INHIBITORY EFFECTS OF SOUTH AFRICAN PLANTS AGAINST MYCOTOXIGENIC PHYTOPATHOGENS

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BLOEMFONTEIN

10 September 2024

DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO INDEPENDENT WORK

I, NTAGI GERALD MARIRI, identity number _____ and student number _____, do hereby declare that this research project submitted to the Central University of Technology, Free State for the Doctor of Health Sciences in Biomedical Technology, is my own independent work, and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State, and has not been submitted before to any institution by myself of any other person in fulfilment of the requirements for the attainment of any qualification.

_____ 10 September 2024

SIGNATURE OF STUDENT DATE

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Dedication

- This work and my life are dedicated to the cherished memory of my beloved late parents **Tsheko** and **Mmantsulwana Mariri**.

Abstract

Mycotoxigenic fungal strains pose a significant threat to food crops consumed by humans and animals, leading to reduced yields and compromised food quality as reported in almost 25% of maize products worldwide. The harmful effects following the ingestion of mycotoxin-contaminated food include inducing cancers, mutagenicity, immune suppression, and toxicities to target organs of the digestive, cardiovascular, and central nervous systems. Synthetic fungicides are associated with a high potential for toxic residues in food and the development of fungal resistance. Genetically modified crops can also reduce crop contamination. However, these strategies are inaccessible and expensive resources for most subsistence farmers. To address these issues, the use of plants as potential biopesticides to counter fungal infections on crops emerges as a possible solution to enhance food safety and security. This study aimed to determine the antifungal activities of *Bauhinia galpinii* N. E. Br., *Combretum caffrum* (Eckl. & Zeyh.) Kuntze, *Markhamia obtusifolia* (Baker) Sprague, and *Maytenus undata* (Thumb.) Blakelock, against mycotoxigenic fungi and potentially develop plant-based bio-fungicides. The leaves of the ethnobotanically selected plants were extracted using methanol and tested for antifungal activity against phytopathogenic strains of *Aspergillus parasiticus*, *Aspergillus nomius*, *Fusarium verticilloides*, *Fusarium graminearum*, *Fusarium oxysporum*, *Penicillium haloterans* and *Cladosporium cladospoides*. The antioxidant activities of the extracts were tested using the 2,2-diphenyl-1-picrylhydrazyl **(**DPPH) and 2,2'-azinobis (3-ethylbenzthiazoline-6-suphonic acid) (ABTS) assays. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) cytotoxicity assay was used to investigate the effects of the extracts on the viability of the African green monkey kidney (Vero) cells and the human colorectal adenocarcinoma (Caco-2) cells. Additionally, the micronucleus test was used to examine the potential of these plants to induce genotoxicity in Vero cells. The identity and quantity of phytocompounds in the plant extracts were analysed using gas chromatography (GC-ToF-MS) mass spectrometry and liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) analysis. The highest percentage extract yield was recorded from *Combretum caffrum* (23%), while *Markhamia obtusifolia* yielded 10.7%. *Bauhinia galpinii* methanol extracts had the lowest MIC value of 0.16 mg/ml against *Furasium graminearum*, *Furasium oxysporum*, *Penicillium haloterans*, and *Cladosporum cladospoides* at 24-hour incubation period. *C. caffrum* had a MIC value of 0.31 mg/ml against *F. vercitilloides* and *F. graminearum*. *C.caffrum* had the highest total activity (TA) of 1437.5 ml/g against the fungal pathogen, *Aspergillus nomius*. In the antioxidant studies, *C. caffrum* had good

antioxidant activity against DPPH with 50% inhibitory concentration (IC_{50}) value of 10 µg/ml, while *B. galpinii* had IC_{50} value of 50 µg/ml against free radicals of ABTS. Based on the ABTS/DPPH correlations (ADC), *M. undata* exhibited a notable ADC correlation of 2.33, which is over three-fold that of the ADC of ascorbic acid (control drug). Furthermore, all extracts were assayed for cytotoxicity and genotoxicity using the MTT and micronucleus assays, respectively. In the cytotoxicity studies, *B. galpinii*, *C. caffrum*, and *M. undata* extracts were cytotoxic to Vero cells at 500 µg/ml. *C. caffrum* and *M. undata* extracts demonstrated cytotoxic effects on human colorectal adenocarcinoma cells in a dose-dependent manner. *C. caffrum* extract significantly reduced cell viability by up to 89.61% at the highest concentration of 500 µg/ml. *M. undata* extract was also cytotoxic at the highest tested concentration of 500 µg/ml. *B. galpinii* extracts increased the viability of the cancer cells, indicating a potential stimulation of cell proliferation. The extract from *M. obtusifolia* had no effect on the cells at the tested concentrations of 250 and 500 µg/ml. These findings suggest that *C. caffrum* and *M. undata* extracts have potential as cytotoxic agents against colorectal adenocarcinoma cells, while *B. galpinii* extracts may not be suitable for cancer treatment. All tested plant extracts were genotoxic at the highest tested concentration of 500 µg/ml. *C. caffrum* showed genotoxicity at 250 µg/ml and reduced cell numbers to less than 50% of the control at 500 µg/ml, indicating its significant cytotoxic potential. Three of the four medicinal plants reported a higher phenolic content (TPC) compared to their total flavonoid compounds. These higher TPC contents may well explain the antimycotoxigenic and antioxidative ability of the medicinal plants selected for the current study. The GC-ToFMS analysis of the selected medicinal plants revealed that all the extracts contained Hexadecanoic acid (HA) and methyl ester. *B. galpinii* and *C. caffrum* exhibited the presence of lupeol, while *M. obtusifolia* and *M. undata* contained phytol. Kaempferitrin was identified following the LC-TOF-MS analysis of both *B. galpinii* and *M. obtusifolia*. Quercitrin was identified from *C. caffrum,* while rutin and pisumionoside were identified from *M. undata*. Overall, the study concluded that the strong inhibitory effects against various fungal pathogens shown by some of the tested plants indicate that investigating plants as potential solutions for fungal infections in crops offers a promising avenue to enhance food safety and security in developing and underdeveloped countries. Therefore, it is important to intensify efforts to explore the use of plants as potential biopesticides. To further advance the findings of the current study, continued research and safety assessments of the tested South African plant extracts are essential to fully exploit their potential benefits in agriculture and healthcare while mitigating any risks to human health and the environment.

Keywords

Antioxidant activity; bio-fungicides; cytotoxicity; flavonoid contents; free radicals; genotoxicity; minimum inhibitory concentrations; mycotoxins; phytochemicals; total phenolic contents.

Preface

List of scientific publications, published and submitted

Mariri, N.G., Dikhoba, P.M., Mongalo, N.I. & Makhafola, T.J. GC-ToF-MS Profiling and In Vitro Inhibitory Effects of the Selected South African Plants against the Important Mycotoxigenic Phytopathogens. Life **2023**, 13, x. https://doi.org/10.3390/life13081660

Mariri, N.G., Mongalo, N.I. & Makhafola, T.J. 2024. The *in vitro* toxicity screening and phytochemical analysis of the four South African plants with good antifungal activity. South African Journal of Botany. Manuscript number: SAJB-D-24-02384. *With editor.*

List of conference presentations

Mariri, N.G., Dikhoba, P.M., Mongalo, N.I. & Makhafola, T.J. 2023. "Inhibitory effects of selected South African plants against mycotoxigenic phytopathogens". 48th Annual South African Association of Botanists (SAAB) Conference. 17–20 January 2023, The Ranch Protea Hotel, Polokwane, South Africa. *Oral presentation.*

Mariri, N.G., Mongalo, N.I. & Makhafola, T.J. 2023. "The *in vitro* toxicity screening and phytochemical analysis of the four South African plants with good antifungal activity". 56th Annual South African Society for Basic and Clinical Pharmacology (SASBCP) conference. 24–27 August 2023, Rhodes University, Eastern Cape, South Africa. *Oral presentation.*

Mariri, N.G., Mongalo, N.I. & Makhafola, T.J. 2023. "The *in vitro* cytotoxicity, genotoxicity and LC-ToF-MS profiling of four South African plants with good antifungal activity". 1st Annual International Congress for the African Phytomedicine Scientific Society (APSS). 09–10 November 2023, University of Pretoria, Gauteng, South Africa. *Poster presentation.*

Mariri, N.G., Mongalo, N.I. & Makhafola, T.J. 2024. "The *in vitro* toxicity screening and phytochemical analysis of the four South African plants with good antifungal activity". 26th Indigenous Plant Use Forum (IPUF). 11-16 August 2024, !*Khwa ttu* San Cultural Centre, Darling, West Coast, South Africa. *Oral presentation.*

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1. Introduction

1.1. Background

Mycotoxigenic phytopathogens, which are a group of fungi capable of producing mycotoxins (Chhaya et al., 2024), pose a severe threat to food safety and security (Kalambate et al., 2024). At present, over 400 variations of mycotoxins have been identified, however, only between 10 and 12 types have been identified to be the most dangerous types concerning public health (Stoev, 2024). These include amongst others, aflatoxins (AFs) with aflatoxin B1 (AFB1) and aflatoxin M1 (AFM1) being the most dangerous, fumonisins (FUMs) with fumonisin B1 (FB1) being the most dangerous, and ochratoxin A (OTA) (Tiwari and Dubey, 2023). These toxins not only reduce the agricultural productivity of developing and underdeveloped countries but also pose health risks to humans and animals, necessitating effective and sustainable solutions to combat their prevalence (Qi et al., 2024). Mycotoxins contaminate an estimated 60-80% of the crops worldwide, yearly (Mesfin et al., 2022; Nagda and Meena, 2024). Consuming mycotoxin-contaminated food or feed can potentially result in various acute and chronic harmful effects for instance cancer, genetic mutations and congenital disorders in both humans and animals (Kalambate et al., 2024).

Naturally, plants produce chemical compounds with diverse physiological and biochemical activities (Rabizadeh et al., 2022). For many years, some plant compounds with antifungal properties have been used in the management of postharvest diseases particularly to prevent fungal spoilage and mycotoxin contamination (Chen et al., 2023). For instance, the bioactive compound; caffeic acid, was reported to inhibit the production of aflatoxin B1 by the pathogen *Aspergillus parasiticus* by 83% (Lorán et al., 2022). Additionally, citric acid extracted from *Nigella sativa* fruits was reported to inhibit the growth of fumonisin B1-producing *Fusarium oxysporum* by between 45.7-62% (Khan et al., 2020). South Africa, with its rich biodiversity, harbours a variety of plant species that have been traditionally used for medicinal purposes. Research into the potential of bioactive compounds derived from plants that could serve as natural alternatives to synthetic fungicides aligns with the first two of the United Nations Sustainable Development Goals (UN SDGs) which are to “end poverty in all its forms everywhere” and to “end hunger, achieve food security and improved nutrition and promote sustainable agriculture” (UN, 2024). Additionally, harnessing the inhibitory effects of South African plants against mycotoxigenic phytopathogens represents a promising strategy to reduce the reliance

on chemical pesticides, thereby minimizing environmental impact and promoting the health of ecosystems for sustainable agriculture. This approach aligns with the national efforts of the South African government to develop sustainable agricultural practices (National Development Plan, 2030).

The investigation into the inhibitory effects of South African plants against mycotoxigenic phytopathogens holds the promise of contributing to both agricultural innovation and public health. The findings could pave the way for the development of novel, eco-friendly biopesticides, ultimately enhancing food security and safety in regions most vulnerable to the impacts of fungal pathogens. This study aims to explore the potential of South African plants in combating mycotoxigenic phytopathogens. The research focuses on identifying plant species with significant antifungal properties, assessing their efficacy and safety as biopesticides and identifying compounds responsible for their bioactivity.

1.2. Problem statement

Mycotoxin contamination has been reported in almost 25% of maize and maize products worldwide, qualifying mycotoxin contamination as a worldwide food safety and public health challenge, with the developing countries affected the most (James and Zikankuba, 2018; Dikhoba et al., 2019; Mahato et al., 2019). The harmful effects following ingestion of mycotoxin-contaminated food and feed include inducing cancers, mutagenicity, immune suppression and toxicities to target organs of the digestive, cardiovascular and central nervous systems. The liver and kidneys are the main target organs affected by mycotoxicosis (Ezekiel et al., 2014). Aflatoxins, and in particular aflatoxin B1, are considered to be carcinogenic to humans and have been seen as the second leading cause of hepatocellular carcinoma worldwide (Kebede et al., 2020; Zavala-Franco et al., 2020) with an estimate of 250 000 deaths relating to hepatocellular carcinomas seen only in sub-Saharan Africa (Wagacha and Muthomi, 2008). In South Africa, the widespread contamination and occurrence of fumonisins observed mainly in maize crops have led to significant exposure to fumonisin B1, which has been suspected to enhance the risk of oesophageal cancer (Chu and Li, 1994; Rheeder et al., 1992). Currently, the most favoured management and treatment strategy to alleviate mycotoxicosis is the application of synthetic fungicides. However, this use of chemicals is deterred by the restrictions on their use because of the high potential of toxic residues in food and, importantly, the development of fungal resistance (Kebede et al., 2020). Additionally, some of the toxic fungal strains

have developed resistance to many of these synthetic fungicides. Genetically modified crops have been relied on to reduce crop contamination. However, both this strategy and synthetic fungicides are not affordable resources to most subsistence farmers (Dikhoba et al., 2019).

1.2.1. Research question

The central hypothesis in this proposed study is that anti-mycotoxigenic plant extracts can inhibit mycotoxin production and contribute to the development of bio-fungicides. It is on this premise that the research question in this study is: Can the selected four South African plants and phytocompounds prevent fungal growth and inhibit mycotoxin production?

1.2.2. Aim of the study

The aim of this study was to determine the antifungal activity against mycotoxigenic fungi of the selected South African plant extracts.

1.2.3. Objectives of the study

The study had the following objectives:

i. To screen the plant extracts for antifungal activity against a panel of mycotoxin-producing toxigenic fungi by determining their MIC values
ii. To determine the antioxidant activity of the selected plant extracts
iii. To determine the *in vitro* toxicity of the plant extracts using a combination of cytotoxicity and genotoxicity methods
iv. To characterize the phytochemical composition of the plant extracts

1.3. Chapter outline

This study is presented in five chapters. The first chapter provides background information to contextualize the study, followed by a statement of the research problem and question, leading to the aim and objectives of this study. Chapter two presents the systemic review of the literature which includes an in-depth analysis of the use of South African plants as antimycotic agents. Chapter three presents the materials and methods. The results obtained from our laboratory experiments are

reported in chapter four. Chapter five presents the discussions of the importance and relevance of the obtained results, and chapter six presents the general conclusions and recommendations. The thesis is completed with the supplementary material not included in the main thesis. This includes the published article.

2 Literature review

2.1. Mycotoxins and mycotoxicosis

Mycotoxins are a group of secondary metabolites produced by various toxigenic strains of fungi from the genera *Aspergillus, Fusarium, Penicillium, Aternaria* and *Claviceps* (Wang et al., 2024). The production of mycotoxins is likely to be influenced by the same environmental factors that impact the production and growth of toxigenic fungi such as temperature, pH, moisture and oxygen concentration (Cimbalo et al., 2020). These bioactive substances are common contaminants of different agricultural commodities worldwide, predominantly affecting developing countries, with an estimated 25% of the world's crops affected every year (Mahato et al., 2019). Mycotoxins have been detected to contaminate various agricultural commodities during the different stages of harvesting, including pre-harvest and post-harvest (Nleya et al., 2018). Mycotoxin contamination is widespread, and consuming contaminated agricultural products can cause mycotoxicosis in both human beings and animals (Phokane et al., 2019). The mycotoxin groups with the greatest occurrence and toxicity include aflatoxins (AFs), fumonisins (FBs) and ochratoxins (OTs) (Kebede et al., 2020). Therefore, the current study focused only on these three classes of mycotoxins.

2.1.1. Mycotoxin classification

2.1.1.1. Aflatoxins

Aflatoxins are by far amongst the most toxic mycotoxins (WHO, 2018) and are produced primarily by the species belonging to the genus *Aspergillus,* specifically *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Nleya et al., 2018; WHO, 2018). The International Agency for Research on Cancer (IARC) has listed aflatoxins and in particular aflatoxin B_1 as carcinogenic to humans (IARC, 2015). Aflatoxin contamination represents the second leading cause of hepatocellular carcinoma worldwide (Kebede et al., 2020; Zavala-Franco et al., 2020) with an estimate of 250 000 deaths relating to hepatocellular carcinomas seen only in sub-Saharan Africa (Wagacha and Muthomi, 2008). Furthermore, consumption of aflatoxin-contaminated diets has been strongly associated with increasing the possibility and risk of the development of liver cancer in Hepatitis B and Hepatitis C carriers (Kebede et al., 2020), as well as reduced growth in children and impaired immune systems

(Probst et al., 2014). These compounds have also been found to be genotoxic since they can cause DNA damage in humans and animals (WHO, 2018). Aflatoxins have been found mostly in cereal crops such as maize, animal feed, milk, medicinal herbal products, and various nuts across various African countries (Kebede et al., 2020).

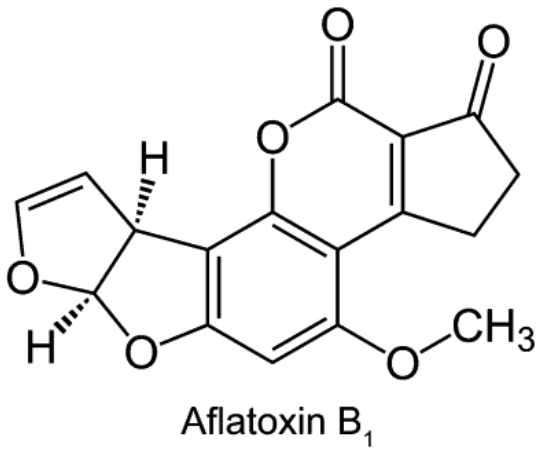

Figure 2.1: Chemical structure of aflatoxin B1 (Dharumadurai et al., 2011)

2.1.1.2. Fumonisins

Fumonisins are toxigenic secondary metabolites implicated in promoting human oesophageal cancer across the world (Rheeder et al., 1992; Chu and Li, 1994). Additionally, these toxins are linked with increased occurrences of neural tube defects in infants born to mothers who consume maize-based products contaminated with fumonisins (Missmer et al., 2006). The prevalence of fumonisin contamination is particularly notable in maize crops cultivated in South Africa, as highlighted by Janse van Rensburg et al., (2017). Fumonisins also pose a threat to livestock, as reported in studies (Kellerman et al., 1990; Glenn, 2007). The initial reports of fumonisins are from mouldy maize in South Africa, having been isolated from *Fusarium verticillioides* (Kebede et al., 2020). *Fusarium verticillioides* account for approximately 95% of all the *Fusarium* strains recovered from maize fields in Africa (Leslie, 2005).

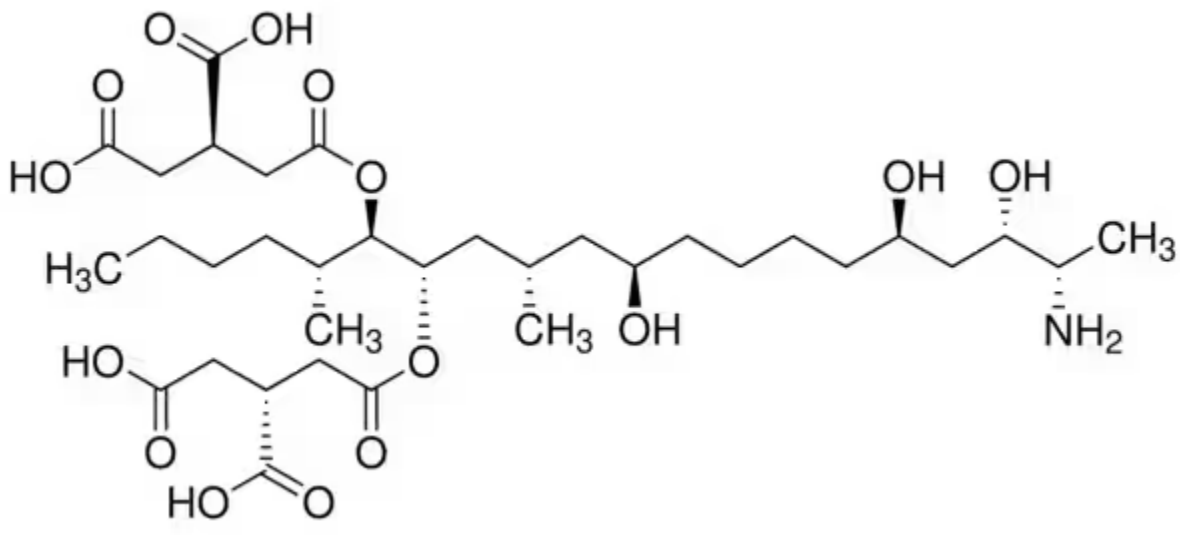

Figure 2.2: Chemical structure of fumonisin B_1 (Wang et al., 2023)

2.1.1.3. Ochratoxins

Ochratoxins are produced by fungi belonging to the genera *Aspergillus* and *Penicillium* such as *Aspergillus alutaceus* (syn. *A. ochraceus*) and *Penicillium verrucossum* (Zavala-Franco et al., 2020). Ochratoxin A is the most toxic mycotoxin within this group and commonly contaminates food products such as maize, cocoa beans, coffee beans, cassava flour, fish, peanuts, dried fruits, wine, milk and poultry eggs (Wagacha & Muthomi, 2008; Zavala-Franco et al., 2020). Ingestion of food contaminated by these compounds has been seen to have toxic effects on the liver and the kidneys with the ability to cause diseases of the upper urinary tract and induce various forms of cancers (Omotayo et al., 2019).

