Determination of the Nutritional and Phytochemical Composition of *Monsonia burkeana* (Special Tea) and Its Potential Anti-Proliferative Effects on Cancer Cell Lines

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SANDILE MANZI NGWENYA

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SUPERVISOR: PROF F.N. MUDAU

CO-SUPERVISOR: PROF L.J. McGAW

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Declaration

Name: Sandile Manzi Ngwenya

Student number: 61953024

Degree: PhD: Agriculture

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Abstract

Traditional medicinal plants have long been used to treat ailments in humans and animals. Africa is rich in such plants, many of which have been studied for bioactivities like anticancer, antimicrobial, and anti-inflammatory effects, though many remain uncharacterized. *Monsonia burkeana* ("special tea") is widely consumed for its health benefits, usually as a decoction. Its antimicrobial properties are known; however, its phytotoxicity and antiproliferative activities remain underexplored. Elucidating these could benefit both traditional medicine and the pharmaceutical industry. This study aimed to investigate the effects of drying methods on the nutritional composition of special tea, identify bioactive compounds in its crude extract, and evaluate antiproliferative activities in cancer cell lines, offering insights for pharmaceutical development and commercial potential. The specific objectives included assessing nutritional composition, profiling bioactive compounds, and testing cytotoxicity against lung, colorectal, and liver cancer cell lines. Samples from Sekhukhune, Lanseria, Brits, and Rietondale were dried using shade, oven, and freeze-drying methods. Nutritional elements were analyzed by inductively coupled plasma mass spectrometry (ICP-MS), phytochemicals profiled using nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS), and anticancer properties evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and caspase-3 activity assays. The results demonstrated a consistent elemental composition across all regions, with all 24 elements present in each sample and collection region. NMR analysis identified phenols, alkyls, esters, and ethers, while GC×GC/TOF-MS detected various bioactive compounds, including oxalic acid, β-caryophyllene, caryophyllene oxide, phytol, squalene, and vitamin E. LC-MS analysis confirmed the presence of additional key phytochemicals such as castalagin, vescalagin, acutissimin B, epicatechin, citroside, trifolin, 1,6-digalloyl-beta-D-glucopyranose, 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-{[3,4,5-trihydroxy-6-

(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one, and puerarin 4',6"-diacetate. The MTT assay revealed significant cytotoxic activity against lung and liver cancer lines, however, activity against the colorectal cancer cell line was lower compared to that demonstrated against the other two cancerous cell lines. In conclusion, the combination of essential micro- and macronutrients, bioactive phytochemicals, and demonstrated cytotoxic effects against cancer cell lines highlights the medicinal value of *M. burkeana* plants. The study provides strong evidence that *Monsonia burkeana* is a promising alternative for cancer treatment. These findings not only validate its traditional use and benefits over the years but also highlight its potential for pharmaceutical applications.

Keywords: African medicinal plants, anticancer, bioactivity, cancer cell lines, caspase-3 activity, metabolomics, *Monsonia burkeana,* nutrition, phytochemicals, phytotoxicity.

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CHAPTER 1

INTRODUCTION

1.1 Background information

Monsonia burkeana (Planch. ex Harv.), mostly called 'special tea', from the Geraniaceae family, native to southern Africa (Woldesemayat et al. 2016). Special tea is a highly regarded and utilized traditional and religious medicinal plant. It is usually harvested whole, and its concoction is used for blood cleansing, treating sexually transmitted infections, and improving erectile dysfunction (Mamphiswana et al., 2011). Special tea plants may have phytochemicals, such as polysaccharides, volatile oils, vitamins, minerals, purines, and alkaloids, e.g. caffeine and polyphenols (Chung et al., 1996). These substances are concentrated in specific plant parts, which include leaves, roots, stems and fruits (Cunningham, 1993). In South Africa, various communities use it for medicinal purposes. This special tea, viewed as a special herb, is used in animal and human health (Mamphiswana et al., 2010).

Plant growth and developmental processes depend on nutrients, which regulate metabolic activity and secondary metabolite production (Kulkarni et al., 2006; Moodley et al., 2012; Andresen et al., 2018). Plant physiological status and growth stage influence secondary metabolite biosynthesis, while environmental conditions further modulate their accumulation by controlling key metabolic pathways responsible for natural compound formation. Environmental and stress-related factors influencing secondary metabolite production include temperature extremes, water limitation, salinity and alkaline soils, pathogen pressure, exposure to ultraviolet radiation, herbicide application, nutrient shortages, mechanical injury, and elevated light intensity (Ramakrishna & Ravishankar, 2011).

High temperatures may influence phytochemical content during the drying process (Saeidi et al., 2016). Drying is employed to prevent microbial degradation during storage and to establish a consistent reference point by measuring the dry weight, which is more reliable than the variable fresh weight (Markert, 1995). Drying also prevents specific phytochemical reactions and enzymatic activities that alter the organoleptic characteristics and preserve active ingredients in tissue cells (Saeidi et al. 2016). Artificial drying, including freeze-drying and oven-drying methods, has been one of the most essential methods for drying in the pharmaceutical industry, which uses dried plants for industrial production (Rahimmalek & Goli, 2013). Freeze-drying (lyophilisation) is a dehydration method in which a frozen material is subjected to reduced pressure, allowing water to be removed through direct sublimation of ice from the solid state to vapour, bypassing the liquid phase (Gaidhani et al., 2015). Oven drying involves forcing air at about 40-60 °C through plant samples for a period. The shade-drying method is a conventional, low-cost method that involves placing plant material under shade, either enclosed to protect against contaminants or left in the open.

The term ‘metabolome’ was introduced in 1998, and metabolomics has been in use since then. Metabolomics, also known as metabolite profiling, is a powerful analytical strategy for detecting and quantifying small-molecule metabolites in biological systems, providing important insights into metabolic pathways and biological network interactions. Additionally, metabolomics contributes significantly to gene annotation and aids in investigating cellular physiological and biological processes in response to both endogenous and exogenous factors (Alawiye & Babalola, 2021). Metabolomics also encompasses the analysis of metabolic intermediates, including lipids, nucleic acids, amino acids, peptides, carbohydrates, organic acids, ketones, aldehydes, amines, steroids, vitamins, signalling molecules, hormones, and secondary metabolites such as flavonoids and polyphenols. Metabolomics generally encompasses two principal strategies: targeted metabolomics,

which focuses on the analysis of predefined metabolites, and untargeted metabolomics, which aims to obtain a broad, unbiased overview of all measurable metabolites (Alawiye & Babalola, 2021).

Since the inception of metabolomics, several techniques have been developed, providing significant assistance to researchers. The field of metabolomics uses technologies such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) with or without chromatography. Mass spectrometry includes gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC).

When integrated with ethnomedicinal research, advanced analytical methods enable the investigation of cultural perspectives on health, disease, and healing, highlighting diverse traditional practices that contribute to human well-being. Medicinal plants and herbal remedies are widely used worldwide and provide therapeutic benefits that support healthcare systems in many countries. Particularly in rural areas, people often depend on traditional medicinal plant remedies due to their accessibility, cultural acceptance, and economic constraints. This reliance on traditional knowledge reflects a historical cultural heritage and a wealth of natural resources passed down through oral and discipleship practices in indigenous communities (Lall & Kishore, 2014; Acharya et al., 2023).

Indigenous and traditional knowledge systems remain important sources of information for the identification and development of new therapeutic agents, including those used in cancer management. Cancer is a major contributor to global mortality, with lung cancer accounting for the highest number of deaths, followed by breast and colorectal cancers (Thandra et al., 2021). Various therapeutic strategies, such as chemotherapy, surgery and radiation, are employed in cancer treatment. While chemotherapy can provide temporary relief to cancer patients, it is just one approach among several in the comprehensive effort to address and manage the disease. Selected medicinal plants exhibiting anticancer activity contribute significantly to the management of

malignancies affecting oral, breast, colorectal, pulmonary, gastric, cervical, hepatic, and haematological tissues. (Chandra et al., 2023). Medicinal plants have demonstrated anticancer potential and may be used as alternatives or as complementary approaches alongside conventional cancer therapies (Merrouni & Elachouri, 2021).

Special tea, as a medicinal herb, is currently attracting the attention of researchers seeking to determine its physiological and phytochemical properties. It has been suggested that special tea harvested from Chuenespoort (south of Polokwane, Limpopo) has a good accumulation capability of nutrients in the stems, leaves and fruits (Mamphiswana et al. 2011). It has also been found to contain polyphenols and tannins, and has shown antioxidant and antimicrobial activity (Tshivhandekano et al. 2014).

1.2 Research problem

Investigations into plants as sources of new therapeutic agents have been conducted over the years due to their potential value in medicine. These investigations led to several modern plant-derived drugs used to treat human diseases. Bioactive compounds in plants are raising interest in consumers, traders, the pharmaceutical industry, as well as traditional healers and herbalists, for their roles in maintaining human health (Shettar et al. 2017). Natural plant products offer the pharmaceutical industry opportunities to develop new drugs, as phytochemical diversity can be used to identify therapeutic properties.

The therapeutic properties of medicinal plants have led to a growing interest in them in South Africa and globally. Indigenous communities have used medicinal plants to treat various ailments; however, for many of these plants, the benefits and potential adverse effects have not yet been scientifically identified or quantified. Among these, special tea has been widely used in traditional medicine in South Africa and other Southern African countries. However, the nutritional, phytochemical, cytotoxicity, and bioactivity profiles of this plant remain underexplored. Scientific investigations detailing the identification, isolation, quantification, and determination of their in vivo activities remain limited, providing an opportunity to explore the special tea bioactive compound attributes, including anticancer activity, cytotoxicity, and phytochemical composition, to generate additional pharmaceutical knowledge and confirm the plant's use for consumption. Comprehensive clinical investigations are essential to elucidate the pharmacokinetic behaviour, therapeutic effectiveness, safety profile, and potential drug interactions of the bioactive compounds. This study aims to investigate procedures for drying plant samples and for the extraction, isolation, and characterization of bioactive molecules from special tea, with a focus on their antioxidant and anticancer activities.

1.3 Research motivation

In recent years, attention has focused on identifying phytochemical activities in plant extracts for the treatment of various ailments. (Vaou et al. 2022). Similarly, extracts of special tea contain compounds with notable antimicrobial activity, underscoring their potential for developing novel therapeutics against infectious diseases (Tshivhandekano et al., 2014). Recent interest in special tea has increased due to its potential antioxidant properties, which are associated with possible anticancer effects. Studies have shown that the leaves and fruits of *M. burkeana* possess significantly higher total phenolic content (TPC) and total antioxidant activity (TAA) than the stems and roots, with the roots exhibiting the lowest values (Mamphiswana et al., 2010).

Given that the bioactive constituents of this medicinal plant comprise a complex mixture of compounds, effective separation and identification techniques are essential (Sasidharan et al., 2011). The growing significance of antioxidants and anticancer agents in developing new pharmaceuticals for both human and animal health underscores the urgent need for innovative therapeutic compounds. This is particularly crucial in addressing the current global health challenges.

In South Africa, much of the phytopharmaceutical trade operates within informal markets, where traditional plant products are sold in unprocessed or semi-processed forms (Mamphiswana et al., 2011). The potential application of bioactive compounds from *M. burkeana* in the pharmaceutical and allied industries requires standardized, reliable procedures for their extraction and characterization. Key steps in utilizing plant-derived bioactive compounds include extraction, pharmacological screening, isolation, and biological evaluation (Sasidharan et al., 2011). Therefore, the present study seeks to determine suitable methodological approaches for natural product research, including sample preparation, extraction procedures, analytical methods, and the isolation and structural characterization of bioactive metabolites with potential pharmaceutical applications.

1.4 Aim of the study

This study aimed to measure the nutritional composition of *Monsonia burkeana*, isolate and characterize its bioactive compounds, and evaluate the plant's anticancer activities, thereby providing critical insights to support its therapeutic application and the potential commercialization of this medicinal plant.

1.5 Specific objectives

The research had the following specific objectives:

1.5.1 To determine the nutritional composition of *M. burkeana* plants harvested from four areas in South Africa using the ICP method.

1.5.2 To determine, isolate and characterize bioactive compounds from *M. burkeana* using NMR, LC-MS and GC-MS techniques

1.5.3 To evaluate cytotoxicity activities of special tea crude extracts on lung, colorectal and liver cancer cells *in vitro* using the MTT Assay.

1.6 Hypotheses

1.6.1 Special tea plant possesses significant nutritional value as determined by the ICP-MS technique.

1.6.2 NMR, GC-MS and LC-MS techniques can successfully isolate and characterize *M. burkeana* phytochemicals.

1.6.3 *M. burkeana* crude extracts have cytotoxicity to lung, colorectal and liver cell lines.

1.7 Overview of the Thesis

This thesis, research authorized by the University of South Africa General Research Ethics Committee with reference number 2017/CAES/185, comprises six distinct chapters, each contributing to the evaluation of the phytochemical composition and bioactivities of the special tea plant, *Monsonia burkeana*.

Chapter 1 provides an introduction to the study, including the problem statement, research aim, and objectives.

Chapter 2 reviews existing research on medicinal plants, emphasizing the application of metabolomics techniques such as NMR, LC-MS, and GC-MS for phytochemical analysis, as well as the use of various bioassays. The chapter concludes by discussing the limitations and opportunities associated with metabolomics in this field.

Chapter 3 focuses on the nutritional composition of the special tea plant and reports the quantification of 24 elements in the plants evaluated using ICP-MS.

Chapter 4 explores the application of metabolomics for phytochemical characterization, utilizing NMR, GC-MS, and LC-MS technologies. This chapter also examines the relationship between identified phytochemicals and their biological activities.

Chapter 5 assesses the cytotoxic effects of special tea extracts on three cancer cell lines using the MTT assay, with further confirmation of activity through the caspase-3 activity assay.

Chapter 6 presents a synthesis of findings, offering a discussion and conclusion drawn from the preceding chapters.

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CHAPTER 2

LITERATURE REVIEW

Abstract

This review explores the application of metabolomics in plant-derived anticancer and antimicrobial activities of African medicinal plants. It also focuses on the challenges faced by the African continent in developing and discovering anti-cancer bioactivities of medicinal plants. The databases utilized in this study included Google Scholar, Mendeley, Science Direct and Scopus. Compound verification and structures were obtained from ChemSpider and PubChem. Nuclear magnetic resonance, liquid chromatography-mass spectrometry, and gas chromatography-mass spectrometry are the most common techniques used to identify plant-derived bioactive compounds and have contributed significantly to the development of novel therapeutics. Antibacterial, antifungal, anticancer and antiviral activities have been identified in plants and developed into valuable and commercial products for the pharmaceutical industry. Inadequate resources, such as funding and research facilities, in Africa prevent it from supporting metabolomics research that could inform and shift policy on medicinal plants. African metabolomics advancement is limited by undefined analytical methods, the costs associated with these spectroscopies, and the lack of reproducibility across laboratories. Metabolomics techniques have increased sensitivity and precision in metabolite identification. The use of metabolomics to identify bioactive compounds from African medicinal plants has significantly advanced the development of commercialized drugs for cancer and microbial diseases.

Keywords: anticancer bioactive compounds, metabolomics, multidrug resistance, precision medicine, traditional medicine.

2.1 Introduction

Researchers have extensively investigated African traditional medicinal plants, Chinese traditional plants, and various others to gain insights into their medicinal properties and potential applications in the pharmaceutical industry. These studies have utilized indigenous knowledge systems to explore historical and current uses, employed advanced techniques such as metabolomics to elucidate phytochemical compositions, and conducted bioassays to evaluate the bioactivities of these plants. The integration of these approaches has significantly advanced the understanding of medicinal plants. Accordingly, this section reviews the relevant literature on the botany, phytochemical composition, and therapeutic benefits of *Monsonia burkeana*.

2.2 Origin and distribution of special tea

Monsonia burkeana is native to southern Africa, where it is also regarded as a valuable indigenous plant with a wide distribution from the Eastern Cape to Limpopo (Lebowakgomo), but also beyond the South African border to Zimbabwe, Angola, Botswana, Mozambique, Lesotho, Swaziland, Namibia and Madagascar` (Tshivhandekano et al., 2014). Distribution areas include regions not commercialised, and often there are low threats to the survival of a species, as is the medicine (Nnzeru et al., 2017). Its common use among traditional communities, such as in along with its application as a natural pesticide for plant health, has contributed to the growing demand for this plant.

2.3 Botanical description of *Monsonia burkeana*

Monsonia burkeana (Figure 2.1) belongs to the family Geraniaceae, which has a rostrate schizocarp and actinomorphic flowers (Aldasoro et al., 2001). This dicotyledonous perennial herb produces annual stems from a woody base, classifying it as a suffrutex. It can reach up to 60 cm in height. The leaves are elongated and narrow, folded along the midrib, and characterized by reddish, hairy, and slightly toothed margins. The stems, petioles, flower stalks, and sepals are also covered with varying degrees of hairiness (Hyde et al., 2016).

Figure 2.1: *Monsonia burkeana* plant. Source: Photograph by the author, 2022.

Several species closely related to *Monsonia burkeana*, both taxonomically and morphologically, include *Monsonia senegalensis* Guill. & Perr. and *Monsonia ovata* Cav. The former, commonly referred to as the pink-flowered crane's bill, is a semi-woody herb distributed across the arid northern regions of Senegal, Niger, and Nigeria, extending to western India. It has traditional medicinal uses, such as serving as an emmenagogue in Guinea and, among the Fula of Nigeria, occasionally forming part of Strophanthus-based arrow poisons. *Monsonia ovata*, native to the Cape Peninsula of South Africa, is valued for its therapeutic applications in treating acute and chronic dysentery, showing particular effectiveness in conditions involving ulceration of the stomach and upper intestinal tract (Nnzeru et al., 2017).

Similarly, a study by (Mamphiswana et al., 2011) reported that M. burkeana contains the following macronutrients: calcium, nitrogen, potassium, magnesium, and phosphorus. The plants also contain the following micro-nutrients: chlorine, iron, copper, zinc, boron and manganese. *M. burkeana* extracts have been reported to have antimicrobial and antifungal activity (Hlokwe et al., 2018).

2.4 Secondary metabolites in plants

Secondary metabolites are biologically active compounds synthesized from primary metabolites in plants. Although not required for primary growth and development, these compounds are vital for plant–environment interactions, contributing to adaptation and protection against herbivores and pathogens. They are responsible for characteristics such as pigmentation, distinct aromas, and taste. These compounds serve as valuable sources of food additives, flavouring

agents, and pharmaceutical products, and are of significant importance in medicinal, nutritional, and cosmetic applications (Alzandi et al., 2021).

Plants are producers of secondary metabolites (Ghasemzadeh & Ghasemzadeh 2011). The formation of secondary metabolites in plants varies with growth stage and physiological status, and these constituents generally occur in trace amounts, commonly below 1% of the plant's dry weight. External environmental factors further shape their biosynthesis by influencing the metabolic networks responsible for natural compound accumulation. Factors impacting secondary metabolite levels include temperature fluctuations, water scarcity, soil salinity and alkalinity, pathogen attack, exposure to ultraviolet radiation, herbicide application, nutrient deficiencies, and mechanical injury. Flavonoids and phenolic acids serve as typical examples of such secondary metabolites (Ghasemzadeh & Ghasemzadeh, 2011).

a) Flavonoids in plants

Flavonoids are a class of secondary metabolites characterized by a polyphenolic structure, synthesized via the phenylpropanoid pathway, with phenylalanine serving as the precursor molecule. They share a common C6-C3-C6 carbon framework, consisting of two aromatic rings (A and B) and a heterocyclic oxygen-containing ring (C). Flavonoids are broadly categorized into six major subgroups: (a) Flavones (e.g., luteolin, apigenin, tangeretin), (b) Flavonols (e.g., quercetin, kaempferol, myricetin, isorhamnetin, pachypodol), (c) Flavanones (e.g., hesperetin, naringenin, eriodictyol), (d) Flavan-3-ols (e.g., catechins and epicatechins), (e) Isoflavones (e.g., genistein, daidzein, glycitein), and (f) Anthocyanidins (e.g., cyanidin, delphinidin, malvidin, pelargonidin, petunidin) (Ghasemzadeh & Ghasemzadeh, 2011; Wang et al., 2018). Other notable flavonoid groups include aurones, xanthones, and condensed tannins. While most flavonoids exist as glycosides, catechins and leucoanthocyanidins are exceptions, typically

found in non-glycosylated forms due to their structural similarity. To date, over 6,000 flavonoids have been identified and isolated through extensive research (Ghasemzadeh & Ghasemzadeh, 2011)

Studies have shown that flavonoids are beneficial compounds that have been used for their anti-viral and antibacterial activities – against the influenza virus, anticancer activities – shown cytotoxicity on cancer cells, anti-ageing – prevention of neurodegeneration, anti-inflammatory, anti-diabetic activities – regulation of blood glucose level and increasing pancreatic cell function (Wang et al., 2018).

b) Phenolic acids in plants

Plant phenolics are defined as natural compounds possessing the capability to influence antioxidant activity. These natural compounds include anthocyanins, phenolic acids, etc (Kiokias et al., 2020). Although phenolic acids primarily support plant growth, development, and reproduction, they also play a significant role in human health by contributing to distinctive taste and flavour and providing various health benefits. Interest in these compounds has grown across agricultural, biological, chemical, and medical research fields, aiming to enhance their production and utilization. Phenolic acids exhibit structural diversity, typically characterized by hydroxylated aromatic rings, and can polymerize into larger compounds such as proanthocyanidins and lignins (Ghasemzadeh & Ghasemzadeh, 2011). There are a few examples of phenolic acids found in plants: a) Caffeic acid – found in herbs and fruits (antimicrobial activities), b) Gallic acid (3,4,5-trihydroxybenzoic acid) – found in berry fruits (anticancer, antioxidants activities), c) Rosmarinic acid – found in species of the Lamiaceae family such as *Rosmarinus officinalis* (anti-inflammatory activities), d) Carnosic acid – found in the Lamiaceae family (antioxidant activity), e) Ferulic acid – found in apples, artichoke and orange (anti-inflammatory activity), f) p-coumaric acid – found in most fruits, tomatoes and

carrots (antimicrobial activities), g) Vanillic acid – found in fruits (anti-inflammatory, antioxidants, anticancer activities) (Kiokias et al., 2020).

c) Alkaloids

Alkaloids are nitrogen-containing organic compounds of natural origin that exhibit biological activity, are found in both plants and fungi, and are derived from amino acids. Even though they have high-end value, they are found in small quantities in plants, and their bioactivities are substantially in low doses (Desgagné-Penix, 2017). Alkaloids are classified into the following groups: i) Amino acid derivatives, e.g. ornithine, histidine, tryptophan, tyrosine, lysine, anthranilic and nicotinic acids, ii) Purines, e.g. zanthine and caffeine, iii) Aminated terpenes, e.g. diterpenoid alkaloid, aconitine and the triterpenoid alkaloid solanine, iv) Polyketides, e.g. coniine and the coccinelollines. Opium poppy (*Papaver somniferum*) and Madagascar periwinkle (*Catharanthus roseus*) are the most studied plants possessing plant alkaloids. Bioactive compounds derived from plant alkaloids include the narcotic analgesic morphine, the cough suppressant codeine, the muscle relaxant papaverine, the antimicrobial agents sanguinarine and berberine, anticancer compounds, stimulants, and, in some cases, poisonous substances. Cocaine, caffeine, quinine, nicotine (used as an insecticide) and strychnine compounds are commonly used and are high-value plant compounds (Desgagné-Penix, 2017).

2.5 Medicinal plants overview

For many centuries, Africa's traditional medical systems have relied extensively on remedies prepared from medicinal plants. Most African communities regularly seek medical attention from traditional health practitioners due to heightened trust in this form of medicine and cultural beliefs. This may be attributed to the fact that the plants are easily accessible, locally grown and

cultivated, considerably cheaper, and being culturally trusted and accepted without the hesitancy that is associated with westernised modern medicine (Mahomoodally, 2013; Oyebode et al., 2016; Quansah & Karikari, 2016; Elansary et al., 2018).

Extracts from traditional medicinal plants have become an important source of contemporary pharmaceuticals, offering potential treatments for a wide range of diseases (Karthikeyan & Balasubramanian, 2014). Plants utilized in traditional African medicine are concoctions that contain a mixture of chemical compounds that act in combination or individually on the body to prevent disease and maintain or rehabilitate health (Nielsen et al. 2012). Medicinal plants contain a diverse range of phytochemical constituents that exert significant biological functions, including antioxidant and antimicrobial effects, inhibition of tyrosinase activity, and provision of essential nutrients, thereby supporting health and reducing the progression of chronic diseases (Lee et al., 2015; Acquah et al., 2021).

2.6 Overview of cancer and anti-cancer drug developments

Over the years, cancer has garnered specialized attention and research to identify potential preventative approaches that may facilitate the discovery and design of therapeutic agents and possibly a cure. Unfortunately, several serious side effects have arisen with current anti-cancer drugs, such as abnormal proliferation, increased risk of endometrial cancer and non-specific cytotoxicity (Chunarkar-Patil et al., 2024). Anti-cancer drugs associated with these reported adverse effects include tamoxifen and paclitaxel. These circumstances have prompted the scientific community to conduct more focused research on indigenous plant-based solutions (Greenwell & Rahman, 2015; Mughees et al., 2020). Bioactive compounds isolated from medicinal plants have been extensively investigated for their ability to inhibit cancer cell

growth, limit tumour development, and induce cell death. In addition, traditionally used medicinal plants remain important in cancer treatment and provide valuable leads for the discovery of novel anticancer drugs (Greenwell & Rahman, 2015).

Cancer is characterized by abnormal cells in the human and animal body that increase continuously without the possibility of control or stoppage (Greenwell and Rahman 2015). Cancer is known for altering normal cellular metabolism (Beger 2013). The initial stage of cancer development begins with the reversal of cell changes, followed by a promotion phase that includes cell clonal proliferation. This process will then progress into the progression phase, which includes the aggressive and metastatic aspects of the disease (Safarzadeh et al. 2014). To date, the human population has many different forms of cancer, with more than 100 types categorized in medicine (Safarzadeh et al., 2014; Greenwell & Rahman, 2015). Interestingly, all forms of cancer share the same or similar insensitivity toward cell growth inhibitors, thereby rendering their replication limitless (Greenwell and Rahman 2015).

Cancer is recognised as a major disease affecting human populations worldwide. The occurrence and mortality of this disease are escalating rapidly worldwide. The worldwide cumulative risk for developing cancer and succumbing to this disease before 75 years has been estimated to be approximately 21.4% and 17.7%, respectively (Bray et al. 2018). A 2019 global report by the World Health Organisation (WHO) ranked tracheal, bronchial, and lung cancers at position 6 among the top 10 global causes of death. The most prevalent types included lung and breast cancers, followed by colorectal, prostate, non-melanoma skin, and stomach cancers (WHO 2021).

Common cancers in Africa include prostate, cervical, breast, gastrointestinal and abdominal cancers (Zouré et al., 2018). While there are many prostate, colon and breast cancer cases in Africa, there has been an upward trend in thyroid, colorectal, lung, prostate and breast cancer

rates from 2002 to 2018. On a positive note, there has been a decrease in fatality rates associated with cervical, breast and prostate cancer; in 2018, across Northern, Central, Eastern, Western and Southern Africa, fatality rates linked to cervical, breast and prostate cancer were 50%, 40% and 30%, respectively (Hamdi et al., 2021). It has also been discovered that cancer prevalence is gender-dependent, with men presenting higher occurrence rates for malignancies in the lung (16.7%), prostate (15.0%), colorectum (10.0%), stomach (8.5%) and liver (7.5%) cancer. In comparison, women exhibit cases of breast (25.2%), colorectal (9.2%), lung (8.7%), cervix (7.9%) and stomach (4.8%) cancer (Alves-Silva et al., 2017).

