In vitro investigation of the anti-hyperglycemic and chemical composition of *Heteromorpha arborescens* (Spreng.) Cham leaf extracts used in the management of diabetes mellitus

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A Thesis submitted to the Department of Botany, Faculty of Science and Agriculture, University of Fort Hare, in fulfillment of the requirement for the award of PhD in Ethnobotany

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Co- Supervisor: Prof AJ Afolayan

September, 2021

DECLARATION

I, Taiwo Oluwafunmilola Abifarin, declare that this thesis, submitted to the University of Fort Hare for the award of PhD in Ethnobotany in the Faculty of Science and Agriculture is my own research work and has not been submitted to any other institution for the award of any academic degree. All citations and sources of information are clearly acknowledged by means of reference.

Again, I declare that I am fully aware of the University of Fort Hare policy on plagiarism, and

I have taken every precaution to comply with the regulations of the University.

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……19/09/2021…….

Taiwo Oluwafunmilola Abifarin Date

DEDICATION

To my loving husband and wonderful parents

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ACKNOWLEDGMENTS

I am very grateful to the almighty God the giver of strength, for it was by his grace I made it this far, he was my source of wisdom, good health and is the reason for my living.

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ETHICAL APPROVAL

Approval for this study given was by the Ethical Committee, University of Fort Hare; with protocol number OTA011SABI01/19/E.

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CERTIFICATION

This dissertation entitled "Evaluation of the *in vitro* anti-diabetic potential of *Heteromorpha arborescens* (Spreng.) Cham leaves used in the treatment of diabetes mellitus in Eastern Cape, South Africa" by Taiwo Oluwafunmilola Abifarin meets the regulation governing the award of PhD of the University of Fort Hare and is approved for its contribution to scientific knowledge and literary presentation.

Supervisor: Dr. GA Otunola

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GENERAL ABSTRACT

Diabetes mellitus is currently a major threat all over the world. It is a chronic disorder characterized by elevated blood glucose levels and disturbance in carbohydrate, fat and protein metabolism which often times leads to several complications such as, atherosclerosis, diabetic nephropathy, retinopathy and neuropathy. Due to the side effects associated with synthetic oral hypoglycemic drugs, herbal remedies have become the alternative in the management of diabetes mellitus. *Heteromorpha arborescens* is one of the many plants used as herbal remedies for the management of diabetes mellitus in South Africa. In addition, the roots, barks and leaves are used in other parts of Southern Africa for the treatment of mental problems, asthma, cough, dysentery and tuberculosis. The roots are fed to malnourished children in Botswana and Swaziland. However, despite the high medicinal importance, there is little or no scientificinformation to prove this claim. Therefore, the basis of this study is to determine the chemical composition as well as perform and invitro investigation of the antihyperglycemic potential of *H. arborescens* leaf extracts to provide information that could validate the ethno-medicinal claims for the use of this plant in the management of diabetes mellitus. Phytochemical contents and antioxidant activity of the leaf extracts were determined. Phytochemical analysis of the acetone, ethanol, aqueous and blanched extracts of *H. arborescens* leaves indicated that the total phenol content of the extracts ranged between 15.10 mg GAE/g- 42.50 mg QAE/g, proanthocyanidin, 459-8402.1 mg QE/g and the flavonoid content, 109.24- 235.79 mg QE/g. In addition, alkaloids (7.65%) and saponin (25.33%) were present in significantamounts. Based on the IC_{50} values, the ethanol extract exhibited the highest total antioxidant activity (0.0125 mg/mL) with highest inhibition against DPPH and ABTS radicals (0.06 and 0.049 mg/mL respectively). Relatively high antioxidant activity may be attributed to the polyphenolic contents which possess hypoglycemic potentials. Minimum

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Inhibitory Concentrations (MIC) of *H. arborescens* leaf extracts against *Bacillus pumilus, Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli* and *Klebselia pneumoniae* was also determined and results indicated considerable antibacterial activity in the acetone, ethanol and blanched extracts with MIC values ranging from 1.563-12.5 mg/mL; however, the aqueous extract was inactive against all the bacteria strains. The antibacterial activity of the leaf extracts could be advantageous against diabetic related infections. The cytotoxicity, anti-obesity and antidiabetic potentials of blanched, aqueous and ethanol extracts of *Heteromorpha arborescens* (Spreng.) Cham leaves were also investigated. The results revealed that both ethanol and aqueous extracts indicated considerable inhibition against α-glucosidase (IC_{50} of 627.29 ± 4.33 µg/mL and 576.46 ± 3.21 µg/mL respectively) while the blanched extract showed weak α-glucosidase inhibition (IC_{50}; 855.38 ± 4.29 µg/mL). The aqueous extract showed the best α-amylase inhibition (IC_{50}; 583.74 ± 5.87 µg/mL) among the assessed extracts. However, weak α-amylase inhibition was observed in the ethanol (IC_{50}; 724.60 ± 4.33 µg/mL) and blanched extracts (IC_{50}; 791.63 ± 3.76 µg/mL) of *H. arborescens* leaves. Some level of glucose utilization in both C3A and L6 cells was also observed for the aqueous and ethanol extracts which may be attributed to the relatively lower toxicity levels present in them, however, glucose utilization was very weak for the blanched extract, which may be due to higher level of cytotoxicity it possessed. Relatively weak lipase inhibition was observed for the ethanol (IC_{50}; 699.3 ± 1.33 µg/mL), aqueous (IC_{50}; 811.52 ± 3.52 µg/mL) and blanched extract ($IC_{50;}$ 1152.7 ± 4.61 µg/mL) as compared to orlistat ($IC_{50;}$ 56.88 ± 0.11 µg/mL). However, there was no reasonable reduction in lipid accumulation observed in all the extract treated cells. The results obtained suggest that *H.arborescens* leaf extracts can serve as a potential tool for the development of new strategies for the treatment of diabetes and obesity. However, further analysis is required to ascertain its anti- obesity potential. Also, caution should be taken in the use of the plant at high concentrations in order to ensure safety

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and efficacy. Analyses were also carried out to determine the nutritional and antinutritional constituents of the plant for possible inclusion in the diet of diabetic patients. Proximate analysis revealed the presence of 8.5 % total ash, 4.92 % crude fat, 8.41 % moisture, 15.74 % crude protein, 21.48 % crude fiber, 40.95 % carbohydrates and 271.04 kcal/100g energy value. Mineral analysis showed that *H. arborescens* leaves are very rich in K, Ca, and Fe. Considerable amounts of Mg, Mn, Na, P, Cu and Zn were also present. Vitamin analysis showed that the plant has a high content of vitamins A, C and E. The anti-nutrients evaluated were phytate, oxalate, saponin, and alkaloids, all of which were below toxic levels except for saponin which was observed in moderately high level. This study revealed that *H. arborescens* leaves are a good source of nutrients and mineral elements, (with low anti-nutrient content) that are highly beneficial to human health especially in diabetic individuals, therefore, encouraging its possible inclusion as a vegetable. Essential oil composition of fresh *Heteromorpha arborescens* leaves were also determined by Solvent-Free-Microwave-Extraction (SFME) and Hydrodistillation (HD) methods and the compositions of both mehods were compared in terms of their chemical compositions, yield, CO_2 emission and energy consumption. Solvent Free Microwave extraction method indicated higher oil yield of 0.7 mL/200 g (0.35 %) as compared to 0.59 mL/200 g (0.295 %), lower energy consumption and CO_2 emission as compared to the hydrodistillation method.

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In conclusion, *H. arborescens* leaves indicated considerable potential efficacy in the management for diabetes mellitus and may require further structural elucidation and characterization in order to identify the bioactive constituents.

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CHAPTER ONE

GENERAL INTRODUCTION

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CHAPTER ONE

1.1. Background of study

Diabetes mellitus is a chronic metabolic disorder associated with low blood insulin level or lack of insulin sensitivity or even both (Arumugan et al., 2013), which alters the metabolism of proteins, fat, and carbohydrates (Akash et al., 2012, Li et al., 2004). It is one of the leading causes of global death and has shown an alarming increase in recent times, ranking it as the fourth or fifth leading cause of death in the world. This is because the excessive accumulation of blood glucose leads to increased risks of complications such as retinopathy, neuropathy, nephropathy, stroke, ischemic heart disease, peripheral vascular disease, and various metabolic disorders, including hyperlipidemia, liver-kidney dysfunctions, and hypertension. These complications lead to a reduced quality of life and decreased life expectancy (Murad et al., 2014).

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Plant extracts contain phytochemical constituents and secondary metabolites, which are very effective with hypoglycemic, antihyperglycemic, and glucose suppressive effects. These polyphenolic compounds have also shown a positive correlation with antioxidant activity (Kifle and Enyew, 2020). Initiation of oxidative stress is a key process in the pathogenesis of various diseases including diabetes mellitus and its complications, and the role of antioxidants in the management of diabetes and its complications by the prevention of oxidative stress has been reported in different studies. Apart from lowering blood glucose effect, these phytochemicals have been reported to restore the damaged β-cells and inhibiting oxidative stress on beta cells in experimental diabetic rats (Alexandru et al., 2007).

A number of studies have reported that phytochemicals such as phenolics have been implicated in reduction of postprandial hyperglycemia and obesity complications as well as inhibitory effects against α-amylase and α- glucosidase (Ibrahim et al., 2014, Buchholz and Melzig, 2016, Kifle and

Eyew, 2020). Obesity is one of devastating risk factors linked with diabetes mellitus as insulin sensitivity and coordination of β-cell function, decreases (Al- Goblan et al., 2014). However, pancreatic lipase inhibitory activities as well as lipid accumulation have been widely used to determine the potential efficacy of natural products as anti-obesity agents (Seyedan et al., 2015). Furthermore, previous studies have indicated that the glucose lowering effect of plant extracts may be attributed to decrease in glucose absorption, elevated glycolysis, increased glucose utilization as well as reduction in both gluconeogenesis and glycogenolysis (Tesfaye et al., 2016, Gong et al., 2020). In addition, diabetes mellitus has been linked with dereased response of T cells, neutrophil function, and weakened immunity. Therefore, diabetic patients have are highly prone to infections and some of the infections associated with the disease include *S. pneumoniae*, *H. pylori*, foot infections, HIV, and bacterial pyelonephritis (Casqueiro et al., 2012). Consequently, plants are currenlt being exploited as safe antimicrobial agents for managing diabetes related infections.

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1.2. Prevalence of diabetes mellitus

The prevalence of diabetes has been on the increase all over the world. As at 2017, there were approximately 451 million persons suffering from diabetes all over the world and it was projected that the number of cases will rise to about 693 million by 2045 (Karuranga et al., 2017). About 80% of people with diabetes and about five million diabetes-related deaths were reported in low-income and middle-income countries (Beagley et al., 2013). Africa is not exempted from the alarming increase in diabetes cases. In 2010, about 12.1 million people were reported to be living with diabetes in Africa and this was predicted to increase to 23.9 million by 2030 (Hall et al., 2011). Recently, it was discovered that approximately 20 million people in sub-Saharan Africa are living with diabetes, with 62% undiagnosed cases, and this number is expected to increase to 41.4 million by 2035 (Azevedo and Alla, 2008).

In South Africa, the prevalence of Type 2 diabetes mellitus has almost doubled from 5.5% in 2000 to 9% in 2009 (Bradshaw et al., 2007, Bertram et al., 2013), although this may likely underrate the current disease burden, as risk factors of the disease have significantly increased in recent years. According to Pheiffer et al. (2021), the prevalence of total and newly diagnosed type 2 diabetes (T2DM), impaired glucose tolerance (IGT), and impaired fasting glucose (IFG) in South Africa among persons 25 years and older was 15.25% for T2DM, 9.59% for IGT, 3.55% for IFG, and 8.29% for newly diagnosed T2DM respectively. Obesity is a major contributor to the diabetes, with excess bodyweight estimated to account for 87% cases in South Africa (Joubert et al., 2007).

1.3. Therapeutic approaches to the management of diabetes mellitus

One of the therapeutic approaches to decreasing diabetes and its complications is to reduce the absorption of glucose and lipid by the inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase and α- glucosidase in the intestine (Hamden et al., 2012).

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Although several synthetic drugs have been identified to be effective in the management of diabetes mellitus, side effects such as hypoglycemia, weight gain, gastrointestinal (GIT) disturbances, nausea, and diarrhea results from excessive use of these hypoglycemic synthetic drugs (Khan et al., 2018). Consequently, as an alternative, the need arises for the search for more novel natural remedies especially from plants which have lower side effects. Furthermore, there is an increase in the use of traditional remedies because of its safety, relatively lower cost, easier accessibility especially by the rural populace, as well as shortage of healthcare systems (Balde et al., 2006). One of the medicinal plants used for the management of diabetes mellitus in South Africa is *Heteromorpha arborescens* (Erasto et al., 2005).

Heteromorpha arborescens (Spreng.) Cham (Figure 1) is a large shrub-like, small, or medium deciduous tree belonging to the Apiaceae family. It is of very great medicinal importance in Africa,

especially in the east and Southern Africa. The leaves are traditionally used for the management of diabetes in South Africa (Odeyemi et al., 2018). In addition, the roots, barks, and leaves are used in other parts of Southern Africa for the treatment of mental problems, asthma, cough, dysentery and tuberculosis (Maroyi, 2018). As a result of its high nutritional value, the leaves are eaten as vegetables in Kenya (Bussman, 2006) and the roots are fed to malnourished children in Botswana and Swaziland (Setshogo et al., 2011).

Figure 1: *Heteromorpha arborescence* (Spreng.) Cham. & Schltdl.

1.4. Problem statement

Diabetes mellitus has threatened the health of millions of people all over the world. Its mortality rate is increasing annually, indicating that the available therapeutic options are inadequate (Al-zuaidy et al., 2018). It was estimated that about 285 million people were living with diabetes mellitus in 2010 and this number is predicted to rise to 439 million adults' cases by 2030 (Shaw, et al., 2010). In industrialized and developing countries such as South Africa the disease is generally acquired in the most productive period of life.

Furthermore, diabetes and its complications are associated with oxidative stress which leads to cellular damage that occurs as a consequence of generation of reactive oxygen species or ineffective antioxidant systems in the human body (Giacco and Brownlee, 2010). Although there are few synthetic drugs used for the management of the disease, their use is limited because of their adverse side-effects, and they are not readily affordable and accessible to the poor (Afolayan & Sunmonu, 2010). This has led to the growing interest in the use of medicinal plants for the prevention and management of the disease, with little or possibly no side effects (Salim, 2005). Moreover, the leaves of *H. arborescens* are traditionally used in the management of diabetes mellitus in the Eastern Cape, South Africa (Odeyemi et al., 2018); however, there are noscientific validations to prove this claim. The leaves are also consumed as vegetable in Kenyabut to the best of our knowledge, there is no scientific documentation of its nutritive and anti- nutritive composition. This study will therefore, provide scientific information on the chemical composition, polyphenolic contents, antioxidant and antibacterial activities of *H. arborescens* leaves. It will also provide information on the safety, nutritional and antinutritional profiles as well as the hypoglycaemic potential of the plant in the management of diabetes mellitus.

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1.5. Aim

The aim of this study is to investigate the *in vitro* anti-hyperglycemic properties and chemical compositions of *H. arborescens* leaves used in the management of diabetes mellitus.

1.6. Specific objectives

The specific objectives are;

1. To determine the phytochemical, antioxidant, nutritional and anti-nutritional contents as well as the antibacterial activities of the plant extracts.

2. To evaluate the cytotoxicity, lipotoxicity, lipid accumulation and glucose utilization potentials of the extracts of *H. arborescens* leaves against C3A cell lines and L6 myocytes.

3. To determine the chemical compositions of essential oil extracted from *H. arborescens* leaves and compare the compositions using hydro-distillation and solvent free microwave extraction methods.

4. To determine the alpha glucosidase, alpha amylase and pancreatic lipase inhibitory potential sof the plant extracts.

1.7. The structure of the thesis

This thesis is composed of seven separate chapters out of which four have been published in various peer-reviewed accredited journals, while one is currently under review. Chapter 1 consists of the general introduction while literature review on the antidiabetic potentials of selected Apiaceae species is presented in Chapter 2. The phytochemical, antioxidant and antibacterial activities of *H. arborescens* leaf extracts are presented in Chapter 3. Chapter 4 presents a comparative study of essential oils obtained from *H. arborescens* leaves using hydrodistillation and Solvent Free Microwave extraction methods, while the nutritional composition and antinutrient contents of the plant are discussed in Chapter 5. The *in vitro* cytotoxicity, lipase inhibition, lipid accumulation and anti-diabetic potentials of *H. arborescens* leaf extracts are presented in Chapter 6, while Chapter 7 covers the general discussion and conclusions.

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CHAPTER TWO

HYPOGLYCEMIC AND ANTIDIABETIC POTENTIALS OF SELECTED APIAECEA SPECIES

This chapter is under review in Journal of Diabetes Research

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HYPOGLYCEMIC AND ANTIDIABETIC POTENTIALS OF SELECTED APIAECEA SPECIES

Abstract

Diabetes mellitus is a metabolic disease that is characterized by hyperglycemia associated with a disorder in carbohydrate, protein, and fat metabolism. It can either be complete insulin deficiency associated with destruction of the pancreatic beta-cells (Type 1 diabetes mellitus), or associated with insulin resistance and beta cell pancreatic malfunctioning (Type 2). It has become a major global threat, affecting about 2.8% of the world's population and is anticipated to cross 5.4% by the year 2025. In spite of many known synthetic antidiabetics, the side effects and associated complications is still a major health challenge. Hence, majority of research work on antidiabetics is directed towards plants- derived products in recent times. It is noteworthy that a good number of the members of the Apiaceae family have been identified to possess potent anti-diabetic agents. Therefore, in this review, an attempt is made to provide an overview of ten plants from the Apiaceae family which has been explored in previous literature for their effectiveness in managing diabetes and its complications. This review could provide hints that may aid in the discovery of novel and improved anti-diabetic drugs.

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Keywords: Apiaceae, diabetes, insulin, hyperglycemia, glucose, novel drugs.

2.0. Introduction

Diabetes mellitus is a chronic metabolic disorder associated with high levels of blood glucose and glucose intolerance from poor insulin secretion or inefficiency of insulin [1]. Oxidative stress caused by excessive production of reactive oxygen species (ROS) in the mitochondria also plays

a vital role in the development of insulin resistance and other complications such as cardiovascular and neurodegenerative diseases [2]. The occurrence of diabetes mellitus is greatly on the increase all over the world especially in developing and underdeveloped countries, making it one of the primary causes of death in developing countries [3].

The prevalence of diabetes is speedily on the increase in South Africa. In 2009, about 9% adults aged 30 years and above were diagnosed with diabetes and the numbers have been increasing in double folds since then [4]. A high percentage of South Africans are overweight, and it was reported in 2000 that excessive body weight was responsible for approximately 87% of diabetes cases in the country [5]. The connection between diabetes mellitus and several complications, places a noteworthy burden on the health structure of South Africa. Recently, diabetes mellitus was considered as one of the leading causes of untimely deaths in the country [6]. Hence, more research is vital in the discovery of more therapeutics for the management of the disease and related complications.

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2.1. Type 1 and Type 2 diabetes

Type 1 diabetes mellitus otherwise called insulin-dependent diabetes is usually characterized by the presence of anti–glutamic acid decarboxylase, islet cell or insulin antibodies which identify the autoimmune processes that lead to beta cell destruction and thus insulin deficiency. Those suffering from type 1 diabetes require insulin therapy to retain good blood glucose levels [7]. However, type 2 diabetes mellitus (non-insulin dependent diabetes), a more common type of diabetes mellitus all over the world is associated with impaired use of insulin and hyperglycemia [8, 9]. Type 2 diabetes greatly contributes to elevated plasma lipids, which is a risk factor for coronary cardiac diseases [10, 11]. Type 2 diabetes mellitus is the most common form of diabetes accounting for 90% to 95% of patients. The prevalence of diabetes among all age groups was approximately 2.8% in 2000 andit has been projected by World Health Organization, that it will rise to about 4.4% and will be theseventh leading cause of death by 2030 [12].

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2.2. Etiology and pathophysiology of type II diabetic mellitus

Type 2 diabetes mellitus can also be referred to as a disorder characterized by insulin resistance and a progressive deterioration in the function of the pancreatic beta-cell associated with increasing hyperglycemia [13]. Type 2 diabetes is initiated as a result of a combination of genetic factors linked to impaired insulin secretion and environmental factors including obesity, high fat intake or calorie dense foods, sedentary lifestyle, stress and aging. Insulin is the major hormone responsible for the control of glucose uptake from the blood to the liver, muscle, and adipose tissues. Hence, the impairment of insulin action in these target organs is a common pathophysiological characteristic of type 2 diabetes [14]. Insulin impairment is step by step and continuous progressionof this condition will lead to permanent elevation of blood glucose, lipid and protein metabolic disorder as well as several complications such as neuropathy, cardiovascular complications, retinopathy and

nephropathy that may eventually lead to death [15]. The likelihood of increased morbidity and mortality observed in Type 2 diabetic patients is because of its frequency, gradual onset, late diagnosis and late intervention especially in under-developed and developing countries like Africa [16].

With regards to the pathophysiology of Type 2 diabetes mellitus, a distruption of the feedback loops between insulin activity and insulin secretion leads to an unusual increase in blood glucose levels. In the instance of β-cell dysfunction, there is a decrease in insulin secretion, thus restraining the ability of the body to maintain normal glucose levels. However, insulin resistance leads to increased glucose production in the liver and reduction in glucose uptake in the muscle, liver and adipose tissue. Eventhough the two processes happen in the premature stage and contributes to the genesis of the disease; the malfunctioning of the β-cell is usually more serious than the case of insulin resistance. Environmental factors, sedentary lifestyle and excessive fat intake resulting to obesity, promotes insulin resistance and impaired insulin secretion in β-cells, muscle, and liver; thus, favoring the development of type 2 diabetes melitus and related complications such as cardiovascular diseases (Figure 1). Although weight reduction for obese individuals is an effective approach to control or prevent the progression of type-2 diabetes. However, accurate diagnosis of diabetes necessitates Fasting Blood Glucose (FBG) which is best done in morning after fasting of over 10 hours and HbA1C and Random Blood Glucose (RBG) which is usually done without fasting. A fasting blood sugar level less than 100 mg/dL (5.6 mmol/L) is normal, from 100 to 125 mg/dL (5.6 to 6.9 mmol/L) is considered prediabetes and if it is 126 mg/dL (7 mmol/L) or higher on two different tests, this can be diagnosed as diabetes. Also, an A1C level of 6.5% or higher on two different tests indicates that diabetes, an A1C between 5.7 and 6.4 % indicates prediabetes and below 5.7 is considered normal.

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A summary of the etiology and pathophysiology of diabetes mellitus is represented in Figure 1.

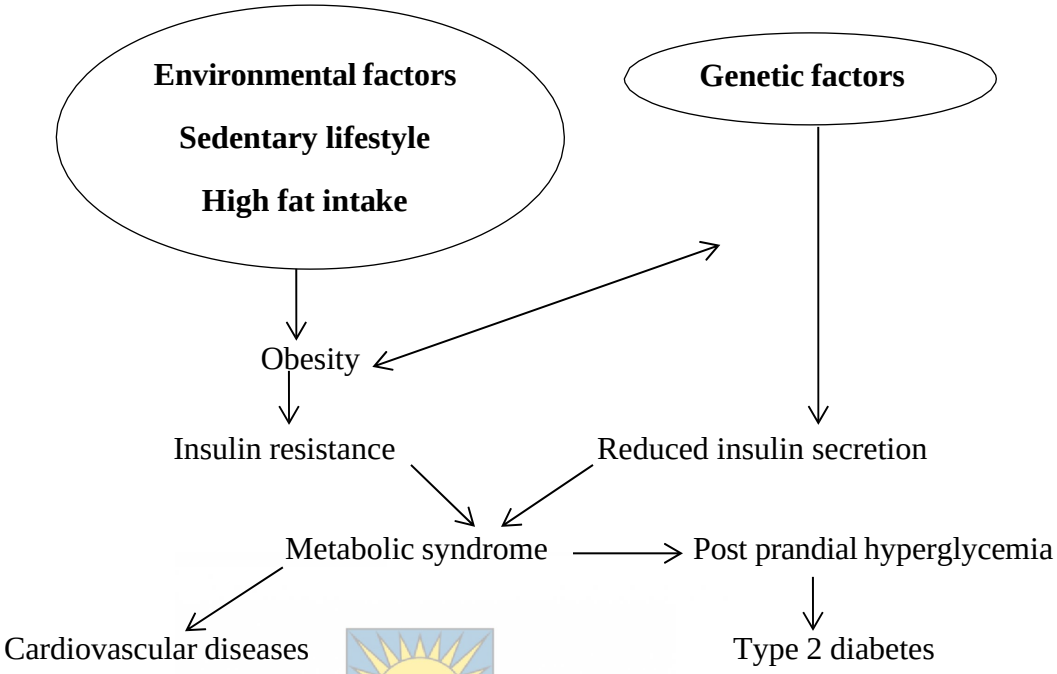

Figure 1: Etiology and pathophysiology of type 2 diabetes [16].