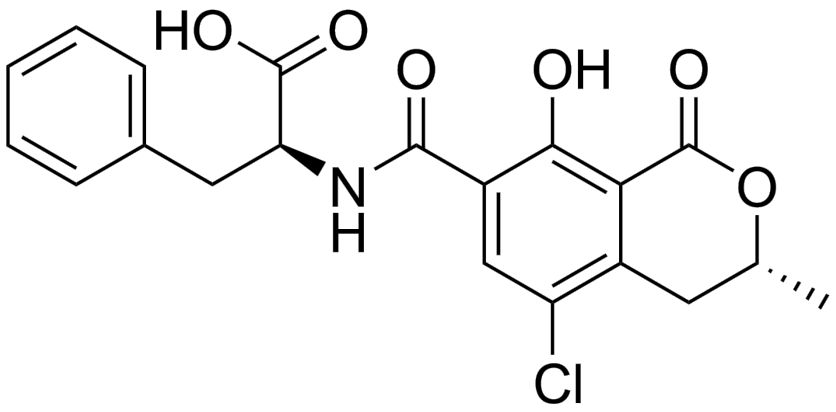

Figure 2.3: Chemical structure of ochratoxin (Dai et al., 2023)

2.1.2. Health and economic impacts of mycotoxin contamination

The toxic effects of mycotoxins on animal and human welfare can be acute or chronic and are referred to as mycotosis. Acute toxicity includes fever, abdominal pain, portal hypertension and death, while chronic mycotoxicosis is strongly associated with liver cirrhosis, kidney failure, immune toxicity and cancer (James and Zikankuba, 2018). Mycotoxins exhibit their adverse effects through various mechanisms following their invasion of the body systems of both humans and animals. The harmful effects following ingestion of mycotoxin-contaminated food and feed include inducing cancers, mutagenicity, immune suppression and toxicities to target organs of the digestive, cardiovascular and central nervous systems. The liver and kidneys are the main target organs affected by mycotoxicosis (Ezekiel et al., 2014). Additionally, fungal infections and mycotoxin contamination of crops and feeds during the pre-and post-harvest periods have had a negative impact on the trade of food and feed worldwide (Ncube, 2008). This is because of the decreased crop yield, not only owing to the crop's physical deterioration but also due to the potential for mycotoxin contamination (Arata et al., 2024). These significant losses in both the agricultural and food industries, further pose serious food security threats and economic challenges to governments across the world, with the African countries incurring the heaviest burden (Imade et al., 2021). The global population is projected to surpass 10 billion by the year 2050, which will heighten the demand for farmers to generate more healthy and safe food (Seepe et al., 2021). Furthermore, it is estimated that food production worldwide should increase by 50% in the next thirty years to satisfy the demands of the global population and "end poverty in all its forms everywhere" and "end hunger, achieve food security and improved nutrition and promote sustainable agriculture" (UN, 2024). However, the food-producing systems may fail to achieve this due to contamination of food by mycotoxigenic fungal strains (Mongalo et al., 2018).

Maize (*Zen mays* L.) is one of the most widely cultivated field crops globally, with the total world production estimated at 1 015 million tons in 2024 (Arata et al., 2024). In South Africa, an estimated 10–12 million tons of maize are produced annually, of which eight million tons are used as food or animal feed (Crop Estimates Committee, 2007). Maize serves as a staple food with an average daily consumption exceeding 300g per day in South Africa (Shephard et al., 2007). Mycotoxins may contaminate maize both before and after harvesting. This contamination arises from infection by mycotoxigenic fungi, commonly from the *Fusarium* and *Aspergillus* species (Phokane et al., 2019).

Consequently, maize consumption in South Africa is higher than in European countries, making the South African population even more susceptible to mycotoxin contamination (Janse van Rensburg et al., 2017). Almost 66.67% of the earth's population relies on cereals, roots and tubers as a source of staple foods. These staples provide the world's population with about 85% of its energy and 10% protein food intake. The diet consumed by almost 40% of the residents in sub-Saharan Africa is made up of roots and tubers (Onyedum et al., 2020).

2.1.3. Control and management of mycotoxigenic fungi

In South Africa, subsistence farmers suffer financial losses because of fumonisin and aflatoxin contamination of grain crops both before and after harvest. This is often associated with inadequate storage conditions that promote the proliferation of mycotoxigenic fungi. Factors contributing to these conditions include poor ventilation, temperatures between 10^{o}C and 40^{o}C and moisture content exceeding 13%, within storage facilities (Phokane et al., 2019). Currently, the most favoured strategy for the control and management of mycotoxigenic fungi is the application of synthetic fungicides. However, this use of chemicals is deterred by the restrictions on their use because of the high potential of toxic residues in food, drinking water systems and within the plant's ecosystem (Kebede et al., 2020; Anaduaka et al., 2023) as well as the development of fungal resistance (Bastos et al., 2021; Piotrowska, 2021). Additionally, synthetic pesticides have been reported to enhance free radicals which are known to induce oxidative damage to several cellular components in mammals (Shukla et al., 2012). There is a strong association between increased levels of oxidative DNA lesions and many tumours, seriously implicating DNA mutation as the cause of most cancers (Makhafola et al., 2016). Alternatively, agronomic practices such as crop rotation and genetically modified crops have been relied on to reduce crop contamination (Dikhoba et al., 2019). However, both these strategies and synthetic fungicides are not affordable resources to most subsistence farmers (Bankole and Adebanjo, 2003). This is of great concern worldwide, because mycotoxin contamination was reported in almost 25% of maize and maize products qualifying mycotoxin contamination as a worldwide food safety and public health challenge (James and Zikankuba, 2018). Combating mycotoxin contamination in crops can substantially reduce crop losses due to pest infestations and subsequently improve agricultural outputs (Ratto et al., 2022). In this way, small-scale farmers who contribute immensely to meeting the demands for food supply can be provided with effective, sustainable and affordable management solutions (Asibe et al., 2023).

2.2. Traditional and medicinal use of plants

Ever since time immemorial, mankind has depended on medicinal plants to provide a source of medicine, and to date, reliance on these traditional medicines for the treatment of different health challenges has continued across the world (Newman et al.,2003). In South Africa, most of the total population depends on the use of certain plant derivatives in catering to primary healthcare needs (UNAIDS, 2006). Medicinal plants have provided an avenue for the development of numerous useful drugs currently in use and remain an important route to new alternative pharmaceuticals some of which have been developed as bio-fungicides and nutraceuticals which are important in the prevention of fungi and mycotoxin contamination (Makhuvele et al., 2020). The popular use of medicinal plants can be attributed to their availability, affordability and the widespread claims about their therapeutic potential (WHO, 2002; Makhafola et al., 2016). Plants are known to be rich in chemically diverse and pharmacologically active compounds commonly referred to as secondary metabolites (El-Shouny et al., 2018; Ilaghi et al., 2021). Secondary metabolites are critical in the defence mechanism of the plant against numerous harmful microorganisms (Ansari et al., 2023). They have different classes, including alkaloids, phenolics, flavonoids, and terpenoids (Verma and Singh, 2020).

2.2.1. Medicinal plants as antimycotic agents

The incidence of fungal infections is steadily increasing, having an impact on more than a billion individuals, and resulting in over 1.5 million fatalities worldwide (Bongomin et al., 2017). Likewise, the agricultural industry faces a significant threat from plant diseases caused by pathogenic fungi, with estimated annual economic losses exceeding $200 billion globally (Wang et al., 2022). Among the prevalent fungal infections and crop diseases are those attributed to *Aspergillus*, *Fusarium* species (Ganesan et al., 2019) and *Penicillium* spp. (Wagacha & Muthomi, 2008). These fungi produce mycotoxins, which can lead to mycotoxicosis in humans when consuming contaminated food (Khanzada et al., 2021). Mycotoxin contamination is challenging to control due to their low molecular weight, non-immunogenic nature, thermostability, low effective concentrations, and wide range of toxicity (Abrunhosa et al., 2016).

The treatment of fungal infections typically involves various antibiotics, each with distinct modes of action. Despite increased awareness and improved treatment strategies, fungal pathogens continue

to develop drug resistance, posing a serious threat to public health and healthcare systems worldwide. Frequent use of antifungal antibiotics may lead to the emergence of resistance (Khanzada et al., 2021). For example, thiabendazole and carbendazim were once effective fungicides against many pathogens but have now become ineffective due to fungal resistance (Seepe et al., 2021). Furthermore, many antifungal agents have been reported to cause complications in host tissues (Mickymaray and Alturaiki, 2018). For example, *Aspergillosis* is commonly treated with azoles, but they may cause hepatotoxicity and visual disturbances as side effects (Schauwvlieghe et al., 2020). Consequently, there is an urgent need to discover novel agents with enhanced antifungal activity and fewer side effects for the treatment of fungal infections.

To control and eliminate mycotoxins in food grains and livestock feeds, detoxification is considered a promising approach, categorized into physical (grading, high temperatures, sorting), chemical (ammonia, ozone), and microbiological (bacteria, enzymes, fungi) processes (Bovo et al., 2013). However, each treatment method has its limitations, necessitating the identification of biologically safe and cost-effective agents for mycotoxin decontamination. Studies have shown that medicinal plants with antifungal properties may also possess decontamination effects (Brinda et al., 2013; Iram et al., 2015). Indigenous knowledge has long recognized the antifungal properties of plants, and modern science is beginning to validate these claims. There is a renewed interest in the indigenous applications of plant products for controlling pests that cause many crop diseases (Ngxabi et al., 2023). This interest is largely due to the continuous capability of medicinal plants to protect various crops against plant pathogens (Mahlo et al., 2010). Developing fungicides from plant-derived compounds is based on their ability to produce a wide range of secondary metabolites, which protect the plant against pathogenic microbes, insects and herbivores (Ansari et al., 2023). These phytochemicals include alkaloids, flavonoids, tannins, terpenoids, phenolic compounds, and which have versatile effects on pathogens (Guerriero et al., 2018). These compounds can inhibit fungal growth through multiple mechanisms, such as disrupting cell membranes, interfering with cell wall synthesis, and inhibiting essential enzymes (Vishwakarma et al., 2024). The action of these phytochemicals against fungal pathogens could be fungicidal (killing pathogens), or fungistatic (inhibiting pathogen development). Therefore, using plant-derived products against fungal pathogens could potentially prevent the development of fungal resistance as a result of the presence of the different constituent antifungal compounds and their synergistic properties (Hassan et al., 2021). Plant-derived bio-fungicides are expected to have low or no toxic effects on human health

and the environment (Dikhoba et al., 2019; Khursheed et al., 2022). They are also easily biodegradable because plant products naturally break down at high temperatures, therefore, they do not remain in the environment for long, unlike conventional synthetic fungicides (Seepe et al., 2021). These are very important traits to possess for any alternative strategy adopted in controlling mycotoxin contamination.

Table 2.1: Examples of medicinal plants with antifungal activity against phytopathogen

Plant species	**Family**	**Plant part used, solvent(s)**	**Organism tested**	**Key findings**	**Reference**
Chamaerops humilis L.	Arecaceae	Leaf, hexane	*A. flavus*	Good AFB_1 inhibitor (43.47%-94.88% reduction)	Boudjaber et al., (2023)
Elionorus latiflorus (Nees Ex Steud.) Hack	Poaceae	Leaf, water	*A. nomius*	Good AFB_1 and AFB_2 inhibitor	Farai et al., (2023)
Heteropterys byrsonimifolia A. Juss	Malpighiaceae	Leaf, methanol	*A. ochraceus*	MIC value of 0.03 mg/ml	Santos Júnior et al., (2014)
Cymbopogon citratus (DC.) Stapf	Poaceae	Leaf	*A. ochraceus*	MIC value of 0.11 mg/ml	Falcon et al., (2024)
Carpobrotus eludis L.	Mesembryanthemaceae	Leaf, 1:1 methanol: dichloromethane	*A. parasiticus*	MIC value of 0.01 mg/ml	Mongalo et al., (2018)
Combretum molle R. Br. ex G. Don	Combretaceae	Leaf, acetone	*A. parasiticus*	MIC value of 0.21 mg/ml	Mogashoa et al., (2019)
Allium sativum L.	Amaryllidaceae	Essential oil	*A. parasiticus*	MIC value of 0.0086 mg/ml and MFCs four-fold greater than MIC	Bocate et al., (2021)
			F. verticilliodes	MIC value of 0.0086 mg/ml and MFCs four-fold greater than MIC	
Combretum erythrophyllum (Burch.) Sond.	Combretaceae	Leaf, ethyl acetate, acetone	*F. verticilliodes*	MIC value of 0.04 mg/ml	Seepe et al., (2020)

Olea europaea L	Oleaceae	Leaf, ethyl acetate, acetone	*F. graminearum*	MIC value of 0.02 mg/ml and MFCs four-fold greater than MIC	Masoko and Makgapeetja, (2015)
Picrasma quassioides (D. Don) Benn	Simaroubaceae	Bark, methanol	*F. graminearum* *F. oxysporum*	Strong inhibitory activity and isolation of five antifungal compounds	Wang et al., (2022)

2.2.2. Selected medicinal plant species

In this study, the ethnobotanical selection of the medicinal plant species studied for their potential antifungal activity against mycotoxigenic fungi, was based on their reported efficacy against plant fungal pathogens (Ma et al., 2016; Dikhoba et al., 2019; Erhabor et al., 2020; Bantho et al., 2023; Molele et al., 2023). The motivation for investigating the leaves of these plants is that they can become a sustainable resource if a product is developed from the extracts.

2.2.2.1. *Bauhinia galpinii* N. E. Br.

Bauhinia galpinii belongs to the family Fabaceae. In English, this plant is commonly referred to as the “pride of De Kaap”. In its natural, untamed form, it presents itself as a medium to large shrub that behaves more like a climber, gracefully clambering through the trees and shrubs of the dense thicket vegetation found across the moister bushveld regions of Gauteng, KwaZulu-Natal, Limpopo, and Mpumalanga provinces in the country (Ahmed et al., 2019). Its leaves are bilobed, meaning each leaf has a symmetrical pair of rounded lobes that are joined symmetrically around a midrib, and a small, soft mucronule can be found in the notch at the tip. The plant blooms with beautiful brick-red flowers for an extended period during the summer months, specifically from September to March, but it may also flower sporadically throughout the rest of the year (SANBI, 2001). The ethnomedical uses of the plant include treating epilepsy, convulsions (Risa et al., 2004), diarrhoea and infertility (Samie et al., 2010). Importantly, Samie et al., (2010) reported the activity of *B. galpinii* against common fungal pathogens in particular the *Candida* species. More recently, Erhabor et al., (2020), reported the relative effectiveness of *B. galpinii* against bacteria and fungi as well as its relative safety.

Figure 2.4: Bauhinia galpinii leaves and flowers (Capegardencentre.co.za)

2.2.2.2. *Combretum caffrum* (Eckl. & Zeyh.) Kuntze

Combretum caffrum belongs to the Combretaceaea family and is native and restricted to the Eastern Cape and KwaZulu-Natal regions in South Africa, where it is commonly known as "*Umdubu*" (Bantho et al., 2023). This tree is characterized as a deciduous, small to medium-sized spreading tree, reaching heights of up to 10 m. Its young branches are covered in dense short hairs and take on a pinkish hue after shedding the bark. The leaves of *Combretum caffrum* are usually arranged in pairs opposite each other, though sometimes they may appear in whorls of three. These leaves are simple and have smooth edges (entire); they lack stipules and are densely covered in short hairs. The leaf blades are narrowly elliptical in shape and have a nearly hairless upper surface, while the underside is densely covered in short hairs, giving them a shiny green appearance. This tree produces spherical spikes that can grow up to 2 to 3 cm in length. Occasionally, these spikes may form short panicles through the suppression of leaves on short shoots (Biodiversity, 2011). In traditional medicine, the plant is used to treat bacterial infections and has antiviral, antiparasitic, cytotoxic and mutagenic

activities (Bantho et al., 2023). Ma et al., (2016) identified the potential of *C. caffrum* as a potent fungicide.

Figure 2.5: Combretum caffrum branch with leaves and seed pods (inaturalist.org)

2.2.2.3. Markhamia obtusifolia (Baker) Sprague

Markhamia obtusifolia is a compact, erect tree from the family Bignoniaceae, which is native to Southern and eastern Africa (Nchu et al., 2011). In South Africa, this species is commonly found in the provinces of Limpopo and Mpumalanga. It typically reaches a height of 8 m, although it can sometimes attain a height of up to 15 m. It features impressive, large yellow flowers and imparipinnate opposite leaves. The tree produces elongated pod-like capsules that encase papery winged seeds (treesa.org). In ethnomedicine, the roots of *M. obtusifolia* are utilized for treating hookworms, especially in Tanzania (Nchu et al., 2011).

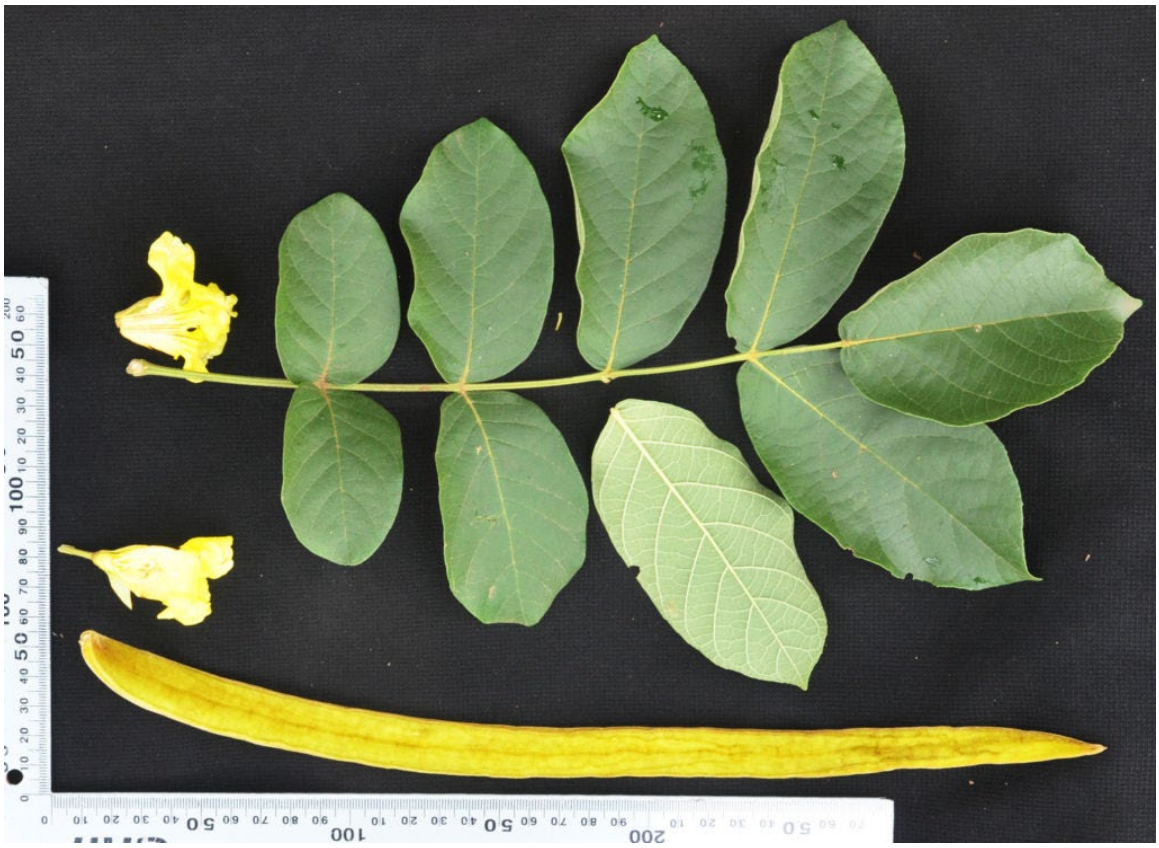

Figure 2.6: Markhamia obtusifolia leaves, flowers and sac encapsulating the seeds (treesa.org)

2.2.2.4. Maytenus undata (Thumb.) Blakelock

Maytenus undata is an incredibly resilient evergreen tree from the Celastraceae family (Mokoka et al., 2013). It exists in both large and small forms, with its height varying significantly depending on the specific environmental conditions in which it grows. In regions with colder climates, the tree tends to be smaller. The bark exhibits a grey-brown hue with sections that flake off, revealing pinkish patches underneath. The foliage, ranging from pale to dark green, is quite diverse in size and colour and emits a slightly musky aroma when rubbed. Throughout the summer months, from September to May, the tree adorns itself with creamy-white flowers. Come April, it yields black seeds ensconced within orange flesh. Commonly referred to as "*Idohame*" in Zulu, "*Umkokane*" in Xhosa, and "*Tshinembane*" in Venda, this tree can be found in various provinces across South Africa, including KwaZulu-Natal, the Eastern Cape, Western Cape, Limpopo, Mpumalanga, Northwest, Gauteng, and the Free State (Random Harvest). In traditional medicine, it is reported to have anti-cancer,

antimicrobial, anti-inflammatory, and anti-ulcer properties, and is used as a treatment for stomach conditions (Mokoka et al., 2013).

Figure 2.7: Maytenus undata leaves and seeds (www.randomharvest.co.za)

2.3. Conclusion

The persistent challenge of mycotoxin contamination in agricultural products, particularly maize, poses significant risks with far-reaching implications for human health, food security, and economic stability. Mycotoxins, which are produced by various toxigenic fungi, contribute to serious health conditions such as cancer, immune system suppression, and chronic diseases. This contamination affects both human and animal populations. As the human global population continues to expand, with projections exceeding 10 billion by 2050, the need for effective and sustainable management of mycotoxin contamination becomes increasingly urgent. Integrating traditional knowledge with contemporary scientific research offers a holistic approach to this challenge. Harnessing the antifungal properties of medicinal plants and advancing our understanding of their mechanisms could provide innovative solutions to mitigate mycotoxin risks, improve public health outcomes, and

support the development of more resilient agricultural systems. The potential of traditional and medicinal plants as bio-fungicides highlights their value in developing sustainable and environmentally friendly alternatives to synthetic fungicides. These plant-derived fungicides not only address the immediate issue of mycotoxin contamination but also align with the broader goals of the United Nations to enhance food security and promote sustainable agricultural practices. The medicinal plants explored in this study include *Bauhinia galpinii*, *Combretum caffrum*, *Markhamia obtusifolia*, and *Maytenus undata,* which have been used in traditional medicine across various cultures and are reported to possess a range of bioactivities.

In summary, this chapter provides a comprehensive literature review, highlighting the critical link between traditional practices and modern science in addressing one of the most pressing challenges in global food safety and security. The subsequent chapter will detail the materials and methods used in this study, including plant selection and collection, extract preparation, various assays performed, and data analysis.

3 Materials and methods

3.1. Plant selection and collection

The leaves of the plant species listed in Table 3.1 were randomly collected from the plants growing in the Lowveld Botanical Garden in the Mpumalanga province of South Africa (30º5758.16" E 25º2642.61" S Long 30.96800Lat-2554669). Leaves without symptoms of fungal or bacterial infections were collected. The identities of the plants were confirmed by Mr Willem Froneman (National Biodiversity Institute, NBI) and matched with the voucher specimens as reported in Table 3.1 below. Only leaves were used for plant conservation considerations.

Table 3.1: Plant species studied and their voucher specimen numbers.

Plant species	Family	Voucher number
Bauhinia galpinii N. E. Br.	Fabaceae	Glow 27/1986
Combretum caffrum (Eckl. & Zeyh.) Kuntze	Combretaceae	Glow 92/1997
Markhamia obtusifolia (Baker) Sprague	Bignoniaceae	Glow 16/1994
Maytenus undata (Thumb.) Blakelock	Celastraceae	Glow 157/1986

3.2. Extract preparation

The plant leaves were air-dried at room temperature and ground into a fine powder using the Genesis Nutrimax blender (Verimark, South Africa). The total weight of 20 g of the powdered leaves was weighed into 500 ml Schott bottles followed by the addition of 200 ml of methanol (Analytical grade, Radchem (PTY) LTD., Alberton, South Africa), making it 1:10 *w/v*. The Schott bottles were shaken uninterruptedly on an orbital shaker (OrbiShake, Labotec, Seoul, Republic of Korea) at 130 RPM for three consecutive days. Thereafter, the resulting supernatant was filtered through Whatman no.1 filter paper and the methanol extracts were then evaporated under reduced vacuum using a Rotary evaporator (Rotavapor R-300, Buchi, Shanghai, China) and then transferred into pre-weighed McCartney Bottles and concentrated to dryness under a stream of cold air under the fume hood for complete dryness. After drying, the McCartney Bottles were reweighed to determine the quantity of the plant material extracted. The dried plant extracts were resuspended in 20% methanol (in distilled water) to stock solutions of 10 mg/ml to be used in subsequent bioassays (Fennell et al., 2004).