One of the major obstacles in cancer treatment is the development of resistance by tumour cells to currently available therapies, including highly effective modalities such as chemotherapy and radiotherapy. Moreover, these interventions are frequently linked to serious adverse effects, require sophisticated healthcare infrastructure, and are financially challenging. Therefore, the present approach is unattainable by most developing third-world nations, especially those consumed by poverty, such as Africa (Safarzadeh et al. 2014). For this reason, there is a continuous need to discover and design potent anticancer therapies that are effective, readily accessible, inexpensive, and act specifically against cancerous cells while remaining harmless to non-cancerous cells, thereby preserving a patient's overall health.

2.7 Metabolomics application and bioactive compound discovery

2.7.1 Metabolomics and Metabolite Profiling

Metabolomics derives from metabolite profiling, which refers to the qualitative and quantitative analysis of compounds of physiological origin. It has developed into a concept of screening, diagnosis and health assessment (Alawiye & Babalola, 2021; Schmidt et al., 2021). Metabolite profiling also focuses on the chemical properties of a sample. It is used in drug research to

describe the catabolic degradation of a chemical, and it is an important scientific practice across many fields of the life sciences (Fiehn, 2002; Wolfender et al., 2015). Identification of organisms in a pathophysiological state, gene function, drug toxicity, and drug efficacy may be achieved through metabolomics. This concept has been considered a unique research strategy for identifying and quantifying metabolites and profiling the whole metabolome, including trace compounds (Viljoen et al., 2015). Metabolomics enables a comprehensive, non-invasive assessment of metabolic biomarkers, facilitating early disease detection, postoperative monitoring of residual disease, evaluation of treatment responses, and identification of early indicators of therapy-related toxicity. This approach has deepened insight into the intricate relationships operating within biological systems and their adaptive responses to internal and environmental factors (Wang et al., 2011). Metabolomics is a valuable approach for deciphering metabolic outcomes associated with phenotypic changes (Su et al., 2014).

The metabolomics workflow (Figure 2.1) comprises several key steps to achieve optimal results. Metabolomics can be divided into (1) untargeted and (2) targeted approaches. In the untargeted approach, there is no prior knowledge of the metabolites, whereas in the targeted approach, the metabolites are already established (San-Martin et al., 2020). A metabolomics study generally involves five essential steps: (1) selecting the appropriate biological sample matrix to extract metabolites relevant to the research question; (2) acquiring data through analytical techniques such as liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) spectrometry, direct infusion mass spectrometry (DIMS), capillary electrophoresis-mass spectrometry (CE-MS), and high-performance liquid chromatography (HPLC), all of which have been successfully used to identify bioactive compounds from medicinal plants; (3) processing the large-scale molecular data through standardization to prepare it for statistical

analysis; (4) applying chemometric statistical and mathematical models, including univariate and multivariate analyses, to interpret the chemical data and reduce the complexity of the features identified; and (5) determining well-known metabolites and correlating them with pathophysiological processes by using algorithms to map the biochemical pathways associated with these compounds (San-Martin et al. 2020).

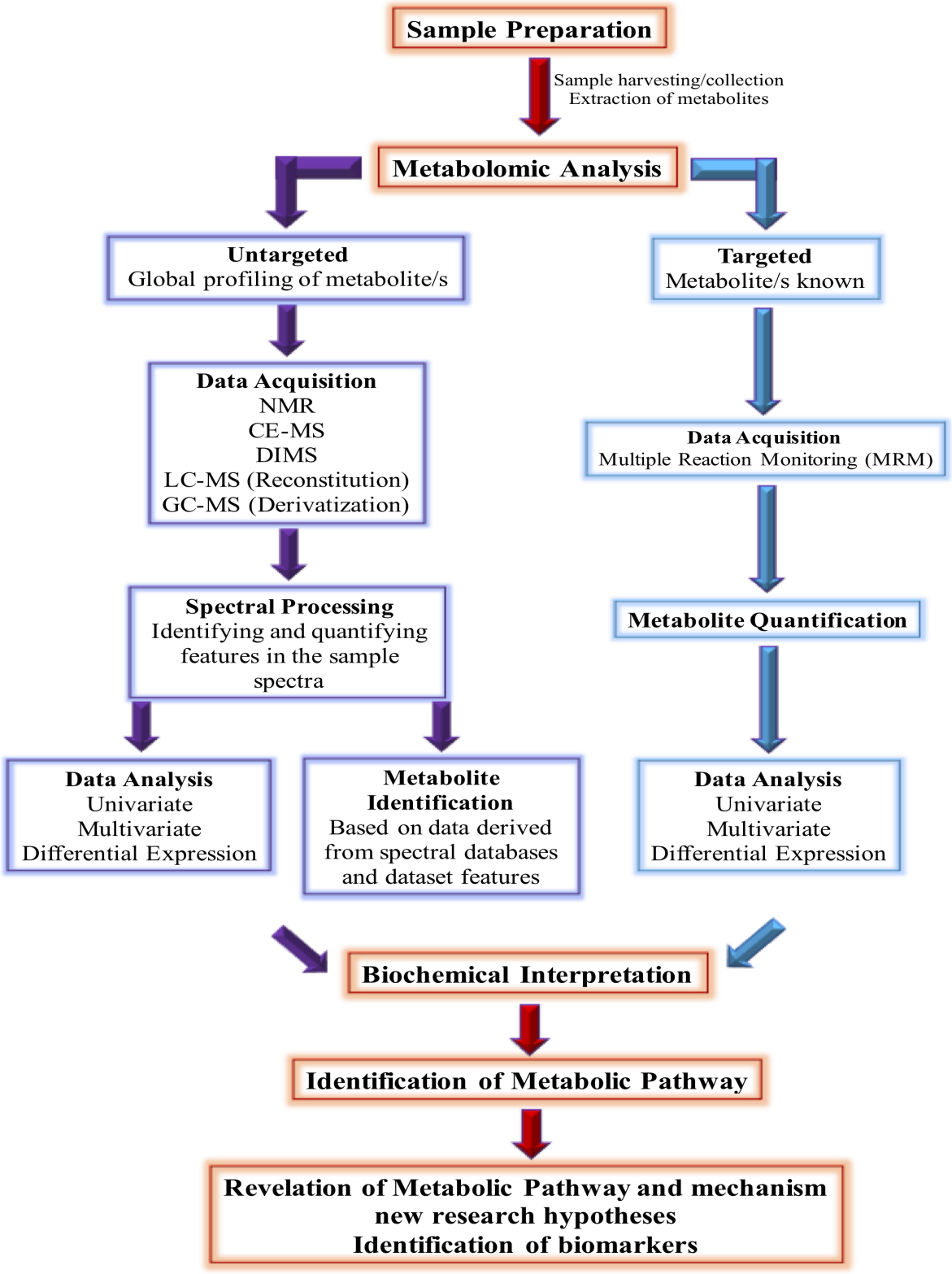


Figure 2.1. Summarized workflow applied during medicinal plant metabolomic studies.

2.7.2 Applications of Metabolomics

New scientific approaches, such as advanced analytical technologies and metabolite imaging, are helpful for metabolomics and may be applied to a broader range of pharmaceutical and biomedical applications. Interestingly, metabolomics provides insights into metabolite biomarkers and potential metabolic mechanisms underlying well-known ailments such as atherosclerosis and cancer. These new scientific approaches have enhanced the identification of previously neglected therapeutic targets, enabling the design of novel, promising therapeutic strategies. Additionally, metabolomics minimizes the cost of toxicological screening, which allows for improved design of clinical trials, thus enabling better patient selection and observation while lessening the duration required for drugs to move through the development pipeline (Olesti et al. 2021).

Metabolomics has over the years emerged as an important component in precision medicine by developing individualized phenotyping and monitoring drug-response reactions. The application of metabolomics in tumour phenotyping and the development and design of modified cancer therapeutics represents the most progressive model of metabolomics-facilitated precision medicine. In plant-based studies, metabolomics may be applied to facilitate drug discovery, identification of herbal medicine, characterization of natural products, ecotype-based differentiation, nutritional evaluation, exposition of biotic and abiotic stress tolerance, and chemotaxonomy (Marchev et al., 2021). The following metabolomics applications using different technologies in bioactive compound discovery are discussed:

a) Nuclear Magnetic Resonance (NMR) spectroscopy

An NMR-based metabolomics approach is an effective technique for investigating and profiling metabolites; it has also been utilized in cancer studies (Zhao et al., 2018). This form of spectroscopy has been shown to possess attractive metabolomics attributes, an important factor

in clinical drug discovery, including non-invasive sample collection, small sample volumes, and minimal sample preparation. Metabolomics has a proven historical track record of identifying inborn errors of metabolism. This approach offers a dynamic perspective on host functional responses under both healthy and diseased states, while enabling more rapid detection and characterisation of disease progression (Mason et al., 2016). High-resolution magnetic angle-spinning nuclear magnetic resonance spectroscopy (HR-MAS-NMR) has enabled direct translation of magnetic resonance spectroscopy (MRS) imaging results (Beger, 2013). NMR-guided metabolomics fractionation is effective in identifying bioactive compounds with anti-HIV activity. This strategy streamlines the discovery workflow and markedly reduces reliance on extensive bioassay testing (Heyman et al., 2015).

Many methods have been developed using NMR spectroscopy, such as non-invasive clinical diagnostics for oral cancer, which utilizes volatile organic compounds found in the exhaled air particles of the individual being examined (Adeola et al., 2017). 1H-NMR spectroscopy has been successfully used in West Africa to identify multiple biomarker metabolites, providing evidence of implications for altered energy-related pathways in hepatocellular carcinoma (HCC) pathogenesis and progression. HCC can also be detected at early stages by identifying urinary metabolites using 1H-NMR (Ladep et al., 2014). *Breonadia salicina* (Vahl) Hepper & J.R.I.Wood is a medium- to large-flowering tree found in many parts of Africa, including South Africa. This plant has been studied and shown to possess strong pharmacological properties, including antioxidant, anti-inflammatory, anti-diabetic, antimicrobial, antiplasmodial, antidiarrheal, anticancer, and antitumor activities. The study carried out the plant analysis using a metabolomics-based approach that coupled proton nuclear magnetic resonance (1H-NMR) and UPLC-QTOF-MS (Tlhapi et al., 2021). Notably, NMR-based metabolomics has provided valuable understanding into the mechanisms and pathways underlying tuberculosis and bacterial

meningitis. This data is instrumental in providing a basis for scientists to effectively design therapeutic strategies that address the shortcomings of current treatments (Mason et al., 2016). Using metabolomics, researchers identified bioactive compounds with anti-HIV, anti-inflammatory, and antimicrobial activities in *Cynanchum callialatum* Buch.-Ham. ex Wight (Karthikeyan & Balasubramanian, 2014).

b) Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS is an analytical technique that couples high-resolution chromatographic separation with sensitive, specific mass spectrometric detection. A typical LC-MS system is a combination of high-performance liquid chromatography (HPLC) with MS using an interface (ionization source). HPLC is an "LC" technique and should be connected to a detector such as a photodiode-array detector (PDA), evaporative light scattering detector (ELSD), or MS. Huge progress has been made in using the LC-MS technique, development of databases and data-handling tools. This method is also non-invasive and requires small sample sizes for analysis, like the NMR method (Mahapatra et al., 2014; Gerona et al., 2019). Metabolite profiling by LC-MS is vital for analysing compounds across a wide range of polarities and molecular weights (Lee et al., 2015). Success in metabolic profiling has recently been achieved through ultra-performance liquid chromatography-mass spectrometry (UPLC–MS), which is also an "LC" technique. Its capabilities include high sensitivity, selectivity in analysis, and structural elucidation for metabolite identification and quantification at low micromolar to nanomolar concentrations. UPLC-MS can rapidly analyse biological samples by providing more specific and comprehensive data and by enabling direct infusion mass spectrometry (DIMS) (Chekmeneva et al., 2018). The advanced application of LC-MS in metabolomics is due to several factors: (a) the widespread availability of instruments, supported by diverse vendor-specific and open-source data processing software, along with a skilled workforce of operators; (b) its broad metabolite

coverage combined with high sensitivity and specificity, which moves the field closer to achieving comprehensive "holistic" metabolic profiling; and (c) the versatility of a single LC-MS setup, which can perform multiple analytical functions (Gika et al., 2014).

Cynanchum callialatum Buch.-Ham. ex-Wight is a non-succulent climber or twiner found in countries such as Madagascar. The LC-MS and GC-MS analysis of this plant's crude extract has successfully shown anti-cancer bioactivity with compounds presence such as betulinic acid, lupeol, germacrone and longiverbenone (Karthikeyan & Balasubramanian, 2014). Interestingly, significant anticancer activity has been observed in plant-derived saponins, including ginsenosides, soy saponins, and saikosaponins.

Improvements in the 'omics' have brought about the use of one or more techniques to identify metabolites. *Lessertia frutescens* (L.) Goldblatt & J.C. Manning is a genus of medicinal plants found in South Africa. South African indigenous people traditionally use this plant as a therapeutic agent against cancer. HPLC–MS analysis of *L. frutescens* crude extract has shown the presence of anti-inflammatory and anti-cancer bioactive compounds (Faleschini et al. 2013; Gouws et al. 2021).

c) Gas Chromatography-Mass Spectrometer (GC-MS)

Gas chromatography coupled with mass spectrometry (GC-MS) is primarily used for identifying volatile compounds, such as those found in essential oils. However, it is less effective for analyzing non-volatile compounds, which require chemical derivatization to become volatile. This modification alters the compound's retention time, complicating its identification. In such cases, liquid chromatography-mass spectrometry (LC-MS) is the preferred technique. *Hellenia speciosa* (J.Koenig) S.R.Dutta is a herbaceous perennial plant native to East Asia, including India, and naturalized in Mauritius. It produces edible fruits, shoots, and roots, and is also valued

for its medicinal properties. GC-MS analysis of its extracts has identified various bioactive compounds, including fatty acids, heterocyclic compounds, steroids, alkaloids, terpenoids, vitamins, octadecanoic acid, n-hexadecanoic acid, caryophyllene, Ar-turmerone, piperine, and squalene, known for their beneficial bioactivities (Ramya, 2022). Similarly, GC-MS technology has been instrumental in identifying antimicrobial, antioxidant, anticancer, hypercholesterolemic, anti-inflammatory, and other activities in *Hibiscus asper* Hook. f. plant (Olivia et al., 2021). Table 2.1 lists previously established findings obtained using metabolomics-guided approaches.

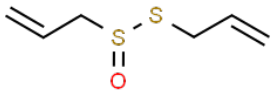
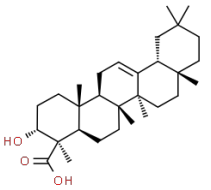
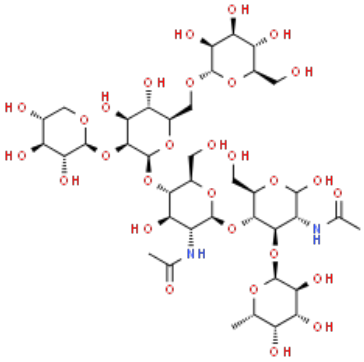
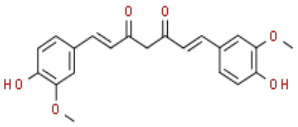
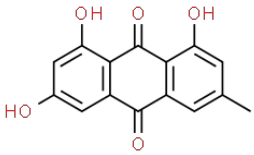
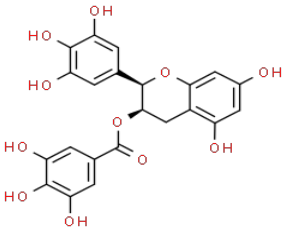
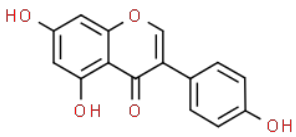
Table 2.1: Common contributions of metabolomics-guided methods in drug discovery

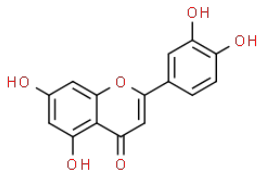
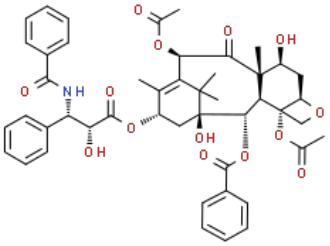
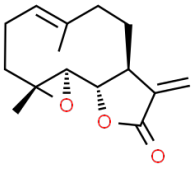
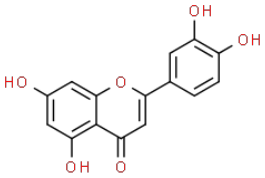
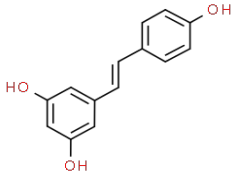
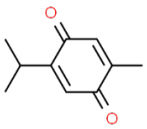
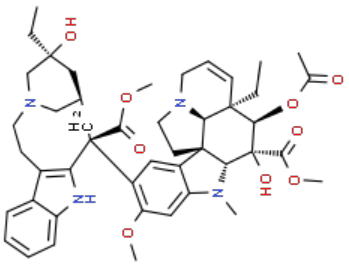
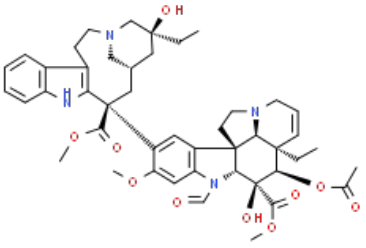
Plant	Location	Method	Activity	Compound	Study Reference
Helichrysum populifolium DC	South Africa	NMR	Anti-HIV	a) 3,4-DCQA b) 3,5-DCQA c) 4,5-DCQA d) 1.3.5-TCQA e) 5-malonyl-1,3,4-TCQA	(Heyman et al. 2015)
Xylopia aethiopica (Dunal) A.Rich.	West-Africa	NMR, HPLC	Anticancer	EOKA	(Choumessi et al. 2012)
Plocamium cartilageneum (L.) P. Dixon	South Africa	NMR, UV, NMR	Anticancer	Halogenated monoterpenes	(Sabry et al. 2017)
Euphorbia guyoniana Boiss. & Reut.	West-Africa	NMR, HPLC	Anticancer	a) 5,6-epoxy-3,8,9,15-tetrahydrox-14-oxo-jatropha-11(E)-ene, b) 3,5,7,9,14,15-hexahydroxy-9-oxojatropha-6(17), 11E-diene, c) 2,5,6,7,8,9,14,15-heptahydroxy-11,12-epoxy-17-ethyl-22-oxojatrophane	(Ghanadian et al. 2015)
Vangueria infausta Burch.	South Africa	NMR	Anti-malarial Antiplasmodial	Morindolide	(Zhao et al. 2018)
a) *Croton grattisimus* Burch. b) *Elaeodendron transvaalense* (Burtt Davy) R.H.Archer	South Africa	NMR	Anti-HIV	-	(Mthethwa et al. 2014)
Hellenia speciosa (J.Koenig) S.R.Dutta	East Asia, Mauritius	GC-MS	Antimicrobial	Caryophyllene, Ar-tumerone	(Ramya 2022)
Ancistrocladus abbreviates Airy Shaw	Cameroon and Ghana	GC-MS	Anti-HIV	Michellamine B	(Bringmann et al. 2008)
Cinchona pubescens Vahl	West African countries	GC-MS	Antimalarial	Quinine	(Canales et al. 2020)

2.7.2.1 Metabolomics in Cancer Drug Development and Treatment

In disease management, drugs are available to treat specific diseases; these may include combinations of more than one therapeutic agent. The use of metabolomics may be necessary for improving knowledge surrounding the discovery, identification and investigation of herbal products (Quansah & Karikari 2016). The discovery of metabolic modifications and challenges about disease status, mechanism, diagnostics, or in response to medical and external treatments is achieved through metabolomics. Factors such as environmental conditions, genetic variation and regulation, changes in the microbiota, and altered enzyme kinetic activity or expression levels have also been monitored using metabolomics. Firstly, the ability to rapidly analyze (identification and quantification of concentrations of all metabolites) tissue or biofluid samples (saliva, blood and urine) with little sample preparation has shown great metabolomics potential for clinical health and cancer treatment (Beger, 2013; Martias et al., 2021). Secondly, metabolomics can detect altered metabolic pathways in cancer and monitor patient status during therapy that targets the specific pathway (Beger, 2013). As a result of this development, about 70% of anti-cancer compounds are natural products or derivatives (Choumessi et al., 2012; Cragg & Pezzuto, 2016) (Table 2.2). Compounds identified during metabolite profiling are found in intermediate metabolic pathways and may serve as biomarkers of exposure or susceptibility to disease (Su et al. 2014; Loras et al., 2018). High treatment costs, limited availability, and significant side effects associated with conventional anticancer therapies emphasise the need to focus research efforts on Traditional African Medicine as a source of effective cancer treatments (Wang et al., 2011; Leighl et al., 2021).

Table 2.2. List of some anti-cancer compounds derived from African medicinal plants (Thomford et al. 2018; Gautam et al. 2020; Talib et al. 2020)

Drug name	Family	Plant Name	Compound structure
Allicin	Liliaceae	*Allium sativum*	
Boswellic Acid	Burseraceae	*Boswellia serrata*	
Bromelain	Bromeliaceae	*Ananas comosus*	
Curcumin	Zingiberaceae	*Curcuma longa*	
Emodin	Polygonaceae	*Polygonum palmatum, Polygonum multiforum*	
Epigallocatechin Gallate	Theaceae	*Camellia sinensis,*	
Genistein	Faboideae	*Trifolium sp, Glycine max*	

Luteolin	Fabaceae	*Sesbania grandiflora, Cajanus cajan*	
Paclitaxel (taxol)	Taxaceae	*Taxus brevifolia, Taxus baccata*	
Parthenolide	Asteraceae	*Tanacetum parthenium, Tanacetum vulgare, Tanacetum larvatum*	
Quercetin	Ginkgoaceae, Sapindaceae, Hypericaceae	*Ginkgo biloba, Aesculus hippocastanum,* and *Hypericum perfuratum*	
Resveratrol	Polygonaceae	*Polygonum cuspidatum*	
Thymoquinone	Ranunculaceae	*Nigella sativa*	
Vinblastine	Apocynaceae	*Catharanthus roseus*	
Vincristine	Apocynaceae	*Catharanthus roseus*	

2.7.2.2. Antiproliferation assays

Multiple assay systems are used to evaluate the anticancer activity of medicinal compounds, with colourimetric and fluorometric methods being the most applied in vitro approaches for measuring cytotoxicity and initial effects on cell viability. Colourimetric assays measure cellular metabolic activity via molecular markers, are rapid, and compatible with various cell types. Fluorometric assays use fluorescent molecules to detect specific compounds in samples. Among these, the MTT assay is commonly used due to its low cost and simplicity. It relies on mitochondrial dehydrogenases, reducing the MTT salt to insoluble purple formazan crystals that correlate with the number of viable cells. However, care must be taken since some phytochemicals can interfere by altering enzyme activity or directly reducing MTT, potentially skewing results. Ensuring no direct interaction between plant compounds and MTT is essential for accurate cytotoxicity assessment (Sanjai et al. 2024).

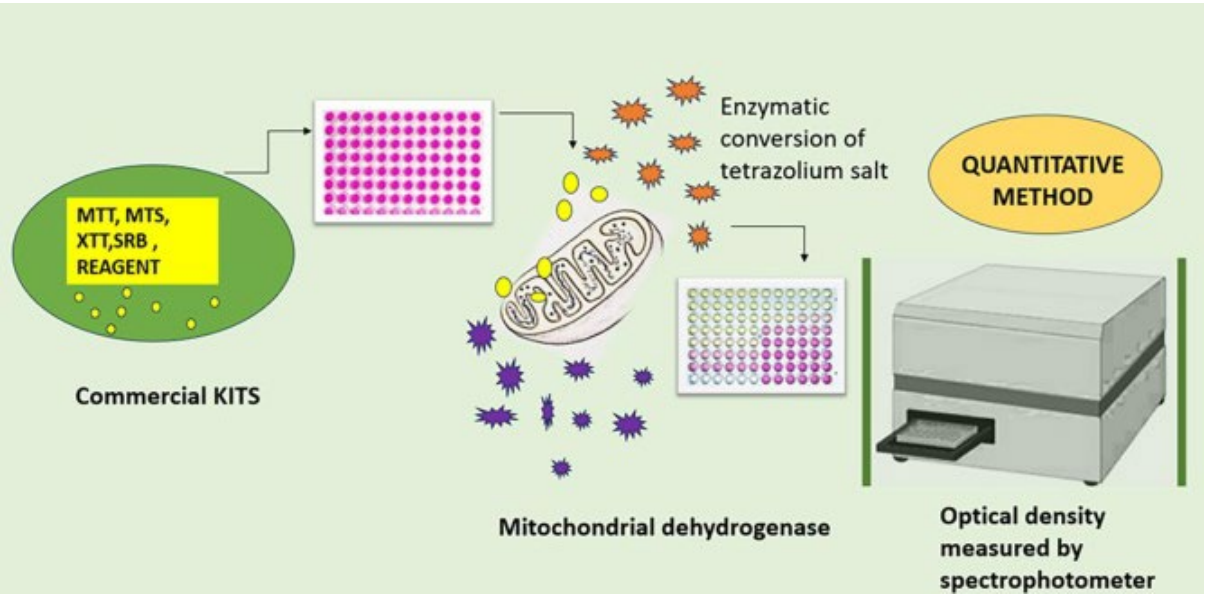


Figure 2.3 Cell viability assay showing the conversion of tetrazolium salt to formazan dye in mitochondria (Sanjai et al., 2024).

2.7.3 Antimicrobial drug discovery from plant bioactive compounds

Diseases such as HIV/AIDS, malaria, and cancer have a continuous infection rate in humanity with increasing mortalities. The anti-cancer drugs taxol (*Taxus brevifolia* Nutt.) and vinblastine (*Catharanthus roseus* (L.) G.Don), and antimalarial drugs such as quinine (*Cinchona pubescens* Vahl.) and artemisinin (*Artemisia annua* L. and *Artemisia afra* Jacq. ex Willd.) were all discovered from natural plant products and are effective in treating these diseases (Table 2.3). Drug discovery from natural products has significantly advanced medicine, subsequently expanding the pharmaceutical industry. The following compounds are medicinal agents identified from natural sources: tetracycline from *Streptomyces aureofaciens* Duggar and doxorubicin from *Streptomyces peucetius* subsp. *caesius* ATCC 27952 and cyclosporine from *Tolypocladium inflatum* Gams (Thomford et al. 2018). Previous phytochemical studies identified compounds such as spermidine, rutin, quercetin, tocopherol, and carotenoids derived from caper (*Capparis spinosa* L.) with antimicrobial, antioxidative, anti-inflammatory, and antiviral activities (Vaou et al. 2021). Curcumin, a bioactive compound isolated from *Curcuma longa* L., is another widely studied natural product with demonstrated anticancer, antimicrobial, and anti-inflammatory properties. Its broad acceptance has led to commercial distribution in multiple product forms, such as tablets, ointments, dietary supplements, soaps, and cosmetic preparations (Sharifi-Rad et al. 2020).

