AMELIORATIVE EFFECT OF *HELICHRYSUM CYMOSUM* EXTRACTS AND BIOSYNTHESIZED ZINC OXIDE NANOPARTICLES ON C3A HEPATOCYTES, L6 MYOCYTES AND TM4 SERTOLI CELLS.

By

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DECLARATION

I, Achasih Quinta Nkemzi, declare that the contents of this thesis represents my personal unaided work and that the thesis has not been submitted for academic examination towards any qualification. All research collaborators indicated in the chapters have been referenced and other assistance obtained have been acknowledged. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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Signed **Date**

ABSTRACT

The current study investigated the antidiabetic, antioxidant, anti-inflammatory, cytotoxicity, and reproductive effect of *Helichrysum cymosum* aqueous, ethanol and zinc oxide nanoparticles using In vitro assays. The shoots of *H. cymosum* were extracted using two solvents; aqueous by infusion and ethanol by extraction. Meanwhile zinc oxide nanoparticle was obtained by green synthesis from the aqueous extract. Bioactive compounds were identified on the aqueous and ethanol extracts using High-Performance Liquid Chromatography and UHPLC-ESI-MS. Cytotoxicity of all tested sample was carried out using MTT (colorimetric assay) on C3A hepatocytes, L6 myocytes and TM4 Sertoli cell, to establish a non-toxic concentration. Antidiabetic effect of aqueous and ethanol, and zinc oxide nanoparticles were tested by enzymatic inhibitory assays; alpha glucosidase, alpha amylase and pancreatic lipase inhibition. Additionally glucose uptake and utilization were assessed on C3A and L6 cell lines. The mouse macrophage cell line, RAW 264.7 was used to assess anti-inflammatory activity of aqueous, ethanol and nanoparticles, by measuring the levels of nitrite produced. The ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay were used to examine the antioxidant activity of aqueous and ethanol extracts, while average cellular CellROX® Orange fluorescent intensity was used to determine the intracellular antioxidant activity of both extracts and nanoparticles on C3A and L6 myocytes cells. The antioxidant enzyme activity, superoxide dismutase, glutathione and other non-enzymatic assays like total antioxidant capacity, reactive oxygen species and morphological changes were performed on TM4 Sertoli cell lines to evaluate the antioxidant activity on reproductive cells.

The findings reveal that the extracts and zinc oxide-mediated nanoparticles demonstrated potent hypoglycemic activity and antioxidant activities, mitigating the effect of oxidative stress induced complications associated with diabetes. The extracts also showed enhanced antioxidants such as glutathione and superoxide dismutase and positively interfered in reactive oxygen species production in reproductive cells indicating that the extracts contain important bioactive constituents which can alleviates oxidative stress induced-infertility complications. The current study suggest that *H.cymosum* extracts and zinc oxide mediated nanoparticles have prospect for future development of antidiabetic and infertility drug. However, additional research in vivo and clinical trials is necessitated.

Keywords: anti-diabetic, oxidative stress, *Helichrysum cymosum*, reproduction, antioxidant, cytotoxicity.

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DEDICATION

This thesis is dedicated to my creator, God almighty in whom I live and have my being, to the entire Nkemzi's family, for their encouragement and support and to my beloved son Rhema N. Ajongakoh, for being my motivation.

PREFACE

The study is structured into six chapters, written and formatted conforming to the journal's submission requirements where it has been submitted for review.

Chapter one presents an introductory overview of the research, outlining the background, problems and motivations, aims and objectives to be accomplish in this study.

Chapter two has been published in the journal "Plant Science Today", thoroughly examined existing research related to the current subject area and identifying gaps to build upon and expand knowledge in the field.

Chapter three presents the first experimental paper title "*In vitro* hypoglycemic, antioxidant, anti-inflammatory activities and phytochemical profiling, of aqueous and ethanol extracts of *Helichrysum cymosum*," has been Published in the journal "Phytomedicine Plus."

Chapter four presents the second experimental paper titled "Antidiabetic, anti-inflammatory, antioxidant and cytotoxicity potentials of green-synthesized zinc oxide nanoparticles using the aqueous extract of *Helichrysum cymosum,"* has been published in the Journal "3 Biotec."

In chapter five is the third experimental paper titled "The *in vitro* protective effects of *Helichrysum cymosum* on TM4 Sertoli cell," the manuscript has be prepared for submission to South African Journal of Botany for consideration.

Chapter six presents the general discussion of the key findings and conclusion of this research and provide possible recommendation for future studies.

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ABBREVIATIONS

ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
AQ	Aqueous
CPUT	Cape Peninsula University of Technology
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
GSH	Reduced glutathione
GLUT2	Glucose transporter 2
HPLC	High-Performance Liquid Chromatography
H2O2	Hydrogen peroxide
IL-1 α	Interleukin-1 alpha
IL-6	Interleukin-6
IL-18	Interleukin-18
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]
NO	Nitric oxide
OS	Oxidative stress
REC	Research ethics committee

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of mean
SOD	Superoxide dismutase
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAC	Total antioxidant capacity
TEAC	Trolox equivalent antioxidant capacity
TM4	Mouse Sertoli cells
TNFα	Tumor necrosis factor alpha
UPLC	Ultra-performance liquid chromatography
WHO	World health organization
ZnO-NP	Zinc oxide nanoparticles

CHAPTER ONE

INTRODUCTION

1. Overview of diabetes mellitus (DM)

Diabetes mellitus (DM) is a well-known chronic metabolic disorder that affects all age groups and has become a global public health burden, with substantial increase in incidence of type 1 diabetes (T1D) and type 2 diabetes (T2D) (Lam and LeRoith, 2012, Machado et al., 2018). T1D known to be insulin dependent diabetes is caused by impaired pancreatic beta cells that hinders insulin production (Akinson et al., 2014, Chiang et al., 2014, Shilleh et al., 2023) while T2D is non-insulin dependent caused by both insulin resistance and insulin deficiency (Olokoba et al., 2012, Hegde and Reddy, 2019). Diabetes affects over 300 million people across the globe, contributing significantly to the high level of mortality and morbidity in the world, with 80% of deaths in the world attributed to diabetes (Machado et al., 2018). Studies have estimated that the global prevalence of diabetes will reach almost 600 million cases by 2035 (Surendar et al., 2017) with 90% of T1D diabetes occurring in children and adolescence while T2D accounts for 90-95% of diabetic adults (Craig et al., 2009, Limbert et al., 2023, Azeez et al., 2024). Factors such as enetics, bad dietary habits, decrease physical activity and stress are the main predictor of diabetes most especially T2D and obesity (Lovejoy, 2002, Olokoba et al., 2012, Wharton et al., 2019).

