are regulated as dietary supplements. The United States Federal Drug Administration (FDA) requires that proper controls are in place to ensure

these products are processed in a consistent manner and meet certain quality standards. However, manufacturers do not have to register their products with the FDA and so many remain unregulated (Jordan et al., 2010).

Heavy metal toxicity is also a concern in South African medicinal plants, some of which are known to accumulate certain metals (Street et al., 2008). For example, *Senecio coronatus* (Thunb.) Harv. (*Asteraceae*) accumulates nickel which can cause allergies, lung fibrosis and cancer (Przybylowicz et al., 1995; Kasprzak et al., 2003). There is also the problem of adulterated or counterfeit products reaching the market. South Africa needs government regulation of traditional medicines to ensure quality, safety and authenticity. Particularly if traditional medicines are in future to be incorporated into treatment plans offered by hospitals, as is the case in Thailand (Politi et al., 2009; Heyman & Meyer, 2012). Unfortunately, comprehensive safety and efficacy data on traditional medicines are lacking, and will take time and money to obtain (Springfield et al., 2005).

However, the testing and standardising of traditional medicines is difficult and could have a significant impact on the current traditional healthcare system and the way it is often practiced. Yet one cannot ignore the poisonings caused by misinformation and the possible microbial or heavy metal contamination of products intended to heal. There must be a way to provide safe and efficacious traditional medicines without changing the system so radically that it loses its identity and the characteristics that define it. In this spirit, the government has begun attempting to regulate the system with the proposed Traditional Health Practitioners Bill of 2003 (Government Gazette No. 24704, 2003). The proposed legislation would require all persons involved in treating patients with traditional medicines to register. It also promotes a regulatory framework which will ensure the efficacy, safety and quality of traditional health care services. As yet this has not been passed. A subsequent Draft National Policy on African Traditional Medicine in South Africa appeared in 2008, which aimed to provide a framework for the institutionalizing of African Traditional Medicine in the South African healthcare system, but this too has yet to be passed into legislation (Draft National Policy on ATMSA, 2008). Registration would provide practitioners with credibility, provide a forum for healers to talk to one another and would also allow only those with the necessary skill to prepare medicines. This sounds like a good idea when faced with child deaths and poisonings, but it would make home remedies illegal and would effectively halt the use of traditional medicines in villages such as Paulshoek. It may also have the negative effect of creating a black market in traditional medicinal plants for those practicing without a license, effectively criminalizing those people it aimed to protect. Even though the WHO has called for the integration of traditional medicines into

mainstream national health systems, this may be difficult to achieve in practice. At the very least it will take a long time and more research is needed before a suitable solution is reached.

4.3 Conservation, bioprospecting and the value of medicinal plants

The value of medicinal plants to the people of Paulshoek is unquestionable. However this resource may be under threat in several different ways, including threats caused by the very same people for whom the plants are so important. In Paulshoek, as in many areas of South Africa and the world, the most common practice is to prepare remedies using plants collected from wild populations (Zschocke et al., 2000). A large number of these are non-renewable resources i.e. when material is collected for the purpose of medicine preparation, the entire plant (or a part vital to its survival e.g. roots, bulbs or bark) is collected (Fennell et al., 2004a). This practice puts pressure on wild populations and as the demand for plant medicine grows, becomes largely unsustainable. In Paulshoek, many medicines are created using leaves alone, and harvesting does not kill the plant or remove it entirely. However, alternatives to wild harvesting may still need to be provided if the practice is to continue or to increase.

In Paulshoek, people are becoming aware of the need to preserve plants. During an interview with one of the healers, the point of not damaging the plant and only taking what is necessary was repeatedly stressed. People also plant gardens near to their houses to provide them with some of the more commonly known medicinal plants rather than collect from the wild. 61.4% of people said that they collect plants from both the veld and from a cultivated garden, which reduces the pressure on wild populations. In a similar study conducted in the Overberg region of the Western Cape province of South Africa, 70.0% of participants said they get their plant materials from their own gardens (or from roadside verges) thereby greatly decreasing the pressure on wild populations (Thring & Weitz, 2006). In some areas stricter, more conservative healers only use wild-grown plants as they consider that wild plants “have more power” or are more potent than their cultivated counterparts (Vermeylen, 2008). But people in Paulshoek don’t seem to be too concerned that they are essentially cultivating the plants in their gardens to make medicine, and still use them in the same way regardless of whether wild or garden grown. However, this cultivation for individual personal use is very different to commercial cultivation, which has been proposed as an alternative source of medicinal plants. Under cultivation situations, environmental conditions can be different to those that would normally be experienced by a plant in the wild. These changes affect the biochemistry of cultivated plants, which is of great importance when the plants are being grown for medicinal purposes (Crozier et al., 2006). Studies done by Fennell et al. (2004b) showed that plants under small-scale cultivation had lowered antihelmintic and antibacterial activity when compared to wild harvest plants. In other instances, however, cultivated species that had been irrigated actually

showed increased antihelmintic activity, making it more potent. Secondary metabolite production can also be significantly altered with the addition of fertilisers containing nitrogen and phosphorus. Genetic, ecological and environmental conditions all need to be consistent and regulated (Bopana & Saxena, 2007). All of these factors make it difficult to prepare plant medicines in any standard way without strict quality control and chemical composition determination at multiple points in the growth phase of those plants.

The cultivation of medicinal plants as an alternative to wild harvesting also faces other challenges besides the biochemistry changes caused by cultivation. For example, strict legal requirements will be needed. Recently, the South African government passed a law requiring anyone developing a commercial product using natural resources (defined to include plants, animals and genetic resources[4]), to obtain a bioprospecting permit (Government Gazette No. 26436, NEMBA, 2004). Essentially, this piece of legislation was intended to prevent unconstrained access to bioresources, to protect the intellectual property rights of indigenous communities and to enforce the need to use South Africa's local bioresources sustainably. Unfortunately, it also had the knock-on effect of preventing would-be farmers from cultivating medicinal plants, among other effects. By shifting to a cultivated alternative the conservation and protection of medicinal plants is still possible, but it is a lengthy process to obtain a permit, and in some cases nearly impossible. This remains a contentious issue, with many strong opinions on all sides (Crouch et al., 2008).

Another alternative to wild harvesting with its potential population-threatening methods of harvesting is to use substitute plant parts or where feasible, substitute plants. However, experiments done by Zscocke et al. (2000) showed that plant part substitution is species-dependent. In some instances roots and twigs of the same plant contain very different chemicals. In other plants chemicals from different parts of the plant may be nearly identical and parts can be used interchangeably in medicine preparation. More common than part substitution is substitution with a different species, usually a closely related member of the same species or family as the traditionally used plant (Dahlberg & Trygger, 2009). For example, a similar smelling plant may be substituted for a species with a particular aromatic bark. This adaptation to what is available is evidence of the "dynamic pharmacopeia" in action, which is essential for the continued survival of these traditions. These substitutions may also occur unwittingly when a very similar looking species is misidentified as a medicinal plant. There is no evidence for a similar plant substitution occurring in Paulshoek, although it is not impossible that this has happened or will happen in the future.

[4] Commercial products based on natural products are not limited to the pharmaceutical industry, but also include foods and beverages e.g. rooibos tea, cut indigenous flowers and additives for the paint and cosmetic industries.

The medicinal plants of the Leliefontein region are also exposed to the threat of landscape degradation due to factors such as overgrazing. Overstocking on communal rangelands has led, in many areas, to the loss of vegetation cover, the increase in woody species such as *Acacia karoo* and *Prosopis* spp., and conversion of previously diverse vegetation to areas of limited biodiversity dominated by stands of *Galenia africana* and other pioneer species (Hahn et al., 2005; Hoffman & Rhode, 2007). This potential reduction is species, particularly those palatable species of *Lamiaceae* and *Geraniaceae*, could in future affect the ability of people to practice traditional healing. Goldberg (1998) found that grazing had no significant impact on the *abundance* of medicinal plants, but the study did not indicate the impact on *diversity* of medicinal plants. The impacts of grazing on plant diversity and soil productivity in general have been studied (Allsopp, 1999; Anderson & Hoffmann, 2011; Anderson & Hoffmann, 2007; Simons & Allsopp, 2007), but the impact on medicinal species *specifically* is not known. While changing land use practices that have been in place for hundreds of years may not be feasible, at least without major social restructuring, if at all, individual cultivation, plant substitution and care when harvesting wild populations may be necessary to ensure a future for traditional medicine in the Paulshoek area.

4.4 The changing pharmacopeia and the illnesses it treats

Over the centuries the pharmacopeia of Namaqualand has changed to accommodate the needs of a changing populace (Grenier, 1998; Dold & Cocks, 2000, Berkes & Turner, 2006). In order to remain a contemporary alternative or addition to Western medical healthcare systems, the healers and the pharmacopeia must remain adaptable to the requirements of the people. This means adapting to treat new diseases, as well as adapting to changing environmental conditions (such as the effects of land degradation and climate change). For example, studies show that the Namaqualand area will experience a general shift to wetter summers and dryer winters over the next 100 years, more typical of the rainfall regime of the Nama-Karoo (MacKellar et al., 2007). Adaption has happened in the past. For example the integration of Nama and Dutch plant remedies to treat new diseases such as TB and when some of the new plants naturalised at the Cape were included into the local pharmacopeia. These are vital steps in maintaining the adaptability of the traditional medicinal system. In Paulshoek, we see evidence of this adaptability not only in the healers, but also in the lay people.

A wide variety of plants is used, but many are used infrequently, with only a handful being the most commonly used. Most people use an average of seven medicinal plants to meet their basic healthcare needs. Despite this rather limited use, this study suggested that the total number of plants known to be useful as home remedies in Paulshoek had increased in recent years. While some of the increase may be explained by a more robust investigation technique uncovering more plants

and more uses, but it is doubtful that this could explain the large increase in the number of plants used. This could be indicative of a pharmacopeia in flux - that new plants are being added, but they are not yet as widely used as the reliable staples people have come to trust. We know that in other areas plants may be substituted if the preferred ones are not available, and there is documented use of alien and weed species for medicinal purposes (Dold & Cocks 2000; van Wyk, et al., 1997). This is common in other areas of South Africa, and there is limited evidence of this in Paulshoek. For example, *Nicotiana glauca* is used in Paulshoek to treat pain and as a bandage. But not only is it not indigenous to the area, it is not indigenous to South Africa at all, having been introduced from South America (Dold & Cocks, 2000). Adopting new plant species into the pharmacopeia seems to be largely the domain of formal traditional healers (McMillan, 2004). During interviews, the healers described how a new plant would be chosen. This ability to determine whether a plant has potential healing power is still seen as a "talent" with spiritual associations and it is not likely that non-specialists who only prepare home remedies would be able to do this. This is a concern, since all of the resident healers in the village have died and there may be no one left to add new material to the pharmacopeia which is likely to remain relatively static under such circumstances.

Despite the variety of plants used, nearly half of them fell into one of four families. *Asteraceae*, *Mesembryanthemaceae*, *Lamiaceae* and *Crassulaceae*. These families are also some of the most speciose in Namaqualand in general, and it is apparent that they would be the most commonly represented. While different species are used in different areas (mostly due to species distribution) the fact that the majority of medicinal plants fall into the same families could indicate that there is some common biological activity or chemical property at a family or genus level that makes them so popular for treating illness (Harborne, 1998).

Plant selection is not random, and it is not just the most common plants that are used - plants are actively sought out. Certain families and species are specifically chosen for certain remedies. Regression analyses for traditionally used medicinal species in the Paulshoek area showed that *Fabaceae*, *Aizoaceae, Lamiaceae* and *Myrtaceae* were preferentially selected for more than one use caterogy, while *Amaryllidaceae*, *Anacardiaceae*, *Malvaceae*, *Ebenaceae*, *Melianthaceae*, *Solanceae*, *Menispermacae* and *Sapindacea* were preferred over other families for one of the 11 use categories investigated. When the second most preferred families are also included, *Asteraceae* and *Viscaceae* emerge as second best in more than one use category. This implies that species in these families should be good candidates for screening as they are most likely to contain bioactive molecules.

Similar results have been found in studies performed in other areas of the world. Seeing as some of the families in this study are largely endemic to the region, they may not appear in lists of "hot

families" from other regions. Nonetheless, some families are consistently preferred for traditional medicinal treatment. *Asteraceae* and *Lamiaceae* are usually among the top medicinal families used (Belize - Treyvaud Amiguet et al., 2006; Mexico - Leonti et al., 2003; India - Kapur et al., 1992, Ecuador - Bennet & Husby, 2008).

Lamiaceae, Asteraceae, Poaceae, Fabaceae, Malvaceae, Rutaceae, Apiaceae, Brassicaceae and *Rosaceae* dominate the list of most used medicinal species in South America (Bennett & Prance, 2000). Weedy families such as *Lamiaceae, Solanceae, Malvaceae* and *Urticaceae* were most preferred in the medicinal flora of Campania, Italy. (Weckerle et al., 2011) and similar results were found for the flora of Sicily and Sardinia (Leonti et al., 2009).

Kapur (Kapur et al., 1992) did a similar analysis of the medicinally used flora of India to see which families were preferred in general, rather than for any specific use category. The top family was *Asteraceae*, then *Lamiaceae* and *Solanaceae*; which is consistent with previous studies. However, they also found that *Malvaceae*, *Scrophulariaceae*, *Menispermaceae*, *Geraniaceae*, *Crassulacae* and *Rutaceae* were marginally preferred - similar results to those found in this study. *Zygophyllaceae*, *Iridaceae*, *Chenopodiaceae*, *Oxalidaceae*, *Apocynaceae*, *Sapindaceae*, *Brassicaceae*, and *Anacardiaceae* were least preferred (Kapur et al., 1992). In a study on medicinal species performed on in South Africa medicinal species in general "hot" orders were identified as *Malpigiales, Fabales, Gentianales, Asterales, Solanales, Malvales and Sapindales* (Douwes et al., 2008).

In this study, *Mesembryanthemaceae* was consistently avoided for traditional use. At first this seems anomalous, as the MIC assays showed that mesemb species were the most active in two of the three assays performed. In addition they are largely succulent species, which was linked to certain types of activity e.g. protease inhibition. But avoidance may be explained by the fact that species of *Mesembryanthemaceae* have strong activity, but the activity causes negative side effects for the illness being treated and are thus avoided for certain types of illness. The same may be true for species in the family *Lamiaceae*. These species are preferred for some types of illness e.g. pulmonary ailments, but avoided for others e.g. dermatological conditions.

Even though the species are mostly from the same families, a wide variety of plant growth forms was recorded. Comparison with Goldberg's (1998) survey also suggests that there has been a general shift to using more trees and shrubs, and fewer perennial herbs. The shift may directly reflect a change in the composition of the vegetation surrounding Paulshoek, or could indicate subtle changes in attitudes towards medicinal plants. As noted earlier, there has been a general decrease in vegetation cover in communal areas due to overgrazing, and an increase in annuals and

invasive species, particularly along river courses (Hoffman & Rhode, 2007). With large areas becoming covered in *Galenia africana*, previously abundant perennial and annual herbaceous medicinal plants may become scarcer, forcing people to use alternatives. As stated previously, while grazing has not seemed to impact on medicinal plant abundance in the past (Goldberg, 1998), the effect of grazing on medicinal plant *biodiversity* has not been fully investigated. People may have also just simply shifted to a more reliable source of medicinal plant parts i.e. shrubs rather than the rainfall-dependant annual and perennial herbs. Even if rainfall is sufficient for a good yield of annuals, they are not available year round and have to be located every year. While this is not a problem for formal traditional healers, who stockpile dried plants for later use, it may be a problem for the non-specialist user as they don't tend to store plants and have to collect as required. Longer lived, larger bushes and succulents, which one can return to year after year without fail, become a much more attractive and reliable option. The ease of use and reliability may also have resulted in the large percentage of people (61.4%) using plants grown in their gardens. The use of geophytes is mostly described by healers only and most home remedies do not usually make use of bulbs or corms. During interviews some people seemed wary of bulbs as there have been poisonings in the village related to ingestion of toxic geophytes. Locating and identifying geophytes is also much more of a specialised skill than locating shrubs, and remains largely the domain of the traditional healers. Despite this, this study recorded a 4% increase in the use of geophytes, indicating that non-specialists are becoming more comfortable identifying these species. This could imply that what was once the preserve of a few formal healers is now becoming more common knowledge.

4.5 Transfer of knowledge (within the community and between the community and outsiders)

The transfer of knowledge of medicinal plants is vital if traditions are to continue. The transfer of knowledge may be within communities, between generations, or to outside entities, such as researchers and ethnobotanists. The results of this study indicate that knowledge is being shared among generations, and that new people are being shown how to collect and prepare medicine. However, few being taught are under 40 years old. It would seem that the general concern in the village that the "youth" will not adopt the traditional ways is one to seriously consider. People are divided on whether the younger people want to learn methods of traditional plant use at all. The younger generation have had the importance of education instilled in them, so much so that most complete their schooling, at least up to Grade 10 (the minimum compulsory level of schooling) which was not the case a generation ago (Duijnstee, 2011; Rhode et al., 2003). However, as mentioned previously, increasing levels of education appear to correspond to a decrease in use of traditional medicines (Giovannini et al. 2011; Satimia et al 1998; Gedif & Hahn, 2003; Karunamoorthi

& Tsehaye, 2012). In other instances the preference for traditional medicines survives thousands of kilometres after immigration. It would seem that the choice to use traditional medicines is influenced by more subtle factors beyond the reach of this study. Plant use is influenced by a range of social and cultural factors that change over time. Education, treatment availability, history of family use and personal knowledge of plants all play a role, but it seems to be more of a personal choice of which system to use rather than any one factor being a direct indicator of future preference.

Knowledge also flows from formal traditional healers to lay people in the village. While there are many secrets not revealed to non-healers, some are passed on and become common place. Of the people interviewed for this study, only 6.6% said they had been instructed by a traditional healer. However, none of the lay people using medicinal plants had unique remedies that they themselves had created.

Knowledge doesn't only transfer within the village. Researchers are welcomed into the village to record traditional knowledge and interview healers, as people value their traditions and believe researchers are able to assist in preserving what they know. This is seen as important as many believe the traditions are dying out - especially since the formal healers living in the village have recently passed away, neither leaving behind a trained apprentice to replace them. With no formal healers in the village, the traditional knowledge of medicinal plants is entirely in the hands of the people and this can easily be lost as people move away or they themselves pass away. But in the process of recording, collating and documenting knowledge, something is transferred back. People are reviving old uses that had been "forgotten" but have been "rediscovered" by researchers investigating historical usage, and subsequently shared amongst villagers. An emerging trend is that people in other rural villages similar to Paulshoek are using published reference books of medicinal plant information, such as those of Gericke or Van Wyk (van Wyk et al., 1997; van Wyk & Gericke, 2000), to create home remedies that may not have been used in those areas for many years or, in some cases ever, before. This may be beneficial to those people using traditional remedies, but it does create a situation where plant use becomes less regional, traditional and culture specific, and more generic across all regions of South Africa, and thus loses it unique, place-specific identity.

Transfer of knowledge is also not unidirectional. Some information has been assimilated into the community "from outsiders" e.g. regarding germs and vaccinations. In other instances knowledge of medicinal plants is accepted by researchers, which could be the start of the commercialisation process e.g. *Mesembryanthemum tortuosum* (kougoed/sceletium), A*gathosma betulina* (buchu), *Aspalathus linearis* (rooibos tea) and *Aloe ferox* (Cape aloe) (van Wyk, 2008b). Information seems to

go back and forth, from healers to villagers, to researchers and vice versa. This allows new understandings and interpretations of illness and health care for all involved, which could eventually result in a new integrated system.

Chapter 3: Evaluation of the Bioactivity of Plant Extracts from Namaqualand

1. Introduction

1.1. What use are bioactive plant extracts to people?

Humans have relied on plants to provide food, shelter, clothing and medicine for thousands of years. With the advent of the modern age, synthetic alternatives have taken the place of plants in many roles. However, this is not the case for medicines. While there are a great deal of chemically synthesised pharmaceuticals available, plants still play a large role in the drug discovery and drug manufacturing processes. An estimated 80% of medicinal products developed up to the year 1996 had their origins in plants, and between 2000 and 2010 plant-derived natural products were involved in producing 50% of all new small molecule pharmaceuticals (Harvey, 2007; Newman & Cragg, 2012).

The detection of bioactive plant extracts and the novel bioactive plant compounds therein is the starting point for their use as medicines (Ghisalberti, 2008). Determination of activity is done using a variety of assays, and is followed by isolation of active compounds. Some plant compounds are used unmodified in the form that they appear in nature e.g. morphine, artemesinin and vincristine (Pan et al., 2009). Other bioactive plant compounds are "lead compounds". They are modified and developed into versions of the original that are more suitable or more effective for human treatment. Certain types of illness have benefitted more from the identification and isolation of natural plant compounds than others. In particular, treatment of infectious diseases and cancer have benefitted from focussed "natural products research" (Baker et al., 2007). Of the 155 anticancer drugs developed between 1940 and 2006, only 27% could *not* be traced back to a natural product, with 47% being either a naturally occurring compound, or a direct derivation thereof (Schmidt et al., 2008; Newman & Cragg, 2006).

The primary healthcare system available to many people living in rural or developing areas is traditional medicine, which often makes use of whole-plant crude extracts for treatment of illness (World Health Organization, 2002). The drawback to using whole plant extracts is that the dose of the active compound present in the plant cannot be quantified. Additionally, as *all* compounds in the plant are ingested, not just the relevant ones, there may be unexpected side effects and toxicity. It is therefore desirable to identify the compounds responsible for activity in a crude plant extract and

develop them into reliable, safe and efficacious pharmaceutical preparations which can be administered in reproducible doses (Macías et al., 2007; Colegate & Molyneux, 2008), although the synergistic effect of compounds in a complex mixture cannot be discounted.

Using a combination of assays, plant extracts can be screened for activity and toxicity. This process often validates historical traditional use and provides a scientific basis for continued use of traditional medicines where pharmaceuticals are not available (Taylor et al., 2001). Such validation has, for example, been done on herbal concoctions sold in markets and "muthi" stores in Kwa-Zulu Natal, where claims to a variety of healing effects have been made. Testing revealed that some products were active in antibacterial, antifungal and antioxidant assays while some had no activity in these tests (Ndhlala et al., 2009). Testing of similar preparations in a separate study revealed that they all had varying levels of toxicity, making safety a concern for those using these and similar products (Ndhlala et al., 2010). The origins and composition of manufactured pharmaceuticals is regulated, whereas those of traditional medicines are not. This highlights the importance knowing the origins and composition of any substance intended for use as a medicine - whether of traditional or pharmaceutical origin.

In the classical approach to natural products chemistry, the screening of plant extracts is followed by bioassay-guided fractionation, and the subsequent isolation and structure determination of pure, bioactive compounds in the active fractions. In rare instances this may produce a compound that is useful as a drug, in which case an evaluation would be carried out on whether the compound could be harvested on a large scale from the plants, or be more efficiently prepared by chemical synthesis. Large scale harvesting of wild stocks can endanger species (Macías et al., 2007), and cultivation of the plants may or may not be viable. Semi-synthetic approaches are a possible alternative, whereby similar compounds to the unavailable bioactive one may be found in other plant species which are more easily cultivated or exist in wild populations not under threat. These may then be harvested and chemically transformed into the compound from the endangered plant (Colegate & Molyneux, 2008).

Natural products are not just a source of drugs for treating human illness. Food production and agriculture also benefit from natural products. Active compounds in plants may be developed into herbicides, pesticides and microbicides (against bacteria and fungi). Some of these isolated compounds are already commercially available: for example cinnamaldehyde, originally isolated from natural sources such as cinnamon (*Cinnamonum zeylanicum*), is synthesised chemically for use as an agricultural fungicide (Dayan et al., 2009). Use of natural microbicides in the food industry is

also possible. For example, research has shown that essential oil of oreganum (*Origanum vulgare*) containing thymol, carvacrol, γ-terpinene and *p*-cymene is active against *Escherichia coli* at concentrations of 0.5 - 1.2 µl per ml (Burt & Reinder, 2003). Consumer tests showed that oregano oil applied to beef fillets at 0.8% v/w (Tsigarida et al., 2000) and beef mince at 1% v/w (Skandamis & Nychas, 2001) had no perceptible changes to flavour, odour or colour of the packaged meat. Thus, switching to this natural microbicide would not have a negative effect on consumer's perceptions of the final product.

Performing simple bioassays can lead to the development of more sensitive analytical assays for particular compounds or for classes of compounds for agricultural and industrial use. One application of these analytical assays is for quality control and screening of toxicity in foods (Colegate & Molyneux, 2008). Bioassays can also be used to isolate and characterise compounds for commercial or industrial use, for example in food production, cosmetics, paint and dye production. Natural alternatives to synthetics have become popular over the past decade, with the move towards "green industry" and "green consumerism" (Burt, 2004). Consumers want fewer synthetic additives in their food and require products with a smaller chemical impact on the environment (Marienhagen & Bott, 2012). This means that more and better assays are needed to identify natural compounds for this purpose and a better method of selecting screening targets, so as to maximise potential positive hits, needs to be in place.

1.2. What is the purpose of bioactive metabolites in plants?

As noted above, it is of great benefit to humans that plants produce bioactive compounds. However, plants do not produce them for our benefit. Bioactive compounds are secondary metabolites, and their production diverts nutrients and energy away from primary metabolism. Their synthesis is costly and the biosynthetic pathways used to produce them are conserved. This implies that they must confer some evolutionary advantage for them to be produced at all (Macías et al., 2007; Wink, 2010). It is currently thought that secondary metabolites play major roles in defence against herbivores, insects and microbes, as signal compounds, in self-regulation and in protection, for example, from UV light-induced damage (Wink, 2010).

Plants are immobile and must defend themselves against attack by herbivores, fungi, bacteria, viruses and other competitive plant species. They do this in different ways, one of which is through producing chemicals that interact with the attacking organism. These chemicals may mimic compounds present in an attacker. For example, some plant-derived alkaloids mimic animal neurotransmitters. They can act as agonists or antagonists in the animal brain resulting in an

undesired effect causing it to stop attacking the plant (Wink, 2000). Humans can use these compounds as medicines precisely *because* of their effect on the animal brain and physiology (Moerman & Estabrook, 2003). It has been suggested that "the only difference between a medicine and a poison is often just the dose" (Patrick, 2005). So if some of these defence compounds are taken in small doses, they may be beneficial. For example, morphine may depress respiration and cause death in high doses, but is an effective painkiller at lower doses. In fact, some of the best known pharmaceuticals, stimulants, narcotics and poisons are alkaloids. There are approximately 12 000 known alkaloids and many more undiscovered alkaloids of potential medicinal value (Zulak et al., 2006).

Plants can also mimic insect compounds to prevent predation. For example, the phytoecdysteroids are structurally similar to key compounds involved in the maturation phase of the insect life cycle. Larvae feeding on the plant ingest these compounds and are trapped in a never-ending immature larval stage (Dinan, 2001). They do not reproduce and insect predation of the plant is reduced. Some insects and animals make use of these secondary metabolites, sequestering them for their own use, e.g. cyanogenic butterflies (Engler-Chaouet & Gilbert, 2007). So the race continues, with adaptations on both sides - plants have evolved to defend against microbes and insects and microbes and insects have evolved to try to overcome the new defences (Macías et al., 2007). This may explain the vast chemical diversity of secondary metabolites.

Bioactive metabolites also serve to attract animals and insects by, for example, producing fragrant monoterpenes or coloured anthocyanins and carotenoids in flowers (Crozier et al., 2006). Some secondary plant metabolites function for self-regulation as phytohormones or for communication between individuals of the same species (pheromones) (Macías et al., 2007). Some secondary metabolites fulfil multiple roles, such as the monoterpenes which attract insects but are also insecticidal and antimicrobial. Some secondary metabolites also carry out physiological functions e.g. alkaloids can transport and store nitrogen, but are also toxic (Wink, 2003). It appears that certain classes of compounds are generally associated with certain activity. It is this that we try to exploit when doing streamlined targeted extraction and testing. If we know that a specific class of compound is most likely to exhibit the desired bioactivity, then extraction methods and assays can be tailored so as to target these classes, thereby increasing hit rate and streamlining the drug discovery process.

1.3. The use of assays to test bioactivity

Biological assays test crude plant extracts for activity. They may also be used on fractions of crude extracts and on pure compounds to test activity. Plants produce a vast number of secondary compounds (for example over 8000 phenolic structures alone have been identified) any of which could yield a breakthrough in medical treatment. Testing every plant for all types of chemicals is laborious and not economically feasible (Firn, 2003). So in order to streamline the process the most likely plant candidates must be chosen, and the appropriate extraction technique for the type of compounds expected (Salis-Lagoudakis et al., 2011). There are different methods of choosing which plants to test, including random selection, ecology based selection and ethnopharmacology informed selection. Ethnopharmacology based selection, where a selection of test plants is chosen using traditional medicine as a guide may provide the best success rate (Martin, 1995). However, it requires extensive inter-disciplinary collaboration which includes biologists, anthropologists, pharmacologists, biochemists, microbiologists and chemists as well as careful negotiation with indigenous people.

Once plant targets are chosen, assays are selected. Simple bioassays can be done on the bench top using test organisms or cells, but need to be accurate, reproducible, unambiguous, simple and inexpensive. (Pieters & Vlietnik, 2005). Larger volumes of extracts can be processed using automated high-throughput screening (HTS), as is practiced by pharmaceutical companies in an attempt to increase the discovery rate for new natural products with drug potential (Littleton et al., 2005). Additional methods are included to increase hit rate, genomics studies for example (Iskar et al., 2012; Feng et al., 2009). However, HTS has been criticized for poor results as analyses have shown that leads originating from HTS methods are the cause of over 60% of clinical failures (Zhang et al., 2012).

1.4. Extent of bioactivity testing

Investigation into the bioactivity of plants has increased over the years as interest in natural products gathers momentum (see Baker et al. 2007 for an overview). This wealth of research has generated numerous textbooks and entire journals are dedicated to research in this field. The Journal of Ethnopharmacology is notable among many similar journals for documentation of research into plant bioactivity. Common approaches to investigating bioactivity include focusing on specific groups of plants e.g. certain families, entire pharmacopeia e.g. of a certain area or group of people or specific traditionally-used preparations e.g. certain traditional Chinese medicines. Testing has been done for a wide variety of activities among them antibacterial, antioxidant, antimalarial, anti-inflammatory, antiviral, antifertility, and antifungal to name a few.

In South Africa, the most commonly performed assays have been antibacterial, antifungal, antihelmintic, antiamoebic, antischistosomal, antimalarial, anti-inflammatory and antioxidant assays (Light et al., 2005). The most common method for testing employed by South African researchers, as reported in the literature, is serial microdilution (Eloff, 1998), but other methods such as the agar disc diffusion method and bioautography (Begue & Kline, 1972; Hamburg & Cordell, 1987) have been used (see Marston, 2011 for an overview of these techniques). Several South African studies have also been done to investigate the neurological effects of bioactive metabolites e.g. the effect of plant compounds on the central nervous system and this forms a significant area of South African research (see Stafford et al., 2008 for an overview). These tests are more difficult to perform and often require live animal test subjects (Stafford et al., 2005; Stafford et al., 2008). Mulholland & Drewes (2004) provide a good overview of research done in South Africa up to 2003. Many more studies have been done since then and entire research units, such as the Ethnobotany Unit of the National Botanical Institute and research groups based at the University of Kwa-Zulu Natal, the University of Johannesburg and at the Council for Scientific and Industrial Research (CSIR), have been established with the aim to discover new bioactive molecules and to validate traditionally used preparations.

In South Africa there has also been a specific focus on testing safety and toxicity of traditionally used preparations (Ndhlala et al., 2010; Elgorashi et al., 2003; Steenkamp & Gouws, 2006; Verschaeve & van Staden, 2008). As the majority of people make use of traditional preparations, it is important to validate them and test their toxicity. There is also the possibility of integrating their use into main stream western healthcare systems (Fennel et al., 2004b). With this in mind, a few indigenous plants have been developed for distribution through health shops and pharmacies. They are available as processed and standardised materials in the form of teas, tinctures, tablets, capsules and ointments. 16 major products[1] have been developed, among them *Aloe ferox* burn gel and laxatives, *Aspalanthus linearis* (rooibos tea), antispasmodics and skin treatment for allergies and eczema and *Sutherlandia frutescens* (Sutherlandia) (van Wyk et al., 2008; van Wyk, 2011). Veterinary applications have not been neglected, and studies have been done to investigate plant based veterinary treatments (McGaw & Eloff 2008 for an overview of SA research and testing in this area; Bagla et al., 2012) as well as to better understand livestock poisonings by plants (Botha & Penrith, 2008; Kellerman et al., 2005).

[1] "Major products" refers to those plants which have been developed as commercial crops i.e. cultivated and managed on a large scale rather than wild harvested

1.5. The importance of this study and its relevance

While some plants have been investigated for bioactive compounds, the vast majority have not. This is particularly true of the South African flora. Traditional use is common, but very few of the species have been tested for activity or phytochemically evaluated. It is important to test for bioactivity in such plants as there is the possibility that novel, highly active compounds may be discovered. In this part of the study, some of the traditionally used plants mentioned during interviews with Paulshoek residents (see chapter 2) were tested in several assays for activity. Some of the other plants not mentioned in interviews were also evaluated for bioactivity in order to test a representative variety of species, growth types etc. from the Namaqualand flora. The types of assays that could be performed are numerous, and for the purposes of this study only a few could be performed. Here follows a short rationale for the choice of assays and their relevance and application in an African context.

1.5.1. Rationale for using an antibacterial assay

As more and more strains of bacteria develop resistance to antibiotics, so the need for novel antibiotics increases. Diarrhoeal diseases, mostly caused by bacterial pathogens, have been recognised as the greatest killer of infants and young children in the developing world. Many diarrhoeal diseases are caused by bacteria in the gastro-intestinal tract, some of which are becoming resistant to drugs (Fawole et al., 2008). Thus, the need for new antibacterials to combat this is great. "Stomach complaints", including diarrhoea, are among the most commonly treated illnesses in Paulshoek. So it follows that there is the potential for discovering good antibacterial activity in the plants of Namaqualand.

Diarrhoeal diseases are not the only diseases of concern in third world countries. Infection with HIV is rampant. While there have been some studies into the use of traditionally used medicinal plants for the treatment of HIV (Tshikalange et al., 2008; Klos et al., 2009), this is not the focus of this study. Rather than treating the HI virus, antibacterials play a large role in the treatment of sexually transmitted infections (STI's). A high HIV infection rate has been directly correlated with a high STI's infection rate. Infection with HIV makes co- infection with STI's more likely. STI's are a serious cause for concern, as left untreated they may result in infertility and even death (Van Vuuren & Naidoo, 2010; De Wet et al., 2012). 26% of deaths in southern Africa in 2000 were as a result of STI's (Johnson, 2008) caused by microorganisms such as *Neisseria gonorrhoea* (gonorrhoea) and *Chlamydia trachomatis* (chlamydia). Van Vuuren & Naidoo (2010) found that 90% of the solvent extracts of ethnomedicinally used plants from South Africa had activity against 6 pathogenic

microorganisms associated with STI. Thus it follows that the plant extracts from Namaqualand may have antibacterial activity relevant to the treatment of STI's.

In addition to these diseases, there is also the concern about tuberculosis (TB) caused by *Mycobacterium tuberculosis*. Multiple drug resistant (those strains resistant to at least two of the major anti-TB drugs) and extremely drug resistant strains (resistant to two of the major drugs, an injectible second line drug, as well as a fluoroquinolone) of TB have emerged (Jones et al., 2008). These strains are prevalent in Africa, and in particular South Africa, where there is an extremely high infection rate (more than 600 cases per 100 000 annually) (McGaw et al., 2008; Green et al., 2010; Eldeen & van Staden, 2007). One in ten cases is resistant to some of the available drugs (Lall & Meyer, 1999). Plants have been recognised as a useful source of highly active antimycobacterial metabolites (Pauli et al., 2005). In South Africa, many studies have focused on developing anti-TB drugs (see McGaw et al., 2008 for a review of research done in South Africa on South African plants, Eldeen & van Staden, 2007).

Antibacterials also have an important veterinary application in South Africa. It has been estimated that 75% of rural livestock owners in the Eastern Cape province of South Africa use plant based remedies to treat their animals (Masika et al., 2000). While many large-scale commercial farmers make use of pharmaceutical preparations, the smaller farmers cannot afford these. Thus, it is important to develop new affordable alternative treatments based on plant compounds. Ethnoveterinary applications are commonly used to treat diarrhoea and gastro-intestinal problems associated with bacterial infection as well as other illness such as eye inflammations, and coughing which may also have a bacterial element (Mcgaw & Eloff, 2008).

1.5.2. Rationale for using an antifungal assay

One of the most common pathogenic fungi is the yeast *Candida albicans*. It is one of the most common opportunistic infections that causes candidosis (thrush) (Motsei et al., 2003). Up to two thirds of HIV infected patients are also infected with *Candida albicans* which causes oral candidosis (McCarthy et al., 1991). Vaginal candidosis affects an estimated 75% of non-HIV infected women at least once in their lifetime (Richardson & Warnock, 1993). Currently used treatments are becoming less effective as the fungus becomes resistant. Thus finding cheap, effective and readily available alternatives is important. Some studies have been done on crude plant extracts using bioautography and MIC to detect compounds with MIC values as low as 0.02 mg/ml (Shai et al., 2008). This suggests that plants are a good source of antifungal agents, and testing the plants of Namaqualand for such activity may yield new antifungal compounds.

Treatment of fungal infections in humans is not the only application for plant sourced antifungal agents. They can also be used to treat fungal infection of crops. For example, fungi are a major cause of seed deterioration of stored seeds (Krishnamurthy et al., 2008). If the seed survives storage without infection, there is still the chance for fungal attack on plants once the seed is sown. Fungal attack of crops is a major cause of losses in agricultural production. *Aspergillus* species cause fruit spoilage and produce toxic compounds that contaminate cotton, corn, peanuts and tree nuts. *Fusarium* blight is the most common fungal disease responsible for wheat and barley loss, and *Fusarium oxysporum* causes wilt disease in tomatoes (Mahlo et al., 2010). *Penicillium* species are also responsible for post-harvest produce deterioration of fruit such as apples, peaches and cherries. Table grapes may also be infected with the fungus *Botrytis cincerea*, which causes gray mould disease. While the wine industry has turned this into a profitable situation (with the production of sweet "desert wines"), fresh produce farmers suffer heavy losses. Fungal diseases in plants can be controlled by using fungicides. However, some fungi have become resistant to the major antifungal agents employed by farmers (Stuardo & San Martín, 2008). Increasingly toxic fungicides are not an alternative as there are adverse health effects to humans and animals consuming the produce (Mahlo et al., 2010). Hence, finding new antifungal agents that are not as toxic yet still effective is imperative for food production as well as human health.

1.5.3. Rationale for using an antioxidant assay

Antioxidants are compounds that prevent other compounds or biological targets from being oxidised. Oxidative damage to cell membranes and DNA by reactive oxygen and nitrogen species (ROS/RNS) has been linked to the development of cancer (Collins, 2005), heart disease (Palace et al., 1999; Kaul et al., 1993), Alzheimer's disease (Mariani et al., 2005) and Parkinson's disease (Joseph, 2008). Natural antioxidants may provide protection against ROS/RNS and prevent oxidative damage associated with these and other diseases (Prior et al., 2005).

For example, Alzheimer's disease is characterised by extracellular deposited amyloid-beta peptide and intracellular hyper-phosphorylated and tangled tau-protein (Choi et al., 2012). Elevated levels of oxidative stress often precede amyloid deposition and neurofibrillary tangles, suggesting that oxidative stress is an early event which leads to development of Alzheimer's disease (Smith et al., 1991; Lovell & Markesberry, 2007; Resende et al., 2008).

Natural antioxidant compounds, such as polyphenols, are able to quench free radical species, removing potentially damaging ROS/RNS. They may also be able to promote cellular pathways that allow cells to produce antioxidant molecules thereby increasing total antioxidant capacity. This can

be done, for example by stimulating the Nuclear factor-erythroid 2-related factor (Nrf)/ antioxidant response element (ARE) pathway (Dumont & Beal, 2011; Choi et al., 2012) Thus there is the possibility that preventing free radical generation, increasing antioxidant capacity and reducing the oxidative stress that often precedes development of the disease may intervene and prevent amyloid deposition and associated cognitive impairment (Ramassamy, 2006). Studies using vitamin E have shown that a dose of 2000 IU per day was effective in improving the cognitive ability of Alzheimer's patients (Sano et al., 1997). Natural extracts containing high levels of flavonoids, such as those from *Ginkgo biloba* are also thought to confer similar protection (Viña et al., 2004; Mecocci & Polidori, 2012). Extract of *Ginkgo biloba*, containing 24% flavonoids and 6% terpenes, is able to scavenge free radicals including NO, hydroxyl radical, superoxide anion and peroxyl radical (Choi et al., 2012). Ginkgo extract also up-regulates several genes that encode antioxidant enzymes, such as haeme oxidase-1 thereby increasing total cellular antioxidant production (Defeudis, 2002).

This is of particular relevance to Africa. While the incidence of Alzheimer's disease in African populations has not been explicitly investigated, studies conducted on groups of African-Americans showed that antioxidant concentrations and levels of oxidative DNA damage differ significantly between adults of African-American ethnicity and adults of white ethnicity (Watters et al., 2008). In addition, African-Americans have a higher mortality rate from cardiovascular disease than whites in America (American Heart Association, 2005; Qian et al., 2007), and they also have a higher risk factor for developing other diseases related to oxidative stress such as cancer (American Cancer Society, 2006). Whether this is true of people of African ethnicity has not been established, but there has been a changing lifestyle profile among South Africans which may suggest that oxidative stress related disorders could be of greater concern in the future. As more South Africans become urbanised, more meat and refined carbohydrates become included in the daily diet. Tobacco and alcohol consumption also increase and these lifestyle and dietary changes lead to higher levels of oxidative stress, which can lead to disease (Reddy et al., 2003). For this reason, identifying natural sources of antioxidants and the development of antioxidant treatment for diseases such as cancer and heart disease is of great importance.

1.5.4. Rationale for using protease and protease inhibition assays

Proteases are enzymes that cleave proteins into amino acids and smaller peptides. They participate in many physiological processes including digestion and blood clotting. Proteases and protease inhibitors may have medicinal use - protease inhibitors are already widely used to treat HIV infection and hypertension (Joseph, 2008). In Africa, traditional medicinal plants are often the only available treatment for many infected with HIV. Investigation into the bioactivity of traditionally used

medicinal plants from around the world has shown that several posses some degree of anti-HIV activity (Tshikalange et al., 2008). Plants can act at different stages of the viral life cycle to halt its progression. For example, by preventing viral attachment (thereby stopping the virus from sticking to host cells) to inhibition of reverse transcriptase (which allows the virus's RNA to be replicated by the host DNA) (Harnett et al., 2005). An important class of drugs targeted at inhibition of HIV protease may arrest the development and proliferation of HIV in infected patients (Tomasselli & Heinrikson, 2000). HIV protease is a virus-specific dimeric enzyme required for cleavage of viral polyprotein precursors into functioning proteins. Intervening in this process prevents the formation of active and infectious virus, which essentially blocks the replication cycle of the virus (Markowitz et al., 1995).

Protease inhibitors (PI) are often synthetic and are quickly metabolised by human cytochrome P450 enzymes, particularly CYP3A. Thus they need to be taken often and at high doses in order to maintain an effective plasma concentration (Treijtel et al., 2009). They are also used in combination therapy with other HIV drugs, as in Highly Active AntiRetroviral Therapy (HAART) (Pawar et al., 2012). Alternatives from natural origins may provide a better solution. A variety of natural products, including alkaloids, flavonoids and lignans, have been found to inhibit HIV specific enzymes and proteins essential for the completion of the virus's life cycle (Cos et al., 2004). For example, *Stauntonia obovatifoliola* subsp. *intermedia*, a plant used in Traditional Chinese Medicine (TCM), contains several active compounds, most notably a triterpenoid that inhibits HIV-1 protease with an IC_{50} value of 8.7 µg/ml (at a concentration of 100 µg/ml) (Wei et al., 2008). Such natural compounds may allow development of improved drugs with better pharmacokinetic profiles. Other natural protease inhibitors may be present in plants from Namaqualand, and thus it is important to test for this bioactivity.

1.5.5. Rationale for using an antihelmintic assay

Helminth infestation is a common and serious problem largely affecting the developing world where people are impoverished and marginalised (Aktar et al., 2000). There has been relatively little research focusing specifically on helminth infestation and helminth infections such as hookworm and schistosomiasis (Jäger, 2005), which are among the core neglected tropical diseases (Hotez et al. 2007; Hotez et al., 2009). There is currently no data on the prevalence of helminth related disease in South Africa. However, high rates of infection have been reported at the provincial level, particularly in Mpumalanga, the Western Cape, and Kwa-Zulu Natal (Aremu et al., 2012).

Currently there are only four basic types of antihelmintics available with four modes of action (Aremu et al., 2012). The first three types are older drugs developed many years ago to which helminths have had time to develop resistance to. They are not always completely effective in severe infestations. The fourth class of drugs has been developed much more recently than the other three classes. However, certain soil dwelling parasitic helminths are already resistant to these drugs (Tritten et al., 2011). Furthermore, drugs of this type often caused complications with hepatitis patients and pregnant women (Savioli et al., 2003). The number of isolated plant bioactive compounds demonstrating antihelmintic activity is low (Aremu et al., 2012) and this route needs to be explored further. Thus, screening the plants from Namaqualand was thought to be of benefit.

Schistosomiasis, also known as bilharzia, is one of the most common helminth diseases. It is estimated that more than 207 million people have been infected worldwide (Steinmann et al., 2006; De Moraes et al., 2010). Plant molluscides have been the focus of investigation with the emphasis on creating cheap, effective and environmentally acceptable alternatives to often unavailable and expensive Western medicines (Clark et al., 1997). Some screening of South African plants, particularly traditionally used medicinal species from Kwa-Zulu Natal has yielded positive results (Sparg et al., 2000; Aremu et al., 2010). Thus it seemed likely that Namaqualand plants may produce similar compounds.

One of the side effects of intestinal helminth infection is chronic diarrhoea (Lewis & Elvin-Lewis, 1977). While this can be deadly to the elderly and the very young, it is rarely associated with mortality in adults. However, a heavy helminth infestation can cause morbidity such as impaired mental and physical development in children, and wasting in adults (Taylor et al., 1995). Another serious implication of helminth infestation is a decline in host immune status, which can increase susceptibility to secondary infections (Borkow & Bentwich, 2006). This can be a serious problem in a person co-infected with HIV or TB. Helminth infection lowers the already lowered immune response and can increase the progression of HIV or TB (Elias et. al, 2006; Wolday et al., 2002; Aremu et al., 2012). In Africa with its high HIV and TB infection rates, reducing helminth infection seems vitally important.

Treating helminth infections is not only of great importance to humans, but also to animals. Antihelmintics dominate the animal pharmaceutical industry and this area of research is probably the only one where efforts and success in animal health exceed those in human health (Hoste & Torres-Acosta, 2011; McKellar & Jackson, 2004; Aremu et al., 2012). The wealth of plants in South Africa means that there is the possibility for development of animal helminth vaccines from plants

(Smith, 1999). Such research is being performed at various institutes in South Africa with some success, and additional plant species from Namaqualand may be useful in this regard.

1.6. Aims/questions and objectives of this section

The main aim of this part of the study was to perform a range of simple biological assays on a sample of plants from the study area in order to assess their biological activity. A selection of medicinal and non-medicinal plant species were collected from the communal area surrounding the village of Paulshoek in Namaqualand for this purpose. A second aim was to use various statistical methods to investigate trends in bioactivity across family lines and various plant features. In summary, the aims of this section of research are to:

1) Assess the biological activity of plants in a series of simple assays
2) Test the validity of the simple assays with more robust minimum inhibitory concentration (MIC) assays
3) Examine trends in activity
    a) across family lines in order to establish the extent to which bioactivity is associated with plant families
    b) among various growth forms, in order to establish whether bioactivity is related to growth form
    c) across seasonality and degree of succulence, to establish whether either of these have a strong relationship with bioactivity
4) Identify potential candidates from the tested species for further phytochemical and metabolomic analysis (see chapter 4)

2. Methods

2.1 Plant collection and extraction

The method for plant collection and sampling was outlined in chapter 2 in the section "*Plant collection and identification*".

Collection took place on several field trips interspersed over a five month period, starting in July (winter) and progressing through to November (start of summer). This was done to ensure that the greatest range of plant species were included in the study as some species have a strong seasonal growth pattern, particularly geophytes and ephemerals (Cowling et al., 1999). Plant sampling yielded a total collection of 124 individual plant species, 91 of which were used to create 102 ethanolic plant

extracts. Some plant species were not extracted due to discovery of insect or fungal damage being discovered upon closer inspection of plant material on return to the laboratory. Some plant species were used to create multiple extracts in order to account for the possibility of different activity in different plant organs e.g. between roots and leaves, or stems and flowers. For example, extracts of both the berries and the leaves of *Solanum burchelii* were created. Plant samples were separated into different plant parts (where necessary) and washed and air dried to remove dirt before extraction in ethanol. Plant extraction followed a slightly modified version of the protocol outlined in the Global Institute for Biological Exploration (GIBEX) "Screens-to Nature" (STN) manual (Joseph, 2008). 2g of each sample was weighed out and macerated by hand in a mortar and pestle with 4ml of 70% EtOH. Ethanolic plant slurry was then filtered through Whatman No. 1 filter paper and stored as liquid ethanolic extracts at 4°C until biological testing was performed. 2ml's of each extract were stored as reference samples, and the remainder used in biological testing.

2.2. GIBEX assays

A range of biological assays developed by The Global Institute for Biological Exploration (GIBEX) were performed on all extracts. These qualitative "Screens-to Nature" (STN) bioassays were developed to be field deployable, simple to perform and requiring little specialised equipment. The idea being that the assays would be available to scientists in the developing world for use in the field, removing the need for a fully equipped laboratory and making it possible to test plants for bioactivity in the areas where they are medicinally used (Andrae-Marobela et al., 2012). The STN bioassays focus on testing for bioactivity relevant to treating diseases prevalent in developing countries. For example antibacterial and antifungal assays indicate potential treatment of infection, an antihelmintic assay indicates potential treatment of parasitic diseases and an assay that tests for enzyme activity and enzyme inhibition indicates potential treatment for certain metabolic diseases and HIV (Joseph, 2008).

A pilot run of the biological assays gave no results as the extracts were too concentrated to allow accurate assessment of results as several of the assays are colorimetric in nature. For example, it could not be determined whether the worms used in the antihelmintic assay were dead or alive after the incubation time had elapsed as the liquid in the wells was too dark to see through. After consultation with Prof. D.W. Gammon (Department of Chemistry, University of Cape Town), it was decided that plant extracts should be diluted 1:3 with 95% EtOH and that these dilutions be used in assays. This was adopted as the protocol for all subsequent samples. Bioassays were performed according to the instructions outlined in the GIBEX manual (and described below) and results recorded. All reagents were obtained from Sigma-Aldrich, unless otherwise specified.

2.2.1. Antibacterial assay

To test the antibacterial activity of the plant extracts under investigation, 300µl of agar solution (containing 1g agar in 30ml water) was placed in each well of a sterile 48 well plate and allowed to solidify. 10µl of human saliva (obtained from the author at least 1 hour after consumption of food or beverages) was placed in each well as a source of bacteria. Human saliva was chosen for this assay as it provides a wealth of bacteria on which to test plant extracts and is easily obtainable in a field situation. Ethanolic plant extracts were tested in duplicate, placing 10µl into each test well. 10µl of penicillin G solution (containing 4mg lyophilised penicillin G powder (Sigma) and 1ml sterile water) was used as the positive control, while the two negative control wells contained only 10µl saliva on agar each. Plates were covered and incubated at 37°C for 24 hours. Bacterial growth was recorded as the percentage of agar covered with bacterial colonies after the incubation time had elapsed. 100% cover indicated no antibacterial activity for that extract, more than 50% cover indicted slight antibacterial activity, less than 50% cover indicated moderate antibacterial activity and no bacterial growth indicated strong antibacterial activity (Joseph, 2008).