Table 2.3. Selected bioactivities of medicinal plants

Plant	Family	Bioactivity	Reference
Adenium obesum (Forssk.) Roem. & Schult.	Apocynaceae	Antibacterial	(Akhtar et al. 2017)
Ananas cosmosus L.	Bromeliaceae	Antimalarial and anti-inflamatory	(Ajayi et al. 2022)
Artemisia absinthium L.	Asteraceae	Anticancer	(Mughees et al. 2020)
Artemisia annua L.	Asteraceae	Anticancer	(Thomford et al. 2016)
Asparagus officinalis L.	Liliaceae	Dysuria, diabetes, epilepsy, night blindness, tumours, and dysentery, enhancing appetite and increasing milk secretion in women	(Elansary et al. 2018)
Capparis decidua (Forssk.) Edgew.	Capparaceae	Antibacterial, antifungal, and antileishmanial	(Sisay et al. 2019)
Cinchona pubescens Vahl	Rubiaceae	Anti-malarial	(Thomford et al. 2016)
Citrullus colocynthis (L.) Schrad	Cucurbitaceae	Cancer, leukaemia, rheumatism, and amenorrhea and also used as insect repellent, anti-diabetic, antipyretic, anti-inflammatory, antibacterial.	(Elansary et al. 2018)
Cryptolepis sanguinolenta (Lindl.) Schltr.	Periplocaceae	TB, antimalarial, anti-cancer, antidiarrheal	(Kirimuhuzya, 2012)
Cymbopogon citratus (DC.) Stapf	Poaceae	Anti-inflammatory agent, insect repellent, antibacterial	(Elansary et al., 2018; Bellaver et al., 2022)
Lantana camara L. (Noxious weed)	Verbaraceae	TB, antimicrobial, antitumor	(Kirimuhuzya et al. 2009)
Launaea taraxacifolia (Willd.) Amin ex C. Jeffrey	Asteraceae	anticancer	(Thomford et al. 2016)
Litsea elliptica Blume *and L. resinosa* Blume	Lauraceae	Antioxidant, anticancer	(Goh et al. 2022)

Manihot esculenta Crantz	Euphorbiaceae	Anticancer	(Samanthi et al. 2021)
Melissa officinalis L., *Mentha spicata* L. and *Salvia officinalis* L.	Lamiaceae	Antioxidant activities and antimicrobial activities	(Silva et al., 2021)
Myrtus communis L. *and Verbena officinalis* L.	Myrtaceae and Verbenaceae	Antibacterial activity	(Sisay et al. 2019)
Ocimum basilicum L.	Lamiaceae	Skin and liver disorders, colds, coughs, fever, and malaria	(Bellaver et al. 2022)
Senna alexandrina Mill., and *Asparagus aethiopicus* L.	Fabaceae and Asparaguceae	Antioxidant, antibacterial, and antifungal activities	(Elansary et al. 2018)
Thymus vulgaris L.	Lamiaceae	Antibacterial	(Bellaver et al. 2022)
Triticum aestivum L.	Poaceae	Anticancer immunomodulatory	(Save et al. 2019)
Zanthoxylum leprieuii Guill. & Perr.	Rutaceae	HIV/AIDS, malaria, urinary infection, rheumatic pain, TB	(Bunalema et al. 2017)

2.7.4 African medicinal plants with pharmacological properties

The following medicinal plants have been found in Africa with pharmacological properties. *Acacia senegal* (L.) Britton. (Leguminosae) common name: Gum Arabic, from the sub-Saharan African region – diarrhoea, gonorrhoea and leprosy. *Aloe ferox* Mill. (Xanthorrhoeaceae) common name: Cape aloe from South Africa and Lesotho – antioxidant, anti-inflammatory, antimicrobial and anti-cancer properties. *Artemisia herba-alba* Asso (Asteraceae), common name: wormwood, from Northern Africa – Antifungal. *Aspalanthus linearis* (Burm.f.) R.Dahlgren (Fabaceae) common name: rooibos from South Africa – antioxidant, anti-diabetic, and anti-mutagenic properties. *Centella asiatica* (L.) Urb. (Apiaceae) common name: centella from Mauritius – anti-inflammatory. *Catharanthus roseus* (L.) G.Don (Apocynaceae) common name: Madagascan periwinkle from Madagascar – anti-cancer property. *Cyclopia genistoides*

(L.) R.Br. (Fabaceae) common name: honeybush from South Africa – TB and antioxidant. *Harpagophytum procumbens* (Burch.) DC. ex Meisn. (Pedaliaceae) common name: devil's claw from South Africa, Botswana and Namibia – cancer, fever, allergies and analgesia. *Momordica charantia* L. (Cucurbitaceae), common name: bitter melon, from South Africa – diabetes. *Pelargonium sidoides* DC. (Geraniaceae) common name: umckaloabo from South Africa – antibacterial (Mahomoodally, 2013) *Zanthoxylum leprieuii* Guill. & Perr. (Rutaceae) common name: sand knobwood, umnyenye, munungu from South Africa and Uganda – HIV/AIDS, malaria, urinary infection, rheumatic pain, TB (Tuyiringire et al., 2020). *Athrixia phylicoides* DC., commonly known as bush tea in South Africa, is traditionally used to manage diabetes, promote bowel regularity, enhance sexual performance, and act as a diuretic (Mudau et al., 2022).

2.7.5 Advantages and limitations of metabolomics

LC–MS, GC–MS, and NMR are the most widely applied analytical platforms in metabolomics. Although LC–MS and GC–MS remain dominant for metabolite identification, recent studies have shown growing interest in the application of NMR spectroscopy for drug analysis (Emwas et al., 2019). Over the years, metabolomics has advanced, and the different metabolomic techniques have distinct advantages. Herein are some of the advantages: sensitivity of detection, metabolite quantification accuracy, precision of variation, reproducibility of results, reduced analytical time to process large samples, improved identification and assignment of molecular structures of metabolites, and convenience of the developed metabolite databases (Ng et al., 2020). The use of small sample sizes for LC-MS and GC-MS, and sample recovery for NMR, are important metabolomics attributes. The most important attribute of techniques is their ability to develop and connect with other techniques to produce better output. Advancements in

metabolite-determination techniques have their limitations. Key challenges include insufficient standardized protocols for the concurrent identification of large numbers of metabolites in the absence of internal standards, difficulties in harmonizing datasets generated from different analytical platforms and laboratories due to independently developed metabolomics methods, and limited reproducibility of biological samples. In addition, NMR spectroscopy exhibits lower sensitivity and higher operational costs compared with other analytical techniques (Kartal et al., 2002; Zhou & Yin, 2016; Chekmeneva et al., 2018; Emwas et al., 2019; Gika et al., 2014; Saigusa et al., 2021). Consequently, biochemical pathway analysis and the creation of associated visualization tools remain at an early developmental stage (Gika et al., 2014).

2.8 Challenges and opportunities of metabolomics and anti-cancer drug discovery

2.8.1 Challenges

Africa is still behind the rest of the world regarding financial and institutional commitment to developing research programmes in omics (Adeola et al. 2017). Insufficient modern scientific research approaches have negatively affected the development of traditional African medicine. The failure to analyze the dynamics of different diseases and the interaction of the biochemical compounds has limited the advancement of this form of alternative medicine (Wang et al., 2011). Africa is estimated to have about 40,000 to 45,000 plant species with a potential for drug development, and only about 5,000 are used for therapeutic purposes (Mahomoodally 2013). This challenge has created a gap between the desperate need to manage the increase in disease prevalence within the African continent and the advancement of new plant-based drug discoveries.

Although metabolomics is considered a revolutionary approach, several challenges continue to limit its full implementation in research. These challenges include the difficulty in systematically profiling the entire metabolome due to its chemical complexity, inherent biological variability among organisms, and the limited dynamic range of most analytical instruments. As a result, multiple complementary techniques are often combined to achieve more comprehensive and detailed data from each analysis (Johnson et al., 2016). In addition, the healthcare sector in Africa is facing a constant challenge of drastically increasing cases of cancer and other diseases, coupled with reduced public health support to fund cancer research and treatments.

2.8.2 Opportunities

Metabolomics studies of medicinal plants and their inherent anticancer bioactivities remain underexplored. Special attention to such studies can yield detailed, informative compound development strategies. Understanding the interaction of microbiome–metabolome and cancer metabolic adaptability in human malignancies is an important consideration that may bring more opportunities to the cancer research system (Oyenihi et al. 2021). Precise determination of pharmacological properties for African medicinal plants can guide scientists to a new path of research and bioactive compound discoveries that can have a direct disease management effect (Bharti et al. 2021).

Further studies are necessary to assess the effects of various plant species, production areas, and nutrient and non-nutrient compositions on plant pharmacological properties (Unati et al., 2016; Bouyahya et al., 2018). While mixtures of traditional medicinal plants are standard for treating various ailments, comprehensive studies to assess the combined bioactivity of compounds, their

toxicity, and appropriate dosing can be illuminating for the pharmaceutical industry. Therefore, the continued advancement of metabolomics in such cases would be invaluable (Mpelangwa et al., 2021).

2.9 Limitations

The current review did not differentiate African medicinal plants by region or country; however, it focused on showcasing the overall potential at the continental level. The current review did not consider issues related to agronomic practices, utilization, type of drugs on demand, and treatment of interest. Anticancer and antibiotic drugs discussed in this study represent only a few phytochemicals from selected African medicinal plants. A detailed analysis of the nutraceutical and pharmaceutical potential of African medicinal plants is provided in Mudau et al. (2022).

2.10 Way Forward: Strategies on African medicinal plants utilization and metabolomics application

Traditional medicinal plant utilization is in high demand in Africa due to the therapeutic properties found in these plants. However, there are associated challenges resulting from such demand, which include: i) possible over-harvesting due to unlimited access of the wild plants, ii) low productivity rate, low nutrient composition, low phytochemical content (mass on mass) in wild plants, iii) excessive market prices due to scarcity of some of the plants, and iv) difficulty in implementing conservation systems (Mudau et al. 2022).

Strategies to eliminate these challenges include:

a) Developing traditional medicinal plant production systems to increase the productivity of the plants. Production must include introducing new varieties that grow well across different agro-climatic regions in Africa and importing other varieties from outside the continent. Developing appropriate fertilizer programmes that will enhance the nutritional and phytochemical composition of the plants is also crucial.

b) Improving conservation strategies for traditional and wild medicinal plants. The depletion of medicinal plants due to high demand and excessive harvesting can be reduced by developing conservation strategies that indigenous users of these plants will welcome. Inclusive discussions with indigenous users can positively impact the successful implementation of these strategies in the short and long term. Limiting harvest to plant parts of interest, rather than removing or uprooting the whole plant, can contribute to sustainable use and help avoid depletion of traditional medicinal plants. Such an undertaking requires regular training for traditional African users.

c) Local communities have access to wild medicinal plants and have been using these plants to treat different ailments. Their culture influences their reliance on these plants (Matlala et al. 2015) while the pharmaceutical industry is developing and introducing new drugs. A shared understanding between the two can expedite the development of new drugs, with the necessary policies, to benefit local residents. There should be an established understanding that research on African Traditional Medicine must be informed by the need to validate the claims made on their use and to develop a scientific understanding of the African Traditional Medicinal plants (Mothibe and Sibanda, 2019).

d) Developing production, marketing and pricing policies, value-adding, facilitating imports and exports, and enhancing utilization of African medicinal plants. This will lead to sustainable utilization of the plants for both traditional and industrial purposes.

2.11 Conclusion

The increase in African medicinal plants utilization necessitates prioritizing the advancement of metabolomics development and application strategies in drug development. A broad-spectrum, universal, unbiased, highly sensitive, high-throughput, and high-capacity metabolism analysis technique should be developed. Techniques with high accuracy and efficiency have been developed; however, there remain opportunities to develop further methods for identifying or annotating metabolites in databases or tools. These holistic techniques and approaches should encompass plant defence responses to biotic and abiotic stresses.

The application of metabolomics for profiling bioactive constituents in medicinal plants has facilitated the discovery of therapeutic agents targeting cancer and infectious diseases. Notable advances have been achieved in Africa through the use of metabolomics in anticancer and antimicrobial drug discovery from indigenous plant species. While studies on plant bioactivities are currently being prioritized, there is a need for studies to provide knowledge on the nutrient composition of special tea plants from different geographical locations, evaluate the phytochemical content of the plants using three metabolomics techniques, and evaluate the antiproliferative activity of the plants on cancer cell lines. This study aims to provide this invaluable information to the pool of knowledge. This will also effectively contribute to Africa's efforts to address two important sustainable development goals: "end hunger, achieve

food security and improved nutrition and promote sustainable agriculture," and, secondly, "Ensure healthy lives and promote well-being for all at all ages."

The government's involvement in funding RDI on medicinal plants can help build the evidence base needed to inform policymaking and develop a regulatory framework for medicinal plants. In this regard, metabolomics offers opportunities for rapid development and identification of plant bioactive compounds needed to build the evidence base. In addition, through collaboration between traditional and modern medicine, the standardization, mainstreaming, and promotion of traditional medicinal products can be achieved, benefiting millions of people who still use traditional medicine in Africa.

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CHAPTER 3

DETERMINATION OF NUTRITIONAL COMPOSITION OF SPECIAL TEA IN FOUR GEOGRAPHICAL AREAS IN SOUTH AFRICA

Abstract

African cultures have long relied on medicinal plants as vital sources of nutrients and therapeutic agents, deeply integrating their use into daily practices for health and well-being. Among these, special tea stands out as a widely consumed herbal tea, renowned for its medicinal properties and valued for its contributions to traditional healing and nutritional support. Previous studies on special tea have primarily focused on one geographical area, providing limited insight into how regional variation may influence its nutritional composition. This study aimed to evaluate the nutritional composition of special tea harvested from Sekhukhune, Rietondale, Brits and Lanseria in South Africa. Plant samples were washed and subsequently dried using two methods: shade-drying and freeze-drying. Nutrient composition was analyzed for 24 elements using the inductively coupled plasma mass spectrometer (ICP-MS) technique. All 24 elements were identified across the plant samples, with freeze-dried samples exhibiting higher nutrient composition than shade-dried samples. Nutritional composition differences between the four locations were minimal, suggesting that the plant has potential as a source of essential nutrients and can be cultivated across these regions.

Keywords: Drying methods, essential nutrients, medicinal plants, *Monsonia burkeana.*

3.1 Introduction

Plant nutrients are an invaluable factor for growth and development, as are key in various metabolic processes (Andresen et al., 2018). Plants require approximately 16 elements for normal growth and the completion of their life cycle. Nitrogen, phosphorus and potassium should be supplied in large quantities to plants, followed by calcium, magnesium and sulphur, as these form the required

macronutrients. Micronutrients include boron, copper, chlorine, iron, manganese, molybdenum, nickel and zinc. These are required in smaller quantities (Njinga et al., 2013; Dalcorso et al., 2014). Soils vary in the amounts of these elements, and some may lack one or more. A shortage of one or more essential nutrients can affect plant growth and yield (Njinga et al., 2013).

Plant's role in transferring essential minerals from the soil to humans and animals cannot be undermined. However, the uptake of these minerals from the soil depends on soil properties (Table 3.1) and plant genotype (Anjum et al., 2015; Sultanbawa & Sultanbawa 2023). It has been concluded that site location may influence element concentrations in Amatungula fruits (*Carissa macrocarpa*) (Moodley et al., 2012). However, nutrients may be a threat to human health if they are consumed at high levels from plants (Moodley et al., 2012).

Table 3.1: Primary forms of nutrient intake by plants (Njinga et al., 2013; Jones & Jacobsen, 2005)

Elements	Symbols	Forms used by plants
Nitrogen	N	$NH4^{+}$ and NO^{3-}
Phosphorus	P	HPO_4^{2-} and $H_2PO_4^{-}$
Potassium	K	K^{+}
Calcium	Ca	Ca^{2+}
Magnesium	Mg	Mg^{2+}
Iron	Fe	Fe^{2+} and Fe^{3+}
Manganese	Mn	Mn^{2+}
Zinc	Zn	Zn^{2+}
Copper	Cu	Cu^{2+}
Molybdenum	Mo	MoO_4^{2-}
Nickel	Ni	Ni^{2+}
Sodium	Na	Na^{+}
Chlorine	Cl	Cl^{-}
Sulphur	S	SO_4^{2-}
Boron	B	H_2BO_3 and H_2BO_3-

Micronutrients in the soil may occur naturally (lithogenic, non-anthropogenic) or anthropogenically (human-induced). Micronutrients in the topsoil are mainly affected by lithogenic processes, whereas anthropogenic macronutrient inputs occur through agriculture and other industrial processes. Several factors, such as soil pH, redox potential, clay content, cation exchange capacity, organic matter, ageing of added elements, and the nature and quantity of elements, significantly affect the phytoavailability of elements. The accumulation, exclusion and translocation of trace element uptake strategies determine the behaviour of plants (Antoniadis et al. 2017; Masanabo et al. 2019). In fulfilling these strategies, elements must be modified, absorbed from the soil, sequestered in the root, loaded into xylem vessels, transported to the plant's aerial parts and distributed among the leaf cells (Dalcorso et al., 2014). A plant's ability to tolerate trace elements is achieved through various mechanisms, including sequestration or compartmentalization within cells, binding or chelation, excretion from above-ground tissues, the action of enzymatic and non-enzymatic antioxidants, protective responses, stress recovery processes, and the repair of damaged proteins. (Antoniadis et al., 2017).

The inductively coupled plasma mass spectrometry (ICP-MS), which offers high sensitivity, low detection limits, and superior accuracy compared with alternative methods such as colourimetric assays, is common for element analysis. The technique is robust and versatile, enabling rapid multi-element quantification of both micro- and macronutrients in plant samples. (Pereira et al., 2010; Santos et al., 2017; Q. Zhang et al., 2018; Masanabo et al., 2019).

Monsonia. burkeana is extensively utilized for medicinal purposes in a number of areas across South Africa and is currently receiving attention from researchers aimed at determining a number of physiological and phytochemical properties, including micro- and macronutrients. While work has been done on the nutritional content of the plant, this study hypothesises that different geographical locations may influence the accumulation of special tea nutrients. Therefore, this study aimed to

determine the nutritional composition of special tea from four different areas in South Africa using ICP-MS.

3.2 Materials and Methods

3.2.1 Sample collection and preparation

Monsonia burkeana plants were collected whole from the wild in four different areas: Sekhukhune (Limpopo province), Rietondale (Pretoria – Gauteng province), Brits (Northwest province), and Lanseria (Gauteng province) (Figure 3.1 and Table 3.1). the plants were uprooted and kept in a cool environment (cooler box) to reduce further biological activities during transport and storage. The plants were transported from the collection's areas to the Horticulture Centre, Science Campus, University of South Africa, Florida. The plant samples were first washed with running tap water and then rinsed with deionised water. Post-harvest processing involved either freeze-drying or shade-drying of plant material. For freeze-drying, samples were enclosed in plastic bags, pre-frozen at −80 °C overnight, and then lyophilized for a duration of seven days. Shade drying plant samples were placed in paper bags to protect them from dust and contamination in the pot shed at the Horticulture Centre, Science Campus, University of South Africa, Florida. The plants were dried for 14 days. Following the drying process, the entire plant samples were finely ground with a blender and securely stored in plastic bags until subsequent analytical procedures.

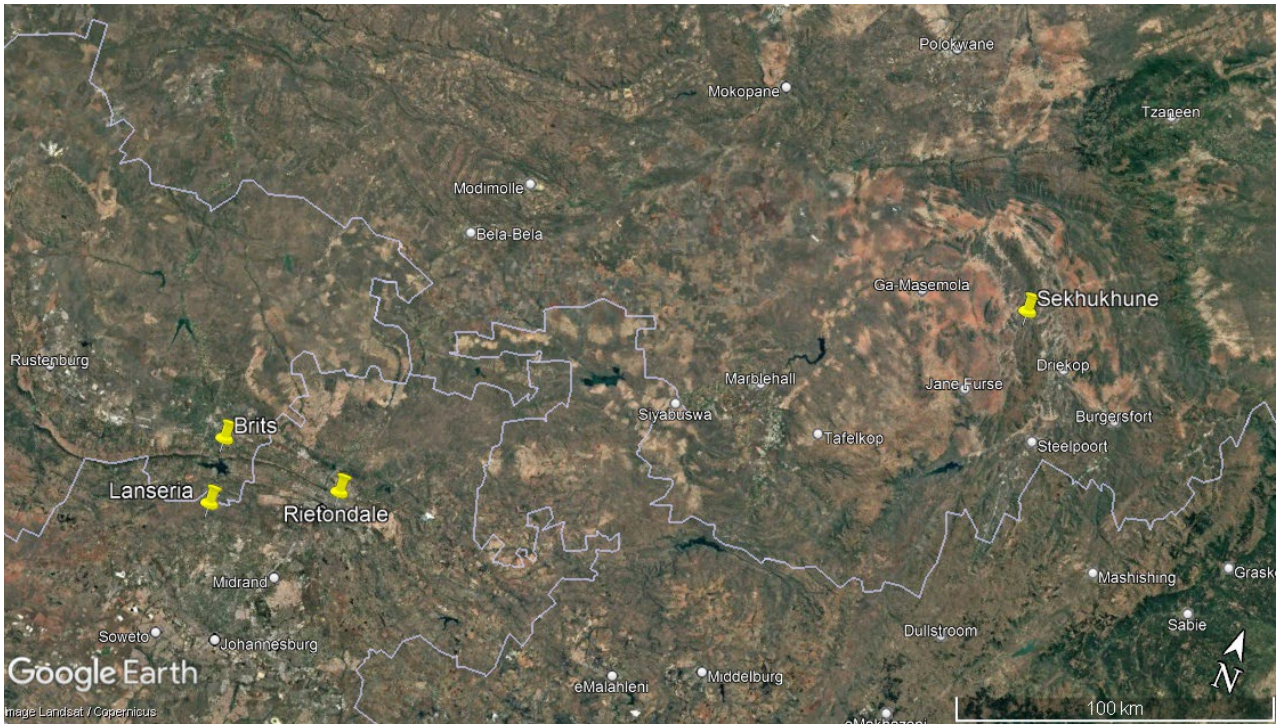

Figure 3.1. Locations where special tea plant samples were collected.

Table 3.1: Geographical collections locations for special tea

Collection area	Coordinates		Altitude (m)
Sekhukhune	24°31'59.8" S	29°58'35.3" E	1166
Rietondale	25°43'45.5" S	28°14'19.7" E	1189
Brits	25°42'42.9" S	27°50'48.8" E	1097
Lanseria	25°53'44.4" S	27°52'43.6" E	1381

3.2.2 Digestion procedure and elements determination

The preparation, digestion, and element analysis using the ICP-MS technique were conducted at the Eureka Building, Science Campus, University of South Africa, Florida, Gauteng, RSA. Two grams of ground plant samples were placed into microwave vessels. Nitric acid (5mL, 65% concentration)

supplied by Sigma-Aldrich Pty. Ltd., Johannesburg, South Africa, was added to each vessel. Microwave digestion was performed at 200 °C for 25 25-minute ramp time and 200 °C for 25 25-minute hold time. The vessels were cooled and then filtered into a 50mL measuring cylinder with a Whatman^{tm} No.1 filter paper. Milli-Q water from a Milli-Q analytical purification system (Millipore, USA) was used to dilute the filtered samples. One mL was extracted into a 10 mL tube and further diluted with MilliQ water. All samples were prepared in triplicate, and a total of 24 samples were digested.

3.2.3 Standard solution preparation

A TraceCERT® multi-element standard solution 24 for ICP (in nitric acid) was supplied by Sigma-Aldrich Pty Ltd, Johannesburg, South Africa. The following standards were used in 1% nitric acid (5 ppb, 10 ppb, 25 ppb, 50 ppb, and 100 ppb), and blanks were added for analysis.

3.2.4 ICP procedure

The plant samples, for nutrient profiling, were tested for a wide range of elements: Al, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, Li, Mn, Na, Ni, Pb, Se, Sr, Te, Ti and Zn. A PerkinElmer inductively coupled plasma mass spectrometer (ICP-MS) (NexION^{tm} 350D) was used for elemental determination. Chemically pure-grade helium gas was used as the collision cell gas for the KED (Kinetic Energy Discrimination) mode. The ICP-MS was calibrated to less than 100 ppb before measurements were made for KED mode to maximize sensitivity and monitored throughout the process for smooth running of analysis. The ICP-MS method used in this study was adapted from Vorster, Greeff and Coetzee (2010).

3.2.5 Statistical analysis

The elemental concentration data obtained from the ICP-MS analysis were first organised and cleaned using Microsoft Excel. The processed data were then subjected to statistical analysis using JMP software (version 15, SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was performed to determine significant differences among treatments, and mean separations were conducted at $p \leq 0.05$.

3.3 Results and Discussion

The results showed that all targeted elements were identified in plants from the four sampling areas. Aluminium and calcium were recorded in the highest concentrations across all areas, followed by sodium, zinc and iron, as shown in Table 3.2. The freeze-drying method resulted in high concentrations of aluminium, zinc, and sodium in samples collected at Sekhukhune, although no significant differences were observed. It also resulted in high concentrations of calcium, iron, boron, barium, chromium, strontium, and tin in samples collected at Brits. The shade-drying method resulted in higher concentrations of calcium, iron, and manganese. Brits showed higher concentrations of several elements, which may be attributed to elevated levels in the surrounding soils. However, this interpretation should be viewed cautiously and requires further investigation as ongoing studies progress. Similarly, the nutrient analysis in this study assessed only total nutrient concentrations in the plants, not the bioavailability of individual nutrients.

Shade-drying results indicated that Rietondale (27.49) and Sekhukhune (17.00) exhibited significantly higher ($p < 0.05$) Na content than Lanseria (9.75) and Brits (8.90). Under freeze-drying conditions, Sekhukhune (17.00) recorded a significantly higher Na concentration than the other three locations. The freeze-drying method for calcium and iron has Brits (85.93, 47.40), Sekhukhune (56.87, 27.44), Lanseria (54.19, 21.98), and Rietondale (35.87, 8.19), respectively, which showed

significant differences between the means. Under shade drying, the corresponding calcium concentrations were Lanseria (52.55), Brits (46.09), Sekhukhune (43.39), and Rietondale (30.67).

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Table 3.2 presents a significant difference between the means of the four areas and the two drying methods. PCA results in Figure 3.2 show variation between the four areas. Between the freeze-drying and shade-drying methods, Sekhukhune showed significant differences in zinc and strontium; Lanseria showed significant differences in boron and manganese; Rietondale showed significant differences in selenium, sodium, and cobalt; and Brits showed significant differences in all elements except selenium, sodium, and lithium. The difference may have been affected by differences in soil properties, which necessitate further synthesis of these outcomes in relation to soil properties across the different areas. Since the special tea is utilized by different people in different geographical areas, the biochemical composition percentage of the plants consumed may differ as well due to the response of the plants to environmental conditions.

The two drying methods differed significantly in element accumulation, with freeze-drying yielding higher concentrations than conventional drying. The PCA in Figure 3.3 indicates variation in the means of the two methods. A study by (Arslan et al. 2010) reported that while drying adds value to the plant product, plants may lose nutritional value during the drying period. While there was no significant difference between the two methods, freeze-drying would be preferred; however, given costs, shade-drying may still be a cost-effective approach for determining element composition in *M. burkeana* plants.