1. 2. Hyperglycemia induced complications

DM is marked by hyperglycemia, a condition indicated by elevated blood sugar level and increase blood sugar intolerance (Gunasekaran et al; 2019, Oguntibeju, 2019). Hyperglycemia manifestation could result in numerous vascular complications such as nephropathy, retinopathy, cardiomyopathy and neuropathy which are the major contributors of morbidity and mortality in diabetes. Additionally, oxidative stress, inflammations and associated pathological pathways have also been implicated during major hyperglycemic episodes and associated vascular complication (Smruthi et al., 2016). Oxidative stress as the main producer of different cellular pathways and subsequent inflammatory incidence, thereby contributing to endothelial damage in the blood capillaries of organs such as the retina, peripheral neurons, and kidneys resulting to retinopathy, neuropathy, and nephropathy (Smruthi et al., 2016).

The development of oxidative stress (OS) is prompted by reactive oxygen species (ROS) which comprise of the following: superoxide anion (O_2), hydroxyl radicals (OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Ravipati et al., 2012, Bryll et al., 2020). According to Torres-Moreno et al. (2019), oxidative stress arises from imbalance of pro-oxidant and anti-oxidant compounds. Pro-oxidants are free radicals and ROS that are produced under normal physiological state in human metabolism. However, overproduction of these free radicals and ROS leads to oxidative stress that could cause enormous damage to cells (Torres-Moreno et al., 2019). Damaging effects caused by excessive production of ROS and reactive nitrogen species (RNS) are peroxidation of membrane lipids, degradation of tissue proteins and membranes, DNA and enzymes damage, which have been linked to pathologies such as arthritis, hemorrhagic shock and coronary diseases, cataract, cancer and AIDS as well as age-related degenerative brain disorders (Aiyegoro and Okoh, 2009, Roy et al., 2017).

Studies have demonstrated a correlation between ROS production, systemic markers of inflammation and their potential association with DM (Luqman et al., 2012, Sevastianos et al., 2020). Increase in inflammatory cytokines such as IL-1, IL-6, IL-18 and TNF-α has been observed in the blood of patients with diabetes (Alexandraki et al., 2008, Zhao et al., 2018). Inflammation is one of the preliminary physiological and immune response characteristics involved in defensive function within the body against potentially harmful stimuli, such as metabolic stress injury and pathogens (Ondua et al., 2019).

1.2.1 Diabetes mellitus and male infertility

Diabetes mellitus is one of the major contributors to male infertility in recent times, with about 35%-51% of males with DM experiences infertility issues (Alabi et al., 2020, Fan et al., 2024). DM is linked to male infertility via chronic inflammation response and oxidative stress induced by hyperglycemia (Barbagallo et al., 2021). Previous studies have reported the correlation of DM with a range of male reproductive health issues such as erectile dysfunction and ejaculation problems, decrease semen volume, sperm count, impaired sperm motility and abnormal sperm (Alves et al., 2013a, Maresch et al., 2019). The pathogenesis of hyperglycemia-induced damage in different organs is mediated by significant pathways such as advanced glycation end product (AGE) formation, polyol pathway flux and activation of protein kinase C (PKC) isoforms through de novo synthesis of diacylglycerol (DAG)(Maresch et al., 2019, González et al., 2023, Pardina et al.,

2024). Among these pathways, the AGEs is exacerbated in diabetic conditions and have earlier been noted as a contributing factor to male infertility (Maresch et al., 2019). DM has a profound impact on reproductive health function by interfering with the different molecular pathways that control sperm production, thereby resulting to significant alteration of sperm cells development and function (Alves et al., 2013b).

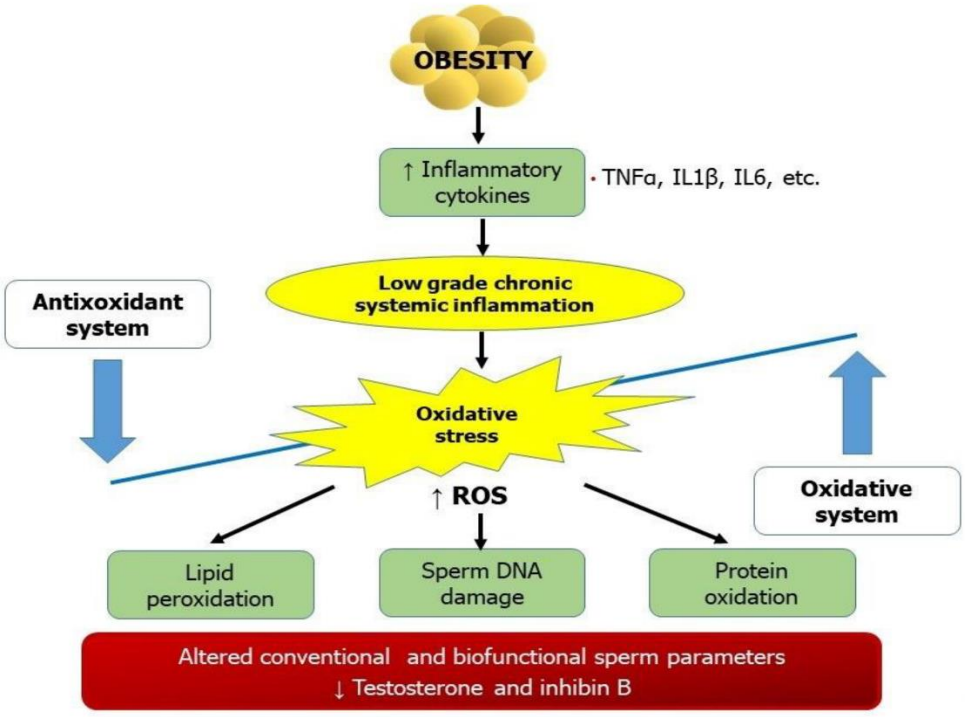

Figure 1.1: Shows a schematic representation of the relationship between oxidative stress, obesity and male reproductive function {adapted without permission from (Barbagallo et al., 2021)}