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2.3. Role of oxidative stress in diabetes mellitus and the effects of antioxidants

Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism**.** Oxidative stress implicated by ROS results in loss of function and structure of normal cells, damage to enzymes, DNA and proteins thus leading to the development of a variety of diseases including insulin resistance [17]. Oxidative stress is implicated in the changes in glucose homeostasis and can lead to the diabetes-associated insulin-resistance. The pathogenesis of insulin resistance is a complicated system of insulin signaling pathways. Binding of insulin to the insulin receptors on the cell surface leads to phosphorylation of insulin receptors and corresponding activation of insulin pathways [18]. Many research studies have indicated higher oxidative damage resulting from increased ROS production and decreased antioxidant defense mechanisms. Antioxidants are defensive substances which serve to neutralize or inhibit excessive free radicals.

Therefore, inhibition of intracellular free radical production will provide a therapeutic approach to prevent oxidative stress and the related diabetic complications.

2.4. Oral synthetic hypoglycemic drugs

Although change in lifestyle, weight control and appropriate diet are required for the management of type II diabetes, antidiabetic drugs are still necessary to provide positive effects. Diabetes mellitus is a complicated disorder and managing it requires complete compliance to prescription. Regulating blood glucose to a normal level is the first step in the treatment of diabetes mellitus [19, 20]. There are some groups of oral drugs which possess antidiabetic activities and exert their hypoglycemic activities through varying mechanisms of action. These could be through decrease in hepatic glucose production, promote pancreatic β- cell insulin secretion, improve insulin sensitivity and inhibit both intestinal digestion and absorption of glucose [21].

These oral hypoglycemics include biguanides, sulphonylureas, thiazolidinediones, non-sulfonylureas secretagogues and α-glucosidase inhibitors [22].

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Biguanidines (e.g. metformin) bring into play their blood glucose lowering effect in Type 2 diabetes mellitus by decreasing gluconeogenesis in the liver in order to restore the sensitivity of peripheral tissues to insulin [22]. This class of hypoglycemic drugs does not directly stimulate insulin secretion; instead they increase insulin action and insulin-mediated glucose utilization in peripheral tissues. However, side effects such as dizziness, anxiety, depression, hypoglycemia, and dyspepsia are associated [23].

Sulfonylureas (glimepiride and glibenclamide) are the most widely used drugs for the treatment of diabetes mellitus. The key effect of this class of drugs is stimulating the sensitivity of β-cells to both glucose and non-glucose secretagogues, which results in the release of more insulin at any

increase in blood glucose [24]. Hypoglycemia and weight gain are the most common side effects of this drug, occurring as a result of high dosage**.** Other side effects associated are dizziness, anxiety, depression, hypoglycemia, and dyspepsia**.** Because sulfonylureas are digested by liver enzymes, with urinary excretion of metabolites, they should be used cautiously in persons suffering from renal or hepatic disorders [25].

Thiazolidinediones (rosiglitazone and pioglitazone) exert hypoglycemic effect by increasing the sensitivity of the muscle and adipose tissues to insulin, decreasing the production of glucose in the liver [26], and increasing beta cell function by reducing free fatty acid levels. Thiazolidinediones activate the nuclear peroxisome proliferator-activated receptor-gamma (PPARG-y) which augments the transcription of various insulin-sensitive genes, improving the action of insulin and somewhat lowering hepatic glucose as well as enhance insulin sensitivity when used in synergy with other antidiabetic drugs [27]. However, some adverse effects associated include hypoglycemia, edema, headaches, bone fracture sinusis, myalgia and pharyngitis [28].

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Non-sulfonylurea insulin secretagogues (repaglinide and nateglinide) function similarly as sulfonylureas although they have different characteristics. These antidiabetic agents act by enhancing the release of insulin from the pancreatic beta cells, and should be reduced in cases with impaired liver function [29]. Negative effects of these drugs include hypoglycemia, weight gain, headaches, upper respiratory tract infection and cardiovascular ischemia [28].

Glucosidase inhibitors (acarbose, miglitol) functions in reducing postprandial hyperglycemia by inhibiting the activity of glucosidase enzymes which are present in the brush borders lining of the intestinal villa which breaks down complex carbohydrates [13]. In addition, besides thefunction of acarbose in decreasing postprandial hyperglycemia, it has also been reported to reduce hypertension and cardiovascular diseases. However, side effects involved include flatulence,

abdominal pain and diarrhea [30].

Although there has been a considerable increase in the development of synthetic drugs for the management of diabetes mellitus, these drugs are expensive, inaccessible to the rural populace and are often associated with several adverse side effects. Some of the side effects associated with continuous use of the synthetic antidiabetic medications are hypoglycemia, obesity, weight gain, gastrointestinal disorders, abdominal pain and fatigue.

Dipeptidyl peptidase 4 inhibitors (DPP1V inhibitors) e.g. sitagliptin, linagliptin, alogliptin promote insulin secretion and inhibit glucagon secretion by increasing endogenous Glucagon-Like Peptide 1 (GLP-1) concentrations without any serious risk of hypoglycaemia since the GLP-1 dependent promotion of insulin secretion occurs in hyperglycaemic conditions. GLP-1 enhances glucose-stimulated insulin secretion and effectively reduces HbA1c with maximum safety from excessive weight gain or cardiovascular complications [31]. Most times DPPIV inhibitors are recommended to be used with lifestyle change and metformin, sulfonylureas, thiazolidinediones, and insulin; nonetheless some patients allergic to the combination therapy or those with serious kidney diseases DPP1V inhibitors can be used as a single therapy. Some adverse effects reported from the use of these medications include dizziness, headaches, acute pacreatitis, chronic abdominal pain [32], inflammatory bowel disease [33] and severe joint pain [34]

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Sodium–glucose cotransporter 2 (SGLT2) inhibitors e.g. dapagliflozin, canagliflozin, empagliflozin, ertugliflozin are potent antidiabetic medications in patients with type 2 diabetes mellitus. They function by inhibiting SGLT2 in the proximal convoluted tube, to avoid reabsorption of glucose and enable elimination through urine. Unlike some other cases of oral hpoglecemic drus, SGLT2 inhibitors are not insulin dependent [35]. They are associated with improved glycaemic control and have been reported to improve cardiovascular and renal health

with other advantages of weight loss and lowering of blood pressure [35]. Reported side effects of this class of drugs include acute kidney injury, increased risk of diabetic ketoacidosis, which are in most cases activated by conditions such as serious insulin deficiency, low glucose diets and excessive consumption of alcohol [36]. SGLT2 inhibitors also inhibit the absorption of glucose from the proximal tubule of the kidney, causing glycosuria thus exposing the patients to urinary tract and genital infections [37, 38]. It was also reported that this class of drugs are likey to affect mineral metabolism and increase the risk for fractures [39, 40].

2.5. The use of medicinal plants as an approach to diabetes mellitus

Medicinal plants are an effective source of both traditional and modern medicines and about 80% of rural populations depend on it for primary health care [41]. Plants are rich sources of anti-diabetic compounds such as alkaloids, flavonoids, phenolic and tannins that increase theefficacy of pancreatic tissues by increasing the insulin secretion or decreasing the absorption ofglucose in the intestine [42, 43, 44, 45]. Herbal medicine is employed for the treatment of diabetes indeveloping countries where the cost of orthodox medicines is a challenge to the population [45].

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The efficiency, lower cost, ease of accessibility (especially to the rural populace) and relatively lower side effects associated with the use of plants for management of wide range of diseases has prompted the increased search for more antidiabetic agents from plants. Several members of the Apiaceae family are popularly known for their antidiabetic potentials. In this review article, an attempt has been made to bring together some Apiaceae plant species with antidiabetic potentials which may be beneficial for the development of alternative medicine to reduce the ever-increasing rate of diabetes mellitus.

2.6. The Apiaceae family

The family Apiaceae is one of the most important families of flowering plants, consisting of over 3000 species and 400 genera [46]. Members of this family are distributed in most parts of the world. But they are commonly found in sub-tropical, north temperate regions [47]. It is also one of the oldest families among the aromatic plants with most of its members been indigenous to the Mediterranean region and Southwest Asia. The Apiaceae species are dominantly herbaceous plants, with generally characteristic aromatic smell, leaves which are alternate, pinnately compound or lobed with reticulate venation with small and simple or compound flowers which are either yellowish or whitish in colour [48]. Several plants belonging to the Apiaceae family are useful as food, food flavors and for their therapeutic purposes in the treatment of abdominal pain and acidity, or treatment of disorders associated with digestive, endocrine, reproductive, respiratory systems [49] and various other ailments [50]. They also possess various compounds, with many biological activities and are considered to have blood glucose lowering effects on humans [51].

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2.7. Selected members of the Apiaceae family with antidiabetic potentials

The hypoglycemic effects of plants are associated with increasing pancreatic secretion of insulin from the islets of Langerhans's or its release from bound insulin [52, 53]. Alpha-glucosidaseinhibitory potential of species of the Apiaceae family suggests their ability to disrupt the breakdown of oligosaccharides, hereby reducing glucose absorption which would prevent increased postprandial hyperglycemia [54]. The α-glucosidase inhibitory potential of the family hasbeen directly linked with the high antioxidants and phenolic components of the species. However, α-amylase inhibition is not in any way associated with the presence of these components [55]. It has been suggested that simultaneous inhibition of both α-glucosidase and α- amylase is not necessary to manage hyperglycemia, in as much as one of the inhibitors exhibit a high activity [56]. However, foods with

high α-glucosidase inhibitors and low or moderate α- amylase inhibitors are better in order to prevent digestive problems from indigestible starch [57]. Since members of Apiaceae family exhibit considerate α-amylase inhibition and good α- glucosidase inhibition, they are considered appropriate for the management of hyperglycemia [58].

The present paper is focused on selected vegetables, culinary and medicinal plants belonging to the Apiaecea family, which have been explored in various literature as having hypoglycemic potentials. These include: *Anethum graveolens,Apium graveolens*, *Carum carvi*, *Coriandrum sativum, Daucus carota, Cuminum cyminum*, *Foeniculum vulgare*, *Ferula gummosa*, *Centella asiatica* and *Pimpinella anisum*.

i. Anethum graveolens

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Anethum graveolens (Dill) is an aromatic annual herb which is native to the Mediterranean countries, South West Asia and South East Europe [59]. The plant is traditionally usedin the treatment of mental disorder, colic pain in babies, flatulence in children and in increasing lactation in nursing mothers. The plant also exhibits antiulceric, antimicrobial, antihyperlipidemic, antihypercholesterol, antihyperglycemic, anticancer, antimicrobial, anti-inflammatory and antihyperglycermic activities [60]. Dill exerts its antidiabetic effect majorly by influencing antioxidant capacity and modifying some genes in the glucose and lipid pathways [60]. It is reported that powdered *Anethum graveolens* decreased blood glucose in diabetic mice by increasing insulin activity and he attributed this activity to the presence of antioxidants, flavonoids and carvone present in the plant [61, 62]. Whereas, another researcher reported that ethanol extract of *Anethum graveolens* significantly reduced blood glucose in diabetic rats [63]. However, the aqueous extracts showed negligible activity. The aerial parts and seeds of the plant were also reported to decrease hyperlipidemia, hyperglycemia and enhance the antioxidant level

of streptozotocin induced rats [64, 65]

ii. Coriandrum sativum L.

Also known as coriander is one of the many documented culinary annual herbs used in traditional medicine, which is native to the Middle East, Latin America and Asia [66]. It is a commonly used food ingredient with medicinal and nutritional properties [67], and widely grown all over the world for its seed, use as spice, or for essential oil production [68]. Although all the parts of the plant are edible, only the seeds and leaves are commonly used for therapeutic purposes [69]. The leaves and seeds are of very high medicinal value, useful as anti-carcinogenic, anti-convulsant, anti-histaminic, hypnotic, diuretic, carminative, antispasmodic, antibacterial and to treat fever, inflammation, nausea and stomach disorders [70]. The seeds are also useful in treating worm infections, insomnia, anxiety, rheumatism, loss of appetite and arthritis [71, 72].

Due to the high hypoglycemic effect exhibited by coriander, it is referred to as "antidiabetic plant" in Europe. Previous studies have shown that ethanol leaf extract significantly lowered blood glucose in alloxan induced diabetic rats [73, 74, 75, 76]. Similarly, ethanolic extract of coriander leaves were found to possess antidiabetic activity at a dose of 400 mg/kg body weight by enhacing and regenerating the β-cell in pancreas and inhibiting α- glucosidase enzyme in the small intestine [76]. Another study also revealed that the methanol extract of coriander leaves exhibited antidiabetic activity in alloxan-induced animals [77] and aqueous extract showed significant hypoglycemic effect in high cholesterol fed diabetic rats. Further studies revealed thatadministering coriander seed powder to streptozotocin-induced diabetic rats resulted in recovery of beta-cells and regulation of both blood glucose and insulin [78]. Conversely, another study showed that coriander did not exhibit hypoglycemic effects in alloxan–induced diabetic rats [79].

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iii. Cuminum cyminum

Cuminum cyminum, commonly known as cumin is a flowering plant which is native from the east Mediterranean to India and majorly cultivated in Europe, Asia, the Middle East, and Africa [80]. The plant is traditionally used for the treatment of jaundice, indigestion and diarrhea and as a stimulant, carminative, and coagulant [81]. It is also widely used in the treatment of chronic diarrhea, hypertension, toothaches, scorpion bites, and gastrointestinal disorders [82]. Reports have shown that essential oil of *C. cyminum* possesses antidiabetic properties [83, 84], and the seed oil possesses inhibitory activities against aldose reductase and alpha-glucosidase in diabetic rats and was attributed to the presence of cuminaldehyde [85]. Methanol extract of cumin seeds was also observed to decrease the blood glucose and serum insulin in alloxan and streptozotocin diabetic rats [51, 86]. Similarly, cumin seeds ethanol extract decreased glycemic levels and improved kidney function in diabetic rats [87].

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iv. Carum carvi L.

***Carum carvi* L.** otherwise called caraway is a well-known traditional herb used as food spice and food products, is native to Asia, Africa and Europe [88]. The fruits are used in traditional medicine as a diuretic and for the treatment of flatulence, indigestion, poor appetite and gastrointestinal disturbance [89, 90, 91, 92]. Aqueous extract of the plant has also been traditionally used for reducing fear and agitation, increasing menstrual flow, lactation in nursing mothers and as an aphrodisiac [93]. Quite a good number of researchers have reported the anti- hyperglycemic potential of *Carum carvi* L. There was a significant reduction in blood glucose with increase in serum insulin levels in streptozotocin induced diabetic rats on administration of ethanol extract of caraway seeds [93, 94]. Similar observations were also made including reduced hyperlipidemia and improved antioxidant status on administration with *Carum carvi* oil [88, 95, 96, 97, 98]. Furthermore, it was also reported that

administration of *Carum carvi* oil exhibited renal protection against diabetic nephropathy in streptozotocin induced diabetic rats [99].

v. Foeniculum vulgare Mill.

Foeniculum vulgare, commonly known as fennel is an aromatic plant, native to the Mediterranean region and Southern Europe. The plant is popularly used for consumption and in traditional medicine to increase lactation in nursing mothers, suppress appetite, ease child birth, enhance sexual activities in men and womenas well as promote menstruation in women [100, 101]. It is also highly reputed for its use for themanagement of diabetes, kidney stones, long-term coughs and bronchitis [102, 103]. Investigation ofthe hypoglycemic activity of *Foeniculum vulgare* essential oil revealed a decrease in blood glucose levels in alloxan induced diabetic rats [104, 105]. Another study conducted showed that the aqueous extract of fennel improved hyperglycemia and considerably lowered blood glucose levels in streptozotocin-induced diabetic rats [106]. Similarly, methanol extract of the aerial parts and seeds showed significant anti-hyperglycemic activity in streptozotocin-induced diabetic rats and alleviated all the complications that resulted from increased blood glucose levels [101]. It was also reported that methanol extracts of the fruits showed blood glucose, triglyceride and cholesterol lowering activities [107] and significantly increased glucose tolerance in diabetic mice [102].

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vi. Daucus carota

Daucus carota popularly called carrot is one of the most cultivated vegetables all over the world. It is traditionally used for the treatment of dysentery, cough, malaria, diarrhea and even cancer [108]. It has also been widely used in traditional medicine as a stimulant; anthelminthic, aphrodisiac, carminative and diuretic [109]. However, very few reports are available for the antidiabetic activities

of carrot. Carrot seed methanol extracts reduces blood glucose, cholesterol and triglycerides, with a significant increase in blood insulin level as result of improving pancreas asinuses and islets [110]. Similarly, more recent studies showed that dichloromethane extract of carrot roots promoted glucose uptake in adipocytes and methanol carrot seed extracts in streptozotocin- induced diabetic rats [111,112].

vii. Pimpinella anisum L.

Pimpinella anisum L. (Anise) is an annual herb which is native to the Mediterranean area and widely cultivated in Southern and Eastern parts of Europe and majorly grown for its seeds and essential oil [113]. Anise seeds employed in the food and meat industries are known to possess analgesic, diuretic, disinfectant, carminative properties [114]. The plant is employed in traditional medicine for the treatment of epilepsy and seizures [115] and has also been reported to increase lactation and reduce gastrointestinal disturbances in babies [116]. The antidiabetic potential of anise seeds has been reported in a number of previous literatures. Considerable inhibitory activities against α- amylase, α- glucosidase and pancreatic lipase enzymes has been indicated in methanol [117] and aqueous extracts [118] of aniseeds. Furthermore, a decrease in fasting blood glucose was observed in diabetic patients treated with anise seed powder and significant increase in glucose absorption in aniseed oil treated diabetic rats. The increased glucose absorption was attributed to the ability of the oil to enhance Na^+- K^+ ATPase which increases the sodium gradient needed for transport of glucose in the mucosal lining [119, 120].

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viii. Apium graveolens L.

Apium graveolens L. (Celery) is an important spice known for its food and medicinal purposes [121.] The plant possesses anti-inflammatory, antioxidant, antihypertensive, hypolipidemic,

hypoglycemic [122, 123], antiproliferative and anticarcinogenic potentials [124]. Some scientific studies have proven that celery seeds are rich sources of flavonoids such a luteolin and apigenin which accounts for their significant antidiabetic activity and potential to prevent the complications associated with diabetes [125, 126, 127, 128].

A significant increase in plasma insulin levels and a decrease in blood glucose levels in celery seeds treated diabetic mice [123, 129]. Similar observations were equally reported on celery leaf extracts on alloxan and streptozotocin induced diabetic rats [79, 122, 128]. Similarly, there was a significant reduction in blood glucose levels observed in elderly pre diabetic patients treated with celery leaf extracts [120]. However, there was no significant increase in their plasma insulin levels. This decrease in blood glucose and increase in insulin levels on treatment with celery extracts may be as result of increased insulin secretion and enhanced repair or proliferation pancreatic beta cells [121]. Conversely, it was indicated that celery exerts its blood glucose lowering activity by altering the absorption of glucose in the intestine and not by enhancing the production of insulin by the pancreas [122, 123.]

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ix. Centella asiatica

C. asiatica (Gotu kola) is an edible plant which is indigenous to Southeast Asian countries, Sri Lanka, China, India, Indonesia, and Malaysia as well as South Africa, Madagascar and other tropical countries [134, 135] and is traditionally used in the management of diabetes in many countries. The plant is a rich source of the pentacyclic triterpenoid saponin which possess a wide range of biological activities, some of which include anticancer [136, 137], antioxidant [138, 139], anti-inflammation [140, 141], wound healing [142] and antidiabetic [143, 144] activities. It is also used in the treatment of pharyngitis and dysmenorrhea convulsion [51].

A good number of researchers have proven the antidiabetic potential of *C. asiatica*. Ethanol extract of *C. asiatica* whole plant, showed significant antidiabetic activity at a dose of 200mg/kg in streptozotocin diabetic rats [145]. In another study, anti-hyperglycemic activity was reported in diabetic rats on treatment with *C. asiatica* powder, with no observation of hypoglycemia [146]. However, there was no significant increase in plasma insulin secretion on administration of the extract at all concentrations tested. It was also indicated that *C. asiatica* is efficient in the inhibition of glucose absorption by inhibition of intestinal disaccharidase, α-amylase enzymes as well as glucose-fiber binding [146]. In another study, it was observed that madecassic acid, which isone of the active compounds in *C. asiatica*, lowers blood glucose levels through stimulation of insulin expression [147.] Agreeably, a significant glucose lowering activity in alloxan induces diabetic mice was indicated on administration of *C. asiatica* leaf extract at 50 mg/kg dose [148]. The results correlate with a more recent that revealed decrease in blood glucose and elevated blood insulin levels in alloxan diabetic mice after day 14 and 21 respectively on treatment with *C. asiatica* leaf extracts and it was concluded that the above results were achieved through the mechanism of stimulating pancreatic beta cells secretion [149]. It was also elucidated that ethanol extract of *C. asiatica* at a dose of 1000mg/kg is the most efficient in decreasing blood glucose levels and inhibition of disaccharidase [146, 150].

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x. Ferula gummosa

F. gummosa (Galbanum) is an aboriginal plant which is native to the Middle Eastand sometimes found in the Northern and Western Himalaya [151]. The plant has been traditionally used as an analgesic, antiseptic, anti-flatulent, and as anti-seizureand anti-inflammation agents [138]. It has also been reported in prior studies that some plants in the Ferula genus possess both anti-hyperglycemic, hypolipidemic effects as well as prevent complications [152, 153]. However, only few reports are

available for the antidiabetic activities of *F. gummosa*. A dose dependent (100-400 mg/kg) antihyperglycemic effect was shown in diabetic rats after administration of ethanol extract of *F. gummosa* oleo-resin [154]. Conversely, there was an insignificant effect on blood glucose levels of diabetic rats [155]. Mechanism of action of selected Apiacaea species used in the management of diabetes mellitus is summarized in Table 1.

Table 1: Possible mechanisms of action of selected antidiabetic Apiaceae species

S/N	Plant names	Parts used	Possible mechanisms of action of hypoglycemic activities	References
1.	*Anethum graveolens*	Aerial parts and seeds	Influences antioxidant capacity and modifies genes in the glucose and lipid pathways.	60
2.	*Coriandrum sativum* L.	Leaves	Enhances and regenerates pancreatic β- cells and inhibits α-glucosidase in the small intestine.	73, 74, 75, 76, 78
3.	*Cuminum cyminum*	Seeds	Inhibits aldose reductase and α-glucosidase enzyme activities.	85, 87
4.	*Carum carvi* L.	Seeds	Inhibition of hepatic glucose production and or enhancing glucose utilization by peripheral tissues. It may also inhibit renal glucose absorption.	156, 157
5.	*Foeniculum vulgare* Mill.	Aerial parts and seeds	Increases glucose tolerance in diabeticconditions. Decreases glutathione peroxidase activity in diabetic conditions.	102, 104

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Table 1 continued: Possible mechanisms of action of selected antidiabetic Apiaceae species

6.	*Daucus carota*	Seeds	Improves pancreas asinuses and islets. Enhances glucose uptake in adipocytes.	110, 111, 112
7.	*Pimpinella anisum L*	Seeds	Inhibition of α-amylase, α- glucosidase and pancreatic lipase. Enhances NaKATPase which increases sodium gradient for glucose transport	117, 118 119, 120
8.	*Apium graveolens* L.	Leaves and seeds	Increases insulin secretion and improves pancreatic cells multiplication and repair. Alters glucose absorption in the intestine.	128 129, 130
9.	*Centella asiatica*	Whole plant	Inhibits glucose absorption by inhibiting intestinal disaccharidase, α-amylase and fiber binding.	146
10.	*Ferula gummosa*	Leaves	Inhibits or slows down diabetic complicationsby inhibiting the development of hyperglycemia and decreasing kidney and liver damage caused by oxidative stress.	154

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Conclusion

Currently, there is an increase in the number of people suffering from diabetes all over the world and there are quite good number oral synthetic hypoglycemic drugs. Although the contribution of synthetic antidiabetics cannot be under-estimated, it is a fact that most of the uses of these drugs are associated with many adverse effects that are detrimental to human health. On the other hand, plants are a rich source of natural hypoglycemic agents that are easily accessible and with minimal side effects. Furthermore, the Apiaceae family is highly reputed for its antidiabetic potential. It is therefore imperative to compile some Apiaceae plant species with antidiabetic potentials.

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CHAPTER THREE

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CHAPTER THREE

PHYTOCHEMICAL, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF HETEROMORPHA ARBORESCENS LEAVES

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RESEARCH ARTICLE

Assessment of the phytochemical, antioxidant and antibacterial activities of *Heteromorpha arborescens* (Spreng.) Cham & Schltdl. leaf extracts [version 1; peer review: 2 approved]

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Abstract

Background: *Heteromorpha arborescens* (Spreng.) Cham. and Schltdl (Apiaceae) is widely used traditionally for the treatment of a wide range of diseases in Southern and Eastern Africa. Although previous studies have reported the biological activities of hexane, ethyl acetate and methanol extracts of *H. arborescens* leaves, there is no scientific information on the phytochemical contents, antioxidant and antibacterial activities of acetone, ethanol, aqueous and blanched extracts. This study is therefore aimed to investigate and compare the phytochemical contents, antioxidant and antibacterial activities of acetone, ethanol, aqueous and blanched extracts of *H. arborescens* leaves.

Methods: Phytochemical analysis for the total phenolic, flavonoid, proanthocyanidin, alkaloid and saponin contents of all the fractions were determined by spectroscopic methods, while the free radical scavenging potential of the extracts were evaluated using DPPH, ABTS radical scavenging and total antioxidant capacity assays. Micro dilution method was used to determine the Minimum Inhibitory Concentrations (MIC) of *H. arborescens* leaf extracts against *Bacillus pumilus, Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*.