Table 3.2: Average mean of *M. burkeana* selected elements concentration in four areas (ppb)

Area	Drying method	Al 27	B 11	Ba 138	Be 9	Ca 43	Fe 57	Cr 52	Mn 55	Zn 66	Cu 63	Ga 69	Se 82	Sr 88	Tl 205	Li 7	Na 23	Co 59	Bi 209	Pb 208
Sekhukhune	Freeze dry	93.94	1.67	0.91	0.01	56.87	27.44	0.81	7.23	17.26	0.74	0.07	0.11	3.14	0.00	0.22	18.20	0.05	0.01	0.03
	Shade dry	75.29	1.02	0.44	0.01	43.39	21.13	0.43	5.04	9.88	0.52	0.02	0.11	1.83	0.00	0.12	17.00	0.04	0.01	0.02
Brits	Freeze dry	68.41	4.72	5.72	0.04	85.93	47.40	2.88	10.29	10.49	0.89	1.45	0.02	4.66	0.03	0.33	13.11	0.08	0.11	0.14
	Shade dry	27.13	3.45	3.42	0.01	46.09	14.29	0.83	5.57	7.14	0.66	0.60	0.07	2.61	0.01	0.19	08.90	0.02	0.04	0.08
Lanseria	Freeze dry	31.95	2.79	0.63	0.01	54.19	21.98	0.22	18.70	9.03	0.64	0.08	0.17	1.29	0.00	0.13	12.88	0.01	0.02	0.06
	Shade dry	34.77	1.95	0.48	0.01	52.55	22.06	0.32	22.59	8.37	0.80	0.04	0.08	1.29	0.00	0.24	9.75	0.01	0.01	0.06
Rietondale	Freeze dry	15.19	0.66	5.39	0.01	35.87	8.19	0.23	7.55	8.84	0.79	0.32	0.00	2.65	0.00	0.98	10.46	0.03	0.00	0.03
	Shade dry	22.51	0.41	3.64	0.01	30.67	10.26	0.22	5.81	8.37	0.59	0.22	0.29	2.15	0.00	0.81	27.49	0.00	0.00	0.03
	S.E	6.99	0.26	0.50	0.002	5.61	2.75	0.16	0.99	0.98	0.09	0.06	0.05	0.37	0.003	0.08	1.53	0.01	0.009	0.009

SE *= standard error of the mean*

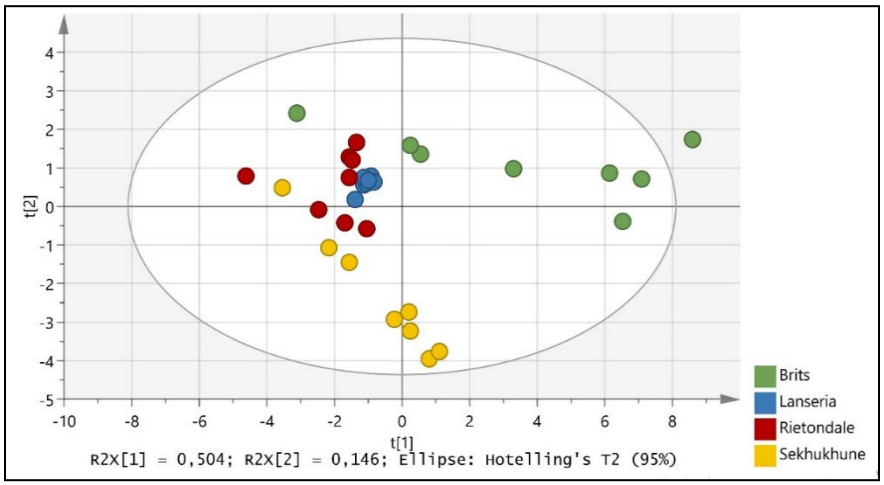

Figure 3.2: PCA results of Brits, Lanseria, Rietondale and Sekhukhune

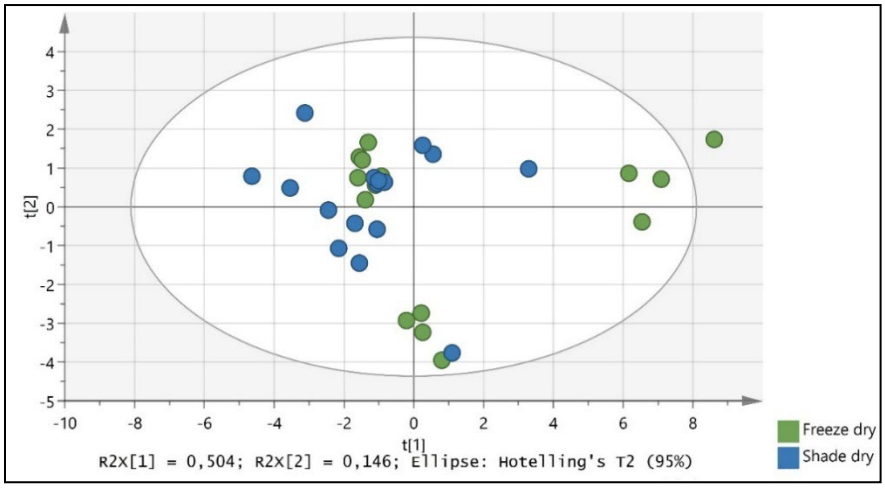

Figure 3.3: PCA results for freeze-drying and shade-drying methods

Special tea had a high concentration of calcium and magnesium (macronutrients). Macronutrient accumulation in the plant indicates that it can absorb available nutrients from the soil and maintain growth regardless of the area/region in which it is growing. A study by Mamphiswana et al. (2011) found that special had accumulated the highest concentration of macronutrients in reproductive and

vegetative organs. Another study reported that calcium and iron had higher concentrations in wild vegetable species than in cultivated species (Flyman & Afolayan, 2006), supporting this study's findings that iron and calcium had higher concentrations in the plants.

Micronutrients were detected at lower levels in all four areas. Iron and aluminium had the highest concentrations among the micronutrients. This is further supported by a study (Mamphiswana et al., 2011) on M. burkeana plants harvested from Chuenespoort, Limpopo Province, South Africa, which found that iron concentrations were high in the roots. Therefore, the microelements in the plant could have resulted from adequate nutrient availability in the four areas. This is despite some of the land, such as Rietondale, having undergone industrial activities, such as grass cutting and bush clearance. This is supported by the fact that soil nutrient availability and supply are required for both plant uptake and rhizosphere microbial activity (López-Arredondo et al., 2013). However, micronutrient deficiencies in plants may arise from factors such as calcareous soils, elevated pH, low organic matter content, salinity, extended periods of drought, high bicarbonate levels in irrigation water, and imbalanced fertilizer use (Malakouti, 2008). Though micronutrients are required in small quantities, as shown by this study, they are highly essential for vital bioprocesses, and their deficiency may pose serious challenges to plant growth, development, and yield, ultimately compromising the health of humans and animals (Malakouti, 2008). The presence of some micronutrients in the soil may affect the concentration of other elements in the plant. An increase in nickel may affect potassium accumulation in plant shoots, as observed in a study of forage grass (Anjum et al. 2015). Nickel was found at a very low concentration in this study, which could be a contributing factor to the high potassium concentration (potassium and magnesium concentrations were above the 100-ppb calibration level; therefore, not presented). Notably, special tea plant samples have shown that toxic

elements, such as lead, were present at low levels (0.009-0.14 ppb). This suggests that the plant is safe for human and animal consumption.

The role of played by plants in contributing significantly to the well-being of both humans and animals has been well documented (López-Arredondo et al., 2013; Anjum et al., 2015), and elements such as iron, zinc and boron act as cofactors in enzymatic reactions (López-Arredondo et al. 2013). Manganese, copper, and zinc are involved in the synthesis of superoxide dismutase, an enzyme that protects against reactive oxygen species and may contribute to the regulation of hypertension. This activity may partly explain the traditional use of this special tea for immune enhancement. These factors may contribute towards *M. burkeana* utilization in improving human health and being grazed by animals for a similar function. This, therefore, positively links the high utilization of this medicinal plant with the presence of these macro- and micro-elements, as the study has shown.

3.4 Conclusion

This study suggests that macro- and microelements were detected in special tea across all areas and drying methods, and that toxic elements, such as lead, were present at levels below the permissible limits. This finding makes special tea an invaluable source of nutrients. Furthermore, the freeze-drying method may be suitable for commercial purposes, as it results in higher nutrient concentrations than shade-drying. The study also suggests that, subject to soil nutrient availability, different geographical areas may not have a direct effect on the nutritional composition of this plant. When produced commercially, this plant's nutritional composition, when drunk as a herb, may be able to supplement daily nutritional requirements in impoverished communities. Further studies on soil analysis across different geographical areas could help determine the relationship between these findings and soil nutrient availability.

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CHAPTER FOUR

EVALUATION OF BIOACTIVE COMPOUNDS IN SPECIAL TEA USING THREE METABOLOMICS TECHNIQUES

Abstract

Special tea is a traditional herbal tea valued for its medicinal properties. Its phytochemical composition across different regions where it is naturally growing has not been widely studied. This study aimed to identify the phytochemical composition of special tea harvested from Sekhukhune, Rietondale, Brits and Lanseria in South Africa using three metabolomics techniques. The tea, including its roots, was harvested from the four regions and dried. Phytochemical extraction was performed using acetone, hexane, and methanol as solvents. The NMR, GC×GC/TOFMS and UPLC-MS techniques were used to analyze the plant crude extracts. Alkyl, benzylic, ether, ester, alcohol, ketone, aldehyde, vinylic and aromatic compounds from the plant samples were identified using NMR spectroscopy. These compounds were then confirmed through the application of GC×GC/TOFMS and UPLC-MS techniques where the following phytochemicals were identified: farnesol, 2-nonanone, benzyl alcohol, β-caryophyllene, caryophyllene oxide, glycerin, glycolic acid, α-humulene, isobutyl acetate, nerolidol, oxalic acid, phytol, squalene, vanillin, vitamin E, grandinin, castalagin, vescalagin, valoneic acid dilactone, acutissimin B, epicatechin, 1,6-digalloyl-beta-D-glucopyranose, chestanin, 1,2,6-Trigalloyl-beta-D-glucopyranose, 1,2,6-trigalloyl-beta-D-glucopyranose, lobetyolinin and citroside A. Additionally, six phytotoxins, listed in the Toxic Plants – Phytotoxin (TPPT) database, were identified, including methyl 2-benzoyl benzoate, β-caryophyllene, caryophyllene oxide, dodecyl acrylate, oxalic acid, and phthalic acid. These findings demonstrate that special tea contains medicinally relevant compounds, while it may also contain

phytotoxins. However, the levels and significance of the phytotoxins in the plants require further analysis, as they may result from the plants' defence mechanisms.

Keywords: Drying methods, medicinal plants, phytochemicals, phytotoxins, solvents

4.1 Introduction

Metabolites are small biological molecules crucial for energy synthesis and conversion, performing important functions during plant interaction with environmental and endogenous stimuli (Alawiye & Babalola, 2021). They also assist in determining plant functions and health during metabolic processes. Insights into these metabolites have advanced metabolomics, a powerful approach essential for assessing plant growth and development, including yield, and for improving overall plant productivity and quality. They have assisted in studying plant responses to abiotic and biotic environmental factors. Metabolomics has applications in medical science, synthetic biology, and agriculture, including the modelling of plant processes and animal and microbial systems. It is instrumental in studying metabolic pathways (Chaudhary et al., 2019; Halket et al., 2005; Alawiye & Babalola, 2021; Patel et al., 2021; Shaw et al., 2021). Recent advances in the 'omics' technologies at the genomic, transcriptomic, proteomic, and metabolomic levels have assisted researchers in gaining new insights into plant genotypes, phenotypes, pathways of metabolism and medicinal properties (Shaw et al., 2021; Waris et al., 2022).

Advancements in metabolomics technologies have enabled the profiling of vast numbers of metabolites, significantly advancing research on photosynthesis, respiration, and other biochemical processes. These developments have also expanded the scope of applied plant sciences, including molecular breeding in agriculture and metabolic engineering to produce valuable traits and bioactive

compounds. Moreover, metabolomics now plays a vital role in plant science, enabling in-depth analysis of plant constituents, assessment of their quality, nutritional and sensory attributes, and their functional and toxicological properties (Faizi et al., 2014; Waris et al., 2022).

Standard metabolomics techniques use mass spectrometry (MS) as a core analytical method, often combined with gas chromatography (GC) or liquid chromatography (LC), as well as nuclear magnetic resonance (NMR) spectroscopy. MS determines the mass-to-charge ratio of ionized molecules, while NMR utilizes the magnetic characteristics of specific atomic nuclei, such as ^{1}H, ^{13}C, and ^{31}P (Kruk et al., 2017).

The targeted and untargeted metabolomics analysis are utilized in the evaluation of metabolites. Although both approaches follow comparable steps in sample collection, preparation, and chromatography, targeted metabolomics is directed at quantifying selected metabolites from specific pathways, whereas untargeted metabolomics seeks to capture a comprehensive overview of all metabolites present within a sample (Patel et al., 2021; Waris et al., 2022).

Metabolomics has been instrumental in uncovering secondary metabolites (SMs) in plants, which have diverse pharmaceutical uses. These compounds are crucial for helping plants cope with environmental stresses. Using metabolomics, around 100,000 SMs have been identified across various plant species and classified into categories such as nitrogen-based compounds, terpenes, thiols, and phenolic compounds.

4.1.1 Nuclear magnetic resonance (H and C-NMR) spectroscopy

NMR spectroscopy involves stimulating nuclear spins in an external magnetic field by rapidly changing the field, followed by detection of the electromagnetic radiation emitted during relaxation.

Only nuclei that resonate at the specific frequency of the applied electromagnetic wave will absorb it. The resonance frequency is influenced by the local chemical environment of each nucleus, allowing the differentiation of nuclei based on the types of atoms surrounding them within a compound (Kruk et al., 2017; Patel et al., 2021).

1D and 2D NMR focus on fluids and tissue extracts; metabolomics can still be used to analyze tissue samples using solid-state nuclear magnetic resonance (ssNMR) spectroscopy. The advantages of ssNMR spectroscopy include minimal sample preparation and results comparable to those obtained with more time-consuming tissue-extraction NMR. ssNMR is non-destructive and requires only small tissue samples, thereby allowing subsequent histopathological examination. However, ssNMR spectra contain numerous peaks, necessitating the use of 2D NMR techniques. Due to dipolar coupling and chemical shift anisotropies, signals are broadened, increasing signal overlap and making identification more challenging (Kruk et al., 2017).

Advantages of NMR spectroscopy include i) no need for preceding separation of purification methods, ii) detects both hydrophilic and lipophilic metabolites, iii) allows for quantitative analysis, iv) fast methods (1-dimensional techniques, solid-state NMR), v) requires small amounts of samples, vi) non-invasive and non-destructive, and vii) reproducible.

4.1.2 Gas chromatography-mass spectra analysis (GC-MS)

GC-MS is widely recognized for its high sensitivity, reproducibility, and excellent peak resolution. Its utility is enhanced by electron impact spectral libraries, which facilitate the identification of diagnostic biomarkers; however, the technique has limitations in analyzing polar and non-volatile compounds (Pasikanti et al., 2008; Wolfender et al., 2015). To address complex mixtures, comprehensive two-dimensional gas chromatography (GC×GC) coupled with TOFMS detectors and

peak deconvolution software is often employed due to its superior resolution and high peak capacity (Li et al., 2009). GC-MS is particularly effective for identifying and quantifying small metabolites such as amino acids, fatty acids, hydroxy acids, alcohols, sugars, sterols, and amines. Because many of these compounds are not naturally volatile, chemical derivatization is required, typically involving two steps: conversion of carbonyl groups to oximes using methoxyamine hydrochloride, followed by trimethylsilylation with reagents such as MSTFA or BSTFA to enhance volatility (Patel et al., 2021; Waris et al., 2022).

4.1.3 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS is a highly versatile tool widely employed in plant metabolomics for the analysis of complex metabolite mixtures. Unlike GC-MS, LC-MS does not require volatilization, making it particularly suitable for high molecular weight compounds (>500 kDa), heat-sensitive or chemically unstable functional groups, and molecules with high boiling points. This technique is effective for profiling secondary metabolites such as alkaloids, phenolics, flavonoids, and terpenes, as well as lipids including phospholipids, sphingolipids, glycerolipids, sterols, and steroids. The choice of ionization method depends on the chemical characteristics of the mobile and stationary phases, with reverse-phase columns (e.g., C18 or C8) commonly used alongside gradient separations involving aqueous-based mobile phases. Atmospheric pressure ionization and electrospray ionization are the most frequently applied techniques in LC-MS (Patel et al., 2021). LC-MS enables accurate determination of molecular structure and weight, allows simultaneous measurement of multiple phytochemicals in a single run, and is particularly advantageous for analyzing polar and non-volatile metabolites compared to GC-MS (Patel et al., 2021; Waris et al., 2022).

4.2 Materials and Methods

4.2.1 Application of NMR spectrometry in metabolite determination

The collection of special tea from four areas, including drying and storage, is detailed in section 3.2.1.

Plant samples were used from the stored samples as stated in 3.2.1. Nuclear magnetic resonance (NMR) spectral studies were performed on the purified compounds using a Bruker 600 MHz NMR spectrometer.

Nuclear magnetic resonance (NMR) spectra were collected using a 600 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA, USA) with 20 scans per sample. The procedures for sample preparation, data acquisition, analysis, and data processing were adapted from Maree and Viljoen (2012). For analysis, 50 mg of powdered special tea samples were placed into 2 mL Eppendorf tubes, followed by the addition of 1.0 mL of a solvent mixture composed of methanol D_4 and deuterium oxide (D_2O) in a 1:1 ratio (pH 6.0). The samples were vortexed at room temperature for 1 minute, ultrasonicated for 15 min to disrupt cell membranes, and then centrifuged for 5 min. About 0.5 mL of the resulting supernatant was transferred to NMR tubes for spectral analysis. All proton (^1H) spectra underwent baseline and phase correction using MestReNova 12.0 software (Mestrelab Research S.L., Santiago de Compostela, Spain). The spectral range from 0 to 10.00 ppm was segmented into 0.04 ppm bins, converted to ASCII format, and imported into Microsoft® Excel 2010 (Microsoft Corporation, Redmond, WA). Multivariate data analysis was conducted using ClustVis with Pareto scaling.

4.2.2 GC×GC/TOFMS spectrometry

4.2.2.1 Extraction procedure for GC×GC/TOFMS

Methanol, *n*-hexane and acetone GC-MS grade (analytical grade) were used as solvents. Fifty mg of each dried plant sample were weighed into 2-mL Eppendorf tubes and 0.75 µL of solvent and 0.25 µL of deionized water were added to the samples. The mixture was vortexed for 1 minute and sonicated for 20 minutes. The sonicated samples were then centrifuged at 184 × g for 20 minutes and syringe filtered (0.4 µL) into GC-MS vials (Mohale et al., 2020). Each sample was replicated three times.

4.2.2.3 GC×GC/TOFMS crude sample analysis

The filtrate samples were analyzed using a gas chromatograph (Agilent Technologies 7890B) equipped with a LECO Pegasus 4-D GC×GC-TOF MS detector and a Gerstel multi-purpose sampler (GMbH and Co. KG, 45473 Mülheim an der Ruhr, Germany). Chromatographic separation was performed on an Agilent HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 µm) housed in the oven. Helium, with a purity exceeding 99.999%, served as the carrier gas at a constant flow rate of 1.5 mL/min. A 1.5 µL sample volume was injected in splitless mode at an inlet temperature of 250 °C. The temperature program began at 35 °C, held for 1 minute, then ramped to 142 °C at 5 °C/min with a 3-minute hold, followed by an increase to 240 °C at the same ramp rate. The transfer line was maintained at 225 °C. The mass spectrometer ion source temperature was set to 200 °C, with a filament bias voltage of −70 V. Mass spectra were acquired over the range 40 to 550 atomic mass units (amu) at 10 spectra per second, with the detector voltage set to 1670 V. The GC's total runtime was 90 minutes. The MS operated in full-scan mode, and peak areas were quantified using ChemStation software (Agilent Technologies). Tentative identification of volatile compounds was

achieved by comparing their mass spectra with the NIST Mass Spectral Data Library (17th edition) (Malongane et al., 2020).

Principal component analysis (PCA) was performed using the Umetrics Suite of Data Analytics Solutions (SIMCA). The heat map was generated using ClustVis, a web-based tool for visualising clustering of multivariate data via Principal Component Analysis.

4.2.3 Sample analysis using LC-MS spectrometry

4.2.3.1 Sample preparation

Dried samples as prepared in 3.2.1 were used in this analysis. About 0.25 g of sample was weighed into a 15 ml falcon tube and extracted with 10 ml 50% methanol/1% formic acid in water with vortexing and sonication. After centrifugation, the supernatants were diluted 5x into 50% methanol/0.1% formic acid and transferred to glass vials for analysis. All samples were replicated three times.

4.2.3.2 Profiling of compounds in electrospray-negative using LC-MS analysis

High-resolution UPLC–MS profiling was performed using a Waters cyclic quadrupole time-of-flight (qTOF) mass spectrometer coupled to a Waters Acquity ultra-performance liquid chromatography system (Waters, Milford, MA, USA). Following chromatographic separation, the eluate was passed through a photodiode array (PDA) detector, enabling simultaneous acquisition of UV and mass spectral data. Ionization was achieved via electrospray in both positive and negative modes. The cone voltage was maintained at 15 V, the desolvation temperature at 275 °C, and the desolvation gas

flow at 650 L/h, while remaining MS conditions were optimized to ensure high sensitivity and mass accuracy.

Spectral data were collected across an m/z range of 100–1500 using both high-resolution and MSE acquisition modes. In MSE mode, low-energy scans (4 V) were used to detect precursor ions, whereas high-energy scans were obtained using a collision-energy ramp of 40–100 V to generate fragment ions. Leucine enkephalin was continuously infused as a lock-mass reference to maintain mass accuracy, and sodium formate was used for external mass calibration.

Compound separation was performed on a Waters HSS T3 column (2.1 × 150 mm, 1.7 μm). Samples were injected at a volume of 0.5 μL, and chromatographic elution was achieved using a binary solvent system consisting of 0.1% formic acid in water (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The gradient program began with 100% A for 1 min, followed by a gradual increase to 28% B over 11 min, a further increase to 40% B within 50 s, a column wash at 100% B for 1.5 min, and re-equilibration for 2 min. The flow rate was set at 0.3 mL/min, and the column temperature was maintained at 60 °C. Quantification was performed using an external calibration curve prepared with ellagic acid standards covering a concentration range of 0.2–5 mg/L.

Data processing of Functions 1 (unfragmented) and 2 (fragmented) from the Waters MSe dataset was performed using MSDial to generate MS1 and MS2 spectra and extracted ion chromatograms with corresponding peak intensities. Due to the lack of calibration standards for most compounds, peak intensities were converted to semi-quantitative concentrations by interpolating against a catechin calibration curve acquired under identical instrumental conditions.

Each deconvoluted feature, along with its corresponding MS1 and MS2 spectra, was exported from MSDial to MSFinder (see Appendix 1). MSFinder then searched several databases (listed above) to identify potential compounds. Using accurate mass and elemental composition data, candidate compounds were identified from these databases and subjected to in silico fragmentation analysis. A matching score, ranging from 0 to 10, was assigned based on the extent to which the in-silico fragments matched the experimental spectra, with the highest-scoring compound considered the most probable identification (assuming a score of at least 4).

After compression, centroiding and application of lock mass correction, the data were processed using MSDIAL and MSFINDER (RIKEN Centre for Sustainable Resource Science: Metabolome Informatics Research Team, Kanagawa, Japan) (Tsugawa et al., 2015; Lai et al., 2018).

4.3 Results and Discussion

4.3.1 Evaluation of special tea phytochemicals using NMR spectrometry

In this study, NMR spectroscopy was employed to identify phytochemicals in special tea plants as a preliminary screening before further analysis by LC-MS and GC-MS. Samples were collected from Brits, Lanseria, and Rietondale and dried using shade-drying and freeze-drying, both of which help preserve phytochemical content during drying. Extractions were performed using acetone and methanol solvents. Figure 4.1 illustrates the peak distributions from the NMR analysis. The results confirmed the presence of various phytochemicals in the samples. After normalizing chemical shifts to highlight the top 40 significant bins for heatmap visualization, apparent differences were observed in the impact of solvent choice and drying method on phytochemical extraction in special tea.

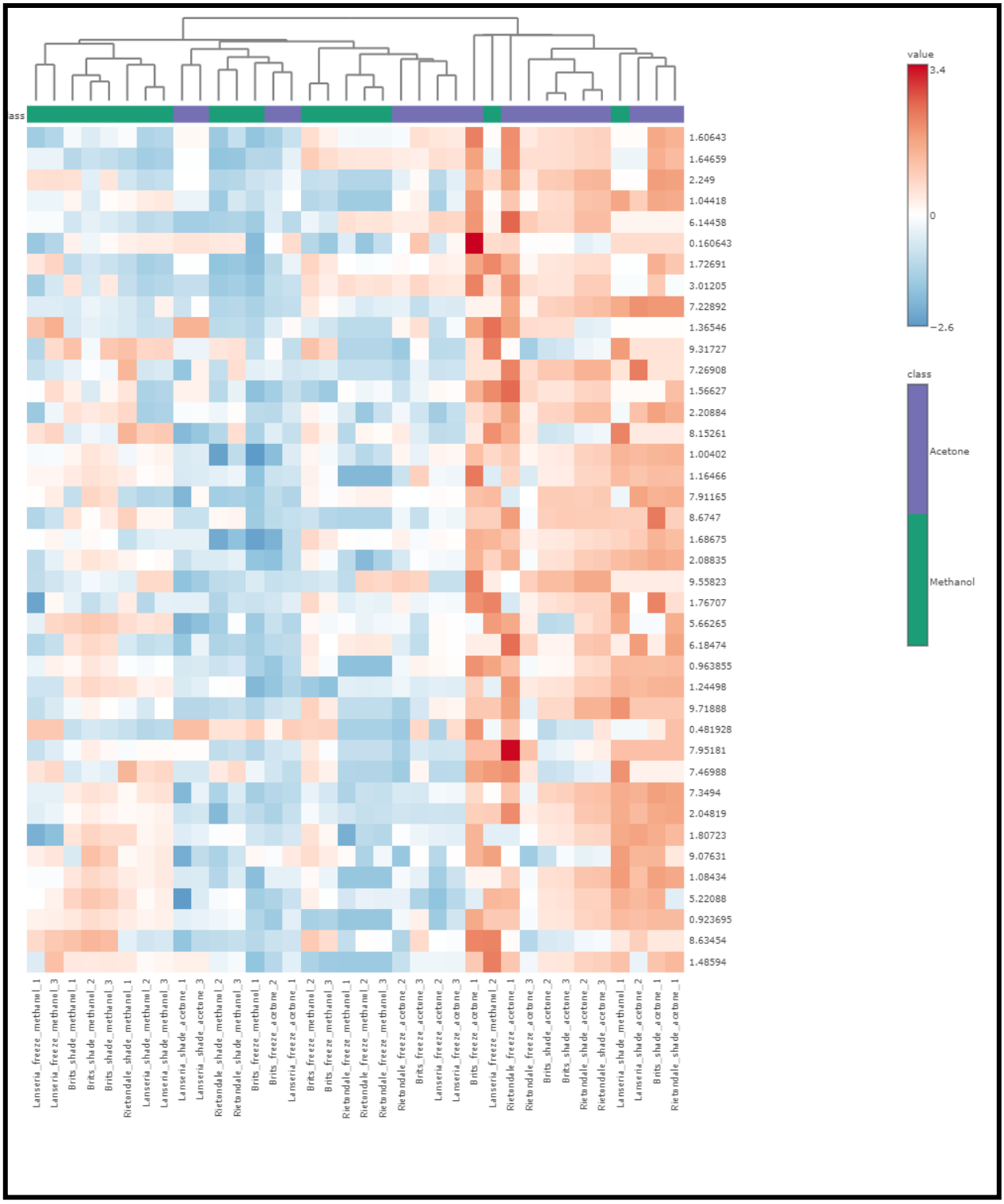


Figure 4.1: Heatmap for NMR spectrometry analysis of special tea samples collected from Brits, Lanseria and Rietondale dried using shade and freeze-dry methods.

Pareto scaling is applied to rows; SVD with imputation is used to calculate principal components. The X and Y axes show principal components 1 and 2, which explain 89.6% and 5.8% of the total variance, respectively. N = 12 data points.