1.2.2 Therapeutic drugs use to manage diabetes mellitus

The existing antidiabetic therapies used for the treatment and management of diabetes includes insulin and oral hypoglycemic drugs such as biguanides (decrease glucose production by hepatic cells); sulfonylureas (promotes insulin secretion from pancreatic islets); α-glucosidase inhibitors (inhibits glucose absorption in the gut); peroxisome proliferator-activated receptor-γ (PPARγ) agonists (enhance the action of insulin) (Padhi et al., 2020; Blahova et al., 2021). Despite the beneficial role of these medications, various side effects have been reported, some of which includes abdominal complications, hypoglycemia, renal failure, weight gain, bloating, diarrhea,

liver injury and impotence etc. (Adhikari et al., 2021, Ansari et al., 2022). Another crucial setback that is associated to this problem is drug resistance and absence of preventive therapies for long standing complications of this disease. Moreover, these limitations combine with the high cost of drug have propelled the search for alternative treatments that is effective with minimal side effects (Gaonkar and Hullatti 2020, Ansari et al., 2022)

1.2.3. Medicinal plants as alternative treatment in the management of DM

Medicinal plants possessing antioxidant potentials have gained enormous interest nowadays owing to the health benefits they provide (Armijos et al., 2018, Singh et al., 2023, Ashraf et al., 2024). Medicinal plants provide a good source of treatment for several illnesses such as diabetes mellitus, male infertility, neurodegenerative diseases and cancer compared to the use of synthetic drugs. Synthetic drugs have been reported to be toxic and have adverse side effects (Erasto et al., 2005, Kumar et al., 2015). Different *in vitro* studies on medicinal plants have shown that they contain vital components that could mitigate protective effect against oxidative stress in biological systems (Armijos et al., 2018). The mechanism through which antioxidant have beneficial effect on health includes direct reaction with and quenching free radicals, chelation of transition metal, reduction of peroxides and stimulation of the anti-oxidative enzyme defense system (Aiyegoro and Okoh, 2009, Fenton-Navarro et al., 2019). Recently, scientists and pharmaceutical industries have developed interest in studying antioxidant-rich herbal plant composition, most especially the effect of free radicals in organisms (Lobo et al., 2010. Shang et al., 2018). As such, the quest for alternative herbal medicine is necessitated due to the bioactive phytochemicals they possess such as alkaloids, steroids, terpenoids, saponins, phenolics, flavonoids (Kumar et al., 2015, Nwozo et al., 2023, Singh et al., 2023).

South Africa is renowned for its rich plant biodiversity (Louw et al, 2002, Street and Princeloo 2013, Ondua et al., 2019). About 80% of South Africans consume medicinal plant compared to pharmaceutical synthetic drugs for their health care needs (Nattrass, 2008). However, several potential medicinal plants in South Africa with prospective novel pharmaceuticals properties still remain uncovered. *Helichrysum* is a well-known medicinal plant that belongs to the genus Asteraceae, and commonly distributed across South Africa. The native name of *Helichrysum* species in isiXhosa is known as “imphepho”, the genus consists of approximately 500 species, of which 246 have been identified to be indigenous to South Africa. The species of the genus

Helichrysum are differentiated based on the following characteristics: hairy of woolly leaves, a persistent flower heads and the shape and size of the flower heads (Hilliard, 1983, Lourens et al., 2008, Popoola, 2015). The consumption of *Helichrysum* species is based on their availability and geographical area. The genus *Helichrysum* have been documented to possess the following compounds phenol, flavonoids, chalcones, phthalides, α-pyrone derivatives, terpenoids, essential oils, volatiles and fatty acids and the biological activities of its extracts has also been reported. Nevertheless, the compounds responsible for their biological activities have only been identified in a few cases (Aiyegoro and Okoh, 2009, 2010). Studies have documented that *Helichrysum* species of southern Africa is used by the indigenous population for the treatment of wounds, infections and respiratory conditions and diabetes (Lourens et al., 2008, Popoola, 2015, Maroyi, 2019).

The specie *Helichrysum cymosum*, is among the indigenous medicinal plant of South Africa (Lourens et al., 2008, Philander, 2011, Maroyi, 2019, Akaberi et al., 2019). *H. cymosum* is traditionally used to treat different illnesses like; coughs, colds, pains, infected wounds, and headaches, has also been reported to induce trances and attract goodwill from ancestors (Heyman, 2013, Maroyi, 2019). Additional medicinal use includes cardiovascular complications, kidney problems, urinary infections, eye infections, vomiting, insomnia, diarrhea, laxatives, blocked noses, as an immune system booster, for controlling flatulence, for weak bones, as an insect repellant, for skin infections, and for treating varicose veins and influenza (Jadalla et al., 2022). Important phytochemical compounds such as sesquiterpenes and chalcones have been identified from this specie (Matanzima, 2014, Maroyi, 2019, Jadalla et al., 2022). Based on adequate contextual evaluation of *H. cymosum*, the current study seeks to evaluate, the anti-oxidant, anti-diabetes, anti-inflammatory and reproductive potentials of this plant. Several biological activities such as antioxidant, anti-inflammatory, antifungal, antiviral, antimicrobial, anti-diabetic, and cytotoxic effects have been document from this plant (Jadalla et al., 2022). However, there is still paucity of data on the effect of bioactive molecule of *H. cymosum* on DM complications and associated reproductive function.

1.3. Problem statement

Oxidative stress is known to be the leading cause of cell damage and has been implicated in several clinical disorders, such as diabetes mellitus, cancer, renal failure, and liver and reproductive

function (Roy et al., 2017). Diabetes and its associated pathologies remain a global health burden to humanity, characterized by elevated sugar level known as hyperglycemia (Machado et al., 2018). Hyperglycemia often results in series of pathological conditions, such as nephropathy, retinopathy, cardiomyopathy and neuropathy, which are the major contributors of morbidity and mortality in diabetes (Smruthi et al., 2016). Studies have also indicated a link between DM, ROS and inflammatory markers (Dludla et al., 2023, Yousef et al., 2023). Therefore, the increase prevalence of clinical pathologies caused by oxidative stress necessitates urgent measures in exploring potential treatment and management of these diseases.