3.3. Antifungal assays

3.3.1. Fungal pathogens

A total of seven phytopathogenic fungal strains belonging to the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Cladosporium* were selected for antifungal activity tests. The freshly prepared fungal cultures of *Aspergillus parasiticus* (PPRI:9153), *Aspergillus nomius* (PPRI:3753), *Fusarium verticilloides* (PPRI:10148), *Fusarium graminearum* (PPRI: 10340), *Fusarium oxysporum* (PPRI:10185), *Penicillium haloterans* (PPRI 25804) and *Cladosporium cladospoides* (PPRI: 10367), were purchased from the Agricultural Research Council, Plant Protection Research Institute (ARC-PPRI) located at Pretoria, South Africa. Dr Adrianna Venter-Jacobs (ARC-PPRI) confirmed their identities. These fungal cultures were then sub-cultured from potato dextrose agar (PDA) slants into freshly prepared potato dextrose broth (PDB) growth medium plates.

3.3.2. Inoculum preparation

Sterile distilled water and 30% glycerol spore suspensions were prepared by gently scrubbing the conidia from the periphery of actively growing 4- to 5-day-old cultures of *Aspergillus*, *Fusarium*, *Penicillium* and *Cladosporium* respectively. Glycerol assists with coating *Aspergillus* spores and prevents them from floating in liquid broth media. Thereafter, a full loop of each spore suspension was transferred into 75 ml of freshly prepared PDB and incubated overnight at 30 °C until slight turbidity was observed. Since *Fusarium* fungal strains sporulate slowly, it was necessary to obtain the suspensions by the exhaustive scraping of the surface using a sterile loop (Petrikkou et al., 2001).

3.3.3. Determination of minimum inhibitory concentrations (MIC) and the total activity of plant extracts

The serial microplate dilution assay developed by Eloff (1998), with further modifications described by Masoko et al., (2005), was used to determine minimum inhibitory concentrations of 10 mg/ml plant extracts dissolved in 20% methanol against the selected phytopathogenic fungi. Briefly, 100 µl of the plant extracts were transferred into the first row of wells in a flat bottom sterile 96 well plate (Merck, RSA, Westfield, South Africa) laid with 100 µl of freshly prepared potato dextrose broth and the contents in the wells were serially diluted two-fold. Aliquots of 100 µl of standardized fungal cultures

at a concentration of $1.1x10^7$ cfu/ml of each tested fungal strain were added into the corresponding wells. Wells with 20% methanol were prepared separately as negative controls, while amphotericin B, Propiconazole and Tebuconazole obtained from Merck, South Africa were used as standard antifungal agents (positive controls). All the wells were then loaded with 40 µl of 0.2 mg/ml of freshly prepared *p*-iodo-nitroterazolium (INT) chloride (Sigma-Aldrich, Darmstadt, Germany), as an indicator for fungal growth. The wells showing purplish colour were indicative of fungal growth, whereas colourless or greenish colour indicated that the plant extract inhibits fungal growth and was reported as the minimum inhibitory concentration (MIC) of the extract (Malhadas et al., 2017). After the addition of INT, the plates were incubated at 30 °C under 100% humidity. The MIC results were read after 24, 48 and 72 hours of incubation, respectively. The extracts were tested in triplicates. Total activity was then calculated by dividing the quantity extracted in milligrams from 1 g of plant material by the MIC value (mg/ml), to determine the total activity level of antifungal compounds of each extract (Masoko et al., 2005).

3.4. Antioxidant activity

3.4.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging activity of the selected medicinal plant extracts was determined using the method previously described by Fadibe et al., (2015), with slight modifications. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is based on the ability of the extract or compound to donate a hydrogen atom, thereby reducing the purplish colour of DPPH to yellowish or colourless (Chintalapani et al., 2018). Briefly, aliquots of plant extracts were serially diluted two-fold, in methanol to yield concentrations 0.5, 0.25, 0.13, 0.06, 0.03,0.02, 0.01, and 0.004 mg/ml, respectively in 100 µl of methanol inside 96 well microplates (Merck, RSA). All the wells were then loaded with 100 µl of a freshly prepared methanol solution of 0.02 mg/ml DPPH (*w/v*) (Merck, RSA) to indicate free radical scavenging activity. Then the plates were incubated in the dark for about 1 hour prior to absorbance reading at 517 nm using the microplate reader (SpectraMax iD3, Separations (Pty) Ltd., San Jose, CA, USA).

3.4.2. 2,2-azinobis (3-ethylbenzthiazoline-6-suphonic acid) (ABTS) radical scavenging activity

The ABTS radical scavenging activity of the selected medicinal plant extracts was measured with modifications of the 96-well plate method previously used by Re et al., (1999). The sterile 96 well microplates were laid with 100 µl methanol followed by the two-fold serial dilution of the tested plant extract to yield different concentrations as used in the DPPH assay above. The ABTS solution was prepared by dissolving 7 mM 2,2-azinobis (3-ethylbenzthiazoline-6-suphonic acid) (ABTS) salt and 2 mM of potassium persulphate in 3 ml distilled water and then incubated in the dark for the entire 16 hours. Then, the solution was diluted 1:60 (*v/v*) with pure-grade fresh methanol. All the wells were then loaded with 100 µl of the methanol solution of ABTS. The plates were then incubated in the dark for 5 minutes and read at 734 nm using the microplate reader (SpectraMax iD3, Separations (Pty) Ltd). Ascorbic acid was used as a positive control in both ABTS and DPPH assays, while wells containing methanol and a test radical were used as negative controls. The percentage of inhibition was then calculated using the formula:

$$\% \text{ inhibition} = 1-(A_t/A_0)\ X100$$

where, A_t equals the absorbance of the treated sample, and A_0 equals the absorbance of the negative control. The concentration of the plant extract leading to a 50% reduction of DPPH (IC_{50}) was then determined from the linear graphs constructed using the percentage of inhibition against the concentration of plant extract, using Microsoft Excel from three experimental replicates.

3.5. Toxicity studies

3.5.1. Cytotoxicity against Vero and Caco-2 cells

In this study, cell viability was tested using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT assay measures the conversion of mitochondrial dehydrogenase enzyme into the purple formazan in live cells (Al-Oqail, 2021). The assay was performed with slight modifications to the description by Mongalo et al., (2018). The MTT is an aqueous yellow solution, and it is reduced by enzymes and other agents present in metabolically active cells, forming formazan, which is blue-violet and insoluble in water. Formazan is extracted with organic solvents and quantified by spectrophotometry, being directly proportional to the number

of living cells (Marrelli et al., 2022). The cytotoxic effects of the selected crude extracts were evaluated against the African green monkey kidney (Vero) cells, and human colorectal adenocarcinoma cell line (Caco-2) (Cellonex, South Africa).

3.5.1.1. Sample preparation

The plant extracts were reconstituted in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA) to a stock concentration of 100 mg/ml. Extracts were sonicated if solubility was a problem and stored at 4°C until required.

3.5.1.2. Cell line maintenance

The African green monkey kidney (Vero) cells, and human colorectal adenocarcinoma cell line (Caco-2), were maintained in 10 cm culture dishes in complete medium (DMEM-LG (Cytiva, Marlborough, MA, USA), 10% foetal bovine serum (FBS), 100I.U./ml penicillin/, and 100 µg/ml streptomycin (Biowest, Nuaillé, France) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

3.5.1.3. 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT)

The Vero and Caco-2 cells were seeded in 96 well plates at 4000 cells/well (100 µl aliquots). The Vero and Caco-2 cells were incubated at 37°C, 5% CO_2 and 100% relative humidity to allow for cell attachment, prior to the addition of plant extracts at concentrations of 125, 250 and 500 µg/ml and treated for 48 hours. Untreated cells (negative control) and positive control (different concentrations of melphalan, Sigma Aldrich, St. Louis, MO, USA) were included in the assay. Following 48 hours of incubation, the treatments were then aspirated.100 µl MTT (0.5 mg/ml) (Sigma Aldrich, St. Louis, MO, USA) in the complete medium was added to each well. The cells were then incubated for three hours, and the MTT reduced by the viable cells to formazan was solubilized with the addition of 100 µl of DMSO. The amount of MTT reduction will be measured immediately by measuring absorbance at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). The percentage of cell viability was calculated using the formula below:

$$\%\text{cell viability} = \frac{\text{Mean absorbace of the sample}}{\text{Mean absorbance of the control}} \text{ X } 100$$

3.5.2. Genotoxicity against Vero cells

In the present study, the *in vitro* micronucleus test was used to estimate the ability of the investigated crude extracts to induce genotoxicity. Genotoxicity refers to the ability that an extract has to cause DNA damage within cells, which can lead to mutations or alterations in the DNA structure. Genotoxic agents can induce various types of DNA damage, including the formation of extra DNA adducts that lead to the formation of micronuclei. Micronuclei serve as indicators of both chromosome breakage and chromosome loss, and an elevated frequency of cells with micronuclei is considered a biomarker of genotoxic effects. Micronuclei are identified using the Hoechst 33342 nuclear dye (Phillips & Arlt, 2009; Luzhna et al., 2013).

3.5.2.1. Sample preparation

The plant extracts were reconstituted in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA) to a stock concentration of 100 mg/ml. Extracts were sonicated when solubility was a problem and stored at 4 °C until required.

3.5.2.2. Cell culture

The African Green monkey kidney (Vero) cells were purchased from Cellonex, South Africa and were cultured in Dulbeccos Modified Eagle Media (DMEM) supplemented with 5% Foetal Bovine Serum (FBS) (GE Healthcare Life Sciences, USA), and incubated in a humidified atmosphere with 5% CO_2 at 37 °C. When 90% confluence was reached, cells were trypsinized (Lonza, USA) (0.25% *w/v*) for subculturing and conducting experiments.

3.5.2.3. *in vitro,* micronucleus test/Genotoxicity evaluation using Vero cells

The Vero cells were seeded in 96 well plates at 3,000 cells/well (100 µl aliquots) and left overnight to attach. The cells were then treated for 48 hours at 37 °C and 5% CO_2 with 31.25, 62.5, 125, 250 and 500 µg/ml of each tested crude extract. Griseofulvin was used as the positive control at a concentration range of 1.875, 3.75, 7.5, 15 and 30 µM. After incubation, cells were fixed using 4% formaldehyde in Phosphate Buffer Solution (PBS) (Lonza, USA) for 15 minutes and aspirated. Hoechst 33342 working solution was prepared in PBS (Ca^{2+} and Mg^{2}) (Lonza, USA) to a final

concentration of 5 µg/ml and 100 µl Hoechst 33342 of the working solution was used to stain the cells for 15–30 minutes and thereafter acquired using the DAPI filter and 10x PlanFluor objective on the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). Nine image sites, covering roughly 70% of the total surface area of the well, were acquired in each well.

3.5.2.4. Data Quantification

Quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) and acquired images were analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Acquired data were transferred to a Microsoft Excel spreadsheet and data were analysed and processed.

3.6. Phytochemical screening

3.6.1. Determination of total phenolics

Total phenolics were determined using the Folin-Ciocalteu method as previously described by Adebayo et al., (2015). Briefly, 25 µl of the freshly extracted samples using 50% methanol were treated with 250 µl Folin-Ciocalteu reagent for 5 min. The reaction was stopped by adding 750 µl 20% anhydrous sodium carbonate and the volume was made up to 5 ml with ultrapure distilled water and incubated in the dark at room temperature for 2 h. The absorbance was then read at 760 nm using the microplate reader (SpectraMax iD3, Separations (Pty) Ltd). The phenolic contents of the selected medicinal plants were then determined from a standard curve of different concentrations of gallic acid dissolved in DMSO and the results were expressed as mg/g gallic acid equivalents (GAE).

3.6.2. Determination of flavonoids

The flavonoid contents of the tested plant extracts were determined according to the method described previously by Adebayo et al., (2015), with slight modifications. Briefly, the 100 µl of each crude extract was dissolved in 300 µl of methanol, to which 20 µl 10% aluminium chloride was added. An additional 20 µl of 1 M sodium acetate was added to the solution. The resulting solution was then made up to 1 ml with ultrapure distilled water and incubated at room temperature for 30 min in a microplate. About 10 mM of quercetin was used as a standard. The absorbance was read at 450 nm

in a microplate reader (SpectraMax iD3, Separations Pty Ltd). The flavonoid contents of each extract were then expressed as mg/g quercetin equivalents (QE)

3.6.3. Phytochemical analysis of crude extracts by Gas-chromatography time of flight (GC-ToF-MS) mass spectrometry

The GC-ToF-MS analysis of organic extracts of the selected plant species was carried out using a method adopted from Mongalo et al., (2023), with slight modification. The small amount of about 0.05 mg/mL of the selected plant extracts was completely dissolved in acetonitrile (GC-MS grade, Sigma Aldrich, Germany) to the lowest concentration. The separation of compounds was performed on a gas chromatography (7890 N GC-ToF-MS, Agilent Technologies, Santa Clara, CA, USA) coupled to a LECO Pegasus HT Flight Mass Spectrometry Time (ToF-MS) obtained from LECO Corporation, Michigan, USA. In short, the prepared samples were loaded into a Gerstel MPS2 Liquid/HS/SPME auto-sampler. For the chromatographic separation, AJ & W capillary column HP-5MS 30 × 0.25 mm I.D with a film thickness of about 0.25 µM was used. The following conditions were applied in the chromatographic separation: About 1 µL of the sample was injected at 250 °C with a spitless injector. The GC oven was programmed at 80 °C for 1 minute, then ramped up at 10 °C per min to 280 °C for 20 min. Helium obtained from Afrox (Johannesburg, RSA) at 99.99% purity was used as a carrier at a constant flow of 1 mL/min. The interface temperature of the GC-ToF-MS was set at 280 °C and the mass spectra were obtained in full scan mode at 70 ev (m/z scan varying from 50 to 550). The collection of data was obtained using ChromaToF, which possesses a NIST 95 library for compound matches.

3.6.4. Phytochemical analysis of crude extracts by Liquid-chromatography time-of-flight mass spectrometry (LC-TOF-MS)

The phytochemical analysis of extracts by LC-TOF-MS was performed with slight modifications of the method previously used by Adu-Amankwaah et al., (2022). Two grams of the crude dried extracts were completely dissolved in 50% methanol/1% formic acid (HPLC grade, Sigma Aldrich, Germany). A Waters Synapt G2 Quadrupole time-of-flight mass spectrometer (QTOF-MS) connected to a Waters Acquity Ultraperformance Liquid Chromatograph (UPLC) (Waters, Milford, MA, USA) system was used for high-resolution UPLC-MS analysis. Column eluate first passed through a Photodiode Array (PDA) detector before going to the mass spectrometer, allowing simultaneous collection of UV

and MS spectra. Electrospray ionization was used in negative mode with a cone voltage of 15 V, desolvation temperature of 275 °C, desolvation gas at 650 l/h, and the rest of the MS settings optimized for best resolution and sensitivity. Data were acquired by scanning from m/z 150 to 1500 *m/z* in resolution mode as well as in MSE mode. In MSE mode two channels of MS data were acquired, one at a low collision energy (4 V) and the second using a collision energy ramp (40−100 V) to obtain fragmentation data as well. Leucine enkephalin was used as lock mass (reference mass) for accurate mass determination and the instrument was calibrated with sodium formate. Separation was achieved on a Waters HSS T3, 2.1 × 100 mm, 1.7 µm column. An injection volume of 2 µl was used and the mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B. The gradient started at 100% solvent A for 1 min and changed to 28% B over 22 min in a linear way. It then went to 40% B over 50 s and a wash step of 1.5 min at 100% B, followed by re-equilibration to initial conditions for 4 min. The flow rate was 0.3 ml/min, and the column temperature was maintained at 55 °C. Compounds were quantified in a relative manner against a calibration curve established by injecting a range of catechin standards from 0.5 to 100 mg/l catechin. The data were processed using MSDIAL and MSFINDER (RIKEN Center for Sustainable Resource Science: Metabolome Informatics Research Team, Kanagawa, Japan) (Tsugawa et al., 2015; Lai et al., 2018).

3.7. Data analysis

Antifungal assays

The results of the antifungal assays were reported as the mean of three independent experiments (n=3). Each experiment was performed under identical conditions to ensure consistency and reliability of the data. The mean values of the minimum inhibitory concentrations (MIC) of plant extracts against the selected phytopathogenic fungi were calculated and recorded.

Antioxidant activity, cytotoxicity and genotoxicity studies

For antioxidant activity, cytotoxicity, and genotoxicity studies, the results were reported as mean ± standard error (SE) to provide a measure of variability and precision of the data. Statistical significance was determined using *p*-values, with values ≤ 0.05 considered significant. In the genotoxicity assay, the quantification of live and dead cells was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). Acquired images were analyzed with the

MetaXpress software and the Multi-Wavelength Cell Scoring Application Module. The percentage of cells with micronuclei was calculated and expressed as mean ± SE.

Phytochemical analysis

In the phytochemical analysis, the results were populated and analyzed using ChromaTOF software, which includes the NIST 95 library for compound matching. The software was used to identify and quantify the chemical compounds present in the plant extracts. The data was processed to ensure accurate identification of the phytochemical constituents.

Statistical analysis

All data were subjected to statistical analysis to determine significance. *p*-values ≤ 0.05 were considered statistically significant, indicating that the observed effects were unlikely to have occurred by chance. Statistical analyses were performed using appropriate software tools to ensure the reliability and validity of the results.

3.8. Conclusion

The current study employed various assays to address the primary research question concerning the inhibitory effects of South African plants against mycotoxigenic phytopathogens. This chapter outlines the comprehensive approach used to investigate the antifungal, antioxidant, cytotoxic, genotoxic, and phytochemical properties of ethnobotanically selected medicinal plants. Plant samples were collected and accurately identified using voucher specimens. The plant extracts were carefully prepared and standardized for subsequent bioassays. The antifungal activity was evaluated using a range of phytopathogenic fungal strains, with the minimum inhibitory concentration (MIC) determinations and total activity calculations, conducted according to well-established protocols. The antioxidant potential was assessed through DPPH and ABTS radical scavenging assays, providing insight into the extracts' efficacy in neutralizing free radicals. The cytotoxic effects were examined using the MTT assay on Vero and Caco-2 cell lines, while genotoxicity was assessed through the *in vitro* micronucleus test, offering a comprehensive view of the extracts' safety and potential health impacts. Phytochemical analyses were performed using advanced techniques, including GC-ToF-MS and LC-ToF-MS, to identify and quantify the chemical constituents of the plant extracts. Data

analysis for antifungal, antioxidant, cytotoxic, and genotoxic assays was conducted with rigorous statistical methods, ensuring the reliability and accuracy of the results.

Overall, this chapter has provided a comprehensive methodological framework for evaluating the potential of medicinal plants as natural bio-fungicides and therapeutic agents. The integration of traditional knowledge with contemporary scientific techniques offers promising avenues for addressing mycotoxin contamination and advancing sustainable agricultural practices. The subsequent chapters will build upon these methodologies, further exploring the results and implications of the study.

4 Results

4.1. Introduction

This chapter presents the results of a comprehensive investigation into the biological activities and phytochemical profiles of selected South African medicinal plants. The primary aim of this study was to determine the antifungal activity against mycotoxigenic fungi of the selected South African plant extracts. The study's objectives included evaluating the extracts' antioxidant, cytotoxic, and genotoxic properties as well as characterising the phytochemical composition of the plant extracts.

The chapter begins with the presentation of the findings concerning the percentage yields of plant extracts, highlighting the most and least productive species. It then details the antifungal activities, reporting the minimum inhibitory concentrations (MICs) and total activity (TA) values of the extracts against various fungal strains. Following this, the chapter reports the antioxidant potential of the extracts, providing insights into their effectiveness in neutralizing free radicals. Subsequent sections then present the findings relating to the cytotoxic effects of the extracts on Vero and Caco-2 cell lines, evaluating their potential safety for therapeutic use. The results of the genotoxicity studies are also included, assessing the potential risks of the extracts to cellular DNA. Finally, the chapter provides a detailed phytochemical analysis of the extracts using GC-ToF-MS and LC-ToF-MS, identifying key compounds and their relative abundances.

These results offer valuable insights into the potential of these South African medicinal plants as natural bio-fungicides and therapeutic agents, enhancing our understanding of their roles in sustainable agriculture and health.

4.2. Percentage extract yields, antifungal activity and total activity

The percentage yield for each of the tested plant extracts was determined by dividing the dried extracted mass after the evaporation of the solvent by the dried plant mass used for extraction multiplied by 100 (Masoko et al., 2005). *Combretum caffrum* yielded 23% of crude extract and was the highest percentage yield recorded from all the tested plant species in this study, followed by *Bauhina galpinii,* which yielded 21.4% of the crude extract, while the lowest percentage yield was recorded from *Markhamia obtusifolia* at 10.7% (Table 4.1). The antifungal activities of the extracts

from the selected medicinal plants are reported in Table 4.1. The methanol extract from *Bauhinia galpinii* leaves exhibited the notably lowest minimum inhibitory concentration (MIC) value of 0.16 mg/mL against *Furasium. graminearum* at a 24-, 48-, and 72-hour incubation period, and a similar MIC against *Cladosporium cladosporioides* at the 24- and 48-hour incubation time. The extract further had a MIC of 0.31 mg/ml against both *Aspergillus parasiticus* and *Asparagus nomium* at all three different incubation periods. The extract from *Combretum caffrum* exhibited an MIC value of 0.31 mg/ml against *Furasium verticilloides*, *F. graminearum,* and *C. cladosporioides* at all tested incubation periods, and the notably lowest MIC value of 0.16 mg/ml against *C. cladospoides* at both the 24- and 48-hours' incubation period. *Markhamia obtusifolia* extract exhibited an MIC value of 0.31 against *A. nomius* and *F. verticilloides* at all tested time intervals while *Maytenus undata* exhibited an MIC value of 1.25 mg/ml against both *F. graminearum* and *Aspergillus parasiticus* at 48- and 72-hours incubation. All the selected strains were more susceptible to Propiconazole, Amphotericin B, and Tebuconazole as antifungal agents.

The total activities (TAs) of the selected medicinal plants are presented in Table 4.2. The extract from *Combretum caffrum* had the highest total activity yielding a TA value of 1437.5 mg/mL against *Aspergillus nomius* at the 24- and 48-hour incubation period, while *Bauhinia galpinii* extract had a total activity of 1337.5 ml/g against *C. cladospoide*s at the 24- and 48-hour incubation times and a similar TA against *Penicillium haloterans* at the 24-hour incubation period. *Maytenus undata* extract exhibited the lowest TA values yielding a TA value of 139.2 against all three *Furasium* (*F. verticilloides, F. graminearum,* and *F. oxysporum*) strains and *A. parasiticicus* at various incubation periods while *M. obtusifolia* exhibited the lowest TA value of 169.84 against both *P. haloterans* and *C. cladospoides* at the 72-hour incubation period.