Results: Total phenol content of the extracts ranged between 15.10 mg GAE/g- 42.50 mg GAE/g, proanthocyanidin was 459-8402.1 mg QE/g, and flavonoid content of 109.24-235.79 mg QE/g. In addition, alkaloids (5.59%) and saponins (23.33%) were present in significant amounts. Based on the IC_{50} values, the ethanol extract exhibited the highest total antioxidant activity (0.013 mg/mL) with highest inhibition against DPPH and ABTS radicals (0.06 and 0.049 mg/mL respectively). Considerable antibacterial activities were observed in the acetone,

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ethanol and blanched extracts with MIC values ranging from 1.563-12.5 mg/mL; however, the aqueous extract was inactive against all the bacteria strains.
Conclusion: The study suggests that *H. arborescens* leaves could be a valuable source of bioactive compounds. Although the blanching process significantly decreased polyphenolic contents and antioxidant activities of the extracts, it increased the antibacterial compounds.

Keywords
Phytochemicals, *Heteromorpha arborescens*, antioxidant, antibacterial, bioactives, blanching, traditional, medicines.

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Introduction

Plants from the Apiaceae family are generally known to be rich sources of phytochemicals and antioxidants and are commonly used as food, flavoring agents and medicine[1]. These bioactive constituents may contribute significantly to the protec- tion of humans from a wide range of diseases[2–4]. *Heteromorpha arborescens* (Spreng.) Cham. and Schltdl otherwise referred to as parsley tree in English, "wildepitersielie" in Afrikaans and "umbangandlala" in Xhosa is a large shrub or small tree thatbelongs to the Apiaceae family[3].

The leaves vary from simple to compound and the flowers are small, greenish to yellowish in colour, occurring in compound umbels, with waxy bark which is smooth or glossy in texture[5]. The species is generally considered as a medici- nally important plant throughout Africa since almost all its parts are traditionally used for the treatment of different ailments[6]. In South Africa, the leaves and roots are used for blood purification, diabetes and shortness of breath[7–9]. Thebark, leaves and roots are also used for respiratory problems in Kenya, Lesotho and Tanzania[9]. The leaves are consumed as vegetables in Kenya[10], while the roots are given to malnourished children in Botswana and Swaziland[11]. The plant is also used to treat abdominal pains, dysmenorrhea, nervous and mental disorders, as well as a vermicide in children[12].

The healing ability of the plant is attributed to the presence of bioactive compounds, such as tannins, phenols, alkaloids, saponin, flavonoids, and proanthocyanidin. Although very scanty, previous scientific reports exist on the phenolic contents, antioxidant[13,14] and antibacterial activities[3] of hexane, ethyl acetate and methanol extracts of *H. arborescens* leaves. To the best of our knowledge no study exists on compari- son of polyphenolic contents, antioxidant and antibacterial activities of blanched, aqueous, acetone and ethanol extracts of *H. arborescens* leaves. The present study was therefore conducted to determine the phytochemical contents, antioxidant and antibacterial activities of blanched, aqueous, acetone and ethanol extracts of *H. arborescens* leaves.

Methods

Ethical approval

Ethical approval was granted by the University of Fort Hare Animal and Plant Use Research Ethics Committee, South Africa with protocol number OTA011SABI01/19/E.

Chemical and reagents

All reagents and chemicals including gallic acid, rutin, quer-cetin, aluminum chloride ($AlCl_3$), ferric chloride ($FeCl_3.7H_20$), Folin-Ciocalteu, oxalic acid, trichloroacetic acid (TCA), sulphu- ric acid (H_2SO_4), hydrochloric acid (HCl), Na_2CO_3, ammonium molybdate, potassium ferricyanide ($K_3Fe(CN)_6$), catechin, 2,2- diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethyl-obenzothiazoline-6-sulphonic acid diammonium salt) (ABTS) are products of Sigma-Aldrich, South Africa. Other chemicals such as anhydrous sodium carbonate, sodium nitrite ($NaNO_2$), ethanol, methanol, n-butanol, sodium acetate, butylated hydrox- ytoluene (BHT), diethyl ether, glacial acetic acid, sulfanilic acid, potassium persulphate, sodium nitroprusside were also purchased from the same company. All the reagents used were of analytical grade. The bacterial strains including; *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus*, ATCC29213, *Pseudomonas aeruginosa* ATCC 19582, *Escherichiacoli* 25922, *Bacillus pumilus* ATCC 14884, *Staphylococcusepidermis* ATCC 12228 were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, South Africa.

Plant material

Fresh leaves of *H. arborescens* were obtained in the month of June, 2019 from an area located on latitude 32° 47′ 50.4″ S, 26° 52′ 41.8″ E along Hogsback road, Alice Town, Eastern Cape, South Africa. The plant was authenticated by a taxono- mist at the University of Fort Hare and a voucher specimen was preserved in the Giffen Herbarium and assigned number Abif2019/03.

Preparation of plant extracts

Fresh *H. arborescens* leaves were oven dried at 40^0C and crushed to coarse powder. 100 g of the powdered plant material was extracted separately in 500 mL acetone, ethanol and water for 24 hours on an orbital shaker. An equal weight of the fresh leaves was immersed into 500 mL of hot water (80^0C) for 5 min to simulate home cooking and ground with a blender. The extracts were filtered under pressure using a Buchner funnel and Whatman filter paper (150 mm); the acetone and ethanol extracts were concentrated to dryness using a rotary evaporator while the aqueous extract and blanched sample were subjected to freeze drying. The extracts were thereafter stored at 4^0C until further analysis.

Phytochemical screening

Determination of total phenolic content. The total phenolic content was determined by spectrometry using Folin-Ciocalteu reagent. Briefly, 0.5 mL of extract (1mg/mL) and gallic acid (0.02 to 0.1mg/mL) were put separately in test tubes. Thereafter, 2.5mL of 10% Folin-Ciocalteu reagent was added into the test tubes, after which 2.5 mL of 7.5% sodium carbonate solution was added to the mixture, stirred and incubated at 40^0C for 30 min. The absorbance was then measured at 760 nm using a Hewlett Packard VR-2000 spectrophotometer. All samples were analysed in triplicate. Total phenolic content was expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) with the standard curve:y =10.875 x+ 0.1025, $R^2 = 0.996$.

Where R is the determined coefficient, x is the concentration, and y is the absorbance.

Determination of flavonoids content. The amount of flavonoids present in the extract was determined using the aluminium chloride ($AlCl_3$) colorimetric assay, as described by Majouli *et al.*[15]. A mixture of 0.5 mL of extract was added to an equal volume of 2% $AlCl_3$ solution. The mixture was incubated for 10 min and vigorously shaken, after which the absorbance was measured at 420 nm. Varying concentrations of the standard (quercetin) were also prepared with the same method. All samples were analysed in triplicates. Flavonoid content was

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expressed as milligrams of quercetin equivalent per gram (mg QE/g) with the standard curve: y = 2.9422x - 0.4438, R^2 = 0.913

Where R is the determined coefficient, x is the concentration, and y is the absorbance.

Determination of proanthocyanidin (condensed tannins). Proanthocyanidin content was determined as described by Sagbo *et al.*[16]. Briefly, a mixture of 3mL of 4% vanillin- methanol solution and 1.5mL of HCl was added to 0.5 mL of each extract. The solution was stirred and incubated at 27°C for 15 min, after which absorbance was measured at 500 nm. All samples were analysed in triplicate and the amount of proanthocyanidin was expressed as mg/g dry weight ofquercetin equivalent (mg QE/g) of the extract with the standard curve: y = 0.0252 x + 0.0482, R^2 = 0.9005.

Where R is the determined coefficient, x is the concentration, and y is the absorbance.

Determination of alkaloid content. Alkaloid content of *H. arborescens* leaves was determined as previously described by Abifarin *et al.*[17]. 0.5 g of the pulverized leaves was mixed with 200mL of 10% acetic acid in ethanol. The mixture was covered, incubated at room temperature for 4 h, filtered and concentrated to about a quarter of its original volume in a water bath. To the extract, concentrated ammonium hydroxide was added in drops till precipitation was complete. After the solu- tion was allowed to settle, precipitates obtained were washed with dilute ammonium hydroxide and then filtered. The resi- due was dried in an oven (40°C), weighed and the alkaloid content was determined using the following formula:

% Alkaloid = weight of precipitate / initial weight of sample × 100.

Determination of saponin content. Saponin content of *H. arborescens* leaves was determined as previously described by Unuofin *et al.*[18]. Briefly, 0.5 g of pulverized *H. arborescens* leaves was measured into 50 mL of 20% ethanol prepared indistilled water. The mixture was heated in a hot water bath for 4 h at 55°C. The mixture was filtered, and the residue extracted again with another 50 mL of 20% ethanol. The two filtrates were combined and reduced to 20 mL over a hot water bath(90°C). The concentrated solution obtained was poured into a 250 mL separating funnel containing 20 mL of diethyl ether. The aqueous layer was collected while the ether layer was discarded. 20 mL of n-butanol was added to the filtrate and then washed thrice with 10 mL of 5% sodium chloride. The mixture was heated in an oven (40°C) to constant weight. The percentage saponin content of the sample was calculated using the following formula:

% Saponins = weight of final filtrate / weight of sample x 100

Antioxidant activity

DPPH radical scavenging activity. DPPH radical scaveng- ing activity for each plant extract was determined as previously described by Ohikhena *et al.*[19]. Briefly, a reaction mixture containing 2.5 mL of DPPH solution (0.13 mM) and 2.5 mL of each plant extract or standard (rutin and BHT) –dissolved in methanol at varying concentrations (0.005, 0.01, 0.02, 0.04, 0.08 mg/mL), stirred and kept in the dark for 30 min. The absorbance was measured at 517 nm and DPPH radical scavenging activity was calculated as:

% DPPH scavenging activity = [(Abs DPPH − Abs Sample) /Abs DPPH)] × 100

Where Abs control is the absorbance of DPPH radical + metha- nol; Abs sample is the absorbance of DPPH radical + sample extract/standard.

ABTS radical scavenging assay. ABTS scavenging activity of the different plant extracts was determined as described by Unuofin *et al.*[20]. A mixture was prepared by reacting 7 mM ABTS solution and 2.45 mM K_2S_2O8 (1:1), which was kept inthe dark for 12 hours to produce a bluish green coloration. The solution was adjusted with methanol until an absorbance of0.700 ± 0.01 at 734 nm was obtained. 1mL of plant extract was allowed to react with 1 mL of the working solution and the absorbance was measured at 734 nm after 7 min. The ABTS scavenging capacity of the extracts were compared with that of BHT and rutin and percentage inhibition was calculated as:

ABTS radical scavenging activity (%) = [(Abs control − Abs sample) / (Abs control)] × 100.

Where Abs control is the absorbance of ABTS radical + metha- nol; Abs sample is the absorbance of ABTS radical + sample extract/standard.

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Total antioxidant capacity (phosphomolybdenum) assay. The total antioxidant capacity was determined by the method described by Abifarin *et al.*[17]. Briefly, 0.3 mL of the extracts and standard (0.025-0.4 mg/mL) were measured into separate test tubes and each was dissolved in 3 mL of reagent solution (0.6 M sulfuric acid, 4 mM ammonium molybdate, and 28 mM sodium phosphate). The test tubes were incubated at 90°C in a water bath for 90 min, allowed to cool to room temperature and the absorbance was measured at 695 nm. Rutin and BHT were used as standards. The percentage inhibition (%TAC) wascalculated as:

%TAC = [(Absorbance of sample − Absorbance of control) / (absorbance of sample)] × 100

Determination of antibacterial activity

Microorganisms and media. The bacteria used in this study were chosen primarily on the basis of their importance as opportunistic microorganisms for humans with diabetes mellitus. The bacterial strains include: *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus*, ATCC 29213, *Pseudomonas aeruginosa* ATCC 19582, *Escherichia coli* 25922, *Bacillus pumilus* ATCC 14884, *Staphylococcus epidermidis* ATCC 12228.

Minimum Inhibitory Concentration (MIC). The MIC of the extracts were evaluated against selected Gram positive (*Bacillus*

pumilus, *Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli*) by microdilution method, as described by Mohsenipour *et al.*[21]. The bacterial concentration in the inoculum was standardized at 0.5 McFarland turbidity scale, (1×10^8 CFU/ml).

Firstly, sterile round bottom 96-well plates were filled with 100 µl of distilled water. 100 µl stock fractions of 50 mg/mL of plant extract and standard (Erythromycin) were then added into the first row. The fractions were serially diluted to make varying concentrations of the plant extracts (0.78125–12.5 mg/mL) and standard (0.0625–1 µg/mL) and then 50 µl of inoculums was added into the wells. Thereafter, 100 µl sterile nutrient broth culture medium plus 50 µl of the culture of each organism was added into each well and the inoculated micro plates were incubated at 37°C for 24 h. 1% DMSO was used as the negative control while 100 µl of inoculums only was used as the growth hormone. The plates were incubated for 24 h at 37˚C, and subsequently 40 µl of 0.4 mg/mL INT dye was added to each well. The plates were gently agitated and incubated for another 30 min. The MICs were then determined as the lowest concentrations at which there was no indication of colour change to pink (no bacterial growth).

Statistical analyses

All data were expressed as mean ± standard deviation (SD)of triplicates and subjected to one-way analysis of variance (ANOVA). Where the data showed significant difference ($p < 0.05$) among the extracts, a mean separation was done using Fischer's Least Significant Difference. MINITAB 17 statistical package was used for analysis.

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Results

Phytochemical contents

The total phenol, proanthocyanidin and flavonoid contents of *H. arborescens* leaf extracts are presented in Table 1. Thehighest phenol content was found in the ethanol extract (42.50 mg GAE/g), followed by the aqueous extract (34.24 mg GAE/g), blanched extract (21.42 mg GAE/g) and acetone extract (15.10 mg GAE/g). Ethanol extract (8402.1 mg QE/g) showed the highest proanthocyanidin content followed by acetone extract (4923 mg QE/g DW), blanched extract (928.6 mg QE/g) and aqueous extract (459 mg QE/g). The concentration of flavonoid was highest in the ethanol extract (235.79 mg QE/g), followed by acetone extract (230.52 mg QE/g), aqueous extract (154.95 mg QE/g) and blanched extract (109.24 mg QE/g). Quantitative determination of the alkaloid and saponin contents of *H. arborescens* leaves also revealed low alkaloid content (5.59%) with considerable amount of saponin (23.33%).

Antioxidant activities

ABTS radical scavenging activity. The ABTS radical scavenging activities of the extracts and standards (BHT and rutin) are presented in Figure 1. The samples exhibited significant ABTS radical scavenging activities, which increased in a concentration dependent manner. Although the standards showed higher ABTS inhibitory potentials than the extracts, the highest inhibitory capacity of the extracts was observed in the ethanol and aqueous extracts. Based on the IC_{50} (Table 2), ABTS scavenging activity of the samples was in the order: BHT > rutin > ethanol & aqueous > acetone > blanched.

Total antioxidant capacity. The total antioxidant capacity of the extracts and standards (BHT and rutin) are shown in Figure 2. There was a dose dependent increase in total antioxidant capacity of the samples, with the ethanol extract showing the highest antioxidant capacity when compared withother extracts. Based on the IC_{50}, (Table 2), the values of the total antioxidant capacity of the standards and extracts were in the order: BHT > ethanol > rutin > aqueous > acetone > blanched.

DPPH scavenging activity. There was a dose-dependent increase in percentage inhibitory activity of the extracts and standards against DPPH radical (Figure 3). Ethanol extracts showed the highest DPPH radical inhibitory activity; even though the standards inhibited best when compared with the extracts. With respect to the IC_{50}, (Table 2) the inhibitory capacity of the standards and extracts were in the order: rutin > BHT > ethanol > aqueous > blanched > acetone.

Minimum inhibitory concentration. Antibacterial activity of erythromycin (standard) and extracts of *H. arborescens*leaves are presented in Table 3. While all Gram-positive and Gram-negative bacteria strains tested were resistant to the

Table 1. Total phenol, proanthocyanidin, flavonoid, alkaloids and saponin contents of *H. arborescens* leaves.

Extracts	Phenol (mg GAE/g)	Proanthocyanidins (mg QE/g)	Flavonoids (mg QE/g)	Alkaloids (%)	Saponins (%)
Aqueous	34.24±0.46[a]	459±49.9[a]	154.95±9.99[a]		
Acetone	15.10±1.38[b]	4923±579[b]	230.52±0.59[b]		
Blanched	21.42±0.28[c]	928.6±90.9[c]	109.24±0.17[c]		
Ethanol	42.50±0.69[d]	8402.1±89.5[d]	235.79±4.52[d]		
Pulverized	*	*	*	5.59±0.45	23.33±2.3

The results are expressed as mean ± standard deviation (n=3). Values with different superscripts are significantly different ($P < 0.05$) across the different extracts. *- not determined.

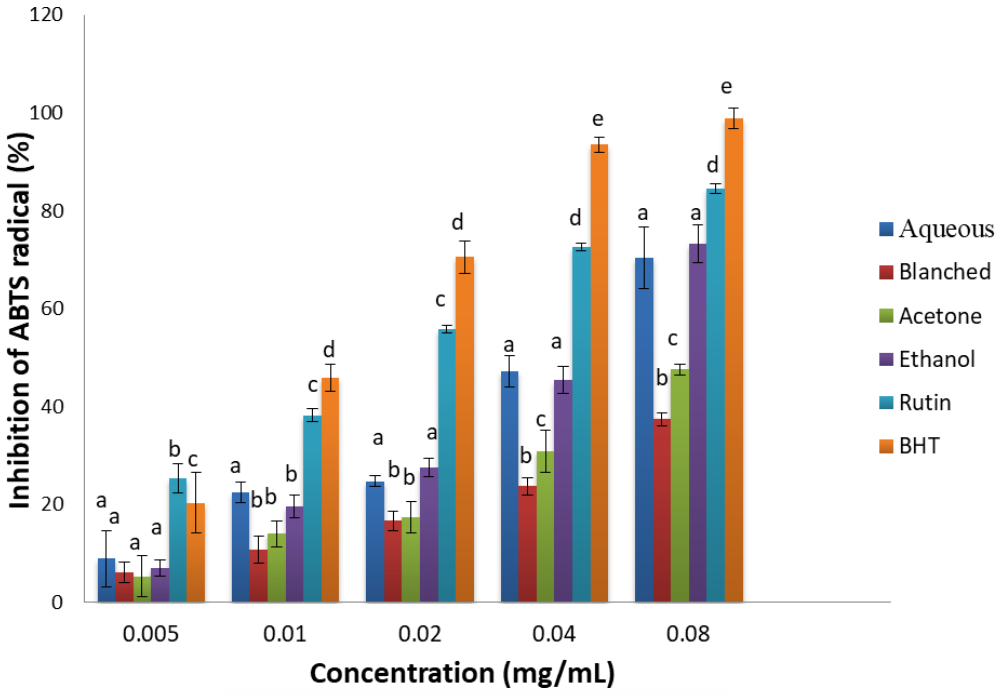

Figure 1. ABTS radical scavenging activity of extracts of *H. arborescens* leaf extracts and standards (rutin and BHT). Results are expressed as mean ± standard deviation (n=3). Columns with different letters are significantly different (P < 0.05) across the different samples.

Table 2. IC_{50} values of various solvent extracts of *H. arborescens* leaf extracts and standards.

Extracts/standard	DPPH (mg/mL)	ABTS (mg/mL)	TAC (mg/mL)
Aqueous	0.1	0.049	0.024
Blanched	0.12	0.109	0.152
Acetone	0.21	0.081	0.046
Ethanol	0.06	0.049	0.013
Rutin	0.026	0.024	0.017
BHT	0.031	0.012	0.012

aqueous extract at all the concentrations tested, the blanched, acetone, and ethanol extracts showed considerable antibacterial activities with MICs ranging from 1.563-12.5 mg/mL. Ethanol extract showed the best antibacterial activity against *B. pumilus*, *S. epidermidis*, *S. aureus* and *P. aeruginosa*, exhibiting a low MIC of (1.563 and 3.125 mg/mL). Generally speaking, the Gram-negative bacteria were observed to be more resistant against the extracts tested.

Discussion

Plant extracts have been reported to have numerous protective roles due to their phytochemical contents, which contributes to a large extent towards their antioxidant and antimicrobial activities[22]. Oxidative stress, which leads to the production of free radicals in the body, plays a major role in the development of chronic and degenerative ailments such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases[23]. Antioxidants play a vital role in deactivating and scavenging of the free radicals in order to reduce the risk of these chronic diseases[24].

In the present study, the phytochemical, antioxidant and antibacterial activities of the acetone, ethanol, aqueous and blanched extracts of *H. arborescens* leaves were assessed. Phytochemical constituents varied significantly in all theextracts, which revealed the presence of phenols, flavonoids

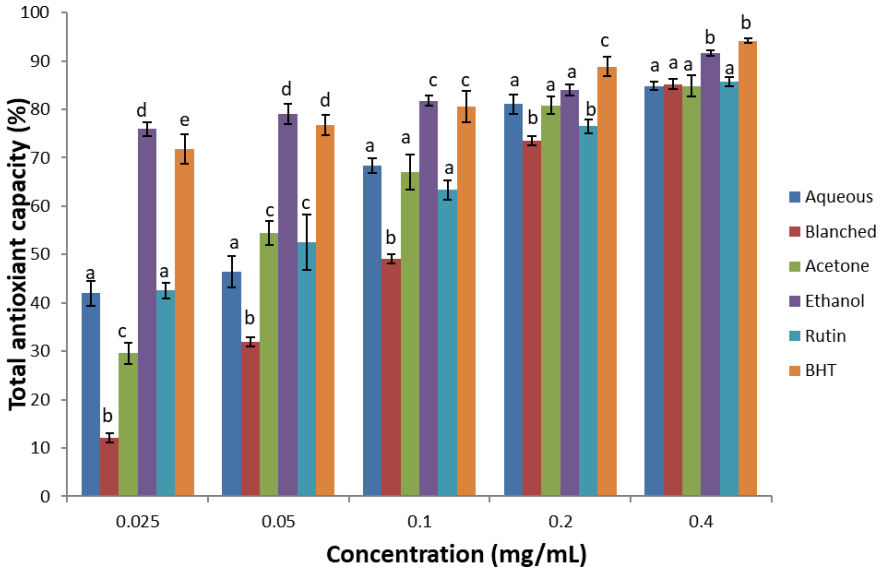

Figure 2. Total antioxidant capacity of *H. arborescens* leaf extracts and standards (Rutin and BHT). Results are expressed as mean ± standard deviation (n=3). Columns with different letters are significantly different ($P < 0.05$) across the different samples.

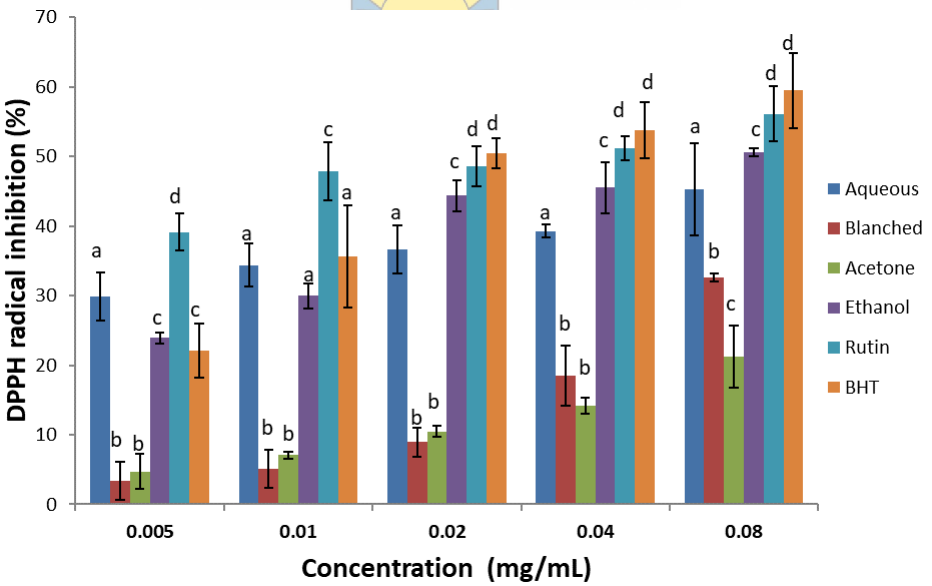

Figure 3. DPPH scavenging activity of *H. arborescens* leaf extracts and standards (Rutin and BHT). Results are expressed as mean ± standard deviation (n=3). Columns with different letters are significantly different ($P < 0.05$) across the different samples.

and proanthocyanidin in considerable amounts. The variation in the phytochemical constituents could be attributed to differences in the extracting capabilities of different solvents. Phenols, a major phytochemical found in plants have been widely studied because of their ability to reduce oxidative stress- related degenerative diseases, including cancer. Flavonoids are a group of hydroxylated phenolics, and their beneficial effects are linked to their antioxidant, antibacterial, anticancer, anti-inflammatory[25,26] anti-hypertensive, and cardio protective activities[27,28]. Proanthocyanidins are a class of compounds belonging to the flavonoid family, with protective effects against tissue damage and cancer, and they also improve blood circulation by strengthening the capillaries, arteries and veins[29].