2.2.2. Antifungal assay

To test the antifungal activity of the plant extracts under investigation, 200µl of yeast solution, containing 50mg dried yeast and 50mg sucrose in 5ml of warm distilled water, was placed in each well of a sterile 24 well plate. Common baker's yeast - *Saccharomyces cerevisiae* - was used in this assay as a model organism for other fungi in the yeast family and as a proxy for general antifungal activity in the plant extracts under investigation. Ethanolic plant extracts were tested in duplicate, placing 50µl into each test well. 50µl of an econozole nitrate solution (containing 300mg econozole nitrate salt dissolved in 1ml dimethyl sulfoxide (DMSO)) was placed into two positive control wells, while 50µl 70% EtOH was placed in each of the two negative control wells. Plates were covered and incubated at 30°C for 24 hours. After 24 hours had elapsed, 20µl of MTT solution (containing 5mg 3-(4,5-dimethylthiazole-3-yl)-2,5-diphenyltetrazolium bromide (MTT) in 1ml sterile water) was added to each well and the plates were incubated for a further 22 hours. Yellow MTT is metabolised by live yeast cells to purple formazan and is widely used to assess cytotoxicity and cell viability (Mosmann, 1983; Van Meerloo et al., 2011). Thus wells containing live yeast cells will turn purple, while those containing yeast cells killed by plant extracts will remain yellow. Extracts in wells remaining yellow after the incubation period were recorded as having strong antifungal activity. Extracts in wells that changed to a yellow-lilac were recorded as having moderate antifungal activity. Similarly light purple indicated mild activity while dark purple was taken to indicate no antifungal activity for that particular extract (Joseph, 2008).

2.2.3. Antioxidant assay

In this assay, ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) was used as an indicator of antioxidant activity of plant extracts. The $ABTS^{\cdot+}$ radical is generated directly in a stable form prior to reaction with any antioxidants present in ethanolic plant extracts. The blue/green $ABTS^{\cdot+}$ chromophore is generated through the reaction of ABTS and potassium persulfate. The coloured radical cation is reduced to colourless ABTS through interaction with antioxidants. Thus the extent of discolouration can be used as an indicator of antioxidant activity. The reaction is fast and is usually complete within one minute (Re et al., 1999).

In order to generate the $ABTS^{\cdot+}$ chromophore, 20µl of potassium persulfate solution (containing 50mg potassium persulfate in 1ml distilled water) was added to 1ml ABTS stock solution (containing 7mg ABTS powder in 1ml distilled water). ABTS stock solution was protected from light and left for 6 hours at 4°C, after which it was diluted to a working concentration with 20ml distilled water.

To test the antioxidant activity of the plant extracts under investigation, 200µl of diluted ABTS solution was added to each well in a sterile 96 well plate. Ethanolic plant extracts were tested in duplicate, placing 10µl into each test well. 10µl of ascorbic acid (containing 17.6mg ascorbic acid powder in 1ml distilled water) was used as the positive control, while 10µl 70% EtOH was used as the negative control. Antioxidant activity was recorded according to colour change. No change in colour from dark blue/black indicated no antioxidant activity in that extract. A change to green indicated mild antioxidant activity, and similarly a change to pale green was taken to indicate moderate antioxidant activity. A change to completely clear indicated strong antioxidant activity (Joseph, 2008).

2.2.4. Protease activity and protease inhibition activity

In this assay, strips of radiograph film coated with gelatine were used to test the protease activity and protease inhibition activity of plant extracts. If protease activity is present, the plant extract will eat away the green top layer of the film exposing the blue layer below (Konarev, 1986).

To test for protease activity in the plant extracts under investigation, 10µl drops of the extracts under investigation were placed at regular intervals along a strip of radiograph film. 10µl of digestive enzyme solution (consisting of 2.5mg trypsin powder dissolved in 1ml sterile distilled water) was used as a positive control. The negative control was a 10µl drop of sterile water. The film was left on a flat surface for 10 minutes and then washed with running water to remove any dried-on remnants of the test extracts. Extracts that had removed the top layer to reveal the blue layer below were recorded as having protease activity.

To test for protease inhibitor activity in the plant extracts under investigation, 10µl drops of extracts under investigation were placed at regular intervals along a strip of radiograph film. The positive control consisted of a 10µl drop of digestive enzyme solution (consisting of 2.5mg trypsin powder dissolved in 1ml sterile water) combined with a 10µl drop of inhibitor solution (containing 3mg trypsin inhibitor powder dissolved in 30µl sterile water). The negative control was a 10µl drop of sterile water. 10µl of trypsin solution was added to each drop of test extract, the film was left on a flat surface for 10 minutes and then washed with running water. Extracts that did not show the blue layer below the surface were recorded as having protease inhibition activity (Joseph, 2008).

2.2.5. Antihelmintic assay

In this assay, a free-living nematode - *Panagrellus redivivus* - was used as a model organism to test antihelmintic activity of plant extracts. Activity against the model organism may indicate lethality to other parasitic species of roundworm e.g. hook worms and pin worms. Worms were cultured in a liquid mixture of oatmeal, and re-cultured once every 10 days. New cultures were left for at least 3 days before use in an assay to allow time for the parasites to multiply.

To test the antihelmintic activity of the plant extracts under investigation, 100µl of worm suspension (consisting of a small spatula scraping of worms from the oatmeal solution in 10ml sterile water) was placed in each well of a sterile 96 well round bottom plate. Roughly an even number of worms were placed in each well. Ethanolic plant extracts were tested in duplicate, placing 5µl into each test well. 5µl copper sulphate solution (containing 160mg $CuSO_4$ salt in 1ml sterile water) was placed in two positive control wells, while 5µl 70% EtOH solution was placed in two negative control wells. Plates were left for four hours at room temperature before evaluating lethality activity. After four hours, wells were examined under 10 x magnification. Those wells containing dead worms were recorded as having extracts with antihelmintic activity.

2.3. Minimum Inhibitory Concentration (MIC) assays

One of the aims of this section of work was to validate the use of GIBEX assays to test for bioactivity. While the assays were developed and thoroughly tested by GIBEX, in collaboration with Rutgers University and the University of Illinois (Andrae-Marobela et al. 2012), it was deemed useful to validate the assays for use with plants from Namaqualand. Namaqualand is a unique bioregion and contains many endemic species, some of which were included in this study (Desmet, 2007; Desmet & Cowling, 1999). Validation studies were done on the basis that these regionally isolated plants may contain unique chemical compounds which may have unforeseen effects on certain assays. Hence, serial microdilution assays (Eloff, 1998) were performed on a subset of the ethanolic extracts

subjected to the GIBEX assays to determine their minimum inhibitory concentration (MIC) values. The original serial microdilution method was intended for use with bacteria only, and slight modifications were made for use with yeast cells in order to test the antifungal activity of plant extracts (Masoko et al., 2005). Results from the MIC assays were then compared to the results for the same extracts obtained in the GIBEX assays. Extracts were selected at random and blind tested.

In addition to determining assay validity, MIC assays allowed quantification of the qualitative results obtained from the GIBEX assays. This meant that the actual levels of bioactivity could be evaluated and the range of efficacious concentrations of the crude ethanolic extracts could be determined.

2.3.1. Organism stock maintenance: bacteria

A stock culture of gram negative (*Escherichia coli*) and gram positive (*Staphylococcus aureus*) organisms was obtained from the American Type Culture Collection (ATCC). The organisms were inoculated into 5ml of Tryptic Soy Broth (TSB), obtained from Sigma-Aldrich in Germany, and incubated at 37°C for 24 hours. Gram and Ziehl-Neelson stains were performed on these cultures to ensure culture purity. 20% sterile glycerol was added to each culture and 500µl aliquots were placed into sterile 1.5ml Eppendorf tubes using plugged 1ml pipette tips. These stocks represent the 'G0' stocks and were stored at -70°C. One of these 'G0' stocks was used to inoculate two further medium cultures, and glycerol stocks were prepared from these cultures by the same method described above. These stocks, designated 'G1', were stored at -20°C and used for all experimentation purposes.

2.3.2. Organism stock maintenance: fungus/yeast

A stock culture of the organism *Candida albicans* was obtained from ATCC. The organism was inoculated into 5ml of Roswell Park Memorial Institute (RPMI) 1640 medium, obtained from Sigma-Aldrich in Germany, and incubated at 30°C for 48 hours. Gram stains were performed on these cultures to ensure culture purity. 20% sterile glycerol was added to each culture and 500µl aliquots were placed into sterile 1.5ml Eppendorf tubes using plugged 1ml pipette tips. These stocks represent the 'G0' stocks and were stored at -70°C. One of these 'G0' stocks was used to inoculate two further medium cultures, and glycerol stocks were prepared from these cultures by the same method described above. These stocks, designated 'G1', were stored at -20°C and used for all experimentation purposes.

2.3.3. MIC assay method

Bacteria (*Escherichia coli* and *Staphylococcus aureus*) from frozen G1 stocks described above were inoculated into 5ml of Tryptic Soy Broth (TSB) medium (consisting of an autoclaved solution of 30g TSB powder in 1L sterile water) each and grown for 24 hours before use. Assays were performed in duplicate in sterile 96 well plates. 100µl of sterile water was placed into all wells. 50µl of each ethanolic extract was tested in duplicate and serial two-fold dilutions were made from the first well down to the desired minimum concentration (Langfield et al., 2004). A 1:200 dilution was made from the day-old bacterial culture using TSB. Plates were inoculated with the diluted bacterial suspension (50µl per well), covered and incubated at 37°C for 24 hours. Ciprofloxacin (concentration 10 µg/ml) was used as the positive control, and appropriate solvent blanks were included as negative controls.

After the incubation period, 10µl Alamar Blue (consisting of 5mg resazurin dissolved in 1ml sterile water, 0.5% (w/v)) was used as an indicator of growth. Alamar Blue is a blue, non-fluorescent, non-toxic indicator dye that is reduced to resorufin, a pink fluorescent molecule, by enzymes present in living cells. It is used as an indicator of cell growth, particularly in assays involving a bacterium or yeast as the test organism (Sarker et al., 2007). A fresh solution of Alamar Blue was made up for each batch of MIC assays. The minimum inhibitory concentration was determined as the lowest sample concentration at which no pink colour, signifying live organisms, appeared after leaving the plate to stand for 1 hour. Plates were returned to the incubator and checked again after another 24 hours incubation to confirm the previously recorded MIC values.

A similar method was followed for testing for antifungal activity. Yeast cells (*Candida albicans*) from similarly prepared and frozen G1 stocks were inoculated into 5ml of RPMI 1640 medium and grown for 48 hours before use. Assays were performed in sterile 96 well plates. 100µl of sterile water was placed into all wells. 50µl of each ethanolic extract was tested in duplicate and serial two-fold dilutions were made from the first well down to the desired minimum concentration (Langfield et al., 2004). A 1:50 dilution was made from the 48 hour old yeast culture using RPMI 1640. Plates were inoculated with the diluted yeast suspension (50µl per well), covered and incubated at 37°C for 24 hours. Nystatin (concentration 50 µg/ml) was used as the positive control, and appropriate solvent blanks were included as the negative controls.

After the incubation period, 10 µl Alamar Blue (consisting of 5mg resazurin dissolved in 1ml sterile water, 0.5% (w/v)) was used as an indicator of growth. A fresh solution of Alamar Blue was made up for each batch of MIC assays. The minimum inhibitory concentration was determined as the lowest sample concentration at which no pink colour, signifying live organisms, appeared after leaving the

plate to stand for 1 hour. Plates were returned to the incubator and checked again after another 24 hours incubation to confirm the previously recorded MIC values.

2.4. Statistical comparison of GIBEX vs. MIC

The results from the GIBEX assays were compared to the results from the MIC assays and statistically evaluated to determine the level of agreement between them. This was done using an *attribute agreement analysis* in Statistica version 11 (Statsoft Inc., 2012). The null hypothesis is that there is no significant difference between two sets of data. The program calculates Cohen's kappa statistic for the data and gives a p-value indicating whether to accept or reject the null hypothesis. Individual agreements analyses were performed to test agreement between the GIBEX antibacterial assay and the MIC assay that used *E. coli* as the test organism, between the GIBEX antibacterial assay and the MIC that used *S. aureus* as the test organism and the GIBEX antifungal assay and the MIC that used *C. albicans* as the test organism.

2.5. Trends in activity across family, growth form, seasonality and succulence

Many investigations into bioactivity have been performed on various floras from around the world. For example, screening of plant extracts traditionally used for medicine in the Peruvian Andes (Neto et al., 2002), and screening of traditionally used medicinal plant extracts from India (Ahmad & Beg, 2001). However, not all of these investigations are successful (Firn, 2003). Plants must be very carefully chosen in order to increase the success rate and number of positive hits resulting from screening efforts (Salis-Lagoudakis et al., 2011). In South Africa, with its varied flora, there are so many potential candidates for screening that selection must be based upon a predetermined set of criteria if maximum positive hit rate is to be achieved.

One way of doing this is to use traditional medicine as a guide. The chance of discovering bioactive compounds of interest is increased if ethnomedicine is used as a guide, particularly if disparate pharmacopoeias use the same, or similar, plants (Upretty et al., 2010). If plants have been independently selected by different cultures for medicinal use, then their case for efficacy is stronger and they become preferred candidates (Salis-Lagoudakis et al., 2011). Using traditional medicine as a guide could be useful for further investigation into bioactivity of Namaqualand plants. However, a large percentage of Namaqualand species are endemic, so the method of selecting the species with cross-cultural use cannot be applied here. A slightly different approach could be used though, where trends in bioactivity across family lines, growth forms and seasonality are used as a guide to plant selection for future screening efforts.

Certain types of compounds responsible for specific bioactivity may be associated with growth strategy, family or life cycle. Uncovering trends in bioactivity, along family lines for example, may indicate which species to focus on when screening for novel compounds with specific activity. The success of future screening endeavours may be enhanced if species are selected on the basis of these trends. In order to establish the trends in bioactivity for species in Namaqualand, the relationships between bioactivity and growth form, seasonality, family and succulence were examined using correspondence analysis and regression analysis.

2.5.1. General method of correspondence analysis

Commonly used multivariate techniques for reducing data complexity and visualising relationships among sets of data include principle components analysis (PCA) and factor analysis (FA). However, these techniques are only applicable to data sets of continuous variables, and cannot be used with categorical data such as the results from the bioassays (Sourial et al., 2010). Correspondence analysis (CA) is an alternative multivariate technique specifically designed for use with categorical data stored in contingency tables (Greenacre, 2007).

CA calculates the chi-square statistic to determine if there is a significant relationship between variables in a contingency table. CA, as with PCA, aims to graphically display any relationship existing between the variables in question and describes the inertia present in the data set. Inertia is a measure of the variance in the data set contained in the contingency table. Thus the larger the inertia, the greater the differences between data points. CA reduces inertia to a small set of orthogonal dimensions. Dimension 1 describes the greatest amount of inertia, Dimension 2 the next largest amount and so on. PCA performs a similar task, but uses principle components to describe the variance between samples. Each dimension has an associated eigenvalue, which describes how much of the inertia it explains. Most analyses retain three dimensions and generally it is accepted to retain the number of dimensions that accounts for >70% of the total inertia i.e. the cumulative percentage eigenvalues must be 70% or more for the retained dimensions (Sourial et al., 2010).

CA generates a 2D correspondence map with Dimension 1 as the horizontal axis and Dimension 2 as the vertical axis. The degree of clustering or closeness of points in the same areas of the map can be used to interpret relationships between variables. Points that are positioned close to one another along the first dimension can be said to be highly related. Similarly, those lying close together along the second dimension may also be said to be related, only slightly less so than those clustered along dimension one. Points that are close together on both dimensions may be said to be very highly related. In general, points that lie between -0.2 and 0.2 along a dimension cannot be said to have

any significant association with that dimension and are usually disregarded when interpreting the correspondence map (Heir, et al.).

2.5.2. Definitions of growth form and classification

For the purposes of this study, plant growth form data were extracted from the Plants of South Africa (POSA) website - an online checklist of current taxonomic data maintained by the South African National Botanical Institute (POSA, 2012). Using the data extracted from POSA, species were grouped into nominal categories following the suggestion of de Almeida et al. (2005) and Douwes et al. (2008): trees, bushes/shrubs, herbs, climbers and geophytes. "Trees" were defined as perennial, woody plants generally with a single stem axis. "Bushes/shrubs" were defined as perennial, woody plants that are shorter than trees and generally have several branches rather than a single stem. "Herbs" were defined as are non-woody plants that are relatively shorter than plants in the "trees" and "bushes/shrubs" categories. "Herbs" grow from seed and have roots. "Geophytes" were defined as herbaceous species growing from an underground rhizome, tuber, bulb or corm. "Climbers" were defined as vine-like creepers that use other plants to support their trailing stems.

In addition to taxonomic data, life strategy data for each species under investigation was also extracted from POSA. "Annuals" were defined as short lived (only persisting for a single growing season) species. Annuals follow an *r* life strategy i.e. they have a high rate of reproduction and growth. "Perennials" were defined as longer lived species. Perennials usually follow a *K* type life strategy i.e. lower reproduction potential but greater ability to survive competitively (de Almeida et al., 2005).

Information regarding the succulence of each of the species under investigation was also extracted from POSA. Plants may be stem or leaf succulents, and some show a combination of woody stems and succulent leaves. Hence, "succulent" was not treated as a distinct growth type, as has been done in other studies, but rather as an attribute related to overall habitat adaptation and choice of photosynthetic pathway. In this study, plants were classified as either "succulent" or "non-succulent". All species under investigation were classified according to these groupings and a contingency table was created for the purpose of performing correspondence analysis on the data.

2.5.3. Analysis of trends in activity

Correspondence analysis was performed in Statistica version 11 (Statsoft Inc., 2012) using a contingency table containing family data extracted from POSA, and bioactivity data from GIBEX test results. A chi-square test was performed as part of the analysis in order to investigate whether there was a significant association between family and bioactivity. Correspondence maps were generated

to visualise the association. For the purposes of this investigation, assay 4 - the test for protease activity - was excluded from the analysis on the basis that only a single species was recorded as having this activity. This was thought to lend unnecessary bias to the data and was therefore excluded.

Similarly, the relationship between growth form and bioactivity was examined using correspondence analysis in Statistica version 11 (Statsoft Inc., 2012). The analysis made use of a contingency table containing growth form data extracted from POSA, and bioactivity data from GIBEX test results. The chi-square statistic was used to investigate whether there was a significant association between growth form and bioactivity. Correspondence maps were generated to visualise the association. Assay 4 was omitted from this analysis for the same reason given in the previous test.

In order to examine the relationship between seasonality and bioactivity, seasonality data extracted from POSA was summed for each assay type. These figures were then plotted in a bar chart and trends extrapolated. Similarly, in order to investigate the relationship between succulence and bioactivity, succulence data extracted from POSA was summed for each assay type. These figures were also plotted in a bar chart and trends extrapolated.

2.6. Choosing candidates for further phytochemical analysis

Of the 102 extracts created and tested for this section of the study, ten were selected for further phytochemical analysis. Species were evaluated on the basis of several criteria - how easy it would be to collect a large sample (plants must be of a suitable size e.g. a species that only grows to a maximum of 5cm would not be a good candidate as hundreds, if not thousands of plants would have to be collected) and how abundant the species is in the Paulshoek area (species must be locally abundant so as a large scale collection would not have a negative impact on populations). Medicinal use was taken into consideration (a good selection of both species with recorded medicinal uses and those with no medicinal uses should be considered). When choosing species of interest, bioactivity was evaluated in two different ways. First, the number of assays a particular extract was active in was considered - activity in four or more assays was taken to indicate a species that merits further investigation. A second evaluation of activity was how strong the activity was in individual assays. This was done so as not to exclude an extract with a single very strong result for one assay, and negative results in other assays. Finally, the literature was reviewed to see if other research groups were working on the same or a closely related species. Those species with no existing literature were deemed more important for investigation. Species were evaluated on all of these criteria and ten were selected for further phytochemical analysis.

3. Results

3.1. Results of the GIBEX assays

The aim of this section was to assess the biological activity of 102 Namaqualand plant extracts using a range of assays. Some of the tested extracts are from plants used traditionally in Paulshoek, and others are from plants with no recorded medicinal use. Results of the selected GIBEX assays that were performed on all 102 extracts are summarised in Table 2. The key to codes used to record results is given in Table 1.

Table 1: Key codes used to record GIBEX assay results

Assay type	Key
antibacterial	0 = no activity, 1 = slight activity, 2 = moderate activity, 3 = strong activity
yeast inhibition	0 = yeast practically unaffected, 1 = some yeast alive, 2 = yeast slightly alive, 3 = yeast dead
antioxidant	0 = no activity, 1 = mild activity, 2 = moderate activity, 3 = high activity
protease activity	0 = no activity, 3 = activity
protease inhibition	0 = no inhibition, 3 = inhibitor activity
roundworm lethality	0 = no activity, 3 = activity

Table 2: A summary of the results of GIBEX assays performed on plant extracts under investigation

Scientific name and plant part extracted	Plant Family	Antibacterial assay	Antifungal assay	Antioxidant assay	Protease activity	Protease inhibition	Round worm lethality
1. Aizoon canariense *(leaves)*	Aizoaceae	3	0	1	0	0	0
2. Aizoon canariense *(roots)*	Aizoaceae	2	0	0	0	0	0
3. Adromischus spp. *(leaves)*	Mesembryanthemaceae	2	0	3	0	3	0
4. Antizoma miersiana *(leaves)*	Menispermaceae	0	0	3	0	0	0
5. Aptosimum indivisum *(leaves)*	Scrophulariaceae	1	0	2	0	0	0
6. Aptosimum indivisum *(rootstock)*	Scrophulariaceae	2	0	2	0	0	0
7. Aptosimum spinescens *(leaves)*	Scrophulariaceae	1	0	3	0	0	0
8. Arctotis fastuosa *(leaves)*	Asteraceae	1	2	1	0	0	0
9. Aridaria noctiflora *(leaves)*	Mesembryanthemaceae	0	0	0	0	0	0
10. Asparagus rubicundus *(leaves, stems & flowers)*	Asparagaceae	0	3	2	0	0	0
11. Ballota africana *(leaves)*	Lamiaceae	2	3	1	0	0	0
12. Berkheya spinosissima *(leaves)*	Asteraceae	1	0	2	0	0	0
13. Bulbine abyssinica *(leaves)*	Asphodelaceae	2	0	3	0	0	0
14. Bulbine abyssinica *(rootstock)*	Asphodelaceae	1	1	3	0	0	0
15. Bulbine praemorsa *(leaves)*	Asphodelaceae	1	0	0	0	0	0
16. Calobota sericea *(leaves)*	Fabaceae	2	0	2	0	0	0
17. Carpobrotus edulis *(leaves)*	Mesembryanthemaceae	2	3	2	0	3	0
18. Cheiridopsis denticulata *(leaves)*	Mesembryanthemaceae	2	0	3	0	3	0
19. Cheiridopsis namaquensis *(leaves)*	Mesembryanthemaceae	2	0	1	0	0	0
20. Chrysocoma ciliata *(leaves & stems)*	Asteraceae	3	3	3	0	0	0
21. Conicosa elongata *(leaves)*	Mesembryanthemaceae	0	0	0	0	0	0
22. Cotyledon cuneata *(leaves)*	Crassulaceae	2	3	3	0	2	0
23. Cotyledon orbiculata var. orbiculata *(leaves)*	Crassulaceae	1	0	3	3	0	0
24. Crassula atropurpurea var. watermeyeri *(leaves)*	Crassulaceae	3	0	3	0	3	0

25. Crassula brevifolia ssp. brevifolia *(leaves)*	Crassulaceae	2	0	3	0	3	3
26. Crassula muscosa var.muscosa *(leaves & stems)*	Crassulaceae	3	0	3	0	3	0
27. Cyphia crenata *(entire plant)*	Lobeliaceae	3	0	3	0	0	0
28. Diascia namaquensis *(leaves & stem)*	Scrophulariaceae	0	0	1	0	0	0
29. Dodonaea viscosa var angustifolia *(leaves)*	Sapindaceae	3	3	3	0	0	0
30. Drosanthemum hispidum *(leaves & stems)*	Mesembryanthemaceae	2	2	1	0	0	0
31. Drosanthemum spp. *(leaves)*	Mesembryanthemaceae	0	0	1	0	0	0
32. Eriocephalus microphyllus *(leaves)*	Asteraceae	1	3	3	0	0	0
33. Erodium cicutarium *(leaves)*	Geraniaceae	0	0	3	0	0	0
34. Euphorbia decussata *(leaves & stems)*	Euphorbiaceae	0	0	3	0	3	2
35. Euphorbia mauritanica *(leaves & stems)*	Euphorbiaceae	1	0	2	0	0	0
36. Euryops multifidus *(leaves)*	Asteraceae	2	3	2	0	0	0
37. Foveolina dichotoma *(leaves)*	Asteraceae	2	2	1	0	0	0
38. Galenia africana *(leaves)*	Aizoaceae	3	3	3	0	0	3
39. Gazania heterochaeta *(leaves)*	Asteraceae	0	0	1	0	0	0
40. Grielum humifusum *(leaves)*	Neuradaceae	1	0	3	0	0	0
41. Heliophila variabilis *(leaves)*	Brassicaceae	1	0	2	0	0	0
42. Hermannia amoena *(leaves)*	Malvaceae	1	1	3	0	0	0
43. Hermannia cuneifolia *(leaves)*	Malvaceae	2	0	3	0	0	0
44. Hirpicium alienatum *(leaves & buds)*	Asteraceae	0	0	3	0	0	0
45. Hypertelis salsoloides *(leaves)*	Molluginaceae	1	0	0	0	0	0
46. Lachenalia anguinea *(leaves)*	Hyacinthaceae	0	0	1	0	0	0
47. Larryleachia cactiformis *(succulent stem)*	Apocynaceae	2	0	0	0	0	0
48. Leipoldtia schultzei *(leaves)*	Mesembryanthemaceae	2	1	3	0	3	0

49. Lessertia diffusa *(leaves & stems)*	Fabaceae	2	2	1	0	0	0
50. Leysera tenella *(leaves)*	Asteraceae	1	0	3	0	0	0
51. Lycium ferocissimum *(leaves)*	Solanaceae	1	0	1	0	0	0
52. Manochlamys albicans *(leaves)*	Chenopodiaceae	1	0	0	0	0	0
53. Mesembryanthemaceae guerichianum *(leaves)*	Mesembryanthemaceae	2	0	0	0	0	0
54. Microloma sagittatum *(leaves)*	Apocynaceae	0	3	1	0	0	0
55. Microloma sagittatum *(flowers)*	Apocynaceae	0	3	2	0	0	0
56. Monoculus hyoseroides *(leaves)*	Apocynaceae	0	1	0	0	0	0
57. Moraea pallida *(leaves)*	Iridaceae	2	0	3	0	0	0
58. Moraea serpentina *(leaves)*	Iridaceae	1	3	2	0	0	0
59. Nicotiana glauca *(leaves)*	Solanaceae	1	0	3	0	0	0
60. Ornithogalum pruinosum *(bulb)*	Hyacinthaceae	1	0	2	0	0	1
61. Ornithogalum pruinosum *(leaves)*	Hyacinthaceae	0	0	3	0	0	0
62. Ornithogalum secundum *(leaves)*	Hyacinthaceae	1	0	1	0	0	0
63. Ornithoglossum vulgare *(leaves)*	Colchicaceae	1	0	3	0	0	0
64. Othonna abrotanifolia *(leaves)*	Asteraceae	1	3	3	0	0	0
65. Othonna cylindrica *(leaves)*	Asteraceae	2	0	2	0	0	0
66. Othonna floribunda *(flowers)*	Asteraceae	1	0	1	0	0	0
67. Othonna floribunda *(leaves)*	Asteraceae	0	0	1	0	0	0
68. Oxalis obtusa *(leaves & stem)*	Oxalidaceae	1	2	3	0	0	0
69. Oxalis pes-caprae *(bulb)*	Oxalidaceae	2	0	2	0	0	0
70. Oxalis pes-caprae *(leaves)*	Oxalidaceae	2	0	1	0	0	0
71. Oxalis pes-caprae *(lower stem)*	Oxalidaceae	1	0	0	0	0	0
72. Pelargonium alternans *(leaves)*	Geraniaceae	2	0	3	0	0	0
73. Pelargonium crithmifolium *(leaves)*	Geraniaceae	0	0	3	0	3	0
74. Pelargonium pulchellum *(leaves)*	Geraniaceae	0	0	3	0	0	0
75. Pentzia incana *(leaves & stems)*	Asteraceae	0	0	2	0	0	0
76. Polymita albiflora *(leaves)*	Mesembryanthemaceae	3	0	3	0	3	0
77. Pteronia divaricata *(leaves)*	Asteraceae	0	3	3	0	0	0
78. Pteronia incana *(leaves)*	Asteraceae	1	3	3	0	0	0

79. Quaqua mammillaris *(stem)*	Apocynaceae	2	0	3	0	3	0
80. Ruschia fredericii *(leaves)*	Mesembryanthemaceae	2	0	3	0	3	0
81. Ruschia fredericii *(stems)*	Mesembryanthemaceae	1	0	2	0	0	0
82. Ruschia robusta *(leaves)*	Mesembryanthemaceae	2	1	3	0	3	0
83. Salvia dentata *(leaves)*	Lamiaceae	3	0	3	0	0	0
84. Sceletium tortuosum *(leaves & stem)*	Mesembryanthemaceae	1	0	0	0	0	2
85. Searsia undulata *(leaves & shoots)*	Anacardiaceae	3	3	2	0	3	0
86. Senecio cardaminifolius *(flower stalks)*	Asteraceae	2	0	1	0	0	0
87. Senecio cardaminifolius *(leaves)*	Asteraceae	0	2	1	0	0	0
88. Senecio cinerascens *(leaves)*	Asteraceae	1	0	1	0	0	0
89. Senecio radicans *(leaves)*	Asteraceae	0	0	0	0	0	0
90. Solanum burchellii *(leaves)*	Solanceae	1	2	2	0	0	0
91. Solanum burchellii *(ripe berries)*	Solanceae	1	0	3	0	0	0
92. Spiloxene ovata *(leaves & bulb)*	Hypoxidaceae	1	0	3	0	0	1
93. Tetragonia fruticosa *(leaves)*	Aizoaceae	0	1	1	0	0	0
94. Thesium lineatum *(leaves & flowering stems)*	Santalaceae	0	0	3	0	0	0
95. Trachyandra tortilis *(bulb & leaves)*	Asphodelaceae	1	0	1	0	0	0
96. Trichogyne polycnemoides *(leaves)*	Asteraceae	0	1	3	0	1	0
97. Tripteris sinuata *(leaves)*	Asteraceae	1	0	1	0	0	0
98. Tylecodon wallichii ssp. wallichii *(leaves)*	Crassulaceae	2	0	0	0	0	0
99. Unidentified spp.	-	2	0	1	0	0	0
100. Viscum capense *(stems & leaves)*	Viscaceae	1	1	3	0	0	0
101. Zaluzianskya benthamiana *(entire plant)*	Scrophulariaceae	0	0	2	0	0	0
102. Zygophyllum foetidum *(leaves)*	Zygophyllaceae	2	3	0	0	0	0

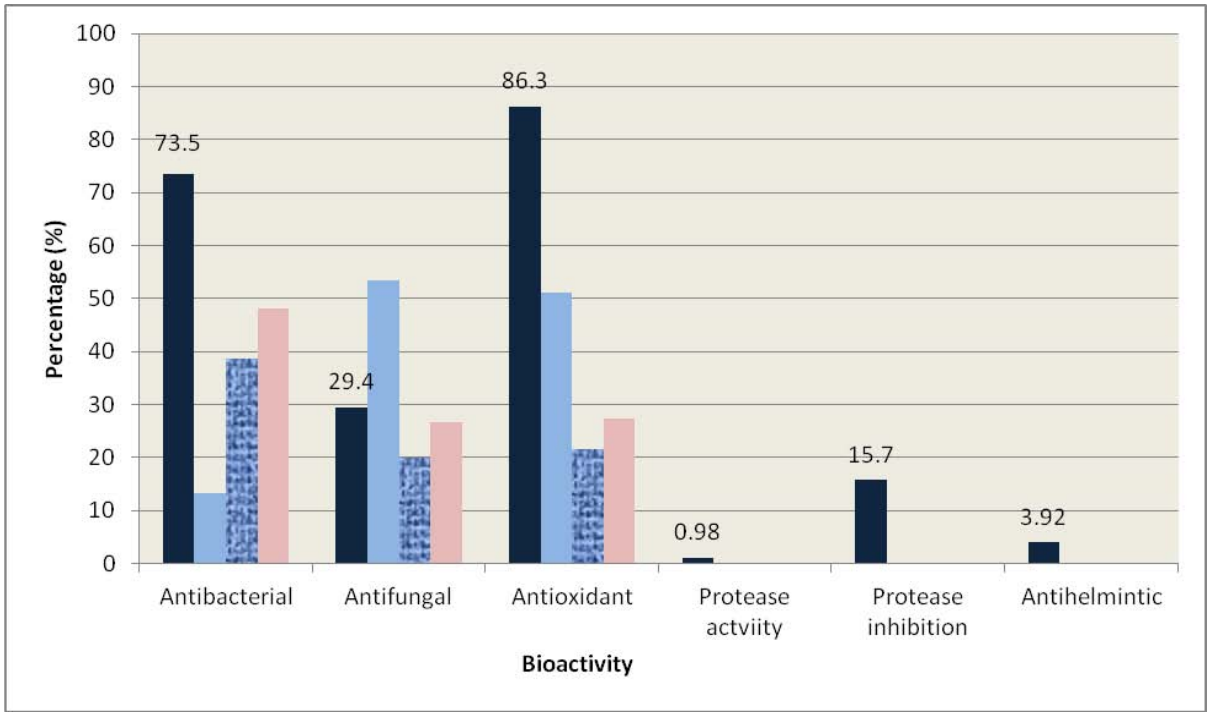


Figure 1: The percentage of tested samples active in each assay type. Additional bars in the antibacterial, antifungal and antioxidant assays indicate the percentage of positives that were strongly, moderately and weakly active (in decreasing order left to right).

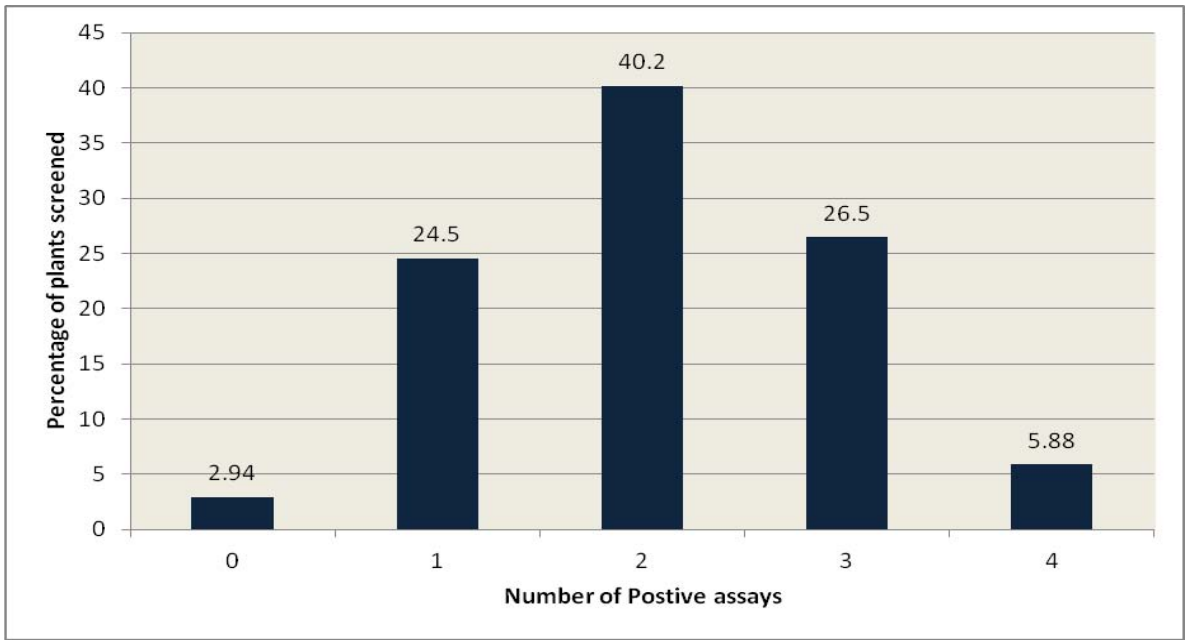


Figure 2: The number of plants that were active in no, single and multiple assays. The majority of screened species were active in two assays.

On examining the results of the assays it is evident that the majority of plants exhibited antioxidant activity (86.3%) and antibacterial activity (73.5%) when tested (Figure 1). Over half of those with antioxidant activity were strongly active (51.1%), while those with antibacterial activity were mostly moderately (38.7%) or weakly active (48.0%) (Figure 1). Very few of the tested extracts were positive for protease activity (0.98%) or antihelmintic activity (3.92%). The majority of extracts were positive in two assays, while very few (2.94%) displayed no activity in any of the assays utilised for this study (Figure 2).

3.2. How do MIC assays compare with GIBEX assays?

The aim of this section was to evaluate how well the simplified GIBEX assays approximate results from more scientifically rigorous assays. A random selection of extracts from the pool of 102 was tested using a more robust serial microdilution MIC assay method. The results of the MIC assays and the results from the GIBEX assays were compared. Extracts having activities with MIC values below 8mg/ml are

Table 3: Minimum inhibitory concentration (MIC) values for all tested crude ethanolic extracts with positive results against various organisms. EC = *Escherichia coli*, SA = *Staphylococcus aureus* and CA = *Candida albicans*. "NT" indicates "not tested" against a particular organism, while "-" indicates a negative result in a particular assay.

Extracted species	MIC CA (mg/ml)	MIC SA (mg/ml)	MIC EC (mg/ml)
Aizoon canariense (leaves)	NT	-	166.7
Arctotis fastuosa (leaves)	NT	166.7	20.8
Berkheya spinosissima (leaves)	NT	166.7	-
Bulbine abyssinica (leaves)	NT	83.3	83.3
Carpobrotus edulis (leaves)	83.3	-	-
Crassula brevifolia ssp. brevifolia (leaves)	NT	20.8	166.7
Galenia africana (leaves)	10.4	NT	NT
Hermannia cuneifolia (leaves)	166.7	NT	NT
Calobota sericea (leaves)	NT	83.3	83.3
Leipoldtia schultzei (leaves)	NT	5.2	83.3
Microloma sagittatum (flowers)	10.4	NT	NT
Microloma sagittatum (leaves)	41.7	NT	NT
Monoculus hyoseroides (leaves)	41.7	NT	NT
Moraea pallida (leaves)	NT	83.3	83.3
Othonna floribunda (flowers)	166.7	NT	NT
Othonna cylindrica (leaves)	10.4	NT	NT
Pentzia incana (leaves & stems)	NT	166.7	166.7
Quaqua mammillaris (stem)	NT	166.7	166.7
Ruschia fredericii (leaves)	2.6	NT	NT
Sceletium tortuosum (leaves & stem)	NT	166.7	-

Table 4: Results of the attribute agreement analysis performed to assess whether there was a significant difference between the results of the GIBEX assays and microdilution MIC assays.

Comparison between assay:	Result	Conclusion
Antibacterial GIBEX and MIC (using *E. coli*)	$p = 0.2910$	Accept H_0
Antibacterial GIBEX and MIC (using *S. aureus*)	$p = 0.0029$	Reject H_0, accept H_1
Antifungal GIBEX and MIC (using *C. albicans*)	$p = 0.5759$	Accept H_0

considered to posses some activity of interest (Fabry et al., 1998) and those with MIC values below 1mg/ml are considered of particular interest (Rios & Recio, 2005). Pure compounds with activity of 10µg/ml or less are considered good candidates for development into pharmaceuticals (van Vuuren, 2008). MIC values ranged between 166.7 mg/ml to 2.6 mg/ml for the assay using *C. albicans* as a test organism, 166.7 mg/ml to 5.2 mg/ml for the assay using *S. aureus* as a test organism and between 166.7 mg/ml and 20.8 mg/ml for the assay using *E. coli* as the test organism (Table 3). *Ruschia fredericii* with an MIC value of 2.6 mg/ml, *Leipoldtia schultzei* with an MIC value of 5.2 mg/ml, and *Arctotis fastuosa* with an MIC value of 20.8 mg/ml were the most active in each of the assays. While none of these crude plant extracts showed exceptional MIC values, the values are low enough to merit further investigation into the active compounds present in these plants.

Any activity against the test organisms was recorded a positive result for comparison against results from GIBEX assays for the same extracts. Attribute agreement analysis performed on each set of results - the GIBEX results and the MIC results - gave *p*-values for each of the tests (

Table 4). In this case, we want to have a non-significant value of *p* at the 5% level i.e. a *p*-value greater than 0.05 in order to accept the null hypothesis (H_0) that the two sets of data are not significantly different. From the results, we can see that H_0 can be accepted for the attribute agreement analyses performed between MIC (using *E. coli*) and the GIBEX antibacterial assay ($p = 0.2910$) as well as for MIC (using *C. albicans*) and the GIBEX antifungal assay ($p = 0.5759$) (Table 4). This is not true for the analysis between MIC using (*S. aureus*) and the GIBEX antibacterial assay ($p = 0.0029$) and the alternative hypothesis, H_1 that there is a significant difference between the results from the two assays, must be accepted. Thus, GIBEX assays may be used as suitable indicators of both antibacterial and antifungal activity. However GIBEX assays may not be equally sensitive to all strains of bacteria resulting in false negatives i.e. extracts may have broad antibacterial activity but are not active against a specific test organism and are recorded as non-active.

3.3. What trends in bioactivity are apparent?

The aim of this section was to examine bioactivity across family, growth form, seasonality and succulence in order to determine if any trends were apparent. Patterns in underlying chemistry have been investigated previously. It has been suggested that plants have two major anti-herbivory strategies and that these are linked to their life strategy and growth form. Plants may either display metabolically inactive defences such as tannins and lignans which reduce digestibility, or chemically active defences such as alkaloids, cardiac glycosides and terpenoids which negatively affect the attacker (Coley et al., 1985). These low molecular weight defence compounds are the ones that most likely to be bioactive.

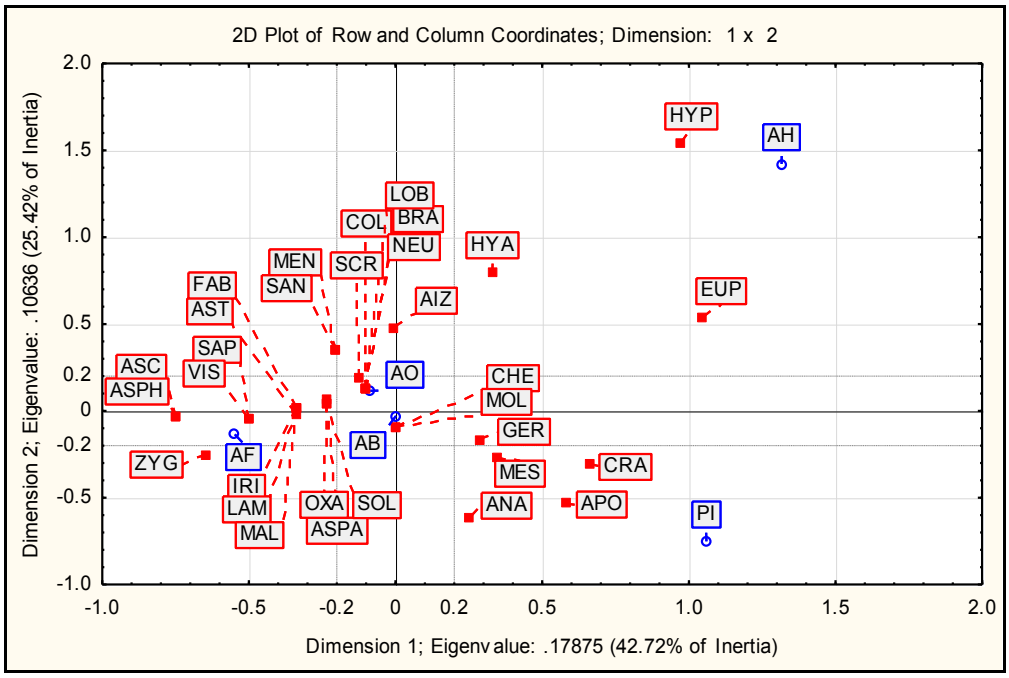


Figure 3: Correspondence analysis map of bioactivity and plant family. Codes used for bioactivity data point labels (represented by open circles) are: AB = antibacterial, AF = antifungal, AH = antihelmintic, AO = antioxidant, PI = protease inhibition. Codes used for family data point labels (represented by filled squares) are: AIZ = Aizoaceae, ANA = Anacardiaceae, APO = Apocynaceae, ASC = Asclepiadaceae, ASPA = Asparagaceae, ASPH = Asphodelaceae, AST = Asteraceae, BRA = Brassicaceae, CHE = Chenopodiaceae COL = Colchicaceae, CRA = Crassulaceae, EUP = Euphorbiaceae, FAB = Fabaceae, GER = Geraniaceae, HYA = Hyacinthaceae, HYP = Hypoxidaceae, IRI = iridaceae, LAM = Lamiaceae, LOB = Lobeliaceae, MAL = Malvaceae, MEN = Menispermaceae MES = Mesembryanthemaceae, MOL = Molluginacceae, NEU = Neuradaceae, OXA = Oxalidaceae, SAN = Santalaceae, SAP = Sapindaceae, SCR = Scrophulariaceae, SOL = Solanaceae, VIS = viscaceae ZYG = Zygophyllaceae

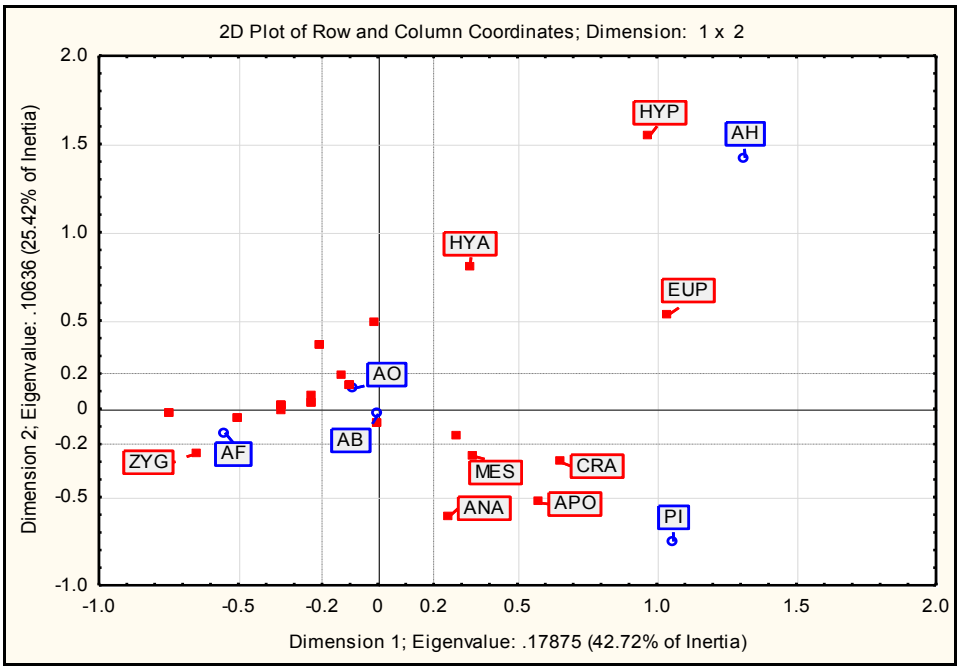


Figure 4: Correspondence analysis map of bioactivity and plant family with non-significant data point labels removed for clarity. Codes for labels for activity and family data points are the same as those used in Figure 3.

Ephemeral or r-selected species, which tend to be opportunistic colonisers and weedy species, seem to rely more on chemical defence (Rhoades & Cates, 1976). Thus, annuals, weeds and herbs are expected to be more bioactive than longer lived shrub and tree species and therefore better targets for screening endeavours (Stepp & Moerman, 2001). Furthermore, studies have shown that alkaloids are twice as likely to be present in annual species as in perennial species (Levin, 1976). Additionally, leaf life may be a predictor of underlying anti-herbivory defence strategy. Plants with long lived leaves rely mainly on more physical defence mechanisms of tannins and lignans. Plants with short lived leaves tend to invest in chemical defence mechanisms (Stepp, 2004). These trends are expected in the species from Namaqualand.

3.3.1. The relationship between bioactivity and family

The chi-square test performed on the data as part of correspondence analysis revealed that there is no significant relationship between family and activity at the 5% level (p = 0.9798). The cumulative percentage of inertia explained by the first two dimensions resulting from correspondence analysis is 68.14%. The correspondence map of these two dimensions clearly shows a lack of relationship between bioactivity and family, as indicated by the chi-square test (Figure 3). The map becomes even clearer

when data points that fall in the range -0.2 to 0.2 on both dimensions (which is the threshold for a significant association with a particular dimension) are removed (Figure 4).

The only relationships are the very weak associations between *Zygophyllaceae* (ZYG) and antifungal activity (AF), and *Hypoxidaceae* (HYP) and antihelmintic activity (AH). But as these are extremely weak associations, they may be disregarded. This confirms the lack of strong associations between families and activities. The lack of any significant relationship or clustering on the correspondence map suggests that bioactivity is not limited to specific families. Thus family cannot be used as an indicator of bioactivity, at least not for the types of bioactivity screens and families examined in this study.

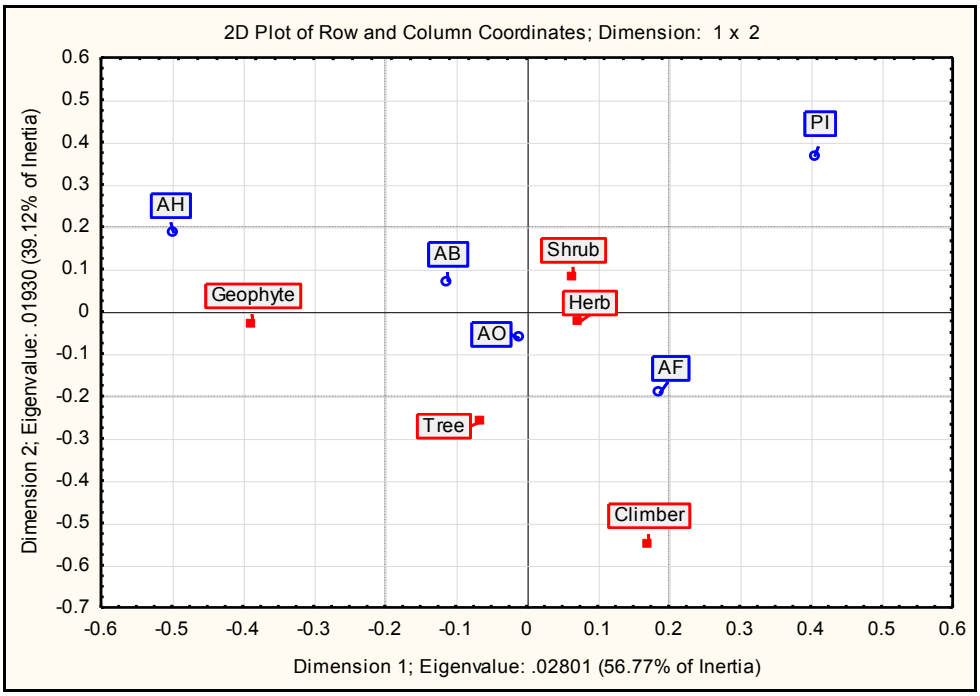


Figure 5: Correspondence map of bioactivity and growth form. Codes used for bioactivity data point labels (represented by open circles) are: AB = antibacterial, AF = antifungal, AH = antihelmintic, AO = antioxidant, PI = protease inhibition.