NMR spectral data revealed signals in the 0–10 ppm range, indicating the presence of various phytochemical groups in special tea. These included alkyls, benzylic compounds, esters, ethers, alcohols, ketones, aldehydes, and phenols, among others (Table 4.1). The detection of these functional groups, consistent with findings by Emwas et al. (2019) and Maulidiani (2023), offers understanding of the chemical complexity of special tea and its prospects for medicinal and pharmaceutical use. NMR spectroscopy, therefore, provided crucial insights into the plant's phytochemical composition, while complementary analyses using GC-MS and LC-MS (Sections 4.3.2 and 4.3.3) further explored the metabolite profile of special tea.

Table 4.1: NMR chemical shift values and aligned proton names.

Type of proton	**Chemical formula**	**Chemical shift (ppm)**	**Presence in special tea**
Primary alkyl	RCH_3	0.8 – 1.0	Yes
Secondary alkyl	R_2CH_2	1.2 – 1.4	Yes
Tertiary alkyl	R_3CH	1.4 – 1.7	Yes
Benzylic	$ArCH_3$	2.2 – 2.5	Yes
Alkyl chloride	RCH_2Cl	3.6 – 3.8	Yes
Alkyl bromide	RCH_2Br	3.4 – 3.6	Yes
Alkyl iodide	RCH_2I	3.1 – 3.3	Yes
Alkyl fluoride	RCH_2F	4.0 – 4.5	Yes
Ether	$ROCH_2R$	3.3 – 3.9	Yes
Ester	$RCOOCH_2R$	3.3 – 3.9	Yes
Alcohol	$HOCH_2R$	3.3 – 4.0	Yes
Ketone	$RCCH_3=O$	2.1 – 2.6	Yes
Aldehyde	RCH=O	9.0 – 10.0	Yes
Vinylic	$R_2C=CH_2$	4.6 – 5.0	Yes
Aromatic	ArH	6.0 – 9.0	Yes
Alcohol Hydroxyl	ROH	0.5 – 6.0	Yes
Carboxulyc	RCOOH	10 – 13	Yes
Phenolic	ArOH	4.5 – 7.7	Yes
Amino	RNH_2	1.0 – 5.0	Yes
Amide	RCNHR=O	5.0 – 9.0	Yes

4.3.2 Determination of special tea phytochemicals using GC-MS spectrometry

Phytochemicals (399) were identified from the plant extract analyses of the special tea using GC×GC/TOFMS. The composition of samples from Lanseria, Rietondale, and Brits was aligned more linearly in the PCA plot, indicating compositional similarity, whereas Sekhukhune samples were more scattered, reflecting greater variability (Figure 4.2). Figure 4.2 also shows that most samples cluster within the central ellipse, indicating substantial overlap among regions and suggesting that special tea from different locations shares a broadly similar phytochemical composition. However, Brits and Lanseria are farther from the central cluster, suggesting regional variation possibly due to plant responses to differences in geographical location and environmental factors, including microclimate conditions that may affect metabolite accumulation. The tight clustering of Rietondale and Sekhukhune samples suggests greater compositional uniformity within the two locations, whereas the broader spread of Brits samples indicates greater compositional heterogeneity.

The clustering patterns in Figure 4.3 suggest that each solvent extracted a different profile of metabolites or phytochemicals, consistent with their polarity differences. For example, hexane, which is a nonpolar solvent, tends to extract lipophilic compounds such as fatty acids and hydrocarbons. Acetone, which has intermediate polarity, extracts a broader range of semi-polar compounds, including flavonoids and some phenolics. Lastly, methanol, a polar solvent, extracts highly polar phytochemicals, such as phenolic acids, glycosides, and sugars. The limited overlap observed among the three solvents demonstrates that solvent polarity significantly affects the type and abundance of compounds extracted from special tea, emphasizing the importance of solvent selection in phytochemical and metabolomic studies. Similarly, the study's results show that the three drying methods did not cause significant variation in the areas studied (Figure 4.4).

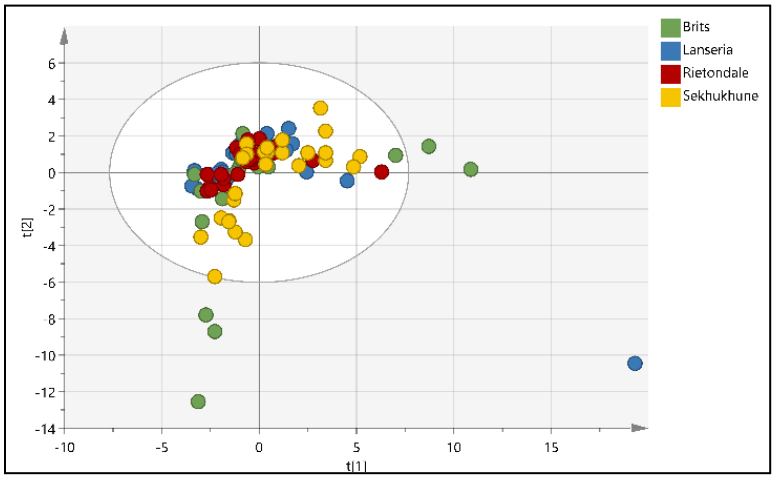


Figure 4.2: PCA showing distribution of special tea compounds for Brits, Lanseria, Rietondale and Sekhukhune areas after drying and extraction.

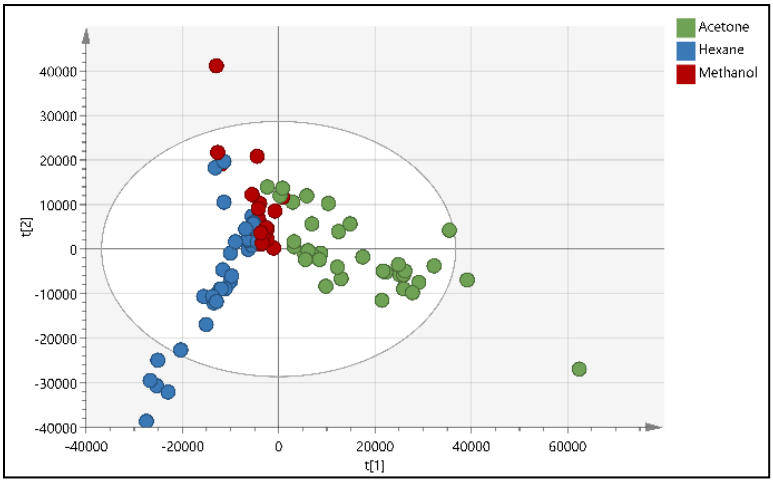


Figure 4.3: PCA showing acetone, hexane and methanol solvents observations for special tea phytochemicals collected in Brits, Lanseria, Rietondale and Sekhukhune and separately dried.

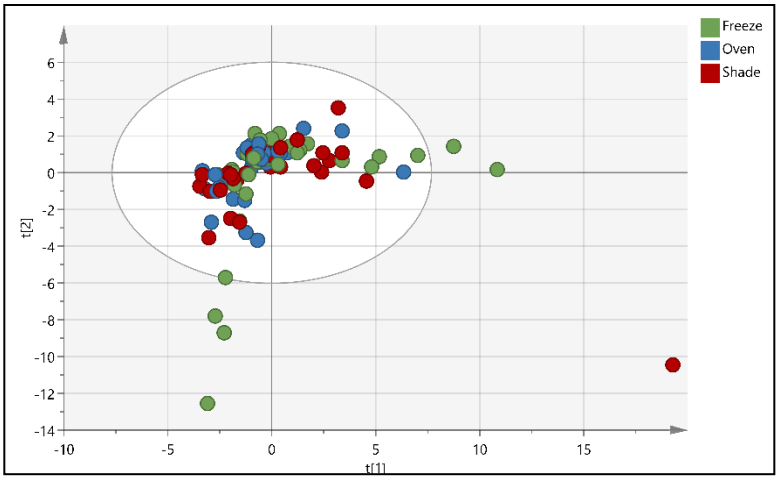


Figure 4.4: PCA showing observations for freeze, oven and shade drying methods of special tea phytochemicals collected in Brits, Lanseria, Rietondale and Sekhukhune.

Figure 4.5 shows a heat map of bioactive compounds by collected area, solvent used, and drying method. The results show a consistent pattern of compound occurrence across all tested areas. A total of 11 bioactive compounds (Table 4.2) and six phytotoxins (Table 4.3) were identified from the samples collected in the four regions. Such compounds include glycerin, oxalic acid, dodecyl acrylate, β-caryophyllene, and Vitamin E. Glycerin was found at high concentrations in acetone-extracted and oven-dried samples. Oxalic acid was present at high levels in methanol extracts and in freeze-dried samples. β-Caryophyllene was present at high levels in both hexane and methanol extracts, and in samples dried in the shade and in the oven. Ethyl vanillin, vanillin, and nerolidol are phytochemicals of interest, but they were present at significantly low concentrations. Plants often synthesize these compounds as a response to environmental stresses, including drought or elevated temperatures, or as a defence strategy against pathogens and herbivores. These metabolites can originate from various plant organs (Kocyigit et al., 2023). The composition of these phytochemicals needs to be determined as part of analyzing the palatability of the plants.

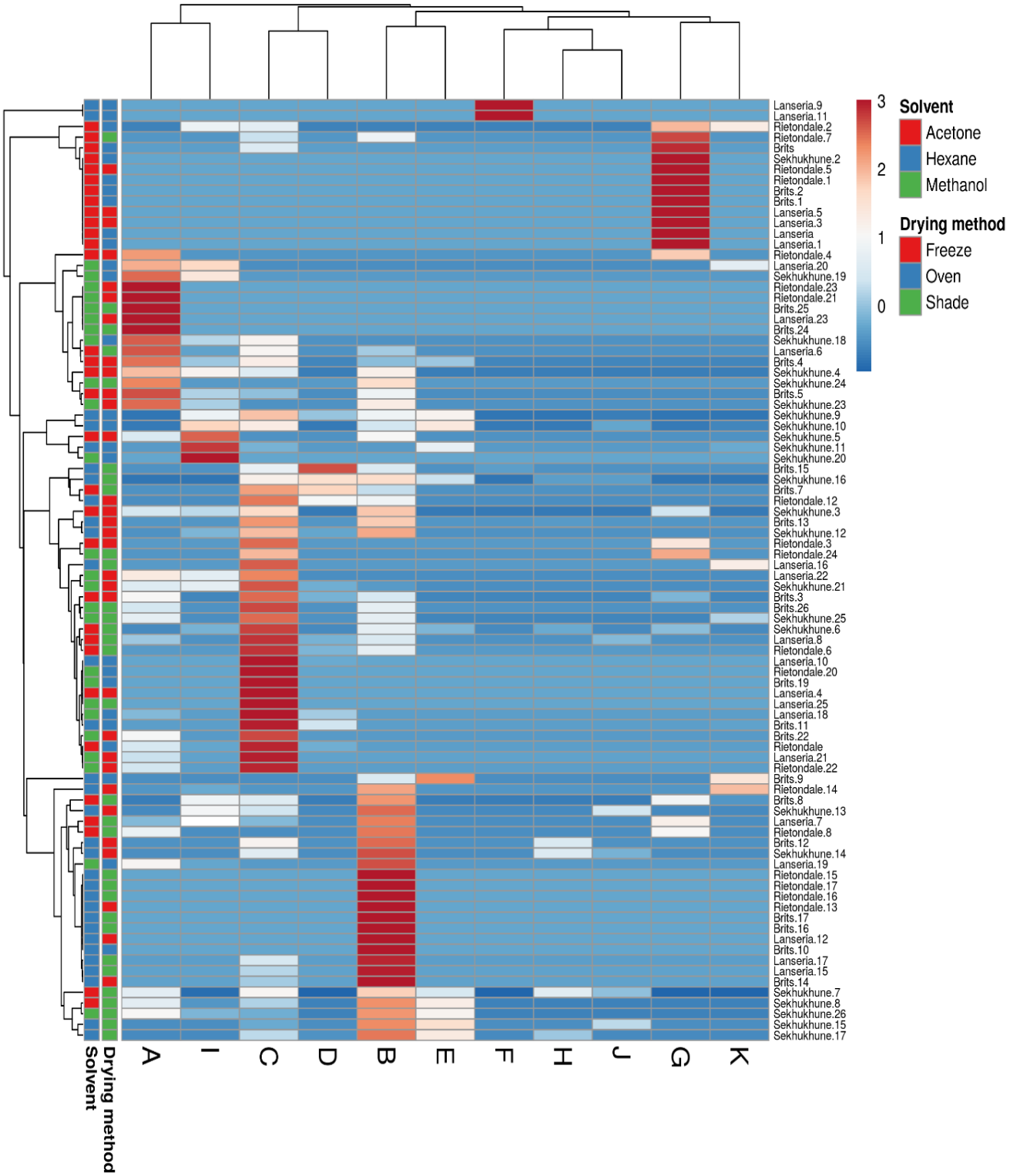
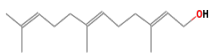
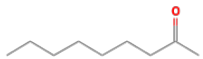
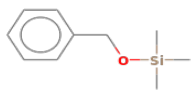
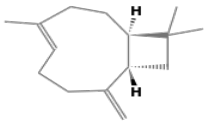


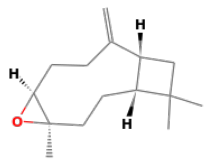
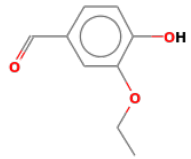
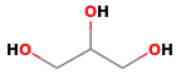
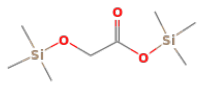
Figure 4.5: Heatmap showing 11 bioactive compounds from four areas in South Africa extracted with acetone, hexane and methanol and dried using oven, shade and freeze-drying methods.

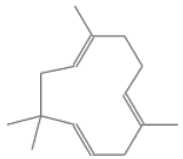
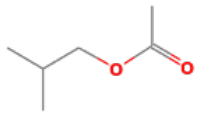
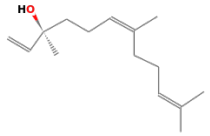
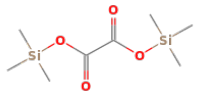
A: Oxalic acid, B: Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-β-Caryophyllene, C: Dodecyl acrylate, D: Methyl 2-benzoylbenzoate, E: Caryophyllene oxide, F: trans-1,2-bis(1-methylethenyl)cyclobutane, G: Glycerin, H: α-Humulene, I: Phytol, J: Squalene, K: Vitamin E

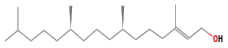
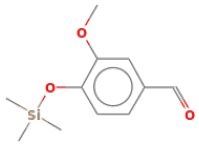
Table 4.2: Special tea bioactive compounds and activities identified using GC×GC/TOFMS installed with internal NIST mass spectral library

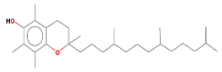
Compound name	**Molecular formula**	**Retention index (sample)**	**Retention index (literature)**	**Molecular mass (g/mol)**	**Compound structure**	**Compound bioactivity**	**References**
Farnesol (2E,6Z	$C_{15}H_{26}O$	2343	2341	222.37		Allergenic, antiadenomic, anticancer (pancreas), anticarcinomic (colon), anticarcinomic (lung), antileukemic, antimelanomic, antispasmodic, apoptotic, flavor, juvabione, nematicide, perfumery, pesticide, pheromonal, sedative, trichomonicide	(Duke, 1992; Booth & Suttie, 1998; Kuete & Efferth, 2013; Lee et al., 2015)
2-Nonanone	$C_9H_{18}O$	1405	1391	142.24		Antiseptic, pesticide	(Duke 1992; Hong et al., 2018)

Benzyl alcohol	C_7H_8O			108.14		Allergenic, anesthetic, antiodontalgic, antipruritic, antiseptic, fungicide, pesticide, sedative	(Duke, 1992; Corcoran & Ray, 2014)
β-Caryophyllene	$C_{15}H_{24}$			204.35		Aldose-reductase-inhibitor, allergenic, analgesic, antiacne, antiasthmatic, antibacterial, anticariogenic, antidermatitic, antiedemic, antifeedant, anti-inflammatory, antileishmanic, antionychyotic, antiproliferant, antispasmodic, antistaphylococcic, antistreptococcic, antitumor, antiulcer, candidicide	(Shimizu et al., 1990; Duke, 1992; Zheng et al., 1992; Muroi & Kubo, 1993; Kim et al., 2008; Dahham et al., 2015)

Caryophyllene oxide	$C_{15}H_{24}O$	1972	1971	220.35		Antiedemic, antifeedant, anti-inflammatory, antitumor, calcium channel blocker, fungicide, insecticide, pesticide	(Duke, 1992; Shimizu et al., 1990; Zheng et al., 1992; Bettarini et al., 1993; Yang et al., 2000)
Ethyl vanillin	$C_9H_{10}O_3$			166.17		Flavour, perfumery, antioxidant	(Duke 1992; Tai et al., 2011)
Glycerin	$C_3H_8O_3$			92.09		Flavouring agent	(Duke, 1992; Carmines & Gaworski, 2005)
Glycolic acid	$C_2H_4O_3$			76.05		Cholesterolytic, diuretic, hepatotonic, irritant	(Duke 1992)

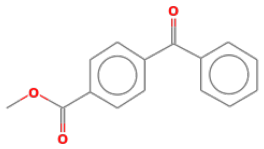
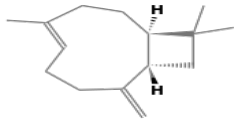
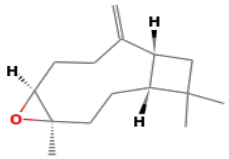
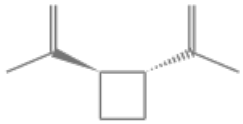
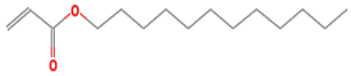
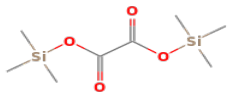
α-Humulene	$C_{15}H_{24}$	1452	1454	204.35		Antimalarial, antiplasmodial, anticancer	(Duke, 1992; Legault & Pichette, 2007)
Isobutyl acetate	$C_6H_{12}O_2$	1011	1012	116.16		Irritant, narcotic, perfumery fragrance	(Duke 1992)
Nerolidol (E) -	$C_{15}H_{26}O$	2946	2027-2050	222.37		Reductase-inhibitor, allergenic, antiacne, antibacterial, anticariogenic, antifeedant, antistreptococcic, flavor, nematicide, perfumery	(Duke, 1992; Chan et al., 2016;)
Oxalic acid	$C_2H_2O_4$	1121	1124	90.03		Acaricide, antiseptic, CNS-Paralytic, fatal, hemostatic, irritant, pesticide, renotoxic, varroacide	(Duke, 1992; Waterman, 1993; Škerl et al., 2011)

Phytol	$C_{20}H_{40}O$	2118	2117	296.5		Cancer-preventive, food additive	(Duke, 1992; Islam et al., 2015 Alencar et al., 2018)
Squalene	$C_{30}H_{50}$	2854	2847	410.7		Antibacterial, antioxidant, antitumor, cancer-preventive, chemopreventive, immunostimulant, lipoxygenase-Inhibitor, perfumery, pesticide, sunscreen	(Duke, 1992; Waterman, 1993; Kim & Karadeniz, 2012)
Vanillin	$C_8H_8O_3$	1419	1390-1430	152.15		Allelochemic, allergenic, antianemic, anticancer, antidrepanocytic, antiescherichic, antilactobacillic, antilisteria, antimutagenic, antioxidant, antipolio, antiradicular 7 x	(Duke, 1992; Tai et al., 2011; Bezerra et al., 2016)

						quercetin, antisickling, antitumor, antitumor-promoter, antiviral, antiyeast, bacteristat, cancer-preventive, candidicide	
Vitamin E	$C_{29}H_{50}O_2$	3112	3111	430.7		5-HETE-inhibitor, allergenic, antitumor, analgesic, antiaging, antialzheimeran, antianginal, antiarteriosclerotic, antiatherosclerotic, antibronchitic, anticariogenic, anticataract, antichorea, anticoronary, antidecubitic, antidermatitic, antidiabetic, antidysmenorrheic,	(Duke, 1992; Thomas, 2006; Yu et al., 2009; Keen & Hassan, 2016)

						antiepitheleomic, antifibrositic	

Table 4.3: Compounds found in special tea plants classified as phytotoxins according to Toxin- Plants – Phytotoxins database (Günthardt et al., 2018)

Phytotoxin	**Molecular formula**	**Molecular Mass (g/mol)**	**Compound structure**
Methyl 2-benzoylbenzoate	$C_{15}H_{12}O_3$	240.25	
β-Caryophyllene	$C_{15}H_{24}$	204.35	
Caryophyllene oxide	$C_{15}H_{24}O$	220.35	
trans-1,2-bis(1-methylethenyl)cyclobutane	$C_{10}H_{16}$	136.23	
Dodecyl acrylate	$C_{15}H_{28}O_2$	240.38	
Oxalic acid	$C_2H_2O_4$	90.03	

The study shows that tea plants grown in different areas exhibit variation in secondary metabolite composition. Similarly, each solvent used in this study can reliably extract specific compounds of interest in special tea. These compounds include those with high-value activities such as anticancer, anticarcinogenic, antitumor (farnesol, β-caryophyllene, caryophyllene oxide, squalene, and vanillin), antibacterial (β-caryophyllene and squalene), antimalarial (α-humulene), antiviral (vanillin), and antioxidant (squalene). These high-value compounds are most likely the reason for the high demand and utilization of special tea as a medicinal plant. Vanillin, farnesol, 2-nonanone, benzyl alcohol, ethyl vanillin, isobutyl acetate, and nerolidol were detected as bioactive compounds in lower quantities in the plant samples. These findings confirm a study reporting the antimicrobial activities of special tea (Tshivhandekano et al., 2014).

Eight compounds were identified in this study, which were classified as phytotoxins by the Toxic-Plants – Phytotoxins (TPPT) database (Günthardt et al. 2018). These compounds of importance, including toxic and antinutritional compounds, may accumulate over time in human bodies through the food chain and subsequently cause long-term undesirable effects due to their toxicity (Guil et al. 1997; Günthardt et al. 2018). Methyl 2-benzoylbenzoate is a hazardous chemical to humans. It can also cause cell death in human peripheral blood mononuclear cells (Tsuboi et al., 2016).

β-Caryophyllene (BCP) was identified as an important compound. The TPPT database records it as a phytotoxin. However, BCP is considered a safe compound currently used in the food industry, as it showed no toxicity in a 700 mg/kg/d trial for 90 days in rats. This compound is used as a food additive in meat products, baked goods, gums, alcoholic and non-alcoholic beverages, and frozen foods (Schmitt et al., 2016). BCP naturally occurs, along with its oxidation product, BCP oxide (Fidyt et al., 2016). Caryophyllene oxide is a representative of an epoxide derivative, which occurs in medicinal and edible plants. Epoxides, due to their

instability, are reactive intermediates that can form covalent adducts with cellular macromolecules (Di Sotto et al., 2013). Caryophyllene oxide is also identified as a phytotoxin. However, it has been found to exhibit important and beneficial bioactive properties (Duke, 1992; Chavan et al., 2010). Studies suggest that caryophyllene oxide compounds in spices and other vegetable products pose a significant risk when high temperatures and long cooking times are used in food preparation (Francis et al., 2012). Studies have shown that BCP oxide exhibits cytotoxic properties against human cancer cell lines. Its cytotoxicity was found to depend on the dose and to be time dependent. However, studies on this compound are limited due to its difficulty in detection and low solubility.

The TPPT database and Dr Duke's Phytochemical and Ethnobotanical Databases identify oxalic acid as a toxic compound. It is also considered an antinutrient factor (Petropoulos et al. 2015; Petropoulos et al. 2019). Its toxicity has been found to be low, with the minimal lethal dose for humans of about 0.0205 g for an adult, with centrilobular hepatic necrosis as an example of an affected area (Guil et al., 1997). Oxalic acid can combine with minerals such as calcium, iron, magnesium, and potassium to form insoluble salts, such as oxalate. These salts reduce the bioavailability of minerals and kidney stones formation (Petropoulos et al., 2015, 2019; Nemzer et al., 2020).

4.3.3 LCMS spectrometry

LC–MS was used to extract and analyze the special tea samples for untargeted metabolite profiling. The PCA plot (Figure 4.6) indicates that different drying methods led to distinct metabolite patterns, whereas samples from different geographical regions showed no significant variation. Among plant parts, flowers and roots exhibited the highest variability in metabolite composition relative to other tissues. In contrast, leaves and stems tended to be more similar, likely because they were more abundant in the samples. Since the roots and

flowers' biomass was being compared to that of stems and leaves, they may have been underestimated; yet they also contain valuable phytochemicals. The OPLS-DA plot (Figure 4.7) further highlights differences in metabolite profiles, as revealed by the LC-MS data. This is also evident in the LC-MS heat map in Figure 4.8, which shows the variation in metabolite levels across the tested samples.