Table 4.1: Minimum inhibitory concentrations (mg/ml) of the selected methanol leaf extracts against the tested fungi recorded after 24, 48 and 72-hour incubation

Plant species	Extraction yield (%)	*F.verticilloides*			*F.graminearum*			*F.oxysporum*			*A.parasiticus*			*A. nomius*			*P.haloterans*			*C.cladospoides*		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	24 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
B. galpinii	21.4	0.63	0.63	**0.31**	**0.31**	**0.31**	**0.16**	**0.16**	1.25	1.25	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	**0.16**	**0.31**	0.63	**0.16**	**0.16**	**0.31**
C. caffrum	23	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	0.63	0.63	0.63	1.25	1.25	**0.16**	**0.16**	**0.31**	**0.31**	0.63	0.63	**0.31**	**0.31**	**0.31**
M. obtusifolia	10.7	**0.31**	**0.31**	**0.31**	0.63	0.63	**0.31**	**0.31**	0.63	1.25	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	**0.31**	0.63	**0.31**	**0.31**	0.63
M. undata	17.4	0.63	1.25	1.25	1.25	1.25	**0.31**	**0.31**	1.25	1.25	1.25	1.25	1.25	0.63	0.63	0.63	**0.31**	**0.63**	1.25	**0.31**	**0.31**	0.63
Amphotericin B	-	0.16	0.16	0.16	0.02	0.04	0.08	0.08	0.16	0.16	0.16	0.16	0.31	0.04	0.04	0.04	0.08	0.16	0.63	0.16	0.16	0.31
Propiconazole	-	0.08	0.16	0.16	0.04	0.04	0.02	0.02	0.02	0.02	0.08	0.16	0.16	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02
Tebuconazole	-	0.08	0.16	0.16	0.04	0.04	0.04	0.04	0.04	0.04	0.08	0.16	0.16	0.02	0.02	0.04	0.04	0.08	0.31	0.02	0.02	0.04

Bold value indicates noteworthy activity.

Table 4.2: Total activity (ml/g) of the selected methanol leaf extracts against the tested fungi recorded after 24, 48 and 72-hour incubation

Plant species	*F.verticilloides*			*F.graminearum*			*F.oxysporum*			*A.parasiticus*			*A. nomius*			*P.haloterans*			*C.cladospoides*		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
B. galpinii	339.68	339.68	690.32	690.32	690.32	690.32	690.32	690.32	690.32	690.32	690.32	690.32	690.32	690.32	690.32	**1 337.5**	690.32	339.68	**1 337.5**	**1 337.5**	690.32
C. caffrum	741.94	741.94	741.94	365.08	184	365.08	184	365.08	184	365.08	184	184	**1437.5**	**1 437.5**	741.94	741.94	365.08	365.08	741.94	741.94	741.94
M. obtusifolia	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	345.16	169.84	345.16	345.16	169.84
M. undata	276.19	139.2	139.2	139.2	139.2	139.2	139.2	139.2	139.2	139.2	139.2	139.2	276.19	276.19	276.19	561.29	276.19	139.2	561.29	561.29	276.19

Bold value indicates noteworthy activity.

4.3. Antioxidant activity

The free radical scavenging activity of the selected medicinal plants against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethylbenzthiazoline-6-suphonic acid (ABTS) assays are presented as an inhibitory concentration leading to a 50% reduction of free radicals (IC_{50}) as summarized in Table 4.3. The extract from *Combretum caffrum* had the lowest antioxidant activity against DPPH with IC_{50} values of 10 and 70 µg/ml against DPPH and ABTS, respectively, while *Bauhinia galpinii* had a notable IC_{50} value of 50 µg/ml against the ABTS free radical. *Maytenus undata* extract yielded an IC_{50} value of 30 µg/ml against the DPPH free radical and a further 70 µg/ml against ABTS, while *Markhamia obtusifolia* exerted a notable antioxidant activity against DPPH, yielding an IC_{50} value of 60 µg/ml. Ascorbic acid exhibited IC50 values of 5.7 and 4.0 µg/ml against DPPH and ABTS free radicals, respectively. The highest ABTS/DPPH value was obtained from the *B. galpinii* extract yielding 9.8, while *M. obtusifolia* and *M. undata* exhibited 2.83 and 2.33, respectively.

Table 4.3: Antioxidant activity (IC_{50} in µg/ml) of selected medicinal plants

Plant species	DPPH	ABTS	ABTS/DPPH
B. galpinii	490±3.11	**50±0.01**	9.8±0.001
C. caffrum	**10±0.06**	**70±0.23**	7.0±0.002
M. obtusifolia	**60±0.08**	170±2.24	**2.83±0.01**
M. undata	**30±0.01**	**70**	**2.33±0.01**
Ascorbic acid	**5.7±0.001**	**4.0±0.01**	**0.70±0.01**

Bold-faceted data show noteworthy antioxidant activity/correlation. Results recorded as mean ±SEM.

4.4. Toxicity studies

4.4.1. Cytotoxicity against Vero and Caco-2 cells

The cytotoxic effects of the crude organic extracts on the African green monkey kidney cell lines and human colorectal adenocarcinoma cell lines are reported in Figures 4.1 and 4.2. *Bauhinia galpinii*, *Combretum caffrum*, and *Maytenus undata* extracts were cytotoxic to African green monkey kidney cells at 500 µg/ml after 48 h of exposure.

Bauhinia galpinii extract reduced the viability of the African green monkey kidney cells by up to 86.74% showing cytotoxicity at the highest tested concentration of 500 µg/ml, which was comparable to melphalan (87.64%).

Maytenus undata crude extract had a comparable effect at 125 and 250 µg/ml, but reduced the viability of the Vero cells by up to 52.52%, showing cytotoxicity at the highest tested concentration of 500 µg/ml.

Combretum caffrum extract was less toxic to the Vero cells, and only inhibited the viability of the cells up to 41.14%. *Markhamia obtusifolia* organic extract had no notable effect on the Vero cells at the highest tested concentrations of 500 µg/ml.

Combretum caffrum extract demonstrated cytotoxicity against human colorectal adenocarcinoma cells in a dose-dependent manner. *C. caffrum* extract inhibited the viability of the Caco-2 cells by up to 89.61%, showing cytotoxicity at the highest tested concentration of 500 µg/ml, which was comparable to melphalan (90.24%).

Maytenus undata crude extract had a similar effect at 125 and 250 µg/ml but reduced the viability of the Caco-2 cells by up to 73.67% showing cytotoxicity at the highest tested concentration of 500 µg/ml.

Bauhinia galpinii organic extract dose-dependently increased the viability of the human colorectal adenocarcinoma cells, which may be an indication that they stimulated the proliferation of these cells.

The organic extract from *Markhamia obtusifolia* had no effect on the Caco-2 cells at the concentrations of 250 and 500 µg/ml.

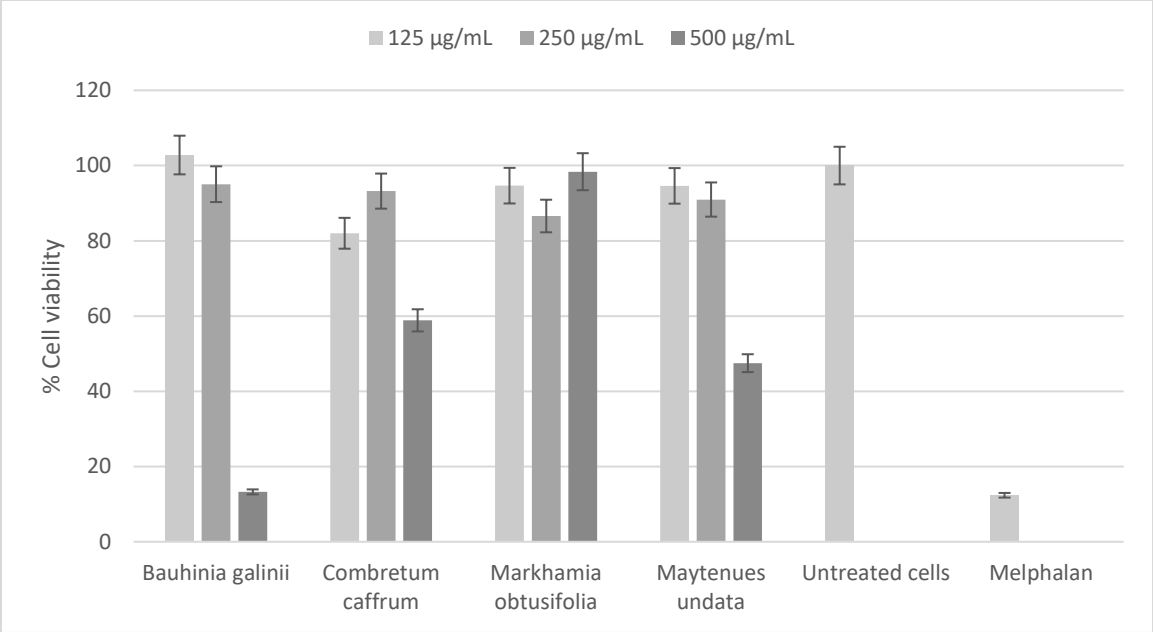

Figure 4.1: Cytotoxicity against Vero cells

Error bars indicate the standard deviation of quadruplicate values done as a single experiment.

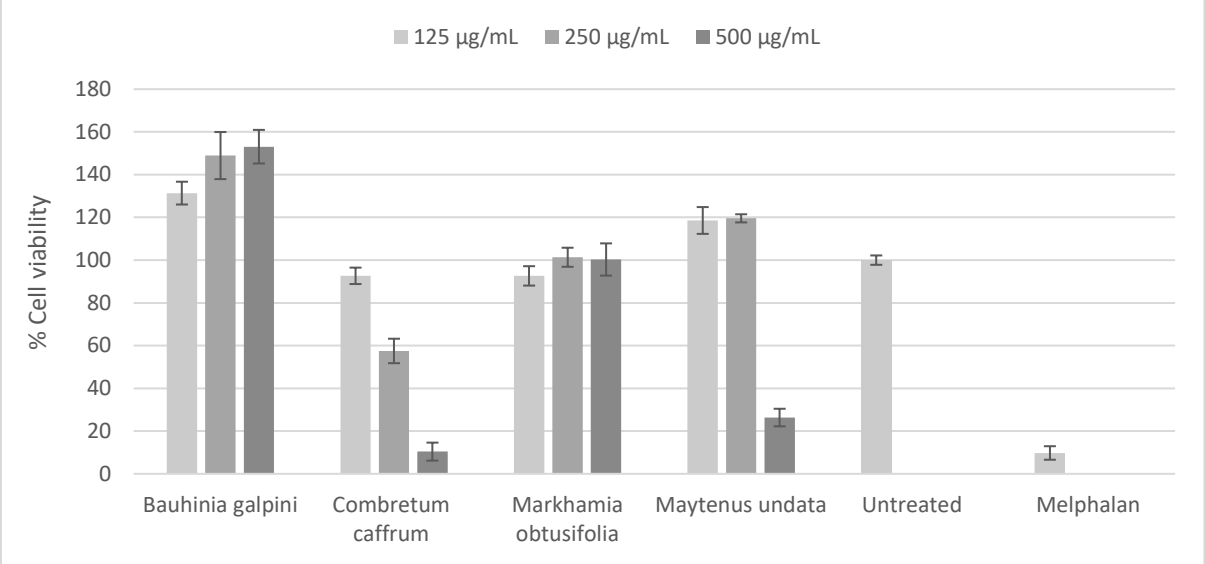

Figure 4.2: Cytotoxicity against Caco-2 cells

Error bars indicate the standard deviation of quadruplicate values done as a single experiment.

4.4.2. Genotoxicity against Vero cells

In the present study, the genotoxicity was assessed using Hoechst 33342 nuclear dye to identify micronuclei. Figure 4.3 illustrates the presence of cytotoxicity, if any, induced by the extracts across

different concentrations. Figure 4.4 displays the percentage of cells exhibiting micronuclei after 48 hours of treatment with a particular sample.

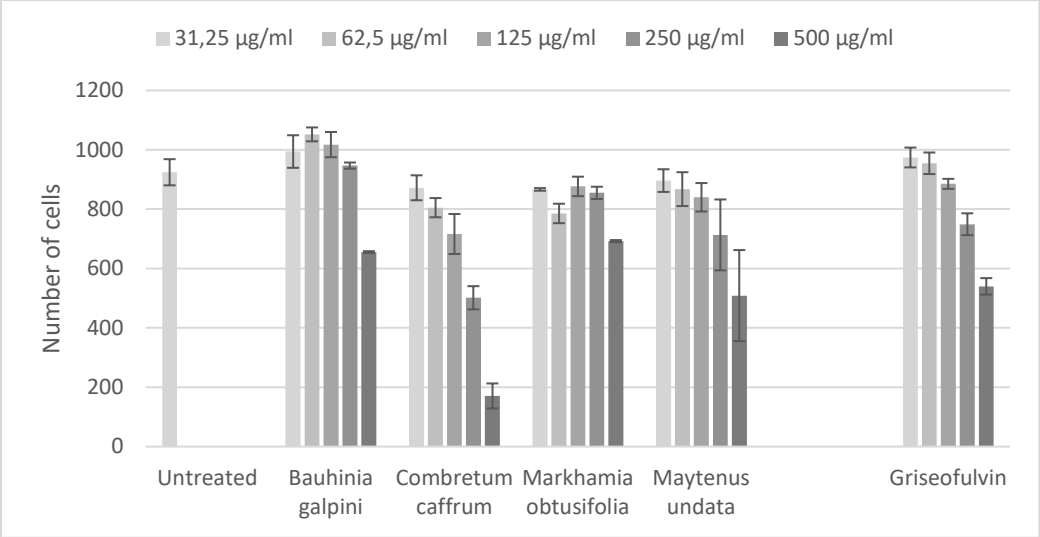

Figure 4.3: Total Vero cell number after 48 hours of exposure to crude extracts

Error bars indicate standard deviation of triplicate values done as a single experiment.

Griseofulvin was used at a concentration range of 1.875, 3.75, 7.5, 15 and 30 µM

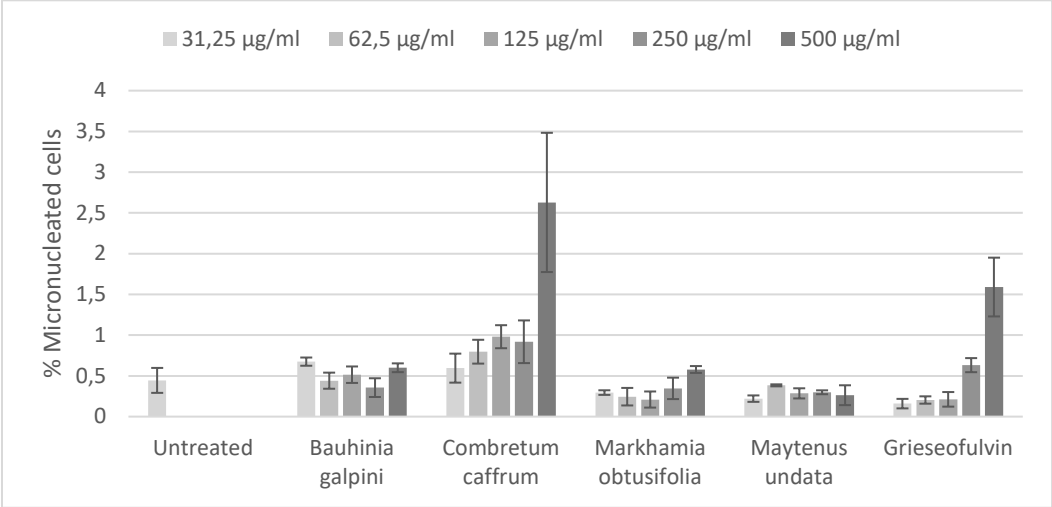

Figure 4.4: Percentage of micronucleated Vero cells after 48 hours of exposure to crude extracts

Error bars indicate standard deviation of triplicate values done as a single experiment.

Griseofulvin was used at a concentration range of 1.875, 3.75, 7.5, 15 and 30 µM

Bauhinia galpinii, *Markhamia obtusifolia* and *Maytenus undata* all showed to exhibit cytotoxicity at the highest tested concentration of 500 µg/ml. *Combretum caffrum* showed cytotoxicity at 250 and

500 µg/ml. *C. caffrum* at 500 µg/ml was the only one that reduced cell numbers to <50% of the control. Therefore, this concentration of *C. caffrum* cannot be considered for genotoxicity. An increase in the % of micronucleated cells was evident after the treatment of Vero cells with *C. caffrum* (62.5, 125, 250 and 500 µg/ml).

4.5. Phytochemical screening

The total phenolic contents (TPCs) and total flavonoid contents (TFCs) of the selected medicinal plants are presented below (Table 4.4). *Combretum caffrum* and *Bauhinia galpinii* extracts exhibited higher TPC values yielding 0.46 and 0.41 mg/g GAE, respectively, while extracts from *Maytenus undata* and *Markhamia obtusifolia* exhibited significantly higher TFC values of 0.39 and 0.13 (*p*-values ≤ 0.05), respectively.

Table 4.4: Total phenolic and total flavonoid contents of selected medicinal plants

Plant species	Total Phenolic Content (mg/g GAE)	Total Flavonoid Content (mg/g QE)
B. galpinii	**0.41±0.11**	0.11±0.01
C. caffrum	**0.46±0.09**	0.09±0.01
M. obtusifolia	0.16±0.04	**0.13±0.01**
M. undata	0.29±0.12	**0.39±0.04**

Bold-faceted data show noteworthy TPC and TFC. Results recorded as mean ±SEM

4.5.1. Phytochemical analysis of crude extracts by Gas-chromatography time of flight (GC-ToF-MS) mass spectrometry

4.4.1.1. GC-ToF-MS analysis of *Bauhinia galpinii*

Bauhinia galpinii revealed a total of thirty-six compounds, twelve compounds of which were present in abundance, identified by a % area greater than one. The chromatogram is presented in Figure 4.5., while the compounds with their retention time and relative abundance (% area) in the crude extract are presented in Table 4.5.

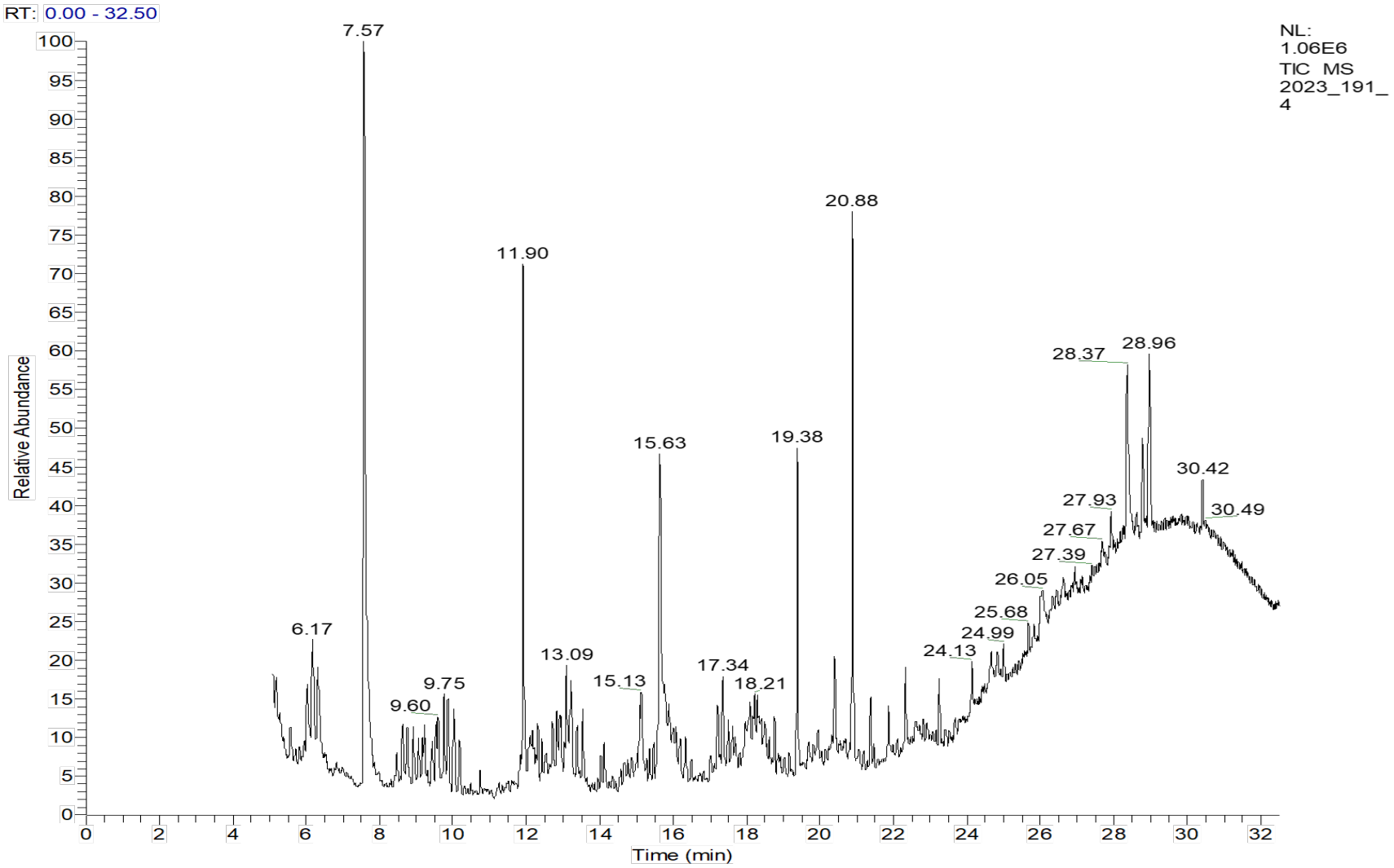

Figure 4.5: GC-TOF-MS chromatogram of crude organic of *Bauhinia galpinii*

Table 4.5: GC-TOF-MS analysis of *Bauhinia galpinii* crude organic extract (% area >1)

Retention time	Area %	Library identification	Identification Match quality %
6,03	1,98	Nonane, 4,5-dimethyl-	55
6,17	**3,10**	**2-Decene, 7-methyl- (Z)-**	**46**
6,32	**2,80**	**Hexane, 2,3,4-trimethyl-**	**50**
7.58	**19,23**	**Phenol, 4-(1,1-dimethylpropyl)-**	**92**
8.63	1,46	Tetracosane	80
8.76	1,57	Heptadecane	72
8,92	1,25	Octacosane	64
9,54	1,13	2-Quinolinecarboxaldehyde, 8-hydroxy	20
9,60	1,47	2-Octene, 4-ethyl	38
9,75	1,93	1-Undecene, 7-methyl	38
9,86	**2,15**	**Tetracosane**	**64**
10,02	1,48	Cyclopentane (2-methylbutyl)-	46
11,91	**7,76**	**6-Phenylisoquinoline**	**64**
13,09	**2,34**	**Cyclohexane, 1,2,4-trimethyl-**	**50**
13,21	1,87	Decanedioic acid, didecyl ester	49
13,54	1,06	Cyclohexane, 1-ethyl-2-propyl-	30
15,08	1,35	Octacosane	86
15,13	1,95	Hexadecanoic acid	98
15,63	**9,07**	**Hexadecanoic acid**	**98**
15,76	1,19	Palmitic Acid	50
17,21	1,20	Oxirane, 2-butyl-3-methyl-	46
17,35	1,47	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R- [R*, R*-(E)]]-	64
19,38	**4,53**	**n-Tricosane**	**90**
20,39	**2,23**	**n-Tetracosane**	**86**
20,88	**8,63**	**Sclerodione**	**83**
22,33	1,18	n-Hexacosane	94
26,05	1,48	3,11-Dimethyl-nonacosane	27
28,37	**3,43**	**Stigmast-5-en-3-ol (3. beta.,24S)-**	**93**
28,78	1,73	Lupenone	64
28,96	**2,99**	**Lupeol (Fagarasterol)(beta-Viscol)**	**90**

Bold-faceted compounds appear abundantly with % area greater than 2%.