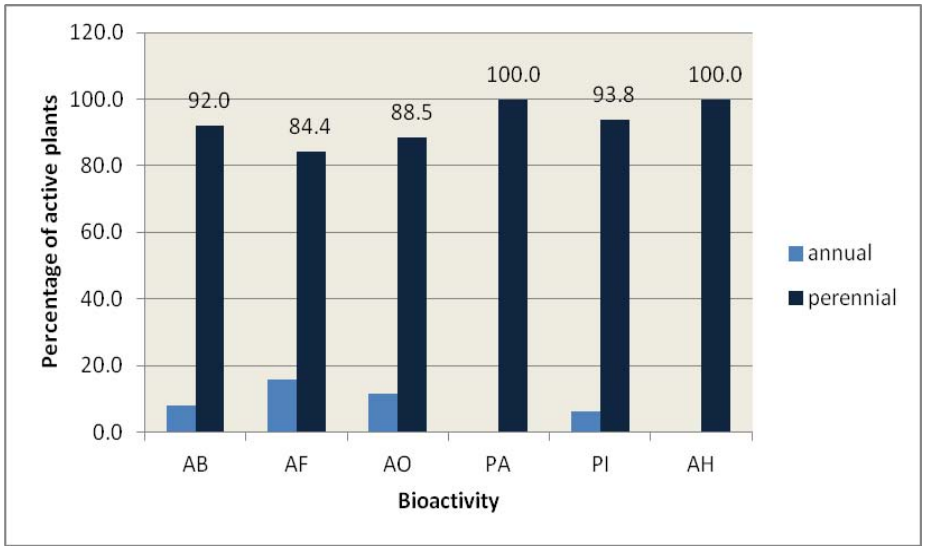


Figure 6: The percentage of annual and perennial species per type of bioactivity. Codes for bioactivity are: AB = antibacterial, AF = antifungal, AH = antihelmintic, AO = antioxidant, PI = protease inhibition.

3.3.2. The relationship between bioactivity and growth form

Similarly to the results of the previous correspondence analysis, the chi-square test revealed no significant association between growth form and activity at the 5% level (p = 0.8359). The cumulative percentage of inertia explained by the first two dimensions resulting from correspondence analysis is 95.89%. The correspondence map of these two dimensions clearly shows a lack of relationship between bioactivity and growth form, as indicated by the chi-square test (Figure 5). The lack of any significant relationship or clustering on the correspondence map suggests that bioactivity is not correlated with any specific growth form. Hence, growth form cannot be used as an indicator of bioactivity.

3.3.3. The relationship between bioactivity and seasonality

The percentage of annual and perennial species exhibiting each bioactivity was plotted in a bar chart (Figure 6). It is clear that many more species with perennial seasonality are bioactive than those with annual seasonality across all categories of bioactivity. Annuals were in the minority, with over a 5:1 ratio of perennials to annuals in all categories.

3.3.4. The relationship between bioactivity and succulence

The percentage of succulent and non-succulent species exhibiting each bioactivity was plotted in a bar chart (Figure 7). From this we can see that non-succulent species were in the majority in the antibacterial (58.1%), antifungal (70.0%) and antioxidant (65.5%) assays. Succulents predominated in the

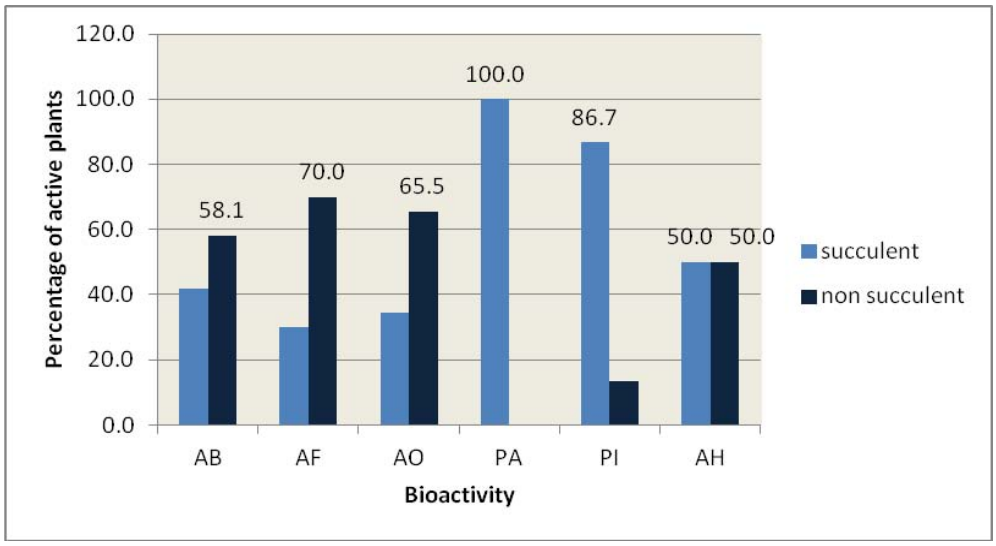


Figure 7: The percentage of the active species that were succulent vs. the percentage that were not per type of bioactivity. Codes for bioactivity are: AB = antibacterial, AF = antifungal, AH = antihelmintic, AO = antioxidant, PI = protease inhibition.

protease activity (100%) and protease inhibition (86.7%) assays. Succulents and non-succulence were equally as active in the antihelmintic assay.

3.4. Choice of candidates for next phase

The aim of this section was to identify potential candidates from species screened in this study for further phytochemical and metabolomic analysis. Criteria for selection were determined and ten species were chosen accordingly (Table 5). The most important selection criterion was bioactivity. Those species that had particularly strong activity or multiple activities were prioritised. Then the ease of their collection and relative local abundance was considered. Species that were readily available, not seasonally constrained, and locally abundant were prioritised. After this the candidates were assessed to see if they were used in the preparation of traditional medicines in Paulshoek, and if there was existing literature on them. Those that are used in traditional medicine and that had no existing literature were prioritised.

Large collections, between 1 and 3kg, of the ten chosen species were gathered during a field trip undertaken in October 2011. Large samples were taken directly back to the laboratory at the University of Cape Town and extracted according to modified version of the GIBEX protocol. Modifications to the protocol involved changes necessary to accommodate the large scale of the samples being processes

and not to modify the extraction process. Samples were washed to remove dirt, separated according to plant part, and extracted with 70% EtOH in a ratio of 1g sample: 2ml EtOH. In order to accommodate the large sample size, parts of interest were macerated in a blender (rather than by hand). The resulting ethanolic plant slurry was filtered through Whatman No. 1 filter paper and stored in sterile brown glass Winchester bottles at 10 °C. Extracts were dried under vacuum at temperatures not greater than 40°C to remove EtOH solvent. Remaining liquid was lyophilized to remove residual water. Powdered crude extracts were stored in glass jars at 4°C until further analysis as outlined in chapter 4.

Table 5: Details of which species were selected for further investigation and the criteria for their inclusion

Species	Activity	Ease of collection/ abundance	Medicinal use	Other research being done
Ballota africana	Strong antifungal activity	Relatively easy, not as locally abundant	Medicinal use in Paulshoek	Existing literature
Chrysocoma ciliata	Strong antibacterial activity	East to collect, locally abundant	Medicinal use in Paulshoek, recorded toxicity in literature	Existing literature
Cotyledon cuneata	Active in four assays	Easy to collect, locally abundant	No medicinal use, flowerstalks reported to be toxic to livestock	No
Crassula brevifolia	Active in four assays	Easy to collect, locally abundant	No medicinal use	No
Crassula muscosa	Strong antibacterial activity	Relatively easy to collect, locally abundant	Medicinal use in Paulshoek	No
Euphorbia decussata	Strong antihelminthic activity	Not as easy to collect (produces latex), locally abundant	No medicinal use, toxic latex	No
Polymita albiflora	Strong antibacterial activity	Easy to collect, locally abundant	Medicinal use in Paulshoek	No
Salvia dentata	Strong antibacterial activity	Easy to collect, locally abundant	Medicinal use in Paulshoek	No
Searsia undulata	Active in four assays	Easy to collect, locally abundant	Medicinal use in Paulshoek	Existing literature
Zygophyllum foetidum	Strong antifungal activity	Relatively easy to collect, less abundant than other species but plants are large	No medicinal use	No

4. Discussion

4.1. Is Namaqualand a potential "bioactivity hot spot"?

Namaqualand is considered one of the 25 top priority "conservation hot spots" globally on the basis of its great endemism and biodiversity (Myers et al., 2000). This biodiversity is thought to stem from a stable local climate during global glacial periods, combined with a series of rapid, adaptive allopatric speciation events (Cowling et al., 2009; Ellis et al., 2006). Thus Namaqualand is home to a unique collection of flora and fauna which may contain novel bioactive compounds of interest. Namaqualand also has a long history of traditional medicinal plant use. It can be assumed that centuries of consistent plant use is indicative of some level of efficacy, and therefore scientifically demonstrable biological effectiveness (Salis-Lagoudakis et al., 2011). This theory was confirmed when 86.3% of species tested for activity in this study showed antioxidant activity, and 73.5% showed antibacterial activity. Most plants were active in two of the six assays performed on extracts. Some (5.88%) were active in four assays and only very few (2.94%) showed no activity in any of the assays. However, this does not mean that they do not possess any biological activity. The few samples inactive in this study may possess a type of biological activity not tested for e.g. anti-inflammatory or antiviral. Nevertheless, 97.1% of tested plants, which consisted of some species with recorded medicinal use and some without, showed activity of one sort or another in this limited battery of tests. Such a high hit rate is sometimes experienced, but usually with sets of extracts which all have traditional medicinal use (i.e. traditional use was used to guide species selection). In this study, some species had traditional medicinal use, while others were selected at random, so we would expect a lower percentage of the randomly selected species to have activity. However, this was not the case. It would appear that almost all species in this study, and possibly in the area generally, may possess activity of varying types and strengths making Namaqualand a "bioactivity hot spot" as well as a centre of endemism and diversity.

The high level of bioactivity in Namaqualand species may be a direct result of the rapid allopatric speciation experienced in the region. Species have adapted to survive Namaqualand's environmental conditions - extreme heat, low rainfall and a high level of UV exposure. Some species have even evolved a different type of photosynthesis - Crassulacean Acid Metabolism (CAM) photosynthesis - in order to thrive in the often harsh environment (Rundel et al., 1999). Adaptations such as CAM introduce new biochemical pathways which produce novel chemical compounds. Such compounds, and those required for protection may confer additional bioactivity not seen in other species in other areas. For example,

flavonols in epidermal cells provide UV protection. (Crozier et al., 2006) and flavonoids (including flavonols) have been linked to antioxidant activity that reduces risk for cancer, diabetes etc, (Clifford & Brown, 2006). Thus species adapted to a hot sunny climate may produce a greater amount of more structurally diverse flavonoids, which could result in a higher level of bioactivity.

In this study, species of *Mesembryanthemaceae* and *Asteraceae* were among some of the most active in antibacterial and antifungal assays (*Ruschia fredericii* with an MIC value of 2.6 mg/ml, *Leipoldtia schultzei* with an MIC value of 5.2 mg/ml, and *Arctotis fastuosa* with an MIC value of 20.8 mg/ml). These families are also some of the most diverse and specious in Namaqualand and species belonging to them are highly adapted to Namaqualand's environment. This suggests that many other plants that have evolved similarly can be expected to have similar activity. Thus Namaqualand species may be worth noting for future screening endeavors.

4.2. Finding potential drug leads from bioactive Namaqualand plants - the way forward?

The Succulent Karoo biome in Namaqualand is home to approximately 3500 species and is one of several unique and speciose South African biomes (Desmet, 2007). Endemism in biomes such as the Succulent Karoo and Fynbos is high and many of the thousands of species in each have never been tested for bioactivity. Testing these plants against multiple screens would be costly and time consuming if conventional laboratory methods of extraction and screening were to be followed. However, the process could be fast tracked if assays were conducted in the field. Portable, field deployable systems, such as the GIBEX set of screens (Josephs, 2008), allow testing of traditional medicinal plants in the field as an initial screen. Only plants with bioactivity of interest are collected and taken back to a laboratory for further testing, reducing time requirements in the field and allowing screening efforts to focus on plants with potential (Prescott et al., 2012). GIBEX assays have been proven to be simple yet accurate indicators of activity. Pilot projects in Botswana and other African countries have proved that they are field deployable with minimal requirements and that local traditional healers are interested to get involved in screening projects and medicinal plant workshops (Andrae-Marobela et al., 2012). A similar portable system for in-field antibacterial testing was successfully employed to test traditional medicinal plants used by the Bulu and Kaulong people of Papua New Guinea (Prescott et al., 2012). With improvements to field kits and better portable equipment researchers can get more reliable and accurate results faster. Field assays used in combination with new and better laboratory assays that are

specific, sensitive and cost effective will improve screening efforts and make screening of more remote locations viable (McChesney et al., 2007).

Other methods of evaluating bioactivity would be useful to identify plants with activity of interest early on. In addition to in-field "pre-screening" other measurable indicators of bioactivity should be considered when selecting plants for extraction and testing. Previous studies have suggested that weedy, herbaceous species are most likely to contain small bioactive molecules (Stepp, 2004; Stepp & Moerman 2001). Herbs and annuals are often the most utilised medicinal species, and perennial trees and shrubs least often (Voeks, 1996). Therefore we would expect herbaceous annual species to contain the most bioactive compounds. However, the results of this study showed that more perennial species are bioactive than annual species. This is a similar result to that found by de Almeida in a study of the medicinal plant of Northeast Brazil, where a greater number and wider variety of active compounds was found in K strategist species (which tend to be woody trees and perennial shrubs) (De Almeida et al., 2005). The results of this study also showed that there was no relationship between growth form and bioactivity, so it cannot be said that a certain type of activity can be found in plants with a certain growth form. Succulence seemed to indicate protease activity and protease inhibition activity, but other than that was also not a useful indicator of bioactivity. This suggests that initial screening efforts be focused on herbaceous and woody perennial species of traditional medicinal plants.

Once a bioactive species is identified, the time consuming process of isolating the active compound and developing a pharmaceutical preparation from this molecule begins. This can take years and is fraught with problems. The lengthy process of isolating the active compound/s from a bioactive medicinal plant may be avoided by simply using the plant as a "botanical". Botanicals may consist of the whole dried powdered plant or the dried powdered crude plant extract. In both cases, the powder may be administered in capsule form or included in an ointment for topical application. Such products are also not subject to the usual process of clinical trials, as they are considered "dietary supplements" rather than pharmaceuticals (Schmidt et al., 2008). Botanicals may also be more readily accepted in communities that are used to using plant medicines. In some areas where a drug has successfully been developed from a medicinal plant, the people who had originally used the medicinal plant did not want to use the drug. For example, a study of a group of rural people in the Amazon forest showed that the majority of them did not like the pharmaceutical and stated that they "wanted to go back to the natural

way" (Wayland, 2004). In such instances a powdered "botanical" may be an acceptable alternative - one that has at least been tested for toxicity.

Botanicals are crude mixtures of phytochemicals in varying concentrations. Bioactivity may not be due to a single compound but to a large number of structurally related and unrelated compounds which contribute to the effect (Heinrich et al., 2005). There is a basic supposition that any plant possessing clinical effectiveness must contain an active principle which can completely replace the whole plant extract (Phillipson, 2001). But this isn't necessarily true. Constituent phytochemicals may work together or with concomitantly administered pharmaceuticals to create beneficial additive or synergistic effects. Additive effects are created when the combined effect is equal to the sum of the effects of the individual components, while synergistic effects are created when the combination of bioactive substances exert effects that are greater than the sum of the effects of the individual components (Schmidt et al., 2008). The idea that compounds work synergistically is not new. In 1936 Szent-Györgyi reported the presence of what he first called ''vitamin P" in citrus fruits and hypothesized that flavonoids and vitamin C worked synergistically to strengthen capillaries (Rusznyák & Szent-Györgyi, 1936). Phytochemicals tend to work synergistically to increase therapeutic effects in one of three ways: 1) by blocking one or more targets of the signal transduction pathway 2) by increasing the bioavailability of another drug or 3) by stabilizing another drug in the system (Hemalswarya & Doble, 2006). For example, quercitin has been found to act synergistically with triazofurin in human ovarian carcinoma cells and can enhance the action of carboxytriazole in human breast carcinoma MDA-MB-435 cells (Shen et al., 1999; Yeh et al., 1995). Natural products have even been shown to to work synergistically with existing antibiotics restoring antibiotic activity against resistant strains of *S. aureus*, *E. coli* and *Shigella* (Sato et al., 2004a; Sato et al., 2004b; Sato et al., 2004c).

Botanicals containing a mixture of compounds may also be beneficial as they are able to affect multiple targets. Studies have shown that drugs that bind to multiple targets may be more effective than those that only bind to a single target. Compounds that bind to multiple targets do so much more weakly than those with a single target, and therefore have a partial effect on the substrate. However, this partial inhibition of multiple targets may still be as effective, if not more effective, than the single compound: single target approach (Csermely et al., 2005). Thus mixtures of compounds may be more effective than a single drug compound as they can affect multiple targets (Schmidt et al., 2007). However, there is the need to characterize the majority, if not all, of the compounds in an extract before this approach can be

used for drug development (Schmidt et al., 2007; Williamson, 2001; Heinrich, 2008). Such analysis may be possible with modern techniques such as metabolomics.

However, synergistic action also causes side-effects and negative interactions with western pharmaceuticals. It is very difficult to determine all the effects of all compounds present in a crude plant mixture and how they will interact with other drug molecules in the human body. Many phytochemicals produce negative side effects such as cardio or hepatotoxicity or they may alter metabolism (Hemalswarya & Doble, 2006). There is also the problem with standard dosing. The concentration of an active compound in a plant extract depends on many factors including environmental and genetic factors affecting the plant, as well as extraction and storage methods used to create the extract. Thus, creating a standard dose is difficult. These problems are avoided when the bioactive compounds are isolated, purified and standardized. Active secondary metabolites isolated from plant material may, with the help of a medicinal chemist, be improved upon to produce a safer, more effective final pharmaceutical product. The process is laborious, and harvesting raw plant material may not be viable for large scale production, but technology such as plant cell suspension and "plant cell factories" may help to speed production of plant compounds of interest. Plant tissue culture for the production of secondary metabolites negates the problems of cultivation or wild harvesting (Moyo et al., 2011). Plant tissues may also be manipulated, through environmental stimuli or genetic alteration, to produce more of the bioactive compounds of interest (Shaik et al., 2011). Liquid suspension of plant cells can also be used to produce antibodies, vaccines, therapeutic enzymes and other proteins (Xu et al., 2011). Activity of compounds produced in these ways seems to be comparable to those of compounds extracted from raw plant material. For example, in a study of the antibacterial plant *Tulbaghia violaceae* (wild garlic) Ncube et al., (2011) found that in vitro and wild extracts were equally effective. This method also has the potential for development of veterinary products (Rybicki et al., 2012). However there are still many problems with scale-up that need to be overcome (Guillon et al., 2006).

Despite the fact that there are benefits and applications for the use of botanicals as well as the increased ease of production of single isolated compounds, drug companies are reluctant to engage in non-targeted bioprospecting because there is a low probability of finding useful compounds (about one in 10 000 plants will show something interesting) (Macilwain, 1998). Therefore, random screening, even in an area of plants with a high level of activity such as Namaqualand, is not preferred. Screening efforts need to be directed in order to maximise hit rate and minimise cost and effort. In many instances it is

more beneficial to isolate and characterise the active compounds from plants used traditionally as medicines, but this process needs to be faster and more reliable than it has been in the past.

Chapter 4: Assessment of Phytochemical Analysis Techniques: "Classic Bioassay-guided Fractionation" vs. "Whole Extract Analysis"

1. Introduction

New chemical compounds are required to address the changing needs of people as humanity evolves and becomes more advanced. These include new and improved medicines, colourants, pesticides, herbicides, preservatives, polymers and plastics as well as compounds for the manufacture of goods, food production, and construction. Chemists and engineers are continually attempting to synthesize and develop new compounds to address these needs and nature is often the starting point for these endeavours. Nature creates a huge diversity of chemical compounds: there are an estimated 200 000 natural compounds currently known and many more remain undiscovered (Dixon & Strack, 2003; Schripsema, 2010). These naturally occurring compounds may themselves have the desired properties, or often provide the inspiration for the development of targeted chemicals. The critical first steps in the process involve discovery and isolation of new compounds from natural sources such as plants, animals and microorganisms.

Of particular interest for the purpose of this study is the isolation and structural elucidation of bioactive molecules, particularly those with medicinal application. Early approaches to his challenge were based on thinking that it was necessary to identify all the compounds present in a botanical extract in order to understand bioactivity (He, 2000). Attempts at complete characterisation of an extract may be possible (although laborious) for commonly used and well researched plants for which standards of the major components can be bought and directly compared with the crude samples (He, 2000). However, this is not so simple for more complex or less studied plants (such as the ones in Namaqualand) where standards are not readily available or the constituent compounds have not previously been characterised. Crude extracts typically contain a wide variety of metabolites e.g. phenylpropanoids, glucosinolates, alkaloids, terpenoids, flavonoids and amino acids. Even the most sensitive and advanced methods of metabolite profiling typically results in long compound lists in which more than half of the detected metabolites are unknown or at best only partially characterised (Van der Hooft et.al., 2012). There is currently no guaranteed, universal approach to the detection and characterising of small molecules in biological extracts, and various

methods are applied in different laboratories most of which are devised on a trial and error basis. This study will use and evaluate three of the major currently used approaches to structural elucidation of small molecules, namely the classic bioassay-guided fractionation approach, and two approaches based on analysis of the whole extract, using either NMR spectroscopy or LC-MS spectrometry techniques. Here follows a short rationale for the choice of these three approaches and a brief outline of the methodology encompassed by each.

1.1 The "classic bioassay-guided approach"

In addressing the challenges of complex mixtures of natural products alluded to above, an approach using bioassay-guided fractionation has been widely adopted, where the objective is the targeted isolation of bioactive compounds (Pieters & Vlietnik, 2005). This still involves many distinct steps including preliminary extraction, drying, dissolving, precipitating, and filtering, followed by multiple chromatographic steps to separate individual components. Along the way, the activity of separated fractions is constantly evaluated using biological assays in order to "weed out" non-active fractions in an effort to eliminate those that are not responsible for observed activity and to finally arrive at the active fractions and/or compounds (Seidel & Taylor, 2004). Some studies are unable to isolate a single active ingredient and draw the conclusion that observed biological activity must be due to synergistic effects between constituent compounds (Engelbertz et al., 2012). Often kilograms of starting material are used up in the process of identifying a single bioactive compound (Kumar et al., 2012). More often than not this is a known compound (Othman et al., 2006; Schwaiger et al., 2011; Küpeli & Yesilada, 2007; Sezik et al. 2005; Freitas et al., 2009), calling into question the value of the considerable effort expended in isolating and identifying it. During the multitude of steps material may be lost and the potential for error increases. With every treatment there is the chance that the original compounds may become chemically altered or degraded. Despite the rash of "failures", the laborious nature of the method and the potential for error, this "classic" method continues to be used today and papers are still being published that detail this approach (Ding et al., 2013 for example). In most instances such literature documents the discovery of known compounds from new sources, and in rare instances new compounds are identified using this method (Ferchichi et al., 2012). Despite this, there *are* new compounds being indentified by this method, thus, while some consider this method outdated, it still has merit and application. The numerous compounds discovered using this approach cannot be discounted and for decades bioassay-guided fractionation, in one form or another, has been the main method employed for compound elucidation. Continued use of this method is driven in part by the significant improvement in separation technologies which allow for more rapid and efficient separations. For these reasons it was included in this study. It should also be noted that more modern approaches, such as metabolomics profiling, rely ultimately

on being able to reference reliably-constituted databases of verified structures, so that the unequivocal identification of a structure of a compound is in fact an indispensable tool.

1.2 Whole extract analysis using LC-MS spectrometry and tandem LC-MS/MS spectrometry

A more recently developed method for determining the constituent compounds in a crude botanical extract makes use of LC-MS spectrometry. In principle, this method requires very little sample material and is able to detect thousands of metabolites relatively quickly and with a high accuracy, although it is rarely possible to determine the full structure of an unknown compound from MS data alone. It is primarily used for the detection, identification and quantitation of small molecules (<1500 Da), many of which have biological activity (Xiao et al., 2012). The method involves efficient HPLC separation of the crude extract, with the higher pressures and control of solvent composition (as compared to the conditions under which bioassay-guided fractionation is performed) significantly reducing run time and optimizing the separations. “Reverse phase” chromatography, using a non-polar solid phase (often C18) and a polar liquid phase, is capable of separating the polar and semi-polar compounds often found in botanical extracts, making this a most efficient method. Effluent from the chromatography column is fed directly into a mass spectrometer, where a variety of ionization methods can be utilized for mass detection. For example, electrospray ionization (ESI) is one commonly used method of ionizing since this approach forms intact molecular ions, allowing initial identification of unknown compounds (Bowen & Northen, 2010; Xiao et al., 2012). When this is performed at high resolution, a molecular formula can be deduced with high certainty from the accurate mass. Untargeted or “global” metabolite detection attempts to identify all compounds present in an extract (Heyman & Meyer, 2012). However, only an average of 10% of detected metabolites obtained in such experiments are known metabolites (Bowen & Northen, 2010). The process of metabolite identification resulting from these untargeted experiments is a significant bottleneck in deriving useful information from such studies (Dunn et al., 2012). Untargeted studies however, do allow for determination of constituent compounds in an extract which are unknown and may be of interest for further study. Subsequent targeted “tandem” LC-MS/MS experiments allow further characterising of these unknowns and acquisition of detailed structural features. Tandem LC-MS/MS provides highly resolved mass spectra for specified ions by carrying out one mass operation after another (He, 2000). Firstly, the specified “parent” or “precursor” ion is isolated. This ion is then subjected to collision-induce dissociation (CID) to form product ions (Xiao et al., 2012) formed by a generally well understood fragmentation process (Dunn et al., 2012). The resulting mass spectra can be compared to those in existing databases, or may be used as the starting point for structural elucidation of the compound if it is unknown (Dunn et al., 2012). This is not a trivial

process and requires considerable expertise and careful handling of extensive data. Despite this, this method is widely used for structural determination of compounds, and can be used to identify bioactive compounds of interest.

1.3 Whole extract analysis using NMR spectroscopy and chemometrics

An alternative and perhaps complementary method for determining the constituent compounds in a crude botanical extract makes use of high field NMR spectroscopy. ^{1}H NMR can in principle be used to detect all organic compounds in a crude extract including carbohydrates, amino acids, organic and fatty acids, lipids, flavonoids, terpenoids and alkaloids (Ali et al., 2011), although the extent to which this can be achieved depends crucially on the field strength and hence resolution of the instrument, and on careful application of a range of 1D and 2D experiments. NMR spectroscopy can however be used to obtain a useful "fingerprint" of the majority of compounds present in a crude extract (Ward et al., 2003). Even simple visual inspection of the spectra can lead to identification of characteristic signals of known compounds, particularly where diagnostic signals are well resolved or found in uncluttered "windows" of the spectrum. For example, where free amino acids may be present in a plant extract, a doublet at δ1.48 (d, J = 7.2 Hz) signals the presence of alanine (the doublet being due to the α-methine proton) while the doublet at δ1.32 (d, J = 6.6 Hz) is characteristic of the corresponding proton in threonine (Kim et al., 2010b). But often in crude biological extracts there are many overlapping signals which make visual assessment difficult. Increasing the field strength of the NMR instrument increases spectral resolution, reducing the number of overlapping signals (Krishnan et al., 2005). 2D NMR experiments, such as heteronuclear single quantum correlation (HSQC) (Xi et al., 2008), correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) (Mannina et al., 2012), total correlation spectroscopy (TOCSY) (Reynolds & Enríquez, 2002) and 2D *J*-resolved experiments (Novoa-Carballal et al., 2011; Liang et al., 2006; Ludwig & Viant, 2010) can also help in teasing apart complex spectra to reveal characteristic signals or spin systems that allow further compound identification not possible from congested 1D spectra alone (Xi et al 2008; Ward et al., 2007).

Another method for dealing with the complexity of crude plant extract NMR spectra is by application of computer-based chemometric methods to reduce the dimensionality of the NMR data for visualisation purposes and to identify patterns between different spectra (Ramadan et al., 2006). Multivariate analysis allows a robust, non-biased, statistics-based interpretation of spectral data. Chemical shift and signal intensities are used to create a data set based on the entire set of variables which can be analysed using multivariate and other computational methods (Defernez & Colquhoun, 2003). Combining ^{1}H 1D NMR spectroscopy with multivariate methods can also be used to identify

characteristic metabolite changes or “markers” that correspond with changes in physiological conditions e.g. growth state, water stress, disease etc. (Ramadan et al., 2006; Lindon et al., 2001).

These methods may be more applicable to studies where the aim is discerning between samples, when performing quality control on herbal medicines for example (Heyman & Meyer, 2012), or discovering biomarkers for disease or growth state. However, NMR-based whole extract analysis methods may also be useful for rapid dereplication and in piecing together the structure of novel compounds. In particular, 2D NMR spectroscopy can link signals from the same spin system, giving insight into structure. Also, NMR spectra tell us what major classes of compounds are present in an extract and their relative quantities. Certain types of compounds are more likely to have certain activity, and this may be useful in discovery of bioactive molecules. For example, tannins and flavonoids often have anti-inflammatory, anti-fungal, antioxidant and healing properties (De Sousa Araújo et al., 2008), and where studies have indicated that these phenolic compounds occur widely in some groups of medicinal plants (such as those in the Caatinga region of Brazil), elevated concentrations in some species that are intensively used by local communities correlate with the therapeutic indications attributed to them (Monteiro et al., 2006). Thus NMR spectra indicating high levels of these types of compounds may be of importance for further study.

1.4 Aims/questions and objectives of this section

The main aim of this section of the study was to use a variety of physical and computational techniques to determine the major metabolites present in selected plant species. A traditional method of chemical structure elucidation - bioassay-guided fractionation - was performed alongside the more recently developed whole extract analysis techniques using NMR spectroscopy, chemometrics and LC-MS spectrometry based metabolomics. This was done in order to determine the major metabolites present in the extracts and to compare the different methods of doing so in terms of ease of use, speed of data acquisition, and cost among other considerations.

2. Methods

2.1 Technical specifications of equipment and reagents used

2.1.1 Reagents and column specifications used for bioassay-guided fractionation

Flash chromatography was performed using a Biotage Isolera One system (Biotage, Uppsala, Sweden). Samples were dry loaded onto a glass column (20mm x 100mm) packed with silica gel (0.063mm particle size, Biotage, Uppsala, Sweden). Open-top chromatography was performed using a glass column (30mm x 550mm) loaded with silica gel (0.2mm particle size, Fluka Analytical)

suspended in, and eluted with, petroleum ether-EtOAc (50:50) containing 5 drops of acetic acid per 10ml mobile phase. Solvents were obtained from Sigma-Aldrich. Those used for flash chromatography and TLC were either of technical grade and redistilled before use (EtOAc, petroleum ether), or of reaction grade and used as they were delivered (MeOH). Solvents used for HPLC were of HPLC grade. Water was deionised water obtained from an in house purification system.

Reagents used in biological testing (ciprofloxacin, nystatin, ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), MTT (3-(4,5-dimethylthiazole-3-yl)-2,5-diphenyltetrazolium bromide), Alamar Blue (Resazurin)) were also obtained from Sigma-Aldrich. Organisms were obtained from the American Type Culture Collection and stored and cultured according to the method outlined in chapter 3.

2.1.2 NMR instrument specifications

A Bruker 400MHz spectrometer was used to acquire all spectra. Spectra were Fourier transformed and processed for phase correction and baseline correction using Bruker's TopSpin™ NMR program (Bruker, Billerica, MA, USA). Deuterated solvents were obtained from Sigma-Aldrich.

2.1.3 LC-MS instrument specifications

High resolution mass spectrometry was performed using a Waters UHPLC coupled with a Waters Synapt G2 mass spectrometer (Waters, Milford, MA, USA) Ionisation was achieved using an electrospray source with a cone voltage of 20 V and a capillary voltage of 3 kV. Continuous internal calibration was achieved using sodium formate, with leucine enkaphalin as the lock mass.

LC-MS and LC-MS/MS spectrometry analyses were conducted using an Agilent 1290 Infinity ultra-performance liquid phase chromatograph (UHPLC) coupled with a diode array detector (DAD) and an Agilent 6530 Accurate Mass Q-TOF mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). An Agilent Poroshell 120 EC-C18 UHPLC column (4.6 x 150mm, 2.7 µm particle size) was used for chromatographic separation. Ionisation was achieved with an electrospray source using a nozzle voltage of 2.0 kV and a capillary voltage of 3.25 kV in the positive mode only. Nitrogen was used as the desolvation gas at 11L/min (660L/H) and the desolvation temperature was set to 340 °C. Accurate mass spectra were acquired in the m/z range 100–1700 at an acquisition rate of 1 spectrum s^{-1}. Continuous internal calibration was performed during analyses with the use of signals at m/z 121.0509 (protonated purine) and m/z 922.0098 (protonated hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazine) as lock reference masses.

2.1.4 TLC specification, reagents and visualising agents

TLC was performed using pre-coated aluminium foil backed TLC plates (0.2mm silica containing a 254nm fluorescent indicator, Fluka Analytical). TLC visureagents: *p*- anisaldehyde (containing equal volumes of anisaldehyde dissolved in 95% EtOH (6% (w/v)) and concentrated H_2SO_4) or sulfuric vanillin (containing vanillin dissolved in 95% EtOH (6.3% (w/v)) to which concentrated H_2SO_4 was added in a ratio of 63.5:1) (Touchstone, 1992; Sherma, 2000).

2.2 Preparation of crude plant extracts

Ten of the 102 extracts previously tested for biological activity during the course of this study were selected for further phytochemical analysis. Large volumes of crude ethanolic plant extract of the selected species were prepared as described in chapter 3. Due to time and resource limitations, five of these ten species were selected for analysis in this section of the study (Table 1). All five were included in whole extract analysis using NMR spectroscopy and LC-MS/MS spectrometry; while only the extract obtained from *C. brevifolia* was subjected to the bioassay-guided fractionation procedure.

2.3 Bioassay-guided fractionation

2.3.1 Flash chromatography

Medium-pressure "flash" column chromatography was performed using 100mg of lyophilised *C. brevifolia* extract adsorbed onto silica and dry loaded for chromatography. Compounds were eluted using an initial mobile phase of 100% EtOAc and a gradient of 0-35% MeOH at a flow rate of 12ml/min. The eluent was monitored by a UV detector at 245nm and 280nm and fractions collected corresponding to UV-active components. Fractions were initially analysed using TLC plates and a mobile phase of 100% EtOAc, with developed plates visualized either under UV (at wavelengths of 254nm and 360nm) or by dipping plates in a reagent solution followed by heating. Chromatographically similar fractions were pooled together into three subfractions and evaporated to dryness under vacuum. Subfraction 1 (designated CB1) appeared to consist of a single compound as visualised by TLC (using a mobile phase of 100% EtOAc). CB1 was resuspended in EtOH at a concentration of 10mg/ml and subjected to biological testing using the antibacterial MIC method outlined in chapter 3. CB1 was also subjected to additional spectroscopic analysis. ^{1}H and ^{13}C NMR spectra were obtained for this sample, as were 2D HSQC, HMBC and COSY spectra. High resolution mass spectrometry data was also acquired in both positive and negative ionisation modes. CB1 eluted from the chromatography column as a single compound using a mobile phase of 50% acetonitrile containing 0.1% formic acid at a flow rate of 0.2ml/min.

Table 1: Species analysed in this section of work

Species	Common name and uses	Uses	Activity
Crassula muscosa* L. var. *muscosa	Hoendervoet (Afr.), slangbos (Afr.), ketting (Afr.), fever bush (Eng.)	Treatment of dysentery and children's illnesses e.g. stomach problems, fevers and colds	Strong antibacterial, protease inhibition
***Cotyledon cuneata* Thunb.**	No common name	Treatment of fever blisters and chapped lips	antibacterial, antifungal, antioxidant, protease inhibition
Crassula brevifolia* Harv. subsp. *brevifolia	Kleinblaarplakkie (Afr.)	No common use	antibacterial, antioxidant, protease inhibition, helminth lethality
***Polymita albiflora* L. Bolus**	Muisoor (Afr.), mouse ear (Eng.)	Treating stomach pain	Antibacterial, antioxidant, protease inhibition
***Zygophyllum foetidum* Schrad. & J.C. Wendl.**	Skilpadbos (Afr.), jakkalspisbos (Afr.), slymbos (Afr.)	Used as a soap, believed to be poisonous	Antibacterial, strong antifungal

Subfractions 2 (designated CB2) and 3 (designated CB3) were of insufficient yield to allow biological testing and further separation (of the type used in this study) and were set aside.

2.3.2 Alternative approach using liquid-liquid extraction followed by open-top column chromatography and bioautography

Prior to column chromatography an initial liquid-liquid separation was performed to separate constituents on the basis of polarity. Lyophilised plant extract (10g) was fully dissolved in water at a concentration of 100mg/ml and subjected to liquid-liquid separation with three equal volume portions of petroleum ether. The collected petroleum fractions were evaporated to dryness under vacuum and the remaining water portion was successively re-extracted with 11 equal volumes of EtOAc. The resulting combined EtOAc fractions were also evaporated to dryness under vacuum and the remaining water portion was frozen at -20°C for further study.

The three subfractions (CBP = petroleum ether fraction, CBE = EtOAc fraction, CBW = water fraction) were subjected to biological testing using the antibacterial MIC, ABTS antioxidant, protease activity and protease inhibition assays outlined in chapter 3. The biologically active fractions were subjected to chromatographic separation using gravity-driven open-top column chromatography.

Chromatography was performed using a mobile phase of petroleum ether-EtOAc (50:50) containing 5 drops of acetic acid per 10ml. Some highly polar compounds remained adsorbed onto the silica

column after this, and were eluted using water as a final column purge. Fractions were analysed by TLC with a mobile phase of 100% EtOAc and chromatographically similar fractions were combined into five sub-fractions (CBE1-CBE5) and evaporated to dryness under vacuum. Fraction 6 (CBE6) consisted of the water-purged fraction. All subfractions were subjected to biological testing in the antibacterial MIC, ABTS antioxidant, protease activity and protease inhibition assays outlined in chapter 3.

Biologically active fractions, with the exception of CBE6, were subjected to direct bioautography (Marston, 2011) rather than further column chromatography and subsequent biological assays. This was done with a view to isolation of active compounds via preparative TLC plates. TLC plates were developed for each of the fractions using 5µl sample spots per plate and a mobile phase of petroleum ether-EtOAc (50:50). In order to test the resulting bands for antibacterial activity, the plates were air-dried and then dabbed with a bacterial suspension containing a 1:200 dilution of 24 hour old *E. coli* culture, prepared as for MIC antibacterial assay use in chapter 3. Plates were sealed in a sterile container and incubated at 37°C for 24 hours. A spot of ciprofloxacin was placed on each TLC plate prior to incubation as a positive control. After the incubation period, plates were air dried and a solution of Alamar Blue (prepared as described in chapter 3) was used as an indicator of growth. Initial tests were inconclusive and bioautography was repeated with 10µl and 15µl sample spots, but still yielded unclear results. Further attempts at isolation of the active components were unsuccessful. A summary of the procedure followed is given in Figure 1.

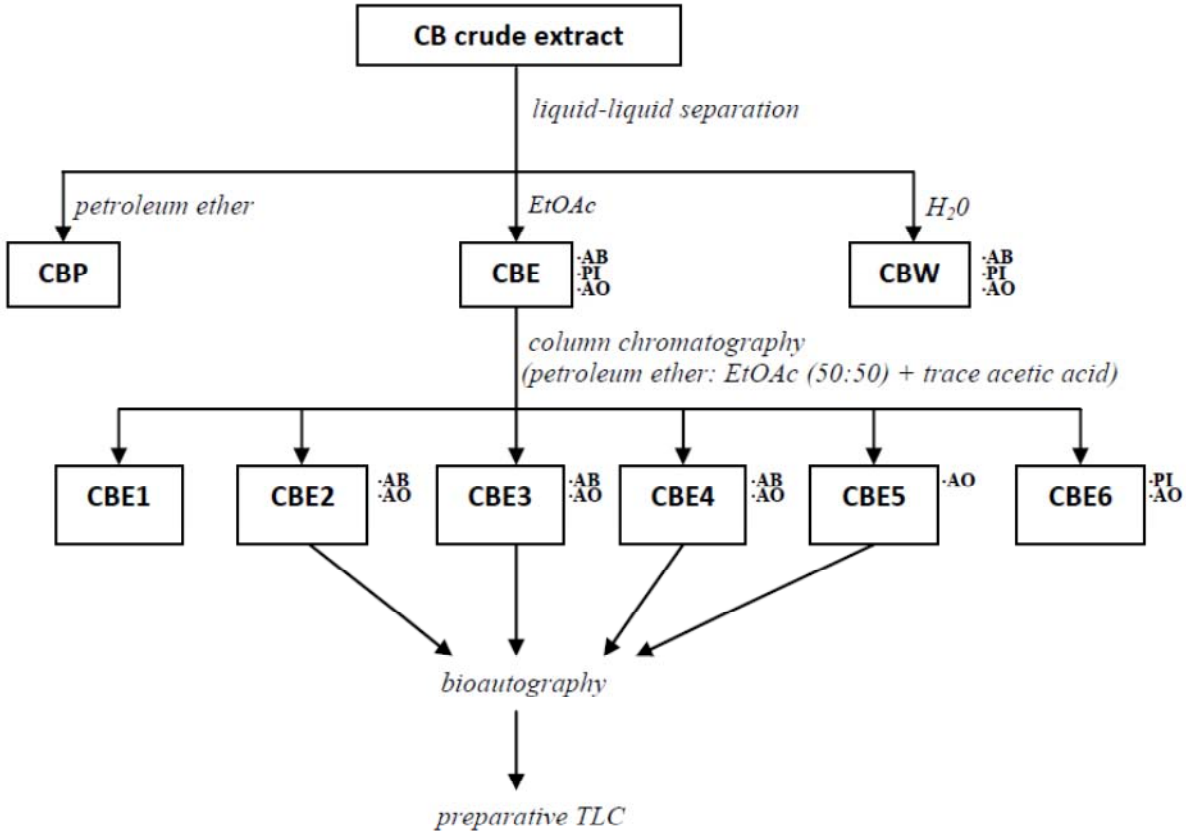


Figure 1: Process followed during bioguided fractionation. AB = antibacterial activity, AO = antioxidant activity, PI = protease inhibition activity.

2.4 Whole extract based LC-MS metabolomics

2.4.1 LC-MS data acquisition and processing

LC-MS and LC-MS/MS analyses were conducted using an injection volume of 4µl of a 10mg/ml test solution for each analysis. A modified version of the protocol suggested by Gómez-Romero (Gómez-Romero et al., 2010) for the chromatographic separation of phenolic containing tomato extracts was followed. The mobile phase, consisting of 97% deionised water with 3% Acetic acid (A) and 90% Acetonitrile and 10% deionised water (B) was pumped at 0.3ml/min into the system with the following gradient program: 0-2 min, 0% B; 2-4.5min, 0-4.5% B; 4.5-9.5min, 4.5-6% B; 9.5-38min, 6-60% B, 38-42min, 60-70% B; 42-44min, 70%B. Subsequently the percentage of B was reduced to the initial run conditions and the column was equilibrated (total run time of 46min).

Absorption spectra of the effluent were acquired at wavelengths of 210, 230, 250, 270, 280 and 350 nm. Data from these runs was subject to analysis using the open-source program MZmine (http://mzmine.sourceforge.net, Katajamaa et al., 2006; Pluskal et al., 2010) following the workflow outline in Figure 2. In brief, Agilent .d files were converted to .mzdata.XML format using Agilent's

MassHunter Workstation software version B.04.00 (Agilent Technologies Inc, 2011). Converted files were imported into MZmine and baseline correction (using algorithms from the statistical program R (The R Foundation for Statistical Computing) was performed in order to facilitate peak detection ("*baseline correction"*). MZmine creates a list of masses from the raw data, which are then used to build chromatograms for each mass that can be detected continuously over the scans ("*mass detection"* and "*chromatogram building"*). Subsequent to this, the built chromatograms are deconvoluted into individual "features" or "peaks" ("*chromatogram deconvolution"*). Once individual peaks have been detected, isotopic peaks corresponding to the same compound are clustered together and the data is filtered to remove duplicates ("*isotope detection"* and "*filtering"*) (Castillo et al., 2011). This results in a list of detected peaks with a single retention time, abundance (given as peak area or height), monoisotopic mass and isotopic distribution for each (Sana et al., 2008). This peak list can be searched for adducts and peak complexes ("*adduct search"* and "*peak complex search"*). In addition, the molecular formula can be predicted for each detected peak using an algorithm that calculates all possible molecular formulae for a given peak within certain user-defined constraints (*"formula prediction"*). Comparison of isotopic abundance of the detected peak's isotopes with predicted isotopic pattern is used as a filter to rate the probability of each formula when several possible formulae are returned (Kind & Fiehn, 2006; Castillo et al., 2011). If parameters are carefully selected following the "Seven Golden Rules" of Kind and Fiehn (Kind & Fiehn, 2007) the correct molecular formula can be assigned up to 98% of the time if the compound in question is known, and up to 65% of the time for novel compounds.

The elements used in the algorithm generating the formula are restricted in order to narrow down the list of suggested formulae per peak and increase the chance of identifying the correct formula. In this study, elements used to generate formulae were restricted to C, H, O, N, S and P, which are the most likely elements to be found in plant secondary metabolites. In addition, an isotopic pattern filter (set to 5ppm with a minimum match score of 75%), a H/C element ratio check, a heteroatom (NOPS/C) ratio check and a multiple element check (which constrains the number of elements in a formula e.g. $C_{26}H_{28}N_{17}OP_3S_8$ is unlikely) were also applied to eliminate unlikely formulae (Kind & Fiehn, 2007). Molecular formulae, RDBE, isotopic peak pattern and retention time can all be used in conjunction with monoisotopic mass to identify ions by comparison with online databases. However, the neutral mass of each ion is calculated from its m/z value and this is used as the primary term for online database searches (Pluskal et al., 2010). For this study, the online databases KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg), PLANTCYC (www.plantcyc.org), LIPID MAPS (Fahy et al., 2007, www.lipdmaps.org) and the METLIN database were searched in order to tentatively identify detected ions. Such identifications based solely on predicted formula and

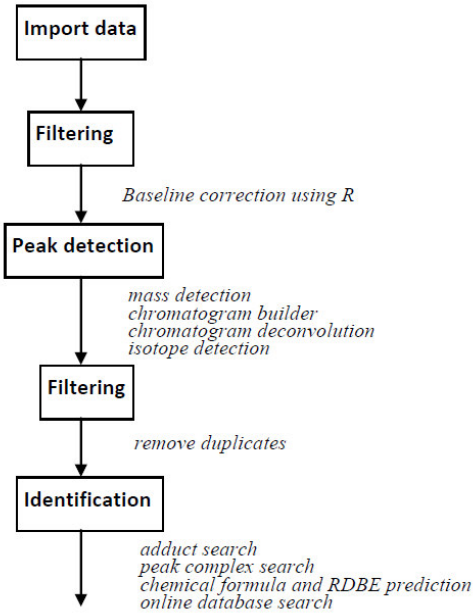


Figure 2: Workflow for identification of precursor ions for LC-MS/MS analysis

monoisotopic mass are of limited use. *A priori* knowledge of biological systems present in the plant may be used to determine if these predictions are likely, but more robust tandem LC-MS/MS or LC-MS-NMR experiments combined with spectral matching to mass spectrum or NMR databases is needed to confirm tentative identifications (Xiao et al., 2012).

2.4.2 LC-MS/MS data acquisition and processing

Lists of detected ions, their m/z values and predicted formulae resulting from analysis of LC-MS spectrometry data gathered during untargeted LC-MS experiments (described in section 2.4.1) were used to specify precursor ions for a subsequent tandem LC-MS/MS experiment (Xiao et al., 2012). For four of the extracts (PA, CC, CM and ZF) only the top ten most abundant ions were selected as precursor ions. The fifth extract (CB) was investigated in more depth and the top 100 ions present were selected as precursor ions. MS/MS spectra of selected precursor ions were acquired using the same column and machine conditions as for the initial untargeted LC-MS data acquisition runs as well as the same mobile phase and elution gradient. To reduce running time and associated costs, a mixture of the five plant extracts, consisting of equal parts of each extract, was created and 4µl of this mixture was injected for analysis. Since compounds may preferentially fragment at different collision induced dissociation (CID) energies, spectra were acquired at six different CID energies: 5eV, 10eV, 15eV, 20eV, 35eV and 45eV. It has been shown that at high CID energies only between 10 and 30% of precursor ions undergo dissociation, resulting in low product ion recovery and few useful spectra. Lower CID energies facilitate better dissociation of precursor ions, particularly for low molecular weight compounds, hence the choice of these six CID energies (Werner et al., 2008).

Spectra acquired from the targeted LC-MS/MS run were analysed using MassBank (Horai et al., 2010; www.massbank.jp). Comparing fragmentation patterns for each of the precursor ions against spectra present in the MassBank database allowed tentative identification of some of the compounds. Confidence in these tentative identifications was based on a percentage match score of how well the experimental MS/MS spectra matched those of database spectra. The percentage match score was generated by comparing fragmentation patterns of the submitted spectrum and the proposed database entry "match". Experimentally obtained daughter ions are only considered to be "matched" to those present in the database fragmentation pattern if their m/z values are within 5ppm of each other. The number of matched daughter ions and their intensities are considered in order to generate the percentage match score. Those compounds not matched to any of the database entries with any degree of certainty were examined for substructure matches against the MassBank database. This allows characteristic structural elements to be matched to the database even if the entire compound has no database match. In some instances this allows suggestions to me made about the likely class of compound e.g. a quercetin glycoside not in the database will have no overall match, but substructure prediction will indicate the likely presence of quercetin. Subtracting the formula of quercetin from that of the most likely predicted formula given will give the formula for the glycoside. Piecing this together allows at least some information to be gathered for unidentified compounds. The remaining ions were unidentified.

2.5 Whole extract NMR spectroscopy -based fingerprinting

NMR spectra were recorded for all five of the crude plant extracts in an attempt to detect and characterise differences in the metabolite composition and to identify common metabolites based on characteristic NMR signals.

2.5.1 Spectral acquisition

A sample (~20mg) of each lyophilised crude plant extract was dissolved in a small volume of methanol-d4, which was filtered and transferred to a 5mm-o.d. NMR tube. Proton spectra were acquired for 64 scans using a standard 1D ^{1}H pulse sequence with water suppression.

2.5.2 Data pre-processing and statistical analysis

Spectra were imported into MestReNova version 6.1.0 (MestReLab Research, S.L.) for further processing. Initially, spectra were aligned and referenced to the ethanol triplet at δ 1.04. Spectral intensities were reduced to a smaller number of integrated regions ("bins" or "buckets") of equal width (0.01ppm) within the regions δ 0.0 and 9.5. This reduced the number of variables available for multivariate statistical analysis to a more manageable 950. Data were normalised to the total area so as to remove variation due to sample concentration (Craig et al., 2006) and exported as a comma-

separated value (.csv) file. The regions between δ1.1 - 1.2, and δ3.6 - 3.7, corresponding to residual ethanol signals, were removed prior to multivariate analysis. Bins with zero values across all samples i.e. areas of the spectrum for which no extract displayed a signal were also excluded from analysis as they did not provide any information useful for multivariate analysis. Principal Components Analysis (PCA) was performed on the standardised data set (after mean-centering and scaling) using the open-source statistical package R version 2.15.1 (The R Foundation for Statistical Computing). PCA scores plots were generated using R and corresponding PC loadings plots were visualised as bar charts using Excel (Microsoft® Office Excel® 2007).

2.5.3 2D NMR spectroscopy

Samples (~50mg each) of lyophilised crude extract of *C. brevifolia* and *C. cuneata* were dissolved in a minimal volume of methanol-d4, filtered, and transferred to a 5mm-o.d. NMR tube. A proton spectrum was acquired for each sample using a standard 1D ^{1}H pulse sequence with water suppression. 2D HSQC, TOCSY and HSQC-TOCSY spectra were subsequently acquired for the each of the whole crude extracts. Acquisition of the TOSCY spectrum was optimised with a 90° pulse sequence and mix time of 120s (Reynolds & Enriquez, 2002). Continuous autoshimming was performed throughout the run.

3. Results and Discussion

3.1 Preparation of plant extracts

Yield of lyophilised crude ethanolic extract varied, with *P. albiflora* having the highest yield (11.13%) and *C. muscosa* having the lowest yield (0.71%). Yields were low overall (Table 2), which is most likely due to a high water content and the succulent nature of the plant species under study.

Table 2: % yield of lyophilised crude plant extract for each species

Species	Mass of raw plant material	Mass of dried ethanolic extract	% Yield	Nature of extract
C. brevifolia	2985.1g	158.7282g	5.31	Red-orange powder
C. muscosa	665.2g	4.7680g	0.71	Dark red powder
P. albiflora	1500.0g	114.1848g	11.13	Red-orange shiny powder
Z. foetidum	2021.3g	24.0002g	1.19	Dark green powder
C. cuneata	1859.9g	44.1861g	2.38	Pale yellow, sticky powder

3.2 Bioassay-guided fractionation of *C. brevifolia* crude extract

Bioassay-guided fractionation was performed in an attempt to isolate potentially novel biologically active compounds from crude plant extracts using column chromatography. Both flash (section 3.2.1) (Weber et al., 2011) and open-top column chromatography (section 3.2.2) were performed, but due to the difficulties of separating complex mixtures, only a single, known biologically active compound could be isolated from the extract of *C. brevifolia* as described in detail below.