Ethanol has been reported to be suitable for extraction of compounds with a wide range of polarity while water is suitable

Table 3. Minimum Inhibitory Concentration (MIC) values of *H. arborescens* leaf extracts against selected bacterial strains.

Samples	Bacterial strains (MIC) mg/mL					
	B. pumilus[a]	*S. epidermidis*[a]	*S. aureus*[a]	*E.coli*[b]	*K. pneumoniae*[b]	*P. aeruginosa*[b]
Aqueous	Na	Na	Na	Na	Na	Na
Blanched	12.5	6.25	12.5	12.5	12.5	12.5
Acetone	6.25	1.563	3.125	12.5	6.25	6.25
Etdanol	3.125	3.125	1.563	6.25	6.25	12.5
Erythromycin (µg/mL)	0.125	0.0625	0.0625	0.0625	0.125	0.125

Bacillus pumilus, Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Klebselia pneumoniae.

Bacteria [a]- Gram positive. Bacteria [b]- Gram negative. Na- not active at tde highest concentration tested. Experiments were performed in triplicates..

for very polar compounds[30]. Consequently, a higher quantityof phenolic compounds, proanthocyanidin and flavonoids were observed in the ethanol samples when compared to the acetone, blanched and aqueous extracts; hence, better activ- ity. This is in agreement with previous reports that ethanol is more suitable for the extraction of phenolic compoundsin plants[30–32]. Furthermore, high phenolic compounds were indicated in ethyl acetate and methanol extracts, suggesting the reason for its high antioxidant activity[13]. Flavonoid con- tent of acetone extract of *H. arborescens* was comparable to results obtained by Elisha *et al.*[13]; however, total phenolic content was much higher as opposed to the current study.

Our results indicate that blanching resulted in a significant decrease in total phenols and flavonoids with significant increase in proanthocyanidin content. This correlates with similar observations by Korus *et al.*[33]. Jaiswal *et al.*[34] and Irondi *et al.*[35]. Losses of polyphenolic compounds upon blanching was also observed in some cruciferous vegetables, such as spinach[36], kale, broccoli[37] and cauliflower[38,39]. The decrease in polyphenolic contents could be attributed to the lossof water soluble vitamins and nitrogen compounds during the blanching process[34]. Contrary to the findings of this study, some researchers have claimed that the heating process may alter the cell membrane, causing a release of some membrane- bound phytochemicals which may increase bioavailability as observed by Jimenez-Monreal *et al.*[40]. Similarly, Ma *et al.*[41] observed an increase in polyphenolic contents of *Daucus carota* L. juice after blanching (at 95^0C for 3 min).

Saponins possess medicinal properties such as anti-inflammatory, antibacterial, anticancer and cytotoxic activities[42]. Alkaloids have also been shown to exhibit antioxidant properties by reducing oxidative damage induced by hydrogen peroxide[43]. The amount of alkaloids and saponin exhibited by
H. arborescens leaves is promising in playing the aforemen- tioned biological roles.

Further assessment of the antioxidant activities of the extracts revealed the free radical scavenging potential of the extracts, which varied among the methods used. This variation was due to the fact that antioxidants are able to neutralize free radicals by different modes of action such as transition metal chelation, singlet oxygen quenchers, and donation of hydrogen[44].

The total antioxidant capacity and scavenging activity of the extracts against DPPH+ and ABTS+ were compared to the standards (BHT and rutin) and the extracts showed consider- able antioxidant activity. Since positive correlation between polyphenolic compounds and antioxidant activities have been reported[43,45,46]; the highest antioxidant activity exhibited by the ethanol extract could be attributed to its high polyphenolic compounds, as shown in this study. In terms of the relativelyhigher DPPH and ABTS inhibitory potential of the ethanol extract, these results are in agreement with antioxidant activity of other Apiaceae species such as: *Coriandrum sativum*[47], *Ammi majus* L.[48],*Daucus carota* L.[49], *Ferula gummosa*[50], *Seseli libanotis* (L.) Koch[51], *Foeniculum vulgare* and *Anethum graveolens*[1]. This strengthens the suggestion that the most important bioactive potentials are observed in the plant extracts with high amounts of phenolics and flavonoids. This finding therefore provides some scientific verification for the biological activities of *H. arborescens* leaves.

In recent years, more attention has been given to the develop- ment of antibacterial drugs from medicinal plants instead of synthetic ones. The antibacterial activities of plant extractshave been confirmed against an array of both Gram-positive and Gram-negative bacteria[52]. Previous studies have established that the crude, hexane, ethyl acetate, chloroform and butanol extracts reveal antibacterial activities against *E. coli*, *S. aureus* and *P. aeruginosa*. However, *P. aeruginosa* was more sus- ceptible to three out of the extracts with the butanol fraction showing the highest antibacterial activity[53]. The resistance
|of all the bacterial strains to the aqueous extract at all concentrations tested and the significant antibacterial activity of the blanched extract obtained in the present study, could be attributed to poor solubility of the antibacterial com- pounds in cold water and high extractive power of boiled water.

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In addition, the higher antibacterial activity observed in the acetone and ethanol extracts could be due to relatively higherflavonoid and phenolic contents present[54].

Our findings are in agreement with McGaw *et al.*[55] who reported that in the evaluation of antibacterial activities of hexane, ethanol and aqueous leaf extracts of *H. arborescens* against *Bacillus subtilis, S. aureus, E. coli,* and *K. pneumoniae*, only ethanol extract exhibited antibacterial activities. These findings correlate with observation made by Wigmore *et al.*[56] that aqueous extracts of plants showed less antibacterial activity when compared with other solvents. Furthermore, in agreement with our findings, the aqueous extract was inactive against all Gram-positive bacteria used. However, contrarily, the aqueous extract was active against *S. aureus* and *S. epidermidis*[3].

Conclusion

Our results clearly indicated that the extraction of phenolic compounds and their antioxidant capacity is highly dependent on the solvent of extraction. The study also revealed that *H. arborescens* aqueous, blanched and ethanol leaf extracts possess various levels and concentrations of phytochemi- cal constituents, which may be essential for human health. A positive correlation between polyphenolic contents, antioxidant and antibacterial activities in extracts of *H. arborescens* leaves was established, which indicates that certain phenolic compounds may be responsible for high antioxidant activity. It was also observed that the blanching process (at 80^0 C) significantly decreased polyphenolic content and antioxidant activities but increased antibacterial activity of *H. arborescens* leaves. This study therefore agrees with its reported use in traditional medicine for the treatment of some bacterial infections and diseases.

Data availability

Underlying data

Figshare: Absorbance values for antioxidant activity.xlsx, https://doi.org/10.6084/m9.figshare.12654479.v1[57].

This project contains the following underlying data:

- Replicate absorbance values for DPPH, TAC and ABTS
- Replicate phytochemical analysis results
- MIC values of antibacterial activity of the extracts

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

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Reviewer Report 02 October 2020

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✔ **Balasubramani Ravindran**
Department of Environmental Energy and Engineering, Kyonggi University, Suwon, South Korea

- **Title:** The title reflects sufficiently, the work done and reported by the authors.
- **Abstract:** Adequate
- **Introduction:** This is adequate with current and relevant citations.
- **Methods:** Methods used were sufficient and reproducible by others.
- **Results** were well presented with Tables and Figures where appropriate. These were also properly captioned and can stand alone.
- **Discussion** projected the results and made appropriate reference to similar works by other researchers.
  Replace part of lines 4 and 5 "Oxidative stress which leads to the production of free radicals in the body," with: Oxidative stress which occurs as a result of imbalance between production and accumulation of free radicals especially reactive oxygen species (ROS),
- **Conclusion** is adequate and reflected the findings of the study.
- I approve that the article be indexed.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Phytochemistry and microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 17 September 2020

https://doi.org/10.5256/f1000research.27806.r70661

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Abimbola Oloye
Department of Veterinary Surgery and Theriogenology, College of Veterinary Medicine, Federal University Of Agriculture Abeokuta, Ogun State, Nigeria

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The work prescribed an ethno-medical alternative to treating oxidative stress and perhaps bacterial infections which are largely considered safer, easy to access, and cheaper when compared to most orthodox prescriptions.
The wideness of the spread of *H. arborescens* especially in southern and eastern Africa makes it fit for consideration as an alternative to some chemically compounded drugs.

The conclusions drawn about the leaf extracts of this plant by the authors, especially in establishing a relationship between the phenolic content of the extracts and their potencies as antioxidant and antibacterial, were appropriate. However, I wished the authors could give consideration to seasonal variation in concentration statuses of polyphenolics and other bioactive compounds of the plant's leaves. The month of June during which the study was carried out in the Eastern Cape, South Africa is the winter period, it would be revealing repeating the study in summer (between November and April) and drawing comparisons.

Studying the effect of blanching was a brilliant one. Indigenes mostly blanch their veggies in the process of making them into edibles or in preparing them as oral medicines for the locals.
Blanching, as against chemical extraction, deactivates bioactive compounds hence reduces the

therapeutic effects associated with the plant. This has been implied in the study. Interestingly, however, the antibacterial effects of the plant seemed enhanced with blanching. The authors attributed this to enhanced extractive proficiency of water at boiling water temperature. In addition to this, however, I also think the heat-stabile bioactive antibacterial factor should be identified and pinned down.

The authors should reconcile the justification for the study enumerated in the abstract with the one stated in the introductory section of the manuscript. The assertion that no literature enumerating the phytochemical contents, antioxidative and antibacterial properties of acetone, ethanol and aqueous exist may not be totally correct. There are few studies on these. (See Citations).[1,2] However, they may be correct about the blanched extract. The justification in the introductory section of the main text appears more appropriate.

The concentrations of saponin and alkaloids determined in the study were said to be enough to elicit the bioactivities associated with the two compounds. Is there any empirical reference that corroborates this assertion especially considering the fact that the authors considered the alkaloid concentration as low?

A minor correction: The MIC of Ethanol extract against *P. aeruginosa* was 12.5 and this did not fall within the range of 1.563- 3.125 stated in the result. This, therefore, revealed an ethanol extract that had a weak antibacterial effect on *P. aeruginosa*. The authors should take a look at this.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

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Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Reproductive toxicology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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CHAPTER FOUR

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CHAPTER FOUR

CHEMICAL COMPOSITION OF ESSENTIAL OILS OBTAINED FROM *HETEROMORPHA ARBORESCENS* (SPRENG.) CHAM & SCHLTDL LEAVES USING TWO EXTRACTION METHODS

This chapter has been published in The Scientific World Journal

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Volume 2020, Article ID 9232810, 6 pages
https://doi.org/10.1155/2020/9232810

Research Article

Chemical Composition of Essential Oils Obtained from *Heteromorpha arborescens* (Spreng.) Cham. and Schltdl LeavesUsing Two Extraction Methods

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Abstract

This study was aimed at comparing the essential oils obtained from *Heteromorpha arborescens* leaves by Solvent-Free Microwave Extraction (SFME) and Hydrodistillation (HD) methods in terms of their chemical compositions, yield, CO_2 emission, and energy consumption. The solvent-free microwave extraction method indicated a higher oil yield of 0.7 mL/200 g (0.35%) as compared to 0.59 mL/200 g (0.295%) obtained through hydrodistillation. GC-MS analysis of the oils revealed a total of 52 chemical components from both methods with the presence of 35 (96.52%) and 30 (71.15%) chemical constituents for HD and SFME, respectively. The major constituents observed in the essential oil extracted by SFME methods include α-pinene (6%), D-limonene (11.27%), β-ocimene (9.09%), β-phellandrene (6.33%), β-mycene (8.49%), caryophyllene (5.96%), and camphene (4.28%). However, in the hydrodistillation method, the oil was majorly composed of α-pinene (4.41%), β-pinene (10.68%), β-ocimene (6.30%), germacrene- D (5.09%), humulene (5.55%), and α-elemene (6.18%). The SFME method was better in terms of saving energy (0.25 kWh against 4.2 kWh of energy consumed), reduced CO_2 emission (200 g against 3360 g of CO_2), a higher yield, and better quality of essential oil due to the presence of higher valuable oxygenated compounds (8.52%) against that of the hydrodistillation method (2.96%). The SFME method is, therefore, a good alternative for extracting the oils of *H. arborescens* leaves since the essential oil yield is higher with more oxygenated compounds, considerable energy savings, lower cost, and reduced environmental burden at substantially reduced extraction time (30 min as opposed to 180 min).

1. Introduction

Plants have a long history of therapeutic use in the management of diseases since time immemorial. Essential oils are natural, complex, and aromatic volatile compounds produced by plants, and they are generally present at low concentrations. These compounds possess antimicrobial and antioxidant activities, and they function in the treatment of various kinds of diseases [1]. They are also beneficial in various food industries (for preserving food against oxidation), as alternatives to artificial chemicals in cosmetics and perfumery industries for the production of various cologne waters, bathing lotions, hair lotions, and shampoos, and as components of disinfectants and insecticides [2].

Among aromatic plant species, the genus *Heteromorpha* (Apiaceae) consists of seven species which are restricted to temperate and subtropical Africa and Southern Yemen [3]. There is an increased interest for Apiaceae, especially in food science, because members of this family such as fennel, celery, dill, carrot, and caraway are commonly used. Apart from the biologically active essential oils found in this family, they contain coumarins, polyacetylenes, flavonoids, sesquiterpenes, and phthalides [4].

Heteromorpha arborescens (Spreng.) Cham. and Schltdl (Apiaceae) can be referred to as a large shrub, small or medium deciduous tree [5]. It is regarded as an important medicinal plant throughout its distribution area in tropical Africa and has been used for the treatment of many ailments including helminthiasis [6], abdominal pains, infertility, nervous disorders, and tuberculosis [5]. The plant is popularly known in parts of Africa for its therapeutic benefits and is therefore included in the monographic treatment of medicinal plants of South African medicine [7].

From previous studies, the volatile oil of *H. arborescens* leaves is known to contain sabinene, δ-3-carene, myrcene, germacrene-D, limonene, (Z)-β-ocimene, β-phellandrene, and α-pinene as major constituents, and it possesses both antibacterial and antifungal activities [5]. Furthermore, the essential oil of *H. arborescens*is is vital in the development of new pharmaceutical and health products in Southern Africa for headache, inhalant, and aromatherapy [8].

Various conventional methods such as hydrodistillation, steam distillation, and cold pressing have been used for essential oil extraction in aromatic plants. However, the use of these methods has been disputable for successive determination of essential oil composition because the long extraction times at high temperatures may cause changes in the essential oil composition or degradation of unsaturated or esterified compounds and the loss of highly volatile compounds [9, 10]. These limitations have led to the development of the solvent-free microwave extraction method in order to reduce the extraction time and obtain better quality of essential oils [9, 10].

The solvent-free microwave extraction (SFME) set up is referred to as a "green technology" which involves a combination of heat from the microwave and dry distillation at atmospheric pressure [11]. It is a sustainable extraction method which relies on reduced energy consumption, ensuring a safe and high quality end product [12]. It involves heating the fresh plant material in a microwave reactor, devoid of water or any solvent at all for 30 min. The short-term heating of the water in the plant material expands the plant cells causing the plant cells and oil producing glands to release the oil from the plant material [9]. SFME method has widely been employed in the extraction of oils from *Melalaica leucadendra* L. [13], *Ocimum basilicum* L. [14], *Kananga odorata* [15], *Pogostemon cabin* [16], *Cuminumcyminum* L. and *Zanthoxylum bungeanum* Maxim [17], and many others. However, until now, there has been no studies on the comparison of the quality and yield of essential oils obtained from *H. arborescens* leaves using hydrodistillation and SFME methods. This work is therefore aimed at making a comparative study in terms of extraction yields and chemical compositions of essential oils obtained from *H. arborescens* leaves using hydrodistillation and solvent-free microwave extraction methods.

1.1. Plant Collection. Fresh leaves of *H. arborescens* were collected in June, 2019, on a site located on latitude 32°47′50.4″ S and 26°52′41.8″ E along Hogsback road, Alice Town, Eastern Cape, South Africa. The plant was authenticated by Prof. Cupido, a taxonomist in the Department of Botany, University of Fort Hare, and a voucher specimen (Abif2019/03) was deposited in the Giffen herbarium.

1.2. Chemicals. Distilled water and n-hexane (chemical formular- C_6H_{14}, MW-86.18 g/mol, BP- 68.73°C, density-655 kg/m^3, and purity-95%) used in the study were of analytical grade.

2. Methodology

2.1. Determination of Moisture Content. 200 g of the fresh *H. arborescens* leaves was weighed and recorded as W_f, dried at 80°C for 72 hours, and the dried leaves were then weighedand recorded as W_d. The moisture content of the leaves was calculated as

$$\text{Moisture content } (\%) = \frac{W_f - W_d}{W_f} \times 100. \quad (1)$$

2.2. Extraction by Hydrodistillation. 200 g of fresh *H. arborescens* leaves was subjected to hydrodistillation with a Clevenger-type apparatus as described by the European Pharmacopoeia and extracted with 1L of water for 180 min at 100°C. The essential oil was collected and analyzed by GC-MS.

2.3. Solvent-Free Microwave Extraction (SFME). 200 g of fresh *H. arborescens* leaves was placed into the reactor without addition of any solvent. Microwave extraction was performed using a Milestone MAO20-A apparatus; a multimode microwave reactor 2.45 GHz with a maximum delivered power of 500 W variable in 5 W increments. The essential oil was completely extracted at atmospheric pressure and 99.85°C (373 K) within 30 min; collected, and subjected to GC-MS analysis.

2.4. Energy Consumption and CO_2 Emission. Power consumption was 500 W and 1400 W for SFME and hydrodistillation methods, respectively. The energy consumption and CO_2 emission were calculated according to previous literature as energy consumption (kWh)Pt/1000 and to obtain 1 kWh from the combustion of coal or fossil fuel, and 800 g of CO_2 will be emitted into the atmosphere [18–20].

2.5. Extraction Yields. The extraction yields of the essential oils obtained from both methods were calculated as

$$\text{Extraction yield}(\%) = \frac{\text{Mass of extracted oil}}{\text{Mass of Fresh Leaves}} \times 100. \quad (2)$$

2.6. Gas Chromatography-Mass Spectroscopy Analysis. The essential oils extracted were separately analyzed by gas chromatography-mass spectroscopy (Agilent 6890 GC, coupled to an Agilent 5975 mass spectrometric detector). Gas

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TABLE 1: Chemical constituents of essential oils obtained from *H. arborescens* leaves by hydrodistillation and solvent-free microwave extraction methods.

S/N	Chemical compounds	Class of compounds	KI	RT	HD (%)	SFME (%)
1	3-Thujene	MH	932	3.805	0.21	—
2	α-Pinene	MH	936	3.893	6.0	4.41
3	Camphene	MH	948	4.028	4.28	0.58
4	2-Thujene	MH	952	4.195	2.33	—
5	β-Myrcene	MH	983	4.293	8.49	—
6	α-Phellandrene	MH	998	4.463	6.33	1.78
7	3-Carene	MH	1002	4.509	1.88	—
8	D-limonene	MH	1027	4.658	11.27	—
9	B-ocimene	MH	1050	4.771	9.09	6.30
10	B-pinene	MH	973	4.236	—	10.68
11	(−)-Myrtenal	MH	1488	5.998	0.72	—
12	o-Cymene	SH	1011	5.376	—	0.42
13	α-Copaene	SH	1390	7.314	3.30	2.30
14	β-cymene	SH	1026	5.038	1.31	0.47
16	Caryophyllene	SH	1419	7.644	5.96	3.64
17	β-Copaene	SH	1102	7.691	2.77	0.44
18	β-Gurjurene	SH	1089	7.393	—	1.42
19	Γ-Terpinene	MH	987	4.896	1.30	—
20	(+)-4-Carene	MH	998	5.127	3.80	—
21	Aromandendrene	SH	1106	7.772	0.31	1.52
22	α-Humulene	SH	1446	7.869	5.55	—
23	c-Muurolene	SH	1442	7.967	—	2.38
24	D-germacrene	SH	1481	8.035	8.92	5.09
25	c-Elemene	SH	1508	8.127	—	6.18
26	β-Cadinene	SH	1126	8.246	2.24	3.98
27	α-Calacorene	SH	1133	8.406	—	1.12
28	(-)-Palustrol	OM	1141	8.603	—	0.96
30	(-)4-Terpinol	OM	1182	5.837	0.69	—
31	α-Terpineol	OM	1197	5.920	0.30	—
32	Spatulenol	OS	1530	8.646	—	1.78
33	3,4-Nonadiene	MH	1048	6.143	0.16	—
34	Geraniol	OM	1065	6.319	0.10	—
35	Caryophyllene oxide	OS	1574	8.696	—	2.0
36	Ledol	OM	1148	8.751	—	2.77
37	Ledene	SH	1151	8.820	—	1.64
38	Isoledene	SH	1375	7.795	0.93	—
39	β-Cubenene	SH	1470	8.329	0.12	—
40	Nerodiol	OS	1133	8.407	0.87	—
41	β-Selinene	SH	1143	8.638	0.10	—
43	Napthalene	SH	1155	8.917	0.34	0.74
44	α-Cardinol	OM	1161	9.063	0.24	—
45	2-Isopropyl-5-methyl-9-methylene[4,4.0]dec-1-ene	SH	1159	8.990	0.41	—
46	Phytol	OD	1949	11.289	0.63	—
47	α-Himachalene	SH	1158	8.983	—	1.98
48	c-Gurjunene	SH	1163	9.092	—	1.57
49	Valencene	SH	1410	2.09	—	2.09
50	α-Bulnesene	SH	1240	10.889	—	1.16
51	Kaur-16-ene	OD	1214	10.994	—	0.91
52	D-Citral	OM	368	10.563	—	0.10
	Monoterpene hydrocarbons				57.17	23.75
	Sesquiterpenes				36.29	38.64
	Oxygenated compounds				2.96	8.52

MH- monoterpenes hydrocarbons, SH- sesquiterpenes hydrocarbons, OD- oxygenated diterpenes, OM- oxygenated monoterpenes, OS- oxygenated sesquiterpenes, KI- Kovatz Index, RT- retention time.

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chromatography-mass spectrometry was combined with a chromatography column HP-5.5% phenylmethylsiloxane, with 30 m length, 0.32 mm film thickness, 0.25 μm internal diameter. The injection port was held at 230°C, while the interface was at 280°C. The temperature was set from 50°C to 280°C at 10°C per minute, using helium as the carrier gas.

The chemical components of the essential oils were identified by comparing their mass spectra and retention

indices with those in PubChem, NIST, and Wiley libraries [21]. The spectrogram of each identified compound was determined by integration of the peak areas.

3. Results

The moisture content of the 200 g of *H. arborescens* leaves was 10.20 ± 0.95%, and a slightly higher oil yield of 0.7 mL/200 g (0.35%) was obtained through SFME after 30 min of extraction as compared to 0.59 mL/200 g (0.295%) obtained after 180 min by the hydrodistillation method. Theoils were pale-yellow liquids with fine aroma. For the energy consumption and environmental footprint, the amount of energy consumed and the quantity of carbon dioxide emitted into the atmosphere were higher for the hydrodistillation method (4.2 kWh and 3360 g CO_2/g of essential oil) as compared to the SFME method (0.25 kWh and 200 g CO_2/g of essential oil).

A total of 52 compounds obtained by hydrodistillation and solvent-free microwave extraction methods are summarized in Table 1. Thirty-five compounds representing 96.52% of the total essential oil present were obtained in the hydrodistillation method. More specifically, the dominant compounds present in the hydrodistilled oil were D-limonene (11.27%), β-ocimene (9.09%), D-germacrene (8.92%), β-myrcene (8.49%), and humulene (5.55%). The SFME method, on the other hand, gave 30 compounds which accounted for 71.15% of the total oil, while β-pinene (10.38%), c-elemene (6.18%), and D-germacrene (5.09%) were the major components. Thirteen (13) of the total components were common to oils extracted by both methods.

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4. Discussion

Some conventional methods used for the extraction of essential oils are hydrodistillation, steam distillation, Microwave-Assisted Extraction (MAE), and solvent extraction. Although these techniques have been used over time, they have some limitations such as longer time of extraction, low extraction effectiveness, reduced oil quality due to degradation of unsaturated or ester com- pounds through thermal or hydrolytic effects, and neg- ative impacts on the environment [22]. SFME and Microwave Hydrodiffusion and Gravity (MHG) methods are two new green techniques employed in the extraction of quality essential oils at a lower cost, reduced time, and environmental footprints [23].

The chemical compositions of the essential oil of *H. arborescens* leaves obtained by the solvent-free microwave extraction method have been compared with those which were obtained by the hydrodistillation method. Our study indicates that the essential oil yield obtained by the SFME method was slightly higher with shorter extraction time (0.35% for 30 min) when compared with that by the hydrodistillation method (0.295% for 180 min). The lower yield obtained by the hydrodistillation method may be attributed to increasing extraction time [24]. The present study also revealed that the SFME method reduced energy consumption and CO_2 emission by about 16 times. Similar observations were made in previous studies [25, 26].