Compounds such as phenol, 4-(1,1-dimethylpropyl) (19.23%) (Figure 4.6), hexadecanoic acid (9.07%) (Figure 4.7), and Stigmast-5-en-3-ol (3. beta.,24S) (3.43%) (Figure 4.8) are amongst compounds that had a higher % area. Hexadecanoic acid exhibited the highest % identification match (98%), followed by Stigmast-5-en-3-ol (3. beta.,24S)— (93%) and Phenol, 4-(1,1-dimethylpropyl)— (92%), while both lupeol (Figure 4.9) and n-Tricosane (Figure 4.10) exhibited a 90% identification match.

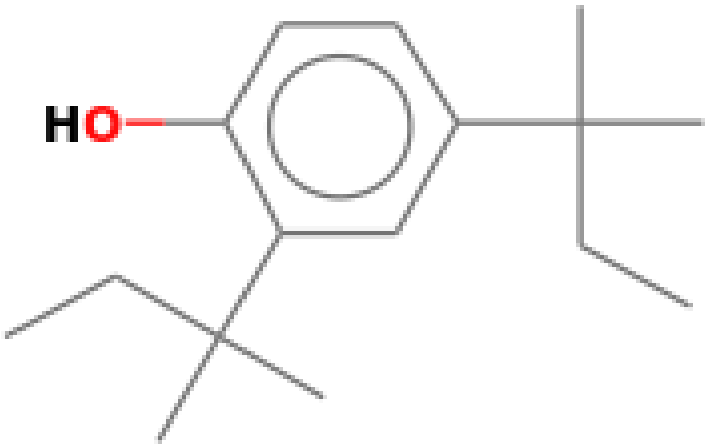

Figure 4.6: Phenol, 2-4-bis (1,1-dimethylpropyl)- (NIST WebBook)

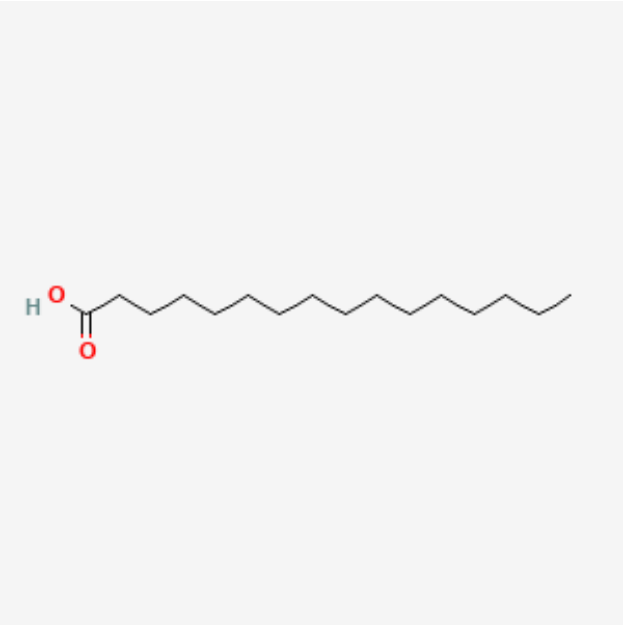

Figure 4.7: Hexadecanoic acid (Pubchem)

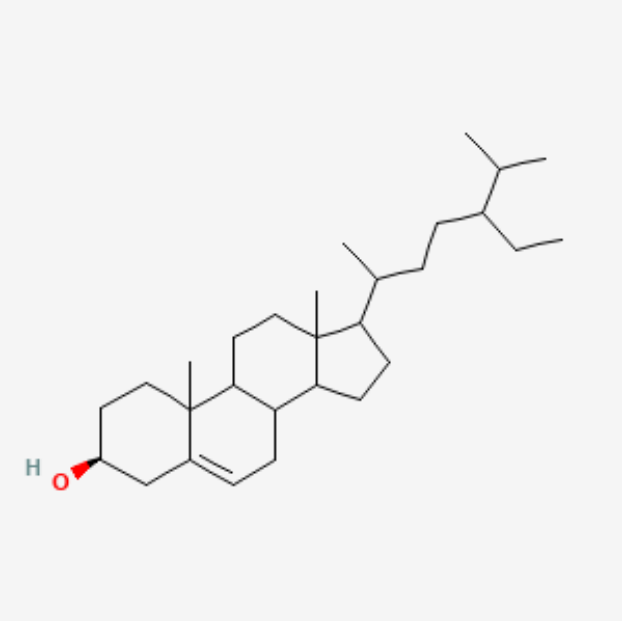

Figure 4.8: Stigmast-5-en-3-ol, 3. beta., 24S (Pubchem)

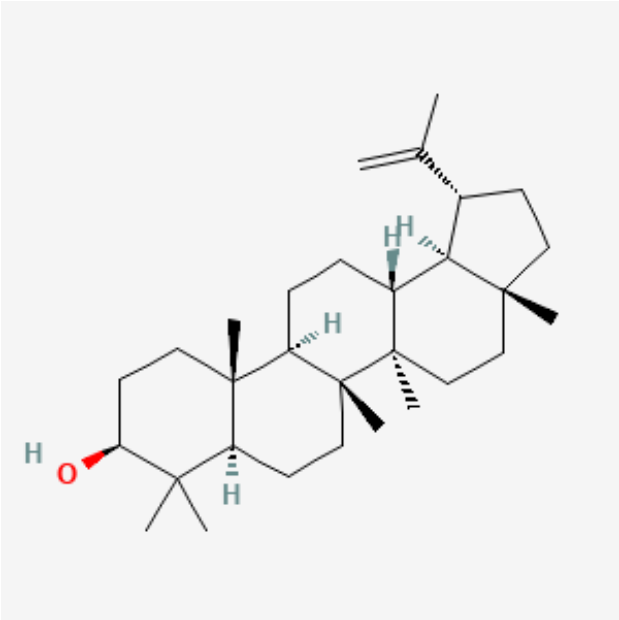

Figure 4.9: Lupeol (Pubchem)

Figure 4.10: n-Tricosane (Pubchem)

4.4.1.2. GC-ToF-MS analysis of *Combretum caffrum*

Fourteen compounds were found to be abundant in the *Combretum caffrum* crude organic extract. The chromatogram is presented in Figure 4.11, while the compounds with their retention time and relative abundance (% area) in the crude extract are presented in Table 4.6. Compounds such as Phenol, 4-1,1-dimethylpropyl (Figure 4.6) (which yielded 22%) and hexadecanoic acid (7.28%) (Figure 4.7) were the most abundant compounds identified in the organic crude extract from *C. caffrum*. Furthermore, compounds such as Croweacin (Figure 4.12), Hexadecanoic acid, 8-Octadecenoic (Figure 4.13) acid, Eicosanoic acid (Figure 4.14), Octadecanoic acid, Stigmast-5-en-3-ol (Figure 4.8), and Docosanoic acid (Figure 4.15) exhibited the highest identification match of 99%.

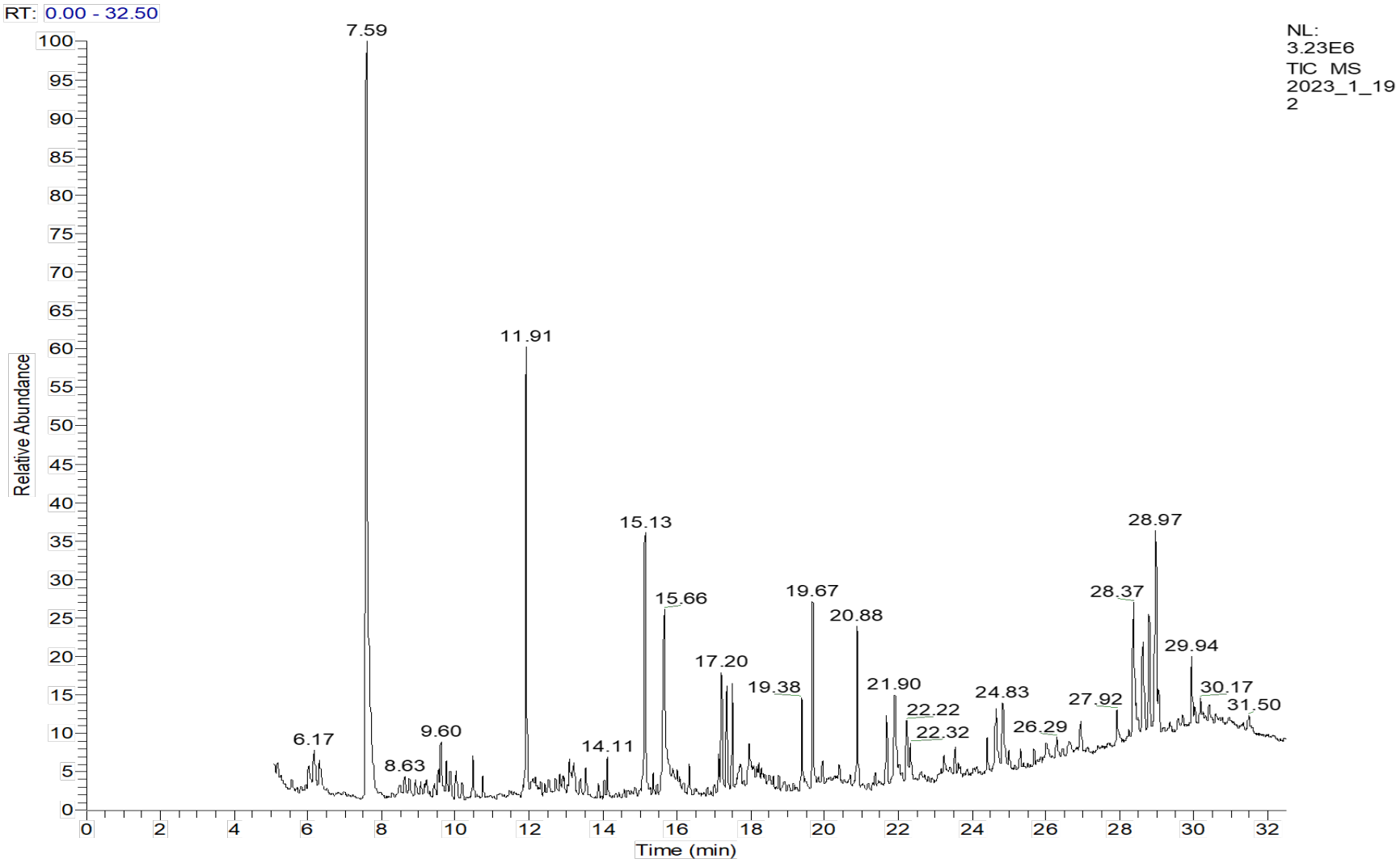

Figure 4.11: GC-TOF-MS chromatogram of crude organic extract of *Combretum caffrum*

Table 4.6: GC-TOF-MS analysis of *Combretum caffrum* crude organic extracts (% area >1)

Retention time	Area %	Library identification	Identification Match quality %
6,17	1,11	2-Decene, 7-methyl- (Z)-	52
7,59	**22,00**	**Phenol, 4-(1,1-dimethylpropyl)-**	**92**
9,60	1,36	Croweacin	99
11,91	**7,23**	**Phenol, 2,4-bis(1,1-dimethylpropyl)-**	**91**
15,14	**4,98**	**Hexadecanoic acid, methyl ester**	**99**
15,66	**7,28**	**Hexadecanoic acid**	**99**
17,21	**2,15**	**8-Octadecenoic acid, methyl ester**	**99**
17,35	1,75	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*, R*-(E)]]-	91
17,50	1,55	Octadecanoic acid, methyl ester	99
19,38	1,57	n-Tricosane	83
19,68	**3,22**	**Eicosanoic acid, methyl ester**	**99**
20,88	**2,55**	**Sclerodione**	**83**
21,68	1,15	Docosanoic acid, methyl ester	99
21,91	**2,58**	**2,2-Dimethyl-4,4,5,5-tetramethoxybiphenyl**	**90**
22,22	1,30	3,8,9-Trimethoxy-6H-dibenzo[b,d]pyran-6-one	80
24,64	**2,05**	**Dimethyl 4,6-dioxo-5,6-dihydro-4H-pyrido[3,2,1-jk] carbazole-5-spirocyclohexane-1,3-dicarboxylate**	**42**
24,83	**2,06**	**Dimethyl 4,6-dioxo-5,6-dihydro-4H-pyrido[3,2,1-jk] carbazole-5-spirocyclohexane-1,3-dicarboxylate**	**59**
27,92	1,03	Stigmasterol	91
28,37	**2,79**	**Stigmast-5-en-3-ol (3. beta.,24S)-**	**99**
28,63	**2,21**	**Viminalol**	**93**
28,80	**2,45**	**Lupenone**	**55**
28,97	**3,96**	**Lupeol (Fagarasterol)(beta-Viscol)**	**83**
29,95	1,31	D: A-Friedooleanan-3-one	70

Bold-faceted compounds appear abundantly with % area greater than 2%.

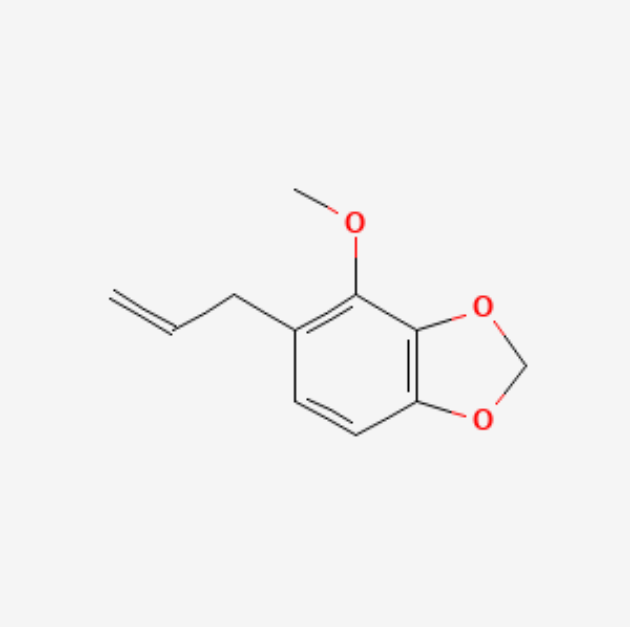

Figure 4.12: Croweacin (Pubchem)

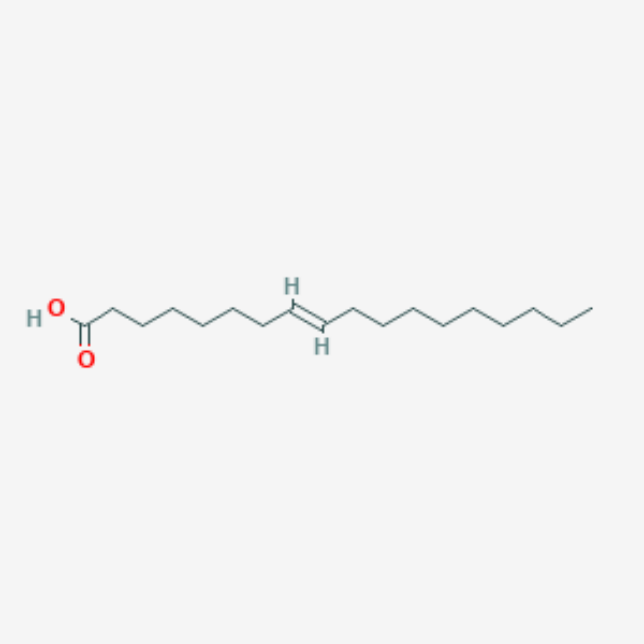

Figure 4.13: 8-Octadecenoic acid (Pubchem)

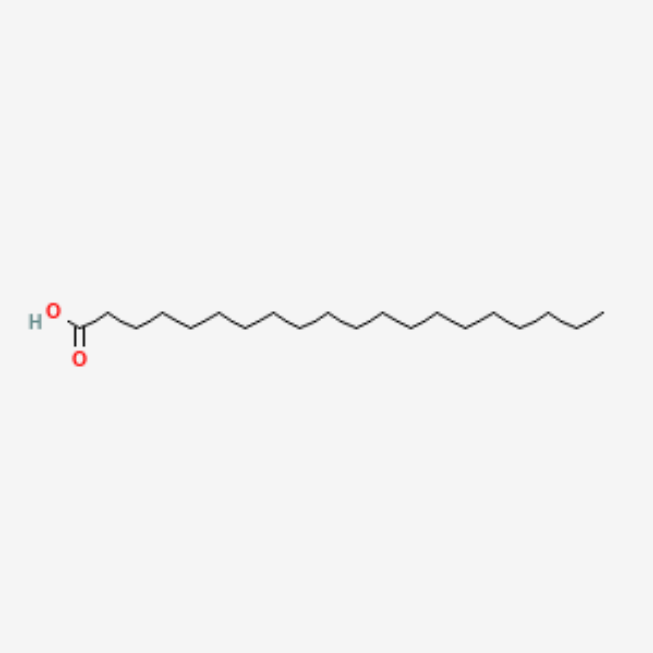

Figure 4.14: Eicosanoic acid (Pubchem)

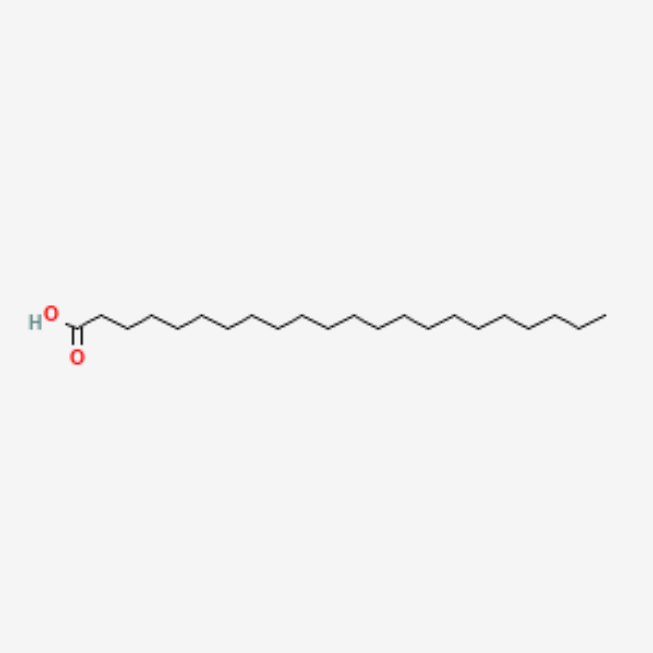

Figure 4.15: Docosanoic acid (Pubchem)

4.4.1.3. GC-ToF-MS analysis of *Markhamia obtusifolia*

From the analysis of the extract from *M. obtusifolia*, a total of 77 compounds were identified and 9 of these compounds were identified to have a % area greater than one and, therefore, are concluded

to be present in abundance. Table 4.7 presents the compounds with their retention time and relative abundance (% area) in the crude extract, while the corresponding chromatogram is presented in Figure 4.16.

The extract had phenol, 4-(1,1-dimethylpropyl)— (20.70%) (Figure 4.6) and hexadecanoic acid (12.05%) (Figure 4.7) as the most abundant compounds; 9-Octadecenoic acid (Z)— (Figure 4.17) was also identified with a % area of 6.97, which indicated its abundance.