3.2.1 Flash chromatography of *C. brevifolia*

Flash chromatography of the crude ethanolic extract of *C. brevifolia* (100g) afforded three subfractions: CB1 (mass 27.7mg), CB2 (mass 63.1mg) and CB3 (mass 5.7mg). CB1 appeared to consist of a single compound, as demonstrated by TLC, while CB2 and CB3 appeared to be complex mixtures of polar compounds. CB1 exhibited antibacterial activity (MIC = 2.5mg/ml) and was subjected to ^{1}H and ^{13}C 1D NMR, HSQC, HMBC and COSY 2D NMR spectroscopy as well as high resolution mass spectrometry. Based on these results it was determined that CB1 consisted of a single compound, isolated as yellow crystals, with [M+H] *m/z* = 291.085 and mp = 151°C. A full discussion of structural determination and NMR spectral assignment for CB1 follows.

3.2.1.1 Discussion of NMR spectral assignment for the unknown compound "CB1"

High resolution mass spectrometry indicated that CB1 has [M+H] = 291.0858 and a proposed molecular formula of $C_{15}H_{14}O_6$ (Figure 3). The ^{1}H NMR spectrum of CB1 gives nine distinct signals, each integrating for a single proton (Figure 4). This only account for nine of the 14 protons in the proposed molecular formula, leaving five unaccounted for by the ^{1}H spectrum. These may be exchangeable hydroxyl group protons, whose existence would be consistent with the solubility of the compound in methanol. The presence of five hydroxyl groups would account for five of the oxygen atoms in the proposed formula, with the sixth likely to be present as a carbonyl group, or as part of a cyclic or acyclic ether group. The presence of methyl ether is ruled out by the absence of a characteristic three-proton singlet at about δ 3.1ppm.

Examination of the proton spectrum in more detail revealed that there are two aliphatic proton signals at δ2.51 (*dd*, *J*=16.1, 8.1 Hz) and δ2.85 (*dd*, *J*=16.1, 5.4 Hz) with the chemical shifts indicating they are not vicinal to oxygen atoms. Slightly further downfield are two signals at δ3.98 (*td*, *J*=7.8, 5.4 Hz) and δ4.57 (*d*, *J*=7.5 Hz) which originate from protons on carbons bearing oxygen atoms or other electron-withdrawing group such as an unsaturated carbon. The spectrum also shows two mutually coupled doublets at δ5.86 and δ5.93, suggesting vinylic or aromatic protons, while a similar set of coupled signals at δ6.72 (*dd*, *J*=8.1, 1.9 Hz), δ6.76 (*d*, *J*=8.1 Hz) and δ6.84 (*d*, *J*=1.9 Hz) suggest a tri-substituted aromatic ring.

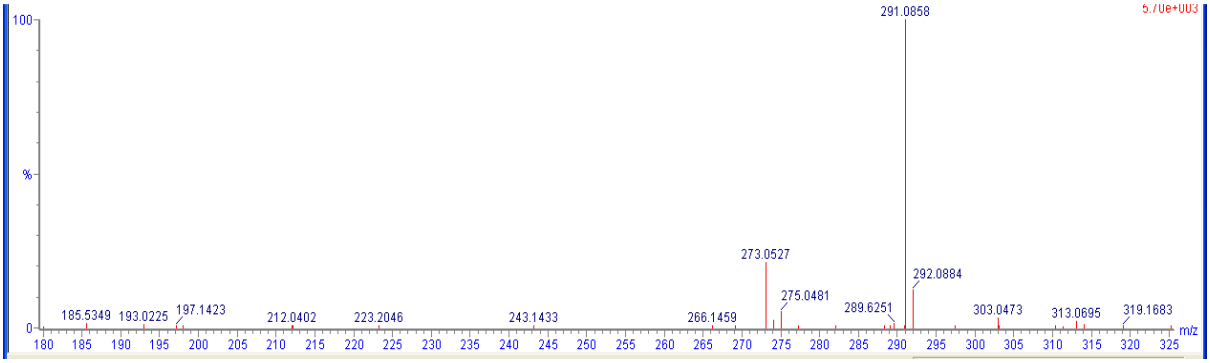


Figure 3: Mass spectrum (in positive ion mode) of CB1 with MS [M+H] *m/z* = 291.0858

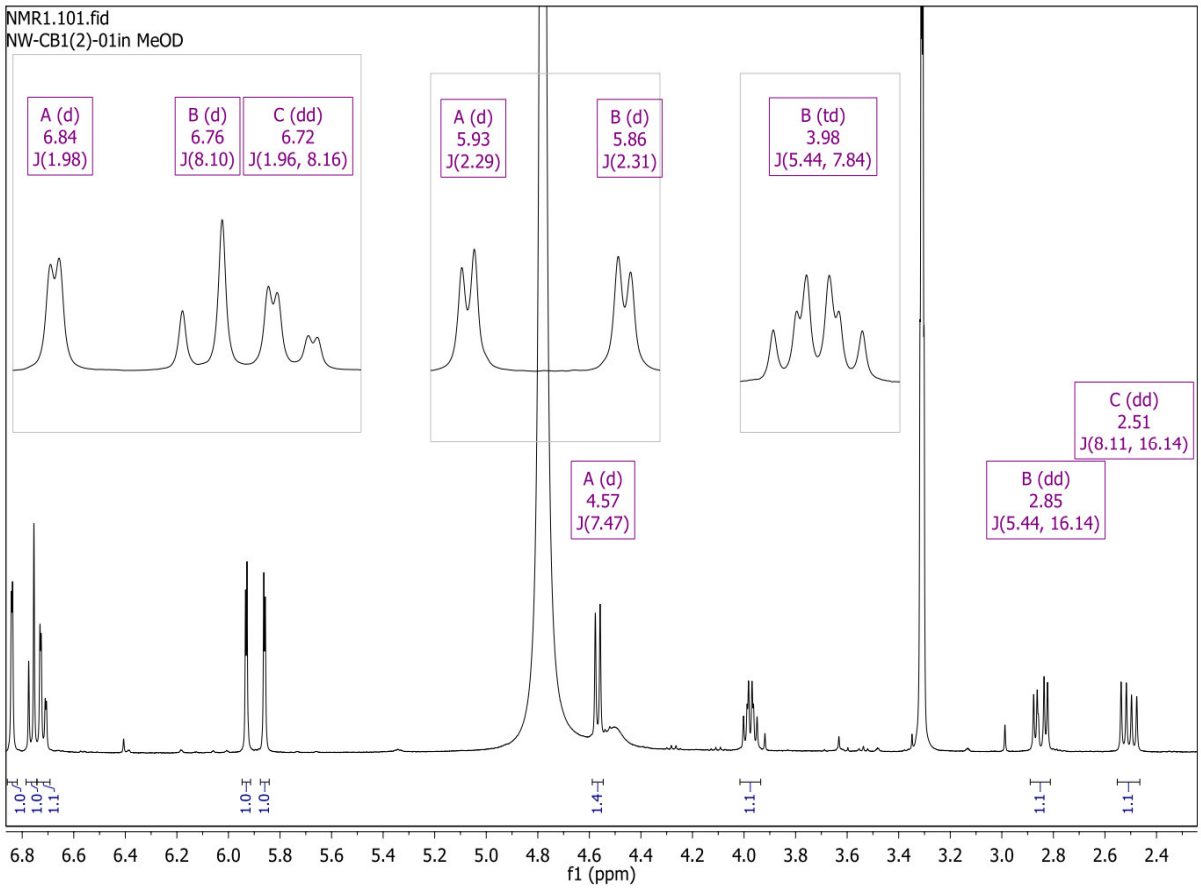


Figure 4: ^{1}H NMR spectrum of CB1. Note that the peaks appearing at δ3.29 and δ4.75 are signals from residual solvents (ethanol and water respectively).

In order to investigate the relationship between these protons, and confirm coupled protons linked in spin systems, a COSY experiment was performed. This confirmed (Figure 5) that the protons resonating at δ2.51, δ2.85 and δ3.98 are mutually coupled, and the latter is coupled to the proton resonating at δ4.57, in a spin system isolated from the rest of the downfield protons ("spin system 1"). This spectrum also showed that the protons resonating at δ5.86 and δ5.93 are coupled in a

second isolated spin system (“spin system 2”) that must contain unsaturated carbons, while the aromatic protons resonating at δ6.72, δ6.76 and δ6.84 form a third isolated spin system (“spin system 3”). There are thus three separate spin systems - 1) four aliphatic protons attached to saturated carbons (with two near to an oxygen and two lacking electron-withdrawing substituents), 2) two protons on unsaturated carbons (possibly an aromatic group) and 3) three protons on a tri-substituted phenyl ring.

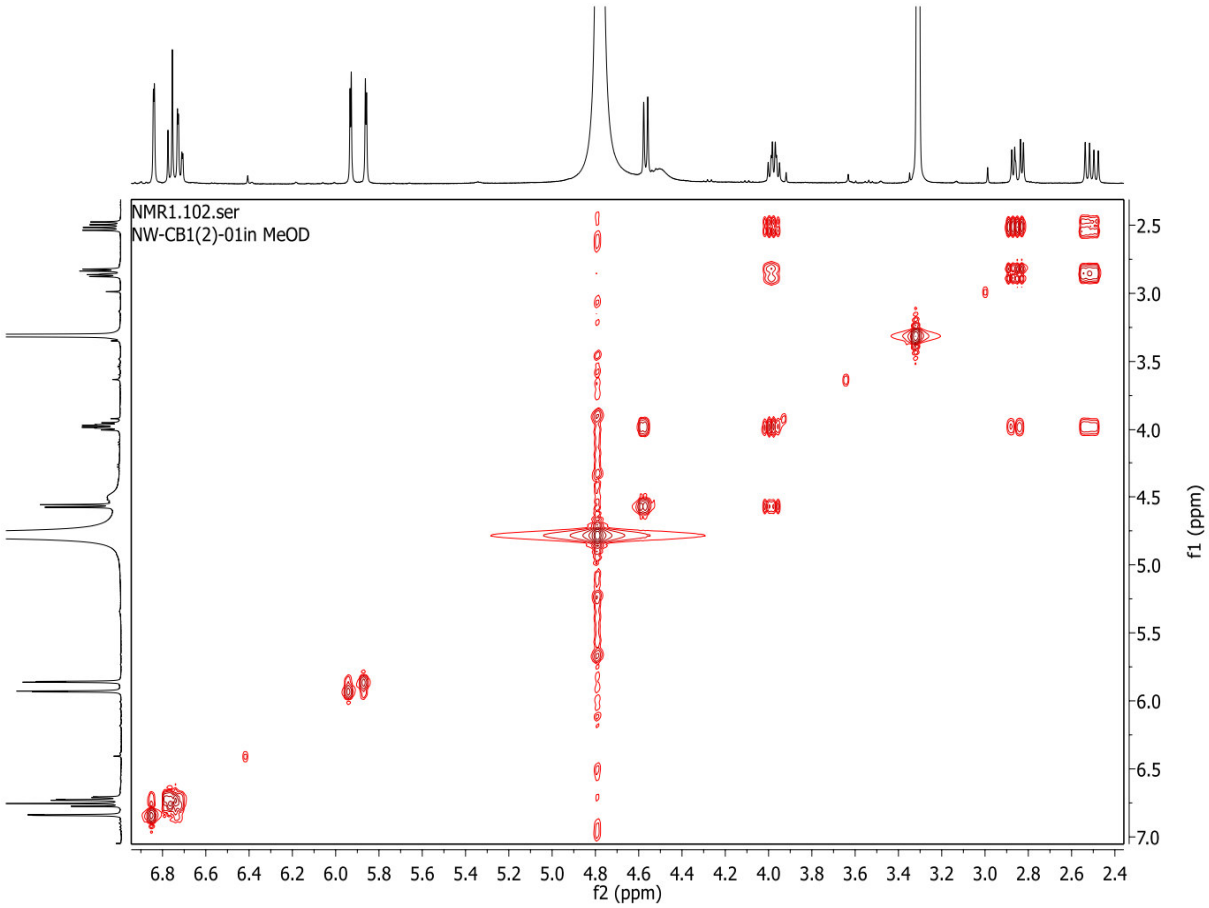


Figure 5: COSY spectrum of CB1

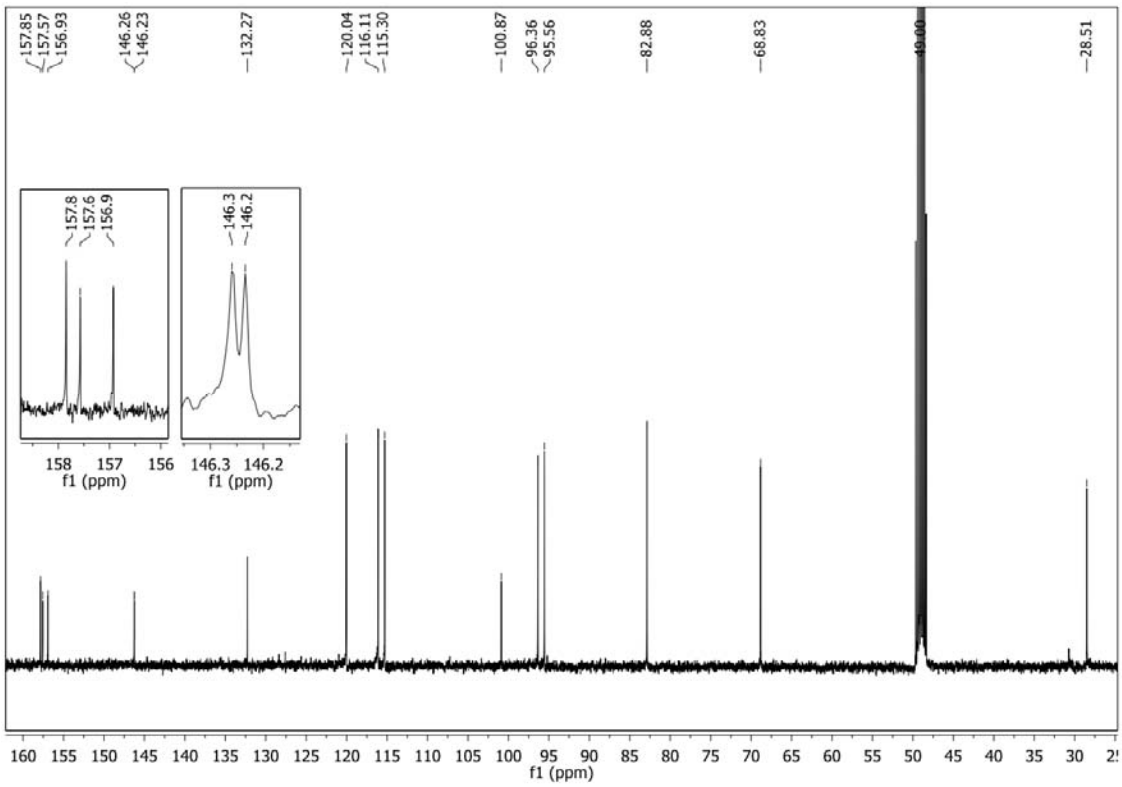


Figure 6: ^{13}C spectrum of CB1

The ^{13}C spectrum shows 15 distinct carbon signals, which is consistent with the formula suggested by high resolution mass spectrometry. It is also possible to deduce the presence of seven quaternary carbons at δ100.9, δ132.3, δ146.23, δ146.26, δ156.92, δ157.57 and δ157.85 (Figure 6), as indicated by their low intensities relative to the other signals in the spectrum, with these identities later confirmed by the absence of C-H correlations for these carbons in the HSQC spectrum.

From a preliminary inspection of the relative chemical shifts some information can be gleaned on the environments that the carbon atoms are located in. Only one upfield carbon at δ28.5 appears to not be vicinal to an electron-withdrawing group. Those with signals at δ68.8, δ82.9, δ95.6 and δ96.4 are likely to be saturated carbons deshielded by adjacent oxygen atoms. The signals at δ100.9, δ115.3, δ116.1, δ120.0, δ132.3, δ146.23 and δ146.26 are characteristic of unsaturated carbons, or those substituted by more than one electron-withdrawing group, and four of these are quaternary carbons. The three signals located at δ156.92, δ157.57 and δ157.85 suggest unsaturated carbons next to oxygen atoms.

In order to fully assign the carbon and proton spectra, an HSQC experiment was performed to reveal one-bond C–H connectivities (Figure 7). This shows that there is only one carbon that is connected to two protons - the saturated carbon at δ28.5 is connected to the protons resonating at δ2.51 and

δ2.85. This can be confirmed by the coupling constants for these protons indicated by the ^{1}H spectrum - a *J* value of 16.1 Hz for both indicates geminal coupling. The remaining carbon atoms are either not connected to protons or are connected to single protons. Originally there was the possibility of CB1 containing a carbonyl group. This possibility can now be eliminated as the three signals in the carbonyl region of the ^{13}C spectrum (δ156.92, δ157.57 and δ157.85) are all shown to connect to protons by the HSQC spectrum. This alone does not eliminate the possibility of a carbonyl group in the structure of CB1 as there may be an aldehyde group present. However, reviewing the proton spectrum it is evident that there are no signals in the δ9.0 - δ10.0 region, which would be characteristic of a proton in an aldehyde functional group.

While HSQC reveals one-bond connectivity between carbon and proton atoms, HMBC reveals longer range two- and three-bond connectivity. This indicates relationships among neighbouring atoms and allows isolated spin systems to be tentatively linked, and importantly the connectivity of quaternary carbons to be determined. From the HMBC spectrum (Figure 8) it is evident that the carbon at δ100.9 is connected to protons in both spin systems one (protons at δ2.51 and δ2.85) and two (the protons at δ5.86 and δ5.93) suggesting that these two systems are linked, possibly via this quaternary carbon. The quaternary carbons at δ156.92, δ157.57 and δ157.85 are also neighbours to

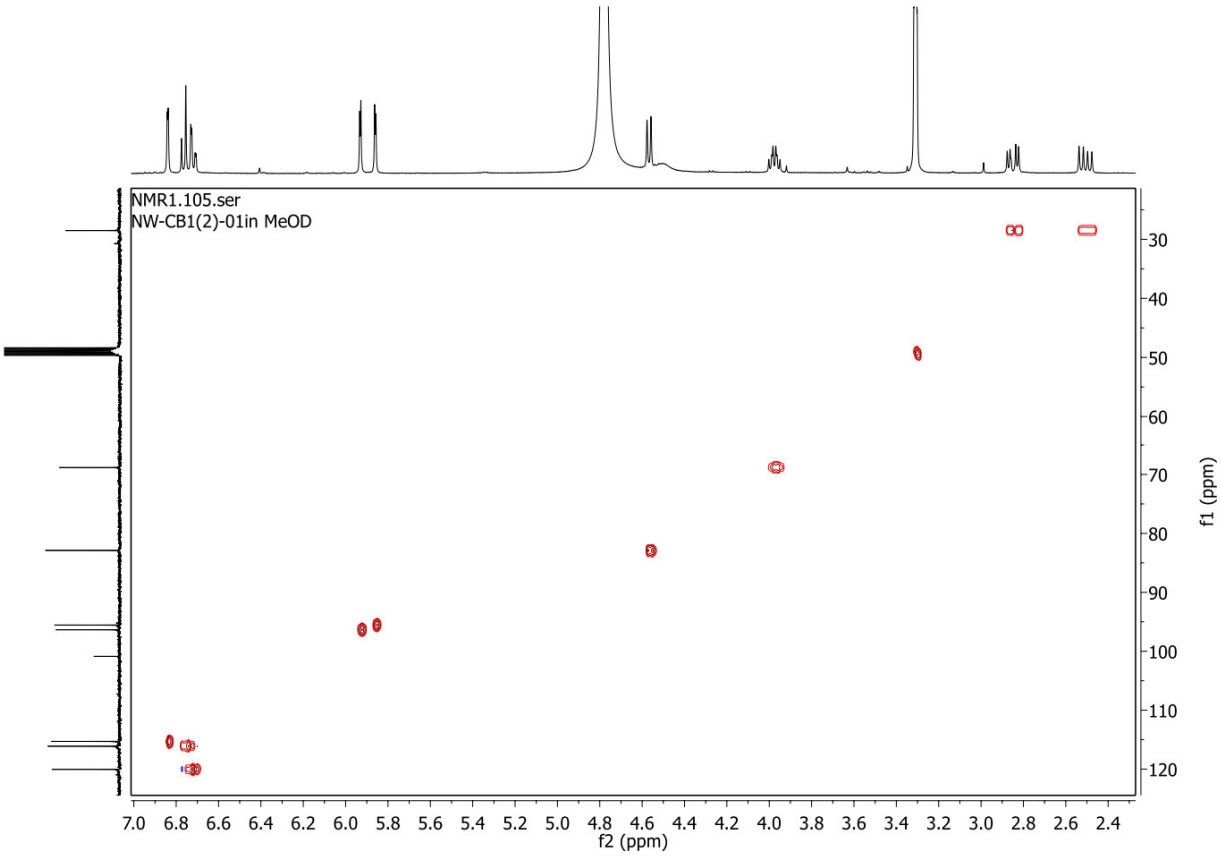


Figure 7: HSQC spectrum of CB1

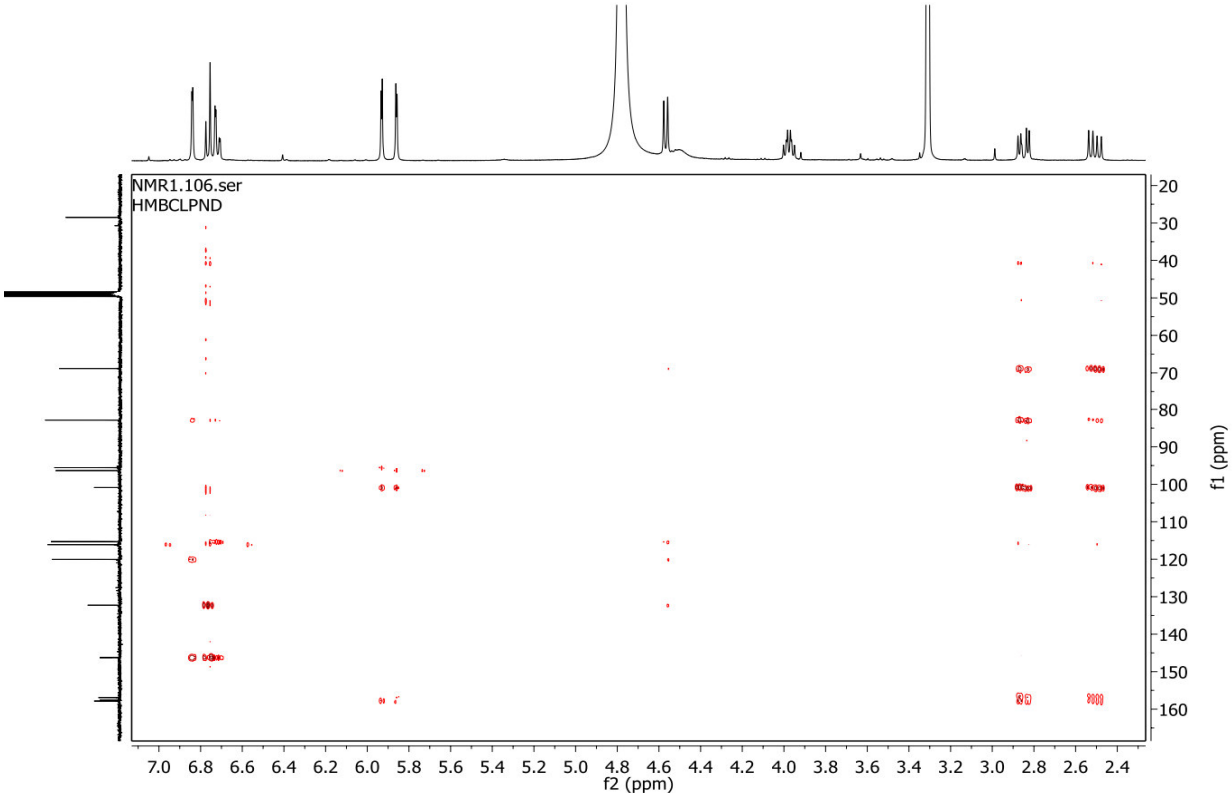


Figure 8: HMBC spectrum of CB1

protons in both spin systems one (protons at δ2.51 and δ2.85) and two (the protons at δ5.86 and δ5.93), further confirming that these two systems are linked.

Similarly the carbon at δ132.3 is connected to protons in both spin systems one (proton at δ4.57) and three (protons at δ6.72, δ6.76 and δ6.84) suggesting that these systems are linked, possibly via this quaternary carbon. Additionally, the carbon at δ82.9 (a saturated carbon next to an oxygen and linked to a single proton) neighbours protons from both spin systems one (protons at δ2.51 and δ2.85) and three (δ6.84) further confirming that these systems are linked. No carbons from spin systems two and three are similarly coupled suggesting that these two are not neighbouring each other, but rather have spin system one between them. The quaternary carbons δ146.23 and δ146.26 are only connected to protons from the aromatic system (those at δ6.72, δ6.76 and δ6.84) suggesting that they are part of the proposed substituted phenyl ring.

A summary of structural information deduced from the experiments described above is as follows:

- the compound is a methanol soluble compound of plant origins
- [M+H] = 291.0858
- proposed formula: $C_{15}H_{14}O_6$ (in seven methine and one methylene group, and five OH groups)
- no carbonyl group present

- possible presence of an oxygen heterocycle (cyclic ether)
- three isolated spin systems
    1) four aliphatic protons attached to saturated carbons (two on carbons near to an oxygen and two geminal protons attached to a saturated carbon not near an oxygen)
    2) two protons attached to unsaturated carbons
    3) three single aromatic protons attached to unsaturated carbons
- spin systems 1 and 3 linked
- spin systems 1 and 2 linked
- the presence of a substituted tri-substituted phenyl ring

This data suggested the structure was likely to be a flavonoid (Figure 9). There are various types of major flavonoid classes, four of which have a ketone group attached at position 4, one with a

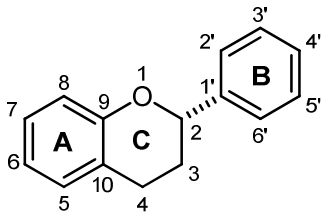


Figure 9: The basic skeleton of a typical flavonoid. Substituents, most often hydroxyl groups, are commonly attached at the 3', 4' and 5' position of the B ring, as well as at the 3, 5 and 7 positions. Sugar moieties may form glycosidic linkages through the substituted hydroxyl groups, most commonly those at positions 3, 5 and 7.

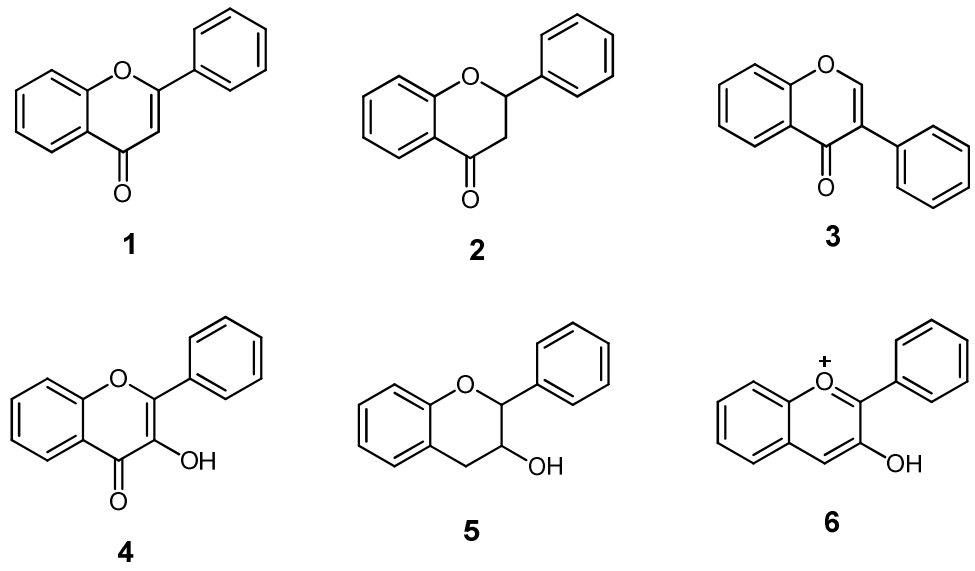


Figure 10: Major categories of flavonoid: 1 = flavone, 2 = flavanone, 3 = isoflavone, 4 = flavonol, 5 = flavan-3-ol, 6 = anthocyanidin (Crozier et al., 2006). The unknown flavonoid CB1 belongs to the flav-3-ol class.

methylene group at that position instead of the ketone group and one with a methine at that position (Figure 10) (Crozier et al., 2006). As there is no evidence of a carbonyl group in CB1 and definite evidence of two geminal protons attached to a single saturated carbon the possible type of flavonoid can be narrowed down to a single class - flavan-3-ols (Kyle & Duthie, 2006). As there are five hydroxyl substituents two of which must be attached to the phenyl ring (as evidenced by the presence of three aromatic protons) and one which must be attached at position 3, the remaining two must be attached to the A ring. This narrows the possibilities of flavonoid and revisiting the spectra suggests that they must be attached at positions 5 and 7. This substitution pattern is characteristic of a catechin type compound and comparison with literature data for common flavonoids of this type revealed that CB1 is indeed catechin (Table 4,Table 5, Figure 11) (Mendoza-Wilson & Glossman-Mitnik, 2006; Lin et al., 2009; Suresh et al., 2012).

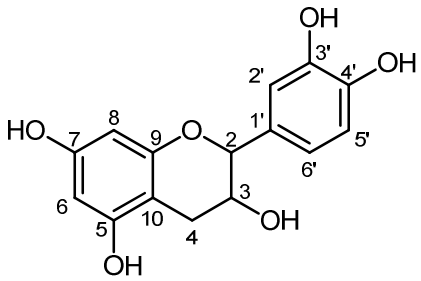


Figure 11: Structure of catechin and final assignment of protons and carbons (see Table 3)

Table 3: Details of ^{1}H and ^{13}C signals: chemical shifts, multiplicity, coupling constants and assignments (solvent is methanol-d4)

1H NMR		13C NMR	
Details	***Assignment***	***Details***	***Assignment***
2.51 (1H, *dd*, *J*=16.1, 8.1 Hz)	H4a	28.51	C4
2.85 (1H, *dd*, *J*=16.1, 5.4 Hz)	H4b	68.83	C3
3.98 (1H, *td*, *J*=7.8, 5.4 Hz)	H3	82.88	C2
4.57 (1H, *d*, *J*=7.5 Hz)	H2	95.56	C8
5.86 (1H, *d*, *J*=2.3 Hz)	H6	96.36	C6
5.93 (1H, *d*, *J*=2.3 Hz)	H8	100.87	C10
6.72 (1H, *dd*, *J*=8.1, 1.9 Hz)	H5’	115.30	C2’
6.76 (1H, *d*, *J*=8.1 Hz)	H6’	116.11	C5’
6.84 (1H, *d*, *J*=1.9 Hz)	H2’	120.04	C6’
		132.27	C1’
		146.23	C3’
		146.26	C4’
		156.93	C9
		157.57	C5
		157.85	C7

Table 4: Comparison of experimental data with ^{1}H NMR literature values for catechin and its isomer epi-catechin (solvent is methanol-d4)

Assignment	CB1	Catechin	Δδ	Epi-catechin	Δδ
H4a	2.51 (1H, *dd*, *J*=16.1, 8.1 Hz)	2.49 (1H, *dd*, *J*=16.0, 8.6 Hz)	+0.02	2.79	-0.28
H4b	2.85 (1H, *dd*, *J*=16.1, 5.4 Hz)	2.82 (1H, *dd*, J=16.0, 1.6 Hz)	+0.03	2.88	-0.03
H3	3.98 (1H, *td*, J=7.8, 5.4 Hz)	3.97 (1H, *m*)	+0.01	3.35	+0.63
H2	4.57 (1H, *d*, *J*=7.5 Hz)	4.56 (1H, *d*, *J*=7.8 Hz)	+0.01	4.17	+0.40
H6	5.86 (1H, *d*, *J*=2.3 Hz)	5.86 (1H, *d*, *J*=2.1 Hz)	0	5.93	-0.07
H8	5.93 (1H, *d*, *J*=2.3 Hz)	5.92 (1H, *d*, *J*=2.1)	+0.01	5.95	-0.02
H5’	6.72 (1H, *dd*, *J*=8.1, 1.9 Hz)	6.7 (1H, *dd*, *J*=8.1, 1.8 Hz)	+0.02	6.73	-0.01
H6’	6.76 (1H, *d*, *J*=8.1 Hz)	6.75 (1H, *d*, *J*=8.1 Hz)	+0.01	6.77	-0.01
H2’	6.84 (1H, *d*, *J*=1.9 Hz)	6.83 (1H, *d*, *J*=1.8 Hz)	+0.01	6.97	-0.13

Table 5: Comparison of experimental data with ^{13}C NMR literature values for catechin and its isomer epi-catechin (solvent is methanol-d4)

Assignment	CB1	Catechin	Δδ	Epi-catechin	Δδ
C4	28.51	29.74	+1.23	29.34	+0.83
C3	68.83	67.05	-1.78	67.46	-1.37
C2	82.88	77.14	-5.74	79.83	-3.05
C8	95.56	96.19	+0.63	95.94	+0.38
C6	96.36	96.58	+0.22	96.45	+0.09
C10	100.87	100.59	-0.28	100.12	-0.75
C2’	115.3	115.33	+0.03	115.33	+0.03
C5’	116.11	116.00	-0.11	115.95	-0.16
C6’	120.04	119.47	-0.57	119.45	-0.59
C1’	132.27	132.12	-0.15	132.28	+0.01
C3’	146.23	145.19	-1.04	145.73	-0.50
C4’	146.26	145.66	-0.60	145.89	-0.37
C9	156.93	157.34	+0.41	157.34	0.41
C5	157.57	158.00	+0.43	157.59	0.02
C7	157.85	158.05	+0.20	157.95	0.1

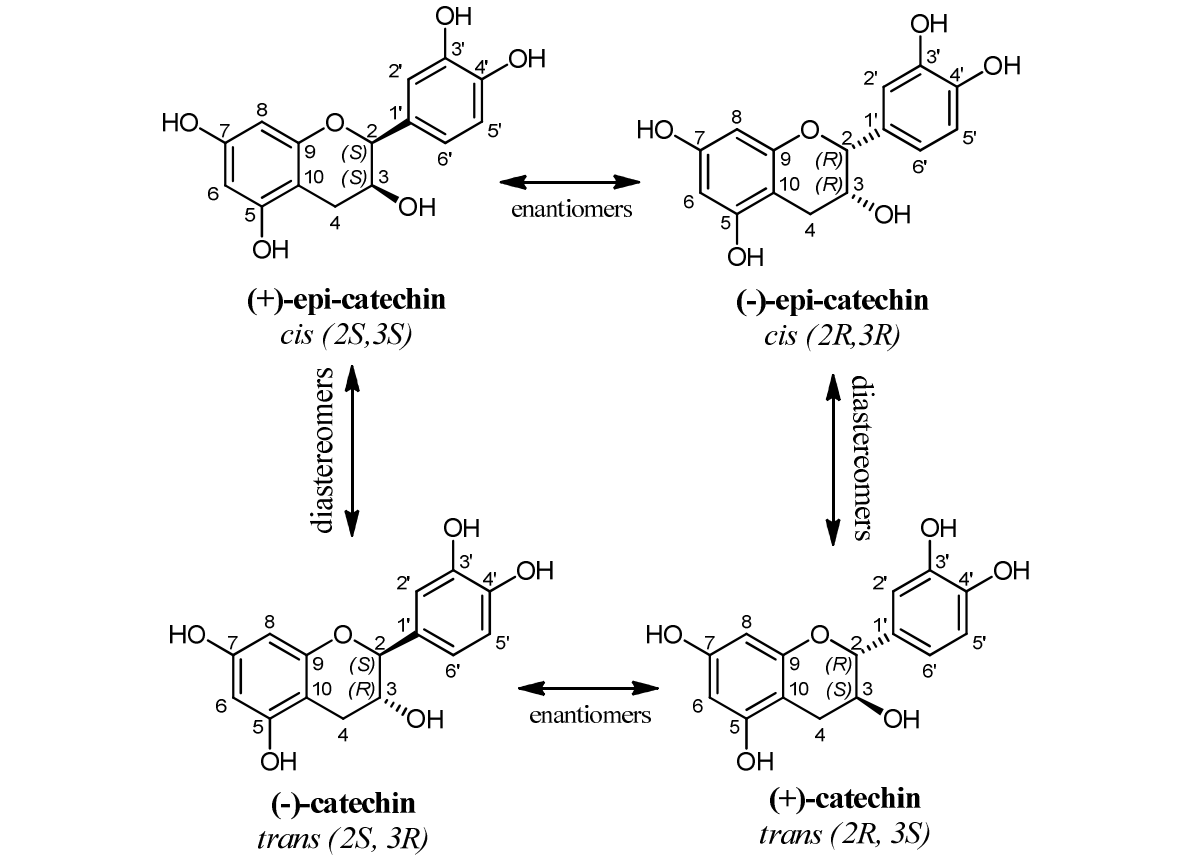


Figure 12: Isomers of catechin and epi-catechin and their stereochemical relationships.

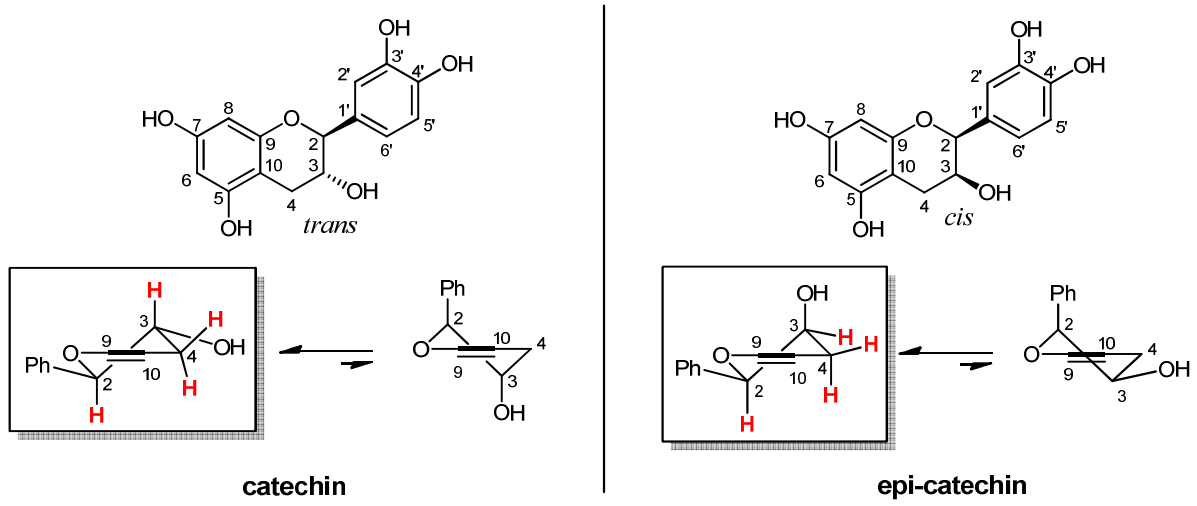


Figure 13: Illustration of conformational preferences and corresponding H–H dihedral angles for the H_2–C_2–C_3–H_3 system.

Having determined that CB1 was the known compound, catechin, a remaining unresolved issue was the question of the relative and/or absolute stereochemistry. The problem is illustrated in Figure 12 which shows the four possible diastereomers arising from the presence of two chiral centres at C2 and C3. The ^{1}H NMR data (Table 4) shows a very close agreement between measured chemical shifts for CB1 and catechin, and the key differences in chemical shift in epi-catechin for H2 and H3 confirm

the assignment of the catechin structure. This conclusion is further confirmed by examining the conformational preferences of catechin and epi-catechin, and considering the implications of this for the vicinal H2 - H3 coupling constants.

In catechin with its 2,3-*trans* geometry (Figure 13), the central ring adopts a half-chair conformation where the phenyl and hydroxyl substituents prefer to be *pseudo*-equatorial. H2 and H3 are thus close to *trans* di-axial and the dihedral angle expected to be close to 180°, leading to a relatively large coupling vicinal coupling constant (*J* ~ 7-10 Hz). In contrast, epi-catechin with its 2,3-*cis* geometry will be predicted to have a preferred half-chair conformation where the large phenyl group is *pseudo*-equatorial, and H-2 and H-3 are therefore *gauche*, resulting in a small dihedral angle and relatively small vicinal coupling constant(*J* ~ 2-4 Hz). The observed coupling constants for H2 and H3 are 7.5Hz and 7.8Hz respectively, consistent with that expected for the *trans* di-axial orientation found in catechin as opposed to the *cis* conformation in epi-catechin (Figure 12). The absolute stereochemistry has not yet been determined, due to loss of material at a crucial stage of the project. This is in principle obtainable from measurement of the optical rotation of CB1 and comparing to known compounds, or by a number of other techniques including formation and structural analysis of adducts of chiral reagents.

3.2.2 Alternative approach using open-top column chromatography and bioautographic testing of *C. brevifolia* crude extract

In addition to flash chromatography already described, open-top column chromatography was performed on the crude ethanolic extract of *C. brevifolia*. Initial liquid-liquid separation of the extract resulted in three subfractions (CBP = petroleum ether fraction, CBE = EtOAc fraction, CBW = water fraction) which were evaluated for activity in a range of biological assays. CBE and CBW showed antibacterial activity, protease inhibition activity and antioxidant activity. CBP showed no activity in any of the biological assays. Despite the fact that both the EtOAc and the water fraction showed activity, only the EtOAc fraction was subjected to open-top column chromatography. Chromatographic separation of a hydrophilic mixture requires more specialised and time consuming reverse phase chromatography, with subsequent lyophilisation of fractions, and the necessary equipment was not consistently available throughout the duration of this study. Thus the bioactive water fraction was frozen at -20°C until further study is possible and only the EtOAc fraction was subjected to chromatographic separation.

“Normal phase” silica gel column chromatography was performed on EtOAc residue (4.07g) resulting from liquid-liquid separation of crude *C. brevifolia* extract. Chromatographic separation afforded five subfractions: CBE1 (13.6mg), CBE2 (11.7mg), CBE3 (37.2mg), CBE4 (45.4mg) and CBE5 (43.4mg).

Fraction 6 (CBE6) was the water purge of the column. All subfractions were subjected to biological testing and it was determined that all fractions possessed antioxidant activity while only CBE2, CBE3 and CBE4 showed antibacterial activity (MIC = 5mg/ml). Only the water fraction, CBE6 showed protease inhibition activity. However, due to the limitations previously mentioned, the active water fraction could not be further evaluated and was frozen at -20°C until further study is possible. A summary of activity is included in the schematic presented earlier in this chapter (Figure 1). Further attempts at isolation of additional bioactive compounds were unsuccessful.

3.3 Whole extract based LC-MS metabolomics

Preliminary untargeted LC-MS spectrometry runs were used to generate chromatograms for each of the five crude extracts *Crassula brevifolia* (CB, Figure 14), *Polymita albiflora* (PA, Figure 24), *Crasssula muscosa* (CM, Figure 25), *Crassula cuneata* (CC, Figure 26) and *Zygophyllum foetidum* (ZF, Figure 27). Analysis of the chromatograms (and the underlying raw data) using MZmine allowed retention times and accurate molecular weights (accurate to 5ppm) to be determined for each detected compound. Formulae were generated for each of these, and the most likely formula for each was determined based on how well the isotopic pattern associated with the peak matched that of the isotopic pattern of the predicted molecular formula (the "*isotope match score*"). The mass difference between the molecular weight of the detected compound and that of the predicted formula was also considered. In some instances, there was a clear "most likely formula", with a high isotope match and a low mass difference. In other instances several formulae were possible, and for some compounds no formula could be predicted using the restrictive filters applied. Certain filters placed on the formula-generating algorithm may have prevented formula prediction in these instances. For example, some South African species such as *Dichapetalum cymosum* ("gifblaar" or "poison leaf") produce fluorine containing compounds such as monofluoroacetic acid (Marais, 1944). This is an unusual case and due to the relative rarity of halogen-containing natural compounds in terrestrial plants, elements such as fluorine and chlorine were not selected for formula generation. Thus the formula-generating algorithm was not able to predict formula for possible halogen-containing compounds.

Subsequent targeted LC-MS/MS spectrometry runs were performed for the most abundant compounds present in the crude extracts of CB, PA, CM, CC and ZF. Spectral matching against database entries present in the MassBank database (www.massbank.jp) allowed tentative identification of some compounds. In many instances where matches occurred, the formula of the matched compound was among those predicted by MZmine. In other instances, this was not the case. Spectral matching was checked manually for select compounds to ensure accuracy. This was

done by comparing the fragmentation patterns of the suggested compound matches to those of the raw LC-MS data in order to confirm the results of automated database matching.

The results of LC-MS analysis and compound identification are presented for the most abundant compounds identified from the extract of *C. brevifolia* in Table 6, and for the top ten most abundant compounds in each of the four other extracts in Table 7 (PA), Table 8 (CM), Table 9 (CC) and Table 10 (ZF). A discussion of each of these tables of results and the associated chromatograms follows.

3.3.1 LC-MS/MS analysis of the crude extract of *C. brevifolia*

Just over a quarter (26.9%) of the most abundant compounds present in the crude CB extract could be identified with a degree of certainty, as indicated by a high database match score (Table 6), and the structures of the 18 most abundant compounds that could be identified are shown in Figure 15. Most of the identified compounds were flavonoids or amino acids. MS/MS fragmentation patterns of several of the unidentified compounds were manually inspected and investigated for substructure matches using MassBank. This allowed for provisional identification of unknown compounds where no database match was available.

The base peak chromatogram resulting from LC-MS spectrometry analysis of CB (Figure 14) is dominated by a few large peaks with intensities greater than 2.0×10^6 (labelled A to L in Figure 14). Most of these consist of several co-eluting compounds of different masses. Using Agilent's MassHunter Qualitative Analysis software version B.04.00 (Agilent Technologies, Inc., Santa Clara, CA, USA, 2011), selected ion chromatograms can be generated to show the different compounds in each composite peak. For example, the earliest eluting composite peak "A" consists of four unidentified compounds (Figure 16), designated 13 (m/z = 110.0089), 23 (m/z = 182.9622), 31 (m/z = 198.9395) and 51 (m/z = 223.9886) in the compound list (Figure 16; Table 6). Furthermore, one of the largest peaks, composite peak "D", was also shown to consist of four compounds (Figure 17). The dominant compound in peak D was identified as catechin with a 93% match to the MassBank fragmentation pattern. Other compounds contributing to peak D were designated 17 (m/z = 139.0385), 41 (m/z = 291.2434) and 86 (m/z=293.3349) (Figure 17, Table 6).

3.3.1.1 The dominance of catechin in CB extract

The dominance of catechin in this extract as demonstrated by LC-MS spectrometry correlated with its isolation in the bioassay-guided fractionation approach described earlier. Dominance of catechin in plant extracts is not unusual and it may be present in quantities of up to 3000mg/kg in certain plants (Obreque-Slier et al., 2010). Chinese Rhubarb, for example, may contain up to 10% catechin by weight (Chunmei et al., 2010). Catechin has previously been found to have antibacterial, antioxidant, and mild antifungal activity (Díaz-Gómez et al., 2013; Brown et al., 2009; Tombola et al.,

2003; Tamura & Ochiai, 2012; Geetha et al., 2004). As it is present in such large amounts in the CB extract it may be responsible for the observed bioactivity of crude CB extract recorded in chapter 3 of this study. Isolated catechin has been shown to be an effective antimicrobial against *E. coli* at levels of between 0.05 and 1.6mg/ml (Chunmei et al., 2010). Crude CB extract tested using GIBEX and MIC assays (see chapter 3 of this thesis) demonstrated activity at levels of 166.7mg/ml against *E. coli*, which may result from the presence of catechin.

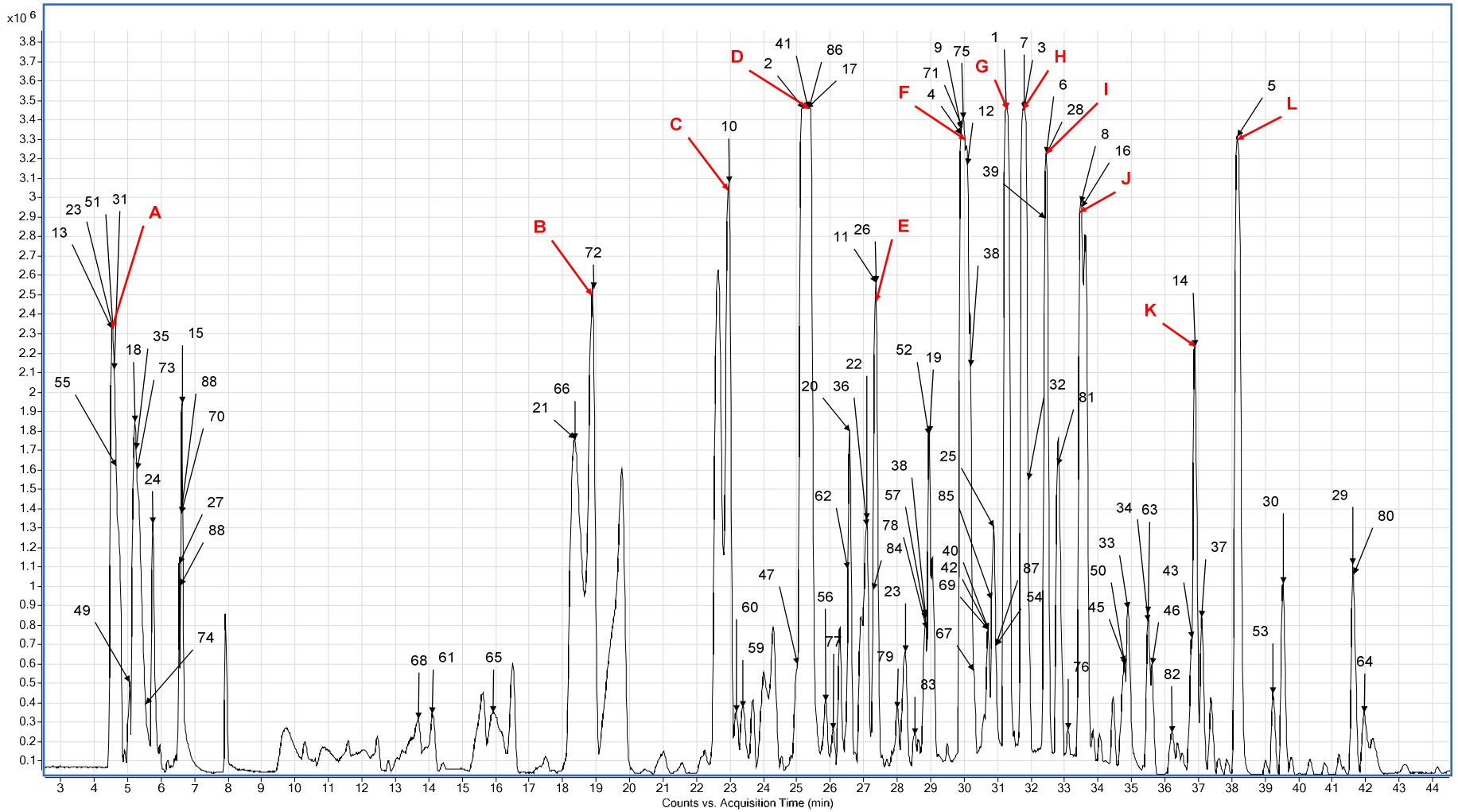


Figure 14: Base peak chromatogram of *C. brevifolia* obtained by HPLC-ESI-QTOF in positive-ion mode. Peak numbering designates the most abundant identified ions. Numbers not indicating a specific peak are signals co-eluting with more intense signals. Lettering indicates the dominant peaks/compounds.