The two oils were characterized with monoterpene hydrocarbons, sesquiterpenes, and oxygenated compounds. However, monoterpenes and sesquiterpenes were dominant in the SFME and hydrodistilled oils, respectively, but higher quantity of oxygenated compounds was present in the SFME-extracted oil. This is in agreement with the essential oil compositions of *Angelica sylvestris* L. var. sylvestris fruits [27], *Ferula glauca*, *Ferula arrigonii*, and *Ferula communis* [28], all members of the Apiaceae family. Lower quantity of monoterpenes with higher sesquiterpenes and oxygenated compounds was observed in the SFME-derived oil. Monoterpene hydrocarbons are less important than oxygenated compounds in terms of their impact on the pleasant aroma of essential oils, while the oxygenated compounds are highly fragrant and, hence, the most important [26]. Nevertheless, monoterpenes are generally useful for therapeutic purposes as antibacterial and antifungal agents [29]. Sesquiterpenes also possess anti-inflammatory and anticarcinogenic properties [1].

Higher oxygenated compounds observed in the solvent-free microwave-extracted oil indicate the superiority of the method over hydrodistillation and could be attributed to reduced water content in the system which would have minimized the thermal and hydrolytic degradation of oxygenated compounds when compared with the hydrodistillation method [30, 31].

The major constituents observed in the essential oil extracted by the SFME method include *a*-pinene (6%), D-limonene (11.27%), β-ocimene (9.09%), β-phellandrene (6.33%), β-mycene (8.49%), caryophyllene (5.96%), and camphene (4.28%). However, in the hydrodistillation method, the oil was majorly composed of α-pinene (4.41%), β-pinene (10.68%), β-ocimene (6.30%), germacrene-(5.09%), humulene (5.55%), and *a*-elemene (6.18%). This is in correlation with the findings of Mwangi et al. [32] and Chagonda et al. [33] in their observations that sabinene, δ-3-carene, myrcene, germacrene-D, limonene, (Z)-β-ocimene, β-phellandrene, and α-pinene are major constituents in *H. arborescens* leaves. The absence of sabinene and δ-3-carene in this study could be as a result of the difference in their geographic areas or some biotic and biotic factors in the environment [34]. It is interesting to note that 22 com-pounds were unique to only the hydrodistillation methodand 17 were distinctive to the SFME methods, while 13 were common to both methods. This buttresses the point that the chemical composition of essential oils is dependent on the extraction methods [35].

5. Conclusions

This study has shown that the chemical composition of the essential oils obtained from *H. arborescens* leaves is dependent on the extraction method. The SFME method resulted in a higher yield in terms of quantity and an essential oil of better quality due to the presence of higher valuable oxygenated compounds than that which was obtained by hydrodistillation. Furthermore, the use of the SFME method

indicated lower energy consumption and CO_2 emission. It can therefore be concluded that the SFME method is considered as a good alternative for the extraction of essential oil from *H. arborescens* leaves at shorter extraction time, reduced energy consumption, lower cost, and environmental footprintwhencomparedwiththehydrodistillationmethod.

Data Availability

All underlying data supporting the results can be found in the manuscript.

Ethical Approval

Ethical approval was granted by the University of Fort Hare Animal and Plant Use Research Ethics Committee, South Africa, with protocol number AFO111SABI01.

Conflicts of Interest

The authors declare that they do not have any conflicts of interest regarding the publication of this paper.

Authors' Contributions

GAO and T.O.A designed the experiments, T.O.A performed the experiments, analyzed data, and wrote the draft. GAO and AJA coordinated and revised the manuscript.TOA, GAO, and AJA read and approved the finalmanuscript.

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CHAPTER FIVE

NUTRITIONAL COMPOSITION AND ANTI-NUTRIENT CONTENT OF *HETEROMORPHA ARBORESCENS* (SPRENG.) CHAM. & SCHLTDL. LEAVES

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ORIGINAL RESEARCH

Nutritional composition and antinutrient content of *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. leaves: An underutilized wild vegetable

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Abstract

The nutritional and antinutrient composition of *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. leaves was reported in this study. Proximate analysis revealed the presence of 8.5% total ash, 4.92% crude fat, 8.41% moisture, 15.74% crude protein, 21.48% crude fiber, 40.95% carbohydrates, and 271.04 kcal/100 g energy value. Mineral analysis showed that *H. arborescens* leaves are very rich in K, Ca, and Fe. Considerable amounts of Mg, Mn, Na, P, Cu, and Zn were also present. Vitamin analysis showed that the plant has a high content of vitamins A, C, and E. The antinutrients evaluated were phytate, oxalate, saponin, and alkaloids, all of which were below toxic levels except for saponin which was observed at moderately high level. The results credibly indicate that *H. arborescens* leaves are nutrient-rich and can contribute effectively to the daily nutrient requirements alongside its therapeuticproperties.

KEYWORDS
Antinutrients, *Heteromorpha arborescens*, malnutrition, mineral, proximate, vitamins

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1 | INTRODUCTION

There are many native medicinal plants which have been reported to be useful as vegetables, spices, and medicine (Okwu 2005; Muhammed et al., 2011). The World Health Organization has confirmed that above 68% of the global population depends mainly on medicinal plants to meet their health needs (WHO 2003), as most of them are nutrient-rich, effective, safe, relatively cheaper, and readily available for the use of people especially the rural populace. Vegetables are very important sources of these nutrients which contribute markedly to food security and healthy diets for humans especially for children, lactating and pregnant women. (Nesamvuni et al., 2001; Yang & Keding, 2009). It is well known that besides providing the satisfaction of fullness, many plants are consumed because of their perceived medicinal needs. However, due to lack of knowledge about some of the medicinal food plants which are necessary to combat malnutrition and meet the required nutrient intake levels, such plants are underutilized. The study of plant foods with nutraceutical, pharmaceutical, nutritional, and functional propertiesis on the increase, and many plants with multi-functional properties are now gaining recognition and usage (Otunola & Afolayan, 2019).

One of such underutilized multipurpose plants is *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. which belongs to the Apiaceae family. It is a large shrub-like, medium deciduous tree widely distributed over tropical and temperate regions in Africa. The plant has gained great importance in parts of Africa especially in east and southern Africa because of its nutritional components, biological activities, and therapeutic efficiency (Maroyi, 2018). The leaves and roots are used for the treatment of inflammation, abdominal pains, and as general analgesic (Mc Graw et al. 1997;

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Lundgaard et al., 2008). Other uses are for intestinal deworming, jaundice, kidney problems, diabetes (Moshi and Mbwambo, 2002; Erasto et al., 2005), to treat mental disorders (Hutchings et al., 1996; Palmer & Pitman, 1972; Van Wyk and Oudtshoorn, 2013), fever, and malaria (Fowler, 2006; Lundgaard et al., 2008; Schmidt et al., 2002; Van Wyk and Oudtshoorn, 2013). As a result of its perceived great nutritional value, the leaves are eaten as vegetables in Kenya (Bussman 2006) and the roots are fed to malnourished children in Botswana and Swaziland (Setshogo and Mbereki, 2011). However, the leaves are not consumed as vegetable in some parts of Africa including South Africa possibly due to lack of information of its nutritional potentials. Furthermore, despite the nutritional and medicinal significance of *H. arborescens* leaves, to the best of our knowledge there is no documented report on the proximate, mineral, vitamins, and antinutritional compositions of *H. arborescens* leaves. Therefore, the present study is aimed at investigating the nutritionalprofile of *H. arborescens* leaves toward an informed validation of its traditional use as a vegetable, while encouraging its possible inclusion in human diets especially in South Africa.

2 | MATERIALS AND METHODS

2.1 | Collection and preparation of plant materials

Fresh leaves of *H. arborescens* were collected from a site located on latitude 32° 47′ 50.4″ S, 26° 52′ 41.8″ E along Hogsback road in Alice, Eastern Cape, South Africa, and it was authenticated by Prof. Cupido, a taxonomist at University of Fort Hare. The freshly collected leaves were washed, oven dried at 40°C, pulverized, and stored at 4°C until further analysis.

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2.2 | Proximate analysis

2.2.1 | Moisture content

An empty glass beaker was oven dried at 105°C for 1 hr, cooled in a desiccator, and weighed (W_1). 2 g of the sample (W_2) was weighed into the beaker, and both beaker and its content were oven dried at 105°C and cooled in a desiccator until constant weight was obtained (W_3). The percentage moisture was calculated as:

$$\text{Moisture (\%)} = \frac{W_2 - W_3}{W_2 - W_1} \times 100.$$

2.2.2 | Ash content

The ash content was determined by as previously described (Unuofin et al., 2017). A crucible was washed, dried (105°C) for 1 hr, and left to cool in a desiccator, and the weight was measured (W_1). Then, 2 g of the dried sample was measured into the weighed crucible and the new weight was measured (W_2). The crucible with its content was then put into a muffle furnace for 1 hr at 250°C and again for another 5 hr (550°C) for complete ashing. The samples were left to cool in a desiccator, and the final weight was measured (W_3). The percentage ash was calculated as:

$$\text{Ash content(\%)} = \frac{W_2 - W_3}{W_2 - W_1} \times 100.$$

2.2.3 | Crude fat

The crude fat content was determined by the AOAC (2016) method. Briefly, 5 g of the pulverized sample was extracted in 100 ml of diethyl ether for about 24 hr. The extract was filtered into a beaker of known weight (W_1). It was thereafter made up to 100 ml with diethyl ether and shaken for another 6 hr; the filtrate was collected into W_1. The ether was concentrated to dryness in a steam bath and oven dried at 40–60°C, and then the beaker was weighed again (W_2). Thecrude fat content was estimated as:

$$\text{\% Crudefat} = \frac{W_2 - W_1}{\text{Original weight of sample}}$$

2.2.4 | Crude fibre content

Crude fibre of *H. arborescens* leaves was determined as described by Unuofin et al. (2017). Briefly, 2 g of the pulverized sample was treated with 100 ml of 1.25% H_2SO_4, and the solution was boiled for 30 min and filtered under pressure, and the residue was washed with boiling water. This residue was further treated and boiled with 100 ml of 1.25% NaOH solution. The final residue was then dried at 100°C and cooled in a desiccator and weighed (W_1). The final residue was thereafter subjected to heating in a muffle furnace at 550°C for 5 hr, transferred to coolin a desiccator, and reweighed (W_2). The percentage crude fibre was calculated as:

$$\text{Crude fibre (\%)} = \frac{W_2 - W_1}{\text{Original weight of sample}}$$

2.2.5 | Carbohydrate content

The carbohydrate content was calculated as:

$$\text{\% Total carbohydrate} = 100 - (\text{\% moisture} + \text{\% total ash} + \text{\% crude fat} + \text{\% crude fibre} + \text{\% crude protein}).$$

2.2.6 | Energy value

The energy value was determined by multiplying the values obtained for carbohydrate, crude fat, and crude protein by 4, 9, and 4 respectively and summing up the products. It was calculated as:

Energy value (kcal/100g)= (carbohydrate× 4)

+(crude fat× 9)+(crude protein× 4).

2.2.7 | Crude protein content

The Kjeldahl method described in the AOAC (2005) was used to determine the protein content. Exactly 2 g of the sample was weighed into a 250 ml digestion flask containing a mixture of 20 ml of concentrated H_2SO_4 and a digestion tablet. The mixture was boiled (until a clear/transparent residue was obtained), allowed to cool, diluted with 250 ml distilled water, transferred into a 500 ml Kjeldahl flask containing 50 ml of 40% NaOH solution, and thereafter, subjected to distillation. 150 ml of the distillate was then collected into a flask containing 100 ml of 0.1N HCl. This was then titrated against 2.0 mol/L NaOH with methyl orange as indicator. The end point was marked by a color change to yellow. The percentage nitrogen content was calculated as:

$$\frac{[(\text{ml standard acid}\times \text{N of acid}) - (\text{ml blank}\times \text{N of base})] - (\text{ml std base}\times \text{N of base})]}{\text{Weight of sample (g)}} \times 1.4007.$$

Where, N = normality, percentage crude protein was obtained by multiplying the nitrogen value by a constant value of 6.25. %; Crude protein = Nitrogen in sample × 6.25.

2.2.8 | Antinutrient content

Phytate

Phytate content was determined as previously described by Talabi et al. (2016). Briefly, 2 g of the sample was soaked in 100 ml of 2% HCL for 3 hr and filtered with Whatman no 1 filter paper. 25 ml of the filtrate was thereafter transferred into another conical flask and to it; 5 ml of 0.3% ammonium thiocyanate solution plus 53.3 ml of distilled water was added. The solution was titrated against standard iron III chloride solution (0.001 95 g of iron per mL) until a reddish brown color which persisted for 5 min was obtained. Phytate content was calculated as: Phytate (%) = Titer value × 0.001 95 × 1.19 × 100.

Oxalate

Oxalate content was determined as described by Ohikhena et al. (2017). Briefly, 1 g of sample was weighed into a conical flask containing 75 ml of 3 M H_2SO_4. The solution was properly mixed and filtered. 5 ml of the filtrate was heated to 90°C and then titrated against 0.05 M of $KMnO_4$ until there was a color change which persisted for about 30 s. The oxalate content was calculated by taking 1 ml of 0.05 M of $KMnO_4$ as equivalent to 2.2 mg oxalate.

2.2.9 | Alkaloid content

Alkaloid content of *H. arborescens* leaves was determined as previously described by Oyeyinka and Afolayan (2019). 0.5 g of the sample was mixed with 200 ml of 10% acetic acid in ethanol. The mixture was covered, incubated at room temperature for 4 hr, filtered, and concentrated to about a quarter of its original volume in a water bath. Thereafter, concentrated ammonium hydroxide was added drop wise to the extract till complete precipitation was attained. The solution was allowed to settle, and the precipitate formed was washed with dilute ammonium hydroxide and then filtered. The resi- due was oven dried at 40°C and weighed, and the alkaloid content was calculated as:

$$\% \text{ Alkaloid} = \text{Final weight of sample} / \text{Initial weight of sample} \times 100.$$

2.2.10 | Saponin content

Saponin content of *H. arborescens* leaves was determined as previously described by Idris et al. (2019). 0.5 g of the sample was measured into a beaker containing 50 ml of 20% ethanol. The solution was heated in a hot water bath for 4 hr at 55°C and filtered, andthe residue re-extracted with another 50 ml of 20% ethanol. The filtrates were mixed together and concentrated to 20 ml over the hotwater bath at 90°C. The solution obtained was transferred into a 250 ml separating funnel containing 20 ml of diethyl ether. The aqueous layer was collected; 20 ml of n-butanol was added to it and then washed thrice with 10 ml of 5% sodium chloride while the ether layer was thrown away. The mixture was oven dried (40°C) to constant weight, and the percentage saponin content of the sample was calculated as

$$\% \text{ Saponin} = \frac{\text{Weight of final filtrate}}{\text{Weight of sample}} \times 100.$$

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2.3 | Mineral analysis

Ca, Mg, K, P, Na, Zn, Mn, Cu, and Fe contents of *H. arborescens* leaves were quantitatively analyzed using Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES; Varian 710–ES series, SMM Instruments, Cape Town, South Africa).

2.3.1 | Vitamin A (retinol)

Vitamin A content of the leaves was determined as described by Famewo et al. (2018). 1.0 g of the pulverized sample was soaked in 20 ml of petroleum ether for 1 hr. The mixture was filtered and evaporated to dryness, and 0.2 ml solution of chloroform-acetic anhydride was then added to the residue. Thereafter, 2 ml of TCA-chloroform (1:1 v/v) was added to the solution, and absorb- ance was measured at 620 nm using UV-3000PC. The standard (Retinol) was prepared in the same manner with gradient concen- tration ranging from 0.2 mg/ml - 1.0 mg/ml. The vitamin A con-tent of thesample was determined as retinol equivalent in mg/g, from the standard curve from the equation: Y = 0.9178 x- 0.02, R^2 = .9979.

TABLE 1 Proximate composition of *Heteromorpha arborescens* leaves

Proximal parameters	Composition (%)
Total ash	8.5 ± 0.15
Crude fat	4.92 ± 0.1
Moisture	8.41 ± 1.10
Crude protein	15.74 ± 1.26
Carbohydrates	40.95 ± 0.03
Energy (Kcal/100 g)	271.04 ± 6.13
Crude fiber	21.48 ± 1.87

Note: Values are expressed as mean ± *SD*, *n* = 2.

2.3.2 | Vitamin C (Ascorbic acid)

Vitamin C content of the sample was determined as described by Njoku et al. (2015). 1 g of the sample was soaked with 20 ml of 0.4% oxalic acid and filtered with Whatman No 1 filter paper.Thereafter, 9 ml of indophenol reagent was added to 1 ml of the filtrate, and the absorbance was measured at 520 nm. The vitaminC content of the sample was determined as retinol equivalent in mg/g, from the standard curve from the equation: $Y = 2.7867x - 0.6521$, $R^2 = .9384$.

2.3.3 | Vitamin E (Tocopherol)

Vitamin E content of the sample was determined as previously described by Njoku et al. (2015). 1 g of the sample was soaked in 20 ml of ethanol and filtered. Thereafter, 1 ml of 0.2% ferric chloride in ethanol and 1 ml of 0.5% α -dipyridyl solution were added to 1 ml of the filtrate. The solution was further diluted with water to 5 ml, and the absorbance was measured at 520 nm. The vitamin E content of the sample was determined as retinol equivalent in mg/g, from the equation: $Y = 0.501 x + 3.2723$, $R^2 = .9661$.

TABLE 2 Mineral composition of *Heteromorpha arborescens* leaves

Minerals	Composition
Calcium	1565 ± 0.03
Magnesium	400 ± 0.01
Potassium	1685 ± 0.02
Phosphorus	55 ± 0.00
Sodium	75 ± 0.00
Zinc	2.1 ± 0.03
Manganese	9.55 ± 0.05
Copper	0.75 ± 0.02
Iron	12.55 ± 1.15

Note: Values are expressed as mean ± *SD*, *n* = 2

3 | RESULTS

3.1 | Proximate composition

The proximal composition of *H. arborescens* leaves is presented in Table 1. The results showed that the leaves of the plant had considerable quantities of total ash, crude fat, moisture, crude protein, crude fiber, and carbohydrate contents with very high estimated energy value.

3.2 | Mineral composition

Nine minerals (Ca, Mg, K, P, Mg, Na, Zn, Mn, Cu, and Fe) from *H. ar- borescens* leaves were analyzed (Table 2).

3.3 | Antinutrient composition

The antinutritional composition of *H. arborescens* leaves is presented in Figure 1. Phytate, oxalate, saponin, and alkaloid contents were present as (3.22 ± 0.02) %, (1.1 ± 0.22) %, (25.33 ± 2.3) %, and (7.65 ± 0.45) %, respectively.

3.4 | Vitamins A, C, and E compositions

Vitamin analyses of *H. arborescens* leaves (Figure 2) revealed the presence of Vitamins A (23.82 mg/100 g) and E (28.77 mg/100 g) was observed in remarkable quantities. However, vitamin C (57.89 mg/100 g) content was the highest.

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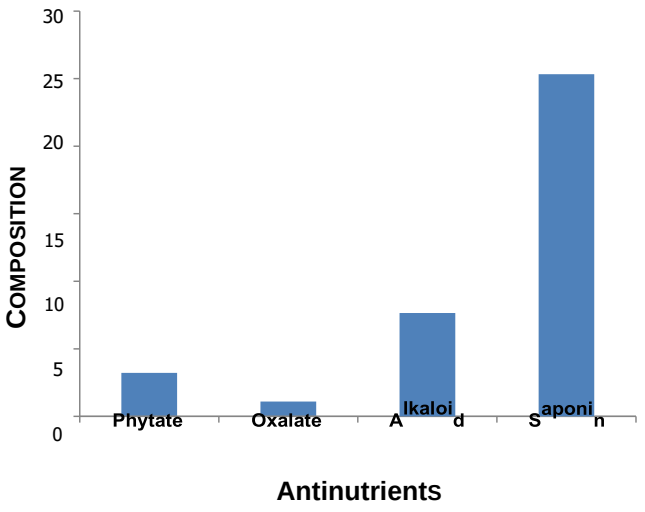

FIGURE 1 Antinutrient compositions of *Heteromorpha arborescens* leaves

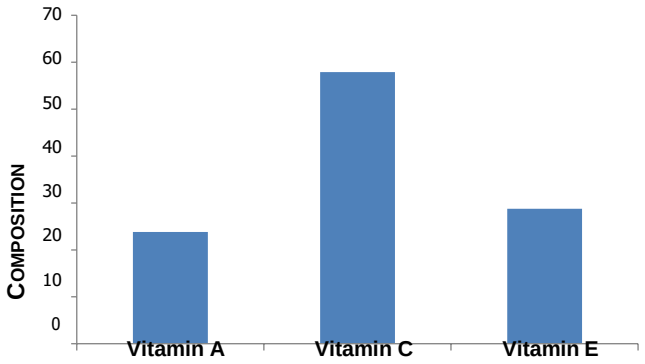

FIGURE 2 Vitamin composition of *Heteromorpha arborescens* leaves

4 | DISCUSSION

Plants are rich sources of nutrients, and there has been an increasing global interest in the investigation of the nutritional and antinutritional compositions of medicinal food plants in order to tackle malnutrition, increase food security, and prevent diseases all overthe world.

Protein is crucial for several body functions such as production of hormones, enzymes, maintenance of fluid balance, boosting the immune system, and body building (Emebu and Anyika, 2011; Achi et al., 2017). The protein content of *H. arborescens* leaves was observed to be moderate, and it can therefore be considereda good source of protein and potential protein supplements in the diet. Carbohydrate content indicates the energy content, and it is effi- cient for oxidation of fats (Omoyeni & Adeyeye, 2009). Considering the substantial quantity of carbohydrate observed in the plant, it can be regarded as a good dietary energy source.

Dietary fiber functions in the regulation of bowel movement, proper diges- tion, and effective eradication of wastes from the body. They also lower the serum cholesterol; the risks of coronary heart diseases, hypertension, constipation, diabetes, colon, and breast cancers (Narzary & Basumatary, 2019; Viuda-Martos et al., 2010). The high fiber content exhibited by *H. arborescens* leaves is indicative of its great health benefitting potential. Vegetables are generally charac- terized by low lipid contents (Achi et al., 2017); therefore, low fat content observed in *H. arborescens* leaves supports this fact and suggests its potential to maintain body weight, for the management of obesity, cardiovascular, and other high fat associated diseases (Adegbaju et al., 2019; Otunola &Afolayan, 2019). The value of ash content is suggestive of its elemental composition. Considerable ash content observed in this study suggests the moderate elemen- tal composition of *H. arborescens* leaves. Likewise, the low moisture content of the samples reveals its long shelf-life and low risk of microbial contamination, since high moisture content could increase microbial action that can lead to spoilage (Olumide et al., 2019; Ooi et al., 2012). The high energy content shown is attributed to the high carbohydrate content, which suggests that it can be a good source of dietary energy.

The results of the present study are comparable to other findings on proximate compositions of selected vegetables belonging to the Apiaceae family; although the fiber content of *H. arborescens* leaves was far higher than values obtained for *Coriandrum sativum* and *Daucus catota* L. According to Ayeni et al. (2018), the proximate analysis on *Daucus catota* L. revealed the presence of moisture (10.23%), ash (12.99%), crude protein (11.75%), carbohydrate (51.81%), energy (358.93%), crude lipid (10.37%), and fiber contents (9.07%). Similarly, Javid et al. (2009) and Ghajarieh Sepanlou et al. (2019) investigated *Coriandrum sativum* and *Eryngium caeruleum* respectively and ob- served the presence of moisture (6.65%, 8.6%), ash (8.03%, 9.45%), crude protein (14.59%, 17.9%), carbohydrate (63.71%, 39.56%), energy (390.39%, 236.78%), crude lipid (9.83%, 1.54%), and fiber contents (5.53%, 22.63%) for *Coriandrum sativum* and *Eryngium caeruleum*, respectively.

Minerals such as potassium, calcium, iron sodium, magnesium, phosphorus, manganese, zinc, and copper are crucial for normal body development and maintenance (Haruna et al., 2015). They helpin sustaining and improving the functions of the, muscles, heart, and brain as well as the production and maintenance of strong bones and teeth (Jequier and Constant 2010). Magnesium is required in the plasma and extracellular fluid to maintain osmotic equilibrium. It also functions in many biochemical reactions including oxidative phosphorylation, glycolysis, and protein synthesis (Gröberet al., 2015; Thomas et al., 2000). Phosphorus plays a vital role in energy generation, maintenance of bones, teeth and muscles, and cell growth and also provides the structural framework for DNA and RNA (Gharibzahedi & Jafari, 2017). It also functions in regulating blood sugar levels and contraction of the heart (Achi et al., 2017). Potassium is crucial in maintaining normal cell functions, regular muscle contraction, and regulation of blood pressure. Calcium is required for bone and mus- cle formation, synaptic nerve impulse transmission and blood co- agulation (Ozan and Akbulut 2008). High intake of calcium is highly recommended especially for children and pregnant women (Insel et al. 2011). Zinc functions in normal body development, protein syn- thesis, and wound healing. It also forms an important part of many enzymes in the human body (Afolayan & Jimoh, 2009). However, ex- cessive consumption of zinc is dangerous to human health (Ogundola et al., 2018). Sodium is involved in the regulation of acid–base bal- ance, normal cell function, transport of metabolites, nerve impulse transmission, and maintaining blood pressure (Unuofin et al., 2017). Iron is essential for hemoglobin formation; energy metabolism, and oxygen transport (Gaeta and Hider 2005; WebMD, 2014).