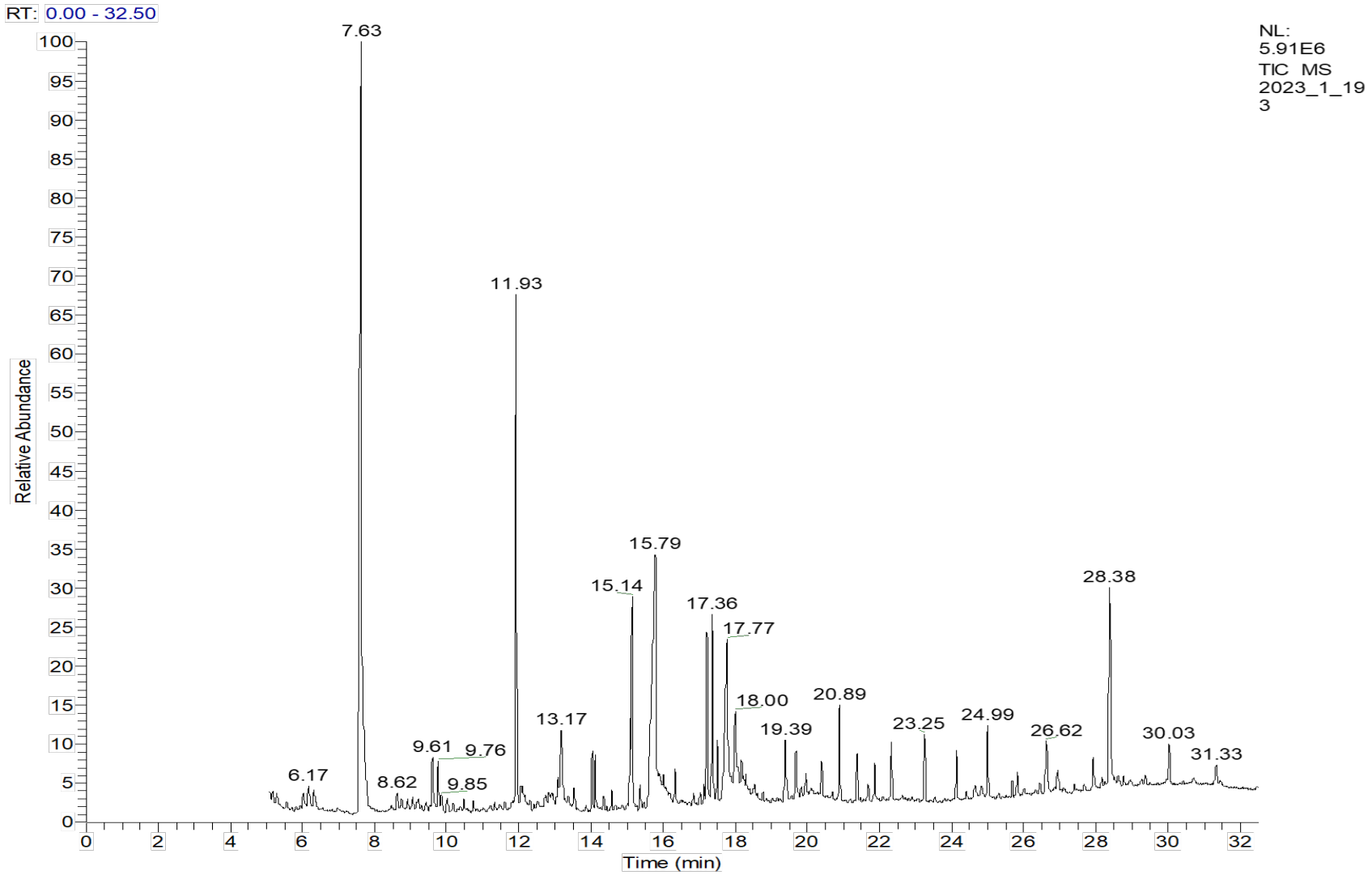

Figure 4.16: GC-TOF-MS chromatogram of crude organic extract *Markhamia obtusifolia*

Table 4.7: GC-TOF-MS analysis of *Markhamia obtusifolia* crude organic extracts (% area >1)

Retention time (mins)	Area %	Library identification	Identification Match quality %
7,63	**20,70**	**Phenol, 4-(1,1-dimethylpropyl)-**	**97**
9,61	1,11	Croweacin	98
11,93	**7,41**	**Phenol, 2,4-bis(1,1-dimethylpropyl)-**	**64**
13,18	1,92	(-)-Loliolide	91
15,08	1,30	9-Hexadecanoic acid, methyl ester (Z)-	90
15,14	**3,02**	**Hexadecanoic acid, methyl ester**	**99**
15,79	**12,05**	**Hexadecanoic acid**	**99**
17,22	**2,51**	**8-Octadecenoic acid, methyl ester**	**99**
17,36	**2,80**	**Phytol**	**78**
17,77	**6,97**	**9-Octadecenoic acid (Z)-**	**97**
18,00	**3,24**	**Octadecanoic acid**	**98**
19,39	1,36	n-Tricosane	83
20,89	1,29	(+-)-scleroderodione	83
23,25	1,07	n-Heptacosane	91
25,00	1,24	n-Nonacosane	93
28,38	**4,06**	**gamma-Sitosterol (Clionasterol)**	**99**

Bold-faceted compounds appear abundantly with % area greater than 2%.

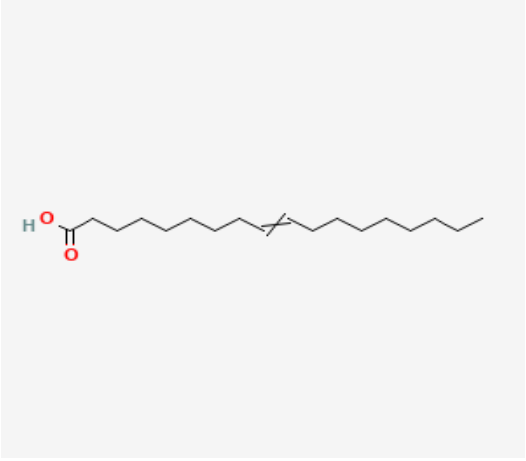

Figure 4.17: 9-Octadecenoic acid (Z)- (Pubchem)

4.4.1.4. GC-ToF-MS analysis of *Maytenus undata*

Maytenus undata revealed a total of eight compounds that were present in abundance. Figure 4.18 presents the chromatogram representing the separated compounds, while the compounds with their retention time and relative abundance (% area) in the crude extract are presented in Table 4.8. The compound with the highest peak area percentage was phenol, 4-(1,1-dimethylpropyl)- (Figure 4.6), accounting for 16.82%. Other compounds that dominated the chromatogram of the organic crude extract were gamma-Sitosterol (Clionasterol) (4.29%) (Figure 4.19) and hexadecanoic acid (3.82%) (Figure 4.7).

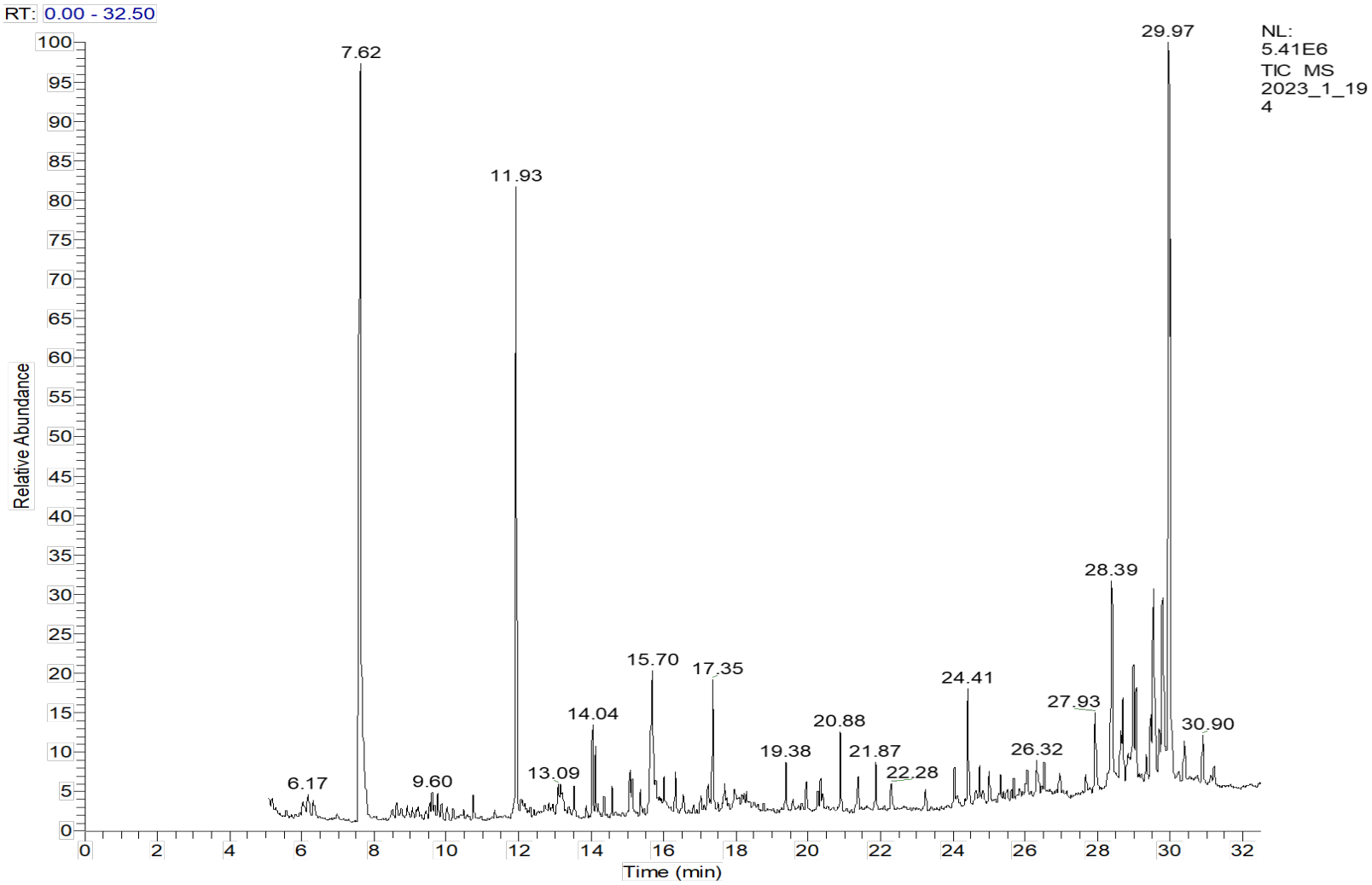

Figure 4.18: GC-TOF-MS chromatogram of crude organic extract of *Maytenus undata*

Table 4.8: GC-TOF-MS analysis of *Maytenus undata* crude organic extract (% area >1)

Retention time (mins)	Area %	Library identification	Identification Match quality %
7,62	**16,82**	**Phenol, 4-(1,1-dimethylpropyl)-**	**97**
11,93	**7,89**	**p- Hydroxymephenytoin**	**64**
14,04	1,04	Neophytadiene	91
15,70	**3,82**	**Hexadecanoic acid**	**99**
17,36	1,74	Phytol	87
24,41	1,59	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-	98
27,93	1,45	Stigmasterol	91
28,39	**4,29**	**gamma-Sitosterol (Clionasterol)**	**99**
28,69	1,43	beta. -Ionone	46
28,85	1,01	Lupenone	30
28,98	1,92	Lup-20(29)-en-3-ol (3. beta.)-	90
29,06	1,54	Simiarenol	86
29,47	1,07	Spirohexane-5-carboxylic acid, 1,1,2,2-tetramethyl-, methyl ester	25
29,55	**3,34**	**Fern-7-en-3beta-ol**	**93**
29,71	1,09	Lup-20(29)-en-3α-ol, acetate	46
29,80	**4,02**	**Longifolenaldehyde**	**53**
29,97	**10,30**	**Friedelan-3-one (Friedelin)**	**45**
30,01	**6,84**	**Phytol acetate**	**91**

Bold-faceted compounds appear abundantly with % area greater than 2%.

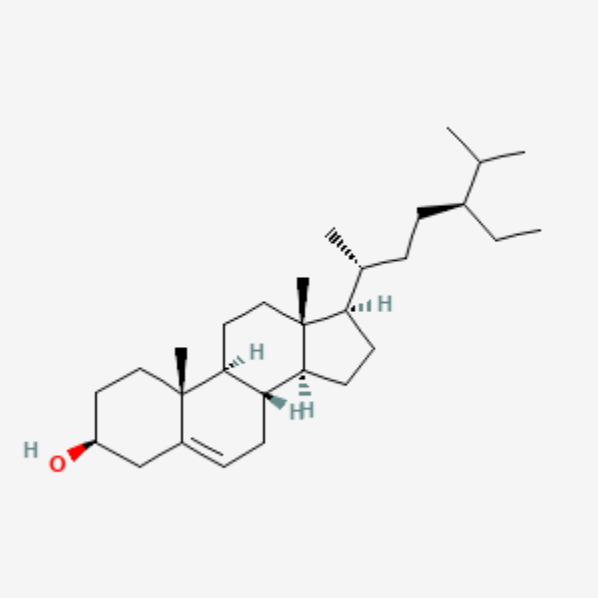

Figure 4.19: gamma-Sitosterol (Clionasterol) (Pubchem)

4.5.2. Phytochemical analysis of crude extract by Liquid-chromatography time-of-flight mass spectrometry (LC-TOF-MS)

4.4.2.1. LC-TOF-MS analysis of *Bauhinia galpinii*

The LC-TOF-MS chromatogram of the crude organic extract of *Bauhinia galpinii* with the mass spectrum of the detected compounds is shown in Figure 4.20. It was observed that the different peaks were obtained at different retention times. In this, the highest peaks at the retention time of 17.80 and 17.85 belonged to Kaempferitrin (Figure 3421), followed by 23.16, 23.19 belonging to UNPD93136, and 24.57 belonging to traumatic acid (Figure 3422).

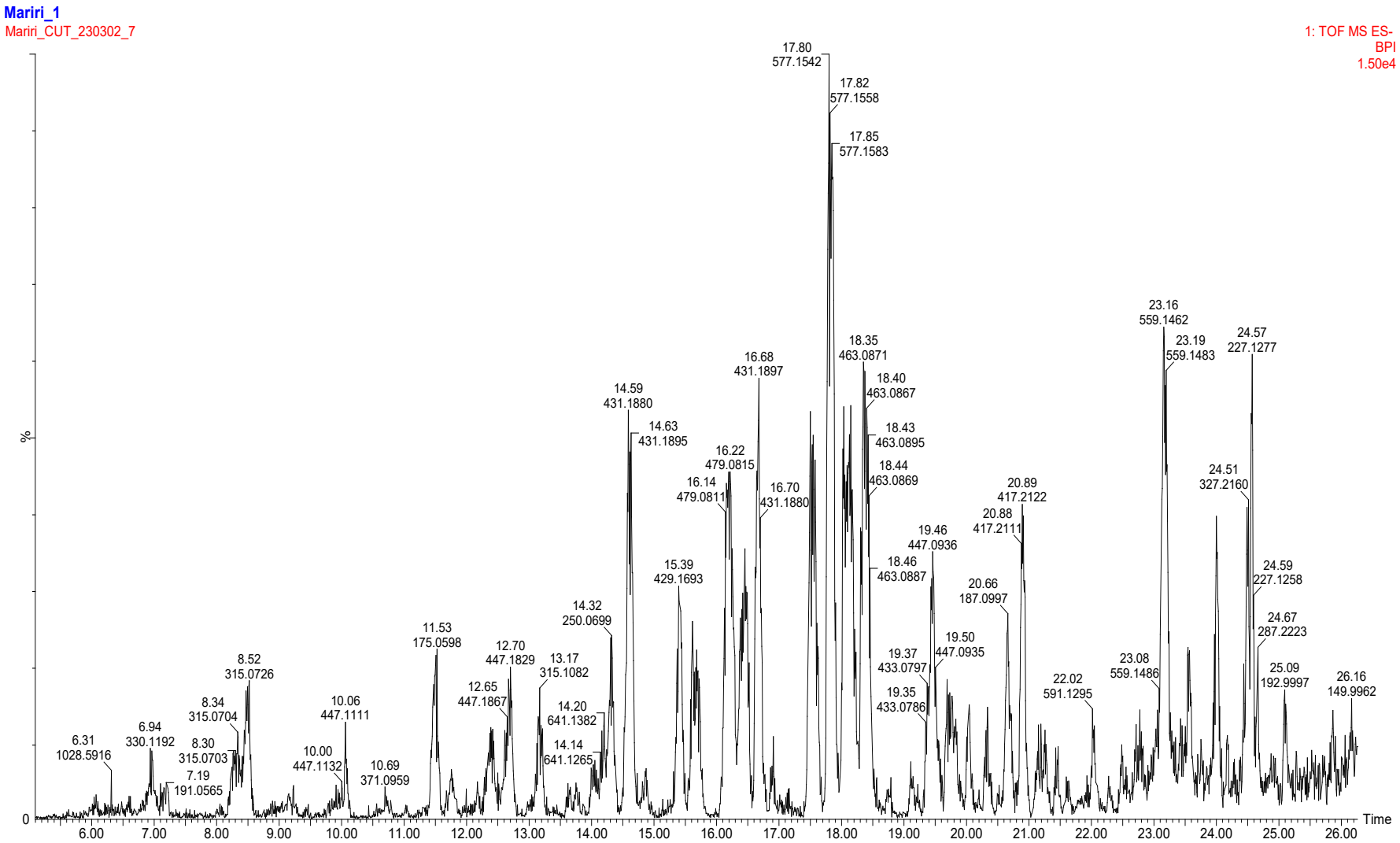

Figure 4.20: LC-TOF-MS chromatogram of methanolic extract of *B. galpinii*

Table 4.9: Important compound identified from methanolic extract of *B. galpinii* by LC-TOF-MS

Peak	Retention time	Name of compound	Molecular formula	m/z	Compound class
6	8.52	Gentesic acid 5-O-glucoside	C13H16O9	315.0726	Phenolic glycosides
8	10.06	UNPD88605	C25H20O8	447.1111	Flavonolignans
10	11.53	2-Isopropylmalic acid	C7H12O5	175.0598	Hydroxy fatty acids
12	12.372	3-[3-(naphthalen-2-yl) prop-2-enoyl]-2H-chromen-2-one	C22H14O3	325.0888	Coumarins and derivatives
13	12.70	4-[4-[1-(4-hydroxyphenyl)-2-phenylbut-2-enyl] phenoxy]butanoic acid	C26H26O4	447.1829	Stilbenes
14	13.17	Vanilloloside	C14H20O8	315.1082	Phenolic glycosides
16	14.20	Myricetin 3,3-digalactoside	C27H30O18	641.1382	Flavonoid-3-O-glycosides
25	17.50	Rhoifolin	C27H30O14	577.1635	Flavonoid-7-O-glycosides
26	17.80	Kaempferitrin	C27H30O14	577.1542	Flavonoid-7-O-glycosides
28	17.85	Kaempferitrin	C27H30O14	577.1583	Flavonoid-7-O-glycosides
29	18.35	Quercetin 3-galactoside	C21H20O12	463.0871	Flavonoid
30	18.40	UNPD93136	C20H32O18	463.0867	Oligosaccharides
34	19.12	cis-3-Hexenyl b-primeveroside	C17H30O10	393.175	Fatty acyl glycosides of mono- and disaccharides
38	19.50	Quercitrin	C21H20O11	447.0935	Flavonoid-3-O-glycosides
39	19.76	649+L463:P463	C22H22O12	477.1038	Isorhamnetin 7-glucoside
42	22.02	Apigenin 7-[rhamnosyl-(1->2)-galacturonide]	C27H28O15	591.1295	Flavonoid-7-O-glucuronides
44	23.16	UNPD93136	C20H32O18	559.1462	Oligosaccharides
45	23.19	UNPD93136	C20H32O18	559.1483	Oligosaccharides
47	24.57	Traumatic acid	C12H20O4	227.1277	Medium-chain fatty acids

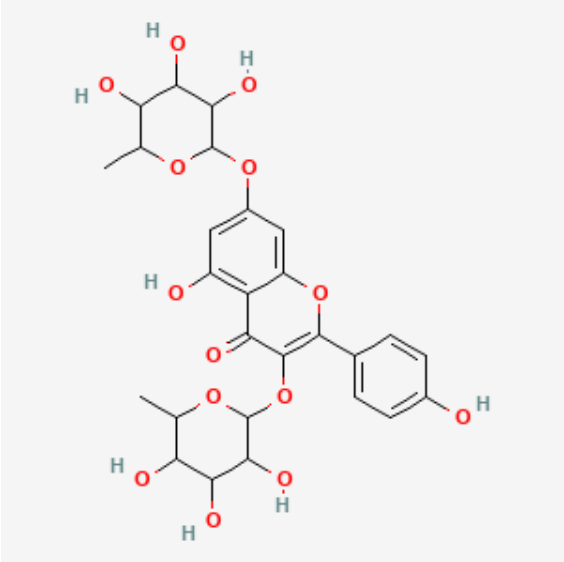

Figure 4.21: Kaempferitrin (Pubchem)

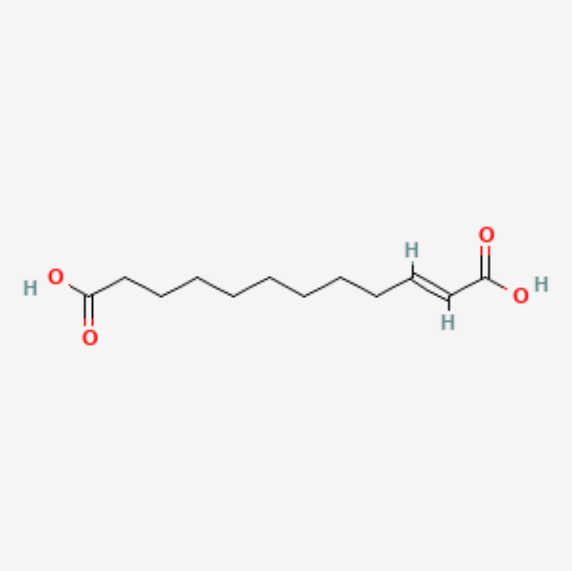

Figure 4.22: Traumatic acid (Pubchem)

4.4.2.2. LC-TOF-MS analysis of *Combretum caffrum*

The LC-TOF-MS analysis of methanolic extract from *C. caffrum* identified a total of 13 compounds. The study revealed the presence of several phytochemicals, including hydrolysable tannins, coumarin glycosides, flavonoid glycosides, bioflavonoids, polyflavonoids, and lignan glycosides. The details of these compounds are illustrated in Table 4.10. The chromatograms for the methanolic *C. caffrum* extract are shown in Figure 4.23. Quercitrin (Figure 4.24) is a flavonoid compound that has tentatively been identified at 16.09 minutes.