Although numerous synthetic drugs have been developed and used, they have not been sufficiently resolved DM and its associated health changes, due to the fact that most of the drugs are expensive and have side-effects. Recently, novel strategies have been developed using phytochemicals found in medicinal plants to mitigate and prevent diseases. This has compelled scientists to further explore different categories of therapeutic agents. *H. cymosum* is one of such plants, a native plant of South Africa, which have been reported to possess the following compounds phenol, flavonoids and chalcones, phthalides, α-pyrone derivatives, terpenoids, essential oils, volatiles and fatty acids. The biological activities of its extract have not fully been documented, and little is known about the modulatory effect, specifically, its antidiabetic activities, anti-inflammatory activities and reproductive potentials. The free radical scavenging activities have been documented but inconclusive. Scientific findings on this plant are also scarce in literature. Therefore, findings from this study will help elucidate the pharmacological potentials, and its anti-oxidant, anti-inflammatory, anti-diabetic and reproductive potentials. The data that will be obtained will contribute to scientific data in the field. Also, it would help in understanding the modulatory effects of these plants and contribute to alternative treatment options for diabetes and reproductive health.

1.4. Aim

This study investigated the ameliorative effect of *Helichrysum. cymosum* on C3A hepatocyte, L6 myocytes and TM4 Sertoli and assessed its anti-oxidant, anti-diabetes, anti-inflammatory and reproductive potentials using *in vitro* microdilutions bioassay.

1.4.1. Objectives

1. To profile the phytochemical compounds present in *H. cymosum* plant extracts and determine the phenolic and flavonoids content using high- performance liquid chromatography and ultra-performance liquid chromatography (UPLC).
2. To determine the antioxidant activities of *Helichrysum cymosum* through the following assays: FRAP, TEAC, DPPH, ABTS
3. Investigate the hypoglycemic, anti-inflammatory and antioxidant activities of aqueous and ethanol extracts on C3A hepatocytes and L6 myocytes.
4. Investigate *H. cymosum* synthesized zinc oxide nanoparticles on C3A hepatocytes and L6 myocytes by performing the following;
    - Characterization of nanoparticles using UV-vis spectroscopy, Scanning electron microscope (SEM), Energy dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD), Transmission electron microscope (TEM), and zeta potential.
    - Assess alpha amylase and alpha glucosidase and pancreatic lipase inhibition by the ZnONPs on C3A hepatocytes and L6 myocytes.
    - Assess glucose uptake and its utilization of ZnONPs on C3A hepatocytes and L6 myocytes.
    - Assess the anti-inflammatory activity on raw macrophage and cellular antioxidant activities.

5. Investigate the protective effect of *H.cymosum* extracts on TM4 Sertoli cell;
    - Assessment of cell viability and morphological changes
    - Assessment of the antioxidant activity on TM4 Sertoli cell lines by analyzing the SOD, GSH, ROS, TAC activities.

1.4.2. Research questions

1 What are the phytochemical compounds present in *H. cymosum* aqueous and ethanol extracts and their concentrations?

2 Would the *H. cymosum* extracts and ZnONPs have an effect on antioxidant and inflammatory markers?

3 Does *H. cymosum* extracts and ZnONPs have potential effects on glucose hydrolyzing enzymes, glucose uptake and utilization in C3A hepatocyte and L6 myocytes?

4 What effect do the extracts have on TM4 Sertoli cell function?

5 What are the effects of the extracts and zinc oxide nanoparticles of *H. cymosum on* C3A hepatocyte, L6 myocytes and TM4 Sertoli cell viability?

1.5. Hypothesis

Helichrysum cymosum extracts and synthesized ZnONPs would demonstrate ameliorative effect on hyperglycemic induced complications such as inflammation, oxidative stress and reproductive functions of the studied cell lines.

1.6. Study rational and justification

Several studies have reported that DM marked by hyperglycemia is highly associated with oxidative stress and inflammatory markers (González et al., 2023, Yousef et al., 2023, Novoselova et al., 2024). Likewise, oxidative stress and inflammation in DM have been linked as major contributors of male infertility (Kim et al., 2015, Fan et al., 2024). The frequent succession of diabetic cases across the world has raised concerns on how to manage and prevent this disease. The use of synthetic drugs has been well documented alongside their shortcoming on human health. This has motivated the use of traditional medicinal plants as treatment options in the management and prevention of diseases, since it is accessible, cheap and believed to have little or no side-effect. Indigenous medicinal plants are well known for their rich anti-oxidant properties which alleviates disease severity. This study is design to investigate *Helichrysum cymosum,* a common medicinal plant used by indigenous South Africans in the treatment of various diseases. This plant has been reported in literature to have antioxidant, anti-inflammatory, antifungal, antiviral, antimicrobial, anti-diabetic, and cytotoxic effects (Jadalla et al., 2022), but there is still paucity of data on the plant. The finding from this study will help elucidate the pharmacological potentials, and its anti-oxidant, anti-inflammatory, anti-diabetic and reproductive properties. The data that would be generated from this study would contribute to new knowledge in the field.

1.7. Ethical consideration

All experimental protocols and procedures of the study were approved by the Research Ethics Committee (REC) of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, South Africa (ethics approval number *CPUT/HWS-REC2021/H1*).