Manganese is necessary for all biosynthetic processes andmaintenance of nerve and muscle electrical potentials. It also functions in oxygen transport from lungs to cells and activation of enzymes reactions associated with the metabolism of carbohydrate, fat, and protein (Jacob et al., 2015). Copper is a vital trace mineral, which is required in several enzymatic reactions, good functioning of organs in the body, collagen synthesis, energy generation, and formation of hemoglobin (DiNicolantonio et al., 2018; Saupi et al., 2009).

Sodium, phosphorus, manganese, iron zinc, and copper were present in considerable amounts while the calcium, magnesium,

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and potassium contents were relatively high. From the result of mineral composition obtained in the present study, it infers that *H. arborescens* leaves can serve as a natural sources of these essential minerals and even as potassium and calcium supplements. The results for mineral analysis obtained in this study are also comparable to previous observations by Tuncturk and Ozgokce (2015).

Antinutrients can be harmful to human health and may pose negative effect by reducing protein digestibility and mineral bioavailability. Low levels of phytate, oxalate, and alkaloids were observed with moderately high level of saponin. However, the presence of moderate level of saponin should not pose a problem if properly processed since processing decreases the level of antinutrients to permissible levels (Jimoh et al., 2010; Ndidi et al., 2014).

Research has linked the consumption of vitamin-rich vegetables to a reduction in the risk of cancers and cardiovascular diseases (Abdou Bouba et al., 2012). Vitamins A, C, and E are great sources of antioxidants and are also vital in proper functioning of the eyes, growth and development, immune function, and reproduction (Shemishere et al., 2018). The significant amount of vitamins A and E and the high vitamin C content of *H. arborescens* leaves suggest that it may be beneficial in the prevention of a wide range of diseases such as coronary heart disease, anemia, heart diseases, cancers, and other degenerative diseases (Mgbeje et al., 2019). The present results revealed higher vitamins A, C, and E contents compared with some other common vegetables in Africa such as *Ocimum gatissimun, Gongronema latifolium, Piper guineense*, *Vernonia amygdalina* (Mgbeje et al., 2019), and *Celosia argentea* (Adegbaju et al., 2019). Vitamin C content of *H. arborescens* leaves has also proven to be higher than *Spinacea oleracea*, *Brassica oleracea*, and *Allium sativum* (Bangash et al., 2011).

5 | CONCLUSION

Plants are good sources nutrients which are required for healthy human diets. This study revealed that *H. arborescens* leaves are a good source of nutrients and mineral elements (with low antinutrient content) that are highly beneficial to human health. Considering the high amounts of protein, fiber, calcium, carbohydrate, and other nutrients observed in *H. arborescens* leaves, it can serve as a good nutrient source to human diets. This finding also encourages its possible inclusion as a vegetable to the diet in South Africa.

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COMPETING INTERESTS

No competing interests were disclosed.

ETHICAL APPROVAL

Ethical approval was granted by the University of Fort Hare Animal and Plant Use Research Ethics Committee, South Africa with protocol number OTA011SABI01/19/E.

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CHAPTER SIX

CYTOTOXICITY, ANTI-OBESITY, AND ANTI-DIABETIC POTENTIALS OF *HETEROMORPHA ARBORESCENS* (SPRENG.) CHAM LEAVES

This chapter has been published in Processes

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Cytotoxicity, Anti-Obesity and Anti-Diabetic Activities of *HeteroMorpha arborescens* (Spreng.) Cham Leaves

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Abstract: This study investigated the cytotoxicity, anti-obesity and anti-diabetic potentials of blanched, aqueous and ethanol extracts of *Heteromorpha arborescens* (Spreng.) Cham leaves. The results revealed that both ethanol and aqueous extracts exhibited considerable inhibition against α-glucosidase (IC_{50} of 627.29 ± 4.62 µg/mL and 576.46 ± 3.21 µg/mL respectively), while the blanched extract showed weak α-glucosidase inhibition (IC_{50}; 855.38 ± 4.29 µg/mL) and the aqueous extract showed the best α-amylase inhibition (IC_{50}; 583.74 ± 5.87 µg/mL). However, weak α-amylase inhibition was observed in the ethanol (IC_{50}; 724.60 ± 4.33 µg/mL) and blanched extracts (IC_{50}; 791.63 ± 3.76 µg/mL). The toxicity of the extracts is indicated by LC_{50} values as 154.75 µg/mL, 125 µg/mL and 90.58 µg/mL for ethanol, aqueous and blanched extracts respectively, indicating the blanched extract to be the most toxic. Moderate glucose utilization in both C3A and L6 cells was also observed for the aqueous and ethanol extracts which may be attributed to the relatively lower toxicity levels present. However, glucose utilization was very weak for the blanched extract, which may be due to higher level of cytotoxicity it possessed. Relatively weaker lipase inhibition was observed for the ethanol (IC_{50}; 699.3 ± 1.33 µg/mL), aqueous (IC_{50}; 811.52 ± 3.52 µg/mL) and blanched extracts ($IC_{50;}$ 1152.7 ± 4.61 µg/mL) compared to orlistat ($IC_{50;}$ 56.88 ± 0.11 µg/mL). However, there was no reasonable reduction in lipid accumulation observed in all the extract treated cells. These observations suggest that ethanol and aqueous extracts of *H. arborescens* leaf are promising as new agents for the treatment of diabetes and its acclaimed anti-obesity potentials are likely due to its lipase, α-amylase and α-glucosidase inhibition.

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Keywords: diabetes mellitus; α-glucosidase; α-amylase; lipase; *Heteromorpha arborescens*; cytotoxicity; glucose utilization

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1. Introduction

Diabetes mellitus is a chronic condition of carbohydrate, fat and protein metabolism impairment, characterized by hyperglycemia and glucose intolerance which is associated with insulin deficiency and/or insulin inefficiency [1,2]. This disease is a primary factor for global mortality, and it is well associated with long term complications such as cardiovascular diseases, retinopathy, neuropathy and oxidative stress [3,4]. α-amylase and α-glucosidase are carbohydrate digestive enzymes which catalyze the degradation of large insoluble starch molecules into digestible oligosaccharide molecules and the final breakdown of the oligosaccharides, respectively. One of the therapeutic approaches for reducing postprandial hyperglycemia is the inhibition of these enzymes to slow down absorption of carbohydrates in the small intestine [5–9]. Diabetes mellitus is also linked to obesity [10] and pancreatic lipase inhibitory activities as well as lipid accumulation have been widely used to determine the potential efficacy of natural products as anti-obesity agents [11]. Even though there is presently no cure for diabetes, there are several groups of artificial drugs which are commonly used for the management of diabetes: including sulfonylureas, thiazolidinediones, non-sulfonylurea insulin secretagogues, biguanides and

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α-glucosidase inhibitors [12]. However, the use of these drugs is limited in that they are expensive; inaccessible for majority of the rural populations and some may have some undesirable aftermath such as excessive weight, diarrhea, nausea and gastrointestinal disorders [12–14]. On the other hand, medicinal plants are readily more available, with lower side effects compared to the conventional drugs and are a safer alternative for the management of diabetes and obesity [15,16]. Previous reports have indicated that some plant extracts possess bioactivity; however, the potency of these extracts may be altered by the cytotoxicity of the plants [17]. Hence the need to ascertain the safety and potency of plants before use.

A frequently used plant for the management of diabetes mellitus in South Africa is *Heteromorpha arborescens* [18–20]. This plant is highly reputed in the South African traditional medicine for the treatment of a wide range of diseases such as rheumatism, cancer, gonorrhea, heart problems, rabies and diabetes [21]. Several biological activities have also been reported for *H. arborescens* such as anticancer and antimalarial [22–25], antibacterial [26], aphrodisiac, antiulcer, anthelminthic, antinociceptive and analgesic [27] and anti-rabies activities [21]. Presumably, only very limited scientific information is available on the efficiency and safety of *H. arborescens* leaves in the treatment of diabetes and its related complications. Therefore, to validate this claim, the cytotoxic, inhibitory activities

against α-amylase, α-glucosidase and lipase in addition to lipid accumulation potentials of the aqueous, blanched and ethanol extracts of *H. arborescens* leaves were determined.

2. Materials and Methods

2.1. Chemicals, Reagents, Cell Lines

Melphalan, Dimethyl Sulfoxide (DMSO), Hoechst 33342, Propidium iodide (PI), p-nitrophenol palmitate (pNPP), dinitrosalicylic acid (DNSA), Triton X-100, Tris-HCl buffer, monosodium and disodium phosphate, acarbose, -nitrophenyl-d-glucopyranoside **p**-nitrophenyl-α-D-glucopyranoside (**p**NPG), sodium carbonate, α-glucosidase, α-amylase, isopropanol, Triton X-100, Metformin, Dulbecco's Modified Eagle Medium (DMEM) and Tris-HCl buffer were purchased from Sigma-Aldrich through Capital Lab Supplies, South Africa. Bovine Serum Albumin (BSA), starch and insulin were purchased from Sigma (St. Louis, MO, USA). Fetal Calf Serum (FCS), Roswell Park Memorial Institute (RPMI) 1640 Medium and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from GE Healthcare Life Sciences (Logan, UT, USA). The C3A/HepG2 cells and L6 cell lines were obtained from Professor Maryna van de Venter, of the Department of Biochemistry and Microbiology, Nelson Mandela University (NMU), South Africa, where the assays were performed.

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2.2. Plant Collection

Fresh *H. arborescens* leaves were collected from Alice, Eastern Cape, South Africa (32°47′50.4″ S, 26°52′41.8″ E). The plant was validated by Prof. Cupido, a Taxonomist, and a voucher specimen (Abif2019/03) was stored in the Giffen Herbarium, University of Fort Hare.

2.3. Preparation of Extracts

Oven dried powdered *H. arborescens* leaves (100 g) were extracted separately in 500 mL of ethanol and water for 24 h on an orbital shaker. For the blanched sample, the same weight of the fresh leaves was extracted in 500 mL of hot water (80 °C) for 5 min to simulatehome cooking, and then ground with a blender. All the extracts were filtered using a Buchner funnel and Whatman filter paper (150 mm) under a vacuum. The ethanol extract was concentrated to dryness using a rotary evaporator while the aqueous extract and blanched samples were subjected to freeze drying.

2.4. Maintenance of Cell Culture

The cell cultures were incubated in a moistened environment with 5% CO_2 at 37 °C. Growth medium (RPMI 1640 medium plus 10% fetal calf serum) was supplied to the C3A cells once in 3 days, and the L6 myoblast cells were cultivated in media containing the growth medium with no antibiotic. The cell lines were sub-cultured after 80% confluence.

2.5. Cytotoxicity Assay

Cytotoxicity of the extracts was determined using Hoechst/PI staining technique. The C3A cells were seeded in 96-well plates at a density of 6000 cells per well in DMEM containing 10% Fetal Bovine serum and allowed to attach overnight. The cells were thereafter treated by replacing the medium with fresh ones at different extract concentrations (12.5–200 µg/mL) and the treated cells were incubated for 48 h. After incubation, the spent media were carefully removed and 50 µL staining solution (5 mL PBS containing2 µL Hoechst solution (10 mg/mL in DMSO) was added to each well. The samples were incubated again at 37 °C for 15 min. Thereafter, 50 µL PI staining solution was added to each well at a concentration of 2 µg/mL and image was acquired using DAPI (4′,6-diamidino-2-phenylindole) and Texas Red filters. Melphalan, a well-known cytotoxic drug was used as the positive control. Cytotoxic activity was calculated as:

$$\% \text{ Cell density} = \frac{\text{A570 nm of treated cells}}{\text{A570 nm of untreated cells}} \times 100 \quad (1)$$

2.6. Alpha-Amylase Inhibition

The α-amylase inhibition assay was performed using the 3,5-dinitrosalicylic acid (DNSA) method. The plant extract was dissolved in very little quantity of 10% DMSO and was further diluted with buffer solution (pH 6.9) in a 96-wells microtiter plate, to obtain varying concentrations between 50–200 µg/mL. 5 µL of α-amylase solution was added separately to 15 µL of the extracts, phosphate buffer (negative control) and acarbose, after which the mixture was pre-incubated for 30 min at 37 °C. The reaction was initiated by adding 20 µL of the starch solution to each well and the resulting mixture incubated for another 30 min at 37 °C. The reaction was thereafter terminated by the addition of 20 µL DNSA reagent and was heated for 10 min in a water bath at 90 °C. The mixture wascooled to room temperature and the absorbance was measured at 540 nm. The α-amylase inhibitory activity expressed as percentage inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{control}}} \times 100 \quad (2)$$

2.7. Alpha-Glucosidase Inhibition

The α-glucosidase inhibition assay was determined as described [28]. Briefly, 5 µL of the extracts (30 60, 120, 240 and 480 µg/mL), 20 µL of 50 µg/mL α-glucosidase solution and 60 µL of 67 mM potassium phosphate buffer (pH 6.8) were reacted together in a 96-well plate. After 5 min of incubation, 10 µL of 10 mM p-nitro phenyl-α-D-glucoside solution (PNPGLUC) was added and then further incubated at 37 °C for 20 min. Thereafter, 25 µL of 100 mM Na_2CO_3 solution was added to the mixture and the absorbance was measured at 405 nm. Acarbose (10 µg/mL) was prepared in separate wells and used as the positive control. The % inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{control}}} \times 100 \quad (3)$$

2.8. Pancreatic Lipase Inhibition

In vitro pancreatic lipase inhibitory assay was performed as described [29,30] with some modifications. Briefly, 20 µL of extracts and orlistat (1 mg/mL stock solution in 10% DMSO) at varying concentrations (30, 60, 120, 240 and 480 µg/mL) was reacted with 100 µL

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of freshly prepared lipase enzyme solution (1 mg/mL). The resulting mixtures were made up to 1 mL by adding Tri-HCl solution (pH 7.4) and then incubated at 25 °C for 15 min. After incubation, 100 µL of the substrate solution (20.9 mg of PNPB in 2 mL of acetonitrile) was added. The mixture was incubated again for 30 min at 37 °C and the absorbance was measured at 405 nm. Orlistat was used as the positive control and controls without orlistat and without the substrate (PNPB) were separately prepared. The percentage inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{control}}} \times 100 \quad (4)$$

2.9. Lipid Accumulation Assay

Lipid accumulation in C3A cells was determined as previously described by [31]. The C3A cells were seeded in 96-well plates at a density of 6000 cells per well in DMEM containing 10% Fetal Bovine serum and allowed to attach overnight. The cells were afterwards treated with different extract concentrations (12.5, 25, 50, 100 and 200 µg/mL) or positive control (Chloroquine) and 1000X LipidTox Green phospholipidosis detection reagent (diluted 1:500 in 5 mL complete growth medium), filtered using a 0.2 µm syringe filter before addition to wells, containing treatment. The treated cells were thereafter incubated for two days. The spent media was removed, washed with 200 µL PBS per well and fixed overnight in 4% formaldehyde. Fifty microliters of staining solution (prepared as 10 mL of PBS plus 10 µL LipidTox Red and 2 µL Hoechst) was added to each well and the mixture was incubated at room temperature for 30min. Image acquisition was then done using DAPI and Texas Red filters.

2.10. Glucose Utilization

Glucose utilization on L6 myoblasts cells was determined as previously described [32], with slight modifications. The cells were grown in DMEM supplemented with 10% FCS and sub-cultured by trypsinization. The L6 cells were further seeded into 96-well culture plates at a density of 5000 cells per well and allowed until 80% confluence of the medium was attained and at least one row was left empty without cells (blanks). The cells were incubated for 5 days to allow sufficient time for full differentiation and thereafter, the spent medium was removed from the differentiated cells and replaced with 200 µL per well of fresh medium at varying concentrations (12.5, 25, 50, 100 and 200 µg/mL). A row of the wells was treated with insulin (12 µM) which served as the positive control. Treatment was continued for two days and the cells were washed once with 50 µL of incubation buffer solution containing RPMI medium diluted with PBS and supplemented with BSA and 8 mM glucose to a final concentration of 0.1%. The plates were thereafter incubated for 2 h at 37 °C. After incubation, 5 µL of the culture medium was transferred to a new 96-well plate and to the wells; 200 µL glucose assay reagent (glucose oxidase/peroxidase colorimetric reagent) was added and incubated a 37 °C for 10–20 min and absorbance was measured at 510 nm. Glucose utilization was calculated as the difference between the wells with no cells and the wells containing cells and the amount of glucose utilized was expressed as a percentage of the untreated controls. Cell viability was then determined as 100 µL MTT solution (0.5 mg/mL in DMEM) was added to each well and further incubated at 37 °C for 1 h. Thereafter, the MTT solution was removed, 200 µL DMSO added, and the wells were again incubated at room temperature for 20 min. Absorbance was then measured at 540 nm and the cytotoxicity expressed as a percentage of the untreated control.

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2.11. Data Analysis

The experiments were performed in triplicates and data were expressed as mean ± standard deviation (SD) values using one way analysis of variance (ANOVA) and Fischer's Least Significant Difference in MINITAB 17 statistical package. Values were regarded as significantly different when $p < 0.05$.

3. Results

3.1. Cytotoxicity

The cytotoxic effect of each sample and standard, as well as the well-known cytotoxic drug (melphalan), is presented in Figures 1 and 2. The effect of the extracts on C3A cells suggests increased toxicity with increased concentration. The three extracts showed a decline in cell density at higher concentrations with the aqueous and ethanol extracts indicating a very sharp drop in cell density at the maximum concentration tested (200 µg/mL) whereas, the blanched extracts showed a sharp decline in cell density from as low as 100 µg/mL and melphalan on the other hand showed normal dose dependent simultaneous increase in dead cells and at the maximum concentration (200 µg/mL), all the extracts exhibited more than 50% cell death. The Lethal Concentration 50 (LC_{50}) were 154.75 µg/mL, 125 µg/mL and 90.58 µg/mL for ethanol, aqueous and blanched extracts respectively, indicating the blanched extract to be the most toxic. The corresponding images of the cells obtained from Hoechst 33,342 staining are presented in Figure 3.

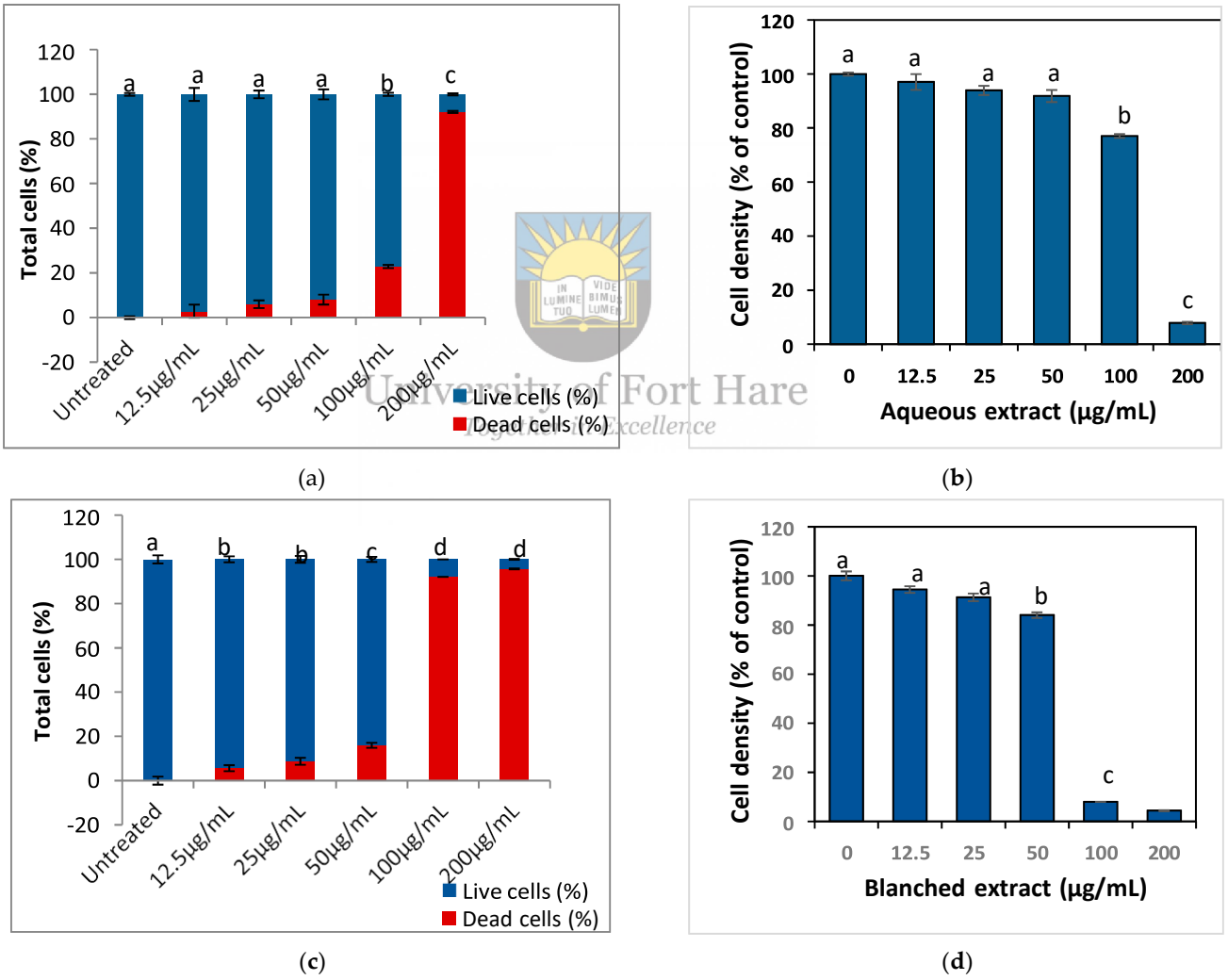

Figure 1. Cytotoxicity screening of aqueous and blanched extracts of *H. arborescens* leaves. (**a**) Total number of C3A cells after treatment with aqueous extract. (**b**) Cell density after treatment with aqueous extract. (**c**) Total number of C3A cells after treatment with blanched extract. (**d**) Cell density after treatment with blanched extract. Dead cells were represented as cells with compromised membrane integrity and subsequent propidium iodide (PI) staining and Live cells obtained from Hoechst 33,342 staining. Values are expressed as mean ± SD (n = 2). Mean separation by SD ($p < 0.05$). Set of bars with different alphabets are significantly different.

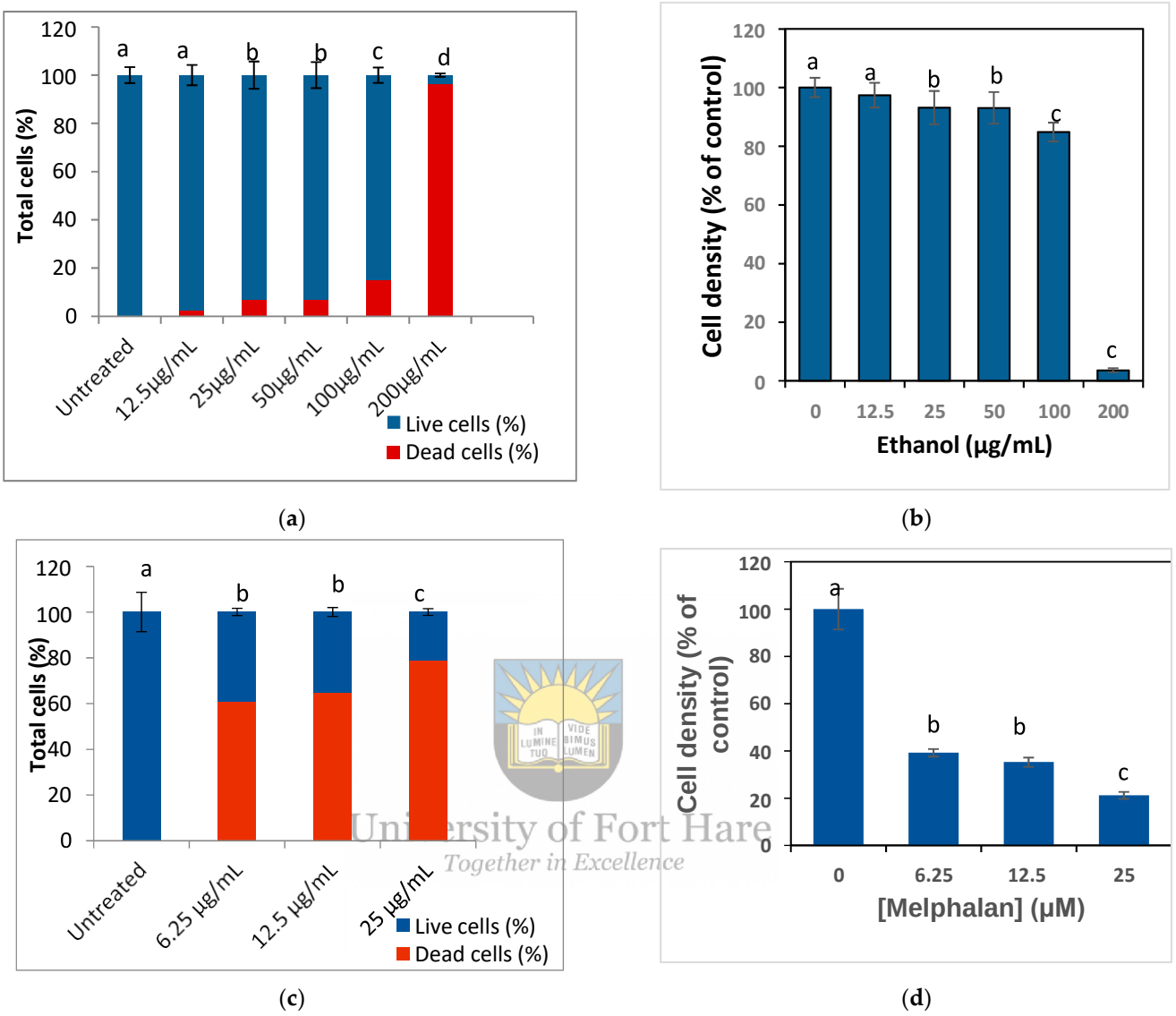

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Figure 2. Cytotoxicity screening of ethanol extract of *H. arborescens* leaves and melphalan (positive control). (**a**). Total number of C3A cells after treatment with ethanol extract. (**b**) Cell density after treatment with ethanol extract. (**c**) Total number of C3A cells after treatment with Melphalan. (**d**) Cell density after treatment with Melphalan. Dead cells were represented as cells with compromised membrane integrity and subsequent propidium iodide (PI) staining and Live cells obtained from Hoechst 33,342 staining. Values are expressed as mean ± SD (n = 2). Mean separation by SD ($p < 0.05$). Set of bars with different alphabets are significantly different.