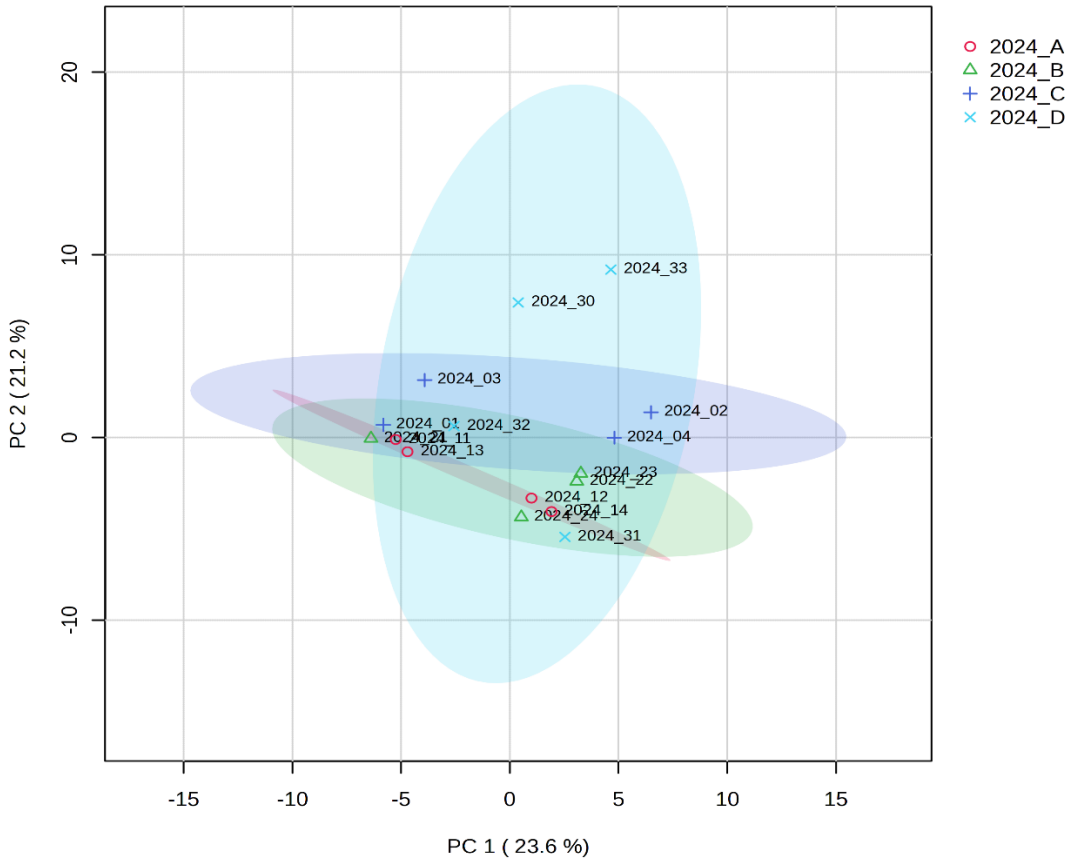


Figure 4.6: PCA plot for LC-MS sample analyses of special tea

202401: Freeze dry-Sekhukhune, 202402: Freeze dry-Rietondale, 202403: Freeze dry-Brits, 292404: Freeze dry-Lanseria, 202411: Oven dry-Sekhukhune, 202412: Oven dry-Rietondale, 202413: Oven dry-Brits, 292414: Oven dry-Lanseria, 202421: Shade dry-Sekhukhune, 202422: Shade dry-Rietondale, 202423: Shade dry-Brits, 292424:Shade dry-Lanseria, 202430:Flowers, 202431: Leaves, 202432: Stems, 202433:Roots

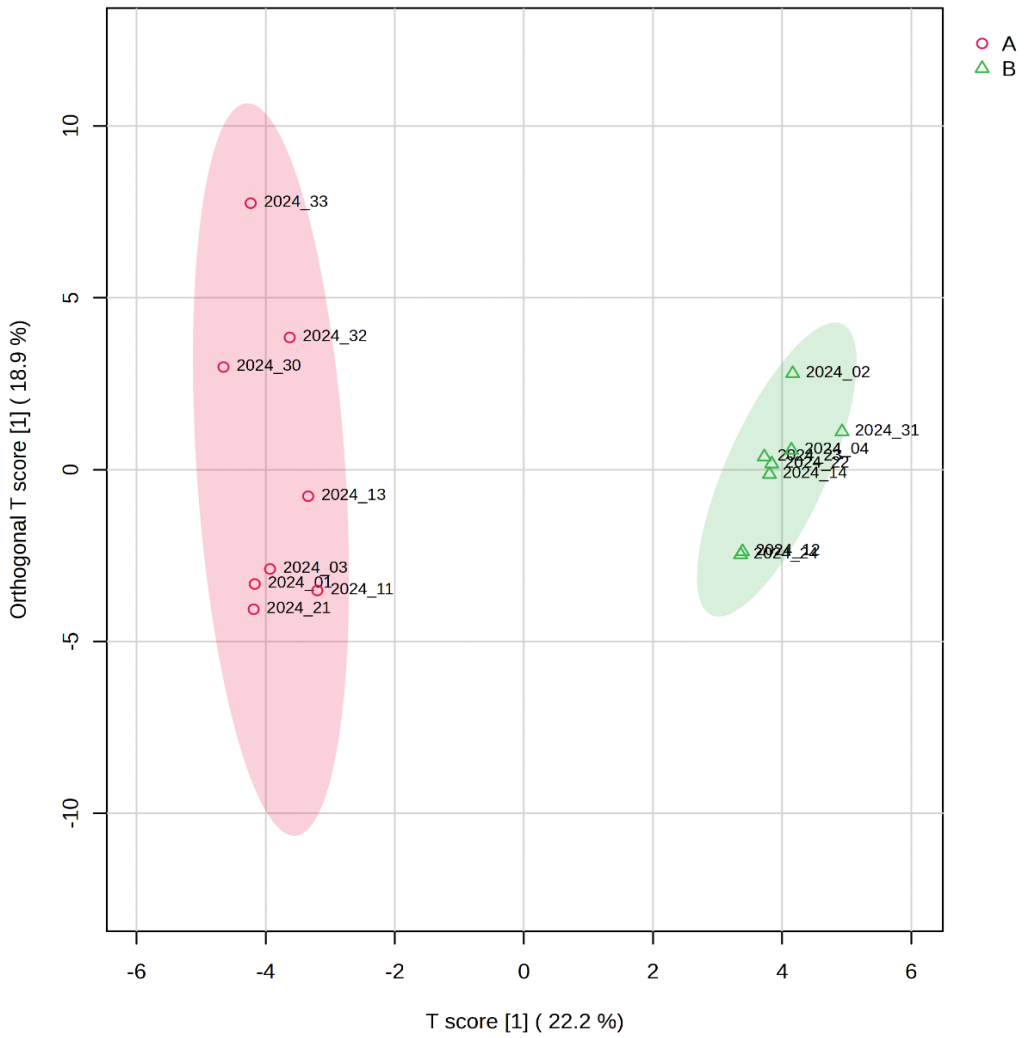


Figure 4.7: OPLSD report on LCMS results

202401: Freeze dry-Sekhukhune, 202402: Freeze dry-Rietondale, 202403: Freeze dry-Brits, 292404: Freeze dry-Lanseria, 202411: Oven dry-Sekhukhune, 202412: Oven dry-Rietondale, 202413: Oven dry-Brits, 292414: Oven dry-Lanseria, 202421: Shade dry-Sekhukhune, 202422: Shade dry-Rietondale, 202423: Shade dry-Brits, 292424:Shade dry-Lanseria, 202430:Flowers, 202431: Leaves, 202432: Stems, 202433:Roots

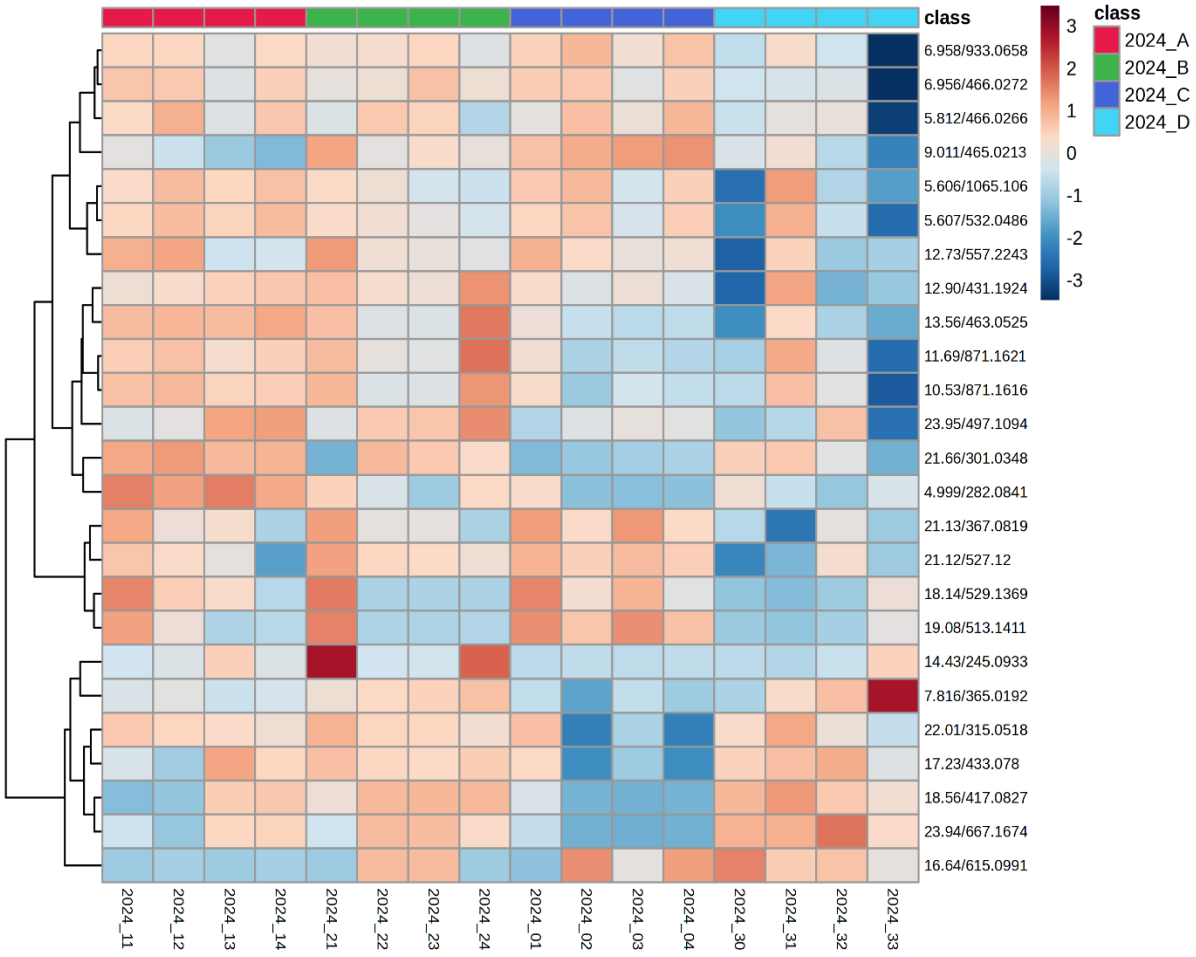


Figure 4.8: LCMS samples heatmap for special tea

202401: Freeze dry-Sekhukhune, 202402: Freeze dry-Rietondale, 202403: Freeze dry-Brits, 292404: Freeze dry-Lanseria, 202411: Oven dry-Sekhukhune, 202412: Oven dry-Rietondale, 202413: Oven dry-Brits, 292414: Oven dry-Lanseria, 202421: Shade dry-Sekhukhune, 202422: Shade dry-Rietondale, 202423: Shade dry-Brits, 292424:Shade dry-Lanseria, 202430:Flowers, 202431: Leaves, 202432: Stems, 202433:Roots

The LC-MS data revealed a relatively consistent metabolite profile across the plant samples, with variation primarily in m/z intensities. This result has confirmed that special tea contains similar compounds across the different areas of interest, though in differing concentrations depending on the drying method and extraction solvent used. Notably, freeze-drying tends to retain the highest level of metabolites in plant extracts. Figure 4.9 illustrates the m/z intensities for four of the tested samples, where similar peaks were observed at retention times of approximately 5.8, 6.9, 15.9, 16.31, and 21.13. These consistent base peaks indicate that the

extracted compounds were uniform across samples. The application of LC-MS techniques effectively separates the compounds, facilitating their identification.

While the peaks for ground samples from different regions were relatively similar, variations in peak positions and intensities were observed across plant parts (Figure 4.10). The roots exhibited higher peaks at retention times of 5.81 (m/z 933.06), 7.81 (m/z 365.0192), and 11.79 (m/z 469.0985). In contrast, the stems showed higher peaks at 6.94 (m/z 615.099), while the leaves had prominent peaks at 14.80 (m/z 598.131) and 15.97 (m/z 463.0896).

The LCMS analysis was expected to identify several key compounds with potential pharmacological significance. The results confirmed the presence of compounds that have been positively linked to the treatment of various ailments (Table 4.4).

Among the biochemicals identified is castalagin, which has also been isolated from berry plants and found to exhibit antitumor properties with significant effects on cancer (Messaoudene et al., 2022). Both castalagin and vescalagin have demonstrated antibacterial activity. Additionally, acutissimin B is known to have biological properties, including antitumor effects through the inhibition of human DNA topoisomerase IIα (Zhang et al., 2015). Epicatechin, a bioactive compound found in plants, has been shown to have medicinal properties, particularly in promoting muscle growth, regeneration, bioenergetics, and strength. It also enhances insulin sensitivity, improves endothelial function, and promotes blood vessel dilation, thereby supporting healthy blood flow and benefiting cardiovascular health (McDonald et al., 2021; Sabarathinam et al., 2023). Chestanin, another compound found in the samples, was reported to have weak efficacy in assays such as the DPPH and ABTS (Esposito et al., 2019). Puerarin 4',6"-diacetate found in *Puerarin lobata* (Willd.) (Shang et al., 2021), and 6"-O-p-coumaroyltrifolin found in *Ginkgo biloba* plants (Tang et al., 2001)

were found in the special tea and have been confirmed to have anticancer efficacy (Asthana et al., 2015).

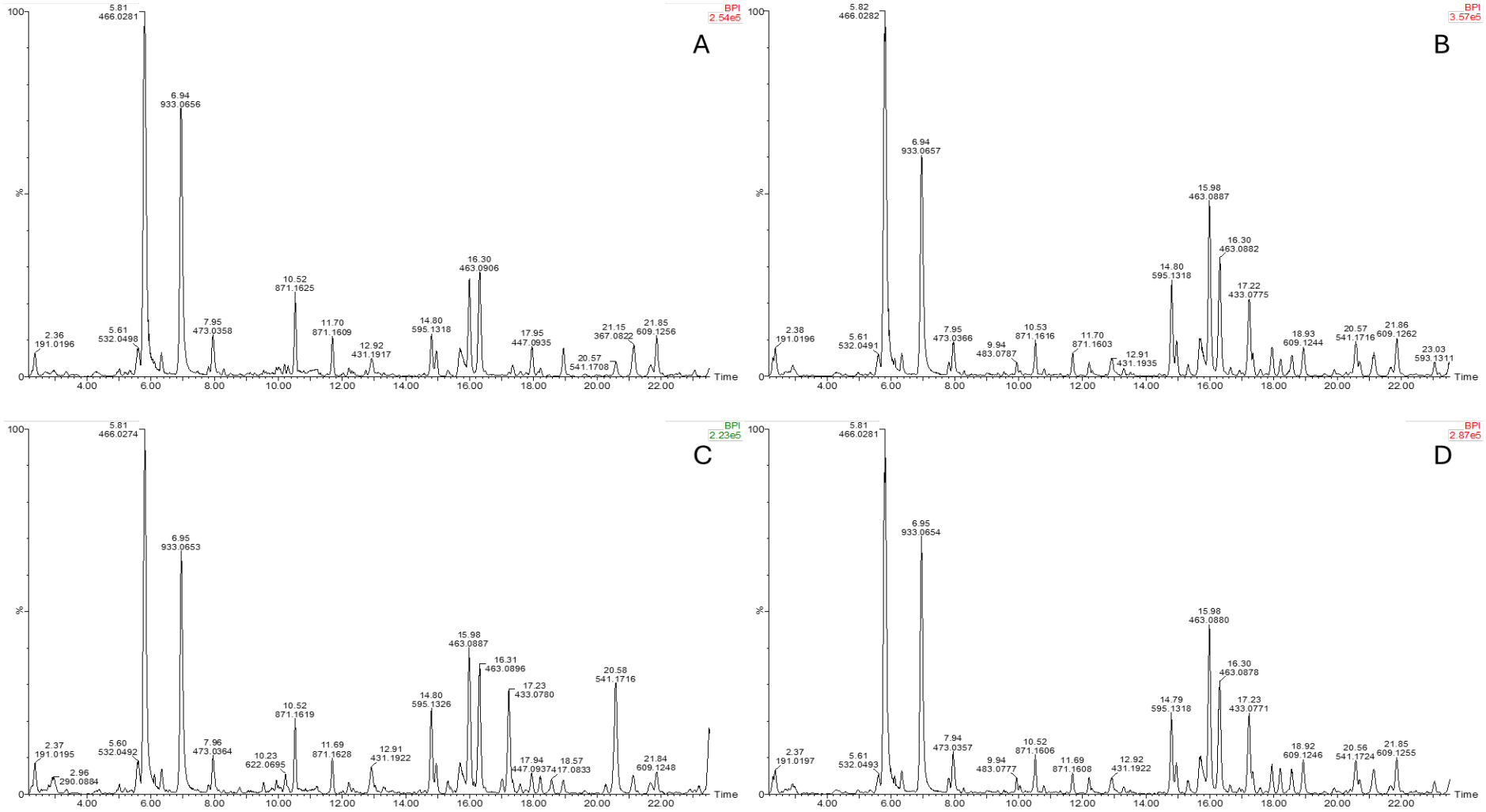


Figure 4.9: Special tea plant samples LCMS m/z intensity for four of the tested samples: A – oven-dry (Sekhukhune), B – Shade-dry (Sekhukhune), C-oven-dry (Brits) and D – shade-dry (Brits).

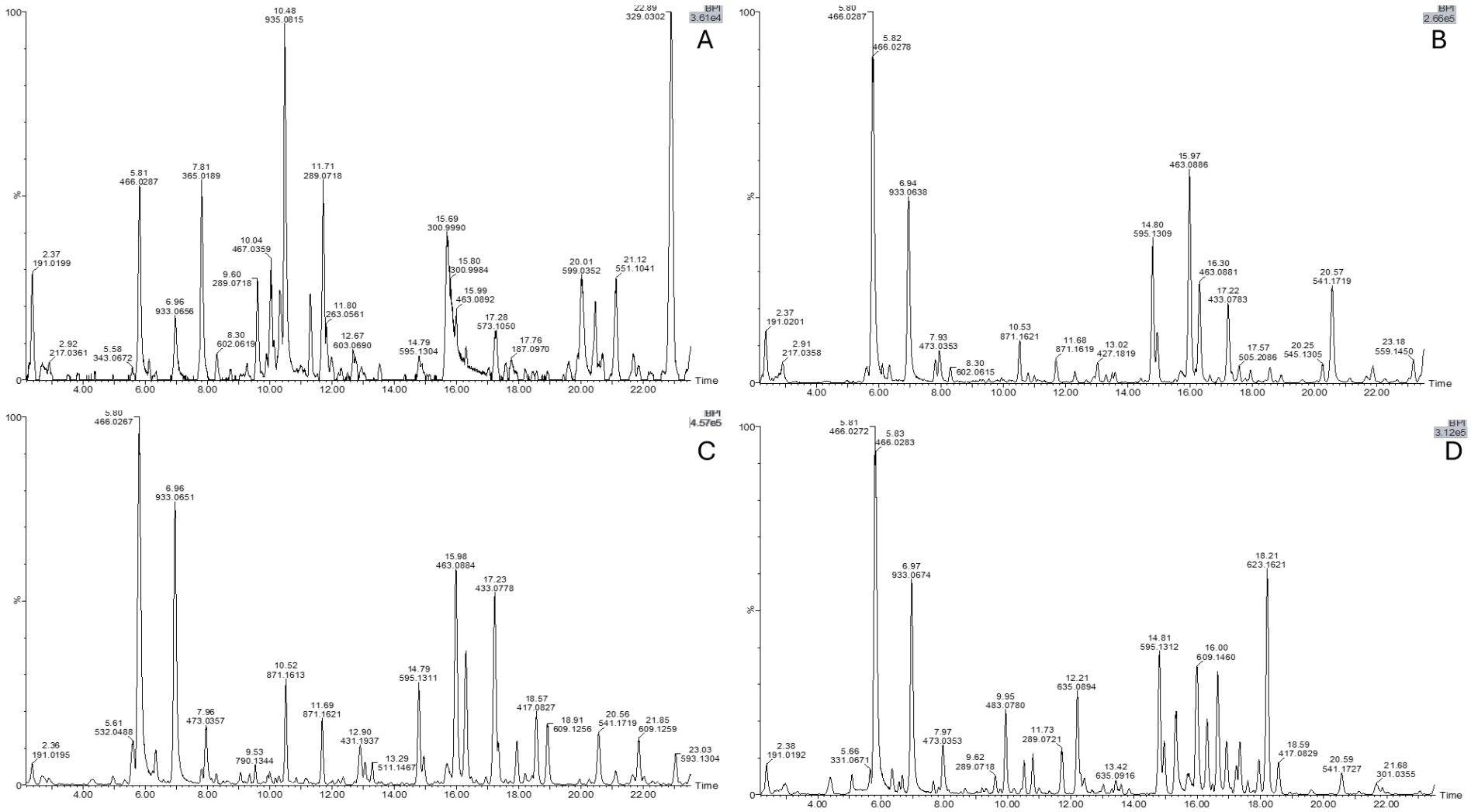


Figure 4.10: Special tea roots – A, stem – B, leaves – C and flower – D samples LCMS m/z intensity

Table 4.4: LCMS results from special tea untargeted metabolites analysis

Alignment	Average Rt (min)	Average Mz	Name	Formula	Ontology	Functional uses	Confirmed other sources	Key reference
2304	5.606	1065.106	Grandinin	C46H34O30	Hydrolyzable tannins	Antioxidants	*Melaleuca quinquenervia*	(Moharram et al., 2003; Qu et al. 2014)
1072	5.812	466.0266	Castalagin	C41H26O26	Ellagitannin	Antitumour, antibacterial, anti-herpesvirus	*Quercus robur*	(Martínez-Gil et al., 2020)
1073	6.956	466.0273	Vescalagin	C41H26O26	Hydrolyzable tannins	Antibactirial	*Punica granatum, Quercus suber*	(Martínez-Gil et al., 2020)
1097	8.128	469.0043	Valoneic acid dilactone	C21H10O13	Hydrolyzable tannins	Antibacterial and anticancer	*Epilobium hirsutum*	(Barakat et al., 1997)
1556	8.306	602.0615	Acutissimin B	C56H38O31	Complex tannins	Antitumour	*Quercus robur*	(Zhang et al., 2015)
1428	9.61	579.1515	Epicatechin	C15H14O6	Catechins	Antioxidant, anti-inflammatory	*Theobroma cacao*	(Martin et al., 2022)
1152	9.939	483.0776	1,6-Digalloyl-beta-D-glucopyranose	C20H20O14	Tannins	Antioxidant, anti-inflammatory	*Camellia sinensis,* L	(Luo et al., 2022)
2211	9.996	937.1828	Chestanin	C40H42O26	Hydrolyzable tannins	Antifungi	*Castanea sativa* Mill	(Neves and Cunha, 2019)
1336	12.736	557.2243	Lobetyolinin	C26H38O13	Fatty acyl glycosides of	*Anti-arrhythmic activity,*	*Codonopsis pilosula*	(Bailly, 2021)

					mono- and disaccharides	*antitumour, antioxidant*		
860	12.908	431.1925	Citroside A	C19H30O8	Terpene glycosides	Anticancer	*Citrus sinensis*	(Afifi et al., 2023)
1952	13.514	757.1848	Quercetin 3-sambubioside-7-glucoside	C32H38O21	Flavonoid-7-O-glycosides	Anti-inflammatory	*Eucommia ulmoides*	(Li et al., 2014)
1044	13.568	463.0525	Ellagic acid glucoside	C20H16O13	Hydrolyzable tannins	Anticancer	*Diplopanax stachyanthus*	(Yan and Guo, 2004)
1518	14.799	595.131	Quercetin 3-lathyroside	C26H28O16	Flavonoid-3-O-glycosides	Antioxidants	*Lathyrus sp*	(Park et al., 2008)
401	15.702	300.9987	Ellagic acid	C14H6O8	Hydrolyzable tannins	Antioxidant and anti-inflammatory	*Dimocarpus longan*	(Wang et al., 2019)
1051	15.98	463.0884	Quercetin 3-galactoside	C21H20O12	Flavonoid-3-O-glycosides	Antioxidants	*Azadirachta indica*	(Rao et al., 2019)
1425	16.474	579.1371	Kaempferol 3-[apiosyl-(1->2)-galactoside]	C26H28O15	Flavonoid-3-O-glycosides	Anticarcinogenic and anti-inflammatory	*Semen Cuscutae*	(Zhang et al., 2018b)
865	17.23	433.078	Quercetin 3-O-alpha-L-arabinopyranoside	C20H18O11	Flavonoid-3-O-glycosides	Antibacterial	*Vaccinium uliginosum L.*	(Jo et al., 2019)
936	17.341	447.0934	Trifolin	C21H20O11	Flavonoid-3-O-glycosides	Anticancer, Antifungal	*Eupatorium perfoliatum*	(Hensel et al., 2011)
1218	17.581	505.2083	Icariside E4	C26H34O10	2-arylbenzofuran flavonoids	Antioxidant, anti-Alzheimer and anti-inflammatory	*Tabebuia roseo-alba*	(Ferreira-Júnior et al., 2015)

1702	17.953	623.1623	Keioside	C28H32O16	Flavonoid-3-O-glycosides	Antioxidants	*Annona coriacea Mart*	(Novaes et al., 2018)
1130	18.316	477.1043	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-{[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxy}-4H-chromen-4-one	C22H22O12	Flavonoid-3-O-glycosides	Antioxidant, anti-cancer, anti-inflammatory, anti-arrhythmic, and vasorelaxant	*Attalea geraensis* Barb. Rodr	(Silva et al., 2023)
785	18.568	417.0827	Kaempferol 3-alpha-L-arabinopyranoside	C20H18O10	Flavonoid-3-O-glycosides	Anti-inflammatory, gout	*Jatropha isabellei*	(Mohapatra et al., 2015)
1603	18.929	609.1243	Rutin	C27H30O16	Quercetin-3-O-rutinoside	Antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activities	*Ruta graveolens*	(Patel and Patel, 2019)
1242	19.081	513.1412	2",6"-Di-O-acetylononin	C26H26O11	Isoflavonoid O-glycosides	Anticancer, antioxidants	*Petroselinum crispum*	(Mallman n et al., 2023)
1301	20.26	545.1301	Puerarin 4',6"-diacetate	C25H24O11	Isoflavonoid C-glycosides	Anticancer	*Puerarin tu berosa*	(Asthana et al., 2015)
1297	20.572	541.1719	Ikarisoside D	C28H30O11	Flavonoid-3-O-glycosides	Antiinflammatory	*Epimedium grandifloru m*	(Ma et al., 2011)

621	21.136	367.0819	Averufin	C20H16O7	Hydroxyanthraquinones	Carcinogenic	*Aspergillus parasiticus*	(Sakuno et al., 2003)
409	21.663	301.0348	Quercetin	C15H10O7	Flavonols	Antioxidant and anti-inflammatory	*Alium cepa*	(Aghababaei and Hadidi, 2023)
4546	22.012	315.0519	Isorhamnetin	C16H12O7	Flavonols	Antitumour, anti-inflammatory, antioxidation	*Ginkgo biloba, Chromolaena odorata L.*	(Chirumbolo, 2014)

4.4 Conclusion

The objective of this study was to investigate the phytochemical composition of special tea using NMR spectroscopy, GC–MS, and LC–MS to identify bioactive compounds and assess their potential benefits for human health. NMR spectroscopy provided detailed information on the plant's phytochemical constituents, including phenols, alkyls, esters, ethers, and amino acids, which are essential for comprehensive compositional analysis. GC–MS and LC–MS further facilitated the identification and characterization of phytochemicals and their associated biological activities. The findings revealed that both geographical origin and solvent choice can influence the phytochemical profile of *Monsonia burkeana*. Variations arising from solvent selection underscore the need for careful optimization when extracting bioactive compounds. Notably, the study was able to assist with the identification of several compounds with proven pharmacological properties, including anticancer agents (vanillin, Puerarin 4',6"-diacetate, 2",6"-Di-O-acetylononin, trifolin), antitumor agents (β-caryophyllene, isorhamnetin, acutissimin B), antibacterial agents (squalene, 1,2,6-Trigalloyl-beta-D-glucopyranose), anti-inflammatory agents (caryophyllene oxide, luteolin hexoside), and antimalarial agents (α-humulene).

This underscores the dual nature of special tea, which can contain both medicinal and potentially harmful compounds. The findings of this study confirm the medicinal value of special tea and its potential as a natural resource for developing pharmacological agents necessary for the pharmaceutical industry. However, further studies are required to quantify the antinutrient composition of the plants, thereby deepening our understanding of their phytotoxicity and ensuring the safe and effective use of their bioactive constituents.

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CHAPTER FIVE

DETERMINATION OF ANTI-PROLIFERATIVE EFFECT OF SPECIAL TEA CRUDE EXTRACT ON THREE CANCEROUS CELL LINES

Abstract

The growing interest in bioactive compounds derived from medicinal plants has increased the focus on special tea. However, despite this growing attention, studies investigating its antiproliferative activities remain limited. This study investigated the cytotoxic effects of special tea on three cancer cell lines. Plant samples collected from Lanseria were shade-dried prior to analysis. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the effects of the samples on lung, liver, and colorectal cancer cell lines, and a caspase-3 activity assay further validated the results. The MTT assay indicated notable cytotoxicity against lung and liver cancer cells, however, it had minimal effect on the colorectal cancer line. Subsequently, the caspase-3 assay confirmed apoptosis in lung and liver carcinoma cells. Water-extracted samples exhibited lower cytotoxicity, whereas acetone- and ethanol-extracted samples showed significant cytotoxicity against the cancer cell lines. In conclusion, this study suggests the therapeutic potential of special tea against cancer cell lines, highlighting its potential value to the pharmaceutical industry.