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CHAPTER TWO

LITERATURE REVIEW

Reproductive, antioxidant, anti-inflammatory, antimicrobial, protective and antidiabetic activities of *Helichrysum* species

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Abstract

South African *Helichrysum* species are known for diverse medicinal use and treatment of different illnesses. Ethnopharmacological studies have revealed the potential use of *Helichrysum* plants in drug discovery. Although some of these species have been documented, there is still paucity of information on most species. This review seeks to provide a compilation of documented traditional uses, reproductive potential, antioxidant, anti-inflammatory, antimicrobial, protective and antidiabetic activities and other therapeutic properties of some *Helichrysum* species of South Africa. Information on *Helichrysum cymosum, Helichrysum foetidum, Helichrysum odoratissimum, Helichrysum patulum* and *Helichrysum petiolare* were collected from such scientific databases as Google scholar, Scifinder, PubMed, Elsevier, Scopus, and Science direct, in the form of journal articles, scientific reports, theses and books from the Library of the Cape Peninsula University of Technology. Our findings show that these species have historic values in traditional medicine through their diverse use for the management and treatment of such illnesses as cold, diabetes, headaches, digestive problems, sores and wounds, HIV, cancer, etc. Additionally, the reported bioactive constituents isolated from these species have been shown to indicate several activities such as antimicrobial, antioxidant, antidiabetic, neuroprotection, reproductive potentials and others. Thus, the current review highlights the phytochemical and bioactive constituents as well as some of the pharmacological properties of the five selected *Helichrysum* species with a view to providing validation for their use in the pharmaceutical drug development process.

Keywords: ethnopharmacology, phytochemistry, therapeutic, biological activities, *Helichrysum*, traditional medicine.

2.1. Introduction

Medicinal plants usage plays an integral role in the treatment of various diseases among ethnic communities, especially in developing countries. South Africa has a robust cultural identity of the medicinal plant use for healing purposes (1, 2). The genus *Helichrysum* Mill. comprises of many medicinal herb species that have long been established across the world, and is grouped under the family Asteraceae, tribe Inuleae and subtribe Gnaphaliinae (3, 4). The genus derives its name from its golden yellow flowers known in Greek as helios ‘sun’ and chryos ‘gold’, which are common features exhibited by most species (4). About 500-600 *Helichrysum* species are known across the

world, in countries such as Asia, Australia, Europe and Africa. However, the highest number of species has been recorded in the African continent (5). In South Africa, about 250 *Helichrysum* species are known and further divided into groups of 30 based on their differential morphological diversity (4, 6, 7, and 8).

Generally, some species of the genus are remarkably scented and grow yearly while exhibiting different levels of polymorphism within the flowers, leaves and habitats. The presence of dense hair with attractive yellow flower heads of different varieties are common features within the genus (6, 9, and 10). Secondary metabolites such as phenolic acids, flavonoids, diterpenes, coumarins, chalcones, polyacetylenes, sterols, pyrenes acylphloroglucinols, humulone derivatives, phthalides and sesquiterpenes have been isolated from the genus *Helichrysum*. These active compounds have exhibited the following activities in experimental studies: anti-oxidant, anti-allergic, anti-inflammatory, anti-microbial, hepatoprotective and protease-inhibition properties, among others (4, 11).

Herbal plant usage has yielded great therapeutic value against many illnesses in traditional medicine, necessitating the exploitation of indigenous South African medicinal plants (1). Many reviews on the traditional use, phytochemistry and biological activities of *Helichrysum cymosum* (L.) D. Don, *Helichrysum foetidum* (L.). Moench, *Helichrysum odoratissimum* (L.) Sweet, *Helichrysum patulum* (L.). Don and *Helichrysum petiolare* Hilliard & B. L Burtt have been published in recent literature, but studies are still ongoing on these plant species and their identified compounds, to explore their diverse underscored biological activities, which has triggered the curiosity of several researchers. The current review is therefore aimed at elucidating and updating the documented medicinal uses, biological activities and phytochemical constituents of selected *Helichrysum* species of South African origin, with a view to assessing their suitability as sources of potential plant-derived pharmaceutical products.

2.2. Materials and Methods

An in-depth literature searches on *Helichrysum cymosum*, *Helichrysum foetidum*, *Helichrysum odoratissimum*, *Helichrysum patulum* and *Helichrysum petiolare* was done using the following scientific databases: Google scholar, Scifinder, PubMed, Elsevier, Scopus, and Science direct. Meanwhile, other information was sourced through online journal articles, dissertations, scientific reports, and books from the Cape Peninsula University of Technology Library. The following

keywords were inputted during the search: *Helichrysum*, phytochemistry, biological activities, ethnopharmacology, botanical description, traditional use and geographical distribution.

2.3 Results and Discussion

2.3.1. Morphological description of the five Helichrysum species

Helichrysum species are differentiated based on their morphological characteristics such as shape, size and flower heads (12). Each species of the plants exhibits distinct features as described in table 2.1 below.

Table 2.1. Botanical description, common names and geographical origin of five South African Helichrysum species.

Species	Common names	Botanical description and distribution	Sources
Helichrysum cymosum subsp. *cymosum*	Gold carpet (English), Goute Tapyt/timie (Afrikaans), Impepho (isiXhosa and isiZulu).	**Botanical characteristics**: A well-branched, groundcover perennial shrubs, and grow up to 1m, having a small greyish-silver leaves and a greyish-white, woolly tinny branches. The plant consists of yellow flowers in flat-topped flower heads. Flowering occurs between September and April. **Geographical origin/habitat:** It is widely spread in South Africa, usually in damp and sandy slopes. They are distributed in the Eastern Cape, KwaZulu-Natal and Western Cape.	(7, 13- 16)
Helichrysum foetidum (L.). Moench	Yellow everlasting (English)	**Botanical characteristics:** Strong biennial shrublet and can grow up to 1 m. Leaves are aromatic, alternate, sticky, oblong-lanceolate, auriculate, sessile and	(17)

		clasping. Upper surface of the leaf is rough and hairy while the leaf base is grey-woolly, clustering at the stem base and withers during flowering. The stems are simple, strong and straight with a cylindrical shape are habitually unbranched at the lower part. The flower heads have many florets with glossy, deep-yellow to cream color, Blossoming: October–May. **Geographical origin / habitat**: Naturally occurs in South Africa and other African countries. Also occurs as invasive weeds or naturalized in other parts of the world: Brazil, Crimea, Hawaii, Netherland, Portugal, and Spain, and the United Kingdom. Can grow in areas like; shrubby valleys, hill, mountain, damp areas and forest margins.	
Helichrysum odoratissimum (L.) Sweet.	Imphepho (isiXhosa, isiZulu) Kooigoed (Afrikaans)	**Botanical characteristics**: The plant is a perennial shrub, scented, with woolly erect and straggling branches, can grow up to 20–200 cm. the surfaces are greyish white wooly on each side of the leaves. The shape of the leaf is generally, linear, oblanceolate, having a thin base or wide and clasping. Has a bright yellow or golden yellow flowers head. Have two	(7, 13, 18)