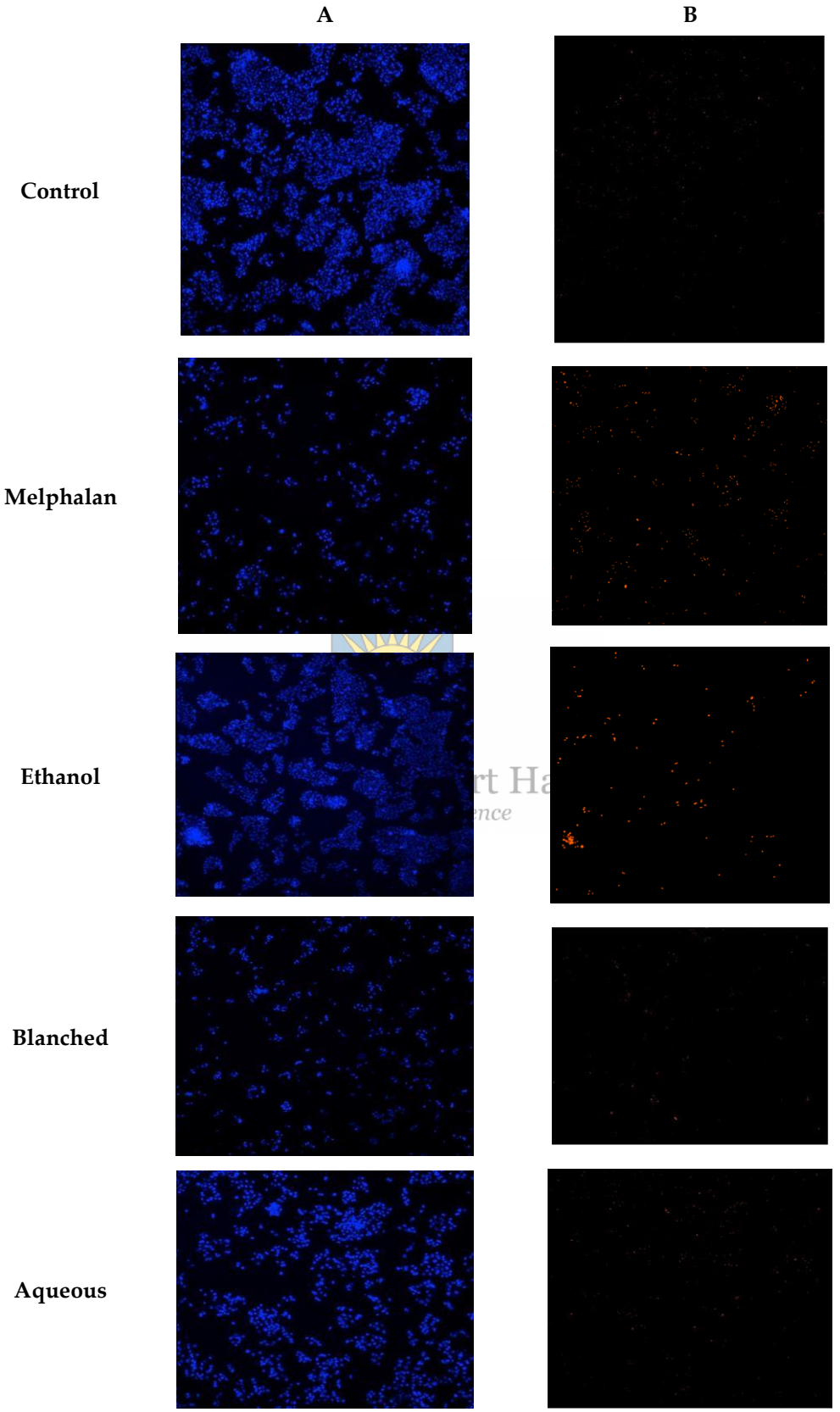

Figure 3. Images of C3A cells after 48 h treatment with 100 µg/mL each of ethanol, blanched and aqueous extracts of *H. arborescens* leaves obtained from Hoechst 33,342 staining. Column (**A**)—all nuclei, Column (**B**)—nuclei of dead cells.

3.2. Alpha-Amylase Inhibition

The α-amylase inhibition of *H. arborescens* leaf extracts and acarbose (positive control) is presented in Figure 4. The extracts displayed a concentration dependent inhibition of the enzyme, but very weak inhibitory potential at the tested concentrations when com- pared to acarbose. Percentage inhibition of α-amylase enzyme ranged from 40.77 ± 1.26% to 85.35 ± 2.18% for acarbose, 2.32 ± 0.45% to 32.46 ± 0.49% for ethanol, 3.04 ± 0.37% to 29.46 ± 1.06% for blanched and 7.48 ± 0.27% to 40.33 ± 3.49% for the aqueous extract. The IC_{50} values (Table 1) were 51.06 ± 1.78, 583.74 ± 5.87, 724.66 ± 4.33 and 791.63 ± 3.76 µg/mL for acarbose, aqueous, ethanol and blanched extracts respectively.

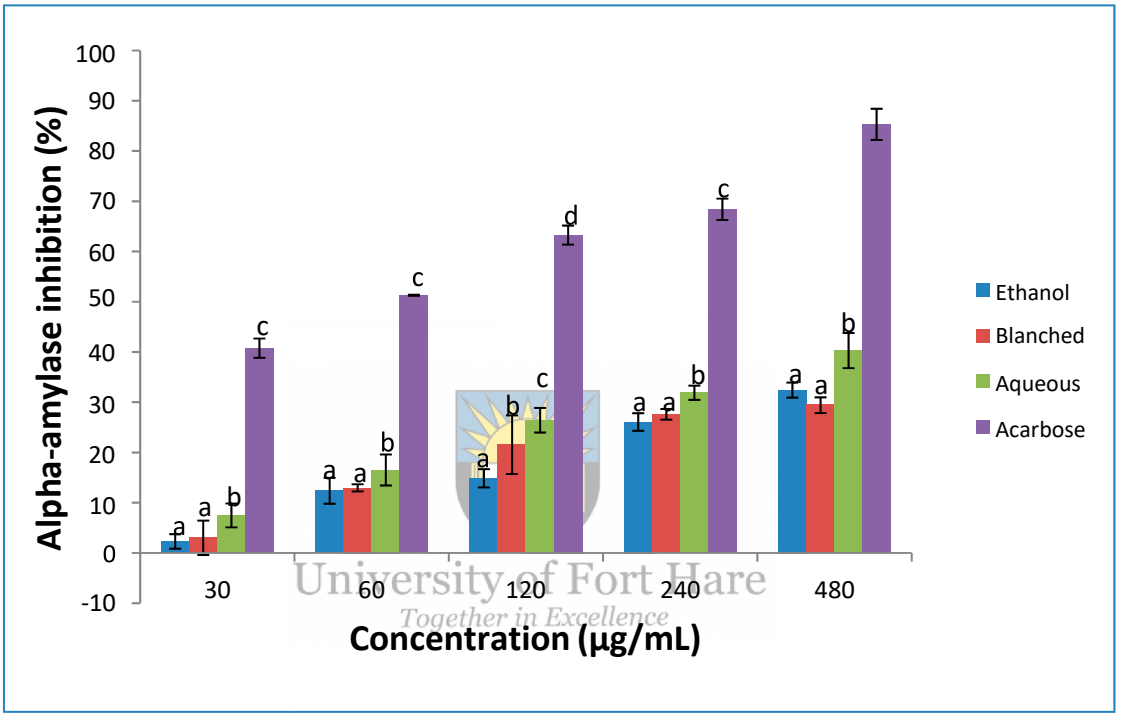

Figure 4. Alpha-amylase inhibitory activity of *H. arborescens* leaf extracts and positive control (acarbose). Values are mean ± SD (n = 3). Mean separation by SD ($p < 0.05$). Set of bars (the same concentration) with different alphabets are different.

Table 1. IC_{50} values for α-amylase and α-glucosidase inhibition by *H. arborescens* leaf extracts and standards (µg/mL).

Extracts	α-Amylase	α-Glucosidase	Lipase
Ethanol	724.66 ± 4.33	627.29 ± 4.62	699.3 ± 1.33
Blanched	791.63 ± 3.76	855.38 ± 4.29	1152.7 ± 4.61
Aqueous	583.74 ± 5.87	576.46 ± 3.21	811.52 ± 3.52
Acarbose	51.06 ± 1.78	45.43 ± 2.31	*
Orlistat	*	*	56.88 ± 0.11

* Not determined.

3.3. Alpha-Glucosidase Inhibitory Assay

The α-glucosidase inhibitory potential of *H. arborescens* leaf extracts and acarbose (positive control) is indicated in Figure 5. The extracts showed considerable dose depen- dent α-glucosidase inhibition, although weaker when compared to acarbose. Percentage inhibition of α-glucosidase enzyme ranged from 44.11 ± 0.19% to 82.47 ± 3.26% for acar- bose, 18.43 ± 0.89% to 40.69 ± 1.03% for ethanol extract, 9.32 ± 0.06% to 31.72 ± 1.13% for blanched extract and 22.63 ± 1.38% to 43.97 ± 0.38% for the aqueous extract. The IC_{50}

values (Table 1) were 45.43 ± 2.31, 576.46± 3.21, 627.29± 4.33 and 855.38 ± 4.29 µg/mL for acarbose, aqueous, ethanol and blanched extracts respectively.

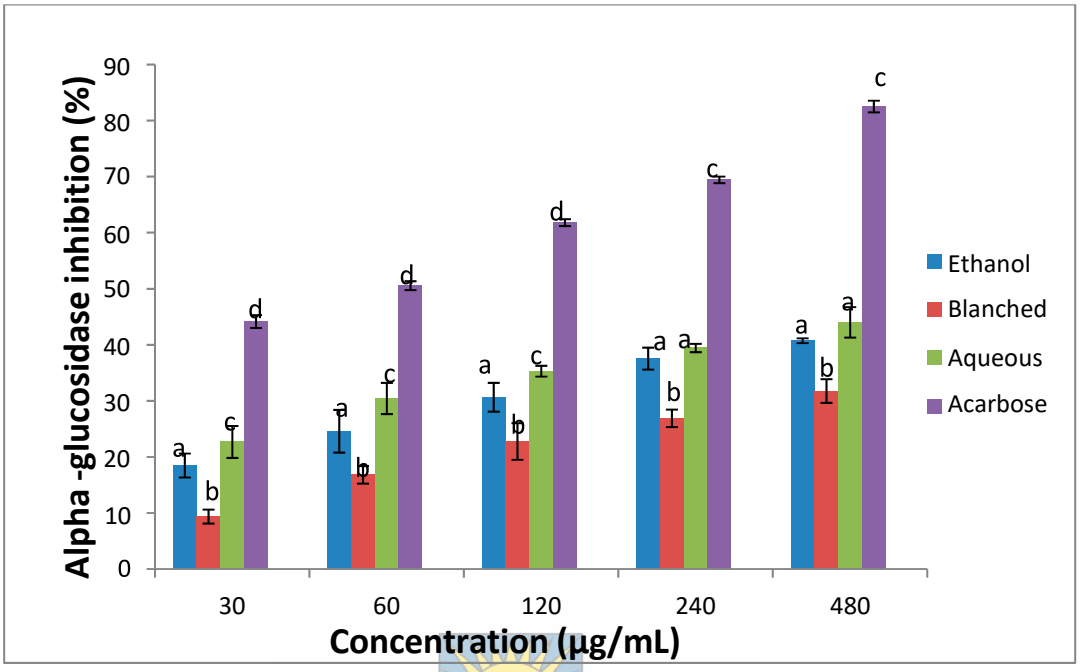

Figure 5. Alpha-glucosidase inhibitory activity of *H. arborescens* leaf extracts and positive control (acarbose). Values are expressed as mean ± SD (n = 3). Mean separation by SD ($p < 0.05$). Bars at the same concentration with different alphabets are significantly different.

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3.4. Porcine Pancreatic Lipase

The inhibitory effects of the *H. arborescens* leaf extracts against porcine pancreatic lipase were evaluated in comparison with orlistat (Positive control) and presented in Figure 6. Although percentage inhibition ranged from 46.33 ±0.76% to 82.07 ±1.41% for orlistat, 12.30 ± 0.85% to 35.87± 1.39% for ethanol, 3.88 ± 1.37% to 25.65 ± 2.33% for blanched and 9.57 ±0.66% to 31.21 ±1.34% for aqueous extract. Among the extracts assessed, ethanol extract showed the most active lipase inhibitory activities with IC_{50} values of 699.3 ± 1.33 µg/mL, followed by aqueous extract (811.52 ± 3.52 µg/mL) and blanched extract (1152.7 ± 4.61 µg/mL). However, orlistat indicated IC_{50} value of 56.8 ± 0.11 µg/mL.

3.5. Glucose Utilization in C3A Hepatocytes

Glucose utilization and corresponding cytotoxicity in C3A hepatocytes are presented in Figure 7. All the samples showed weak glucose utilization in C3A hepatocytes compared with metformin treated cells which showed the highest glucose uptake. At 50 µg/mL, only the blanched extract showed a very sharp drop in glucose uptake. Whereas, at the highest concentration tested (100 µg/mL), very weak or no glucose utilization was observed for all the three extracts. The results have also shown that higher concentrations of the extracts drastically reduced the viability of the cells, with the greatest reduction observed in the blanched extract.

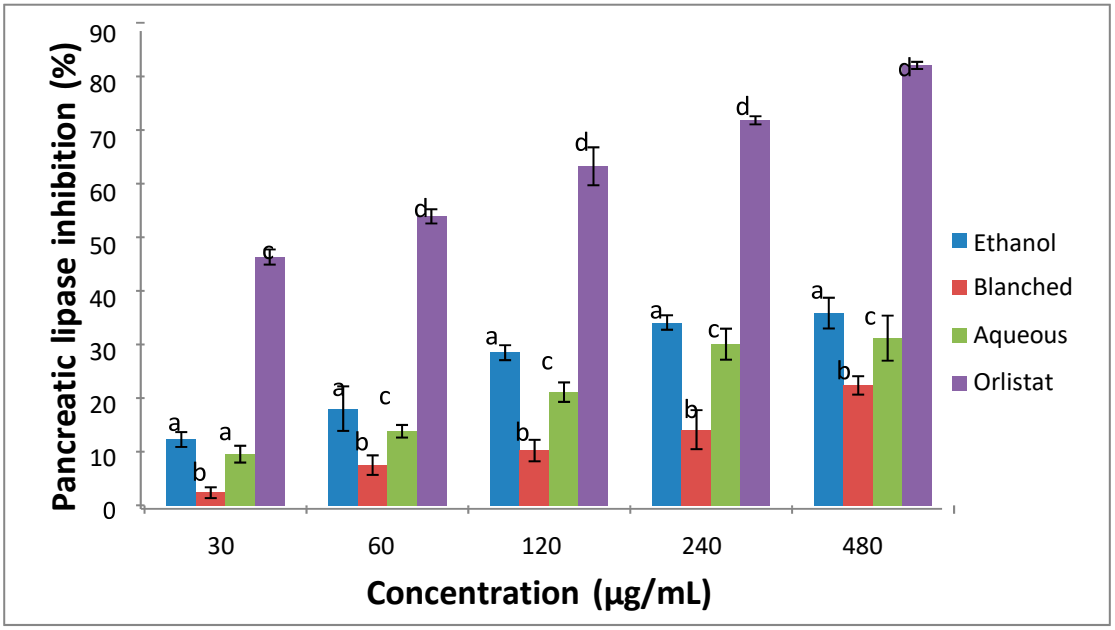

Figure 6. Pancreatic lipase inhibitory activity of *H. arborescens* leaf extracts and positive control (orlistat). Values are expressed as mean ± SD (n = 3). Mean separation by SD (p < 0.05). Set of bars (the same concentration) with different alphabets are different.

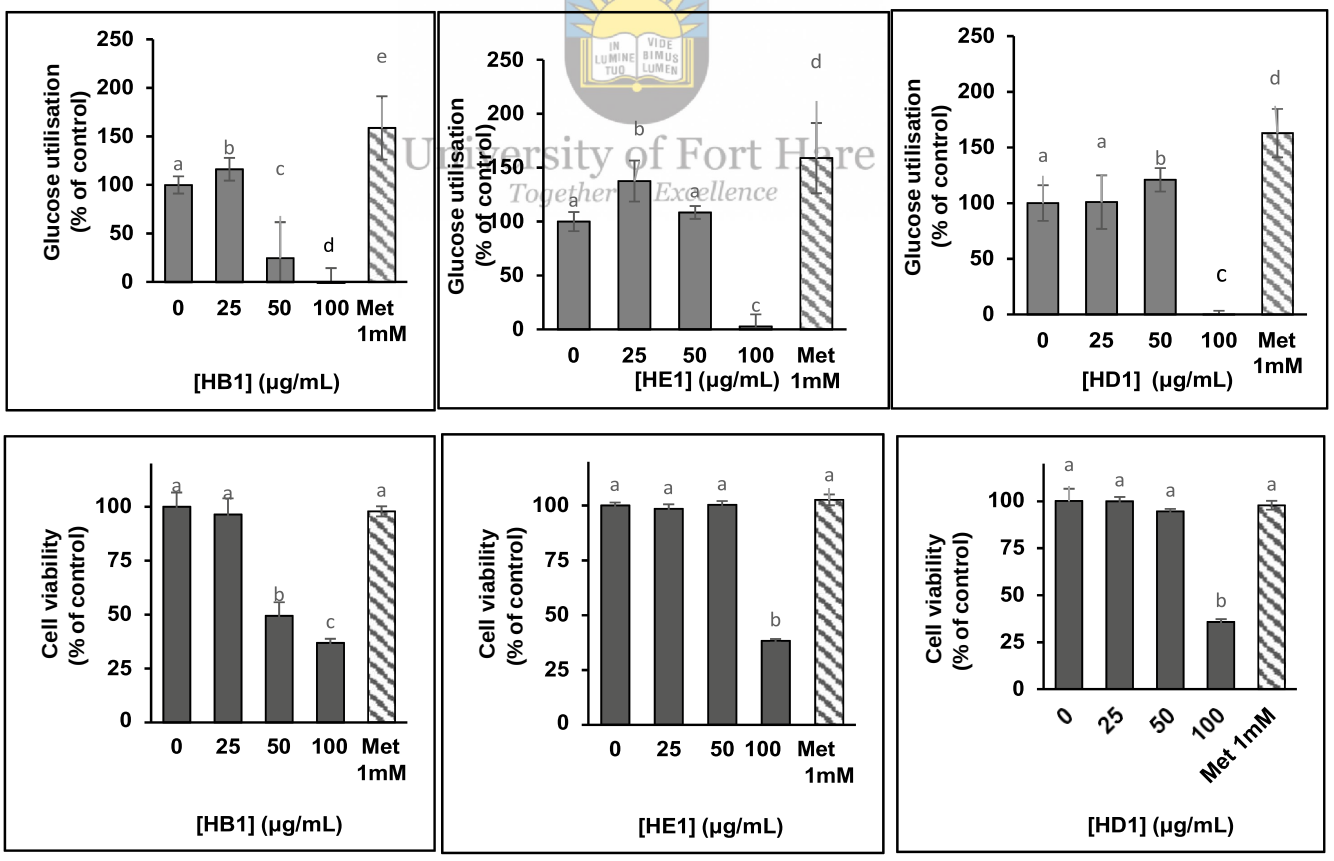

Figure 7. Effects of *H. arborescens* leaf extracts on glucose utilization in C3A cells and their corresponding effects on the C3A cell viability (MTT cytotoxicity) compared to the untreated cells. (Data are expressed as % of control ± SD, n = 3). HB—Blanched; HE—Ethanol and HD—Aqueous extracts; Met—Metformin. Mean separation by SD (p < 0.05). Set of bars with different alphabets are significantly different.

3.6. Lipid Accumulation

Lipid accumulation effect of *H. arborescens* leaf extracts on C3A cells compared with the control is depicted in Figure 8. A great increase in the levels of neutral lipids and phospholipids was obvious for the blanched extract (75.95% at 50 µg/mL and 45.45% at 100 µg/mL respectively). However, for the ethanol extract, a great increase in neutral lipid accumulation (63.11%) was only observed at the highest concentration (100 µg/mL). Onthe other hand, a relatively weaker, but statistically significant ($p > 0.05$), increase in neutrallipids (18.07%) and phospholipids (22.02%) at 50 µg/mL was observed in the aqueous extract. The corresponding images of the C3A cells treated with leaf extracts are indicated in Figure 9.

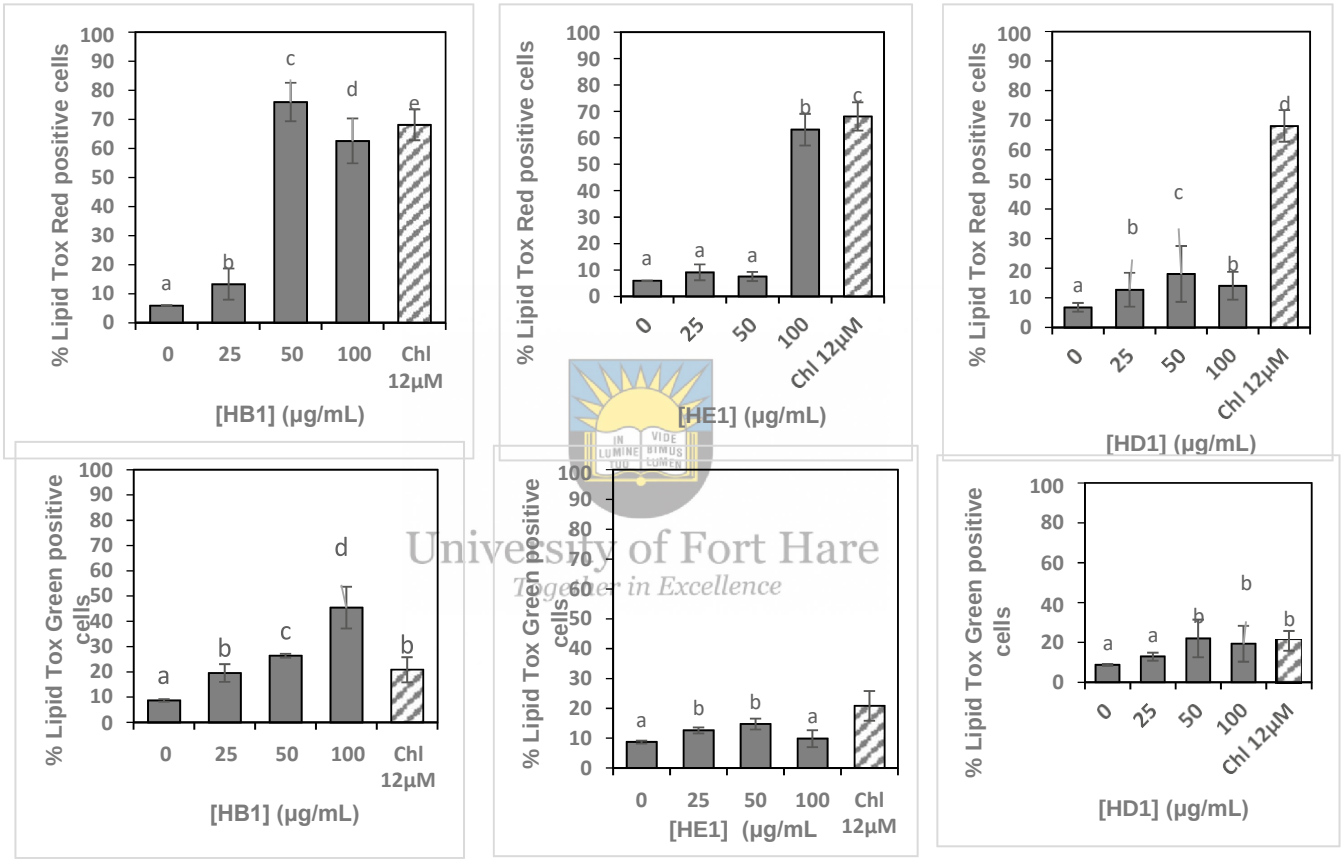

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Figure 8. Neutral lipid (LipidTox Red) and phospholipid (LipidTox Green) accumulation in C3A cells treated with indicated extracts and chloroquine (positive control) for 48 h. HB—Blanched; HE—Ethanol and HD—Aqueous extracts; Chl.–Chloroquine; Data is expressed as a percentage of cells staining positive for each treatment. Mean separation by SD ($p < 0.05$). Set of bars with different alphabets are significantly different. The corresponding images of the C3A cells treated with *H. arborescence* leaf extracts are indicated in Figure 9.