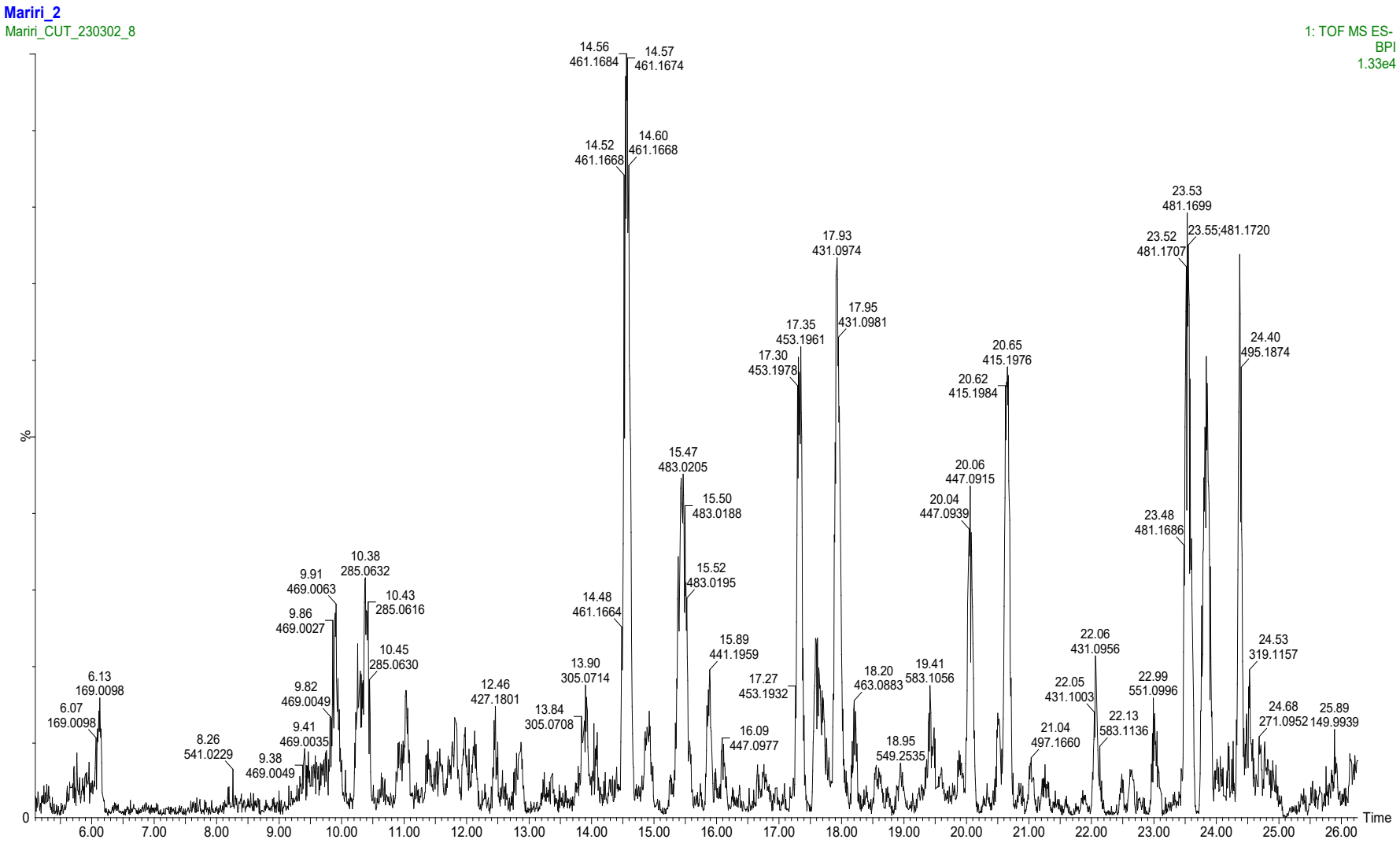

Figure 4.23: LC-TOF-MS chromatogram of methanolic extract of *Combretum caffrum*

Table 4.10: LC-TOF-MS analysis of methanolic extract of *Combretum caffrum*

Peak	Retention time	Name of compound	Molecular formula	m/z	Compound class
7	9.86	Valoneic acid dilactone	C21H10O13	469.0027	Hydrolysable tannin
12	12.46	Garcinone D	C24H28O7	427.1801	8-prenylated xanthones
13	12.86	Scopolin	C16H18O9	353.0883	Coumarin glycosides
16	14.52	Verbasoside	C20H30O12	461.1668	O-glycosyl compounds
19	15.47	methyl (S)-flavogallonate	C22H12O13	483.0205	Hydrolysable tannins
25	16.09	Quercitrin	C21H20O11	447.0977	Flavonoid-3-O-glycosides
27	17.30	Butyl (S)-3-hydroxybutyrate [arabinosyl-(1->6)-glucoside]	C19H34O12	453.1978	Fatty acyl glycosides of mono- and disaccharides
29	17.93	5,7-dihydroxy-2-(4-hydroxyphenyl)-3-[(3,4,5-trihydroxy-6-methyloxan-2-yl)oxy]-4H-chromen-4-one	C21H20O10	431.0974	Flavonoid-3-O-glycosides
33	19.41	Philonotisflavone-4-methyl ether	C31H20O12	583.1056	4-O-methylated flavonoids
42	23.01	Bilobetin	C31H20O10	551.1017	Biflavonoids and polyflavonoids
45	23.53	6-O-(4-Methoxybenzoyl)-ajugol;(-)-6-O-(4-Methoxybenzoyl)-ajugol	C23H30O11	481.1699	Iridoid O-glycosides
47	23.85	2alpha-hydroxymaprounic acid 2,3-bis-p-hydroxybenzoate	C44H56O8	711.3909	Scalarane sesterterpenoids
48	24.40	(7R*,8R*)-3-Methoxy-3,4,7,9,9-pentahydroxy-8,4-oxyneolignan 4-xyloside	C24H32O11	495.1874	Lignan glycosides

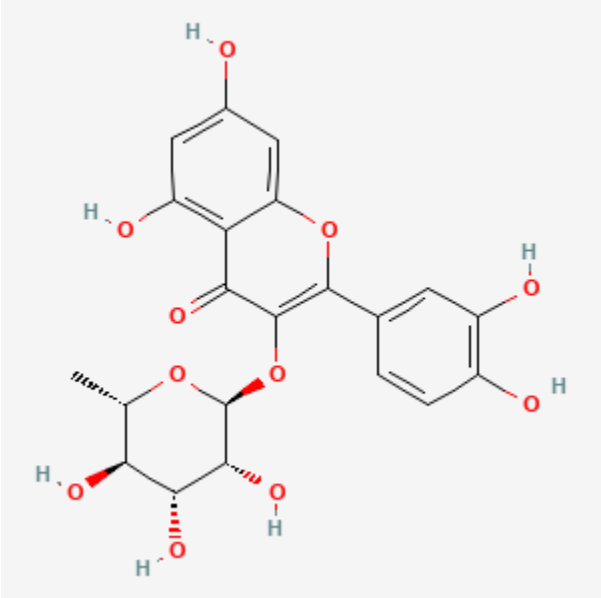

Figure 4.24: Quercitrin (Pubchem)

4.4.2.3. LC-TOF-MS analysis of *Markhamia obtusifolia*

In the present study, LC-TOF-MS analysis was used to tentatively identify compounds in the *M. obtusifolia* extract. Fourteen major compounds were identified based on their retention periods, mass and chemical formula as illustrated in Table 4.11. The corresponding LC-TOF-MS chromatogram is presented in Figure 4.25. Keioside (Figure 4.26) is a flavonoid-3-O-glycoside that has tentatively been identified at 18.32 minutes.

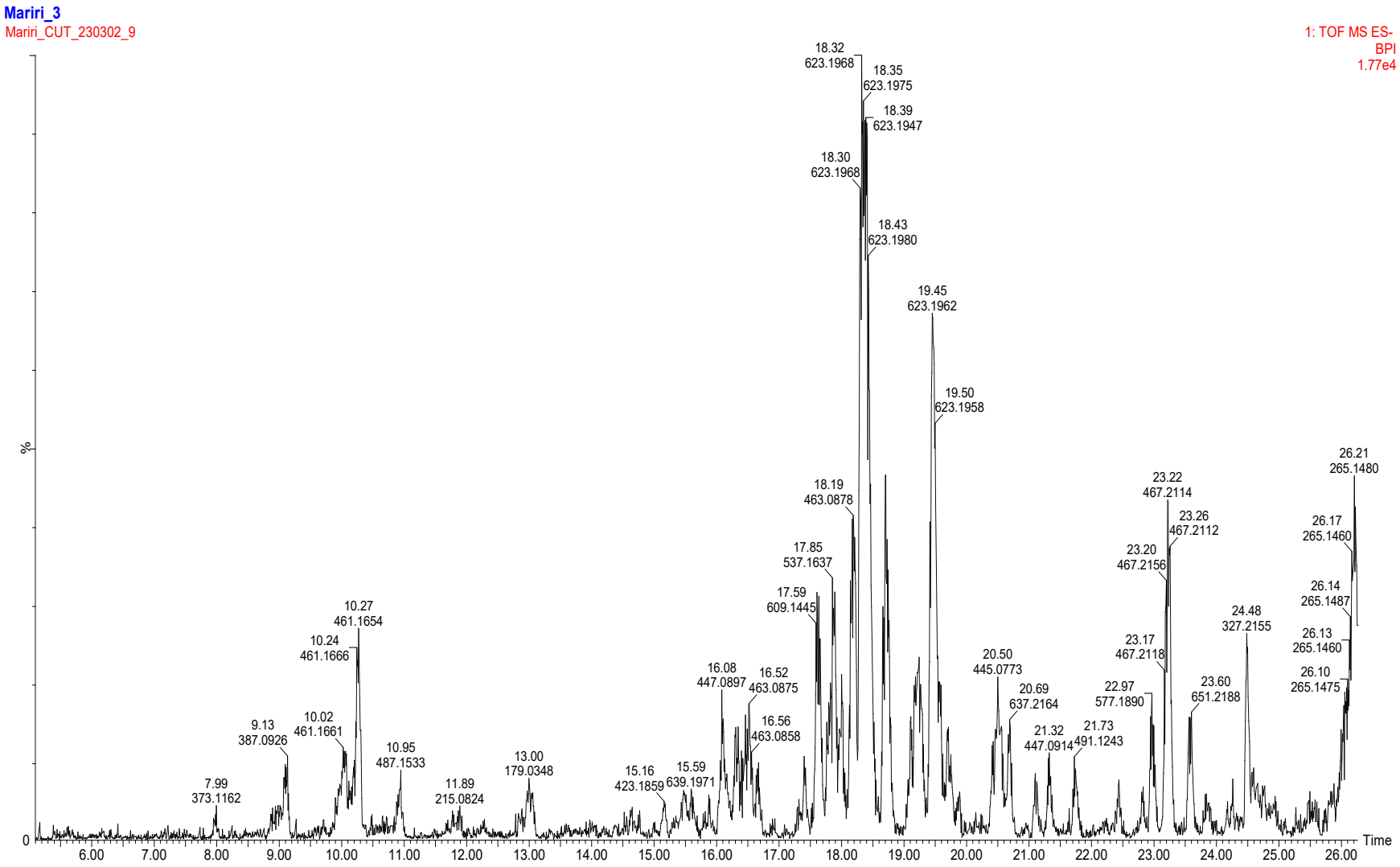

Figure 4.25: LC-TOF-MS chromatogram of methanolic of methanolic extract of *Markhamia obtusifolia*

Table 4.11: LC-TOF-MS analysis of methanolic extract of *Markhamia obtusifolia*

Peak	Retention time	Name of compound	Molecular formula	m/z	Compound class
4	10.24	Verbasoside	C20H30O12	461.1666	O-glycosyl compounds
6	10.95	UNPD67703	C21H28O13	483.1533	Phenolic glycosides
9	15.16	UNPD132978	C18H32O11	423.1859	Fatty acyl glycosides of mono- and disaccharides
15	17.85	Minecoside	C25H30O13	537.1637	Coumaric acids and derivatives
18	18.32	Keioside	C28H32O16	623.1968	Flavonoid-3-O-glycosides
22	19.11	(7R)-(+)-Lyoniresinol 9-glucoside	C28H38O13	581.2257	Lignan glycosides
25	19.70	Kaempferitrin	C27H30O14	577.1557	Flavonoid-7-O-glycosides
26	20.50	Baicalin	C21H18O11	445.0773	Flavonoid-7-O-glucuronides
27	20.69	Jionoside D	C30H38O15	637.2164	Coumaric acids and derivatives
28	21.12	Tectoridin; Shekanin; Shekkanin; Tectorigenin 7-O-glucoside	C22H22O11	609.1201	Isoflavonoid O-glycosides
31	22.44	(6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl]oxy}-3,4,5-trihydroxyoxan-2-yl)methyl (2E)-3-(4-hydroxyphenyl)prop-2-enoate	C30H26O14	609.1201	Flavonoid 3-O-p-coumaroyl glycosides
35	23.22	(6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl]oxy}-3,4,5-trihydroxyoxan-2-yl)methyl (2E)-3-(4-hydroxyphenyl)prop-2-enoate	C30H26O14	467.2114	Flavonoid 3-O-p-coumaroyl glycosides
37	23.60	Bosistoabiflavanone dimer	C38H36O10	651.2188	8-prenylated flavanones
38	24.48	Corchorifatty acid F	C18H32O5	327.2155	Linoleic acids and derivatives

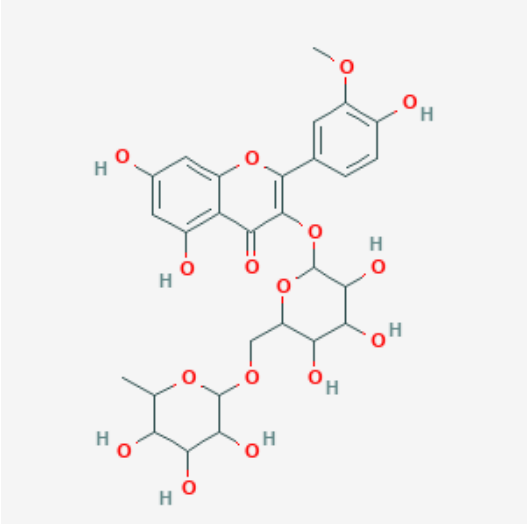

Figure 4.26: Keioside (Pubchem)

4.4.2.4. LC-TOF-MS analysis of *Maytenus undata*

The results of the LC-TOF-MS analysis of *M. undata* uncovered the presence of flavonoids, specifically rutin (Figure 4.28) at 17.57 minutes and camelliaside B (Figure 4.29) at 17.66 minutes, as can be seen in Table 4.12. Furthermore, the LC-TOF-MS analysis of *M. undata* also tentatively identified pisumionoside (Figure 4.30) at 12.90 minutes.

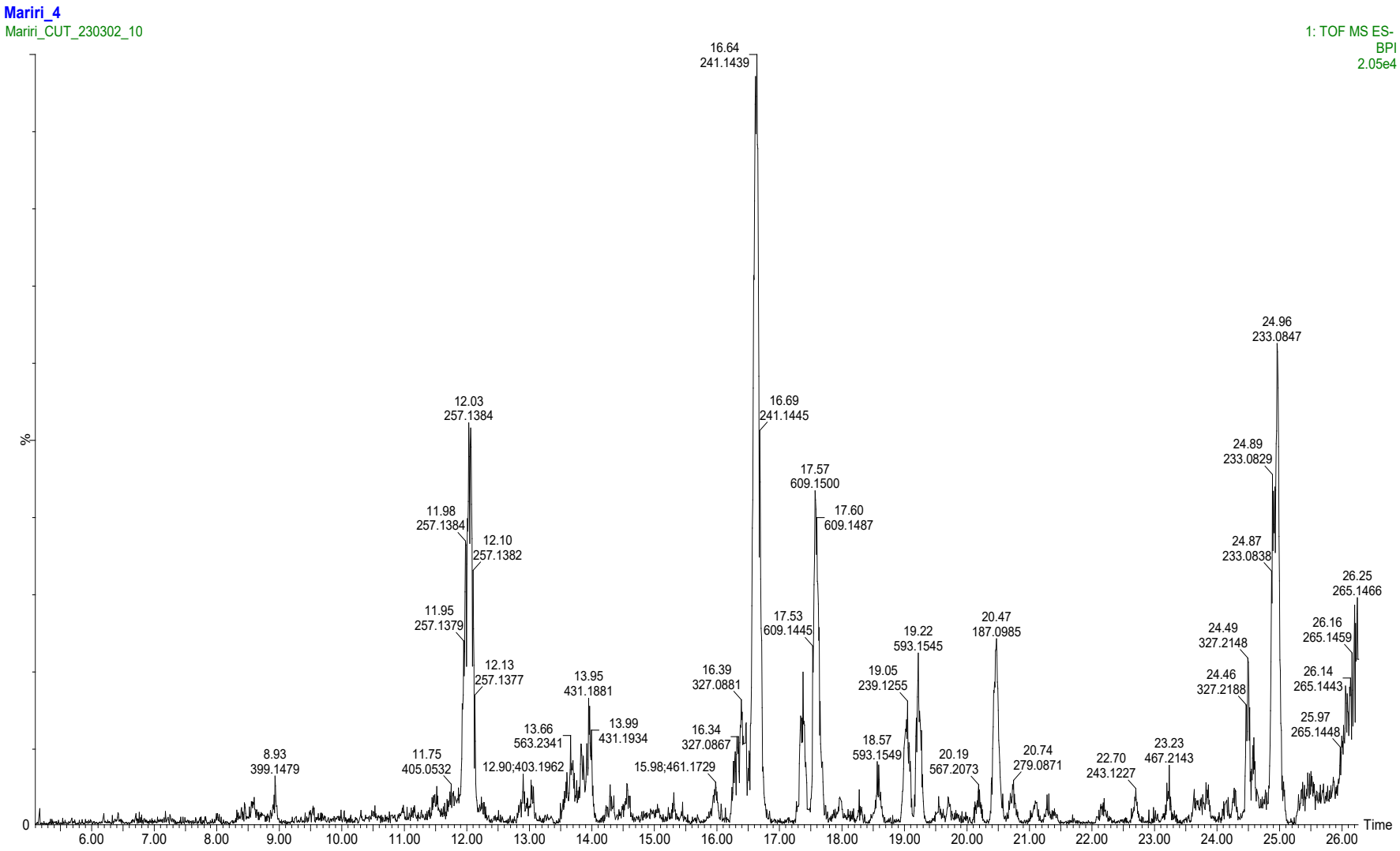

Figure 4.27: LC-TOF-MS chromatogram of methanolic extract of *Maytenus undata*

Table 4.12: LC-TOF-MS analysis of methanolic extracts of *Maytenus undata*

Peak	Retention time	Name of compound	Molecular formula	m/z (mass)	Compound class/ (ontology)
1	8.93	(-)-Deoxypodorhizone; Dihydroanhydropodorhizol; (-)-Yatein;Yatein	C22H24O7	399.1479	Dibenzylbutyrolactone lignans
5	12.03	UNPD54493	C13H22O5	257.1384	Fatty alcohols
8	12.90	Pisumionoside	C19H32O9	403.1962	Terpene glycosides
9	13.66	UNPD30531	C25H40O14	563.2341	Fatty acyl glycosides of mono- and disaccharides
10	13.95	aspulvinone H	C27H28O5	431.1881	1-hydroxy-2-unsubstituted benzenoids
12	15.98	Phenylethyl primeveroside	C19H28O10	461.1729	O-glycosyl compounds
14	16.39	Frenolicin B	C18H16O6	327.0881	Benzoisochromanequinones
15	16.64	A factor	C13H22O4	241.4139	Gamma butyrolactones
18	17.57	Rutin	C27H30O16	609.1500	Flavonoid-3-O-glycosides
20	17.66	Camelliaside B	C32H38O19	725.1992	Flavonoid-3-O-glycosides
21	17.97	MINEs-338436	C20H34O11	449.2039	Oligosaccharides
24	19.22	Astragalin 7-rhamnoside	C27H30O15	593.1545	Flavonoid-7-O-glycosides
27	20.74	Xestodecalactone B;(+)-Xestodecalactone B	C14H16O6	279.0871	Oxocins
29	23.23	1-Octen-3-yl primeveroside	C19H34O10	467.2143	Fatty acyl glycosides of mono- and disaccharides
30	23.82	Microlenin	C29H34O7	493.226	Sesquiterpene lactones
31	24.49	Corchorifatty acid F	C18H32O5	327.2148	Linoleic acids and derivatives
32	24.96	Traumatic acid	C12H20O4	233.0847	Medium-chain fatty acids

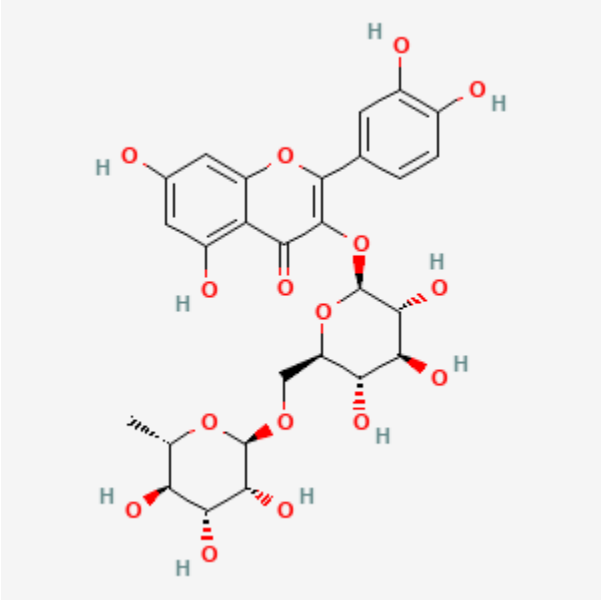

Figure 4.28: Rutin (Pubchem)

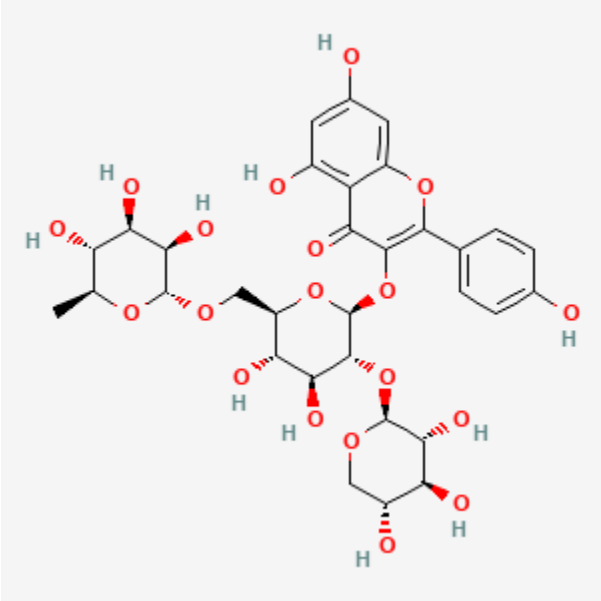

Figure 4.29: Camelliaside B (Pubchem)

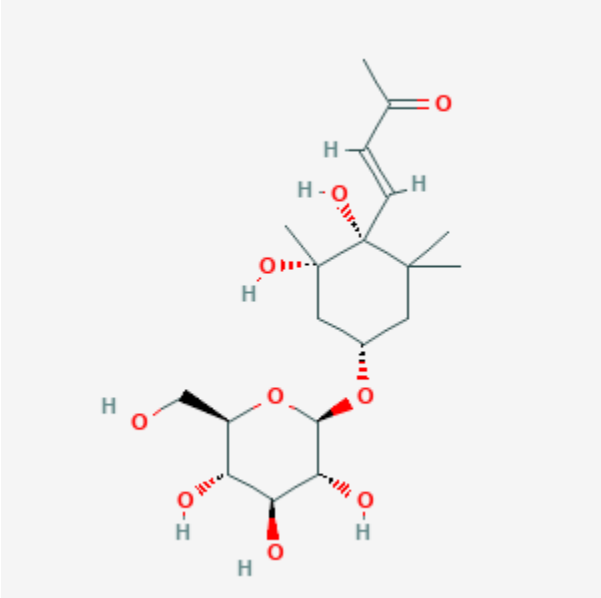

Figure 4.30: Pisumioside (Pubchem)

4.6. Data analysis

In the antifungal assays, the results are reported as a mean of the three independent experiments (n=3), while in the antioxidant activity, cytotoxicity, and genotoxicity studies the results are reported as mean±SE. P-values ≤0.05 were referred to as significant. In the genotoxicity assay quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) and acquired images were analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. In the phytochemical analysis, the results were populated and analysed by ChromaTOF, which possesses a NIST 95 library for compound matches.

5 Discussions

5.1. Antifungal and total activity

Numerous mycotoxigenic fungal strains negatively impact the production of food crops consumed by humans as food. Such strains may well reduce both the crop yields (quantity) and general composition (quality) of foods and contribute to overall hunger, compromised food security, and devastating illnesses when consumed (Balendres et al., 2019; Phokane et al., 2019). The situation is further compounded by the projection that the total global food demand is expected to increase by 35% to 56% between 2010 and 2050, hence observed in many African countries (Mann and Loginova, 2023; Singh et al., 2023). The use of plants as inexpensive and readily available possible pesticides/fungicides to counter various fungal infections on crops may well be a possible solution to counter food infections, thereby increasing both yields and quality and thereby alleviating hunger in both developing and underdeveloped countries (Muñoz et al., 2013; Bondareva and Fedorova, 2021; Ngegba et al., 2022a; Ngegba et al., 2022b).