Table 6: Tentative identification of the most abundant compounds in the ethanolic extract of *Crassula brevifolia* (in order of abundance)

No.	m/z	Neutral mass	RT (mins)	Proposed formula/e (MZmine)	Mass difference	Isotope match score (%)	Tentative identification (MassBank)	Database match score (%)	Major fragments
1	435.0928	434.0855	31.27	$C_{33}H_{10}N_2$ **$C_{20}H_{18}O_{11}$** $C_{21}H_{14}O_7N_4$	0.0011 **0.0006** 0.0007	96.80 **83.14** 82.62	Quercetin-3-arabinoside ($C_{20}H_{18}O_{11}$)	84.27	435, 303/304, 73
2	291.0909	290.0836	25.28	$C_{12}H_6N_{10}$ $C_{16}H_{10}O_2N_4$ **$C_{15}H_{14}O_6$**	0.0014 0.0013 **0.0001**	90.12 89.91 **88.89**	Catechin ($C_{15}H_{14}O_6$)	93.00	291, 165, 147, 139, 123
3	303.0500	302.0427	31.78	**$C_{15}H_{10}O_7$** $C_{16}H_6O_3N_4$	**0.0000** 0.0013	**84.22** 80.85	Quercetin ($C_{15}H_{10}O_7$)	89.17	285, 257, 229, 155, 153, 137, 81, 68
4	451.1238	450.1165	29.92	**$C_{21}H_{22}O_{11}$** $C_{22}H_{18}O_7N_4$ $C_{34}H_{14}N_2$ $C_{18}H_{14}O_5N_{10}$	**0.0003** 0.0010 0.0008 0.0016	**95.08** 94.18 88.57 88.64	Marein (Okanin-3-O-glucoside) ($C_{21}H_{22}O_{11}$)		451, 289, 207
5	303.0502	302.0429	38.18	**$C_{15}H_{10}O_7$** $C_{16}H_6O_3N_4$	**0.0002** 0.0011	**96.22** 92.29	Morin ($C_{15}H_{10}O_7$)	98.03	229, 155, 153, 137, 68
6	587.1039	586.0966	32.44	$C_{27}H_{22}O_{15}$ $C_{28}H_{18}O_{11}N_4$ $C_{41}H_{10}N_6$	0.0007 0.0006 0.0001	97.24 91.60 81.87	Quercetin xyloside	89.26	587, 304, 285, 153, 115
7	449.1083	448.1010	31.78	$C_{21}H_{20}O_{11}$ $C_{22}H_{16}O_7N_4$ $C_{34}H_{12}N_2$ $C_{18}H_{12}O_5N_{10}$	0.0004 0.0009 0.0010 0.0018	96.62 95.83 86.93 85.18	*Unknown: likely a flavonoid coumaroyl ester*	-	449, 287, 147, 119, 91
8	493.1349	492.1276	33.49	**$C_{23}H_{24}O_{12}$** $C_{24}H_{20}O_8N_4$ $C_{20}H_{16}O_6N_{10}$ $C_{36}H_{16}O_1N_2$	0.**0008** 0.0005 0.0022 0.0013	**98.17** 95.87 91.89 82.25	Iristectorin A ($C_{23}H_{24}O_{12}$)	71.34	493, 331, 316
9	289.0706	288.0633	29.92	$C_{15}H_{12}O_6$ $C_{16}H_8O_2N_4$ $C_{12}H_4N_{10}$	0.0001 0.0014 0.0013	96.99 93.60 92.11	Unknown	-	289, 179, 153, 67

10	579.1504	578.1431	22.95	**$C_{30}H_{26}O_{12}$** $C_{31}H_{22}O_8N_4$ $C_{14}H_{26}O_{17}N_8$ $C_{43}H_{18}O_1N_2$	**0.0007** 0.0007 0.0015 0.0012	**99.98** 89.56 88.48 83.27	Procyanidin B1 ($C_{30}H_{26}O_{12}$)	60.23	579, 409, 287, 163, 139, 127, 123
11	451.0879	450.0806	27.36	$C_{20}H_{18}O_{12}$ $C_{21}H_{14}O_8N_4$ $C_{17}H_{10}O_6N_{10}$ **$C_{21}H_{22}O_{11}$** $C_{33}H_{10}O_1N_2$	0.0008 0.0006 0.0021 **0.0005** 0.0013	95.71 93.83 90.99 **87.55** 80.19	Eriodictyol-7-O-glucoside ($C_{21}H_{22}O_{11}$)	87.02	451, 290/289, 153
12	479.0826	478.0753	30.17	**$C_{21}H_{18}O_{13}$** $C_{22}H_{14}O_9N_4$ $C_{18}H_{10}O_7N_{10}$ $C_9H_{22}O_{20}N_2$	**0.0006** 0.0008 0.0019 0.0013	**95.27** 93.36 91.45 89.14	Quercetin-3-glucuronide ($C_{21}H_{18}O_{13}$)	88.16	303, 257, 229, 153, 137, 113, 85, 73
13	110.0089	109.0016	4.55	no formulae	-	no score	*Unknown: similar to proline*	-	111, 70, 68
14	597.1609	596.1536	36.90	$C_{30}H_{28}O_{13}$ $C_{31}H_{24}O_9N_4$ $C_{32}H_{20}O_5N_8$ $C_{27}H_{20}O_7N_{10}$	0.0006 0.0007 0.0021 0.0020	89.66 89.29 88.88 84.73	Eriodictyol-7-O-neohesperidoside ($C_{27}H_{32}O_{15}$)	64.00	597/598, 435, 289
15	140.1432	139.1359	6.62	$C_9H_{17}N$	0.0002	98.00	*Unknown: possibly decahydroquinoline*	-	140/139, 96
16	289.0706	288.0633	33.49	**$C_{15}H_{12}O_6$** $C_{12}H_4N_{10}$	**0.0001** 0.0013	**95.33** 93.53	Eriodictyol ($C_{15}H_{12}O_6$)	70.32	289, 153, 137, 107
17	139.0389	138.0316	25.32	$C_7H_6O_3$	0.0001	97.79	*Unknown: likely to be protocatechualdehyde*	-	nd
18	104.1074	103.1001	5.22	**$C_5H_{13}NO$**	0.0004	96.52	Choline ($C_5H_{14}NO$)	99.51	104, 60, 59, 58
19	611.1608	610.1535	28.96	$C_{23}H_{26}O_{14}N_6$ $C_{24}H_{22}O_{10}N_{10}$ **$C_{27}H_{30}O_{16}$** $C_{28}H_{26}O_{12}N_4$ $C_{29}H_{22}O_8N_8$ $C_{15}H_{34}O_{23}N_2$ $C_{11}H_{30}O_{21}N_8$	0.0028 0.0015 **0.0001** 0.0012 0.0026 0.0017 0.0010	96.11 95.70 **94.61** 92.59 90.53 89.68 82.74	Rutin ($C_{27}H_{30}O_{16}$)	92.73	611, 465, 303

20	481.0981	480.0908	26.58	**$C_{21}H_{20}O_{13}$** $C_{22}H_{16}O_9N_4$ $C_{34}H_{12}O_2N_2$ $C_{18}H_{12}O_7N_{10}$	**0.0004** 0.0009 0.0009 0.0018	**92.89** 92.73 88.37 83.00	Myricetin-3-galactoside ($C_{21}H_{20}O_{13}$)	89.13	481, 319, 245, 153, 85
21	307.0815	306.0742	18.36	$C_{15}H_{14}O_7$ $C_{16}H_{10}O_3N_4$	0.0002 0.0011	94.46 92.73	*Unknown: possibly gallocatechol*	-	307, 139, 131
22	731.1601	730.1528	27.09	$C_{34}H_{22}O_{10}N_{10}$ $C_{37}H_{30}O_{16}$ $C_{21}H_{30}O_{21}N_8$	0.0008 0.0006 0.0003	95.88 94.34 81.16	*Unknown: likely a catechin polymer*	-	731, 411, 271, 247, 153, 139
23	182.9622	181.9549	4.55	no formulae	-	no score	Unknown	-	182, 139/138, 108
24	287.1233	286.1160	5.76	$C_{12}H_{18}O_6N_2$	0.0005	96.76	Unknown	-	287, 207, 198, 85
25	443.0967	442.0894	30.88	$C_{22}H_{18}O_{10}$ $C_{19}H_{10}O_4N_{10}$	0.0006 0.0008	97.44 85.97	*Unknown: possibly catechin gallate*	-	443, 333, 153 97
26	319.0450	318.0377	27.36	**$C_{15}H_{10}O_8$**	**0.0001**	**96.93**	Myricetin ($C_{15}H_{10}O_8$)	74.19	319, 182, 164, 153
27	465.2078	464.2005	6.54	$C_{19}H_{32}O_{11}N_2$ $C_{20}H_{28}O_7N_6$	0.0001 0.0014	92.12 90.31	Unknown	-	465, 303, 132
28	285.0606	284.0533	32.44	$C_{12}H_{12}O_8$ $C_{13}H_8O_4N_4$	0.0001 0.0013	95.00 93.31	*Unknown: possibly a kaempferol derivative*	-	285, 245, 85, 71, 57
29	611.1763	610.1690	41.64	$C_{31}H_{30}O_{13}$ $C_{32}H_{26}O_9N_4$ $C_{44}H_{22}O_2N_2$	0.0004 0.0010 0.0009	98.47 98.03 82.71	Unknown	-	611, 187
30	585.1609	584.1536	39.53	$C_{29}H_{28}O_{13}$ $C_{30}H_{24}O_9N_4$	0.0006 0.0006	94.33 92.31	Unknown	-	nd
31	198.9395	197.9322	4.60	no formula	-	no score	Unknown	-	198, 154, 124, 84
32	603.1339	602.1266	31.93	$C_{25}H_{18}O_9N_{10}$ $C_{28}H_{26}O_{15}$ $C_{12}H_{26}O_{20}N_8$	0.0008 0.0006 0.0003	95.12 95.32 81.79	*Unknown: possibly a substituted hydroxyflavanone*	-	603, 435, 315, 171, 153, 74
33	571.1086	570.1013	34.91	$C_{40}H_{14}O_3N_2$ $C_{27}H_{22}O_{14}$ $C_{29}H_{14}O_6N_8$ $C_{28}H_{18}O_{10}N_4$	0.0009 0.0003 0.0023 0.0001	95.65 86.64 85.45 85.13	*Unknown: possibly a malvidin derivative*	-	nd

34	601.1194	600.1121	35.51	$C_{28}H_{24}O_{15}$ $C_{29}H_{20}O_{11}N_4$	0.0006 0.0008	95.39 93.33	Unknown	-	nd
35	203.0522	202.0449	5.27	$C_4H_6O_4N_6$	0.0002	95.71	Unknown	-	203, 110, 98, 68
36	441.0817	440.0744	27.07	$C_{22}H_{16}O_{10}$ $C_{23}H_{12}O_6N_4$ $C_{19}H_8O_4N_{10}$ $C_{10}H_{20}O_{17}N_2$	0.0001 0.0013 0.0014 0.0018	97.70 95.73 89.83 86.94	*Unknown: possibly an apigenin glycoside*	-	271, 153
37	627.1711	626.1638	37.10	$C_{31}H_{30}O_{14}$ $C_{32}H_{26}O_{10}N_4$ $C_{15}H_{30}O_{19}N_8$	0.0002 0.0011 0.0011	95.24 93.13 81.36	Unknown	-	nd
38	603.0982	602.0909	28.85	$C_{27}H_{22}O_{16}$ $C_{28}H_{18}O_{12}N_4$ $C_{11}H_{22}O_{21}N_8$	0.0001 0.0012 0.0010	95.48 93.44 81.74	Unknown	-	602, 319, 153, 125, 81, 69
39	419.0975	418.0902	32.41	$C_{20}H_{18}O_{10}$ $C_{17}H_{10}O_4N_{10}$ $C_{21}H_{14}O_6N_4$	0.0002 0.0016 0.0011	96.39 91.37 89.68	Unknown	-	nd
40	171.0286	170.0213	30.72	$C_7H_6O_5$	0.0002	96.26	*Unknown: possibly gallic acid*	-	171, 153
41	291.2434	290.2361	25.32	$C_{18}H_{30}O_1N_2$	0.0003	89.74	Epi-catechin	84.97	291, 207, 165, 147, 139, 123
42	199.0598	198.0525	30.72	$C_9H_{10}O_5$	0.0003	97.31	*Unknown: possibly dihydroxyphenyllactate*	-	nd
43	289.0706	288.0633	36.80	$C_{15}H_{12}O_6$ $C_{12}H_4N_{10}$	0.0001 0.0013	95.17 93.54	Unknown	-	289, 153
44	579.1492	578.1419	28.23	$C_{30}H_{26}O_{12}$ $C_{27}H_{18}O_6N_{10}$ $C_{13}H_{30}O_{21}N_4$ $C_{14}H_{26}O_{17}N_8$	0.0005 0.0008 0.0016 0.0003	95.15 93.92 81.07 80.72	Unknown	-	579, 163, 139, 127
45	281.0656	280.0583	34.80	$C_{13}H_{12}O_7$ $C_{10}H_4O_1N_{10}$	0.0000 0.0013	95.55 93.42	Unknown	-	nd
46	571.1448	570.1375	35.61	$C_{28}H_{26}O_{13}$ $C_{29}H_{22}O_9N_4$ $C_{25}H_{18}O_7N_{10}$	0.0002 0.0012 0.0015	95.00 92.99 92.31	Unknown	-	nd

				$C_{16}H_{30}O_{20}N_2$ $C_{12}H_{26}O_{18}N_8$	0.0017 0.0010	87.71 81.23			
47	340.2595	339.2522	25.02	$C_{18}H_{33}O_3N_3$	0.0000	96.77	Unknown	-	340, 322, 227, 161
48	617.1137	616.1064	30.29	$C_{41}H_{16}O_5N_2$ $C_{28}H_{24}O_{16}$	0.0005 0.0000	87.15 72.67	Unknown		nd
49	175.1185	174.1112	5.07	**$C_6H_{14}O_2N_4$**	**0.0005**	**82.65**	Arginine ($C_6H_{14}O_2N_4$)	-	175, 130, 116, 70
50	299.0761	298.0688	34.8	$C_{14}H_{10}O_4N_4$ $C_{13}H_{14}O_8$ $C_{10}H_6O_2N_{10}$	0.0014 0.0001 0.0013	94.74 94.64 89.45	Unknown	-	nd
51	223.9886	222.9813	4.55	no formulae	-	no score	Unknown	-	223, 182, 138, 114, 68
52	303.0498	302.0425	28.96	**$C_{15}H_{10}O_7$** $C_{16}H_6O_3N_4$ $C_{12}H_2O_1N_{10}$	**0.0002** 0.0015 0.0012	**95.36** 93.63 92.96	Quercetin ($C_{15}H_{10}O_7$)	90.15	303, 229, 153, 137, 68
53	555.1501	554.1428	39.24	$C_{28}H_{26}O_{12}$ $C_{29}H_{22}O_8N_4$ $C_{25}H_{18}O_6N_{10}$ $C_{16}H_{30}O_{19}N_2$ $C_{12}H_{26}O_{17}N_8$	0.0004 0.0010 0.0017 0.0015 0.0012	95.61 93.53 91.93 87.31 80.19	Unknown	-	555, 503, 271, 175
54	731.1604	730.1531	30.95	$C_{37}H_{30}O_{16}$ $C_{38}H_{26}O_{12}N_4$ $C_{34}H_{22}O_{10}N_{10}$	0.0003 0.0016 0.0011	96.77 94.43 92.98	Unknown	-	731, 619, 580, 271, 153
55	214.9170	213.9097	4.65	no formulae	-	no score	Unknown	-	214, 170, 140, 84
56	387.0713	386.0640	25.86	$C_{20}H_{10}O_5N_4$ $C_{19}H_{14}O_9$	0.0011 0.0002	83.82 81.75	Unknown	-	387, 371, 227, 209, 141, 89
57	285.0604	284.0531	28.85	$C_{12}H_{12}O_8$ $C_9H_4O_2N_{10}$	0.0001 0.0012	96.60 92.26	Unknown	-	285, 213, 171, 153, 68, 57
58	118.0864	117.0791	6.58	$C_5H_{11}O_2N$	0.0001	no score	*Unknown: possibly valine*	-	118, 72, 55
59	747.1548	746.1475	23.4	$C_{34}H_{22}O_{11}N_{10}$ $C_{37}H_{30}O_{17}$ $C_{21}H_{30}O_{22}N_8$ $C_{20}H_{34}O_{26}N_4$	0.0005 0.0008 0.0000 0.0014	99.74 97.82 86.11 85.17	*Unknown: possibly apigenin with two rhamnoside substuents*	-	747, 579, 441, 303, 271, 153

60	188.0703	187.0630	23.2	$C_{11}H_9O_2N$	0.0003	98.07	*Unknown: similar to tryptophan*	-	170, 146, 127, 118, 91
61	139.0388	138.0315	14.12	$C_7H_6O_3$	0.0002	92.90	*Unknown: likely to be protocatechualdehyde*	-	139, 109, 97
62	141.0544	140.0471	26.51	$C_7H_8O_3$	0.0002	98.36	*Unknown: possibly dihydroxyanisole*	-	141, 124, 97, 56
63	153.0182	152.0109	35.51	$C_7H_4O_4$	0.0001	98.12	Unknown	-	153, 127, 98, 81
64	228.2317	227.2244	41.97	$C_{14}H_{29}O_1N_1$	0.0005	89.55	Unknown	-	228, 192, 109, 95
65	595.1446	594.1373	15.90	$C_{43}H_{18}O_2N_2$ $C_{30}H_{26}O_{13}$	0.0005 0.0000	95.49 80.39	*Unknown: possibly a caffeoylglucoside of kaempferol*	-	595, 425, 287, 127
66	139.0388	138.0315	18.36	$C_7H_6O_3$	0.0002	98.94	*Unknown: likely to be protocatechualdehyde*	-	139, 126, 109, 98, 81, 68
67	315.0710	314.0637	30.29	$C_{13}H_{14}O_9$ $C_{14}H_{10}O_5N_4$ $C_{10}H_6O_3N_{10}$	0.0001 0.0014 0.0013	96.46 94.77 91.73	Unknown	-	302, 274, 228, 153
68	171.0285	170.0212	13.68	$C_7H_6O_5$	0.0003	94.84	*Unknown: possibly gallic acid*	-	171, 153, 127, 109, 81, 53
69	127.0389	126.0316	30.72	$C_6H_6O_3$	0.0001	99.08	*Unknown: possibly phloroglucinol*	-	127, 96
70	175.0235	174.0162	6.58	No formulae	-	no score	Unknown	-	175, 129, 101, 85
71	153.0180	152.0107	29.92	$C_7H_4O_4$	0.0003	95.51	*Unknown: similar to naringenin*	-	153, 125, 111, 93, 79, 51
72	206.0445	205.0372	18.95	$C_{10}H_7O_4N$	0.0003	81.91	*Unknown: possibly methoxyindoleacetic acid*	67.89	206, 188, 160
73	233.0630	232.0557	5.28	$C_5H_8O_5N_6$	0.0001	94.11	Unknown	-	233, 110, 98, 82, 68
74	138.0547	137.0474	5.56	**$C_7H_7O_2N$**	**0.0003**	**no score**	Trigonelline ($C_7H_7O_2N$)	86.50	138, 92, 78, 65, 53
75	465.2989	464.2916	29.97	$C_{22}H_{16}O_8N_4$ **$C_{21}H_{20}O_{12}$** $C_{18}H_{12}O_6N_{10}$ $C_{34}H_{12}O_1N_2$	0.0012 **0.0001** 0.0015 0.0006	95.17 **93.46** 88.86 82.21	Hirsutrin ($C_{21}H_{20}O_{12}$)	78.24	465, 303, 110

76	440.2486	439.2413	33.1	$C_{19}H_{37}O_{10}N$ $C_{20}H_{33}O_6N_5$	0.0004 0.0018	93.91 93.74	Unknown	-	441/440, 271, 153, 119
77	581.1650	580.1577	26.1	$C_{30}H_{28}O_{12}$ $C_{31}H_{24}O_8N_4$ $C_{27}H_{20}O_6N_{10}$ $C_{43}H_{20}O_1N_2$	0.0004 0.0017 0.0010 0.0001	92.50 89.54 87.00 80.84	Naringin ($C_{27}H_{32}O_{14}$)	80.19	581/580, 291, 273, 127
78	267.0497	266.0424	28.85	$C_{12}H_{10}O_7$ $C_9H_2O_1N_{10}$	0.0003 0.0011	96.09 90.72	Unknown	-	267, 437, 109, 95, 71, 57
79	465.1029	464.0956	28.00	$C_{22}H_{16}O_8N_4$ **$C_{21}H_{20}O_{12}$** $C_{18}H_{12}O_6N_{10}$ $C_{34}H_{12}O_1N_2$	0.0012 **0.0001** 0.0015 0.0006	95.17 **93.46** 88.76 82.21	Hyperoside ($C_{21}H_{20}O_{12}$)	91.09	303, 85, 61
80	287.0552	286.0479	41.64	$C_{15}H_{10}O_6$ $C_{16}H_6O_2N_4$	0.0002 0.0012	95.87 94.14	Unknown	-	287, 271, 123
81	205.0703	204.0630	32.79	$C_8H_{12}O_6$ $C_5H_4N_{10}$	0.0004 0.0010	97.74 83.10	Unknown	-	205, 165, 121, 109
82	233.2009	232.1936	36.22	$C_{15}H_{24}N_2$	0.0003	97.42	Unknown	-	233, 113, 57
83	317.1018	316.0945	28.53	$C_{17}H_{16}O_6$ $C_{18}H_{12}O_2N_4$ $C_{14}H_8N_{10}$	0.0002 0.0015 0.0012	95.61 93.84 92.46	Unknown	-	317, 165, 123
84	453.3435	452.3362	27.32	$C_{24}H_{44}O_4N_4$ $C_{23}H_{48}O_8$ $C_{25}H_{40}N_8$	0.0001 0.0013 0.0014	96.93 96.50 87.49	Unknown	-	453, 291, 287, 255, 153
85	631.0928	630.0855	30.82	$C_{28}H_{22}O_{17}$ $C_{25}H_{14}O_{11}N_{10}$ $C_{29}H_{18}O_{13}N_4$ $C_{12}H_{22}O_{22}N_8$ $C_{41}H_{14}O_6N_2$	0.0002 0.0011 0.0015 0.0006 0.0003	88.54 87.13 86.62 76.77 72.76	Unknown	-	631, 459, 345, 253, 221, 167
86	293.0919	292.0846	25.32	$C_{17}H_{12}O_3N_2$	0.0002	88.25	Unknown	-	293, 207, 125, 99
87	441.0819	440.0746	30.95	$C_{22}H_{16}O_{10}$ $C_{23}H_{12}O_6N_4$	0.0003 0.0011	82.84 81.91	Unknown	-	203, 110, 98, 68

				$C_{19}H_8O_4N_{10}$ $C_{10}H_{20}O_{17}N_2$ $C_6H_{16}O_{15}N_8$	0.0016 0.0016 0.0011	76.95 70.16 65.04			
88	157.0128	156.0055	6.56	$C_6H_4O_5$	0.0004	98.23	Unknown	-	158, 84/83, 57

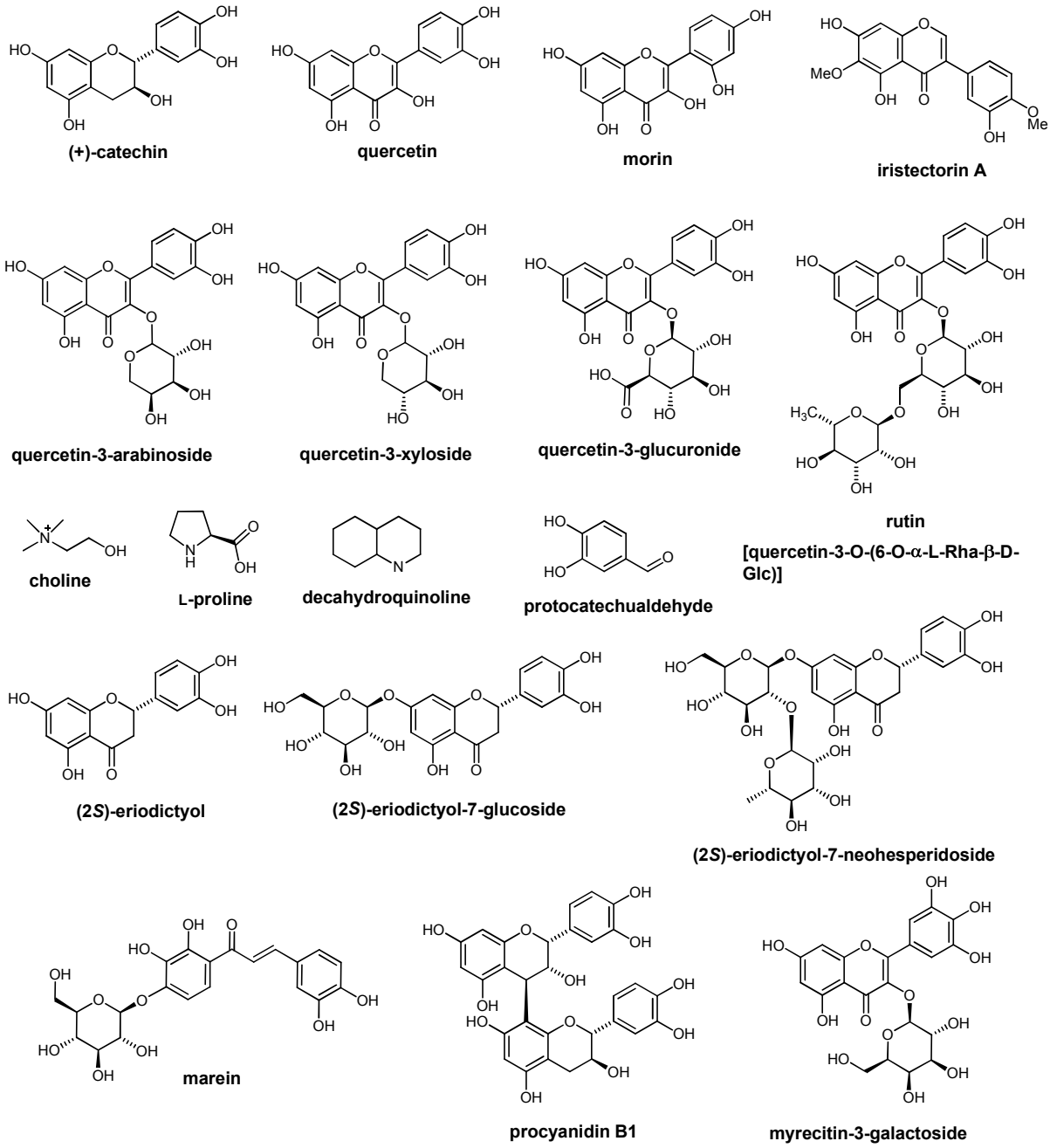


Figure 15: Structures of the most abundant compounds tentatively identified from LC-MS/MS of the extract of *Crassula brevifolia*

Pure catechin has also been shown to possess bioactivity that the CB extract was not tested for during this study. For example, catechin reduces cardiovascular and cancer risk (Mittal et al., 2004) and protects against neurodegenerative diseases like Parkinson's and Alzheimer's diseases (Teixeira et al., 2013; Smith et al., 2010; Berletch et al., 2008). Semi-synthetic catechin derivatives have also been shown to inhibit strains of human influenza, including H9N2 "bird flu" for which there is currently no treatment (Song et al., 2007). Thus, catechin as well as the whole extract of *C. brevifolia* has the potential for future drug development, either in its naturally occurring form or with modifications to enhance its bioactivity. Further testing of CB extract, and other species from

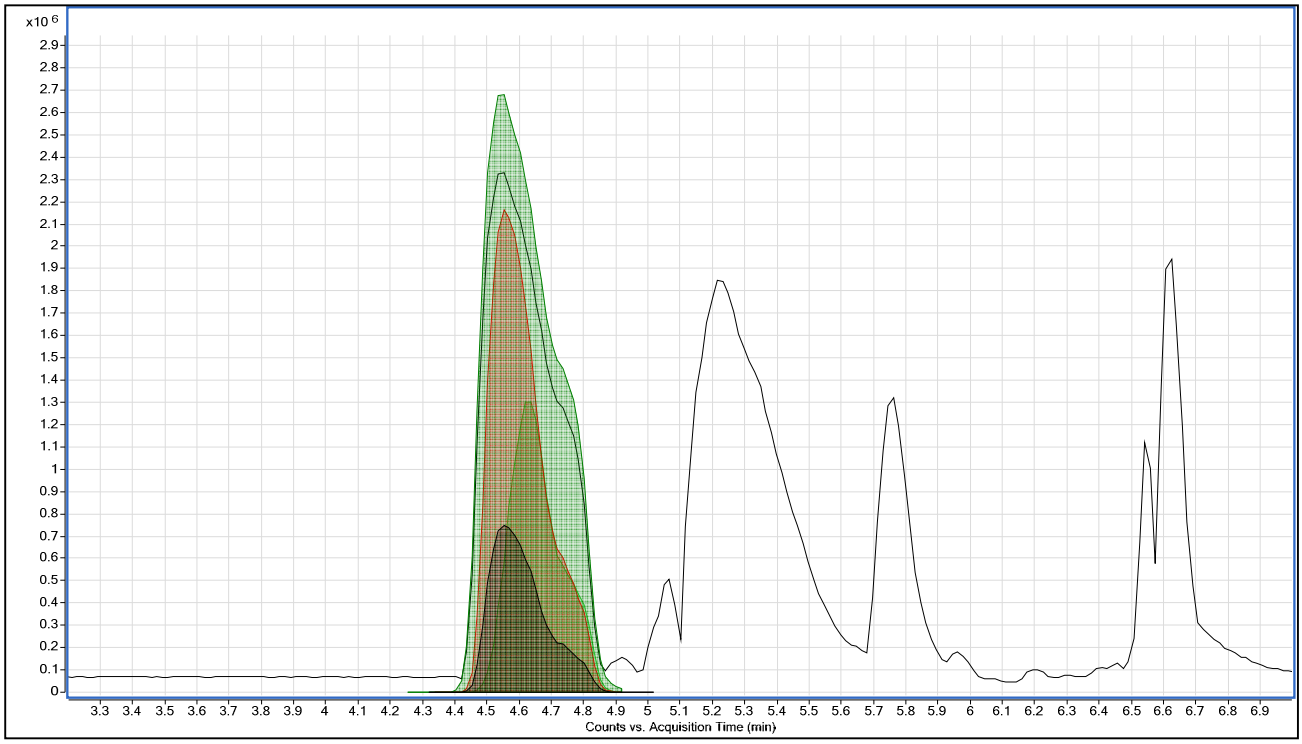


Figure 16: Expansion of peak A to show the constituent compounds from largest to smallest: compound 13 (dark green), compound 23 (orange), compound 31 (light green) and compound 51 (grey).

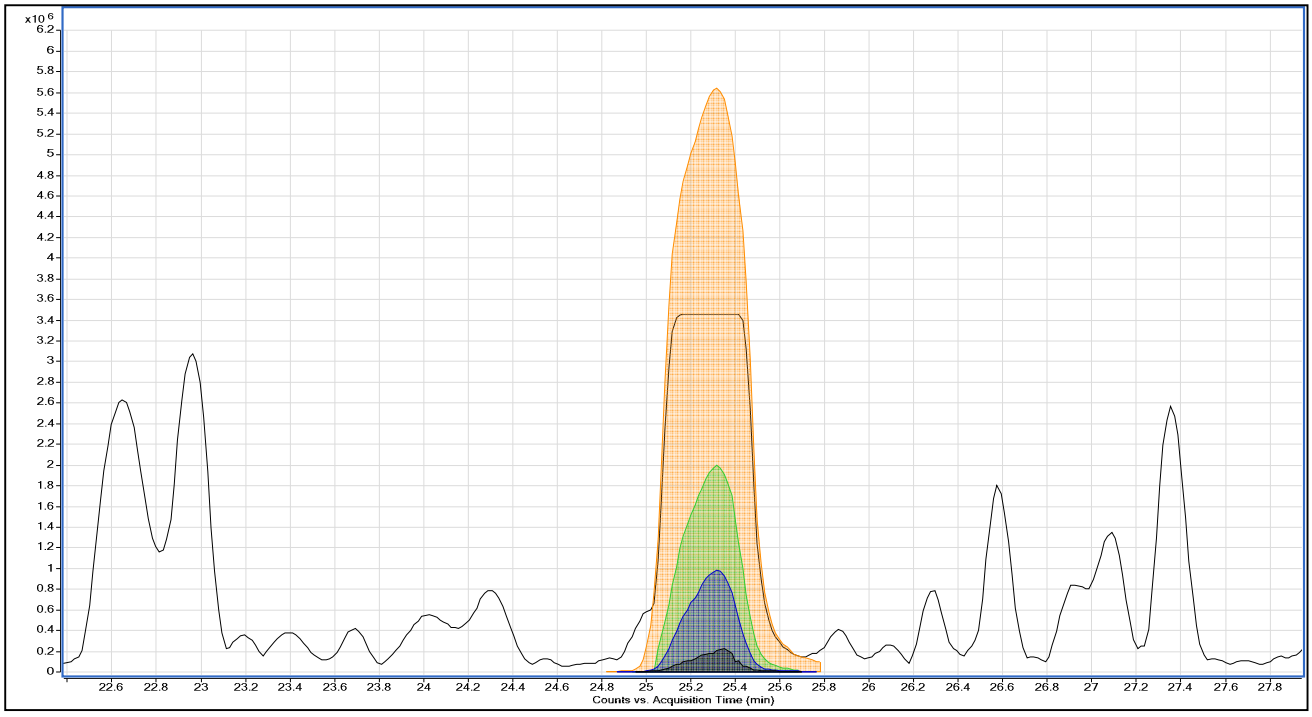


Figure 17: Expansion of composite peak D showing the dominance of catechin (the orange peak). Other minor peaks shown are compounds 17 (green peak), 41 (blue peak) and 86 (grey peak).

Namaqualand, in additional assays may reveal multiple bioactivities not discovered using the selection of assays used in this study. Additional bioactive compounds may also contribute to the activity of CB, and this deserves further investigation.

3.3.1.2 Analysis of additional "most abundant" compounds present in CB extract

Based on the analysis of the LC-MS data presented above, it was evident that flavonoids and their glycosides featured prominently among the most abundant compounds in the extract. This analysis could therefore be validated with reference to the literature on flavonoid mass spectral fragmentation patterns, and noting that our choice of ESI-MS in the positive mode was in line with observations that first-order mass spectra in the positive ion mode contain more structural information than those obtained in the negative ion mode (Cuyckens & Claeys, 2004).

Fragment ions from flavonoid glycoconjugates are denoted according to the nomenclature introduced by Domon and Costello (Figure 18) (Domon & Costello, 1988). Ions containing the aglycone are labelled $^{k,l}X_j$, Yj, and Zj, where the superscripts k and l indicate the cleavages within the carbohydrate rings, and j is the number of the interglycosidic bond broken, where the glycosidic bond linking the glycan part to the aglycone is numbered 0. When the charge is retained on the carbohydrate residue, fragments are designated $^{k,l}A_i$, B_i and C_i, where i (≥1) represents the number of the glycosidic bond cleaved, counting from the non-reducing end.

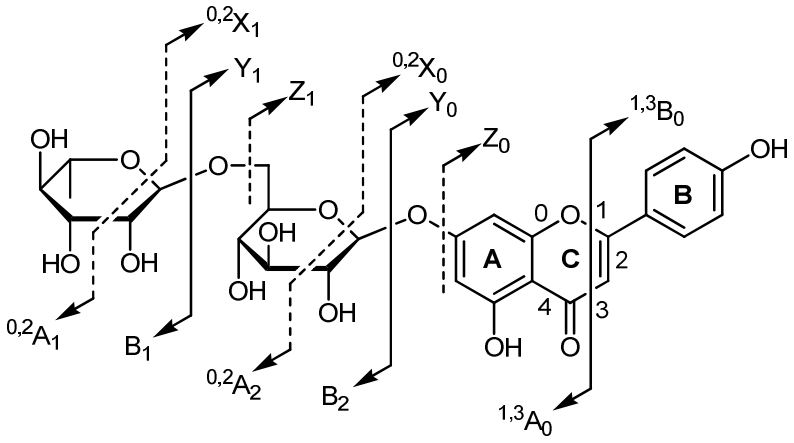


Figure 18: Ion nomenclature used for flavonoid glycosides (illustrated on apigenin-7-O-rutinoside) (Domon & Costello, 1988)

The most useful fragmentations in terms of flavonoid aglycone identification are those that require cleavage of two C-C bonds, or a C-O bond and a C-C bond, of the C-ring, resulting in structurally informative $^{i,j}A_0^+$ and $^{i,j}B_0^+$ ions (Figure 18). The $^{1,3}A_0^+$ ion, which is observed for all flavonoid groups, is generally the fragment most readily formed and often constitutes the most abundant fragment ion. It is most often found at m/z 153 or, in the absence of the 4-keto group, i.e. for flavanes and

flavanols, at m/z 139 (Wolfender et al., 2000), and also confirms the degree of hydroxylation of the A ring. These distinctive fragmentation patterns were observed in many of the spectra obtained for compounds present in CB extract, confirming compound identification. For example, the spectrum of quercetin obtained by LC-MS/MS (Figure 19), belonging to the flavonol class, displays the molecular ion [M+H] at 303 while the $^{1,3}A_0$ ion is clearly visible at m/z = 153, as is the $^{0,3}A_0$ ion at m/z = 137. The spectrum of catechin, belonging to the flavan-3-ol class, (Figure 20) shows the molecular ion at [M+H] = 291, and the typical $^{1,3}A_0$ ion for flavonoids lacking a 4-keto group appears at m/z = 139.

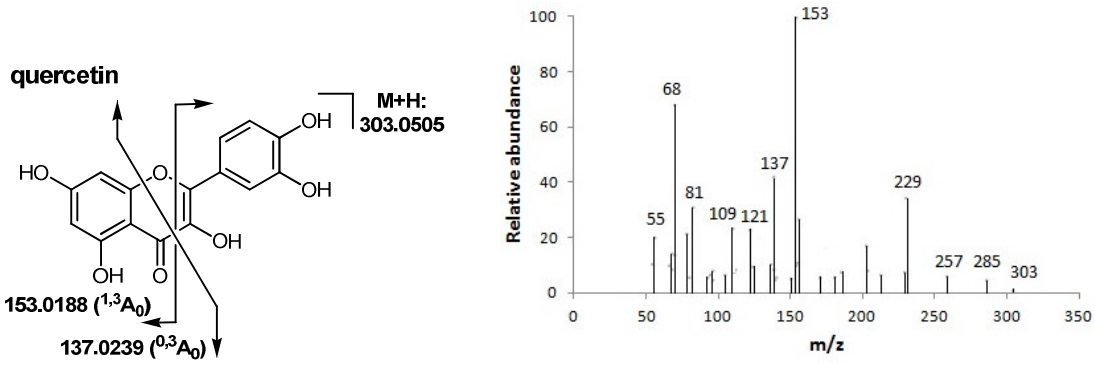


Figure 19: Fragmentation patterns of quercetin

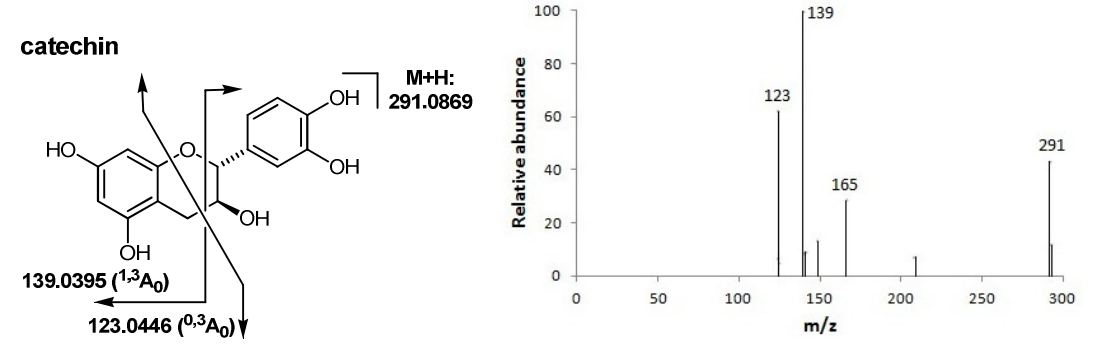


Figure 20: Fragmentation patterns of catechin

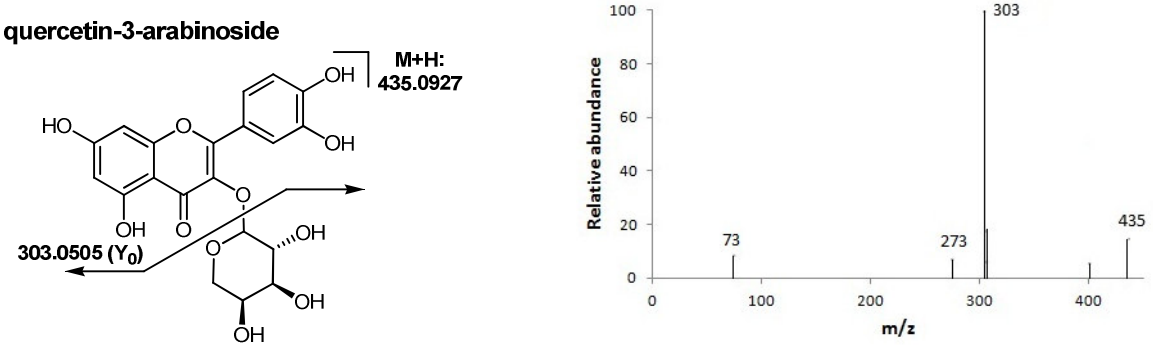


Figure 21: Fragmentation patterns illustrating the cleavage of a monosaccharide unit from the flavonoid skeleton in quercetin-3-O-arabinoside

Differentiation between O-glycosides, C-glycosides and O, C-diglycosides can be made by examining first-order positive ion spectra or low-energy CID spectra. The protonated O-diglycosides give rise to Y_1^+ and Y_0^+ ions which are formed by rearrangement reactions at the interglycosidic bonds. With flavonoid O-glycosides cleavage at the glycosidic *O*-linkages with a concomitant H-rearrangement leads to the elimination of monosaccharide residues, i.e. the loss of 162 u (hexose), 146 u (deoxyhexose), 132 u (pentose) or 176 u (uronic acid), allowing the determination of the carbohydrate sequence (Wolfender et al., 1992). Fragmentation patterns arising from such cleavages are evident among the compounds identified from CB extract. For example, quercetin-3-O-arabinoside (Figure 21) shows the molecular ion [M+H] = 435, as well as the Y_0 ion at m/z = 303 resulting from the cleavage of the pentose sugar (132 u) from the quercetin skeleton.

In a second example, eriodictyol-7-O-glucoside (Figure 22) gives a molecular ion at [M+H] = 451 and the Y_0 ion at m/z = 289, corresponding to the loss of the glucose residue of 162 u. As a final example, the mass spectral data for rutin (quercetin-3-O-rutinoside) illustrates how the order of sugar

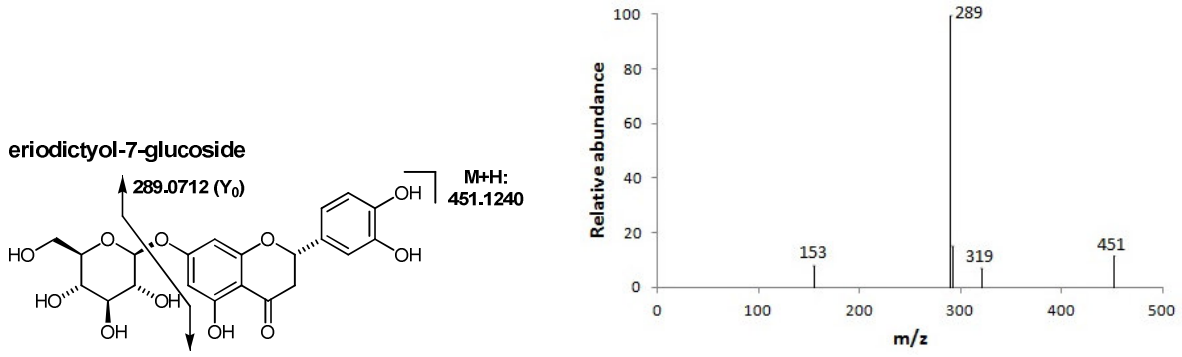


Figure 22: Fragmentation patterns illustrating the cleavage of a monosaccharide unit from the flavonoid skeleton in eriodictyol-7-O-glucoside

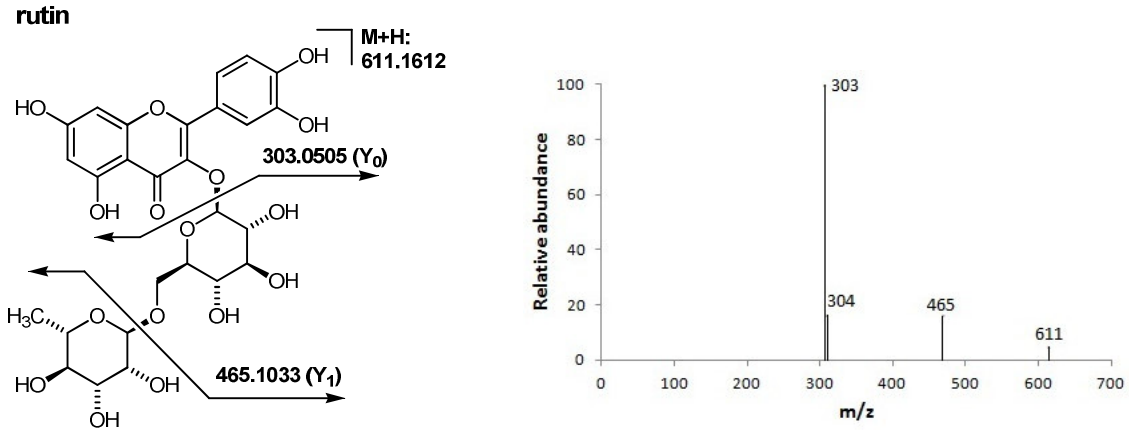


Figure 23: Fragmentation patterns illustrating cleavage of monosaccharide units from the flavonoid skeleton in rutin

residues in a disaccharide can be determined from fragmentation patterns (Figure 23). In this example, the molecular ion is visible at [M+H] = 611. The Y_1 fragment appears at m/z = 465, corresponding to the elimination of a terminal 6-deoxyhexose of 146 u and the Y_0 fragment appears at m/z = 303, corresponding to the elimination of a disaccharide, comprising a 6-deoxyhexose linked to a hexose (146 + 162 u). This confirms that the order of sugar residues in rutin is rhamnose-glucose-3-O-quercetin.

Many of the other "most abundant" compounds detected, and tentatively identified using mass fragmentation patterns, are flavonoids (Table 6). The majority of them belong to the same "family" of flavonoids and share a similar skeleton varying only in the position and degree of hydroxylation (Figure 15). Despite these compounds having similar structures, they exhibit a wide variety of biological activities. Some are specifically active in a single area while others have multiple bioactivities. For example, Iristectorin A is thought to have antitumor effects (Fang et al., 2008) and marein has been shown to have antioxidant capacity (Dias et al., 2012). Rutin, on the other hand has several pharmacological activities including antiallergic, anti-inflammatory, antihemorrahagic, antitumour, antibacterial, antiviral, antiprotozoal, antioxidant and free radical scavenging, hypolipidaemic, cytoprotective, antispasmodic and anticarcinogenic activities (Rosane et al., 2006; Calabrò et al., 2005; Chat et al., 2011; Yang et al., 2008; Casa et al., 2000; Webster et. al, 1996) and morin has chemoprotective, antimutagenic, anti-inflammatory and antioxidant activity and is a free radical scavenger (Chen et al., 2012; Kim et al., 1999; Raso et al., 2001; Parihar et al., 2007; Kawabata et al., 1999; Makris & Rossiter, 2002). It may be possible that CB extract would demonstrate some of these activities if tested in additional bioassays.

In general, flavonoids have been associated with sensory, taxonomic, nutritional and pharmacological properties of plants (Monagas et al., 2005). These characteristics of flavonoids are thought to be related to their chemical structure, the variation on the basic structure (hydroxylation, methoxylation etc.), type and position of conjugation (glycosylation, malonylation, sulphonation) and degree of polymerisation (Kumar et al., 2009). Observed bioactivities are thought to arise through biological interaction with these chemical variations on the basic flavonoid skeleton (Havsteen, 1983). For example, the antibacterial activity of flavonoids has been linked to be to their chemical structure, particularly the number and positions of methoxyl and hydroxyl groups (Hidalgo et al., 2010). Flavonoids lacking hydroxyl groups on the B-ring have been shown to have a higher degree of antibacterial activity (Cowan, 1999). However, more hydroxyl substituents on the B-ring has been linked to improved radical scavenging ability (Tsimogiannis & Oreopoulou, 2006). Substitution patterns on the A-ring have also been shown to positively affect the antioxidant activity

of flavonoids (Sghaier et al., 2011). C-ring configuration also appears to be a key factor affecting bioactivity. The presence of a C2-C3 double bond and a carbonyl group at position C4 increases antioxidant activity and the presence of these features is also thought to contribute to increased cytotoxic activity (Santos et al., 2011). An investigation of the antiviral activity of flavonoids on the influenza virus showed that a carbonyl at position C4 and a C2-C3 double bond as well as hydroxylation at the C4' and C7 positions were essential for high viral inhibition. Glycosylation of flavonoids can greatly affect bioactivity. For example, glycosylation has been shown to diminish antioxidant activity (Sghaier et al., 2011), and the presence of a glycosylation group greatly reduced the ability of flavonoids to inhibit neuraminidase action (one of two surface glycoproteins that facilitates movement of the influenza virus) (Liu et al., 2008).

Thus the basic skeleton may be considered an "essential pharmacophore" with the arrangement and type of substituents unique to each individual flavonoid conferring bioactivity of different types. Strong biological activity means that flavonoids may be novel leads for drug development and flavonoids extracted from cultivated plants or obtained as industry waste at low cost, e.g. as byproducts from the citrus industry, have the potential as a low cost feedstock for drug manufacture. Flavonoids are increasingly becoming the subject of medical research (Boudet, 2007; Havsteen, 2002). For example, eriodictyol, while only moderately antifungal (with an IC value of 162.71 mgL^{-1} against *F. graminearum* (a crop pathogenic fungus) (Wang et al., 2010a)), has been shown to significantly suppress diabetes-related lipid peroxidation in rats (Bucolo et al., 2012). It also acts as an agonist of the transient potential vanilloid 1 receptor (TRPV1) which is involved in modulation of pain signalling (Rossato et al., 2011) and could therefore be developed into a future pain management medication. One of its glycosides, glycoside eriodictyol-7-O-glucoside, confers protection against cisplatin-induced toxicity and may have application as a co-administered chemoprotective drug during breast cancer treatment (Hu et al., 2012).

While many flavonoids are highly active in their naturally occurring form, slight modification to the natural structure often increases bioactivity. For example, caffeic acid *n*-butyl ester, a synthetic cinnamic acid derivative, was found to be a highly active in an in vitro study of antiproliferation of human and murine tumour cell lines (Cárdenas et al., 2006). Rutin is an efficient antioxidant and free radical scavenger (Yang et al., 2008), but its activity can be increased by several orders of magnitude by complexation with transition metals such as iron and copper (Afanas'eva et al., 2001).

Catechin has been shown to be the dominant compound present in CB extract. However, the other compounds present in CB may contribute to the observed bioactivity of CB, either individually or synergistically. Biological testing using GIBEX and MIC assays during this study showed CB extract to

have antibacterial, antioxidant and antihelmintic activity as well to be a protease inhibitor (see chapter 3). This activity can be linked to several of the top 20 most abundant compounds. Of these, catechin (Díaz-Gómez et al., 2013; Tamura & Ochiai, 2012), eriodictyol and its glycosides (Chatzopoulou et al., 2010; Liu et al., 2011) and quercetin and its glycosides (Calabrò et al., 2005) are all known to have antibacterial activity. Catechin (Geetha et al., 2004; Lee et al., 2011), marein (Dias et al., 2012), eriodictyol and its glycosides (Chatzopoulou et al., 2010; Koleckar et al., 2008; Yao et al., 2006), myricetin and its glycosides (Wang et al., 2010b), quercetin and its glycosides (Turan et al., 2007; Makris & Rossiter, 2002) and procyanidin B1 (Saito et al., 2005; Saito et al., 2009) have also been shown to have strong antioxidant and free radical scavenging ability. Procyanidin B1 has possible antihelmintic activity against intestinal nematodes (Hoste et al., 2006), which may have resulted in the observed antihelmintic activity of the crude CB extract.