Keywords: Antiproliferative, bioactive compound, medicinal plants, *Monsonia burkeana,* phytochemicals

5.1 Introduction

Cancer is characterized by the uncontrolled proliferation of abnormal cells arising from the expansion of a clonal population (Ganogpichayagrai and Suksaard, 2020). Its development involves six key alterations in cell physiology: self-sufficiency in growth signals, insensitivity to

anti-proliferative cues, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and the ability to invade tissues and metastasize (Dupré and Malik, 2018). The progression of cancer occurs in three stages: initiation, promotion, and progression. Globally, cancer is the second most prevalent disease after cardiovascular conditions (Majrashi et al., 2023; Siegel et al., 2023). In 2022, the World Health Organization reported 20 million new cases and 9.7 million deaths worldwide, with projections suggesting that by 2025, cases could rise to 35 million, a 77% increase (WHO, 2024).

Cancer cell-based assays are crucial for investigating the mechanisms of action of chemotherapeutic agents prior to preclinical validation in animal models and clinical trials. These assays support drug discovery by targeting diverse cellular processes to evaluate anticancer efficacy. Among them, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is widely used and considered a gold standard for measuring cell viability and cytotoxicity (Kumar et al., 2018).

Cell viability assays measure various cellular functions, including enzyme activity, membrane integrity, and ATP production. They can be categorized into dye-exclusion, colourimetric, fluorometric, luminometric, and flow cytometric methods. Dye-exclusion assays, such as trypan blue staining, are time-consuming but straightforward for large sample volumes. Colourimetric assays, such as the MTT assay, detect metabolic activity via spectrophotometry and are more sensitive and cost-effective (Riss et al., 2004; Kamiloglu et al., 2020). Flow cytometry provides detailed insights into cell morphology and death progression by assessing membrane asymmetry and permeability.

When selecting a viability assay, factors such as cost, speed, sensitivity, and equipment availability are important. An ideal assay should be safe, reliable, efficient, and not interfere with the tested compound. Consistency in the cell source and careful optimization of reagent concentrations and

incubation times are critical for accurate results (Kamiloglu et al., 2020). In this study, the response of cancer cells to crude extracts of special tea was assessed using the MTT assay.

The MTT assay is a sensitive, quantitative, colorimetric method. Dehydrogenase enzymes reduce the yellow tetrazolium compound (MTT) in viable cells, forming an insoluble purple formazan product that accumulates within intact cells (Figure 5.1). The amount of formazan produced is proportional to the number of living cells. After solubilizing the formazan in dimethyl sulfoxide (DMSO), its concentration is measured spectrophotometrically at 540 nm (Sylvester, 2011; Vega-Avila & Pugsley, 2011; McCauley et al., 2013; Kamiloglu et al., 2020).

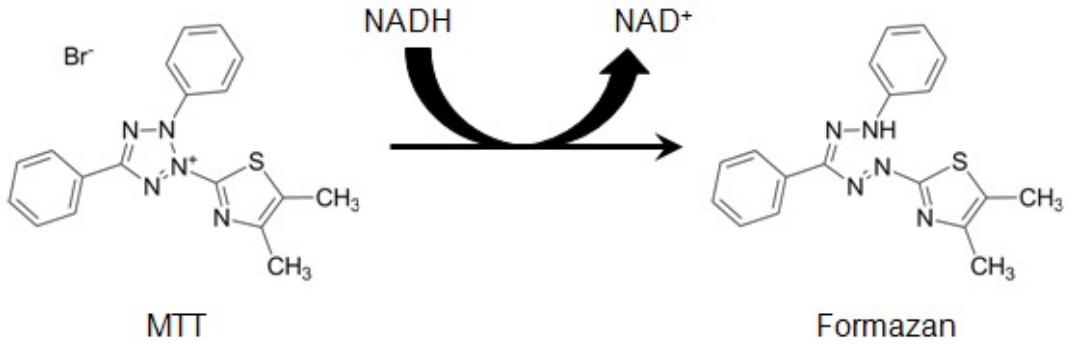

Figure 5.1: Structures of MTT and coloured formazan product after reduction (Riss et al., 2011).

Apoptosis, a programmed cell death mechanism, is a central target of many anticancer therapies, which exploit its activation to eliminate cancer cells (Weigel et al., 2000; Yadav et al., 2021). Cancer cells' ability to evade apoptosis poses a significant challenge for effective treatment. Caspases, a family of cysteine proteases, are crucial regulators of apoptosis, existing initially as inactive proenzymes that are activated to execute cell death. They are classified into initiator caspases (e.g., caspase-2, -8, -9, -10), executioner caspases (e.g., caspase-3, -6, -7), and inflammatory caspases (e.g., caspase-1, -4, -5, -11, -12). Initiator caspases trigger downstream activation of executioner caspases, with caspase-3 playing a central role in dismantling cellular components, mediating DNA fragmentation, chromatin condensation, and formation of apoptotic bodies (Weigel et al., 2000; Chandler et al., 2004; Patel et al., 2021; Yadav et al., 2021).

Activating caspase-3 has emerged as a promising strategy for inducing cytotoxicity in cancer cells, and numerous compounds have been developed to activate this pathway. In this context, special tea contains macro- and micronutrients and bioactive compounds (Chapters 3 and 4), and this study hypothesizes that it may exert anti-proliferative effects on lung, liver, and colorectal cancer cell lines through apoptosis induction. Investigating such natural products offers potential for the discovery of novel anticancer agents that exploit the caspase-mediated apoptotic pathway (Yadav et al., 2021).

5.2 Materials and Methods

5.2.1 MTT assay determination

5.2.1.1 Reagents

Unless otherwise specified, all reagents and chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Minimal Essential Medium (MEM), and phosphate-buffered saline (PBS) with and without calcium and magnesium ions were sourced from Cytiva (Marlborough, MA, USA). Foetal Bovine Serum (FBS) and penicillin/streptomycin antibiotics were purchased from Biowest (Nuaillé, France). The MTT reagent was also sourced from Sigma-Aldrich (St. Louis, MO, USA).

5.2.1.2 Sample preparation

Shade-dried, ground plant samples were used for this study. The samples were then extracted by decoction at a 1:10 (w/v) ratio. Acetone, ethanol and water were used as solvents. While ethanol and acetone are highly volatile, their ability to yield high concentrations of metabolites proved invaluable for this study. The plant samples (25 mg) were weighed and added to 250 mL of the solvents. The mixture was heated to boiling and boiled for 5 minutes, then removed from the heat to cool. To enhance extraction, the samples were shaken overnight. Filtration through a Whatman

No. 1 filter was then performed to obtain the supernatant. The solvents were evaporated from the supernatants using a rotary evaporator. The resulting crude extracts were collected and stored in refrigerated vials for further analysis.

Determination of anti-proliferative activity against cancer cells was performed at Nelson Mandela University's Bioassaix Labs in Gqeberha, Eastern Cape, South Africa. Extracts were solubilized using dimethyl sulfoxide (DMSO) to yield a stock concentration of 100 mg/mL. Extracts were stored at 4°C until used.

5.2.1.3 Cell line maintenance

Lung cancer (A549), liver cancer (C3A), and colorectal cancer (Caco2) cell lines were utilized for cytotoxicity testing. A549 and Caco2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS). For C3A cells, the growth medium was Minimum Essential Medium (MEM) supplemented with nonessential amino acids, penicillin-streptomycin, and 10% FBS. All cells were grown in 10 cm culture dishes and incubated at 37°C in a humidified environment containing 5% CO_2.

5.2.1.4 Screening protocol

The experiment followed the procedure described by Teo et al. (2020). Cells were plated in 96-well microtiter plates at 5,000 cells per well, with 100 µL of medium per well. Plates were incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO_2 to allow cell attachment before treatment. Cells were exposed to extract concentrations of 15.625, 31.25, 62.5, 125, and 250 µg/mL, with 50 µM melphalan used as a positive control. After 48 hours, the medium was removed, and 100 µL of MTT solution (0.5 mg/mL) was added to each well, followed by a 3-hour incubation. The MTT solution was then aspirated, and 100 µL of DMSO was added to dissolve the resulting formazan crystals. Absorbance was measured at 540 nm using a BioTek® PowerWave

XS spectrophotometer (Winooski, VT, USA), and IC_{50} values were determined using GraphPad Prism 4.

5.2.2 Cancer cytotoxicity confirmation

5.2.2.1: Sample preparation

Extracts as prepared in 5.2.1.2 were used for this experiment. However, only ethanol was used as the extraction solvent for this assay. Reagents used are described in 5.2.1.1.

5.2.2.2: Cell line maintenance

Liver cancer (C3A, purchased from the ATCC) and lung carcinoma (A549, purchased from Cellonex, RSA) cell lines were used. The colorectal cancer cell line was not tested because it showed the least cytotoxicity in the MTT assay in this study. Complete growth medium consisted of DMEM supplemented with 10% FBS for A549 cells. The complete medium consisted of MEM supplemented with 1x nonessential amino acids and 10% FBS for C3A cells. Cells were grown in 10 cm dishes with complete medium at 37°C in 5% CO_2.

5.2.2.4 Caspase -3 activation protocol

Cells were plated in 96-well microtiter plates at 4,000 cells per well, with 100 µL of medium per well for each cell line. Plates were incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO_2 to allow cell attachment before the addition of test extracts. Treatments consisted of ethanolic extracts at the IC_{50} concentration for each cell line, with Melphalan as a positive control. Cells were exposed to the treatments for 48 hours. Subsequently, cells were fixed overnight 12 hr) at 4°C using 4% formaldehyde and permeabilized with ice-cold methanol. Blocking was performed in PBS containing 0.5% BSA, which was removed by aspiration, followed by washing with PBS. To detect activated caspase-3, cells were incubated with a cleaved caspase-3 (Asp175) monoclonal antibody (Cell Signalling Technology) diluted 1:300 for 1 hour at 37°C. After washing, cells were

exposed to an Alexa 488-conjugated secondary antibody (1:1000) for 30 minutes at 37°C in the dark. Hoechst stain (5 µg/mL) was added to each well and incubated for 20 minutes. Finally, imaging was conducted using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices).

5.2.2.5 Data quantification

The relative levels of activated caspase-3 were quantified using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). Hoechst 33342 and Alexa 488 signals were captured using DAPI and FITC filters, respectively. Images were taken at nine different sites per well using a 10x objective, covering approximately 70% of the well's area. The collected images were analyzed with MetaXpress software using the Multi-Wavelength Cell Scoring Application Module. The data were exported to an Excel spreadsheet for further analysis and processing.

5.3 Results and Discussion

5.3.1 Cytotoxic activity using MTT Assay

The cytotoxicity of three extracts of *M. burkeana*, prepared using water, ethanol and acetone, was evaluated on A549 (lung cancer), C3A (liver cancer), and Caco-2 (colorectal cancer) cell lines using the MTT assay. Non-cancerous cells were not included at this stage, as follow-up assays were planned for subsequent evaluation. The water extract showed no cytotoxic activity against any of the tested cell lines. This lack of cytotoxicity may be attributed to the limited or absent phytochemicals extracted by water. However, other studies have demonstrated that water can be an effective solvent for in vitro and in vivo analyses of cancer bioactivity (Zandi et al., 2010; Shanab et al., 2012; Wadhwa et al., 2013). Additionally, cold water extraction has been reported to induce apoptosis in cancer cell lines (Jung, 2014), and water-ethanol extracts have been found to be effective in anticancer activity assays (Mohammed et al., 2021). Although this study did not

demonstrate biological activity in the three cancer cell lines using the water-extracted samples, the cytotoxicity assay is crucial for determining the solvent for anticancer analyses.

The acetone and ethanol extracts induced cytotoxicity in a dose-responsive manner after 42 hours of exposure, as indicated in Figures 5.2, 5.3, and 5.4. The acetone and ethanol extracts were most cytotoxic against the lung cancer cell line (A549) and least cytotoxic against the colorectal cancer cell line (Caco2). This result is confirmed by Jawad et al. (2014), who stated that the cytotoxicity of *Salvia officinalis* L. samples, extracted with acetone and methanol, yielded positive effects with varying responses in the three cancer lines studied. Another study confirms that acetone extracts of *L. ferrugineus* Roxb showed a positive effect for antibiotics, antioxidants, and cervical anticancer compounds (Juwitaningsih et al. 2022) and acetone extract of *Angelica sinensis* had anti-proliferative activity (Cheng et al., 2004).

This study demonstrates significant activity against lung and liver cancer cell lines, confirming the presence of anticancer bioactive compounds in specific tea plants. The lung and liver cancer cell lines showed an average of less than 50% cell viability after exposure to the ethanol extract, whereas lung cancer cells had approximately 60% cell viability (Figures 5.2 and 5.3). While the cells were treated using 15.625, 31.25, 62.5, 125 and 250 µg/mL (Appendix Table A1), this study reveals that the three highest concentrations (62.5, 125 and 250 µg/mL) are the ones which gave the most cytotoxicity against the cancer lines (Figure 5.4), highlighting the dose-dependent response. The colorectal cancer cells exhibited the lowest cytotoxic response to the plant samples. Further studies may be necessary to determine the degree of response.

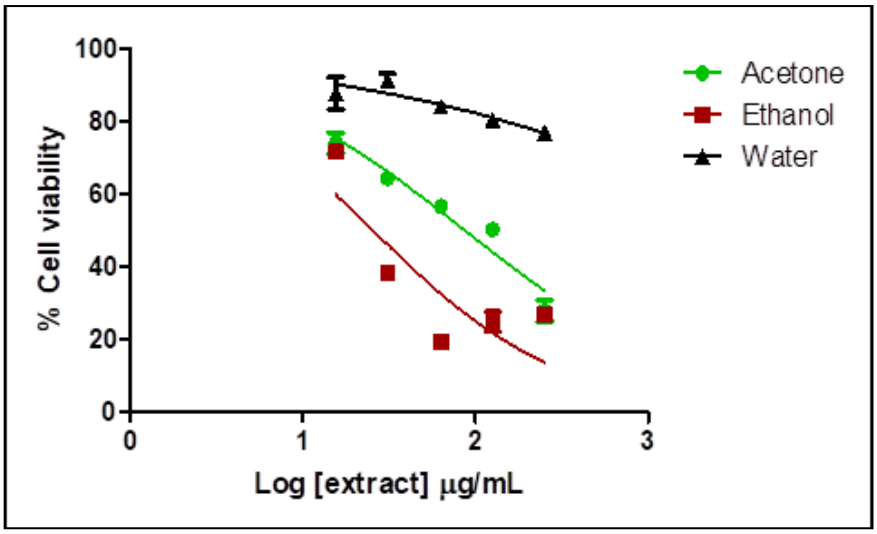

Figure 5.2: Dose–response curves for IC_{50} determination of water, ethanol, and acetone extracts of *Monsonia burkeana* against A549 lung cancer cell lines.

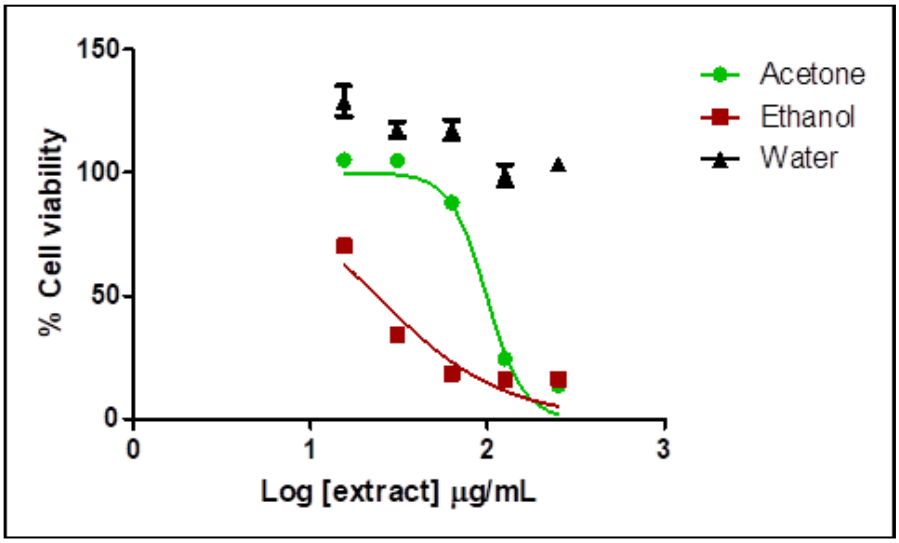

Figure 5.3: Dose–response curves for IC_{50} determination of water, ethanol, and acetone extracts of *Monsonia burkeana* against HepG2 (C3A) liver cancer cell lines

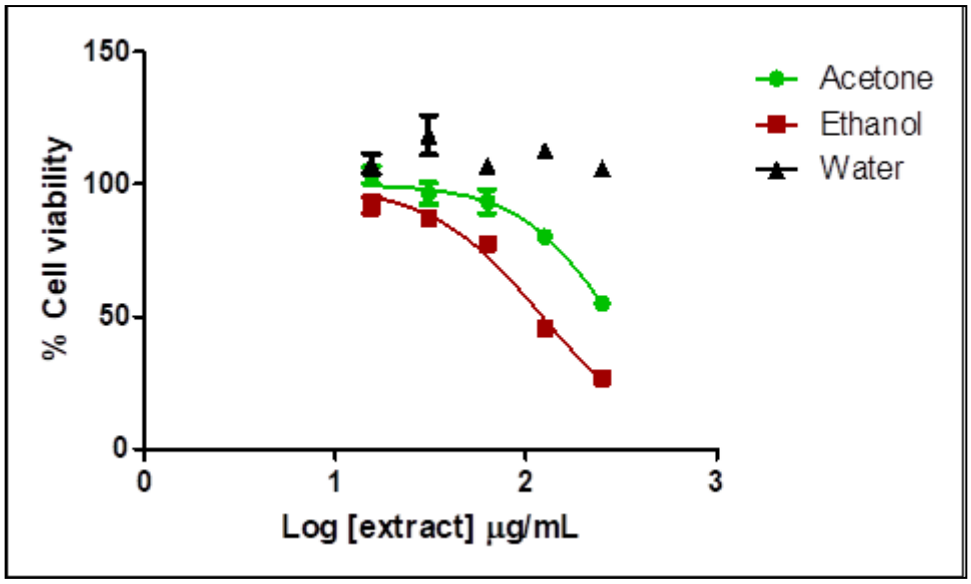

Figure 5.4: Dose–response curves for IC_{50} determination of water, ethanol, and acetone extracts of *Monsonia burkeana* against Caco-2 colorectal cancer cell lines

IC_{50} determination was performed using GraphPad Prism (Appendix 2 and Appendix 3). The summarized generated IC_{50} values are shown in Table 5.1. The ethanol extract had the lowest overall IC50 value, and the water extract could not be evaluated because it did not exhibit cytotoxicity against any cell line tested (Table 5.1). The R^2 values in Table 5.1 indicate the accuracy of the analysis; values above 0.9 (90%) are considered accurate. According to Gertsch (2009), a physiologically relevant IC_{50} value of a crude plant extract is said to be <100 µg/mL.

Table 5.1: IC_{50} (µg/mL) R^2 determination of three plant extracts of *Monsonia burkeana* against three cell lines

Treatment	**Lung**		**Liver**		**Colorectal**	
	IC_{50} (µg/mL)	R^2 values	IC_{50} (µg/mL)	R^2 values	IC_{50} (µg/mL)	R^2 values
Acetone	86.8	0.89	98.92	0.97	280.9	0.90
Ethanol	25.44	0.67	23.78	0.87	122.6	0.97
Water	ND	/	ND	/	ND	/

ND: means not detected

5.3.2 Cytotoxic activity confirmation using caspase-3 activity assay

Caspase-3 activation was assessed by immunohistochemistry using antibodies specific for the activated (cleaved) form of caspase-3. An increase in the mean integrated fluorescence intensity indicates a higher level of activated caspase expression. Figure 5.5 illustrates the experimental results after 48 hours of treatment exposure. The ethanolic extracts were evaluated for their ability to trigger caspase-3 activation, a marker of apoptotic cell death. Treatment with the extracts resulted in increased caspase-3 activation, as evidenced by enhanced green fluorescence (W2) in both A549 and C3A cells. Notably, A549 cells treated with the ethanol extract exhibited the most pronounced increase in green fluorescence (Figure 5.6). These findings indicate that apoptosis, evidenced by caspase-3 activation, is the primary mode of cell death in both cell lines.

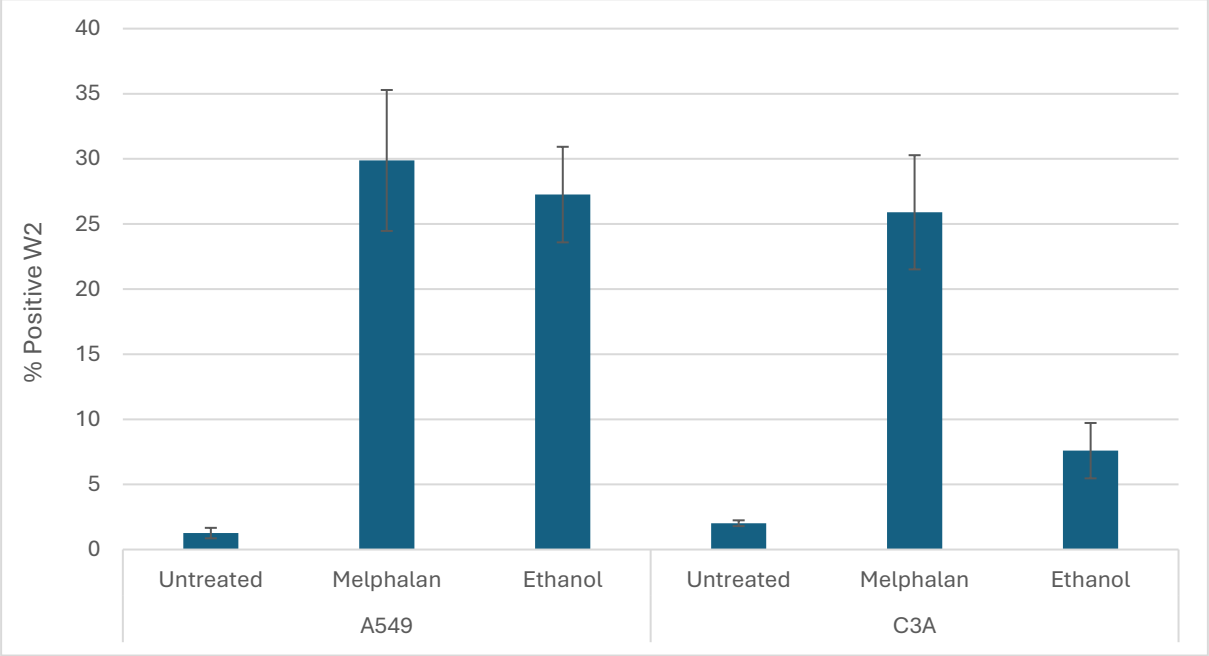

Figure 5.5: Cleaved caspase-3 analysis using the % positive e wavelength 2 (W2) parameter (i.e. percentage of cells that stained positive with anti-active caspase 3 antibody).

Bar graph represents the average of one individual experiment performed in quadruplicate. SD is represented as error bars.

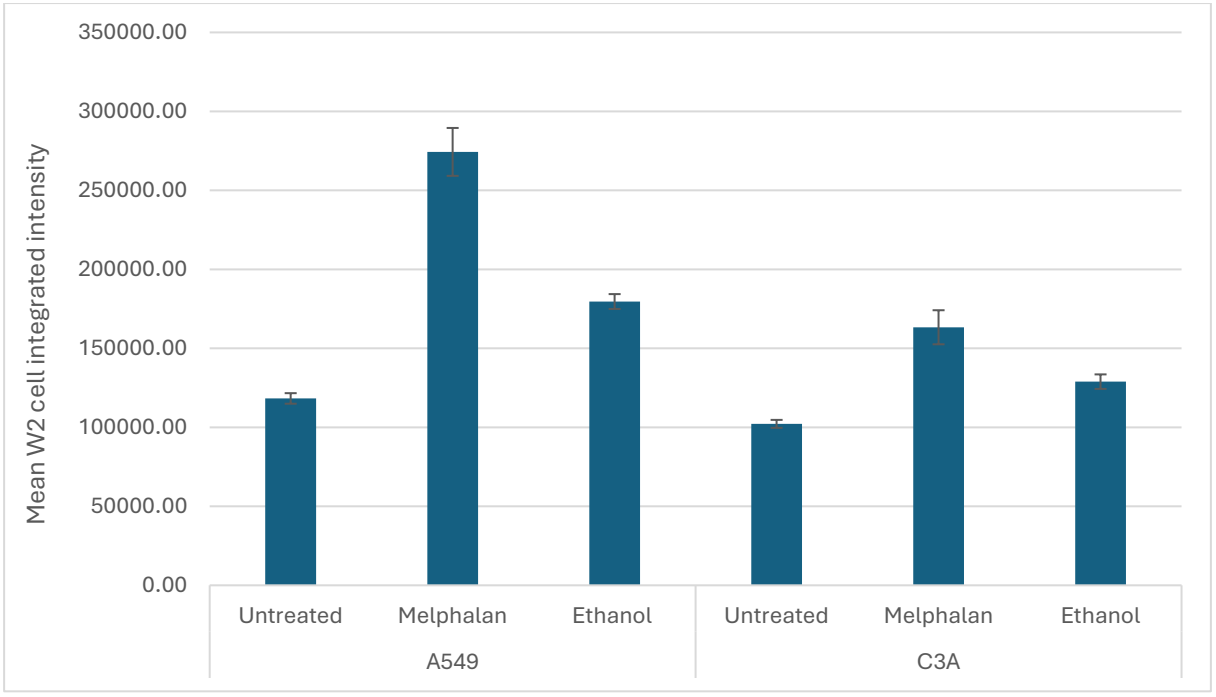

Figure 5.6: Cleaved caspase-3 analysis using the mean wavelength 2 (W2) cell integrated intensity parameter.

The bar graph represents the average of one individual experiment performed in quadruplicate. SD is represented as error bars.

5.4 Conclusion

The current study demonstrated that acetone and ethanol extracts of special tea plants exhibit potent anticancer effects against lung and liver cancer cell lines, with limited sensitivity in colorectal cancer cell lines. The positive anticancer activity was further confirmed by a caspase-3 activity assay in liver and lung carcinoma cell lines, highlighting the potential role of special tea in reducing cancer prevalence. However, further investigations are required using the water extract and the colorectal cancer line to determine their responses in alternative assays.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1 General discussion

The Republic of South Africa, like the African continent as a whole, is rich in medicinal plant diversity, with numerous species recognized for their phytochemical significance, while others remain largely unexplored. Among these, Special tea is widely used within South Africa and is particularly valued for its therapeutic properties (Woldesemayat et al., 2016). Currently, this plant is utilized for both medicinal and religious purposes. Notably, this plant is used by rural folk to boost the immune system and for consumption as a therapeutic agent for their ailments, most importantly, cancer. Evaluation of this plant's nutritional and biochemical composition is important for both the rural people and the pharmaceutical industry. It has been identified not only as a therapeutic agent but also as a potential nutritional supplement due to its antioxidant and antimicrobial bioactivities, among other potential health benefits (Woldesemayat et al. 2016). In rural areas, the plant is commonly prepared fresh or shade-dried, with decoctions made by boiling in water to extract its medicinal properties.