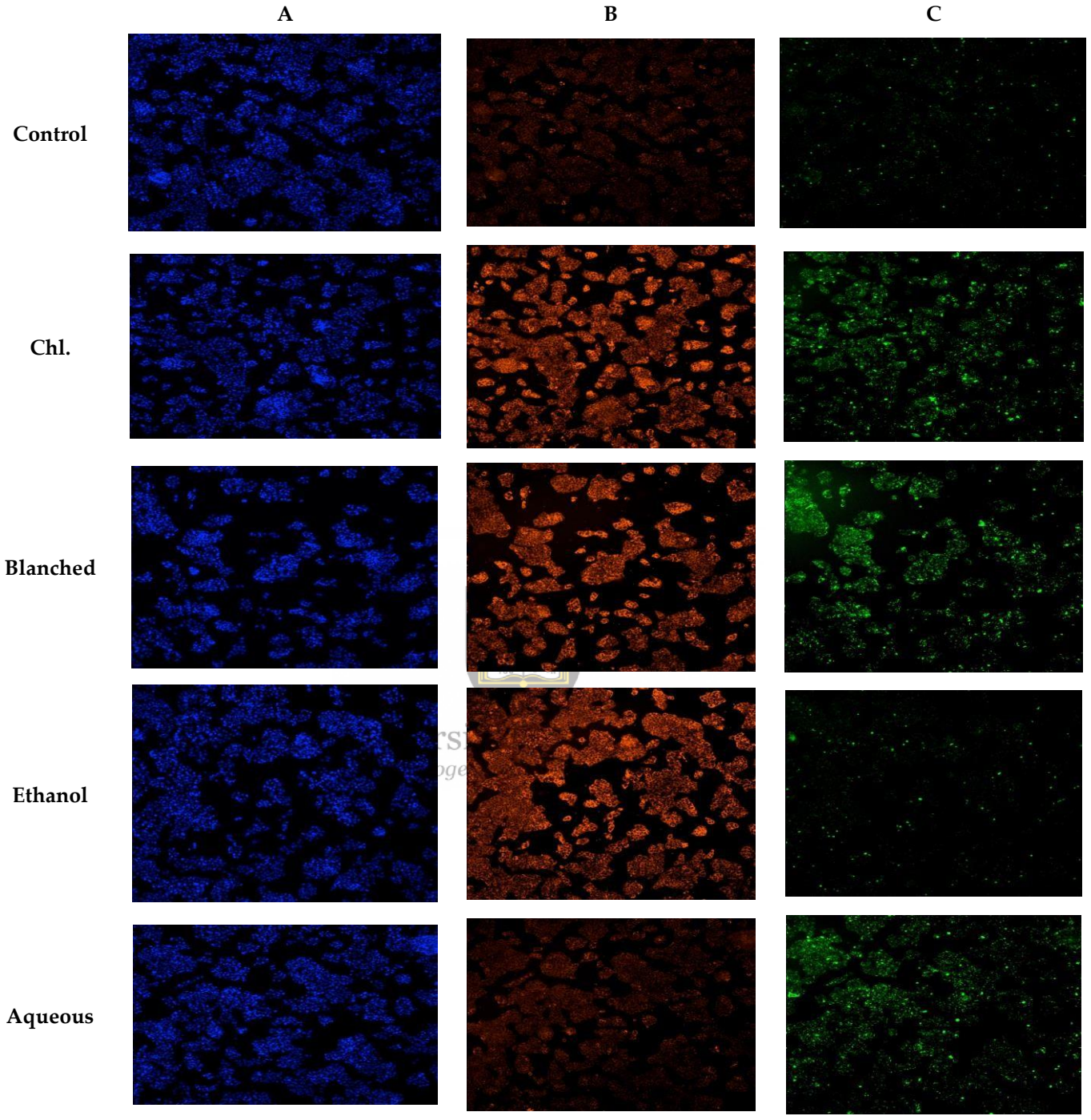

Figure 9. Images of C3A cells after 48 h treatment with 100 µg/mL each of ethanol, blanched and aqueous extracts of *H. arborescens* leaves obtained from Hoechst 33,342 staining. Column (**A**)—All nuclei, Column (**B**)—LipidTox Red (neutral lipids), Column (**C**)—LipidTox Green (phospholipids).

3.7. Glucose Utilization in L6 Myocytes

The effect of *H. arborescens* leaf extracts on glucose utilization in L6 myocytes, and its resultant cytotoxicity are revealed in Figure 10. Data was expressed as a percentage of the untreated control, and insulin was used as the positive control. While the insulin treated cells showed significantly higher glucose utilization than the extracts, a slight increase was observed only at 50 and 100 µg/mL for aqueous and ethanol extracts, with no significant increase for the blanched extract. However, for the aqueous extract there was a decline in glucose utilization from 154.89% at 50 µg/mL to 124.39% at 100 µg/mL; while for the

ethanol extract treated cells, glucose utilization only increased from 95.11% at 25 µg/mL to 107.82% at 50 µg/mL with no further increase at 100 µg/mL. Furthermore, the cytotoxicity of the extracts on L6 cells measured and expressed in comparison with the control, showed significant levels of toxicity at high concentrations.

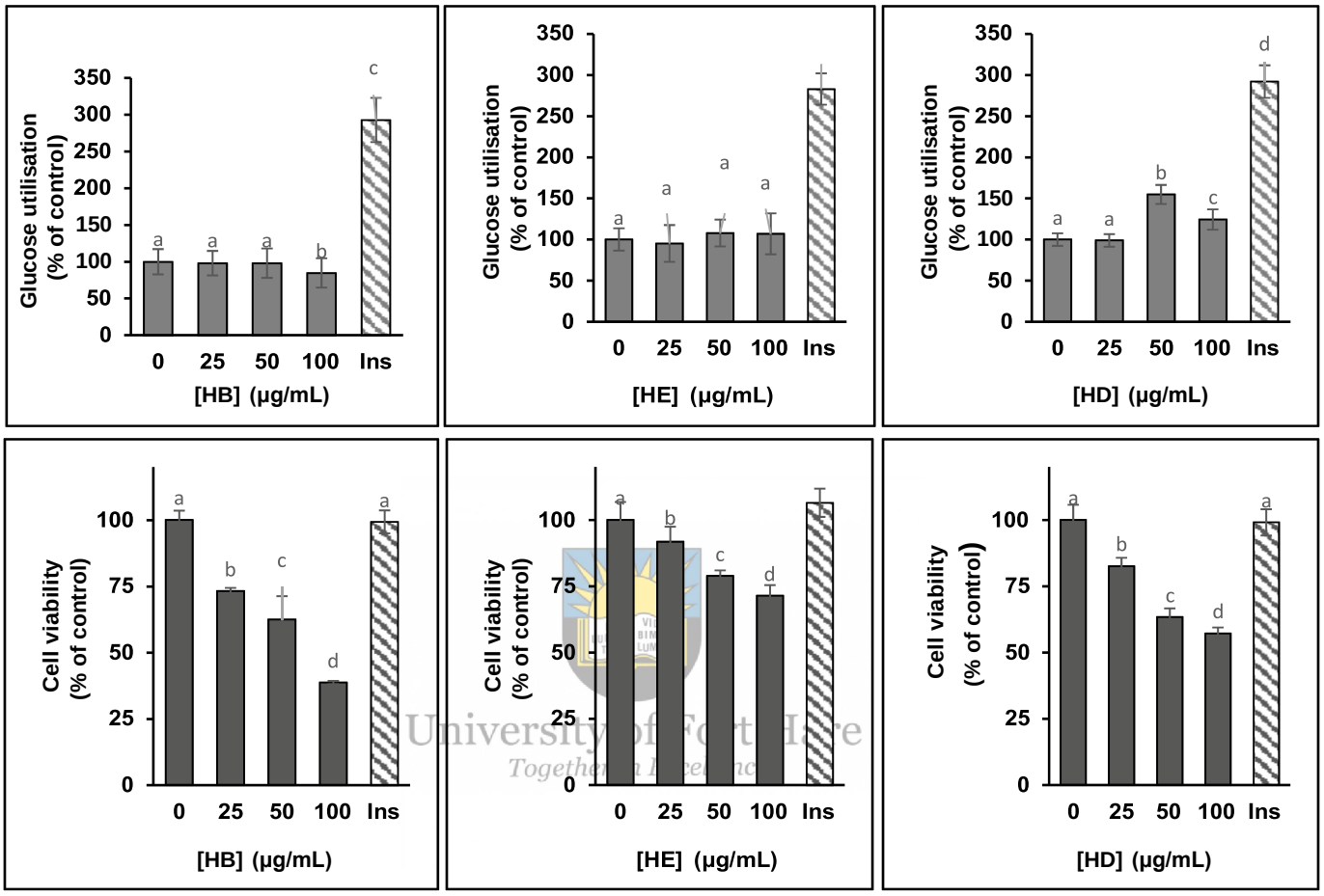

Figure 10. Effects of *H. arborescens* leaf extracts on glucose utilization in L6 cells and their corresponding effects on the L6 cell viability (MTT cytotoxicity) compared to the untreated cells. (Data are expressed as % of control ± SD, n = 3). HB—Blanched; HE—Ethanol and HD—Aqueous extracts; Met—Metformin. Mean separation by SD ($p < 0.05$). Set of bars with different alphabets are significantly different.

4. Discussion

The upsurge in cases of diabetes mellitus has become a global health challenge. Herbal medicines have been employed in the management of diabetes because they have reduced negative consequences when compared with the oral hypoglycemic agents such as sulfonylurea, metformin and troglitazone [2,32]. However, the use of these herbal remedies without proper scientific validation of their safety and acclaimed activity can be detrimental and sometimes deadly [33].

H. arborescens is a common medicinal plant traditionally used in South Africa for the management of diabetes mellitus [18,19]. Cytotoxic evaluation using cell lines is a common method for the screening of pharmaceutical products and synthetic organic compoundsfor their potential in the treatment of diseases [34]. The dose dependent but weak decline in cell density observed for both aqueous and ethanol extracts suggests that the risk for toxicity is minimal at physiologically relevant dosages as opposed to the blanched extract with more prominent decrease in cell density even at lower concentration. In addition, the sharp drop in cell density for all the extracts at higher concentrations may be due to the loss of propidium iodide positive cells during the staining procedure or due to a loss in

proliferation and hence the absence of meaningful PI staining at lower concentrations. The cytotoxicity of the extracts also further explains the reason for the low glucose utilization observed for the extracts.

A-glucosidase and α-amylase are key enzymes for intestinal carbohydrate digestion and the inhibition of these enzymes have been recognized as one of the therapeutic approaches for the regulation of postprandial hyperglycemia; an initial metabolic defect that occurs in the onset of diabetes mellitus [35–38]. Our previous studies by GC-MS analysis have documented the presence of α-pinene, β-pinene, β-ocimene and D-limonene in *H. arborescens* leaves [39] and these components have been established to show some hypoglycemic activities [40,41]. Phenolic compounds such as phenols, terpenoids and flavonoids are possible natural sources of α-glucosidase inhibitors which have been reported to repress glucose release from the liver as well as enhance hepatic glucose uptake [30,42]. Our previous work has established the phenolic constituents of *H. arborescens* leaves [43] and based on previous reports, the α-glucosidase inhibitory activity of the three extracts observed in the present study could be attributed to the presence of polyphenolic compounds [44,45]. The high inhibition of α-amylase enzyme by acarbose is linked to several unpleasant side effects such as abdominal disturbances, flatulence and diarrhea [46]. Therefore, the weak α-amylase activity with corresponding stronger inhibition against α-glucosidase observed for the *H. arborescens* leaf extracts is advantageous [47–49] and agrees with previous reports which proposed that phytochemicals are stronger inhibitors of α-glucosidase compared to α-amylase [44,50]. Moderate α-glucosidase inhibitory potential obtained in the present study is comparable with previous results on hexane, ethyl acetate and methanol extracts of *H. arborescens* leaf and bark [51] as well as in some other members of the Apiaceae family such as *C. Asiatica* [52], *A. graveolens* [53], *P. Anisum* [48,54,55] and *C. cyminum* [56,57].

One mechanism through which phenolic compounds control glucose metabolism is to stimulate the muscle and fat cells to enhance their glucose utilization activities [58]. However, this study did not indicate a direct relationship between phenolic compounds and glucose utilization in L6 cells, as this was relatively weak for all the extracts especially the blanched extract, even though the extracts showed high phenolic compounds, with ethanol having the highest phenolic compounds. Arguably, the slight increase observed for ethanol extract at a concentration of 50 µg/mL might be attributed to the presence of higher hypoglycemic phenolic compounds [59] compared to the aqueous and blanched extract. Similarly, in C3A cells, glucose utilization declined at higher concentrations especially for the blanched extract and this could be because it proved to be the most toxic among the extracts assessed.

Excessive weight gain is a major risk factor for diabetes mellitus and lipid accumulation is used as a tool to check the level of adipogenesis in 3T3-L1 cells [60]. A reduction in lipid accumulation and inhibition of pancreatic lipase; an enzyme responsible for digestion of dietary fat, delaying fat deposition into adipose tissue, play a crucial role in reducing weight gain and obesity [61]. In this study, although lipase inhibition was quite low compared with orlistat (positive control), the lipase inhibitory activity obtained suggests that *H. arborescens* leaves may be a promising tool in managing excess weight and obesity. On the other hand, the results obtained for lipid accumulation in C3A cells with the use of Hoechst 33342/PI staining has shown that the three extracts do not significantly reduce lipid accumulation in C3A cells. These results suggest that the mechanism of action of *H. arborescens* leaves extracts may not be through reducing lipid accumulation.

5. Conclusions

This study presented new findings and established the pharmacological potential of *H. arborescens leaves* in controlling of diabetes and obesity. The cytotoxic activities as well as glucose utilization in L6 cell lines were evaluated. The results suggest a reasonable toxicity profile for the aqueous and ethanol extracts with limited risk for hepatotoxicity at physiologically relevant concentrations. Although relatively weak glucose utilization was obtained for the plant extracts, when compared to metformin, significant inhibition against

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α-amylase and α-glucosidase enzymes was observed for the ethanol and aqueous extracts. Our results have shown that both extracts may possibly exert their anti-diabetic properties by stimulating the inhibition of the carbohydrate digestive enzymes may therefore be promising for the management of diabetes mellitus. In addition, the extracts indicated considerable lipase inhibitory potential but no reasonable reduction in lipid accumulation in C3A cell lines.

Presumably, there is little or no study to justify the traditional use of *H. arborescens* leaves in the management of diabetes and obesity. Very importantly, precautions should be taken in the use of *H. arborescens* leaves at high concentrations to ensure safety and efficacy.

Author Contributions: T.O.A. and G.A.O. designed the experiments, performed the experiments, analyzed data, and wrote the draft. G.A.O. and A.J.A. supervised the study, coordinated, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee, University of Fort Hare Animal and Plant Use Research Ethics Committee, South Africa with protocol number OTA011SABI01/19/E.

Data Availability Statement: The data that support the findings of this study are available in this article.

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Conflicts of Interest: The authors declared no conflict of interest with respect to the research, authorship, and/or publication of this article.

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CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS

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GENERAL DISCUSSION AND CONCLUSION

Discussion

Diabetes mellitus is a very significant disease condition with very high-cost implication. A major concern with this disease is the projected increase in mortality and morbidity which is associated with the complications of the disease (Sena et al., 2010). The global use of herbal medicines and phytonutrients or nutraceuticals is tremendously on the increase with many people now resorting to the use of plants for the treatment and management of a myriad of diseases including diabetes mellitus, and this is centered on the evidence that plants contain bio actives that can promote health and alleviate diseases (WHO, 2004, Bora & Sharma, 2011). Although there are many plants used in traditional medicine with acclaimed therapeutic properties, there is no proof to thisclaim. Also, some plants with great medicinal potentials and high nutritional value are greatly underutilized due to dearth of scientific information on their pharmacological importance, insufficient evidence of safety, and potency (Mohammed, 2012).

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Heteromorpha arborescens is one of the plants used in the management of diabetes mellitus in the Eastern Cape Province, South Africa. However, there are little or no scientific reports on the chemical composition and the antihyperglycemic activity of this plant to proof this claim. Therefore, the basis of this study is to determine the chemical composition as well as perform and invitro investigation of the antihyperglycemic potential of *H. arborescens* leaf extracts so as to provide information that could validate the ethno-medicinal claims for the use of this plant in the management of diabetes mellitus.

The development of diabetic complications can be revealed by inflammation, lipid peroxidation and the accumulation of glycation adducts. Therefore, reduction of hyperglycaemia and increasing

antioxidants status plays a key role in prevention of diabetic complications. The phytochemical contents and antioxidant activity of *H. arborescens* extracts were determined and a higher quantity of phenolic compounds, proanthocyanidin and flavonoids were observed in the ethanol samples when compared to the acetone, blanched and aqueous extracts. A positive correlation between polyphenolic contents and antioxidanta c t i v i t i e s in extracts of *H. arborescens* leaves was established, indicating that some phenolic compounds may be responsible for the high antioxidant activity (Baharfar et al., 2015). Such polyphenolics possess great antioxidant properties elicited by mechanisms involving free radical scavenging, chelation of metal ions involved in free-radical reactions, or an increase in antioxidant enzyme activities (Asmat et al., 2015). The relatively high antioxidant activity observed in the extracts was of great interest in its potential of allieviating oxidative stress because suppression of ROS in diabetic conditions restores cell function and insulin sensitivity (Kaneto et al., 2010). Diabetes mellitus has been linked with reduced response of T cells, neutrophil function, and reduced immunity. Thefore, diabetic patients have very tendencies to develop infections (Khazaal et al., 2020). Considerable antibacterial activity observed with the leaf extracts may be advantageous in contributing to the management of diabetes mellitus.

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Diet plays a huge role in inducing multiple metabolic processes and changes the metaabolism homeostatasis. Antioxidant nutrients including vitamins, minerals and related bioactive compounds from fruits and vegetables have been reported to provide protective roles against oxidative stress (Matough et al., 2012). It is therefore pertinent to identify and make suitable dietary solutions that will enhance the health of diabtetic patients and alleviate the prevalence of diabetes and its related complcations. Since *H. arborescens* leaves are in some cases consumed as vegetable, the nutritional and antinutrient composition of *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. leaves were further assessed to ascertain its nutitional importance in diabetic

mellitus and its complications. The results indicate that *H. arborescens* leaves arenutrient-rich and can contribute effectively to the daily nutrient requirements especially in diabetic conditions.

Furthermore, plants contain essential oils which are valauable natural products, with theurapeutic uses. The chemical composition of essential oils from *H. arborescens* leaves were investigated and observed to contain sabinene,δ-3-carene, myrcene, germacrene-D, limonene, (Z)-β- ocimene, β-phellandrene, and α-pinene as major constituents, which possess antibacterial activities (Abifarin et al., 2020). This study has shown that the chemical composition of the essential oils obtained from *H. arborescens* leaves is dependent on the extraction method. The SFME method resulted in a higher yield in terms of quantity and an essential oil of better quality due to the presence of higher valuable oxygenated compounds than that which was obtained by hydrodistillation. Furthermore, the SFME method resulted in a reduced consumption of electricity and CO_2 emission.

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The strategy of reducing carbohydrate digestion by regulating the activity of α-amylase and α-glucosidase to control postprandial hyperglycemia (Gong et al., 2020). The use of carbohydrate digestive enzyme inhibitors from natural resources could be a possible approach to block dietary carbohydrate absorption with reduced negative effects than synthetic drugs (Exteberria et al., 2012). Phytochemicals such as flavonoids and phenols have been reported to have antidiabetic effects (Arukwe et al., 2012). Therefore, considerable phytochemical contents of *H. arborescens* leaves seemed promising for its antidiabetic activity. Therefore, to establish the claimed antidiabetic potential of *H. arborescens* leaf extracts, the antihyperglycemic activity was investigated invitro. Results revealed that both ethanol and aqueous extracts exhibited considerable inhibition against α-glucosidase (IC_{50} of 627.29 ± 4.62 µg/mL and 576.46 ± 3.21 µg/mL respectively), while the blanched extract showed weak α-glucosidase inhibition (IC_{50}; 855.38 ± 4.29 µg/mL) and the aqueous extract showed the best α-amylase inhibition (IC_{50}; 583.74

± 5.87 µg/mL). However, weak α-amylase inhibition was observed in the ethanol (IC_{50}; 724.60 ± 4.33 µg/mL) and blanched extracts (IC_{50}; 791.63 ± 3.76 µg/mL). The toxicity of the extracts is indicated by LC_{50} values as 154.75 μg/mL, 125 μg/mL, and 90.58 μg/mL for ethanol, aqueous and blanched extracts respectively, indicating the blanched extract to be the most toxic. Moderate glucose utilization in both C3A and L6 cells wasalso observed for the aqueous and ethanol extracts which may be attributed to the relativelylower toxicity levels present. However, glucose utilization was very weak for the blanched extract, which may be due to higher level of cytotoxicity it possessed. Relatively weaker lipase inhibition was observed for the ethanol (IC_{50}; 699.3 ± 1.33 µg/mL), aqueous (IC_{50}; 811.52 ± 3.52µg/mL) and blanched extracts ($IC_{50;}$ 1152.7 ± 4.61 µg/mL) compared to orlistat ($IC_{50;}$ 56.88 ± 0.11 µg/mL). However, there was no reasonable reduction in lipid accumulation observed in all the extract treated cells. These observations suggest that ethanol and aqueous extracts of *H. arborescens* leaf are promising as new agents for the treatment of diabetes and its acclaimed anti-obesity potentials may are likely as due to its lipase, α-amylase, and α-glucosidase inhibition.

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Conclusions

1. *H. arborescens* ethanol extracts exhibited the highest phytochemical, antioxidant and antibacterial activities as compared to the aqueous, blanched and acetone extracts. The aqueous extract was not active against the Gram positive bacteria (*Bacillus pumilus, Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram negative bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli*) at concentrations between 0.78-12.5 mg/mL.
2. Although *H. arborescens* is wild and somewhat underutilized, it could be a good source of nutrients and mineral elements due to its abundance in protein, fiber, calcium, carbohydrates and other vital elements. It may therefore help to combat malnutrition in South Africa.

3. The plant extracts showed weak glucose utilization compared to metformin, there was a significant inhibition against alpha-amylase, alpha-glucosidase and alpha-lipase for the ethanol and aqueous extracts. These observations suggest that ethanol and aqueous extracts of *H. arborescens* leaf are promising as new agents for the treatment of diabetes and its acclaimed anti- obesity potentials may are likely as due to its lipase, α-amylase, and α-glucosidase inhibition. Also, a reasonable toxicity profile was observed for only the aqueous and ethanol extracts with limited risk of hepatotoxicity at concentration. However, further *in vivo* studies needs to be done to ascertain its safety.
4. *H. arborescens* leaves contain sabinene,δ-3-carene, myrcene, germacrene-D, limonene, (Z)-β-ocimene, β-phellandrene, and α-pinene as major essential oil constituents and solvent-free microwave extraction method is better in extracting quality oil from *H. arborescens* leaves at reduced electricity consumption and CO_2.

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APPENDIX


IN LUMINE TUO
VIDE BIMUS LUMEN
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ETHICAL CLEARANCE CERTIFICATE
AREC-150311-008

Certificate Reference Number: OTA011SABI01/19/E

Project title: **Evaluation of the *in vitro* anti-diabetic potential of *Hetromorpha arborescens* (Spreng.)Cham leaves using cell line and enzyme based models**

Nature of Project: PhD in Botany

Applicant: Taiwo Oluwafunmilola Abifarin

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Supervisor: Dr. G.A. Otunola
Prof. A.J. Afolayan

On behalf of the University of Fort Hare's Animal Research Ethics Committee (AREC) I hereby give ethical approval in respect of the undertakings contained in the above-mentioned project and research instrument(s). Should any other instruments be used, these require separate authorization. The Researcher may therefore commence with the research as from the date of this certificate, using the reference number indicated above.

Please note that the AREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the document;
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Researcher must report to the AREC in the prescribed format, where applicable, annually, and at the end of the project, in respect of ethical compliance.

The AREC retains the right to

- Withdraw or amend this Ethical Clearance Certificate if
  - Any unethical principal or practices are revealed or suspected;
  - Relevant information has been withheld or misrepresented;
  - Regulatory changes of whatsoever nature so require;
  - The conditions contained in the Certificate have not been adhered to.

- Request access to any information or data at any time during the course or after completion of the project.

- In addition to the need to comply with the highest level of ethical conduct principle investigators must report back annually as an evaluation and monitoring mechanism on the progress being made by the research. Such a report must be sent to the Dean of Research's office.

The Animal Research Ethics Committee wished you well in your research.

Yours sincerely

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Dr. Craig Tambling
AREC Chairperson

07 September 2019