Flavonoids have been shown to interact synergistically in complex plant extracts, conferring greater activity to the crude than can be explained by the sum of the activity of the constituent compounds (Hidalgo et al., 2010).The combination of multiple active compounds could contribute to a greater activity than that demonstrated by any single compound mentioned above. For example, the co-occurrence of quercetin and catechin in an extract has been shown to increase the antioxidant activity of a mixture more than the sum of the antioxidant activity of the two individual compounds (Turan et al., 2007). Similar synergistic effects may exist in the CB extract with its abundance of bioactive flavonoids, but this remains to be investigated.

The majority of the compounds in the CB extract could not be readily identified using the LC-MS technique with reference to available data-bases. These may be novel structures or known structures that have not yet been listed in existing databases and this is clearly a significant limitation of this approach. Identification depends largely on reliable and comprehensive databases which, by virtue of their size and complexity, need to be compiled through effective collaboration of many research groups and institutions. Current projects are underway to do just this, such as PlantCyc and MassBank itself, but they are far from comprehensive. Additional problems in creating such an amalgamated database arise, such as the fact that there is as yet no standardardized methodology for spectral acquisition and lesser known metabolites, or those that are not readily available to purchase, are often neglected, while more common metabolites are repeatedly analysed in different laboratories resulting in multiple database entries under different conditions. The advent of the metabolomics era has stimulated renewed interest in resolving these challenges, but it will require further, concerted effort.

3.3.2 Results of LC-MS spectrometry analysis of PA, CM, CC, and ZF extracts

The remaining four extracts, PA, CM, CC and ZF were investigated on a more preliminary basis than CB. Some of the most abundant compounds found to be present in these extracts were the same compounds found in CB. All of the extracts demonstrated antibacterial activity in assays performed during this study (see chapter 3). All four extracts have among their most abundant compounds known compounds with antibacterial activity (particularly catechin and quercetin derivatives), which could explain the observed bioactivity. ZF extract contains rutin, quercetin, and 5-oxoproline (Urbanek, 2012), and PA extract contains catechin. CM extract contains hyperoside and isorhamnetin-3-O-glycoside (both quercetin glycosides) and CC extract contained both quercetin and quercetin glucuronide. Catechin and quercetin and their derivatives are also known to possess potent antioxidant activity. The presence of these compounds, as well as peltatoside and hyperoside (also known antioxidants (Agbo et al., 2013; Xing 2011)), may also explain the antioxidant activity observed in the four extracts.

Antifungal activity was also observed in two of the extracts - ZF and CC extracts. Quercetin-3-O-rhamnoside has been shown to be antifungal (Miles et al., 2013). Other glycosides of quercetin may have variable antifungal activity, which could explain the observed antifungal activity in CC extract. However, there are many more compounds present in these extracts than this precursory examination covers which could be responsible for observed activity.

Other compounds with the same activities, or different activity not tested for during the course of this study, may also be present. For example, syringetin (syringetin-3-O-glucoside is present in PA extract) has been shown to inhibit the proliferation of colorectal epithelial adenocarcinoma cells. (Gómez-Alonso et al., 2012). Peltatoside (present in CC extract) has been shown to inhibit dipeptidyl-peptidase IV (DP-IV), a key enzyme of blood glucose homoeostasis which suggests it may have application in treatment of diabetes (Eidenberger et al., 2013). Hyperoside (present in CM extract) demonstrated antithrombotic activity and has the potential to be developed into an anticoagulant (Ku 2013) as well as having an osteogenic effect, with potential application in treatment of osteoporosis (Yang et al., 2011). Essential oils containing D-limonene have been shown to have antifungal, antimicrobial and antitumor activity (Graebin et al., 2010) and it stands to reason that derivatives of limonene, such as that present in ZF extract, may also possess similar activity. Further study of these extracts using a similar whole-extract analysis approach to that used to analyse the CB extract may yield interesting results in the future.

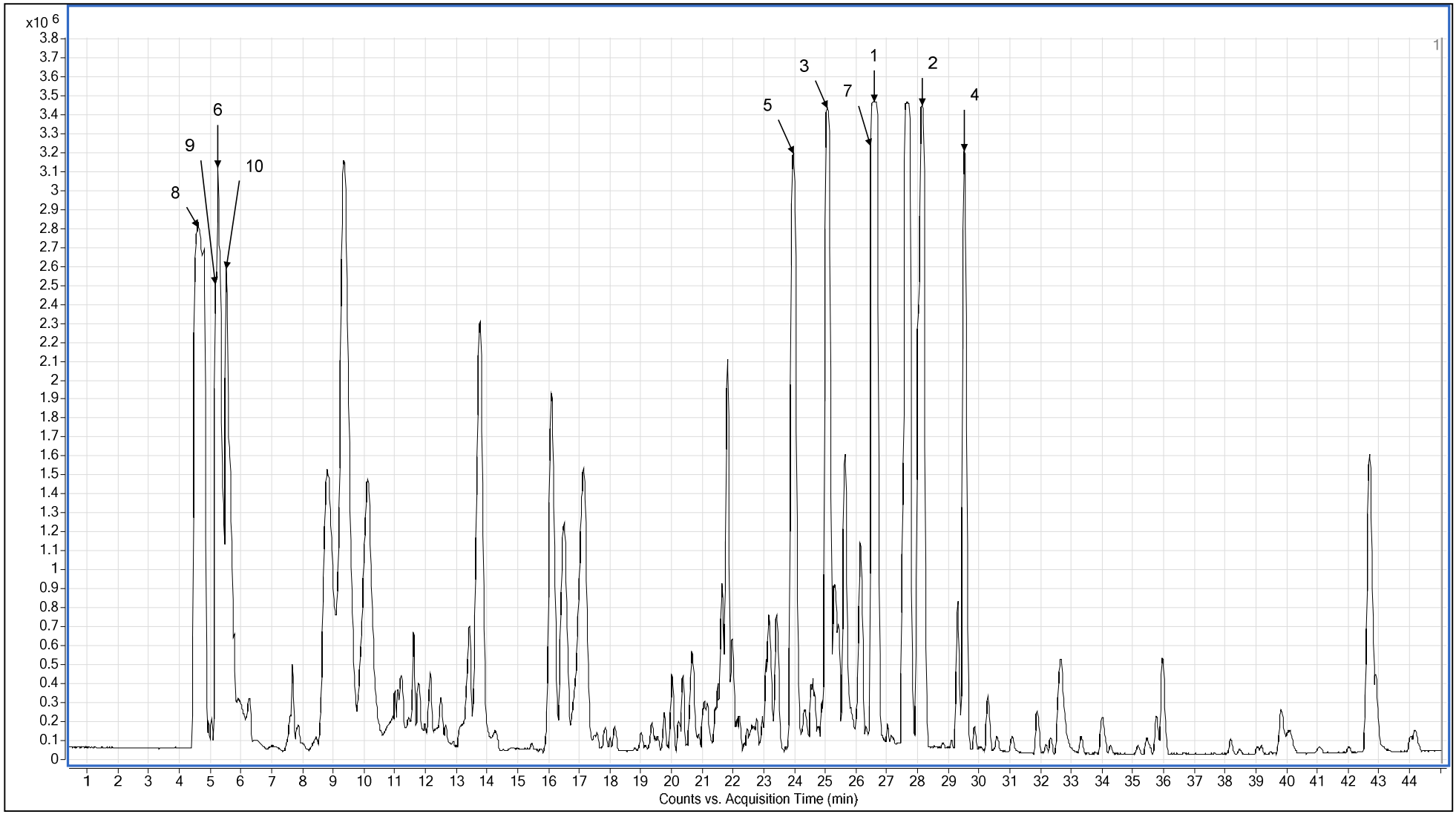


Figure 24: Base peak chromatogram of *P. albiflora* obtained by HPLC-MS in positive-ion mode. Peak labelling designates the most abundant identified ions (See Table 7). Numbers not indicating a specific peak are signals co-eluting with more intense signals.

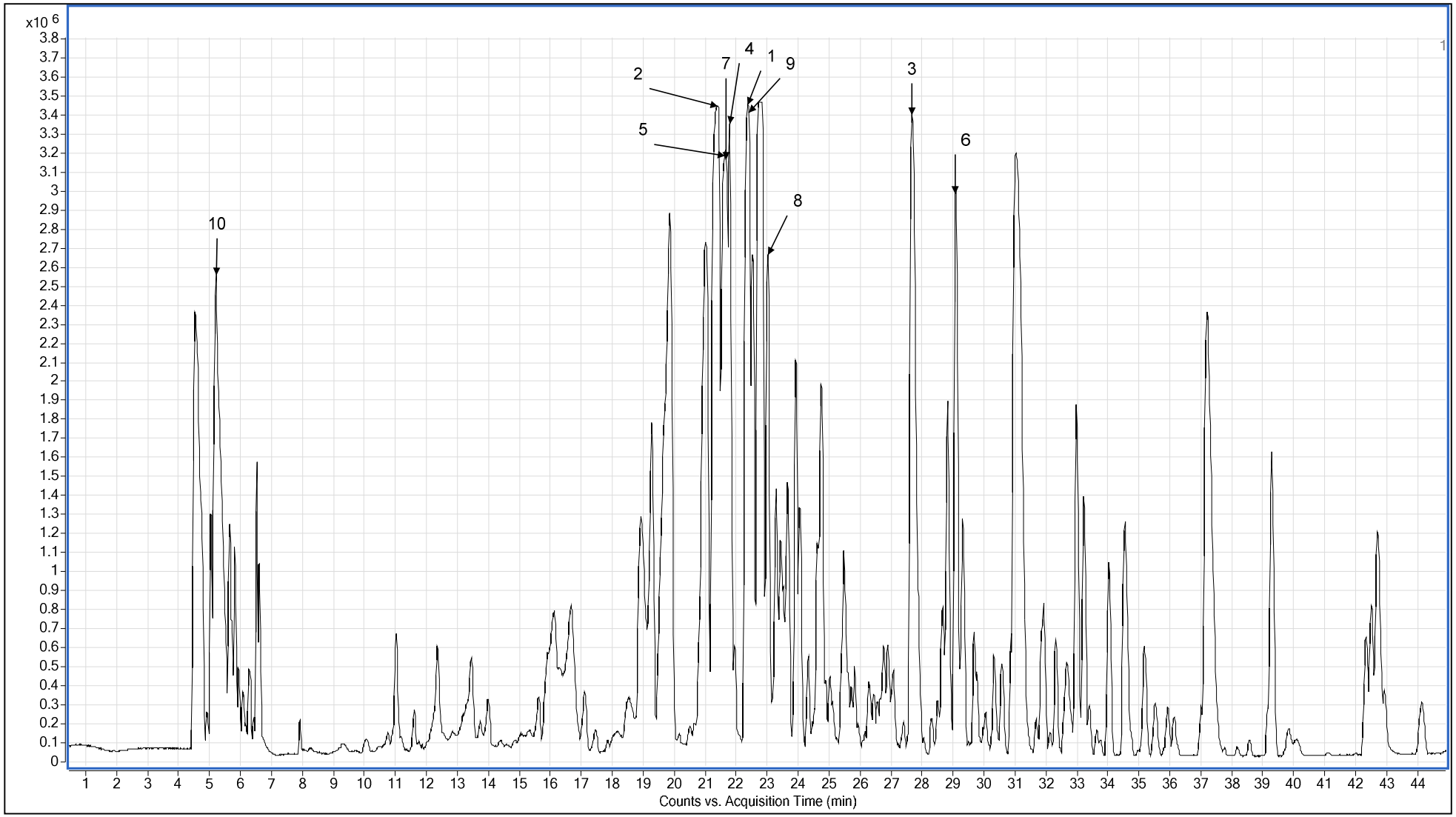


Figure 25: Base peak chromatogram of *C. muscosa* obtained by HPLC-MS in positive-ion mode. Peak labelling designates the most abundant identified ions (see Table 8). Numbers not indicating a specific peak are signals co-eluting with more intense signals.

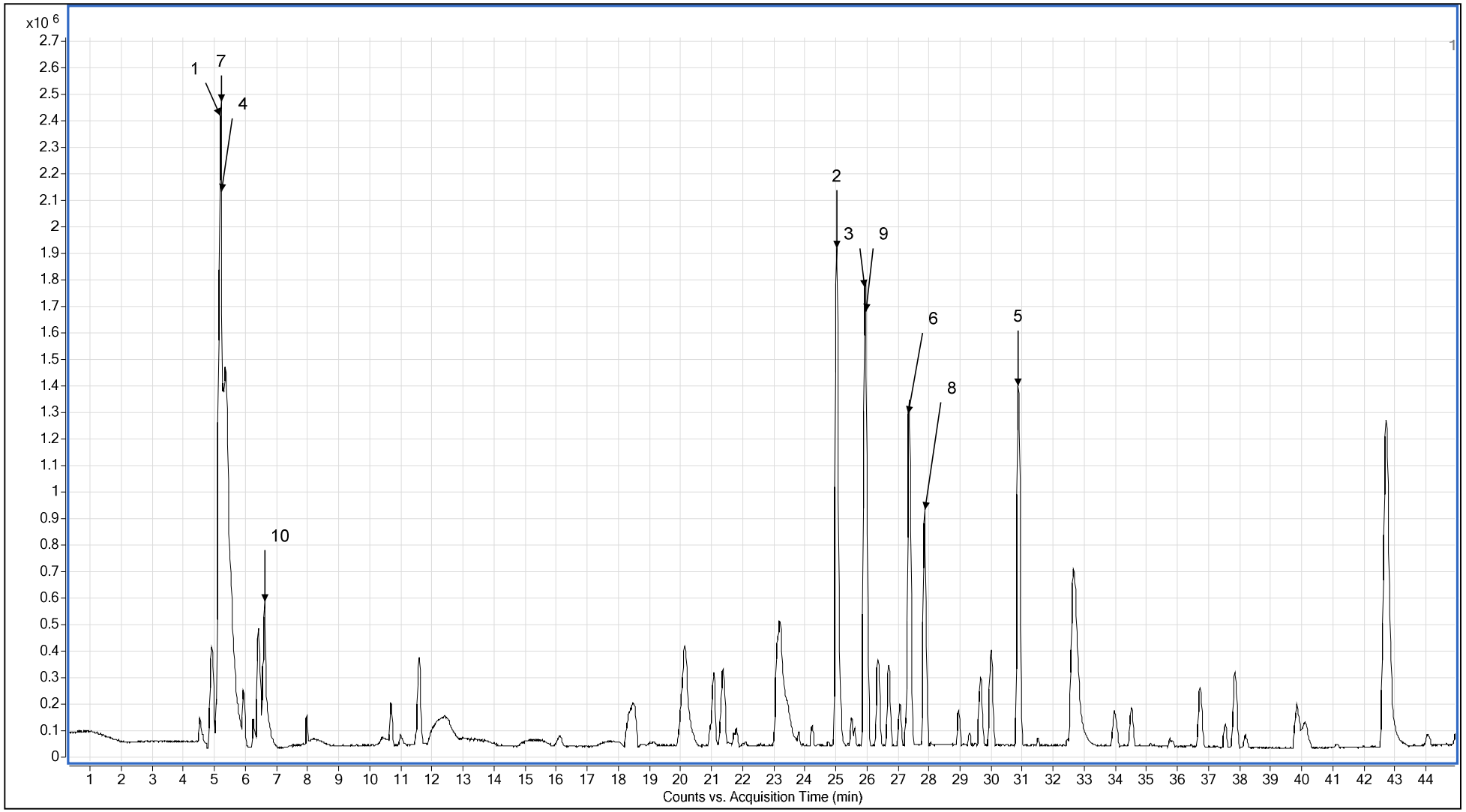


Figure 26: Base peak chromatogram of *C. cuneata* obtained by HPLC-MS in positive-ion mode. Peak labelling designates the most abundant identified ions (see Table 9). Numbers not indicating a specific peak are signals co-eluting with more intense signals.

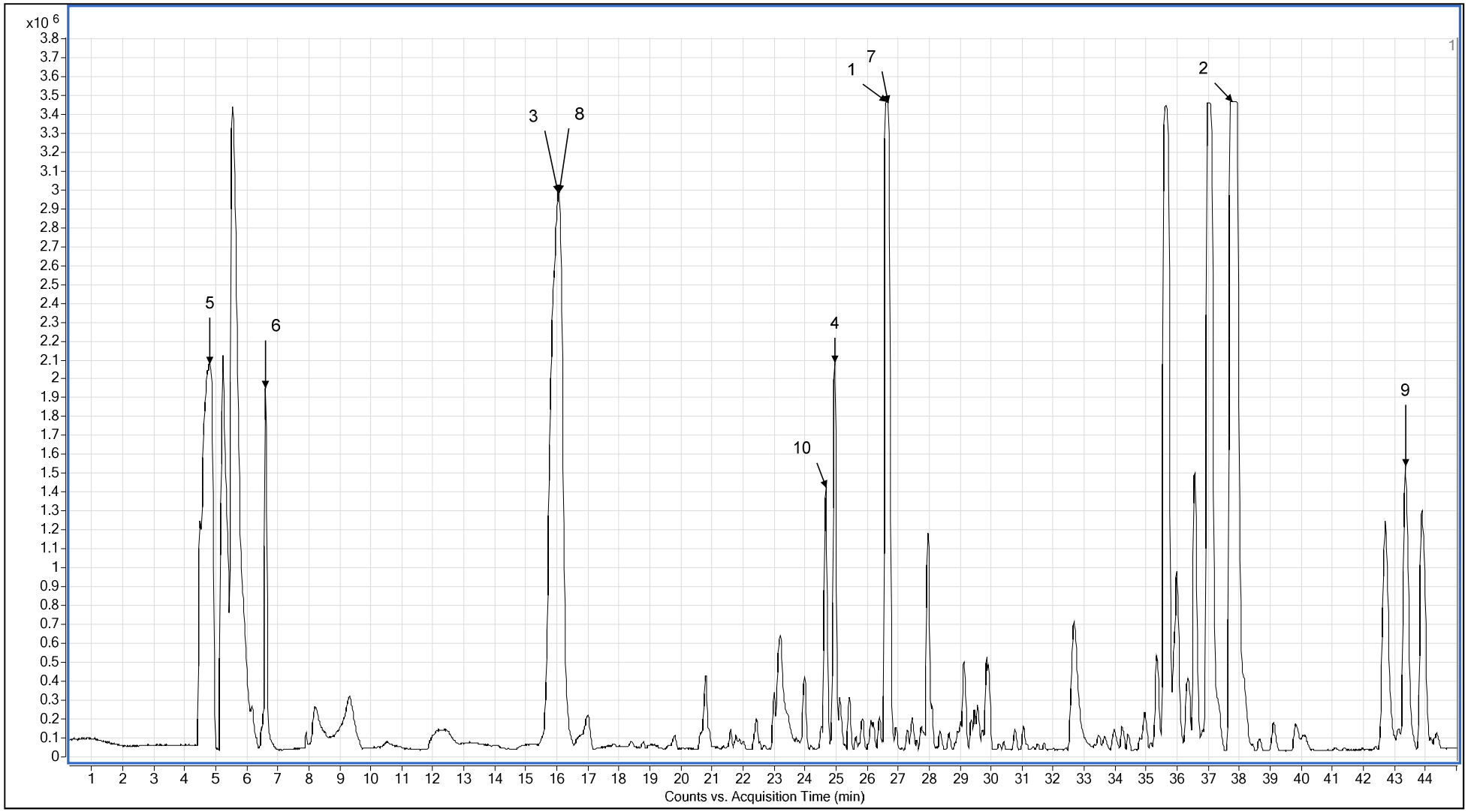


Figure 27: Base peak chromatogram of *Z. foetidum* obtained by HPLC-MS in positive-ion mode. Peak labelling designates the most abundant identified ions (see Table 10). Numbers not indicating a specific peak are signals co-eluting with more intense signals.

Table 7: The top 10 most abundant compounds present in the crude extract of *P. albiflora*.

Plant	No.	m/z	Neutral mass	RT	Proposed formula	Mass difference	Isotope match score (%)	Tentative identification	Database match score (%)	Major fragments
PA	1	495.1202	494.1129	27.65	$C_{29}H_{14}O_3N_6$ $C_{16}H_{22}O_{14}N_4$	0.0002 0.0004	no score	Unknown	-	495, 333, 97, 85
PA	2	655.1876	654.1803	28.15	$C_{47}H_{26}O_4$ $C_{42}H_{26}O_6N_2$ $C_{43}H_{22}O_2N_6$	0.0028 0.0012 0.0001	94.47 87.10 86.81	Unknown	-	655, 509, 347, 332, 85, 71
PA	3	292.0934	291.0861	25.08	no formulae	-	no score	Catechin ($C_{15}H_{14}O_6$)	64.57	292, 208/207, 166, 148, 139, 123
PA	4	509.1296	508.1223	29.52	$C_{36}H_{16}O_2N_2$ $C_{23}H_{24}O_{13}$ $C_{24}H_{20}O_9N_4$	0.0011 0.0006 0.0007	95.60 81.35 80.74	Syringetin-3-O-glucoside ($C_{23}H_{24}O_{13}$)	82.61	347, 332, 85
PA	5	581.1616	580.1543	23.97	$C_{38}H_{20}O_3N_4$	0.0008	65.42	Unknown	-	581/580, 429, 411, 291, 139, 127
PA	6	217.0684	216.0611	5.26	No formula	-	No score	Unknown	-	217, 199, 127, 98
PA	7	642.1757	641.1684	26.59	$C_{21}H_{31}O_{18}N_5$ $C_{22}H_{27}O_{14}N_9$ $C_{26}H_{31}O_{16}N_3$ $C_{34}H_{23}O_7N_7$	0.0020 0.0007 0.0020 0.0025	93.33 93.05 91.74 81.33	Unknown	-	642, 496, 334, 318, 85, 71
PA	8	110.0092	109.0019	4.58	No formulae	-	No score	Unknown	-	110, 68, 66
PA	9	203.0524	202.0451	5.28	$C_4H_6O_4N_6$	0.0000	no score	Unknown	-	203, 110, 98, 68
PA	10	104.1075	103.1002	5.23	$C_5H_{13}NO$	0.0005	97.16	Choline ($C_5H_{14}ON$)	99.51	104, 60, 59, 58

Table 8: The top 10 most abundant compounds present in the crude extract of *C. muscosa*.

Plant	No.	m/z	Neutral mass	RT	Proposed formula	Mass difference	Isotope match score (%)	Tentative identification	Database match score (%)	Major fragments
CM	1	641.1719	640.1646	22.37	no formulae	-	no score	Unknown	-	641, 479, 461, 359, 91, 85, 61
CM	2	329.0889	328.0816	21.4	$C_{16}H_8O_1N_8$ $C_{15}H_{12}O_5N_4$	0.0005 0.0008	no score	Unknown	-	329, 275, 263, 247, 209, 181
CM	3	465.1035	464.0962	27.69	$C_{21}H_{20}O_{12}$ $C_{22}H_{16}O_8N_4$	0.0007 0.0006	91.95 91.33	Hyperoside ($C_{21}H_{20}O_{12}$)	91.09	465, 303, 127
CM	4	511.2289	510.2216	21.79	$C_{24}H_{34}O_{10}N_2$ $C_{25}H_{30}O_6N_6$ $C_{26}H_{26}O_2N_{10}$ $C_{36}H_{30}O_3$	0.0003 0.0011 0.0024 0.0021	93.76 93.08 92.45 89.77	Unknown	-	511, 205, 188, 146, 118
CM	5	145.0859	144.0786	21.66	$C_7H_{12}O_3$	0.0000	97.44	Unknown	-	145, 127, 109, 99, 81, 71
CM	6	479.1193	478.112	29.1	$C_{35}H_{14}O_1N_2$ $C_{22}H_{22}O_{12}$ $C_{23}H_{18}O_8N_4$ $C_{24}H_{14}O_4N_8$	0.0014 0.0005 0.0009 0.0018	92.03 91.36 90.47 83.42	Isorhamnetin-3-O-glucoside ($C_{22}H_{22}O_{12}$)	89.09	479, 317, 302, 285, 85
CM	7	127.0754	126.0681	21.66	$C_7H_{10}O_2$	0.0000	98.88	Unknown	-	127, 109, 81, 71, 53
CM	8	655.1508	654.1435	23.02	$C_{25}H_{22}O_{12}N_{10}$ $C_{28}H_{30}O_{18}$ $C_{29}H_{26}O_{14}N_4$ $C_{16}H_{34}O_{25}N_2$ $C_{12}H_{30}O_{23}N_8$	0.0016 0.0003 0.0011 0.0016 0.0011	95.81 94.59 92.54 89.61 82.46	Unknown	-	655, 479, 461, 359
CM	9	199.06	198.0527	22.54	$C_9H_{10}O_5$	0.0001	97.20	Unknown	-	199, 167, 155, 137, 125, 107, 97, 77, 65, 55
CM	10	104.1074	103.1001	5.21	$C_5H_{13}ON$	0.0004	98.91	Choline ($C_5H_{14}ON$)	99.51	104, 60, 59, 58

Table 9: The top 10 most abundant compounds present in the crude extract of *C. cuneata*.

Plant	No.	m/z	Neutral mass	RT	Proposed formula	Mass difference	Isotope match score (%)	Tentative identification	Database match score (%)	Major fragments
CC	1	104.1075	103.1002	5.21	$C_5H_{13}ON$	0.0005	96.28	Choline ($C_5H_{14}ON$)	99.51	104, 60, 59, 58
CC	2	449.1078	448.1005	25.03	$C_{21}H_{20}O_{11}$ $C_{18}H_{12}O_5N_{10}$ $C_{22}H_{16}O_7N_4$ $C_{34}H_{12}N_2$	0.000 0.0013 0.0014 0.0005	96.95 93.99 91.23 81.24	Unknown	-	147, 119, 91
CC	3	303.0501	302.0428	25.95	$C_{15}H_{10}O_7$ $C_{12}H_2O_1N_{10}$ $C_{16}H_6O_3N_4$	0.0001 0.0015 0.0012	94.79 93.40 91.83	Quercetin ($C_{15}H_{10}O_7$)	90.15	303, 229, 153, 137, 68
CC	4	249.0371	248.0298	5.36	$C_8H_4O_4N_6$	0.0004	94.18	Unknown	-	249, 219, 158, 130, 98
CC	5	440.2494	439.2421	30.86	$C_{19}H_{37}O_{10}N$ $C_{20}H_{33}O_6N_5$	0.0004 0.0010	91.97 90.17	Unknown	-	440, 295, 271, 159, 163, 153, 145, 127, 85, 69
CC	6	567.1343	567.1343	27.36	$C_{22}H_{18}O_9N_{10}$ $C_{25}H_{26}O_{15}$ $C_{26}H_{22}O_{11}N_4$ $C_9H_{26}O_{20}N_8$	0.0012 0.0002 0.0015 0.0007	95.19 95.15 93.17 82.40	Unknown	-	567, 435, 303, 229, 211, 115, 97
CC	7	233.0632	232.0559	5.26	$C_5H_8O_5N_6$ $C_6H_4O_1N_{10}$	0.0003 0.0011	91.88 80.21	Unknown	-	233, 110, 82, 68, 55
CC	8	479.0822	478.0749	27.86	$C_{21}H_{18}O_{13}$ $C_{22}H_{14}O_9N_4$ $C_{18}H_{10}O_7N_{10}$	0.0002 0.0012 0.0015	95.61 93.73 90.46	Quercetin-3-glucuronide ($C_{21}H_{18}O_{13}$)	88.16	302, 274, 229, 153, 85, 61
CC	9	597.1451	596.1378	25.95	$C_{26}H_{28}O_{16}$ $C_{10}H_{28}O_{21}N_8$	0.0001 0.0009	94.59 81.78	Peltatoside	82.58	597, 435, 303
CC	10	140.1431	139.1358	6.62	$C_9H_{17}N$	0.0003	98.45	*Unknown: possibly Decahydroquinoline*	98.00	140/139, 68, 53

Table 10: The top 10 most abundant compounds present in the crude extract of *Z. foetidum*.

Plant	No.	m/z	Neutral mass	RT	Proposed formula	Mass difference	Isotope match score (%)	Tentative identification	Database match score (%)	Major fragments
ZF	1	611.1614	610.1541	26.63	$C_{41}H_{18}ON_6$ $C_{27}H_{30}O_{16}$	0.0001 0.0007	84.20 69.41	Rutin ($C_{27}H_{30}O_{16}$)	92.73	611, 465, 303
ZF	2	439.3687	438.3614	37.83	$C_{29}H_{46}ON_2$	0.0004	no score	Unknown	-	
ZF	3	120.0806	119.0733	16.06	C_8H_9N	0.0002	92.15	Unknown	-	120, 103, 91, 77
ZF	4	169.1216	168.1143	24.96	$C_{10}H_{16}O_2$	0.0007	98.72	Limonene dioxide	72.65	151, 137, 123, 109, 85, 81, 69, 57
ZF	5	214.9175	213.9102	4.67	no formula	-	No score	Unknown	-	214, 170, 140, 84
ZF	6	130.0496	129.0423	6.61	$C_5H_7O_3N$	0.0003	98.06	5-oxoproline ($C_5H_7O_3N$)	78.38	130, 86, 84
ZF	7	303.0496	302.0423	26.64	$C_{15}H_{10}O_7$ $C_{12}H_2O_1N_{10}$	0.0004 0.0010	95.96 93.78	Quercetin ($C_{15}H_{10}O_7$)	90.15	303, 229, 153
ZF	8	166.0856	165.0783	16.07	$C_9H_{11}O_2N$	0.0007	94.19	Phenylalanine ($C_9H_{11}O_2N$)	94.51	121/120, 103, 93
ZF	9	439.3572	438.3499	43.36	$C_{30}H_{46}O_2$	0.0001	95.69	Unknown	-	
ZF	10	400.2178	399.2105	24.67	$C_{16}H_{33}O_{10}N$ $C_{17}H_{29}O_6N_5$	0.0001 0.0013	93.20 91.44	Unknown	-	400, 295, 251, 163, 145,85

3.4 Whole extract NMR spectroscopy -based fingerprinting

NMR fingerprinting was performed on each of the five crude ethanolic plant extracts of *Crassula muscosa* (CM), *Crassula brevifolia* (CB), *Crassula cuneata* (CC), *Polymita albiflora* (PA) and *Zygophyllum foetidum* (ZF) in an attempt to characterise the major classes of metabolites present in each extract, and possibly provide tentative identification of certain metabolites possessing characteristic NMR peaks. In addition, multivariate statistical analysis was performed using NMR spectral data in order to compare extracts.

3.4.1 Visual analysis of spectra

^{1}H NMR spectra of the five crude extracts under investigation are shown in Figure 28. The spectra of the five extracts are understandably complex, but with identifiable differences and similarities. The signals are clustered in three major regions: an aliphatic region (δ0.0 - δ3.0), a region corresponding

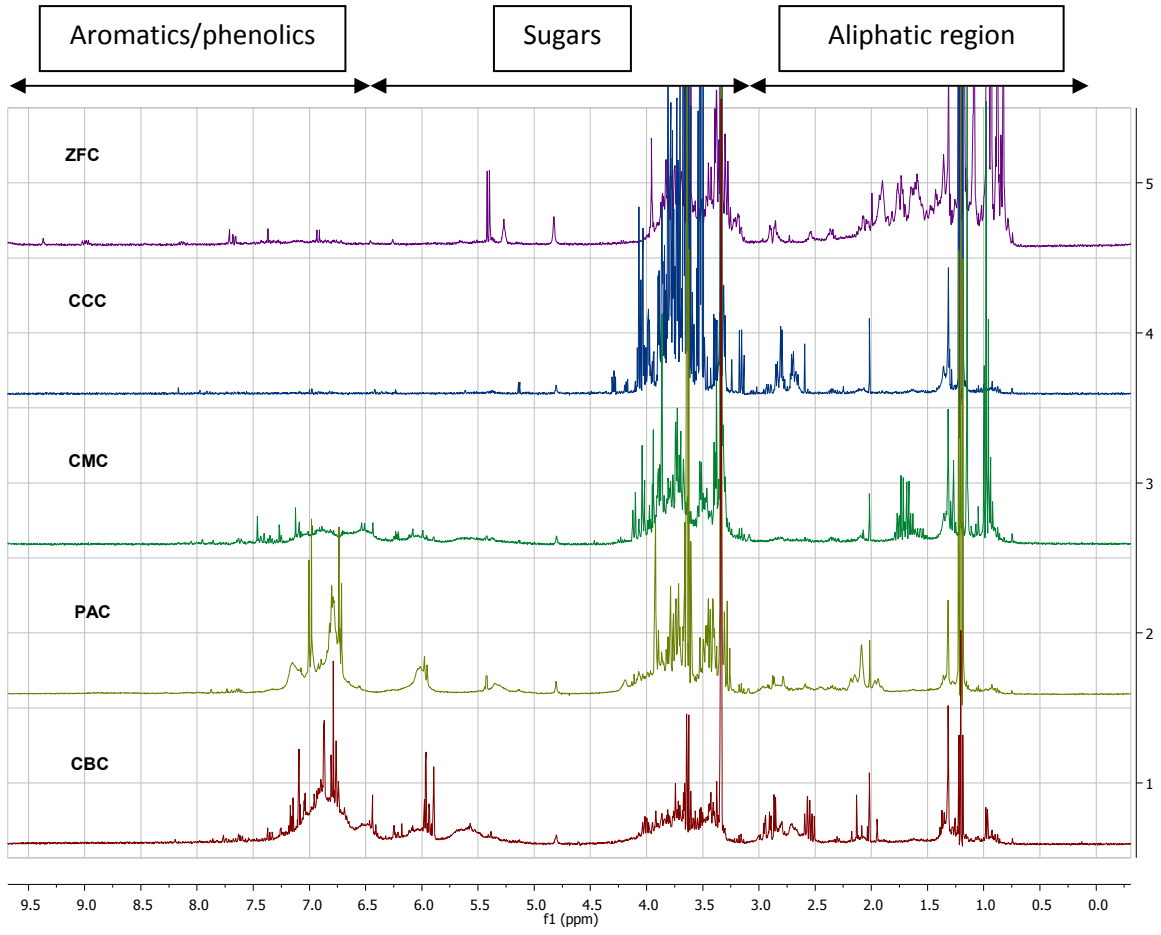


Figure 28: All ^{1}H NMR spectra stacked to show commonalities and differences between species (CBC = *C. brevifolia*, PAC = *P. albiflora*, CCC = *C. cuneata*, CMC = *C. muscosa*. ZFC = *Z. foetidum*) (solvent is methanol-d4).

to moderately deshielded protons, as found in oxygenated hydrocarbons and sugars (δ3.0 - δ5.5) and an unsaturated/aromatic/phenolic region (δ5.5 - δ8.0) (Figure 28). The spectra also show residual solvent peaks of ethanol (a triplet at δ1.2 and a quartet at δ3.6) as well as a peak at δ3.3 which is characteristic of methanol and results from impurities in the deuterated solvent used for NMR analysis.

Polar plant extracts of different plant tissues have characteristic NMR spectra determined by compounds specific to those organs and the functions they perform (Le Gall et al., 2003; Fan et al., 1997; Fan et al., 2001). Visual analysis shows that the experimentally determined spectra are typical of those of most green tissue polar extracts i.e. they are dominated by signals from carbohydrates (sugars), amino acids and organic acids (Choi et al., 2004). While high concentrations of these primary metabolites are present in all spectra, varying concentrations of other types of compounds, such as aromatics, are indicated by the spectra. An initial visual inspection of the spectra suggests that *Crassula brevifolia* (CB) and *Polymita albiflora* (PA) are the most similar across all regions, implying that they contain similar types of compounds of all classes. Additional similarities and differences exist between spectra, as detailed in the following paragraphs.

3.4.1.1 "Aliphatic" region

An expansion of the aliphatic region of each extract's spectrum is given in Figure 29. This region contains many signals from non-aromatic amino acids (such as alanine and leucine) and organic acids (such as citric, acetic and succinic acids) (Yang et al., 2012). No single amino acids or organic acids could be unequivocally assigned due to signal overlap in this region. The strong signal around δ1.29 - δ1.30 present in all spectra could be attributed to fatty acids, but could not be attributed to individual fatty acids (Farag et al., 2012). Common fatty acids may be saturated or simple unsaturated C16 or C18 chains e.g. stearic acid (saturated) and linoleic acid (unsaturated) (Harborne, 1998). Multiple methylene and methine signals from such chains result in large distinctive signals such as those visible in the spectra. The clear signal at δ2.02 present in all spectra may also result from methylene signals of unsaturated fatty acid chains (Frédérich et al., 2004).

In, general, *Zygophyllum foetidum* (ZF) and *Crassula muscosa* (CM) appear to be the most aliphatic-compound rich, displaying signals in this region of the spectrum (particularly in the region between δ0.7 - δ1.8) that are absent from *Crassula cuneata* (CC), PA and CB. CC, PA and CB do display signals in this region, but further downfield in the region δ2.5 - δ2.9.

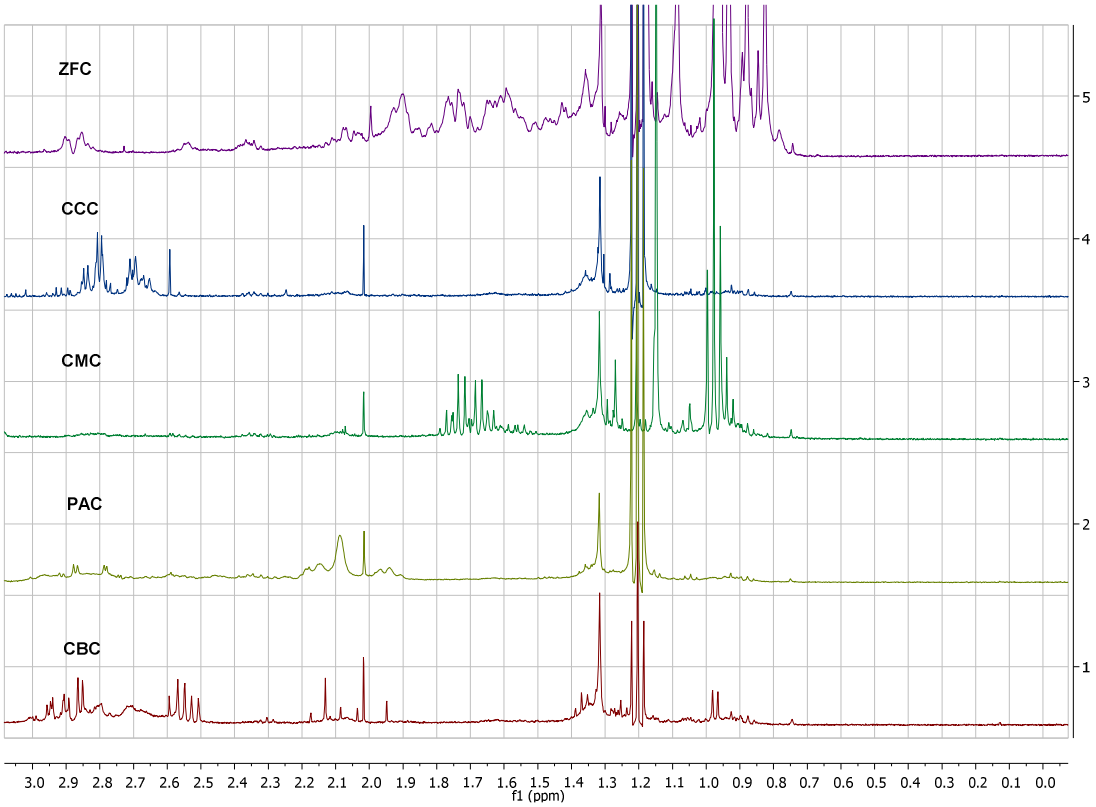


Figure 29: Expansion and comparison of the aliphatic regions of the five ^{1}H NMR spectra

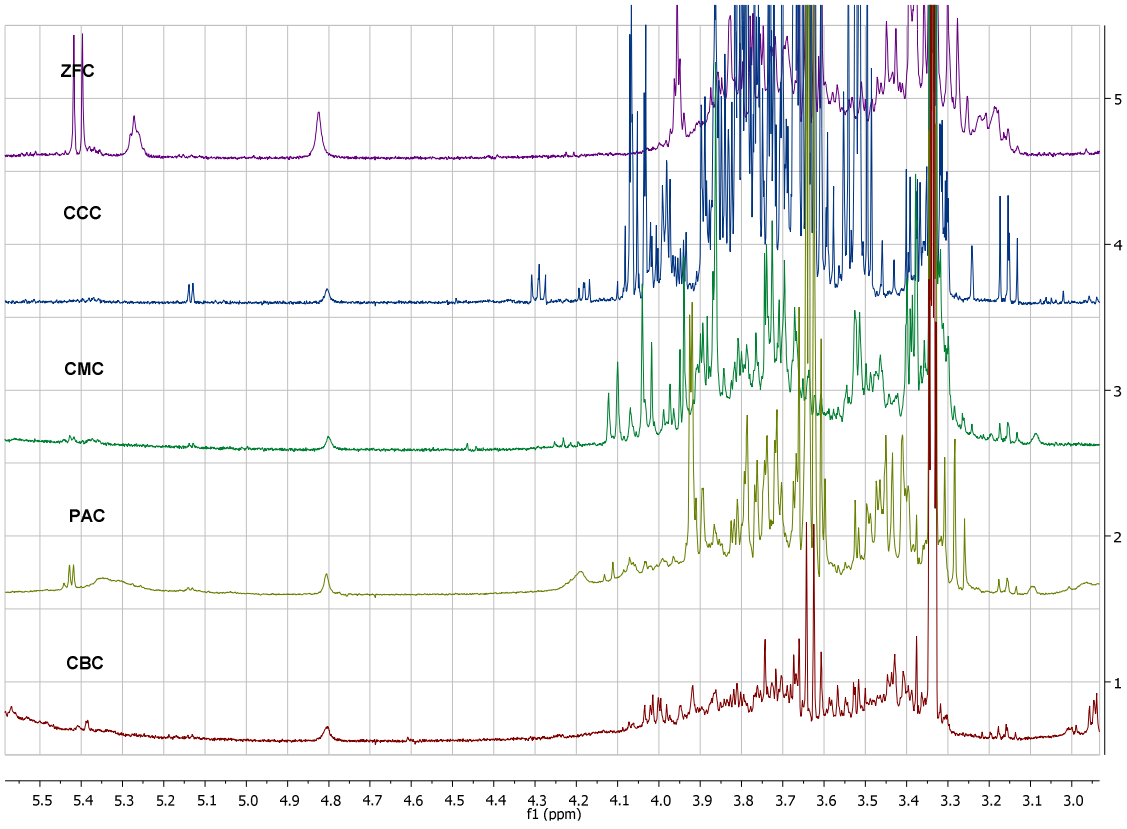
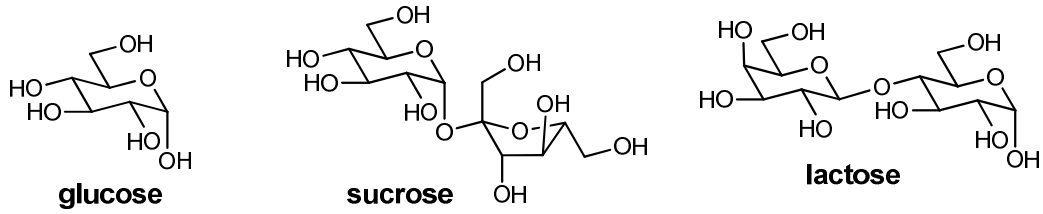


Figure 30: Expansion and comparison of the "sugar" regions of the five ^{1}H NMR spectra

CB, PA and ZF also show additional signals in the region between δ1.8 and δ2.5, which are absent in CC and CM. This suggests that CB, PA and ZF contain aliphatic compounds of a different type to those present in CC and CM. These additional compounds are most likely to have a higher degree of saturation (e.g. saturated fatty acids) or may be unsaturated aliphatic compounds containing electronegative atoms (such as in saponins, which are polar glycosides of oxygenated triterpenes and steroids) (Oleszek & Bialy, 2006). These species, particularly ZF, demonstrated presence of saponins during preparation of extracts from fresh material by producing foam in aqueous solution. Additionally, one of the traditional uses for ZF is as soap (Table 1).

3.4.1.2 "Sugar" region[1]

An expansion of the sugar region of each extract's spectrum is given in Figure 30. This region is busy in all spectra with many overlapping signals, strongly indicating the presence of glycosylated metabolites and free sugars (Moco et al., 2008). CC appears to have the highest concentration of sugars, while ZF the lowest. Sugars are usually the most common metabolites in polar plant extracts and can often be identified from the crude NMR spectrum by the distinctive shifts and coupling constants of their anomeric protons e.g. sucrose at δ5.40 (*d*, *J*=3.82 Hz), fructose at δ4.16 (*d*, *J*=8.6 Hz), α-glucose at δ5.18 (*d*, J=3.9 Hz) and β- glucose at δ4.58 (*d*, J= 7.8 Hz) (Gerogiev et al., 2011; Choi & Yoon, 2007). However, in this instance, only the characteristic doublet of the ubiquitous disacharride sucrose was clearly visible in CB (δ5.39), PA (δ5.42) and CM (δ5.42) (Kim et al., 2010b). Additionally, lactose was tentatively identified in CB and CM with clear signals at δ3.65 and δ3.22 (Tarachiwin et al., 2008) and α-glucose was identified from signals present in CC, PA and CM (Liang et al., 2006). These ubiquitous mono- and disaccharides were possibly present in concentrations too low for detection in all samples.



In general, the sugar region appears to follow a similar pattern for all spectra (except in the spectrum of CC) - that is a region of intense signals between δ3.25 and δ3.55, followed by a local absence of signals, followed by a second region of intense signals between δ3.58 and δ4.01. CM and CC appear to have an additional region of sugar signals not observed in CB and ZF between δ4.11

[1] This region is referred to as the "sugar region" even though the signals encompassed in the region do not originate exclusively from sugar molecules. This is following the convention of Kim et al., 2010a.

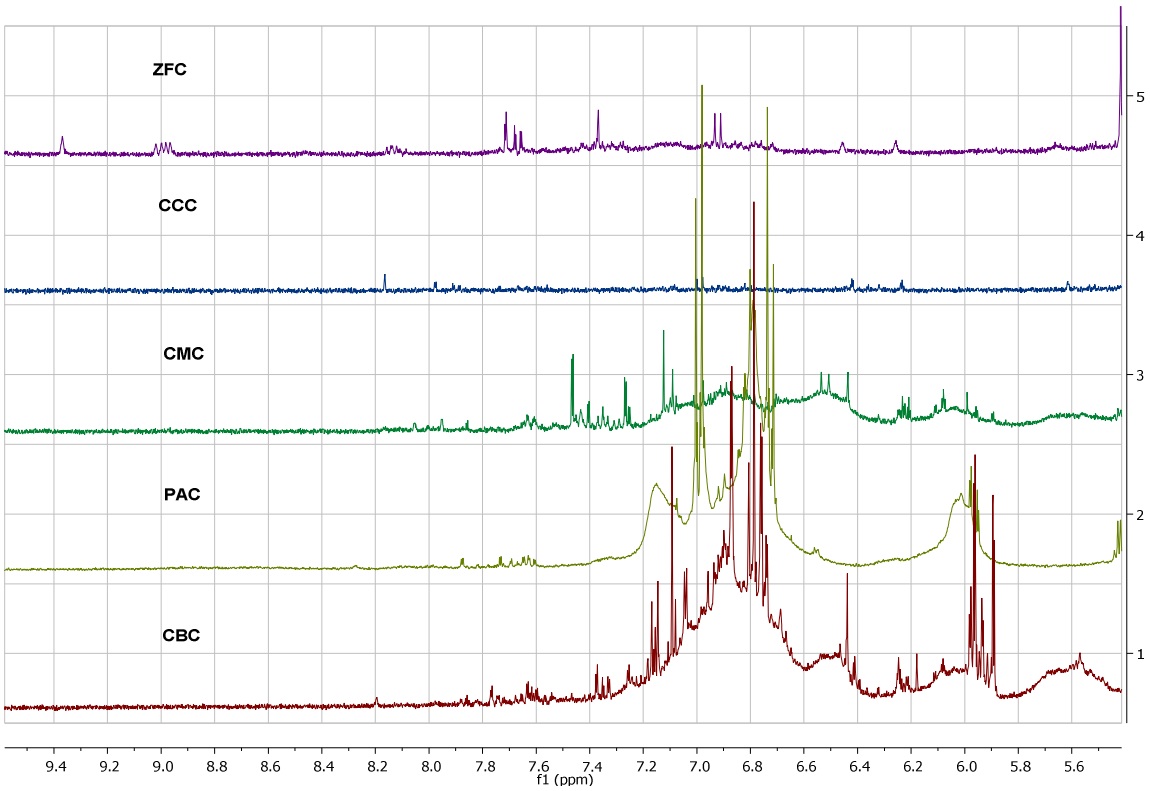


Figure 31: Expansion and comparison of the "aromatic/phenolic" regions of the five NMR spectra

and δ4.3 (which could be due to sugars such as fructose). A few weak signals are observed in this additional sugar area of the spectrum of PA, but the concentrations are extremely low. This suggests that CC and CM are more sugar rich than the other species investigated. The additional sugar signals present in the spectra of CC, CM and to a lesser extent PA indicate either a higher *concentration* of carbohydrate compounds or a wider *variety* of these types of compounds may be present in these extracts. In addition, some organic acids show chemical shifts in this region of the spectrum e.g. malic acid (δ4.34, *dd J*=6.6 Hz, 4.7 Hz) which may be present in these extracts, but this cannot be confirmed without further analysis (Yang et al., 2012). PA and CB also have an additional region of intense signals even further downfield towards the aromatic region (between δ5.8 - δ6.0), which is absent in the other spectra. This in combination with presence of signals in the aromatic region of the spectrum (see 3.4.1.3) region could indicate the presence of hydroxycinnamic acids, particularly those conjugated with tartaric acid (Ali et al., 2011).

3.4.1.3 "Unsaturated/Aromatic/Phenolic region"

An expansion of the "aromatic/phenolic" region of each extract's spectrum is given in Figure 31. CB and PA, and to a lesser extent CM, contain the highest concentration of aromatic compounds, as evident by the large number of intense signals in this region (particularly the region between δ6.1 -

δ7.3). ZF and CC contain the lowest concentrations of aromatic compounds, with CC being almost devoid of signals in this region of the spectrum. Signals in the aromatic/phenolic region of the spectrum may be due to primary metabolites such as aromatic amino acids e.g. tyrosine and tryptophan, as well as phenylpropanoids such as coumaric and caffeic acids and their tartrate esters (Crozier et al., 2006) among others. The possibility of the presence of phenylpropanoids (hydroxycinnamic acids) was suggested in the previous paragraph, but this could not be confirmed from ^{1}H NMR spectra alone. Certain hydroxybenzoic acids also give signals in this region of the spectrum, such as syringic acid and gallic acid, which was tentatively identified from the strong singlet at δ7.04 in the spectrum of CB.

The combination of high concentrations of sugar signals and numerous strong signals in the aromatic region of the spectra of CM, CB and PA suggest that these extracts contain glycosidic flavonoids. Most flavonoids in plant cells are present as glycosides (Stobiecki, 2000). Sugar substitution on the flavonoid skeleton may occur at different positions, resulting in hundreds of variations of the same few skeletons. For example, there are over 200 variations of the flavonol kaempferol alone (Crozier et al. 2006). While it is doubtless than many classes of flavonoid and the multiple variations thereof are present in these extracts, none could be identified from the ^{1}H NMR spectra alone. Additionally, the lack of strong downfield signals in the region δ9.00 - δ11.00 suggests a general absence of alkaloids from the extracts.