The investigation of bioactive phytochemicals in medicinal plants remains a critical area of research, focusing on compounds exhibiting antimicrobial, anticancer, antioxidant, and anti-inflammatory effects. However, substantial work is still needed to explore the range of medicinal benefits these plants offer to both the pharmaceutical industry and local people to address ailments such as mastitis, boils, haemorrhoids, congestion, headaches, hepatitis, liver disorders, vertigo, stomatitis, and kidney issues (Agidew, 2022; Rabizadeh et al., 2022; Rao et al., 2023).

Understanding these benefits of medicinal plants through phytochemical investigations using metabolomics technologies is essential for interpreting their pharmacological effects, including those of plants such as special tea (Mabona and Van Vuuren, 2013). Metabolomics, a key component of the 'omics' sciences, is instrumental in studying metabolites and is widely applied to analyze the biochemical composition of medicinal plants. The success of the metabolomic application depends on precise sample preparation, including drying and extraction processes. Techniques such as LC-MS, NMR, and GC-MS are currently employed, using both targeted and untargeted approaches to identify plant metabolites accurately. To determine the therapeutic value of medicinal plant metabolites, many studies have focused on anticancer phytochemicals, amongst other bioactivities (Roy et al., 2022). These anticancer bioactive compounds have been shown to modulate proliferative and apoptotic pathways, potentially preventing the growth of cancer cells. Therefore, their investigation of phytochemicals of interest in the development of new anticancer drugs is invaluable within the research industry.

The traditional use of special tea underscores the need for a detailed phytochemical profile analysis to identify the compounds responsible for the therapeutic applications reported by indigenous people. In addition, there is a knowledge gap regarding the benefits of these phytochemicals and potential phytotoxins that may endanger human and animal health. Therefore, this study focused on determining the nutrient content, phytochemicals, and anticancer activity of special tea, and on identifying variations associated with growth regions, drying methods, solvent extraction processes, and metabolomic techniques.

Special tea plants were collected from four regions in South Africa: Sekhukhune, Lanseria, Brits, and Rietondale. After being rinsed with distilled water, the plants were dried using three methods: shade drying, oven drying and freeze drying. Once dried, the

samples were ground into a fine powder and stored. Nutrient analysis was performed by microwave-assisted digestion, enabling quantification of 24 elements by ICP-MS. Additionally, phytochemical profiling using the NMR, GC-MS, and LC-MS techniques was conducted. The anticancer activity of the plant extracts was assessed using the MTT assay. The confirmation of apoptosis induction as a mechanism of anti-proliferative activity was conducted using a caspase-3 activation assay.

Special tea plants were subjected to two drying methods for nutritional composition: shade-drying, commonly used by traditional communities to preserve plants after harvesting, and freeze-drying, known for its superior retention of phytochemicals. The freeze-drying method yielded a significantly higher element concentration compared to shade-drying. However, despite these differences, the elements present in shade-dried plants could still benefit special tea consumers, as they retain these elements at lower concentrations. With frequent consumption, the difference in element concentration between the methods becomes less significant. Therefore, traditional communities could continue using shade-drying, as it remains a more cost-effective and sustainable method in traditional systems. The presence of these elements across four regions, despite variations in concentration, suggests that the plant can be successfully harvested wherever it grows, with great potential for production in most areas at both subsistence and commercial scales.

There is a clear distinction between the extraction yield of a solvent and the sensitivity of the resulting extract. A higher extraction yield from plant samples does not necessarily correlate with greater sensitivity of the extract obtained from that solvent. For instance, a study on *Salvia officinalis* L. demonstrated that although acetone yielded a low amount of extract, it exhibited a potent cytotoxic activity against cancer cells. However, this heightened sensitivity cannot be universally applied across all cell lines, as some may exhibit lower sensitivity (Alzeer et al., 2014). Consequently, the effectiveness of a

particular solvent for cytotoxicity analysis depends on the specific cell line and the method or assay employed. Special tea showed that ethanol extracts had greater cytotoxicity than acetone extracts against liver and lung carcinoma cell lines, whereas water extracts were inactive against any of the three cancer lines. When using the caspase-3 activity assay on ethanol-extracted samples, apoptosis was confirmed as the mechanism of cytotoxicity in the plant samples.

The determination of the nutritional composition of any plant consumed for food or medicinal purposes is important in the desire to fight against food insecurity and malnutrition (Li et al., 2023; Shahid et al., 2023; Yimer et al., 2023). This study employed ICP-MS to determine the plant's nutritional composition. The results showed that the 24 elements evaluated were present in the plant harvested from all areas. The freeze-drying method yielded a high elemental yield. However, shade-drying also revealed all the tested elements to be present. Shade drying is a cheaper, readily available technique for preserving plants for storage. The results indicate that, subject to soil nutrient availability, geographical regions may not directly affect the nutritional composition of special tea plants. The elements tested have varying importance in the body, such as psychological function and disease prevention, among many positive attributes (Shahid et al. 2023). Therefore, allowing it to be produced across different geographical areas or environments to meet the nutritional demand of rural people and the pharmaceutical needs, as the world's agricultural products have not yet reached a point of meeting the nutritional demands and sustainable foods for the world (Li et al., 2023). Primary metabolites in special tea were successfully profiled, indicating the presence of secondary metabolites, which were subsequently explored using NMR, GC-MS, and LC-MS.

NMR, GC-MS, and LC-MS techniques have been used in this study to determine the phytochemical composition and bioactive compounds of special tea plants. The application of these three techniques has greatly assisted in the identification,

quantification, and classification of phytochemicals in plants, and in the subsequent assignment of names to databases (Vinaixa et al., 2016). NMR spectroscopy showed the availability of phytochemicals in the plant. These include phenols, alkyls, esters, and ethers, which were important for the identification and characterisation of phytochemicals using GC-MS and LC-MS (Chhikara et al., 2021).

The GC×GC/TOFMS technique identified several important phytochemicals from special tea, including oxalic acid, bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, [1R-(1R*,4Z,9S*)]-, β-caryophyllene, dodecyl acrylate, ethyl 2-benzoylbenzoate, caryophyllene oxide, trans-1,2-bis(1-methylethenyl)cyclobutane, glycerin, α-humulene, phytol, squalene, and vitamin E. These phytochemicals have been identified to significantly contribute towards human health, exhibiting high-value biological activities such as anticancer, anticarcinogenic, and antitumor effects (e.g., farnesol, β-caryophyllene, caryophyllene oxide, squalene, and vanillin). They also demonstrate antibacterial (β-caryophyllene and squalene), antimalarial (α-humulene), antiviral (vanillin), and antioxidant (squalene) properties.

However, certain phytotoxins were also detected, including methyl 2-benzoylbenzoate, β-caryophyllene, caryophyllene oxide, trans-1,2-bis(1-methylethenyl)cyclobutane, dodecyl acrylate and oxalic acid. While these compounds have been classified as phytotoxins by the TPPT, they may also be a potential source of benefits for human health. It is essential to acknowledge that these substances are not necessarily harmful toxins; they may instead be plant stress-induced chemicals with varying biological effects. (Roy et al., 2022).

The LC-MS technique successfully identified several high-value phytochemicals with activities against cancer, bacteria, and inflammation, as well as antioxidant activity. Amongst the list of identified bioactive phytochemicals are the following polyphenols:

castalagin, vescalagin, acutissimin B, epicatechin, citroside, trifolin, 1,6-digalloyl-beta-D-glucopyranose, 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-{[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one, and puerarin 4',6''-diacetate.

The MTT assay has been employed to assess the metabolic activity of crude extracts in lung, liver, and colorectal cancer cells. (Ghasemi et al., 2021). The assay demonstrated minimal to no cytotoxicity in samples extracted with boiled water. This is particularly relevant, as the plant is commonly consumed as a tea prepared with boiled water, suggesting that users may be exposed to only limited quantities of key anti-proliferative phytochemicals. These findings warrant further investigation using additional assays and cancer cell lines to more conclusively determine the efficacy of water as an extraction solvent. In contrast, extracts obtained with acetone and ethanol exhibited significant cytotoxicity against all three tested cancer cell lines—lung, liver, and colorectal—with colorectal cancer cells showing the least sensitivity. While the assays could have limits such as MTT concentration and incubation time, and cell density, it remains the most versatile assay for the determination of cell activity (Ghasemi et al., 2021). The special tea plant's positive cytotoxic activity was validated through a caspase-3 activity assay, supported by studies demonstrating the ethanolic extracts' anti-proliferative properties and caspase-dependent effects on cancer cell lines (Gull et al., 2022; Chanu et al., 2024).

Kaempferol, a polyphenolic flavonoid belonging to the largest group of secondary plant metabolites, is known for its potent antioxidant properties. Over 104 types of flavonoids have been identified, with demonstrated activities beyond antioxidation, including hepatoprotective, antimicrobial, renoprotective, antidiabetic, cardioprotective, anti-arthritic, neuroprotective, gastroprotective, and anti-mutagenic effects (Periferakis et al., 2022). Similarly, isorhamnetin exhibit pharmacological properties, such as cardiovascular protection, anti-inflammatory, antitumor, antioxidant, antibacterial, and antiviral effects. Studies indicate its potential in managing cardiovascular diseases,

various tumours, and neurodegenerative conditions like Alzheimer's disease. Additionally, it shows promise in treating hyperuricemia and pulmonary fibrosis (Gong et al., 2020). Furthermore, isorhamnetin demonstrates significant antitumor activity, including tumour size reduction in leukaemia, inducing cell cycle arrest and apoptosis in colorectal cancer, and reducing pulmonary metastasis and metastatic nodules in hepatic carcinoma (Ganeshpurkar & Saluja, 2017). Research on rats indicates that large dosages of epicatechin might induce liver damage, characterized by hepatomegaly and a reduction in total body weight (Sabarathinam et al., 2023). These findings demonstrate the richness of special tea as a source of flavonoid secondary metabolites with several therapeutic applications.

The phytotoxins identified in the special tea could be present at all growth stages. When such plants are consumed, the phytotoxins they contain may harm both human and animal health. Cooking may lead to either beneficial or harmful changes, such as the leaching of nutrients or bioactive compounds into the cooking water, softening of plant tissues, alterations in colour and aroma development, and the breakdown of anti-nutritional factors, which can enhance the bioavailability of specific components. Reports have shown that medicinal plants remain a relatively safer source of medicine, despite the presence of possible phytotoxins, which may pose risks depending on their concentration (Uddin et al. 2020). Oxalic acid is one of the phytochemicals that may be transformed or decreased during the processing of the food (Arias-Rico et al., 2020).

6.2 Conclusions

Nutritional and phytochemical composition are important considerations for any plant consumed as food or for medicinal purposes. Many plants in the African context have been used for both purposes, and further research is needed in this regard. Special tea is an important medicinal plant consumed by rural people and understanding its nutritional and phytochemical properties and bioactive compounds was a critical component of this study. The plants' nutritional, phytochemical, and anti-proliferative properties were examined.

The freeze-drying method was more effective at retaining nutrients than shade-drying. However, special tea plants, as practised by traditional folks, can be harvested and dried using the shade-drying method, as elements are retained during the drying process. The plants can be produced in different areas, as shown, because nutrients are available regardless of the growing area. The study revealed that special tea plants have all 24 elements tested present. This is an important indication that the special tea plant can contribute to nutritional intake through its use as a medicinal plant. Ingesting it through a decoction in hot water or by any method may yield the desired nutrient uptake.

Phenols, alkyls, esters, ethers are some compounds identified from the plants, and further analysis identified important bioactive compounds needed by the human body including vanillin, puerarin 4',6"-diacetate, 2",6"-Di-O-acetylononin, trifolin, antitumor agents (β-caryophyllene, isorhamnetin, acutissimin B), antibacterial agents (squalene, 1,2,6-Trigalloyl-beta-D-glucopyranose), anti-inflammatory agents (caryophyllene oxide, luteolin hexoside), and antimalarial agents (α-humulene). These identified compounds from special tea belong to various classes of bioactive phytochemicals, primarily

polyphenol and terpenoid families, which are recognised as secondary metabolites associated with antioxidant, antimicrobial, anti-inflammatory, and anticancer activities.

The presence of phytochemicals in the special tea plants is consistent with the positive activity against cancer cell lines. Therefore, it is concluded that *Monsonia burkeana* plants are indeed highly valued plants for consumption by rural folk and for consideration by the pharmaceutical industry for commercial purposes. The plant's beneficial nutritional composition is another important advantage to be noted.

6.3 Recommendations

The *Monsonia burkeana* plant has demonstrated valuable attributes that may benefit human health. While there are tangible benefits from this plant, further studies are necessary to evaluate production aspects, including propagation, agronomic practices, and overall growth and development. Postharvest processing, such as packaging and the development of more efficient, cheaper tea bags, should be investigated. This research may enhance the production of this plant and aid in the attainment of Sustainable Development Goal 2, which seeks to eradicate hunger, ensure food security and improved nutrition, and promote sustainable agriculture.

Additionally, further studies should measure the response of this plant's phytotoxin accumulation to various external stimuli during different growth stages. More studies are needed to investigate other cancer activities to expand from the findings from this study in order to have a holistic bioactivity understanding of the plant. This invaluable research will determine the plant's usability and help develop processing systems to mitigate phytotoxin levels.

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APPENDICES

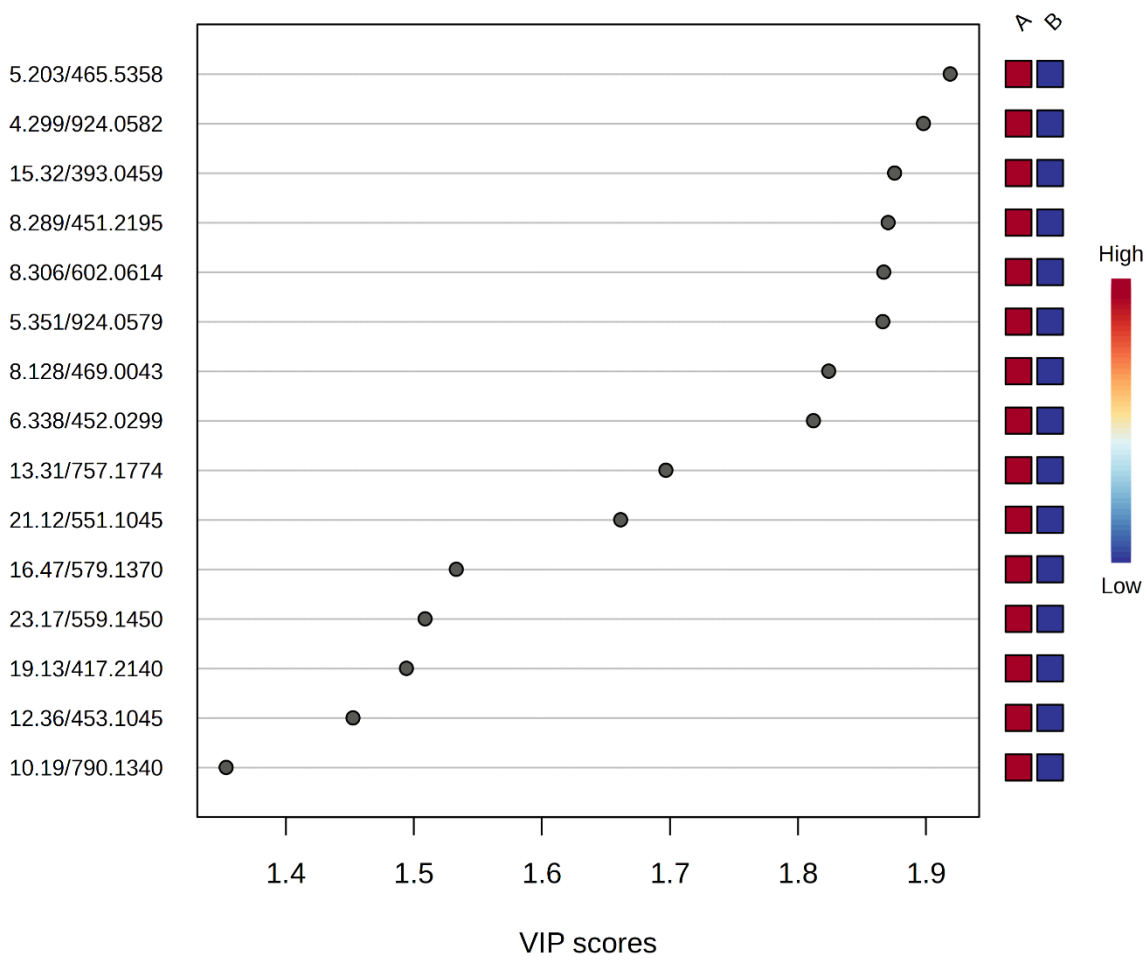

Figure A1: VIP scores for LCMS data results

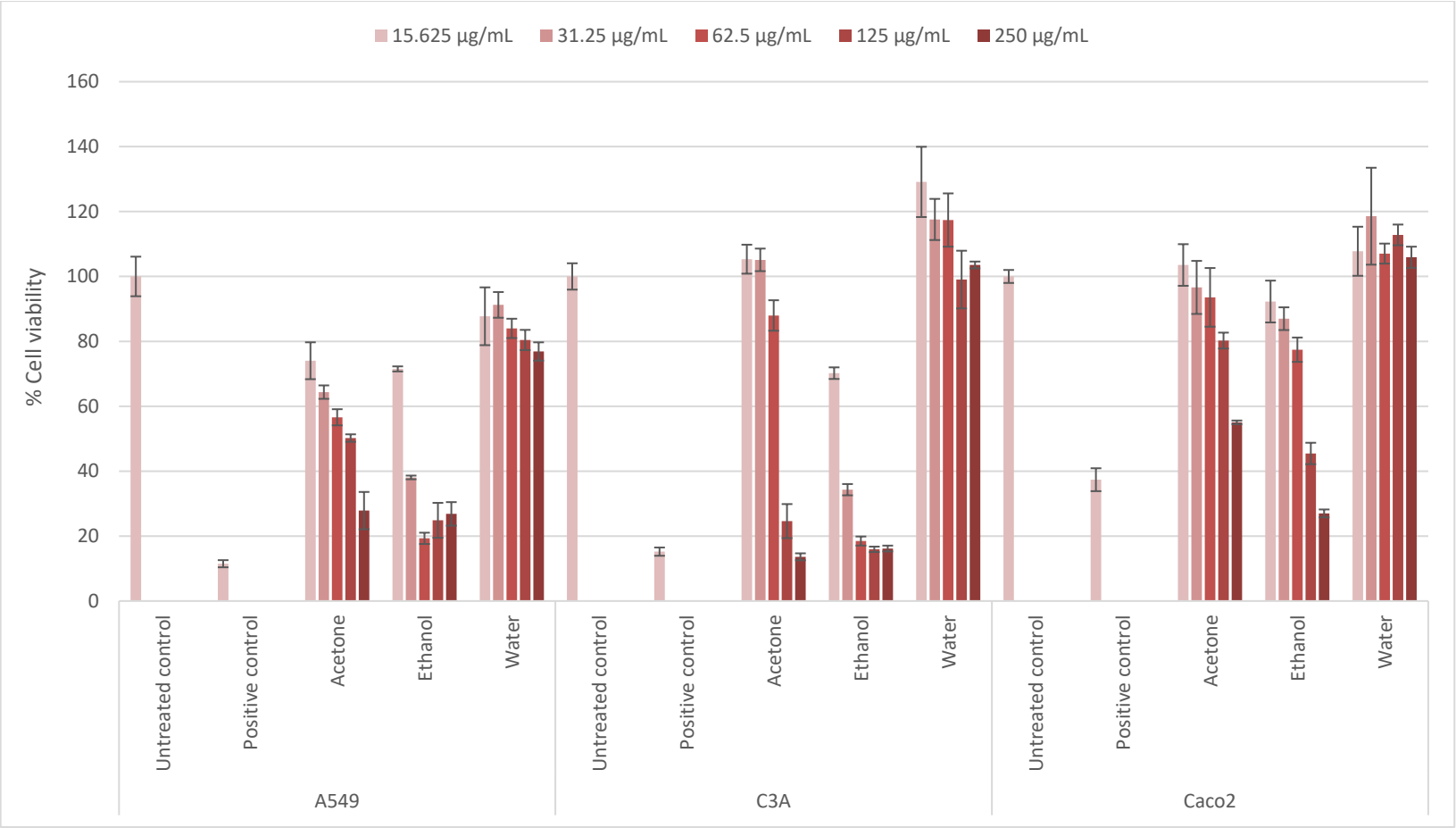

Figure A2: Cytotoxicity of three samples tested against 3 cell lines after 48 hours of treatment. Error bars indicate the standard deviation of quadruplicate values done as a single experiment.

Table A1: Colourimetric presentation of IC_{50} from the three cell lines and control (blank)

A549	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.42	0.959	0.977	0.87	0.735	0.335	1.054	0.544	0.287	0.459	0.45	0.432	540
B	0.41	1.15	0.905	0.818	0.729	0.335	1.028	0.56	0.277	0.39	0.407	0.406	540
C	0.403	1.085	0.933	0.782	0.748	0.501	1.034	0.549	0.31	0.306	0.38	0.411	540
D	0.406	1.111	0.928	0.822	0.708	0.449	1.043	0.561	0.249	0.291	0.324	0.414	540
E	0.411	1.15	1.287	1.16	1.121	1.065	0.158	1.4	1.374	0.053	0.053	0.405	540
F	0.414	1.242	1.274	1.227	1.141	1.108	0.149	1.371	1.367	0.054	0.053	0.406	540
G	0.414	1.253	1.346	1.237	1.2	1.139	0.181	1.52	1.48	0.056	0.055	0.397	540
H	0.433	1.457	1.399	1.261	1.215	1.159	0.18	1.604	1.515	0.058	0.056	0.401	540

C3A	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.279	0.728	0.758	0.608	0.152	0.103	0.499	0.227	0.139	0.12	0.116	0.267	540
B	0.256	0.705	0.734	0.582	0.146	0.102	0.508	0.257	0.138	0.113	0.122	0.255	540
C	0.254	0.763	0.709	0.63	0.169	0.09	0.478	0.241	0.125	0.106	0.11	0.257	540
D	0.255	0.773	0.762	0.66	0.227	0.089	0.494	0.242	0.119	0.11	0.108	0.249	540
E	0.254	0.869	0.79	0.831	0.697	0.738	0.104	0.657	0.681	0.053	0.051	0.25	540
F	0.258	0.652	0.813	0.745	0.764	0.733	0.12	0.72	0.734	0.055	0.053	0.252	540
G	0.257	0.998	0.893	0.876	0.717	0.727	0.099	0.687	0.739	0.054	0.051	0.251	540
H	0.267	0.863	0.818	0.857	0.614	0.721	0.106	0.699	0.721	0.059	0.053	0.27	540

Caco2	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.427	1.443	1.44	1.395	1.11	0.719	1.342	1.214	0.97	0.562	0.341	0.413	540
B	0.412	1.445	1.227	1.263	1.038	0.735	1.218	1.144	1.08	0.593	0.358	0.403	540

C	0.412	1.294	1.208	1.175	1.049	0.734	1.148	1.146	0.999	0.664	0.379	0.407	540
D	0.412	1.3	1.241	1.121	1.052	0.727	1.178	1.102	1.051	0.588	0.351	0.397	540
E	0.413	1.371	1.456	1.377	1.512	1.346	0.538	1.33	1.308	0.053	0.055	0.38	540
F	0.408	1.321	1.464	1.409	1.43	1.402	0.519	1.306	1.296	0.051	0.054	0.393	540
G	0.407	1.469	1.493	1.408	1.514	1.409	0.491	1.331	1.298	0.051	0.055	0.388	540
H	0.421	1.545	1.865	1.474	1.517	1.451	0.431	1.368	1.353	0.054	0.051	0.4	540

A549	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A		SA					SE					
B		acetone					Eth					
C		L				H						
D												
E		SW					melp	untreated				
F		water										
G												

Table A2: Caspase -3 activity report

Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											
Description												
Acquisition												
User	MolDev											
Z Step	1											
Measurement	Total Cells (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		505.5556	372.6667	337.4444	2088.556							
D		521.1111	354	301.4444	2197.333							
E		539.5556	332.8889	292.8889	2230.667							
F		549.7778	312.7778	270.2222	2040.889							
G												
H												
Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											
Description												
Acquisition												
User	MolDev											
Z Step	1											
Measurement	% Positive W2 (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		9.566093	31.50609	34.96055	1.84469							

D			10.86674	28.53001	34.12589	0.924754						
E			11.15142	26.15299	25.66643	1.119414						
F			10.73909	22.84129	24.75344	1.169115						
G												
H												
Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											
Description												
Acquisition												
User	MolDev											
Z Step	1											
Measurement	All W2 Mean Nucl Integr Intens (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		152903.7	156350.8	235141.5	119007.7							
D		161061.1	151510.6	240714.5	114462.3							
E		167649.1	159330.1	233361.8	116931							
F		173671.2	168932.1	243617.3	117406.4							
G												
H												
Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											
Description												
Acquisition												
User	MolDev											
Z Step	1											
Measurement	All W2 Mean Cyto Integr Intens (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12

A												
B												
C		12340.93	28843.28	51944.72	3974.34							
D		13869.88	26806.3	46519.6	644.6618							
E		13634.75	14673.03	24104.01	327.9323							
F		8506.281	12115.15	22012.9	430.8431							
G												
H												
Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											
Description												
Acquisition												
User	MolDev											
Z Step	1											
Measurement	All W2 Mean Cell Integr Intens (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		165244.6	185194.1	287086.2	122982							
D		174931	178316.9	287234.1	115107							
E		181283.8	174003.1	257465.9	117258.9							
F		182177.5	181047.2	265630.2	117837.3							
G												
H												
Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											
Description												
Acquisition												
User	MolDev											

Z Step	1											
Measurement	Total Cells (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C								328.1111	245.5556	39.22222	1028.667	
D								346.6667	243.8889	33.77778	1071.444	
E								289.7778	234.4444	38.44444	961.7778	
F								306.1111	223.7778	39	997.4444	
G												
H												
Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											
Description												
Acquisition												
User	MolDev											
Z Step	1											
Measurement	% Positive W2 (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C								6.223461	6.889864	26.90341	1.814132	
D								4.530657	7.206373	31.44105	2.044577	
E								3.811578	5.655787	24.11707	2.322123	
F								5.266654	10.61996	21.1279	1.904364	
G												
H												
Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											

Description												
Acquisition												
User	MolDev											
Z Step	1											
Measurement	All W2 Mean Nucl Integr Intens (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C								137717.8	117234.9	142335.7	98588.35	
D								136497.7	127158.6	162611.3	100573.9	
E								143757.1	129918.9	148296.2	104212	
F								145572	127432.2	142354.3	102464.9	
G												
H												
Barcode	N/A											
Plate Name	Sandile Caspase 3											
Plate ID	36544											
Description												
Acquisition												
User	MolDev											
Z Step	1											
Measurement	All W2 Mean Cyto Integr Intens (MultiWaveScoring)											
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C								4087.155	4884.286	14734.04	730.6897	
D								2822.423	3378.961	16489.17	526.9119	
E								1907.258	2283.494	13163.71	885.7307	
F								1241.777	3401.784	13394.63	806.9011	
G												
H												

Barcode	N/A
Plate Name	Sandile Caspase 3
Plate ID	36544
Description	
Acquisition	
User	MolDev
Z Step	1
Measurement	All W2 Mean Cell Integr Intens (MultiWaveScoring)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C								141805	122119.2	157069.8	99319.04	
D								139320.2	130537.6	179100.4	101100.9	
E								145664.3	132202.4	161460	105097.7	
F								146813.8	130834	155749	103271.8	
G												
H												