The antifungal activity of some selected medicinal plants is reported in Table 4.1. *Bauhinia galpinii* methanol extract exhibited a notable minimum inhibitory concentration (MIC) compared to other medicinal plants. This extract had the lowest recorded MIC value of 0.16 mg/ml against a plethora of pathogens that includes *F. graminearum*, *F. oxysporum*, *P. haloterans*, and *C. cladospoides* at a 24-hour incubation period. As there are no validated endpoint criteria for *in vitro* testing of plant extracts (Mongalo et al., 2015; Mongalo et al., 2016), Souza et al., (2007) propose that extracts with a MIC < 0.5 mg/ml be considered strong inhibitors, while those with a MIC between 0.5 and 1.6 mg/ml were considered moderate inhibitors, and extracts with MIC > 1.6 mg/ml were considered weak inhibitors for at least two different temperature regimes (Soyingbe et al., 2018). Using the standard above, the methanol extract from *Bauhinia galpinii* remains the strongest inhibitor of *F. graminareaum* and *C. cladospoides*, yielding a MIC value of 0.16 mg/ml at 24 and 48 hours.

Recently, *F. graminearum* was reported to cause *Fusarium* head blight (FHB) on wheat, barley and other grains by secreting hundreds of unknown putative effectors, which may well favour the progression of the disease through interference with crop immunity, thereby promoting the formation of higher quantities of mycotoxins (Chen et al., 2019; Hao et al., 2020). Although the extracts had a

notable inhibition of the fungal strain, there is a need to quantify the mycelium growth inhibition and inhibition of mycotoxin production further in an *in vivo* experimental setup. Furthermore, the mode of action needs to be explored. According to other studies, the mode of action is believed to be through mycelial growth inhibition (Dikhoba et al., 2019). The acetone extract from *B. galpinii* leaves was reported to have good antifungal activity against a plethora of both human- and crop-infecting fungal strains (Ahmed et al., 2012; Ahmed et al., 2019; Manyarara et al., 2016). However, these results are not comparable with the findings of the current study due to differences in terms of the extractant used, the nature and origin of the strains, the collection site of the plant specimen, and several other environmental conditions.

The methanol extracts of *Combretum caffrum*, *Markhamia obtusifolia*, and *Maytenus undata* also had strong inhibitory effects against at least one fungal strain at two different incubation periods. *C. caffrum* had a MIC value of 0.31 mg/ml against two *Fusarium* strains: *F. vercitilloides* and *F. graminearum*. According to reports, the extracts with a similar MIC at two different incubation periods might mean that the extracts are fungistatic and, if used in a ploughing field to encounter mycotoxin infection, should be administered frequently until the fungal strains die off (Rocha-Miranda et al., 2019; Molele et al., 2023). *Fusarium verticilloides* mainly infect maize and are notoriously known to produce harmful secondary metabolites known as fumosinin mycotoxin, which causes severe human and animal diseases (Blacutt et al., 2018; Murillo-Williams et al., 2008). The acetone extract from *C. caffrum* was reported to exhibit a potent antifungal activity, both *in vitro* and *in vivo*, against a plethora of both human- and crop-infecting pathogens, and hence possesses other biological activities (Mapfunde et al., 2016; Masoko et al., 2010; Masoko et al., 2006). Additionally, the compounds from *C. caffrum* leaves such as combrestatin A-4 and Combrestatin A-4P and some derivatives thereof were found to be highly active against a plethora of pathogens such as *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Pyricularia oryzae*, and *Rhizoctonia solani* (Yan et al., 2016).

The methanol extracts from the selected medicinal plants exhibited some varying degrees in their total activity, which serves as a guide to the concentration at which the plant extract could be diluted and still inhibit the fungal growth (Eloff, 1998) (Table 4.2). *Combretum caffrum* exhibited the highest total activity (TA) of 1437.5 ml/g. This means the extract (1 g) could be diluted with such a high volume of water and still inhibit the growth of the fungal pathogen, *Aspergillus nomius* (Fadipe et al., 2015; Ahmed et al., 2014). It is crucial to note that the TA is dependent on several factors, which include the solubility of the plant extract in a specific solvent and the activity of such an extract against

the selected microorganisms (Dikhoba et al., 2019). The results in the current study may well explain that these plant extracts can mitigate the risk of mycotoxin contamination by inhibiting fungal growth. Although the tested extracts had good to moderate antifungal activities, there is still a need to explore the minimum fungicidal/fungistatic concentrations to determine and further explore whether the extracts are fungicidal or fungistatic. Furthermore, there is a need to explore their potential in inhibiting mycotoxin production and secretion.

5.2. Antioxidant activity

Reactive oxygen species (ROS) arise in edible crops consumed as food daily as a by-product of several metabolic processes that are in different cell compartments, or because of the inevitable escape of electrons to oxygen from the electron transport activities of chloroplasts, mitochondria, and plasma membranes (Chaves et al., 2020; Mansoor et al., 2022). It is important to note that these reactive species are formed in chloroplasts, mitochondria, plasma membranes, peroxisomes, apoplasts, the endoplasmic reticulum, and cell walls. The free radical scavenging activities of the methanol extracts from the selected plants are presented in Table 4.3, against 2,2-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethylbenzthiazoline-6-suphonic acid) (ABTS) free radicals. The DPPH assay is common and widely used in the antioxidant capacity screening of plant extracts, isolated compounds, and their respective derivatives, fruits and vegetable juices or extracts thereof, for it is easy and rapid, and requires only a UV-vis spectrophotometer to test (Xu et al., 2017). Compared with the ABTS assay, the DPPH radical is commercially available and does not have to be generated before the assay. *Combretum caffrum* exhibited a noteworthy and the lowest 50% inhibition (IC_{50}) of DPPH yielding an IC_{50} value of 10 µg/ml, while *Markhamia obtusifolia* exhibited an IC_{50} value of 70 µg/ml against the ABTS free radical. According to More and Makola (2020), medicinal plant extracts that are regarded as potent in an antioxidant assay should yield the lowest IC_{50} value of ≤20 µg/ml. Other authors corroborate the similar benchmark (Mathew & Subramanian, 2014; Pandey et al., 2020), while others have reported an IC_{50} as high as over 200 µg/ml as in the case with *B. galpinii* in this study (Sindhu et al., 2014; Ayoub et al., 2019). The fractions from the 70% acetone extract of *B. galpinii* leaves were reported to have DPPH inhibition, with IC_{50} as low as 0.89 and 4.30 µg/ml (Ahmed et al., 2012). Besides speculating that the antioxidant compounds might be much more soluble in 70% acetone, the results could not be compared to our current study due to differences in terms of the extracting solvent and several other parameters. The

noteworthy antioxidant activity of *B. galpinii* against the ABTS free radical, in this study, can be attributed to the presence of Stigmast-5-en-3-ol (3. beta.,24S) (Figure 4.8) tentatively identified through the GC-ToF-MS analysis of the extract. Ahmed et al., (2021) report that Stigmast-5-en-3-ol (3. beta.,24S) further possesses cancer-preventive properties. Nonetheless, in this study, *B. galpinii* increased the viability of the human colorectal adenocarcinoma cells. Judging by the ABTS/DPPH correlations (ADC), *M. undata* exhibited a notable ADC correlation of 2.33, which is over three-fold that of ADC of ascorbic acid, which was used as a control drug. From these findings, except in the case of *B. galpinii*, the extract exhibited higher inhibition compared to ABTS, contrary to other authors (Chaves et al., 2020).

Phenolic compounds found within medicinal plants are sought after more often as inhibitors of oxidative stress, hence accounting for various biological activities of such plants. In the phenolic content analysis (Table 4.4), three of the four tested plants had a higher phenolic content (TPC), compared to their total flavonoid compounds. A similar trend has been reported previously (Saeed et al., 2012; Aryal et al., 2019; Shi et al., 2022). These higher TPC contents may explain the antioxidative ability of the medicinal plants selected for the current study.

5.3. Toxicity studies

5.3.1. Cytotoxicity against Vero and Caco-2 cells

While plants, particularly those utilized in traditional medicine, are generally considered safe, it is essential to acknowledge that many of them may possess inherent toxic properties. Therefore, thoroughly examining the toxicity levels of these plants before advocating their use is of the utmost importance (Makhuvele et al., 2020). Moreover, conducting toxicity studies serves the purpose of verifying that any observed biological activity of the plant is not a result of general metabolic toxic effects (Dzoyem et al., 2016). *Bauhinia galpinii* extract had a toxic effect on the African green monkey cells, which could be attributed to the presence of the flavonoid compound, kaempferitrin (Figure 4.21) detected with the LC-ToF-MS analysis of the crude extract of the plant (Table 4.9). Govindarasu et al., (2022) report this flavonoid compound to have the ability to inhibit the viability of colon cancer cells, induce apoptosis, and reduce the generation of reactive oxygen species in a concentration-dependent manner. However, in this study, the extract of *Bauhinia galpinii* stimulated the proliferation of the human colorectal adenocarcinoma cells. This is rather surprising, considering the high

phenolic content (0.41 mg/g gallic acid equivalents) we previously associated with the good antioxidant activity (IC_{50} values of 50 µg/ml) of *B. galpinii* extract against free radicals of ABTS. As a result, the methanol extract from *B. galpinii* is not a good lead for the development of new anticancer agents to be used in the treatment of human colorectal adenocarcinoma.

The crude extract from *Maytenus undata* had cytotoxic effects on both the Vero cells and the Caco-2. The LC-ToF-MS analysis of *M. undata* tentatively identified pisumionoside (Figure 4.30), which has been reported by Liu et al., (2022) to demonstrate antagonistic effects on cancer cells. Therefore, we can attribute the antagonistic effect of *M. undata* on both the Vero cells and the Caco-2 cells to the presence of pisumionoside. Additionally, we previously found *M. undata* to have a high content of flavonoids equating to 0.39 mg/g quercetin equivalent. Sylvie et al., (2014) report the ability of natural antioxidants to protect the human body from free radicals and slow down the progress of many chronic diseases like cancer. Therefore, the detection of flavonoids, particularly rutin (Figure 4.28) and camelliaside B (Figure 4.29) in the *M. undata* extract could account for its anticancer properties (Li et al., 2021; Moravkar et al., 2023).

Combretum caffrum extract was less toxic to the Vero cells but demonstrated cytotoxicity against Caco-2 cells in a dose-dependent manner. The cytotoxic effects of the *C. caffrum* extract can be attributed to the presence of valoneic acid dilactone, which was tentatively identified through LC-ToF-MS analysis (Table 4.10). Ahmed et al., (2022) claim that valoneic acid dilactone may exert strong antioxidant properties and deactivate radicals through the single electron transfer mechanisms. This antioxidant activity can help prevent cellular and DNA damage, which is associated with various chronic diseases, including cancer. Additionally, the GC-ToF-MS analysis of *C. caffrum* revealed the abundant presence of phenol, 4-(1,1-dimethylpropyl)- (Figure 4.6). Polyphenolic compounds have been proven to have chemopreventive and suppressive activities against cancer cells by inhibiting many metabolic enzymes involved in the activation of potential carcinogens or arresting the cell cycle (Sylvie et al., 2014). Furthermore, the observed cytotoxicity of *C. caffrum* can be attributed to the presence of hexadecanoic acid (palmitic acid) (Figure 4.7) (dos Santos et al., 2012), which has been identified through the LC-ToF-MS analysis of the plant (Figure 4.23). Dos Santos et al., (2012) further claim that saturated fatty acids induce DNA damage and cause apoptotic cell death in insulin-producing beta-cells. In addition, *C. caffrum* extract has been found to possess potent antioxidant activity against DPPH, with IC_{50} values of 10 µg/ml. Literature reports have attributed the ability of medicinal plants to prevent or control diseases to the antioxidant

properties of their constituents, commonly associated with molecules such as phenols and flavonoids (Sylvie et al., 2014). In this study, we found the organic extract from *C. caffrum* to have high phenolic contents, yielding 0,46 mg/g gallic acid equivalents.

5.3.2. Genotoxicity against Vero cells

The majority of documented toxic effects arising from the consumption of herbal medicines and dietary supplements are primarily linked to liver toxicity. However, various other adverse effects have been reported, such as those affecting the kidneys, nervous system, blood, cardiovascular system, and skin, as well as instances of mutagenicity and carcinogenicity (Makhafola et al., 2014). Taking these concerns into consideration, we investigated the potential genotoxic effects of the extracts of the selected medicinal plants. The presence of quercitrin (Figure 4.24) identified through the LC-TOF-MS analysis of *C. caffrum* crude extract (Table 4.10) may potentially contribute to its genotoxicity. Quercitrin is well-known for its potent antioxidant activity, which allows it to scavenge oxygen-free radicals and protect against DNA damage (Li et al., 2023). The GC-TOF-MS analysis of the *C. caffrum* extract further identified the presence of hexadecanoic acid (palmitic acid) (Mariri et al., 2023), which is a saturated fatty acid. Dos Santos et al., (2012) claim that saturated fatty acids induce DNA damage and cause apoptotic cell death in insulin-producing beta-cells.

5.4. Phytochemical screening

5.4.1. Phytochemical analysis of crude extracts by Gas-chromatography time of flight (GC-ToF-MS) mass spectrometry

In the GC-ToF-MS analysis, the plant extracts yielded varying compounds at varying time intervals and identification matches (Tables 4.5-4.8). Comparing the spectra of the selected medicinal plants, all the extracts contained phenol, 4-1,1-dimethylpropyl (Figure 4.6), Hexadecanoic acid (HA) (Figure 4.7) and methyl ester. Recently, HA was identified in a variety of medicinal plants and was reported to possess an enormous antimicrobial and antioxidant activity (Mohadjerani et al., 2016; Mongalo et al., 2018; Shaaban et al., 2021; Rzhepakovsky et al., 2022). Both *B. galpinii* and *C. caffrum* exhibited the presence of lupeol (Figure 4.9) at a % area of 2.99 and 3.96, respectively, while *M. obtusifolia* and *M. undata* contained phytol at a % area of 2.80 and 1.78, respectively. Besides its known

antioxidant activity, Lupeol and its derivatives were reported to possess enormous anticancer, wound healing, and anti-inflammatory activities (Le et al., 2021; Santiago et al., 2014; Malinowska et al., 2021). Elsewhere, phytol was found to possess notable immunostimulant and antimicrobial activities (Saha & Bandyopadhyay, 2020). The GC-ToF-MS analysis of *C. caffrum* revealed the presence of Octadecanoic acid (Figure 4.17), which has been reported to possess antihypertensive effects as well as to increase HDL (high-density lipoprotein) and decrease LDL (low-density lipoprotein) cholesterol (Ahmed et al., 2021).

5.4.2. Phytochemical analysis of crude extracts by Liquid-chromatography time-of-flight mass spectrometry (LC-TOF-MS)

Various phytocompounds were identified from the LC-TOF-MS analysis of the methanolic extracts of the investigated plants and reported in Tables 4.9-4.12. Kaempferitrin (Figure 4.21) was identified from the analysis of the LC-TOF-MS chromatogram of the crude organic extract of both *Bauhinia galpinii* and *Markhamia obtusifolia*. Kaempferitrin is a flavonoid compound that has demonstrated the ability to inhibit colon cancer cell viability, induce apoptosis, and reduce the generation of reactive oxygen species in a concentration-dependent manner (Govindarasu et al., 2022). The methanolic extract from *C. caffrum* identified the presence of Quercitrin (Figure 4.24), which is recognized for its strong antioxidant activity, which enables it to scavenge oxygen-free radicals and provide protection against DNA damage. Quercitrin also exhibits anti-proliferative activity against various types of cancer cells (Li et al., 2023). The LC-TOF-MS analysis of *M. undata* revealed the presence of flavonoids, specifically rutin (Figure 4.28) at 17.57 min and camelliaside B (Figure 4.29) at 17.66 minutes. Rutin, a flavonoid-3-O-glycoside, demonstrates favourable bioavailability and biodegradability, which contribute to its effective anti-cancer properties. It is suggested that the anti-tumour activity of rutin may be attributed to its interactions with various pathways, including the Wnt signalling pathway, mixed-lineage protein kinase 3, mitogen-activated protein kinase, apoptotic pathways, as well as proinflammatory protein signalling. Moreover, the xylosyl moiety of camelliaside B can be hydrolysed into kaempferol-3-O-rutinoside (Nikotoflorin) and kaempferol-3-O-glucoside (astragalin). Kaempferol-3-O-glucoside holds promise as a potential therapeutic candidate in lung cancer and other types of cancer, such as leukaemia, hepatocellular, skin, and gastric cancers (Li et al., 2021; Moravkar et al., 2023). Furthermore, the LC-TOF-MS analysis of *M. undata* also tentatively

identified pisumionoside (Figure 4.30), which has been documented to exhibit antagonistic effects on cancer cells (Liu et al., 2022).

6 Conclusions and Recommendations

6.1. Introduction

Mycotoxigenic fungal strains significantly impact the production of food crops consumed by humans and animals, leading to reduced crop yields and compromised food quality. This issue contributes to hunger, jeopardizes food security, and causes severe illnesses when contaminated food is ingested. With global food demand expected to rise in the coming years, the problem becomes even more critical, particularly in African countries. In this context, using medicinal plants as potential fungicides or pesticides to combat fungal infections on crops emerges as a possible solution to improve food safety and security. This research aimed to determine the antifungal activity of the selected South African plant extracts against mycotoxigenic fungi. This chapter presents the significant results and conclusions of the study, along with its recommendations and limitations.

6.2. Significant results from the study

The study documented four South African plants, detailing their descriptions and ethnobotanical uses. It also described the methods for extract preparation and the assays performed. The results indicate that several tested plants displayed promising antifungal activity. Notably, *Bauhinia galpinii* methanol extract exhibited particularly strong inhibitory effects against various fungal pathogens. Additionally, *Combretum caffrum* showed the highest total activity which indicated the mass of the extract that could be diluted with water and still inhibit the growth of the fungal pathogen, *Aspergillus nomius.* The selected medicinal plants also demonstrated significant antioxidant activity, which is crucial for combating reactive oxygen species and protecting crops from oxidative stress. Specifically, *C. caffrum* and *M. obtusifolia* demonstrated potent antioxidant capabilities. The evaluation of the possible cytotoxicity revealed that *B. galpinii*, *C. caffrum*, and *M. undata* extracts were cytotoxic to Vero cells at the highest tested concentration. While the extracts from *C. caffrum* and *M. undata* exhibited cytotoxic effects on cancer cells, *B. galpinii*'s organic extract showed potential stimulation of cell proliferation, warranting caution in its use for developing anticancer agents. Genotoxicity assessment showed cytotoxic effects for *B. galpinii*, *M. obtusifolia*, and *M. undata* at higher concentrations, and for *C. caffrum* at a lower concentration. Furthermore, three of

the four medicinal plants reported a higher phenolic content compared to their total flavonoid compounds. Further phytochemical analyses using GC-ToF-Ms and LC-ToF-MS identified various compounds with antifungal, antioxidant and anticancer activities, highlighting the potential value of these plants in agriculture and healthcare.

6.3. Recommendations

The findings from this study suggest that these plant extracts have the potential to reduce the risk of mycotoxin contamination. Some of the plants also show promise for use in colon cancer therapy. However, further studies are necessary to determine the exact mechanism of action of these plant extracts and to establish whether they are fungicidal or fungistatic. It is also important to investigate their ability to inhibit mycotoxin production and secretion. Additionally, the safety profiles of these extracts should be thoroughly assessed and compounds such as mycotoxins should be quantified using advanced technologies and recent analytical tools. Given the observed toxicity, it is crucial to exercise caution regarding the quantities used in traditional medicine.

6.4. Contributions of the study

This study advances the understanding and documentation of indigenous knowledge related to the alternative management and control of phytopathogenic fungi. It highlights the potential for developing safe, affordable and environmentally friendly fungicides derived from medicinal plants.

6.5. Limitations

This study faced several challenges, although these were significantly outweighed by its overall success. The main difficulties included:

Plant collection and extract preparation

Only leaves were collected and tested due to plant conservation considerations and only methanol was used as a solvent for extraction. This limitation makes it challenging to compare some of the current results to those in other studies, which may have used different plant parts or extracting solvents.

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Annexure A.

Published article number 1.

life

MDPI

Article

GC-ToF-MS Profiling and In Vitro Inhibitory Effects of Selected South African Plants against Important Mycotoxigenic Phytopathogens

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Abstract: The harmful effects following the ingestion of mycotoxin-contaminated food include the induction of cancers, mutagenicity, immune suppression, and toxicities that target organs of the digestive, cardiovascular, and central nervous systems. Synthetic fungicides are generally associated with a high toxic residue in food and the development of excessive fungal resistance. This study aimed to determine the antifungal activities against mycotoxigenic fungi of selected South African plant leaves and potentially develop plant-derived bio-fungicides, and, furthermore, to explore the in vitro antioxidant activity and the phytochemical spectra of the compounds of the selected medicinal plant extracts. The extracts were tested for antifungal activity against phytopathogenic strains using a microdilution broth assay. *Bauhinia galpinii* extracts exhibited the lowest minimum inhibitory concentration (MIC) against *C. cladospoides* and *P. haloterans* at 24 h incubation periods. *C. caffrum* had good antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) with 50% inhibitory concentration (IC_{50}) values of 0.013 mg/mL while *B. galpini* had IC_{50} values of 0.053 against free radicals of 2,2'-azinobis (3-ethylbenzthiazoline-6-suphonic acid (ABTS). The antimycotoxigenic and antioxidant activity exerted by both *B. galpinii* and *C. caffrum* may well be attributed to high TPC. In the GC-ToF-MS analysis, all the selected medicinal plants exhibited the presence of Hexadecanoic acid at varying % areas, while both *B. galpinii* and *C. caffum* exhibited the presence of lupeol at % area 2.99 and 3.96, respectively. The compounds identified, particularly the ones with higher % area, may well explain the biological activity observed. Although the selected medicinal plants exhibited a notable biological activity, there is a need to explore the safety profiles of these plants, both in vitro and in vivo.

Keywords: mycotoxins; biofungicides; minimum inhibitory concentrations; antioxidant activity; total phenolic contents

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1. Introduction

Mycotoxins are a group of secondary metabolites produced by various toxigenic strains of fungi from various fungal genera that can contaminate different agricultural commodities across the world and mostly affect developing countries, with an estimate of 25% of the world's crop affected every year [1]. The toxic effects of mycotoxins on animal and human welfare can be mild or severe, otherwise acute or chronic, and are referred to as mycotosis. Acute toxicity may include fever, abdominal pain, portal hypertension, and death, while chronic mycotoxicosis is strongly associated with liver cirrhosis, kidney failure, immune toxicity, and cancer incidences [2]. There have been numerous

Annexure B.

Accepted article number 2.

From: em.sajb.0.8df9e6.8f939916@editorialmanager.com on behalf of South African Journal of Botany
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Subject: Decision on submission to South African Journal of Botany
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The in vitro cytotoxicity, genotoxicity and LC-ToF-MS profiling of four South African plants with good antifungal activity

Dear Mr Mariri,

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