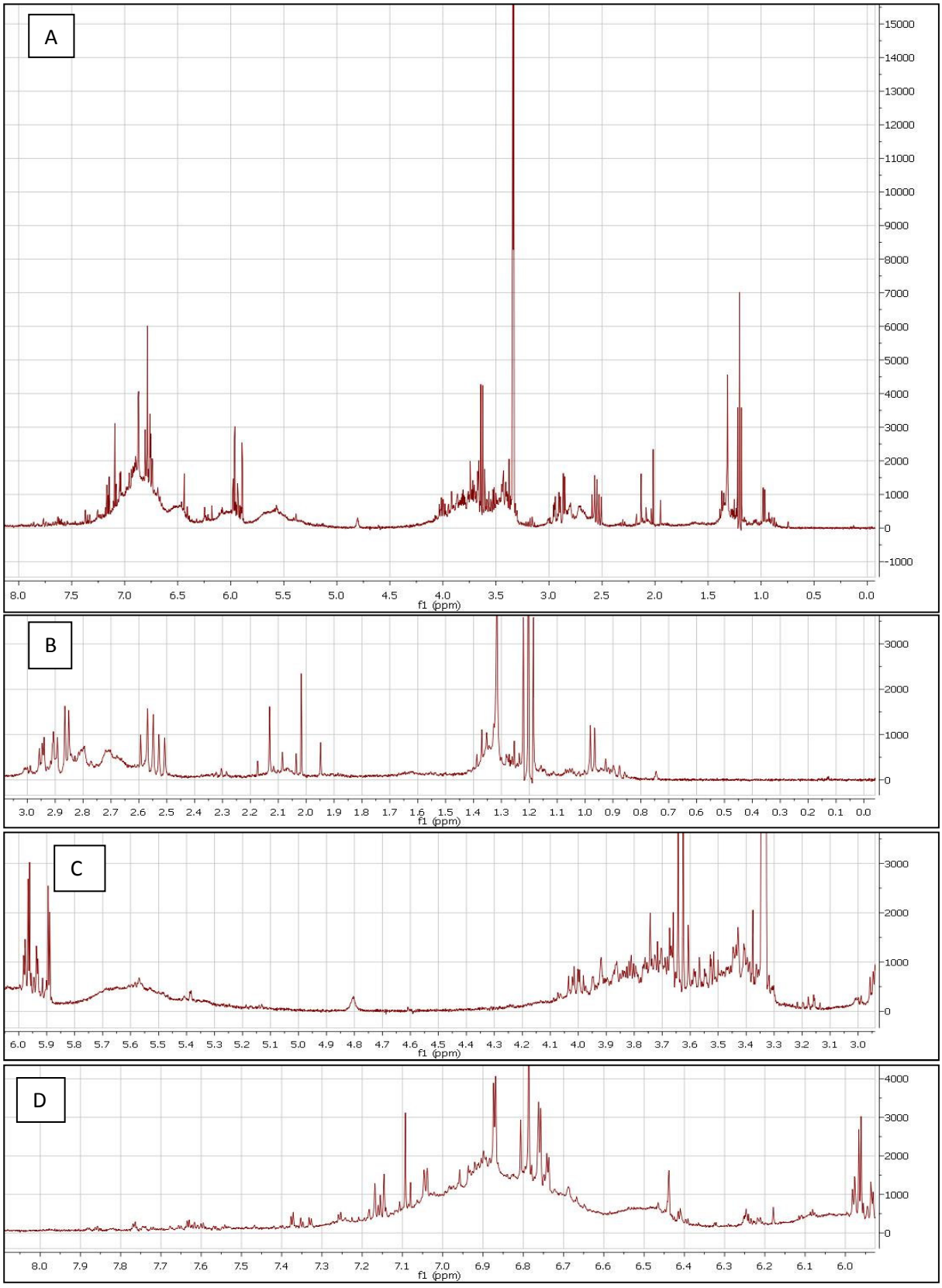


Figure 32: ^{1}H NMR spectrum of *C. brevifolia* recorded in methanol-d4, showing (A) the full spectrum and (B-D) expansions of the main spectral regions.

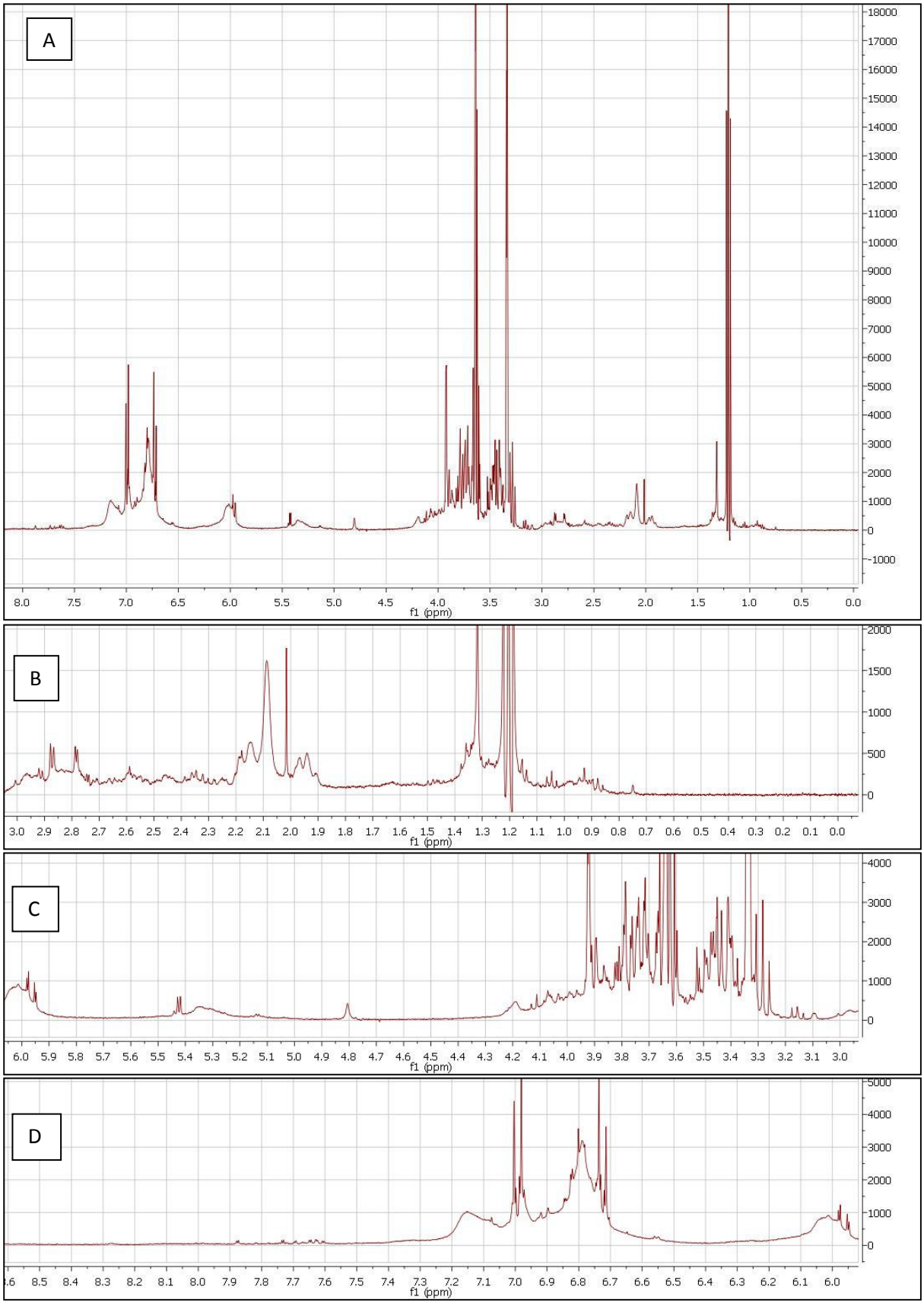


Figure 33: ^{1}H NMR spectrum of *P. albiflora* recorded in methanol-d4, showing (A) the full spectrum and (B-D) expansions of the main spectral regions.

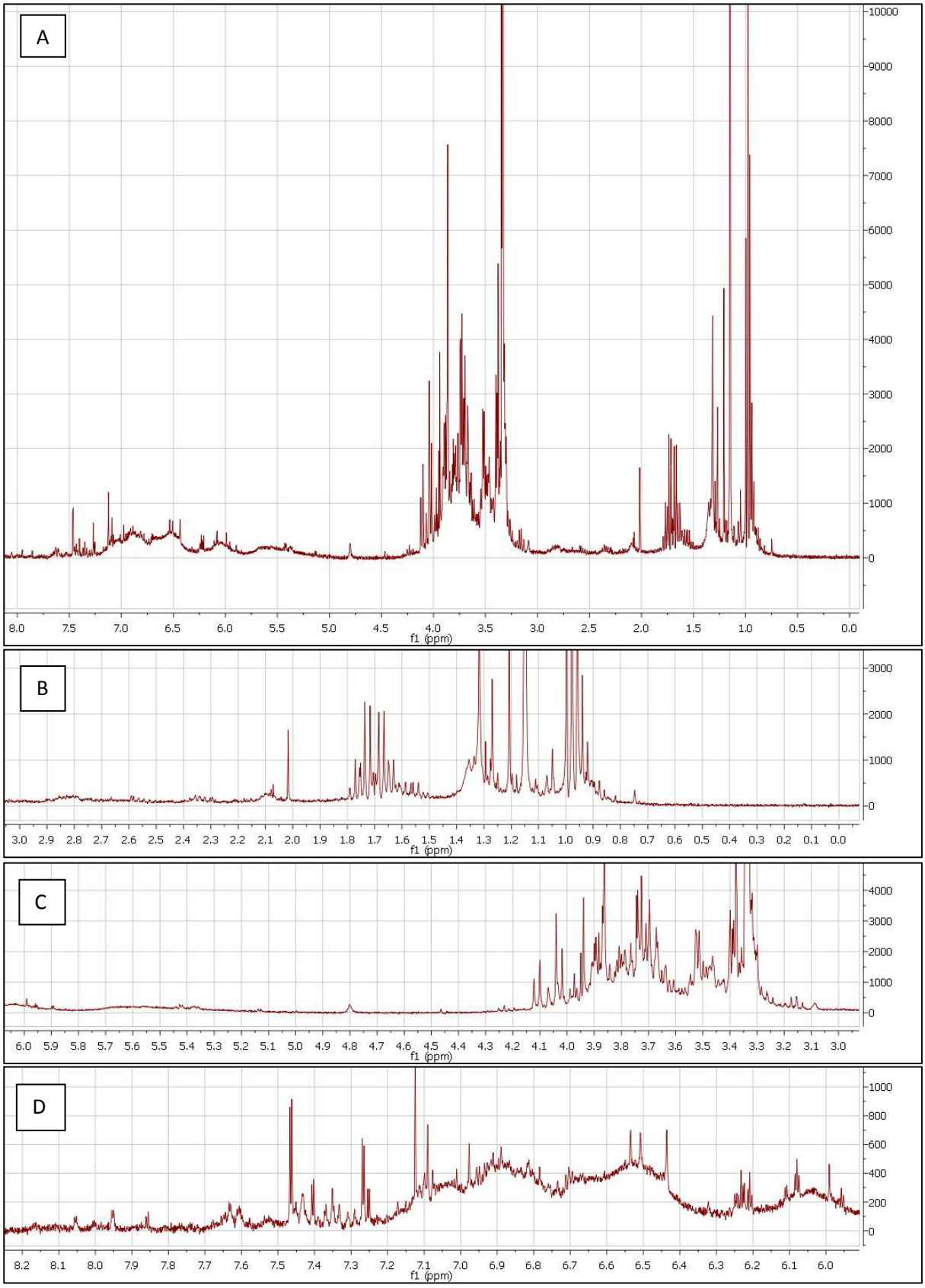


Figure 34: ^{1}H NMR spectrum of *C. muscosa* recorded in methanol-d4, showing (A) the full spectrum and (B-D) expansions of the main spectral regions.

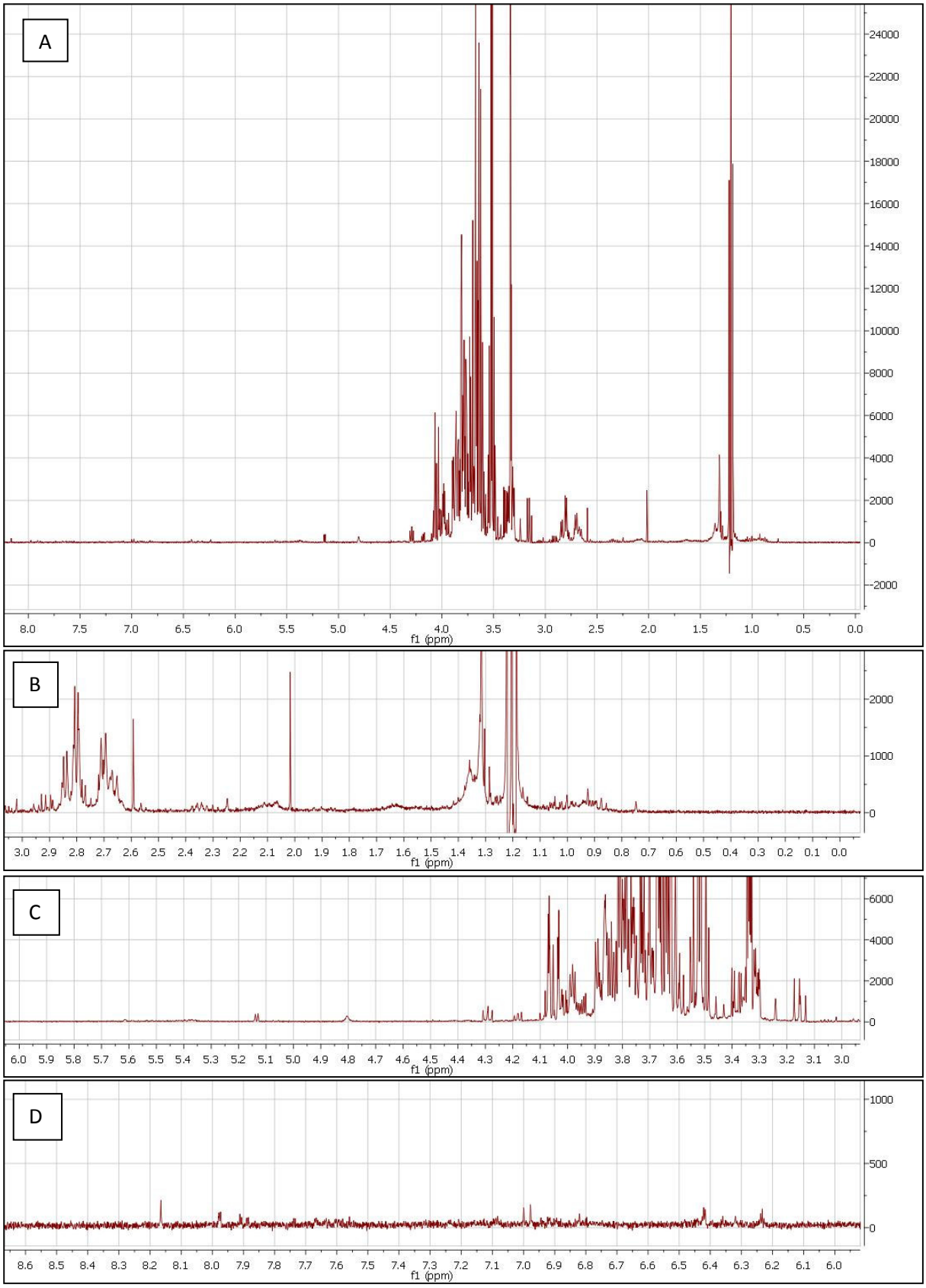


Figure 35: ^{1}H NMR spectrum of *C. cuneata* recorded in methanol-d4, showing (A) the full spectrum and (B-D) expansions of the main spectral regions.

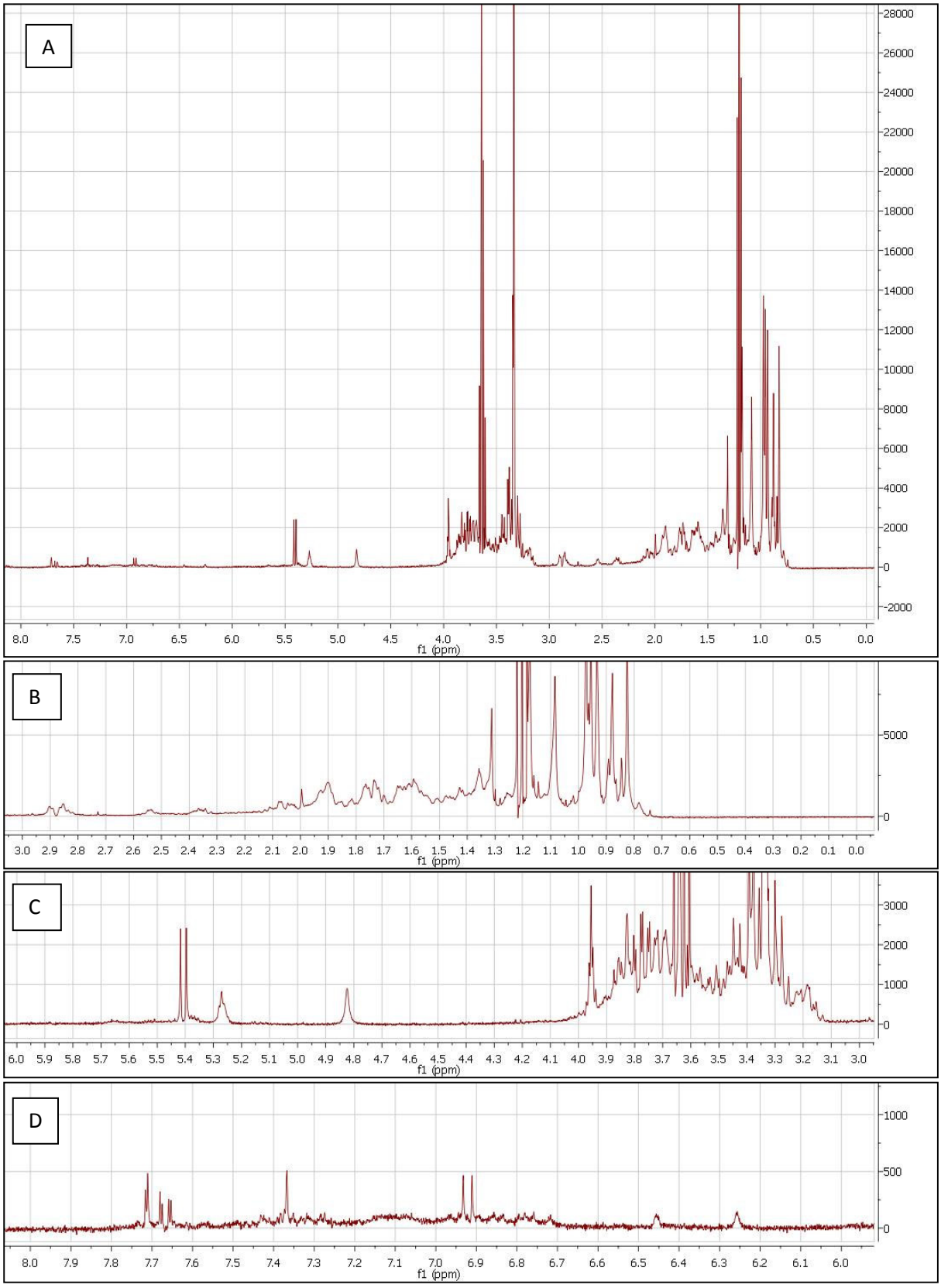


Figure 36: ^{1}H NMR spectrum of *Z. foetidum* recorded in methanol-*d4*, showing (A) the full spectrum and (B-D) expansions of the main spectral regions.

3.4.2 Evidence of the presence of catechin in the NMR spectrum of CB

Analysis of the crude CB extract using tandem LC-MS/MS revealed catechin to be one of the most abundant compounds present, dominating the chromatogram (Figure 14; Figure 17). Catechin was also the only compound successfully isolated using bioassay-guided fractionation (section 3.2 of this chapter) suggesting it is present in this extract in large quantities.

The presence of catechin in CB extract can be further confirmed by analysis of the NMR spectra. However, it is important to note that the sample for this analysis was prepared from the original ethanol extract, which had been dried and then re-suspended in deuterated methanol. An insoluble residue was removed by centrifugation, and the solution submitted for NMR analysis was therefore not completely representative of the original extract. Notwithstanding this, the presence of catechin is apparent from comparison of the ^{13}C NMR spectra of CB1 (identified as catechin) with the whole CB extract (Figure 37). Similar confirmation is obtained by overlaying the HSQC spectra catechin on that of the whole crude extract (Figure 39). The overlay of the ^{1}H NMR spectrum of catechin on that of the whole crude CB extract additionally confirms the *dominance* of catechin in this extract (Figure 39). Proton signals arising from catechin are clearly distinct in the spectrum of the crude extract. In some instances, such as in the aromatic region between δ6.7 and δ6.9 for example, similar signals

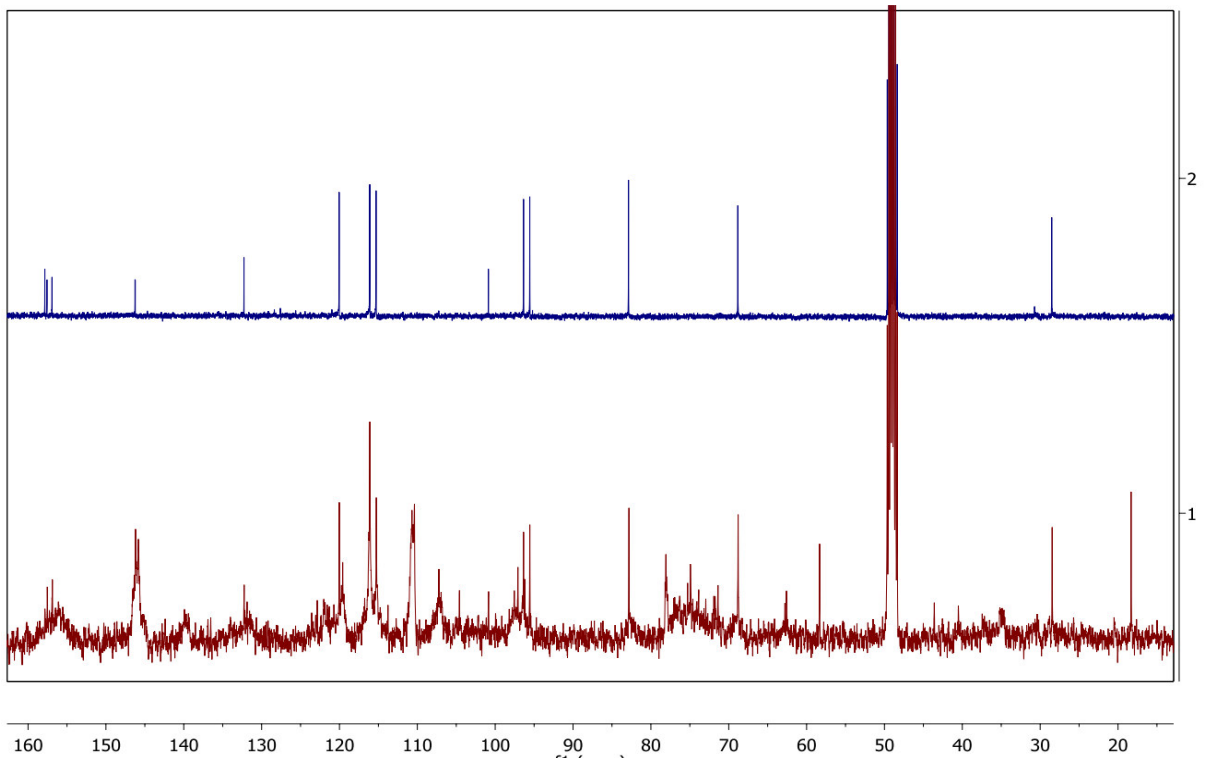


Figure 37: Comparison of ^{13}C spectra of crude CB (bottom) and that of the isolated compound CB1, catechin (top). Note that the large peak at δ49.0 results from residual solvent.

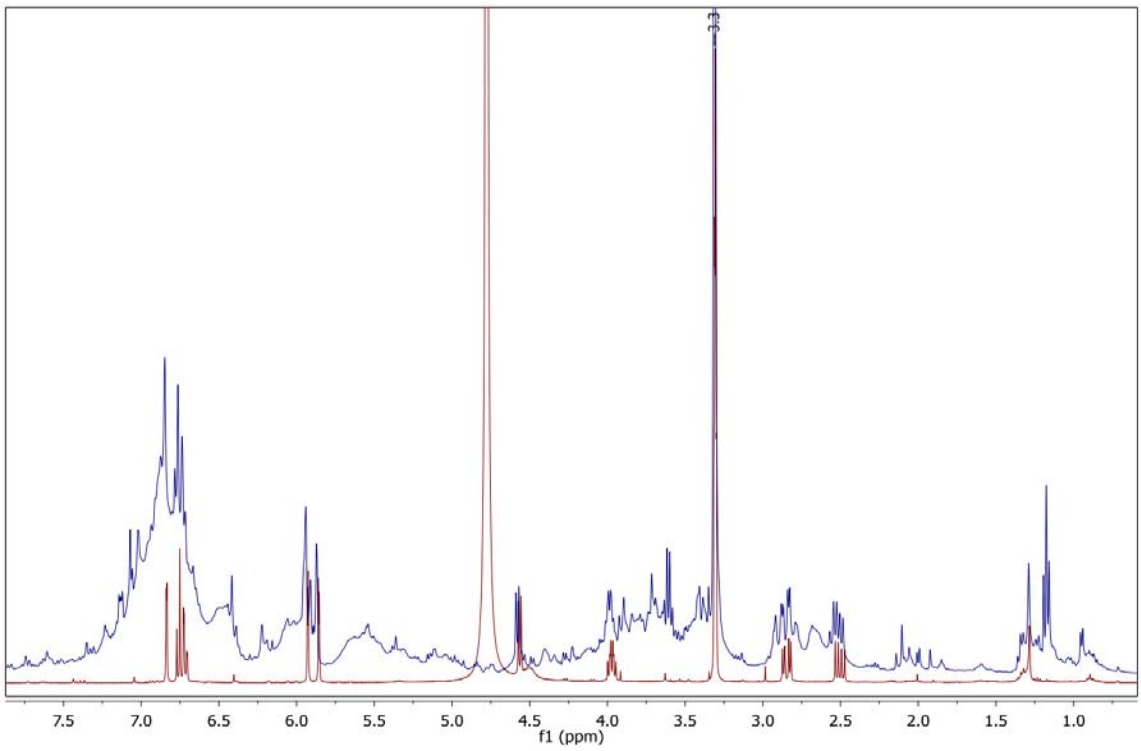


Figure 38: Comparison of ^{1}H NMR spectrum of crude CB (upper blue spectrum) and the ^{1}H spectrum of the isolated compound CB1, catechin (lower red spectrum). Note that the peaks appearing at δ3.3 and δ4.8 are signals from residual solvents (methanol and water respectively).

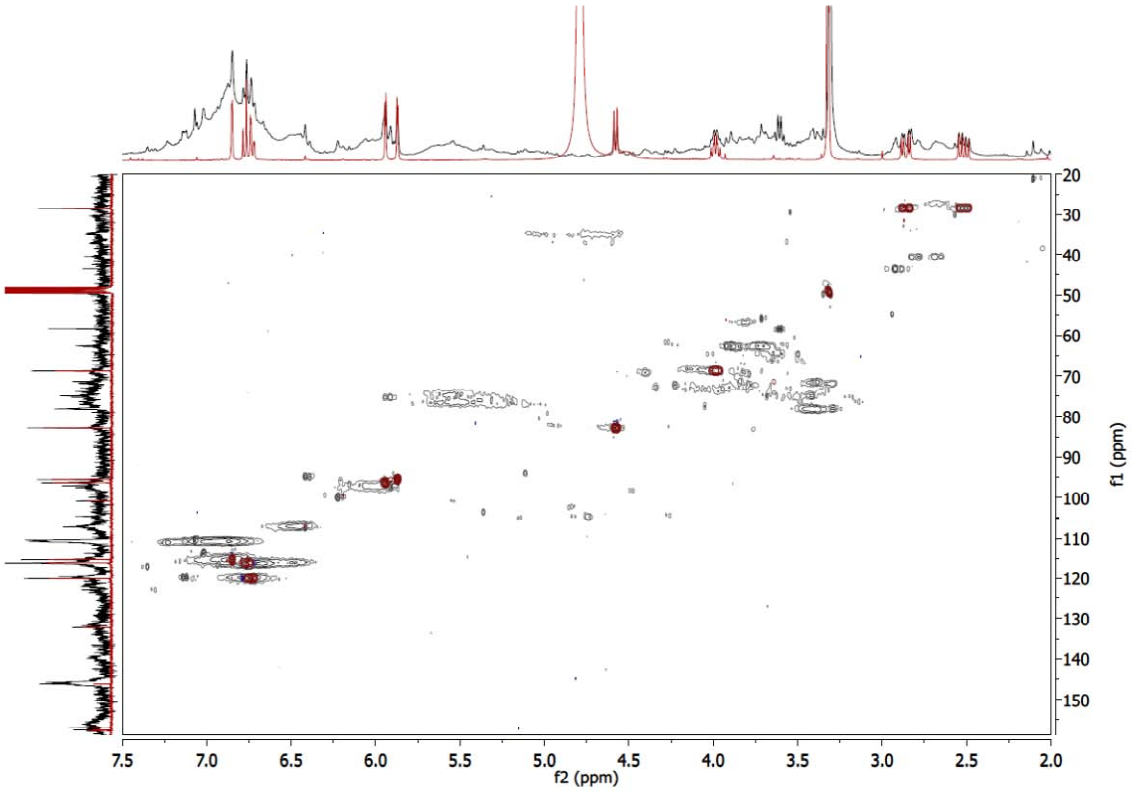


Figure 39: Overlay of HSQC spectrum of catechin (red) on that of CB whole extract.

from other compounds occur near to those originating from catechin's protons, resulting in much larger complex signal patterns than those resulting from catechin alone. This is to be expected given the fact that there are numerous flavonoids with similar structures to catechin present in the crude extract (as suggested by LC-MS spectrometry).

The signals arising from the protons on the B-ring of similar flavonoids gives rise to a complex aromatic region in which it is difficult to distinguish individual compound proton signals. In other areas of the spectrum the proton signals arising from catechin are clearly visible. The signals at δ2.51, δ2.58, δ3.98 and δ4.57 are particularly distinct in the crude extract spectrum, and are characteristic of the "catechin" class of flavonoids, the flavan-3-ols, to which catechin itself belongs. The presence of relatively few signals in this upfield region implies that the numerous other flavonoids present in the extract must belong to different classes. This is confirmed by the list of most abundant compounds in CB extract (Table 6). Many of the compounds are flavonols and flavones, and lack the two geminal protons on C4 that are characteristic of flavan-3-ols and give rise to distinct signals, H4a and H4b. In the case of catechin these appear at δ2.51 and δ2.58 (Figure 38). The NMR analysis results thus confirm those from LC-MS/MS spectrometry, suggesting that catechin is the major flavan-3-ol present in the extract. The presence of the additional suggested flavonoids can be confirmed by mapping of literature NMR data onto the cross peaks of the HSQC spectrum (Table 11, Table 12, Table 13), on the basis that distinctive chemical shifts are associated with substitution patterns in the core flavonoid structure and the C-ring in particular (Figure 10). Thus additional classes of flavonoids present in a crude extract can be identified relatively quickly, without knowledge of the specific compounds or the need to isolate them. For example, the proton attached to C2 in flavanones (such as eriodictyol) couples with the two geminal protons attached to C3 in those compounds, giving rise to a doublet of doublets with a signal between δ5.0 and δ5.5. These two C3 protons are reminiscent of the two geminal C4 protons present in flavan-3-ols, which also give rise to doublets of doublets in the aliphatic region of the proton spectrum between δ2.0 and δ3.0. Flavanone H3a and H3b signals can be distinguished from signals arising from H4a and H4b in flavan-3-ols in the HSQC spectrum - those signals arising from H3a and H3b of flavanones are shifted downfield in the ^{13}C spectrum due to the influence of the nearby electron withdrawing ketone group. Thus they form a cluster of signals separate from those arising from the flavan-3-ols, which are attached to an unsaturated carbon remote from an oxygen atom. Flavonols (such as quercetin) have no protons attached to C2, C3 or C4 and give no such signals.

Signals arising from protons attached to C6 and C8 give similar crosspeaks between δ5.5 and δ6.4 and δ95.0 and δ115.0 for almost all flavonoids present, indicating general C5 and C7 substitution in

Table 11: ^{1}H NMR spectral data for some of the flavonoids identified from CB extract (data compiled from Scharbert et al., 2004; Tachakittirungrod et al., 2007; Ragab et al., 2010; Dudek-Makuch & Matławska, 2011; Zhang et al., 2006; Güvenalp et al., 2006; Alivi et al., 2009; Mendoza-Wilson & Glossman-Mitnik, 2006; Lin et al., 2009; Suresh et al., 2012). Data are recorded in DMSO-d6, except for catechin, which is in methanol-d4.

Flavonoid class		Flavan-3-ol	Flavanone		Chalcone
Compound		***Catechin***	***Eriodictyol***	***Eriodictyol 7-O-glucoside***	***Marein***
Aglycone	H2	4.57 (1H, *d*, *J*=7.5 Hz)	5.26 (1H, *dd*, *J*=13.0, 3.0 Hz)	5.45 (1H, *dd*, *J*=12.6, 3.0 Hz)	7.30 (1H, *d*, *J*= 2.0 Hz)
	H3	3.98 (1H, *td*, *J*=7.8, 5.4 Hz)	H3a: 2.68 (1H, *dd*, *J*= 17.0, 3.0 Hz)	H3a: 2.75 (1H, *dd*, *J*=17.1, 3.0 Hz)	
			H3b: 3.06 (1H, *dd*, *J*=17.0, 3.0 Hz)	H3b: 3.29 (1H, *dd*, *J*=17.1, 12.6 Hz)	
	H4	H4a: 2.51 (1H, *dd*, *J*=16.1, 8.1 Hz)			
		H4b: 2.85 (1H, *dd*, *J*=16.1, 5.4 Hz)			
	H5				6.85 (1H, *d*, *J*=8.2 Hz)
	H6	5.86 (1H, *d*, *J*=2.3 Hz)	5.89 (1H, *d*, *J*=2.2 Hz)	6.13 (1H, *d*, *J*=2.2 Hz)	7.23 (1H, *dd*, *J*=8.2, 2.0 Hz)
	H7				
	H8	5.93 (1H, *d*, *J*=2.3 Hz)	5.87 (1H, *d*, *J*=2.2 Hz)	6.15 (1H, *d*, *J*=2.2 Hz)	
	H2'	6.84 (1H, *d*, *J*=1.9 Hz)	6.91 (1H, *dd*, *J*=0.7, 1.3 Hz)	6.89 (1H, *brs*)	
	H3'				
	H4'				
	H5'	6.72 (1H, *dd*, *J*=8.1, 1.9 Hz)	6.78 (2H, s, H5' + H6')	6.79 (2H, *s*, H5' + H6')	6.77 (1H, *d*, *J*=9.2 Hz)
	H6'	6.76 (1H, *d*, *J*=8.1 Hz)			7.78 (1H, *d*, *J*=9.2 Hz)
Sugar	H1''			4.99 (1H, *d*, *J*=7.6 Hz)	4.92 (1H, *d*, *J*=7.2 Hz)
	H2''			3.22 (1H, *dd*, *J*=7.6, 9.2 Hz)	3.72
	H3''			3.27 (1H, *dd*, J=9.2, 9.1 Hz)	3.38
	H4''			3.17 (1H, *t*, J=9.1 Hz)	3.18
	H5''			3.39 (1H, *m*)	3.45
	H6''			3.67 (1H, *dd*, *J*=11.5, 3.2 Hz)	H6'a: 3.72 (1H, *d*, *J*=11.6 Hz)
				3.45 (1H, *dd*, *J*=11.5, 5.5 Hz)	H6'b: 3.57
					α = 7.70 (1H, *d*, *J*=15.4 Hz)
					β = 7.72 (1H, *d*, *J*=15.4 Hz)

Table 12: ^{1}H NMR spectral data for some more of the flavonoids identified from CB extract (data compiled from Scharbert et al., 2004; Tachakittirungrod et al., 2007; Ragab et al., 2010; Dudek-Makuch & Matławska, 2011; Zhang et al., 2006; Güvenalp et al., 2006; Alivi et al., 2009; Mendoza-Wilson & Glossman-Mitnik, 2006; Lin et al., 2009; Suresh et al., 2012). Data are recorded in DMSO-d6, except for myricetin-3-O-glycoside, which is in CD_3COD.

Flavonoid class		**Flavonol**			
Compound		***Morin***	***Myricetin 3-O-galactoside***	***Quercetin***	***Rutin***
Aglycone	H6	6.19 (1H, *s*)	6.23 (1H, *d*)	6.27	6.20 (1H, *d*, *J*=1.8 Hz)
	H8	6.39 (1H, *s*)	6.41 (1H, *d*)	6.47	6.39 (1H, *d*, *J*=2.2 Hz)
	H2'		7.4 (2H, *s*)	7.82	7.66 (1H, *d*, *J*=1.8 Hz)
	H3'	7.68 (1H, *s*)			
	H5'	6.87 (1H, *d*)		6.97	6.86 (1H, *d*, *J*=8.0 Hz)
	H6'	7.63 (1H, *d*)	7.4 (2H, *s*)	7.72	7.60(1H, *dd*, *J*= 8.0, 1.8 Hz)
Sugar	H1''		5.23 (1H, *d*, *J*=7.7 Hz)		5.09 (1H, *d*, *J*=7.8 Hz)
	H2''		3.88 (1H, dd)		3.25-3.47 (4H, *m*, H2, H3, H4, H5)
	H3''		3.89 (1H, *d*)		
	H4''		3.51 (1H, *dd*)		
	H5''		3.66 (1H, *dd*)		
	H6''		3.61 (2H, *m*)		H6a'': 3.38 (1H, *m*)
					H6b'': 3.80 (1H, *d*, *J*=10.5 Hz)
	H1'''				4.51 (1H, *d*, J=1.8 Hz)
	H2'''				3.63 (1H, *dd*, *J*=3.5, 1.5 Hz)
	H3'''				3.53 (1H, *dd*, *J*=9.5,3.5 Hz)
	H4'''				3.28 (1H, *m*)
	H5'''				3.44 (1H, *m*)
	H6'''				1.11 (3H, *d*, J=6.0 Hz CH3-6''')

Table 13: ^{13}C NMR spectral data from some of the flavonoids identified from CB extract (data compiled from Scharbert et al., 2004; Tachakittirungrod et al., 2007; Ragab et al., 2010; Dudek-Makuch & Matławska, 2011; Zhang et al., 2006; Güvenalp et al., 2006; Alivi et al., 2009; Mendoza-Wilson & Glossman-Mitnik, 2006; Lin et al., 2009; Suresh et al., 2012).

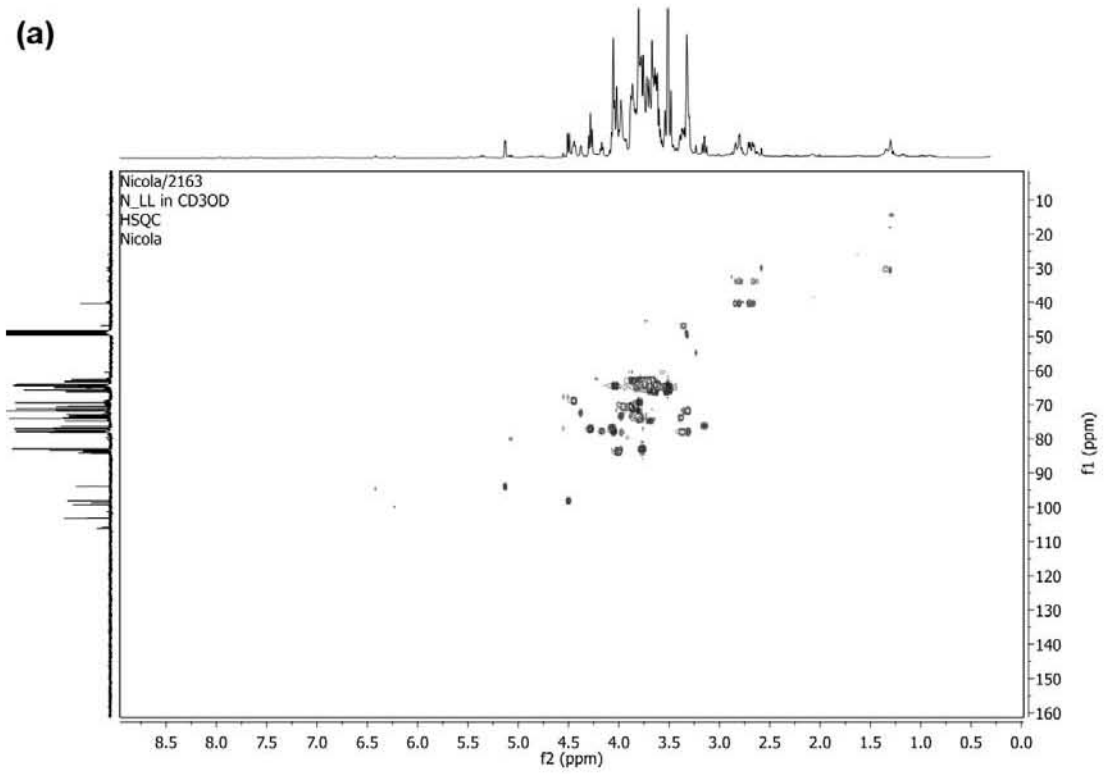
Flavonoid Class		Flavan-3-ol	Flavanone		Flavonol				Chalcone
Compound		***Catechin***	***Eriodictyol***	***Eriodictyol 7-O-glucoside***	***Morin***	***Myricetin 3-O-galactoside***	***Quercetin***	***Rutin***	***Marein***
Aglycone	C1								126
	C2	82.88	80.5	78.75	144.73	157.8	148	158.5	116.4
	C3	68.83	44.1	42.18	132.10	136.4	137.21	135.6	146
	C4	28.51	197.8	197.17	177.96	179.3	177.33	179.4	149.5
	C5	157.57	165.4	162.93	161.80	162.2	162.5	162.5	116.1
	C6	96.36	97	96.44	98.58	99.2	99.25	99.9	122.9
	C7	157.85	168.4	165.29	164.71	165.4	165.34	166.0	
	C8	95.56	96.2	95.43	93.41	94.1	94.4	94.8	
	C9	156.93	164.9	162.75	157.02	157.2	158.22	159.3	
	C10	100.87	103.3	103.27	104.46	104.8	104.52	105.6	
	C1'	132.27	131.8	129.21	121.94	120.9	124.15	123.1	118
	C2'	115.3	114.7	114.44	158.02	109.6	115.99	117.6	153
	C3'	146.23	146.5	145.21	114.90	137.2	148.75	145.8	135
	C4'	146.26	146.9	145.82	148.73	145.4	146.21	149.7	151
	C5'	116.11	116.2	115.35	115.84	137.2	116.22	116.1	106.8
	C6'	120.04	119.3	118.09	121.78	109.6	121.67	123.5	121.9
Sugar	C1''			99.58		104.5		104.7	101.3
	C2''			73.01		66.5		75.7	74
	C3''			76.31		69.2		77.2	76
	C4''			69.49		76.2		71.4	71
	C5''			77.08		74.3		78.1	78
	C6''			60.57		60.9		68.6	61

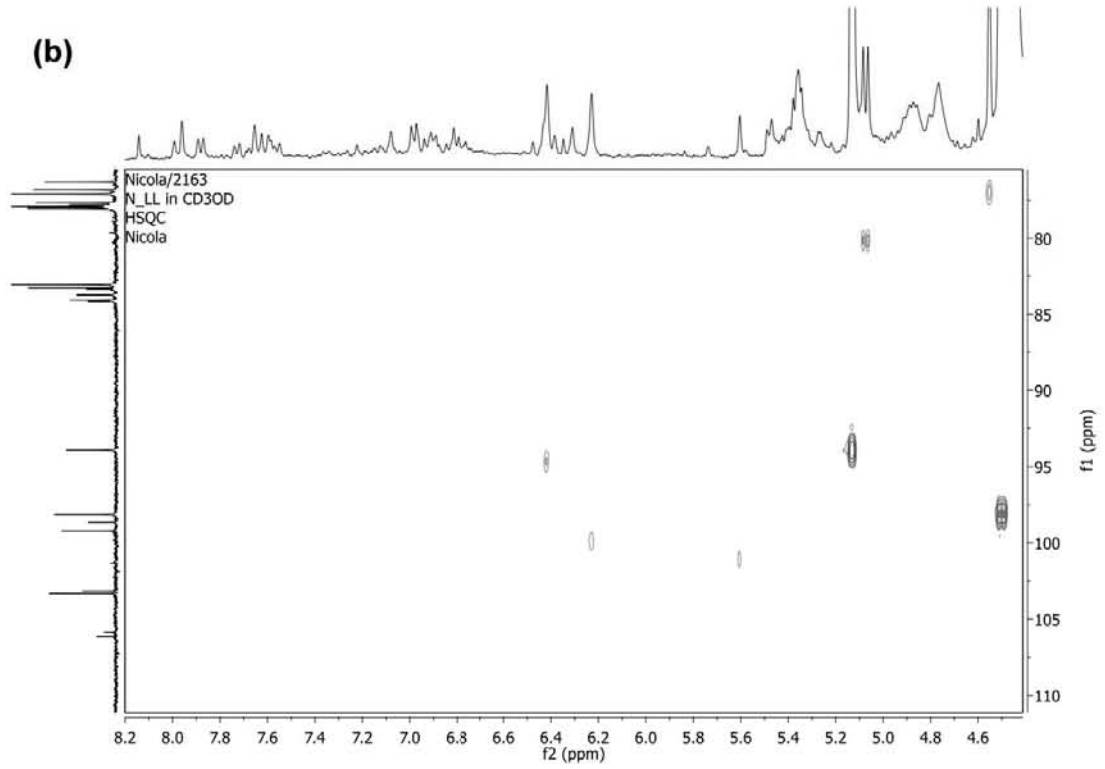
									C=O, 192.5
									α = 117.8
									β = 145.8
	H1'''							102.4	
	H2'''							72.0	
	H3'''							72.2	
	H4'''							73.9	
	H5'''							69.7	
	H6'''							17.9	

the suggested flavonoids. Lack of such substitution results in the signals being shifted upfield in the ^{13}C spectrum to the region between δ70.0 and δ85.0. This is not highly informative; however differing substitution patterns and the degree of hydroxylation on the B-ring giving rise to different signals is potentially informative. A higher degree of hydroxylation shifts the aromatic signals of B-ring protons downfield, and a 2', 4' dihydroxyl pattern of substitution (such as that present in morin) results in signals being shifted even further downfield into the δ7.0 - δ7.5 region. Thus, when examining the HSQC spectrum, the likely classes of flavonoids and relative degree of substitution can be determined.

As alluded to earlier, one of the major drawbacks to this simple assessment of NMR data to gain insight into types of compounds present in the way described above is that the sample submitted for NMR spectroscopy may not necessarily be representative of the extract as a whole, due to limited solubility in the solvent required for the experiment. In addition, high resolution is required in order to make meaningful deductions from the overlapping signals in the spectra. This is illustrated for the case of the crude extract of CC which was analysed in the same way as the CB extract. LC-MS spectrometry analysis suggested the presence of flavonoids such as quercetin in this extract. However, HSQC spectra (Figure 40) show the conspicuous absence of signals in the aromatic region while the spectra are dominated by signals which strongly suggest the presence of sugars. The presence of sugars is more clearly evident from Figure 41 which shows the ^{13}C spectrum of CC juxtaposed with the ^{13}C spectra of some sugars commonly occurring in plants (the latter spectra obtained from http://www.bmrb.wisc.edu/metabolomics/metabolomics_standards.shtml). This shows clearly the signals in the distinctive regions of the spectrum for anomeric carbons (δ94.0 - δ107.0), ring carbons involved in the inter-glycoside linkages (δ82.0 - δ86.0) and the general ring carbons and C-6's (δ62.0 - δ79.0).

While the scope of this study precluded further investigation of this extract, the results illustrate that LC-MS spectrometry is more reliable than NMR spectroscopy in profiling the whole extract, but that the combined used of LC-MS spectrometry and NMR spectroscopy provides complementary structural information if interpreted carefully in the light of the history of the sample being analysed. Further examples of the benefits of combining the two approaches follow in the next section.


(a)
Nicola/2163
N_LL in CD3OD
HSQC
Nicola
f2 (ppm)
f1 (ppm)


(b)
Nicola/2163
N_LL in CD3OD
HSQC
Nicola
f2 (ppm)
f1 (ppm)

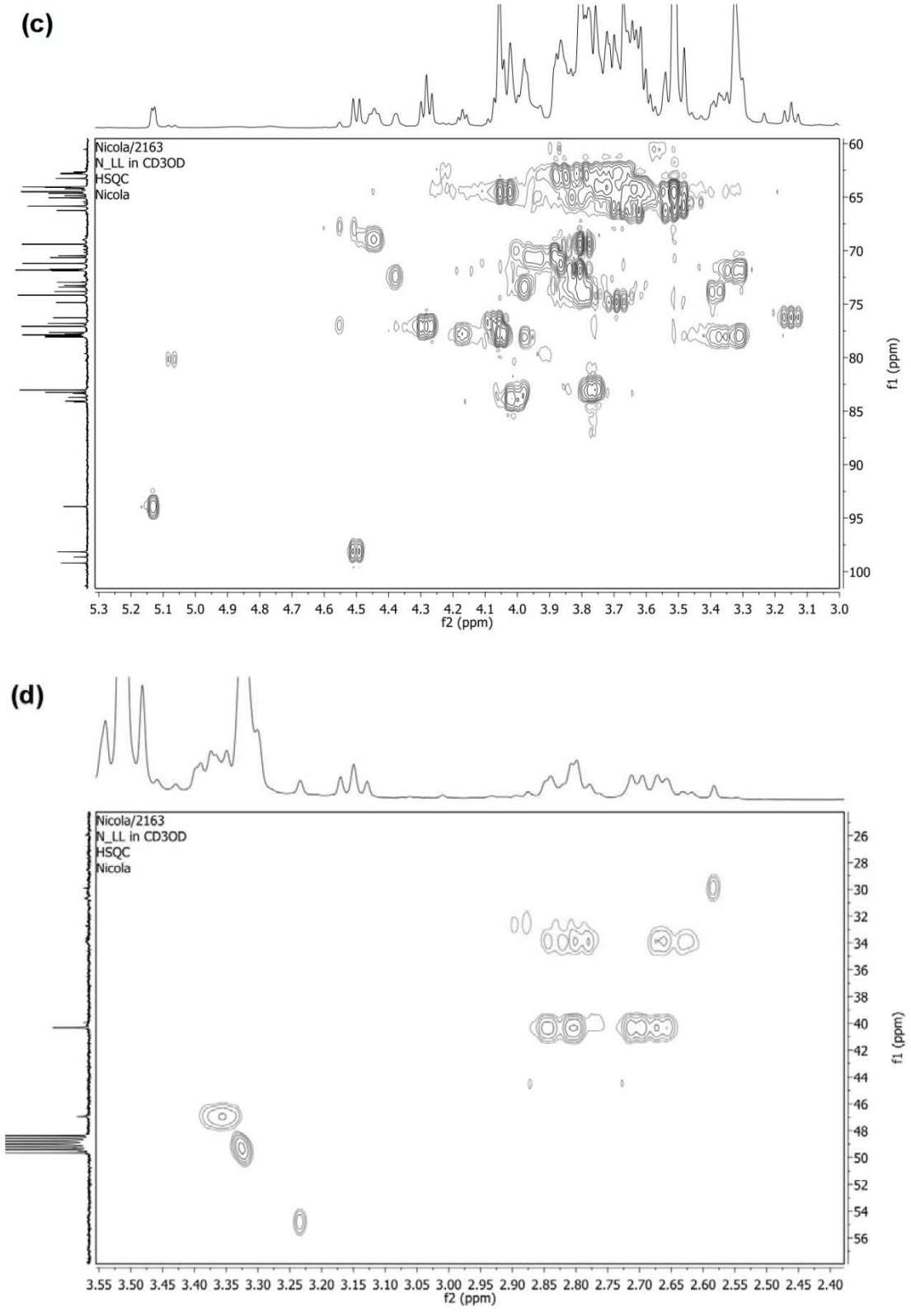


Figure 40: 2D NMR spectra of the CD_3OH-soluble portion of the ethanol extract of CC: (a) full HSQC spectrum; (b) expansion of upfield region, where the intensity of the aromatic region of the ^1H spectrum has been significantly increased; (c) and (d) expansions of other selected regions.