		flowering season in South Africa based on the region: western and Eastern Cape from August to December while others like Kwazulu-Natal, Mpumalanga, Limpopo, Lesotho and Swaziland from January. **Geographical origin/habitat**: South Africa, Mozambique, Zimbabwe, Malawi. Commonly found in grassland areas, forest margins, and along roadsides reaching 5 m to 3050 m above the sea.	
Helichrysum patulum (L.) D. Don (H. pat.)	Phefu (Sotho) Imphepho (Xhosa, Zulu) Kooigoed (Afrikaans)	**Botanical characteristics**: it is a well-branched subshrub and can attain a height of 1m, the leaves are small and hairy, the flower head is medium having Compact inflorescence Cream bracts, which blossom from September to February most especially between December-January. **Geographical origin/habitat**: Endemic to South Africa and grow on coastal dunes and mountain slopes reaching 600 m above sea level.	(17)
Helichrysum petiolare Hilliard & B. L. Burtt	Silver bush everlasting, (English), Hottentotskooigoed, Hottentotskruie,	**Botanical characteristics:** Aromatic, perennial shrub. The leaves are greenish-yellow or greyish, round to ovate form and covered by silver gray hairs on each	(16, 19)

kooigoed, and Kruie (Afrikaans),	side. The flower-heads is tiny having creamy or pale- yellow color
Imphepho (Xhosa, Zulu)	**Geographical origin/habitat**: The plant is prevalent in South Africa and other African countries like; Mozambique, Zimbabwe and Malawi. In South Africa, the species are distributed across Eastern Cape, Free State, Northern Cape, and Western Cape Provinces.

2.3.2. Traditional uses

A large number of South Africans living mostly in rural communities rely on herbal remedies because these are affordable, accessible and easy to prepare and administer (14, 20-22). These medicinal remedies are often used as complementary or alternative treatments to Western medication (23) and the *Helichrysum* genus is one of the many plants used extensively in traditional medicine for centuries across the world (4, 11, 17). These plants have been used for treating complications such as sores, wound dressing during circumcision, stress-related ailments, bruises and cuts, etc. (4, 22, 24). Common traditional approaches of application include decoctions, infusions (using water), inhalation of smoke or vapour forms as well as the external application of leaf extracts to wounds as shown in Table 2.2 (4, 10, 25-27).

Table 2.2. Traditional uses of H. cymosum, H. foetidum, H. odoratissimum, H. patulum and H. Petiolare.

Scientific name	Part and method of preparation	Traditional uses	Sources

H. cymosum	**Leaves:** Boiled as tea, burnt and smoke inhaled, wound dressing, boiled and vapour inhaled.	Treatment of cough and cold, pains, Infected wounds, headache, induces trances and goodwill to ancestors.	(4, 16)
H. foetidum	**Leaves:** Poultice, wound dressing, extract. **Roots:** Extract. Whole plant: Extract drunk, smoke inhaled**.**	Treatment of Sores, infected wounds, herpes, influenza, eye problem, sedative Induced trances, menstrual pain**.**	(11, 28, 29)
H. odoratissimum	**Leaves:** Tea, infusion, wound dressing, smoke inhaled, ointment, burnt as incense, eye drop **Stems:** Smoke inhaled **Ariel part**: extracts	Used in the treatment of, coughs and colds wounds and burns, headache, tonic for pregnant women, incense to invoke goodwill to ancestors, ointment for pimples, fumigate sickrooms.	(4, 11, 13)
H. patulum	Infusion	Treatment of, asthma, influenza, heart trouble, backache, kidney disease, bladder infections, gynaecological disorders, bedding and Hyperpiesa,	(4, 11)

H. petiolare	**Leaves:** Taken as tea	Used to treat, coughs, colds, catarrh, headache, fever, menstrual disorders, urinary tract infections, bedding, wound dressing, heart conditions, stress, hypertension, anxiety.	(4)

2.4. Phytochemistry

Previous studies have established the chemical components of large proportions of South African *Helichrysum* species (4, 30), and the occurrence of flavonoids, alpha pyrenes, coumanins, phoroglucinol and terpenoids compounds (29, 31), with some species known to contain an abundance of diterpenes and pyrenes derivatives (32). Despite, the wide variety of chemical compounds present in the *Helichrysum*, the presence of terpenoids appears to characterize this genus with a typical aromatic smell. Typically, all the species are also known to have hydroxylated/methylated flavonoids, as well as prenylated and O-prenylated flavonoids (29). Ring-A methoxylated flavonols have been described in European and South African *Helichrysum* species and sesquiterpenes with different alcohol and monoacid bicyclic derivatives, have also been documented in the South African and Australian species (29).

Flavonoids and other phenolic constituents are often associated with the medicinal potential exhibited by the *Helichrysum* plants especially for treating various infections (33), including their anti-oxidant, anti-bacterial, anti-inflammatory, anti-diabetic, anti-cancer, hepatoprotective and other effects (34). Likewise, phoroglucinol derivatives (arzanol) have indicated antibacterial and anti-viral potentials (10, 29).

The active compounds in the *Helichrysum* species are mostly responsible for the healing properties attributed to these plants (35), which also accounts for the bioactivity acclaim, medicinal value and general acknowledgement of this genus in traditional medicine (29, 34, 35).

2.4.1. Major classes of compounds isolated and characterized from five Helichrysum species.

The bioactive compounds isolated from the *Helichrysum* species selected for this review have been well documented. *H. cymosum* contains flavone, 5-hydroxy-8 methoxy-7-prenyloxyflavonone

helihumolone, helichromachalcone and phloroglucinol derivatives (4, 7, 14). *H. foetidum* contains chalcones (6-methoxy-2′, 4, 4’-trihydroxy-chalcone, 6-methoxy-2′, 4-dihydroxy-chalcone-4′-O-β-D-glucoside), diterpenoid (kaur-16-en-18-oic acid), flavonoids (apigenin-7-O-β-D-glucoside), 7, 4’ dihydroxy-5-methoxy-flavanone (28, 31), while *H. odoratissimum* contains numerous chalcone and flavanol compounds, diterpenes, phloroglucinol and pyrones (4, 7). Swartz et al, (36) isolated the following compounds from *H. patulum*: carboxylic acids (hexadecanoic acid and tetradecanoic acid), glucosides (arbutin), several terpenoids, sesquiterpenes (viridiflorol, β-caryophyllene, (-)-alloaromadendrene, γ-gurjunene), monoterpenes (pinene), terpineol and limonene, while *H. petiolare* was found to contain the flavonoid derivatives, pyrenes and diterpenes (4). However, these are not only compounds that are restricted to these *Helichrysum* species, additional compounds pertaining to these plants have been listed in Table 2.3 below.

Table 2.3. Phytochemical compound reported from five Helichrysum species.

Phytochemicals (%)	***H.cmy. (a)***	***H. foe. (b)***	***H. odora. (c)***	***H. pat. (d)***	***H. pet. (e)***	**References** ***(a) (b) (c) (d) (e)***
Acridine-9-carbaldehyde	-	-	+	-	-	38[c]
Ar-curcumene	-	-	-	-	+	39[e]
cis-Alloocimene	+	-	-	-	-	13[a], 14[a], 37[a]
α-Amorphene	+	-	+	-	-	16[a,c],37[a], 40[c]
Aromadendrene	+	+	+	-	+	13[a,c,e], 14[a], 17[b], 34[e] 37[a], 41[c]
(+)-Aromadendrene	+	-	-	-	-	37[a]
Allo-aromadendrene	+	+	+	+	+	13[c], 16[a,e], 17[b,d], 41[c]
(−)-Alloaromadendrene	-	-	+	-	-	38[c]
Alloaromadendrene epoxide	+	-	-	-	-	37[a]
Benzaldehyde	+	-	-	-	-	13[a], 14[a], 42[a]
Benzylacetone	+	-	-	-	-	13[a], 14[a], 37[a]
Bicyclogermacene	+	-	-	-	-	37[a]

Borneol	+	-	+	-	+	13[a,e], 14[a], 16[e], 37[a], 39, 40[c], 42[a]
Bornylene	+	-	-	-	+	37[a]
Bornyl acetate	-	-	+	-	+	13[c], 16[c,e], 40[c], 41[c]
Bornyl formate	-	-	+	-	-	16[c]
β-Bourbonene	+	+	+	-	-	14[a], 17[b], 37[a], 40[c]
α-trans-Bergamotene	-	-	+	-	+	16[c,e]
trans-β-Bergamotene	-	-	+	-	-	13[c], 41[c]
β-Bisabolene	-	+	+	-	+	16[c,e], 17[b]
cis-α-Bisabolene	-	-	+	-	-	13[c], 41[c]
γ-Bisabolene	-	-	+	-	-	13[c], 41[c]
Bicyclogermacrene	-	+	+	-	-	17[b], 40[c]
Bifloratriene	-	-	+	-	-	16[c]
α-Bisabolol	-	-	-	-	+	16[e], 34[e]
epi-α-Bisabolol	-	-	-	-	+	16[e], 34[e]
β-Bisabolol	-	-	+	-	+	13[c,e], 16[c], 34[e], 41[c]
Bulnesol	-	-	+	-	-	16[c]
α-Bulnesene	-	+	+	-	+	16[e], 17[b], 34[e], 43[c]
Cadalene	-	-	+	-	+	13[c], 16[c,e], 34[e], 41[c]
Cadinene	-	+	-	-	-	17[b]
trans-Cadina-1,4-diene (Cubebene)	+	-	+	-	+	16[a,c,e]
Cadina-1,4-diene	-	-	+	-	-	44[c]
α-Cadinene	+	-	+	-	+	16[c,e], 34[e], 37[a], 44[c]
γ-Cadinene	+	-	+	-	-	13[c], 37[a], 41[c]
ε-Cadinene	-	-	+	-	-	44[c]
δ-Cadinene	+	+	+	+	+	13[c,e], 16[a,c,e], 17[b,d], 34, 37a, 39[e], 41[c], 45[c]
trans-γ-Cadinene	+	-	+	-	+	16[a,c,e]
epi-α-Cadinol	+	-	+	-	+	16[a,c,e], 34[e]

α-Cadinol	+	-	+	-	+	13[a,e], 14[a], 16[a,c], 34[e], 37[a], ,39[e],44[c]

Table 2. 3 continued

Phytochemicals	***H.cmy.* *(a)***	***H. foe.* *(b)***	***H. odora.* *(c)***	***H. pat.* *(d)***	***H. pet.* *(e)***	**References** ***(a) (b) (c) (d) (e)***
δ-Cadinol	+	-	+	-	-	37[a],44[c]
T-Cadinol	-	-	+	-	+	13[c,e], 39[e],41[c]
β-Calacorene	+	-	-	-	-	13[a], 14[a], 16[a], 37[a]
Cis-calamenene	+	-	-	-	+	14[a], 37[a], 42[a], 46[e]
Camphene	+	+	+	+	+	13[a,c,e], 14, 16[a,c,e], 17[b,d], 37[a], 39[e], 41[c],
Camphor	+	-	+	-	+	16[a], 37[a]
α-Campholenal	-	-	+	-	+	16[c,e], 34[e]
δ-4-Carene	-	-	+	-	-	40[c]
Δ-3-Carene	+	-	-	-	-	37[a]
Carotol	-	-	-	-	+	16[e], 34[e]
Carvone	-	-	+	-	-	13[c], 41[c]
Carvacrol	+	-	+	-	+	13[c,e], 16[a,c], 34[e], 39[e], 41[c]
cis-Carveol	-	-	+	-	-	13[c], 41[c]
cis-Carvyl acetate	-	-	+	-	-	13[c], 41[c]
trans-Carvyl acetate	-	-	+	-	-	13[c], 41[c]
trans-Carveol	+	-	+	-	+	13[a,c,e], 14[a], 16[a], 37[a], 39[e], 41[c]
Caryophellene	-	-	+	-	-	38[c]
α-Caryophyllene	-	-	+	-	-	38[c], 45[c]
β-Caryophyllene	+	+	-	+	+	13[a,e], 14[a], 16[a,e], 17[b,d], 34[e], 37[a], 39[e]
trans-Caryophyllene	+	-	-	-	-	37[a]
Caryophylladienol I	+	-	-	-	-	13[a], 14[a], 42[a]
Caryophylladienol II	+	-	-	-	+	13[a,e], 14[a], 34[e], 39[e], 42[a]