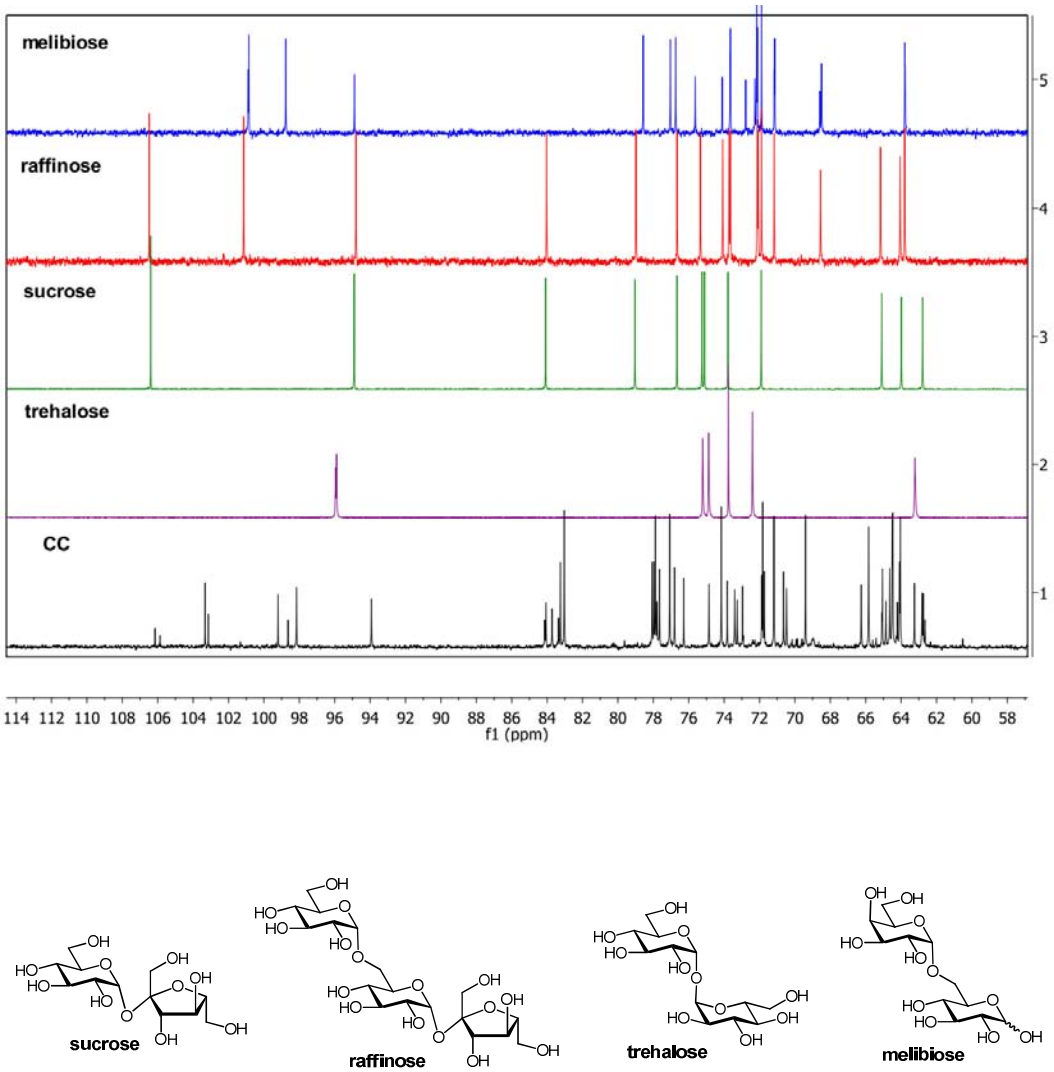


Figure 41: Comparison of the ^{13}C NMR spectrum of CC extract with database spectra for four sugars commonly occurring in plants; structures of the di- and trisaccharides are shown below the spectra.

3.4.2 Identifying compounds using a combination of NMR spectroscopy and LC-MS spectrometry

Initially, the presence of catechin was determined using bioassay-guided fractionation. This often lengthy and complex process to isolate and identify compounds may be avoided if a combination of LC-MS/MS spectrometry and NMR spectroscopy is used, though the success of this approach depends critically on on the extent of the database of known structures available, or, if new compounds are to be identified, on the resolution and/or deconvolution of the spectroscopic data

Table 14: Comparison of eriodictyol literature values (da Silva et al., 2010) and cross peak values from experimentally obtained CB HSQC spectrum (solvent is methanol-d4).

Eriodictyol (literature values)				CB extract (experimental values)	
^{1}H details (δ)	***Assignment***	***^{13}C details (δ)***	***Assignment***	***HSQC cross peak***	***Assignment***
2.68 (1H, *dd*, *J*= 17.0, 3.0 Hz)	H3a	44.1	C3	2.90/43.79	H3
3.06 (1H, *dd, J*=17.0, 3.0 Hz)	H3b	80.5	C2		
5.26 (1H, *dd*, J=13.0, 3.0 Hz)	H2	96.2	C8	5.29/77.12	H2
5.87 (1H, *d*, J=2.2 Hz)	H8	97.0	C6	5.88/95.80	H8
5.89 (1H, *d*, *J*=2.2 Hz)	H6	103.3	C10	5.91/ 97.27	H6
6.78 (2H, s)	H5' + H6'	114.7	C2'	6.77/116.30	H5'
6.91 (1H, *dd*, J=0.7, 1.3 Hz)	H2'	116.2	C5'	6.77/120.11	H6'
		119.3	C6'	6.90/115.00	H2'
		131.8	C1'		
		146.5	C3'		
		146.9	C4'		
		164.9	C9		
		165.4	C5		
		168.4	C7		
		197.8	C4		

available. In this study, LC-MS/MS spectrometry and a database of reference spectra was used to confirm the presence of catechin. The presence of catechin was then confirmed by the presence of diagnostic signals in the ^{13}C and ^{1}H NMR spectra of the crude CB extract. The presence (or absence) of other compounds, and particularly other flavonoids, were confirmed in a similar way, using either LC-MS spectrometry or NMR spectroscopy or a combination of the two to analyse the whole crude extract. Compounds whose presence was suggested by LC-MS/MS spectrometry, but not confirmed through database matching, could be confirmed using NMR spectroscopy.

An example of this approach was the initial identification of the presence of eriodictyol in CB extract by LC-MS/MS spectrometry, with a 70.32% match to reference spectra present in MassBank. This was then confirmed by comparing literature data for proton and carbon signals characteristic of eriodictyol (da Silva et al., 2010) with cross peaks present in the HSQC spectrum of the whole CB extract (Table 14).

The analysis can be extended by combining data gained from LC-MS spectrometry and NMR spectroscopy experiments with knowledge of biological systems and metabolic pathways to allow confirmation of the presence or absence of other compounds suspected to be present in CB extract. For example, another of the compounds in CB tentatively identified by LC-MS spectrometry was

phloroglucinol with m/z = 127.0389 (compound 69 in Table 6). However, the MS data for compound 69 did not have a statistically significant match with reference spectra. It is known that reductive cleavage of some flavonoids yields free phenols and phenolic acids (Harborne, 1998). Phloroglucinol (1,3,5-trihydroxybenzene), a free phenol, is commonly formed by the degradation of phloridzin, a dihydrochalcone (Gosch et al., 2009). However, it has also been shown that eriodictyol is metabolised to phloroglucinol and 3,4-dihydroxyphenylproprionic acid by bacteria (Miyake et al., 1997) (Figure 42). It is therefore possible that a similar metabolic process may occur in plants resulting in the presence of phloroglucinol in crude plant extracts. Free phloroglucinol can also be used as a building block in secondary metabolite forming pathways, such as phenylpropanoid forming pathways (Andersen et al., 2008) and it is used to produce phlorotannins by similar pathways as those used to produce gallic acid and ellagic acid derived hydrolysable tannins (Clifford & Brown, 2006). Phloroglucinol and its derivatives occur naturally in ferns and brown seaweeds as well as several families of angiosperms such as *Rutaceae* (Okada et.al., 2004; Wollenweber, 1998; Auzi et al., 1997), but phloroglucinol has not previously been documented in species of *Crassula*.

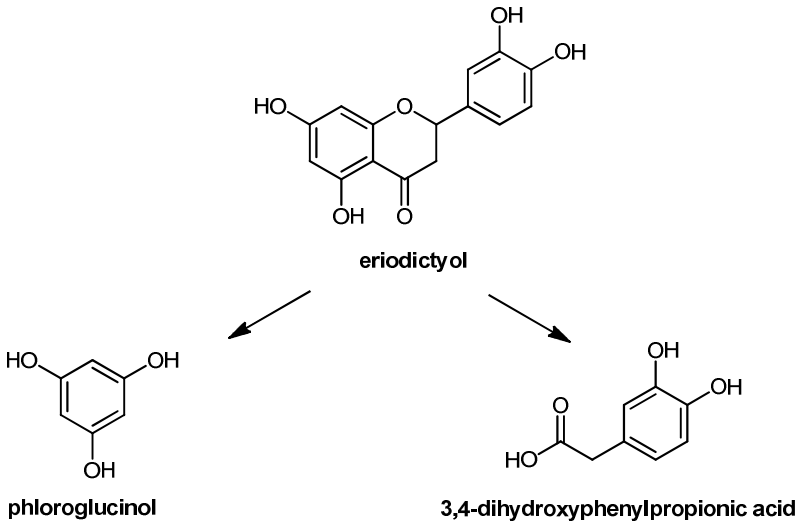


Figure 42: Metabolism of eriodictyol by bacteria (Miyake 1997). A similar process may occur in plants.

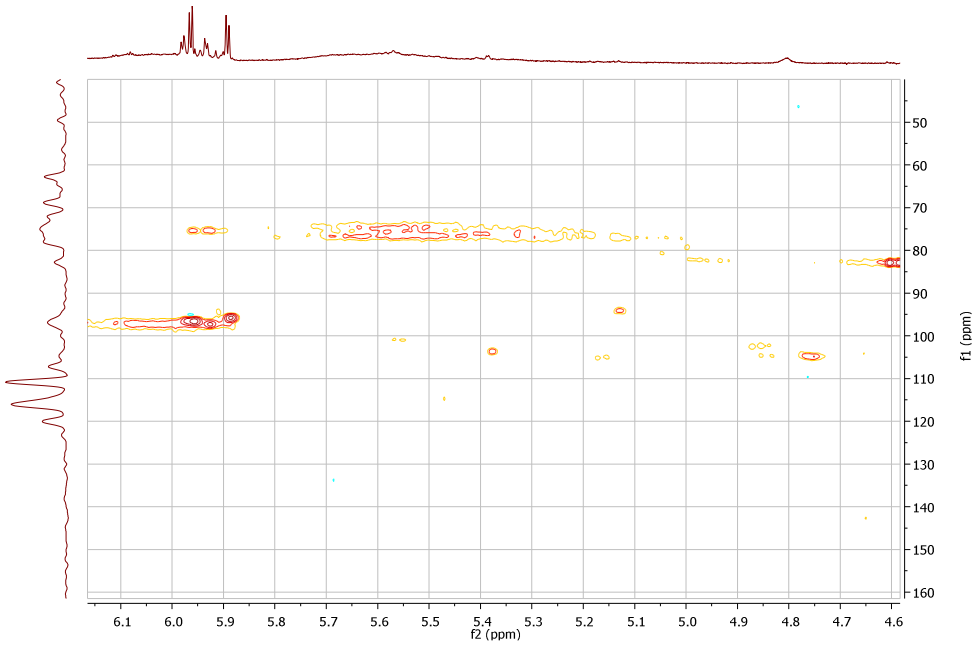


Figure 43: Expansion of the HSQC spectrum of CB to show the absence of the phloroglucinol cross peak at δ5.85 and δ86.1

Literature suggests that phloroglucinol gives rise to a proton signal at δ5.82 (3H, *br s*, H2, H4 and H6) in the ^{1}H NMR spectrum and two carbon signals at δ86.1 (C2, C4 and C6) and δ150.4 (C1, C3 and C5) (Miyake et.al., 1997). Examination of the HSQC spectrum of CB extract reveals that the cross peak at or near δ5.82 and δ86.1 is absent (Figure 43), suggesting that phloroglucinol is NOT present in CB as previously suspected, though the possibility that it was not solubilized by the deuterated methanol cannot be ruled out. However, the combination of LC-MS/MS spectrometry and NMR spectroscopy suggests that a false identification was avoided and the absence of phloroglucinol from the extract was confirmed.

4. General discussion and conclusions

The aim of this section was to use various methods, including bioassay-guided fractionation and whole-extract analysis, to attempt to determine the major metabolites present in selected plant species from Namaqualand. The approaches used to do this were evaluated and compared in order to find an optimal approach for the determination of secondary plant metabolites.

4.1 How do the methods compare?

4.1.1 How efficient are the different approaches? Comparison of the amount of sample required, waste produced and equipment sensitivity

Classic bioassay-guided fractionation (CBF) requires much more raw plant material than required by whole extract approaches. In this study, tens of grams of crude lyophilised plant extract extracted from kilograms of plant material were needed to isolate and characterise a single active compound, catechin. Powdered material was wasted as it adhered to the silica used for separation and material was lost at every step in the chromatographic separation when fractions were transferred between flasks, beakers and vials. CBF relies on the use of TLC to visualise and confirm chromatographic separation of compounds, and even quick checks on TLC use up small amounts of isolated material. Analysis of the whole extract (using NMR spectroscopy and LC-MS/MS spectrometry) requires far less material. Just 50g of plant tissue is enough for detection of the majority of metabolites present in an extract using NMR spectroscopy, and just 10mg of plant tissue is enough for detection by LC-MS spectrometry (Krishnan et al., 2005). Much less material is lost during transfer steps as plant material can be extracted into a small volume of solvent, filtered and directly analysed. In the case of NMR spectroscopy, plant material can be extracted directly into deuterated solvent. As soon as cells are ruptured during extraction enzymes start to degrade and change the metabolites, creating artefacts. The quick approach used for NMR spectroscopy and LC-MS spectrometry analysis removes the need for storage, refrigeration and drying, meaning that the most accurate "snapshot" of the secondary metabolites present can be obtained. However, as seen in with CC extract in this study, compounds may not be as soluble in the different solvents used. If plant material is not extracted directly into deuterated solvent, but rather extracted in one solvent, dried and redissolved in a different deuterated solvent, not all compounds may be available for spectral acquisition. This results in an inaccurate representation of the constituent compounds.

An additional benefit of using less raw plant tissue is that some plants are endangered or rare and harvesting large amounts from the wild is not feasible. Cultivation is a possible solution, but this takes time and some species are difficult to cultivate (e.g. *Sutherlandia frutescens* (Albrecht et al., 2012; Oksman-Caldentey & Inze', 2004). Alternatives to cultivation are plant tissue culture and other micropropagation techniques, such as preparation of plant cell cultures in large bioreactors (Moyo et al., 2011), but these methods are expensive and can take a long time.

In recent years, chemical waste and environmental concerns have come to the forefront, with "green" chemistry methods becoming more popular (Burt, 2004). The amount and type of solvents used during experimental procedures is a concern. CBF uses the most solvent - many litres per

chromatographic step - and large volumes of solvent are used up during the multiple drying and solvation steps involved in compound separation. Contaminated silica waste from CBF must also be disposed of. LC-MS spectrometry is essentially a high pressure streamlined chromatographic separation system. It uses less solvent than CBF as high pressure is used to force much smaller volumes of solvent through a much smaller column. There is no silica waste produced using this method as the column is sealed and reused. NMR spectroscopy requires the least solvent - typically only 0.5ml solvent is usually required per sample.

Comparing the methods in terms of sensitivity, CBF relies on TLC and visual detection methods, which is the least sensitive approach of those investigated. NMR spectroscopy and LC-MS spectrometry are much more sensitive. ^{13}C and ^{15}N NMR spectroscopy are less sensitive than ^{1}H NMR spectroscopy, which has a detection level of perhaps 5nmol. This is several orders of magnitude less sensitive than LC-MS spectrometry, which has a detection threshold of 10-12 µmol (Sumner et al., 2003; Krishnan et al., 2005). Mass spectrometry has a very high sensitivity and large dynamic range and is capable of provided accurate molecular weights (<5ppm) and retention time information for all compounds. The disparity in sensitivity means that a combined approach is often the most useful. NMR spectroscopy is able to detect a limited number of compounds (currently about 100-200 compounds per crude extract using a 500 MHz spectrometer (Colquhoun, 2007)), making the data easier to manage and analyse. Thus NMR spectroscopy is suggested as the first tool to use in analysing the metabolome, followed by the much more sensitive LC-MS spectrometry analysis to give greater detail (Verpoorte et al., 2005).

4.1.2 Comparison of the costs involved in the different approaches

NMR spectroscopy uses the least amount of solvent of the approaches investigated, but it is often the most expensive solvent as only deuterated solvents are used for NMR spectroscopy. As LC-MS spectrometry is performed under high pressure HPLC grade, degassed solvents must be used to prevent blockages in the column and wear and tear on the system. These can also be expensive. If using a CBF approach, much cheaper grades and types of solvent can be employed but larger amounts are required. Glass columns, silica, TLC plates and detection reagents are relatively inexpensive but setting up any system other than a silica one can be more costly. For example, a reverse phase C18 set up can cost ten times more than the equivalent silica one. However, these set up costs are minimal when compared to the costs involved in setting up an NMR spectroscopy or LC-MS analysis system. The NMR instruments, HPLC pumps and columns, mass spectrometers, photodiode arrays and UV detectors used for whole extract analysis are sensitive and expensive equipment which require constant maintenance by specialist technicians.

4.1.3 How easy are the different approaches to carry out? Comparison of ease of use and data interpretation

CBF requires minimal training, and even a relative novice to the chemical laboratory can perform the steps involved. Some experience may be required to optimise solvent systems for separation of complex mixtures, but comprehensive literature is available to guide selection. Some knowledge of microbiology is needed to perform bioassays and cell culturing can be tricky. However, biological testing can be outsourced.

NMR spectroscopy also requires minimal training to perform. Sample preparation is relatively easy and machine operation can be learned in a morning. Optimising 2D spectral acquisition and the removal of residual solvent peaks to obtain good results takes longer to master. Interpretation of the resulting spectra benefits greatly from experience. NMR spectroscopy becomes more complicated when multivariate methods are used to analyse NMR data. While commercial statistical software packages are capable of performing multivariate analysis, better results are obtained when the user can specify variables and parameters explicitly. This often requires knowledge of computer programming in order to obtain meaningful results.

LC-MS spectrometry requires the most training, often requiring a specialist technician to set up the experiments and optimise the separation system. As the equipment operates at high pressures, constant system monitoring and optimising may be required. Experimental conditions may need to be altered to obtain the desired separation and many settings and parameters need to be specified in order to do this. Interpretation of the data can also be tricky and requires knowledge of computer software and experience in interpreting the resultant spectra.

4.1.4 How long does it take to obtain data? Comparison of the speed of each method

Of the three approaches, NMR data can be obtained the fastest, as preliminary ^{1}H spectra can be obtained in minutes. 2D spectra take longer - spectral acquisition can take several hours or overnight to obtain good peak resolution. However, the acquisition time can be reduced from 5 hours to just 5 minutes if a 900 MHz spectrometer equipped with a cryoprobe is used, but this equipment is expensive and not readily available to all researchers (Colquhoun, 2007). Interpretation of the often complex natural products spectra can also take time, unless one is experienced, and analysis of 2D spectra can be even more time consuming. The amount of time required for analysis can also be substantially decreased if multivariate methods are used. New computer code may need to be generated to analyse data, depending on the type of analysis performed and the desired outcomes.

The time required to obtain data using LC-MS spectrometry is comparable to that for NMR spectroscopy, with spectra obtained in as little as a few minutes if Ultra High Pressure Liquid

Chromatography is employed. Once again though, it is the interpretation of resulting spectra that takes the time. Once chromatograms have been analysed and mass spectra have been extracted, matching to reference spectra in databases and confirming possible identifications takes time, patience and expertise if the results are to be meaningful.

CBF takes the longest to obtain results, with multiple steps often performed over several days required before a single compound can be characterised. The additional bioassays in between steps can take days to complete if microorganisms, which must be cultured, are involved. However, interpretation of assays and structural determination can be quick if compounds are small molecules.

4.1.5 Overall comparison of pros and cons of each method

There are additional pros and cons to each approach that have not already been discussed. For example, NMR spectroscopy cannot distinguish between isomers and often even different glycosides of a single flavonoid cannot clearly be discerned. However, NMR spectroscopy allows the determination of substitution positions of functional groups, which is not possible using LC-MS spectrometry data alone. In this study, LC-MS spectrometry suggested the flavonoid eriodictyol-7-O-glucoside to be present in CB extract. However, LC-MS spectrometry can be ambiguous and cannot be used to distinguish glycosidic substitution patterns. By comparing literature values for NMR spectra of eriodictyol-7-O-glucoside with crosspeaks of the experimentally determined HSQC spectrum of CB, it could be confirmed that it is indeed the 7-O-glycoside that is present. Without HSQC, the identification of the compound could not be definitively confirmed using LC-MS spectrometry alone, and without the LC-MS spectrometry data as a guide to the type of compounds present, we would not have easily been able to discern that eriodictyol-7-O-glucoside was present from the NMR data alone. In this way, the two approaches complement each other, allowing greater insight into the composition of the crude extract that would otherwise not be possible using any single technique.

A distinct advantage of using NMR spectroscopy is that it is non-destructive and sample preparation is simple (Ali et al., 2011). Despite this, results are not guaranteed. Low resolution and peak overlap in the spectra may hinder metabolite identification (Safer et al., 2011), but these problems can be overcome by using a cryo-probe to overcome low sensitivity for example (Kim et al., 2010b) or using a higher strength magnet (600MHz or stronger) for greater peak resolution (Ward & Beale, 2006). Residual solvent peaks may obscure important signals and care must be taken to suppress these signals if possible or to remove as much solvent as possible without exposing the sample to extreme temperatures or pressures which may cause compound degradation or artefact formation. Ideally,

natural extracts should not be exposed to temperatures higher than 40 °C, which presents a barrier to complete solvent removal using standard laboratory methods. However, the clear advantage of NMR spectroscopy over LC-MS spectrometry is that NMR spectroscopy allows detection of metabolites that are undetectable using LC-MS spectrometry due to incomplete ionisation (Ali et al., 2011).

Variable ionization is a major problem when using an LC-MS spectrometry approach to analyse secondary metabolites (Ward & Beale, 2006). Some compounds are unable to ionize at all in positive and/or negative polarity, while other compounds predominantly form di- and trimeric ions (Nielsen et al., 2011). If compounds do ionize, they may form multiple adducts e.g. sodium adduct ions, which are detected along with the standard protenated form of the compound complicating spectra and confusing identification (Brown et al., 2009a). Predetermined biosynthetic information concerning potential compounds present in a sample can make peak identification easier (Tohge & Fernie, 2009). The use of computational and informatics tools can also accelerate the identification process and reduce costs, however these tools are limited by computational power, proficiency of the operator and coverage of spectral databases (Xiao et al., 2012).

A “typical” metabolomics database has increase in recent years from containing hundreds of samples described by tens or hundreds of variables, to databases that may contain thousands of samples defined by tens of thousands of biological descriptors, which adds to the overall complexity of the data sets (Holmes & Antti, 2002). Even though there is a wealth of spectra in metabolomics databases, the lack of standardised instrumental conditions means not all spectra can be compared unilaterally. Additionally, there are only a few thousand commercially available analytical standards, making the creation of “standard” reference spectra for comparison difficult (Bowen & Northen, 2010). Good methods exist for the separation of most major classes of compounds isolated from plants using LC-MS spectrometry. However, there is no universal extraction method for all types of compounds, mainly because of variable solubility of the classes of compounds. What is required is that a few well tested and reliable methods be universally adopted, and that comprehensive, well documented, central databases be created. The incorporation of systems or biological data would be of great benefit for such databases, allowing better use and faster connections to be made.

While NMR spectroscopy sensitivity is much less than LC-MS spectrometry, structural information and reproducibility are superior to mass spectrometry (Schripsema, 2010). NMR spectroscopy is perfectly reproducible, unlike LC-MS spectrometry which may vary from day to day with pH, temperature or other factors. Ideally, an online system combining LC-MS spectrometry with NMR spectroscopy could provide maximal compound identification and quantification (Moco et al.,

2007a), but many research institutions do not have the resources to establish this type of system. Thus, the combination of NMR spectroscopy and LC-MS spectrometry approaches is optimal. NMR spectroscopy can be used to detect compounds that do not ionize completely and to determine substitution patterns and configuration of isomers, while LC-MS spectrometry can be used to detect those compounds whose signals are obscured by residual solvent peaks in the NMR spectra, those compounds that are variably soluble in deuterated solvents and those that are present in too low concentrations for NMR spectroscopy to detect.

Table 15: Summary of the comparison of the bioassay-guided fractionation approach and the two whole extract approaches using NMR spectroscopy and LC-MS spectrometry.

Category	CBF	NMR	LC-MS
Amount of plant material used	· Large amount - up to kg's	· Small amount - 50g	· Very small amount 10mg
Detection limits	· mmol of material	· nmol of material	· µmol of material
Waste	· Litres of solvent · Silica waste	· Virtually none	· Some solvent (less than CBF)
Cost	· Relatively cheap, common solvents used · Inexpensive, reusable equipment	· Requires expensive deuterated solvents · Requires expensive machinery connected to a computer.	· Requires expensive HPLC grade solvents · Requires very expensive equipment connected to high end computer · Requires specialist software required. · Columns need to be replaced occasionally, which can be expensive
Training	· *Minimal*: 2nd year chemistry student could perform this. · Some knowledge required to optimise solvent systems for complex separations.	· *Average*: operating the equipment can be simple, but interpreting the resulting spectra can be tricky	· *Extensive*: Machine conditions need optimising for every use. · Sensitive high pressure equipment requires a specialist to maintain. · Training required to use software for data interpretation.
Speed	· Can be slow	· Quick data acquisition (as fast as 10 minutes). · Spectral interpretation can take time unless experienced in NMR spectroscopy.	· Relatively quick data acquisition (up to an hour). · Time required for data interpretation can be extensive.
General con's	· No way to tell if isolated	· Low resolution and peak	· Constant optimisation for

	compound is known until the process is complete. · Still needs NMR spectroscopy for structural determination.	overlap in complex spectra. · Insensitive · Poor database coverage. · Can't distinguish between isomers.	each separation. · Variable ionization. · Adduct formation. · Poor database coverage. · Destructive
General pro's	· Can be effective if time is not a concern.	· Non destructive. · Can discern substitution patterns on flavonoids for example. · Set up the equipment once and maintain - no constant optimising.	· Very sensitive and accurate - can obtain formulae and masses for compounds.

4.2 How can identification of bioactive compounds be improved using these methods?

New analytical methods for the structural elucidation of bioactive plant compounds need to be developed that are faster, more reliable and more sensitive than the "classic" approaches developed last century. Whole extract analysis of crude mixtures is one such newly developed approach. Due to the complexity and diversity of plant metabolites (the actual size of the metabolome for a plant cell is unknown and the estimate of 5000 metabolites may be conservative (Trethewey, 2004)) it is unlikely that one single analytical method could generate information about all metabolites present in a plant. It is probable that a multiple sources of data need to be combined to optimise the process (Liang et al., 2006).

An ideal experimental set up is to couple LC-NMR-MS in an online system (Corcoran & Spraul, 2003). Effluent is diverted post column to NMR and MS for analysis and to a fraction collection in order for bioassays to be performed (Moco et al., 2007b). NMR spectroscopy alone often cannot distinguish isomers, while LC-MS spectrometry cannot be used to distinguish compounds with the same molecular mass (isobars). Coupling LC to NMR allows isomers and isobars to be characterised individually (Bobzin et al., 2000). As this method does not rely entirely on databases of reference spectra, it can be used for *de novo* compound identification. However this can be a complicated and very expensive arrangement, requiring dedicated machinery and careful attention to detail (Moco et al., 2007a). A whole extract approach using a combination of LC-MS spectrometry and NMR spectroscopy running in parallel may be an alternative for rapid dereplication of known compounds, as described in this chapter. What is known and what is unknown can be quickly determined using a

combined approach. As demonstrated in this study, a simple visual analysis can give insight into the types of compounds, in this instance the classes of flavonoids present were quickly confirmed using LC-MS spectrometry and HSQC NMR spectroscopy of the crude CB extract.

Bioassays may be performed if fractions are collected post column in 96 well plates or in storage loops (Van der Hooft et al., 2012). NMR spectra for bioactive fractions of interest may also be obtained allowing structural determination of unidentified compounds. Integration of contextual information, such as biochemical pathways, into the process can potentially reduce ambiguity in metabolite identification (Xiao et al., 2012) and in some instances, such as was demonstrated in this study with the confirmation of the *absence* of phloroglucinol from CB extract, prevent false identification.

Alternatively, a variety of computational methods are also available that do not require biological testing and the often complicated process of *de novo* structural determination. Known compounds can be identified using LC-MS and NMR data and spectral databases, in a process known as dereplication. Researchers often perform this step to quickly eliminate known compounds when searching for new secondary metabolites, continuing on with other methods for structural determination of the unknowns. However, computer modelling, docking studies and ADME analysis can be done for known compounds to predict their potential bioactivity and suitability as drugs (Yu & Adedoyin, 2003; Eddershaw et al., 2000). Targeted "virtual screening" can be done testing for drug-like molecules. For example target fishing and docking studies were conducted on previously identified compounds present in Chinese herbal medicine to identify those structures that may be useful leads for drug development (Barlow et al., 2012). All of this can be done without laborious separation - enough data to feed into drug metabolism and pharmacokinetic studies can be obtained from 50mg of plant material in a single day. Researchers often focus on finding new compounds but perhaps we should turn our attention to what is already known. Flavonoids have been shown to be potential scaffold for drug development as bioactivity is directly related to the number and type of substituents attached to the basic flavonoid skeleton.There are thousands of known secondary metabolites, perhaps mining these libraries is a viable alternative to expensive and complicated metabolomics and whole extract analyses.

Chapter 5: General Discussion and Conclusions

A return to natural products based drug discovery

There has been recent interest in returning to plants as a source of new drugs after many pharmaceutical companies abandoned their natural products research in the late 1990s and early 2000s. However, for plants to become a relevant and a viable option for drug discovery, the process by which active natural compounds are identified and characterized must be streamlined. Historically, hit rate for bioactive plants has been low, even when HTS approaches are employed (Zhang et al., 2012). Testing every available plant for every type of activity is not a viable endeavour when considering the vast numbers of unscreened species and the available resources (Firn, 2003). Additionally, the often laborious isolation and determination of an active compound once a hit has been found has often proved fruitless - either resulting in the "rediscovery" of a known compound or just failing outright. Thus, for plant-based drug development to continue, new approaches are required. New ways of thinking about plant based drug discovery are needed and better methods for selecting screening targets and structural determination of active compounds are needed (Salis-Lagoudakis et al., 2011).

Incorporating traditional knowledge to guide selection of screening targets

How did people in ancient times find the important drugs which are still at the core of our pharmacology? Traditional medicine is a good starting point for selecting plants for screening (Verpoorte et al., 2005). It is well known that ethnopharmacological sampling, which involves sampling and screening only plants that are used for traditional medicinal purposes, is likely to produce positive bioactive hits (Martin, 1995). Despite this, traditional medicine is often seen as "unscientific", on account of being based on non-testable hypotheses and beliefs. However parallels can be drawn between the "traditional scientists" using "traditional selection criteria" and "research based scientists" using "assays, statistics and computers". Both aim to discover plant-based medicines; they just use different approaches to do so. For example, constituents present in the plants used for digestive complaints are responsible for both astringent taste and efficacy in the treatment of intestinal disorders. (Etkin 2001; Ankli et al., 1999; Brett & Heinrich, 1998). Traditional methods of selection highlight these species for use because of their taste, but scientific methods use laboratory analysis and biological assays to indicate their suitability for treating gastro-intestinal illness. Researchers need to link broad-level ethnobotanical and anthropological studies with the

more focused metabolomic and phytochemical studies in order to understand the pharmacological basis of culturally significant plants. This study attempted to addresses this by examining medicinal plants from different perspectives in an effort to highlight the areas where they complement and overlap each other.

Specificity is needed to guide plant selection in drug discovery efforts. Understanding why different plants produce different secondary metabolites is also an important consideration to facilitate optimising plant selection (Douwes et al., 2008). Investigation into evolutionary mechanisms that regulate bioactivity highlights the links between systems (metabolic pathways) and bioactive chemicals. In some instances, certain chemicals are linked to specific life strategy adaptations embedded in the phylogeny of families or genera, and these may give rise to specific bioactivity (Wink, 2003). Traditional knowledge, when considered from a scientific point of view, can act as a proxy for detecting bioactive molecules (Gottlieb et al., 2002) and the preferential selection of certain families for medicinal use may be used as an indicator of underlying bioactive phytochemistry. Repeated selection and use in traditional preparations over hundreds of years could indicate some underlying efficacious phytochemical compounds.

The work reported in this thesis demonstrated that plant selection is not random: species of certain families are actively sought out (or avoided) by the people of Paulshoek for treating certain types of illness. Regression analysis was used to show which plant families are preferred in the preparation of traditional medicine. For example, regression analysis performed on the traditional knowledge recorded during interviews with the people of Paulshoek indicated that species of the family *Lamiaceae* are traditionally preferred to treat pulmonary ailments including asthma, coughs and TB. One of the species of *Lamiaceae* used for this purpose is *Ballota africana*. Studies have shown that compounds present in this plant, such as premarrubiin, are effective expectorants and antibacterial assays performed during the course of this study showed the extract to have high antimicrobial activity. This is an example of the use of “hot families” to indicate which species have underlying chemistry of with potential for selecting candidates for future screening (Douwes et al., 2008).

Certain classes of compounds are sometimes restricted taxonomically. As a general rule, a few major secondary metabolites tend to dominate a given clade, and usually almost all members of a monophyletic clade share the same chemicals (Wink, 2003). For example, species in families *Fabaceae* and *Anacardiaceae* have been shown to contain a large percentage of flavonoids. In contrast, species of the families *Asteraceae* and *Euphorbiaceae* are known to contain large amounts of terpenoids (Balandrin et al., 1985). Dominance of certain types of compounds may infer particular bioactivity in certain families, and thus *general* bioactivity (of different sorts) may be indicated by

family. However, the results from this study showed that there is no relationship between *specific* bioactivity and family i.e. there is no direct correlation between family and antibacterial activity for example. Traditional preference can be used to indicate which families are most likely to contain bioactive species, but cannot in all instances indicate the specific type of bioactivity. For example, plants of a certain family that contains a high percentage of flavonoids may each have febrifuge, anti-inflammatory or antibacterial activity, and may all be used to treat influenza and associated secondary infections. The family is *preferred* for treatment of influenza but the species all have different modes of activity for alleviating influenza symptoms (and thus will have different bioactivity when screened). However, plants that are preferred to treat urinary tract infections (UTI's) are, for example, much more likely to contain compounds that work in a similar manner (as UTI's are more specifically caused by bacteria or fungus), and would therefore have similar antibacterial or antifungal activity. The same is true of the previous example of *B. africana*.

Detection of localized bioactive phytochemicals through the use of indicators such as traditional preference should enhance the rate of natural product drug discovery from plants. Additional indicators of bioactivity, such as those that indicate more specifically which types of bioactivity are likely in a given category of plants, would be greatly beneficial in further refining screening choice to maximize hit rates. This study showed that more perennial species than annual species tended to have bioactivity. Thus screening efforts should focus first on traditionally preferred families, and on perennial species in those families. Succulence could also be used to indicate type of activity (e.g. protease activity), and these plants should be prioritized when searching for new protease inhibitors. However, growth form could not be directly linked with bioactivity of a specific type. Surprisingly little is known about the relationships of natural products with morphology, ecology and evolution of their plant source (Gottlieb et al., 2002). More systems level studies are needed to fill in these gaps and to link all available data together. This approach could be similarly applied on a larger scale to identify "hot" areas or ecosystems, where screening efforts should be focused.

Aligning the biomedical objectives with those of traditional medicinal systems can further increase the chance of positive hits from plant sources. This occurs naturally in certain areas. For example, in the course of this study it was demonstrated that in Paulshoek medicinal plants are mainly used to create home remedies to treat "everyday" ailments such as colds and stomach upsets. These types of illnesses are largely microbial in nature. With the increasing resistance of microbes to mainstream antibiotics, drug discovery research has focused on finding novel, highly effective antimicrobial compounds. Thus the aims of the traditional medicine overlap with the biomedical ones and the

chance of finding active compounds of this nature are increased. Other overlapping areas of interest are in treating diabetes and high blood pressure as well as ethnoveterinary applications.

Once target species have been selected, screening and identification of species for full analysis can be fast tracked by the use of in-field test kits such the GIBEX "screens-to-nature" (STN). During the course of this study, results of simplified GIBEX assays were shown to correlate with more sophisticated MIC assays performed in a laboratory. Thus, in-field testing gives a rough indication of activity so that the number of plants harvested and taken to a laboratory for phytochemical analysis is reduced, thereby streamlining the process. Local satellite stations could be established to get communities involved in testing plants and free up time for researchers to focus on other aspects of the process. This has already been proven to be successful in pilot projects in Botswana (Andrae-Marobela et al., 2012). Interviews conducted in the study area during the course of this research, indicate that similar projects are viable in South Africa. Inhabitants of Paulshoek attended community meetings to discuss the use of traditional plant medicines and expressed an interest in learning more about the plants they use and how to test them themselves using in-field assay kits. The presence of a trained para-ecologist (resident Ms. Marianna Lot) in the village to guide such testing would enable broad scale preliminary plant extract screening in the communal area of Namaqualand.

The method of identifying "hot" families using regression analysis can also be applied to existing literature, removing the need for extensive field work altogether. Research into this has shown that correlations between ancient and current plant use practice may indicate efficacious plants (Buenz et al., 2004; Buenz et al., 2005). Mining old texts has proved useful in identifying good targets for screening for flora in other parts of the world. However, this method may not be as applicable in South Africa as there is oral tradition rather than written history and historical medicinal texts are limited.

Using whole extract analysis to improve the isolation and characterization of bioactive compounds

Better target selection increases the chances of discovering bioactive plant species. But in order to streamline characterizing of the active constituents, the chemical screening process should be optimized so as to rapidly identify already known compounds so as to avoid unnecessary isolation (Wolfender et al., 2003). Thus "dereplication" of already known compounds is a vital part of the discovery process that saves time and resources and allows isolation efforts to be focused on novel or unusual compounds (Nielsen et al., 2011). This study demonstrated that a good method for doing this is a combined approach using LC-MS spectrometry and NMR spectroscopy to analyze whole

crude extracts. An ideal experimental set up to achieve this, as discussed in chapter 4, is to couple LC-NMR-MS in an online system (Corcoran & Spraul, 2003). Effluent is diverted post column to NMR and MS for analysis and to a fraction collection in order for bioassays to be performed (Moco et al., 2007b). This optimizes rapid dereplication, and also allows biological testing, and possible further characterizing, of unknown compounds of interest. Unfortunately such systems are costly to set up and run and alternatives must be considered.

In this study it has been demonstrated that parallel use of NMR spectroscopy and LC-MS spectrometry for whole crude analysis can yield a wealth of information about constituent compounds in a short amount of time, and is therefore a viable alternative to an online system. The crude extract of *Crassula brevifolia* was rapidly dereplicated and the major constituent compounds were characterized using LC-MS/MS spectrometry and 1D and 2D NMR spectroscopy methods. The combined use of these approaches has several benefits most notably that it requires very little sample material and is quick to obtain results. The combined use of these methods compensates for the inherent weaknesses in each. For example, NMR spectroscopy sensitivity is low, peaks can be obscured and compounds must be fully dissolved in a deuterated solvent. LC-MS spectrometry is much more sensitive and is performed on the whole crude extract in the extraction solvent. In the case of the *C. brevifolia* extract NMR spectroscopy was used to confirm the presence of flavonoids tentatively identified from LC-MS/MS data. Additionally, NMR spectroscopy confirmed glycosidic substitution patterns of flavonoids, where LC-MS spectrometry was not able. Additionally, NMR spectroscopy was used to determine the absence of a compound tentatively suggested to be present by LC-MS/MS data. Conversely, LC-MS/MS data was used in the case of *C. cuneata* extract to determine the presence of flavonoids, where relatively weak NMR signals in the aromatic region were obscured due to scaling and solubility problems. These examples highlight how the approaches complement each other and can be used to effectively determine what is known and what remains unknown in a plant extract. With the addition of a fraction collector, this approach can also be used to test bioactivity of unidentified compounds present in the crude extract. Repeat runs can be used to accumulate enough of the bioactive fractions for NMR spectroscopy to be performed to characterize any new unknown bioactive compounds.

Metabolic profiling could also be used in a number of areas to provide biological information beyond the simple identification of plant constituents (Sumner et al., 2003). The development of databases of metabolite information can lead to a better understanding of biochemical composition and contributes to a greater overall insight into the functioning of the biological system. A variety of biological functions have been assigned to certain classes of secondary metabolite - those involved

in pollination, photoprotection and defense for example (Moco et al., 2007b). Placing metabolites in context of the metabolic systems allows us to infer their use in plants, and their possible use in humans. Knowing which metabolites have which biological function and which species they are likely to occur in facilitates better target selection for screening. Knowledge of compound class proportions in plants could potentially be applied in addition to regression analysis to prioritise candidate taxa for bioactivity evaluation, thereby further increasing the likelihood of success (Douwes et al., 2008).

Making better use of what is already known - data mining and computational methods

There is a considerable body of literature and numerous journals dedicated to bioactive natural products, phytochemistry and ethnopharmacology. This immense resource could be used more effectively to improve drug discovery and development from natural products. Although the traditional method of analyzing plant extracts is still widely used to isolate active compounds for further drug development, many researchers are now turning to virtual screening of existing data. Bioactive chemicals exert their function through binding to one or more protein targets. Therefore, identifying important target proteins for medicinal compounds is a vital step in drug discovery. Computational drug-target prediction is an attractive option for exploring the interaction between potential protein targets and bioactive chemicals (Iskar et al., 2012). For example, a target based virtual screening approach was proven to be effective in the search for neuraminidase (NA) inhibitors for influenza viruses A and B. The 3D structure of NA was used for the enzyme conformation, and an existing NA inhibitor (Zanamivir) was used as a reference inhibitor structure. Target structure and shape was reviewed and molecular docking and post docking was computed for known natural products and synthetic compounds (Baker et al., 2007).

There are various other “virtual” approaches to developing new potentially highly active drug molecules from natural products. For example, natural products can be used as structural scaffolds. Active structural motifs can be identified from biologically active molecules and new compounds can be developed by diversifying around the central active core (Nielsen, 2002). The common core structure present in a number of natural products with the same or similar bioactivity must be identified. Combining this with protein structure similarity clustering to identify similar ligand-binding cores in different proteins allows the possibility of using the scaffold to develop multiple drugs (Zhang & Wilkinson, 2007; Koch et al., 2005). In this study we saw that the central skeleton of flavonoids identified in the whole extract of *C. brevifolia* could possibly be used in this way. Flavonoids have multiple sites for chemical modification on the basic structure, and activity is linked

to substitution type and pattern. Thus, the central skeleton could be used as a starting point for virtual drug development.

Known natural products can also be used as building blocks in the creation of larger molecules. Fragments are defined as low molecular weight (<300 Da), moderately lipophilic highly soluble organic molecules which typically bind with low affinity (Chessari & Woodhead, 2009). Such molecule fragments that bind to the active sites of a specific target protein can be identified using molecular modeling and docking software. Two or more fragments binding at different sites can possibly be linked into one highly active, high affinity molecule (Challis & Hopwood, 2007). Thus new drugs may be developed without the need for extensive field or laboratory work. However, these methods require specific training and experience to obtain meaningful results.

Many combinatorial libraries of "created" potential drug molecules have now been create by these methods. Lipinski's "rule of five" was introduced as a way of reducing the number of molecules in libraries - removing molecules that are too large, too polar or too flexible as this makes them less likely to make good orally bioavailable drugs (Zhang & Wilkinson, 2007). Orally bioavailable small-molecule drugs are desirable because of the huge advantage of requiring the patient to take a pill rather than having an injection administered (Challis & Hopwood, 2007). This narrows the search for new drugs using this method and allows medicinal chemists to focus on optimizing only the top candidates created via virtual methods.

Alternatively, multivariate analysis of NMR spectroscopy or LC-MS spectrometry data could be used as a first pass screen to rapidly determine and characterize differences in molecular composition of plant samples (Ward et al., 2003). Patterns in the data can be visualized, interpreted and compared to distinguish differences or establish similarities between samples prior to full phytochemical analysis. Extraction and isolation efforts could be focused on plants with similar profiles, if similar compounds are desired or on species with unusual fingerprints if completely novel structures are to be discovered. When compared with reference spectra of bioactive plant species, such "fingerprints" could also give insight into potential biological activity (Heinrich, 2008).

Similarly, multivariate analysis of NMR spectra acquired from "botanicals", tinctures and other herbal products can be used to ensure batch homogeneity and for quality control (Heyman & Meyer 2012; Politi et al., 2009). For example, angostura bark (*Galipea officinalis*) is a South American tree which is traditionally used to treat dysentery and to make bitter liquor (Jacquemond-Collet et al., 1999). However, products sold in markets and herbal shops as "angostura bark" often contain "false angostura". Multivariate analysis of the NMR spectrum of "false angostura" as compared with reference spectra showed that it should to be a sample of *Strychnos nux-vomica* root bark (Frédérich

et al., 2004). Species of *Strychnos* belonging to the family *Loganiaceae* often contain dangerous levels of strychnine. Thus the identification of this toxic adulterant in traditional preparations was vital in preventing illness and possible death as a result of ingesting "angostura". An additional application of multivariate analysis of NMR spectra is found in taxonomy. Classification based on the chemical profiles of species can help correctly place species in the correct families and assist with species, genus and family level delimitation. This method has been successfully used to identify and separate species of *Glycorrhiza* (liquorice, Yang et al., 2010) as well as species of American *Ilex* (mate, Kim et al., 2010a). Any way one views it, fingerprinting could be used as a guide to plant extraction and screening if the traditional "wet" method is to be followed.

An additional application of NMR fingerprinting is to identify biomarkers of toxicity in crude plant extracts. As many people make use of traditional medicinal preparations, it is important to validate them and test their toxicity (Fennel et al., 2004b). Problems with the isolation of a single active ingredient and acceptance of drugs developed from traditionally used medicinal plants mean that botanical preparations have become an alternative to pure compound drugs. Even in Paulshoek, some people when interviewed stated that they believe plants are better and would not take the developed product. PCA can be used to screen botanicals for toxicity markers and can also be used to select candidates for cultivation studies if a botanical product is viable.

An integrated approach to plant based drug discovery?

Can a study of medicinal plants from several different views allow us to find an integrated approach to drug discovery from natural products? How do the different ways of approaching medicinal plants and natural products overlap and complement each other?

The pharmacopeia and traditions of many regions of South Africa, and the world, remain unexplored. Namaqualand (and the Succulent Karoo) is a biodiversity hotspot. But other hotspots of diversity and endemism exist in South Africa. For example, the fynbos kingdom exists only in the Cape region of South Africa and the area surrounding Van Rhynsdorp is the "bulb capital of the world" with approximately 20 000 bulbs per cubic metre of soil. This study demonstrated how bioactive plants from Namaqualand can be identified for screening using traditional knowledge and how the active components can be rapidly determined using combined whole-crude analysis methods. This method (Figure 1) can be used as a model for similar studies in other regions in South Africa.

Natural products are not just a source of drugs for treating human illness. Food production and agriculture also benefit from natural products. Active compounds in plants may be developed into

herbicides, pesticides and microbicides (against bacteria and fungi) for use in agriculture. This method can also be used to identify active compounds for these purposes. In particular, species traditionally preferred for the treatment of veterinary illnesses may yield compounds of interest, but this was beyond the scope of this research.

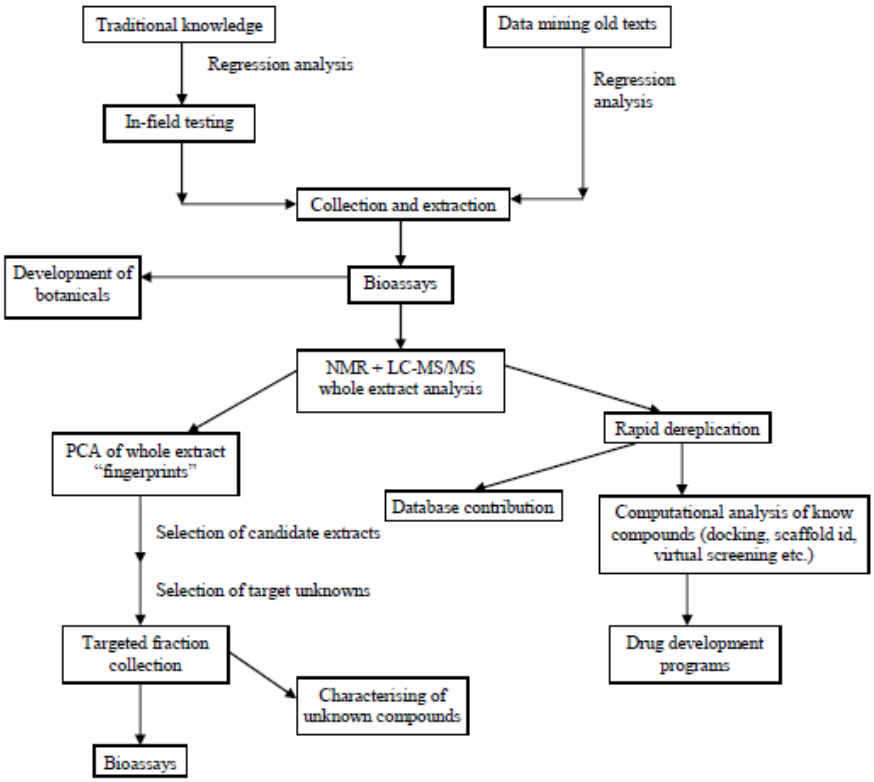


Figure 1: Suggested workflow for future studies of bioactive medicinal plants combining traditional knowledge, whole extract metabolomics and pc based analysis methods.

An evaluation of the demographics of the people using traditional medicinal plants, their thoughts and attitudes towards traditional medicine were contrasted with the aims of drug development research. Ultimately it is the aim of both traditional medicine and drug development studies to identify and create safe and effective medicines. Ideally, a pluralistic medical system would be developed, as has been done in other areas of the world, such as Thailand. In areas where "Western" pharmaceuticals are not readily accepted by local communities, standardized botanical extracts become a viable alternative. The use of botanicals allows people to retain their culture and tradition while providing them with good healthcare options. This also allows the continued evolution of traditional medical systems, which is vital if the suggested integrated approach to drug discovery is to be implemented.

This study has therefore proposed that traditional knowledge used in combination with modern analytical techniques, such as those discussed earlier in this chapter, may accelerate natural product-based drug development, since it allows for a more rapid, efficient and focused effort on potentially efficacious compounds.

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Appendix A

Question set one - Basic Household Use of Medicinal Plants

House number:

Explain the purpose of the project:

- The project is about the use of medicinal plants that are still used in households on a regular basis. Which plants are used for which ailment and which plants are preferred? Are certain plants better (preferred to) than Western medicine/medicine from the clinic? We would like to find out how many people still use the traditional ways of making medicine and what their opinions of traditional plant medicine are.

Ask if there are any questions about the project.

Answer the following questions:

1) Have you used medicinal plants for any purpose in the past year?

2) If yes, did you collect them from the wild (veld) or from the garden?

3) What did you use the plants for (which illnesses)?

4) Please give the names of the plants (common/local names) and what they are used for.

5) How often do you use medicinal plants?

6) In which season do you use medicinal plants the most?

7) Do you have anything else to add – about medicinal plants, history, personal experiences? Any comments, insight or questions?

Thank you

Question set two – Follow Up Questions

House number:
Name of interviewed person:
Answer the following questions: 1) How many people live in your house? 2) How many are younger than 18? What are the ages of the other people (older than 18) in the house? 3) How many people in the house use medicinal plants to treat sickness? 4) Who collects the plants? How old are they? 5) Who prepares the medicine? How old are they? 6) How did the person/people who collect and/or prepare the medicine learn about medicinal plants? 7) Do you think than the young people will continue to use the medicinal plants in the old way? 8) Has anyone consulted a kruiedoktor (traditional healer) in the past year? 9) Are there sicknesses that you would rather use plants to make your own medicine rather than visit the kruiedoktor (traditional healer)? 10) Has anyone visited the clinic in the past year? 11) Are there sicknesses that you would rather use plants to make your own medicine rather than visit the clinic? 12) Would you like to know more about the plants and their uses at the end of the study? 13) Are there any comments or questions?
Thank you