Caryophyllenyl alcohol	+	-	-	-	+	16[a], 34[e]
Caryophyllene alcohol l	+	-	-	-	-	16[a], 37[a]
α-Caryophyllene alcohol	+	-	-	-	-	37[a]
β-Caryophyllene alcohol	+	-	-	-	+	13[e], 16[a], 34[e], 37[a], 39[e]
Caryophyllene oxide	+	+	+	-	+	13[a,c,e], 14[a], 16[a,c,e], 17[b], 34[e], 37[a], 39[e], 41[c], 45[c]
Caryophylla-4 (14),8 (15)-dien-5-ol	+	-	+	-	-	16[a,c], 37[a]
Caryophyllenol-I	+	-	+	-	+	13[a,c,e], 14[a], 37[a], 41[c],
Caryophyllenol I	+	-	-	-	-	13[a], 14[a], 42[a]
Caryophyllenol II	+	-	-	-	+	13[a,e], 14[a], 34[e], 39[e],
Cedren-13-ol	-	-	+	-	-	38[c]
β-Chamigrene	-	-	+	-	+	34[e]
chrysanthemumate	-	+	-	-	-	17[b]
1,8-Cineole	+	-	+	+	+	13[a,c,e], 14[a], 16[a,c,e], 17[d], 34[e] , 31[a], 39[e], 40[c], 41[c]
Clovenol	+	-	+	-	+	13[a,c,e], 14[a], 39[e],41[c]
α-neo-clovene	-	+	-	-	-	17[b]
α-Copaene	+	+	+	+	+	13[a,c,e], 14[a], 16[a,e], 17[b,d], 34[e] ,37[a], 39[e], 45[c]
β-Copaene	+	+	+	-	+	13[e], 16[a,c], 17[b], 34[e], 37[a], 39[e]

Table 2.3 continued

Phytochemical	***H. cym.* *(a)***	***H. foe.* *(b)***	***H. odora.* *(C)***	***H. pat.* *(d)***	***H. pet.* *(e)***	**References** *(a) (b) (c) (d) (e)*
Cubenol	-	-	+	-	+	16[c], 34[e]
1-epi-Cubenol	+	-	+	-	+	13[c], 16[a,c], 34[e] , 41[a]
α-Curcumene	-	-	+	-	-	44[c]
γ-curcucumene	-	-	+	+	-	13[c], 17[d], 41[c], 45[c]
Cyclosativene	+	-	+	-	-	16[a,c], 37[a]

Cyclooctanone	-	-	+	-	-	38[c]
p-Cymen-8-ol	+	-	+	-	+	13[a,c.e], 14[a], 39[e], 41[ce], 42[a]
Cyperene	-	-	-	-	+	34[e]
o-Cymene	+	-	+	-	+	16[a,c], 34[e]
p-Cymene	+	+	+	-	+	13[a,c], 14[a],17[b], 34[e], 37[a], 41[c]
p-Cymenene = α	-	-	-	-	+	13[e], 34[e]
p-Cymenene=α,p-Dimethylstyrene	-	-	+	-		13[c], 41[c]
p-Dimethylstyrene (2E,4E)-Deca-2,4-dienal	-	-	-	-	+	34[e]
Decanal	+	-	+	-	+	16[a,c], 34[e]
(E)-2-Decenal	-	-	-	-	+	34[e]
1,10-Di-epi-cubenol	+	-	-	-	+	16[a], 34[e], 37[a]
α, p-Dimethylstyrene	-	-	-	-	+	39[e]
5,7-Di-epi-a-eudesmol	-	-	+	-	-	16[c]
2,7-dimethyl-2,6-Octadiene	-	-	+	-	-	38[c]
3,5-Dimethylcyclohex-1-ene-4-carboxaldehyde	-	-	+	-	-	38[c]
3α,7α-dimethyl-hexahydro-2(3H)-Benzofuranone	-	-	+	-	-	38[c]
19,19-Dimethyl-eicosa-8,11-dienoic acid (%)	-	-	+	-	-	43[c]
Docosanoic acid methyl ester	-	-	+	-	-	38[c]
1,22-Docosanediol	-	-	+	-	-	38[c]
Dodecanal dimethyl acetal	-	-	+	-	-	38[c]
Drimenol	-	-	+	-	-	45[c]
Dodecanal	+	-	-	-	-	16[a], 37[a]
β-Elemene	+	+	+	-	-	17[b], 37[a] ,40[c], 45[c]
δ-Elemene	+	-	+	-	-	16[c], 37[a]
Epiglobulol	+	-	-	-	-	13[a], 14[a]

α-Eudesmol	+	-	-	-	-	37[a]
β-Eudesmo	+	-	-	-	-	37[a]
cis-1,2-Epoxy-terpin-4-ol	-	-	-	-	+	13[e], 34[e], 39[e]
2-ethyl-1,4-dimethyl-benzene	-	-	+	-	-	38[c]
5-ethyl-m-xylene	-	-	+	-	-	38[c]
10-epi-y-Eudesmol	-	-	+	-	+	16[c,e]
(E,E)-Farnesol	-	-	+	-	-	47[c]
Farnesene	-	-	+	-	-	44[c]
(E)-β-Farnesene	-	+	+	-	-	17[b], 44[c]
E, e-α-Farnesene	+	-	-	-	-	37[a]
α-Fenchone	+	-	-	-	+	13[a,e],14[a], 39[e], 42[a]

Table 2.3 continued

Phytochemistry	***H. cym.* *(a)***	***H. foe.* *(b)***	***H. odora.* *(C)***	***H. pat.* *(d)***	***H. pet.* *(e)***	**References** ***(a) (b) (c) (d) (e)***
Fenchyl alcohol	+	-	-	-	+	13[a,e], 14[a], 34[e], 37[a]
endo-Fenchol	+	-	-	-	-	37[a]
Furfuryl alcohol	+	-	-	-	-	37[a]
β-Fenchyl alcohol	+	-	-	-	-	37[a]
Fenchyl acetate	-	-	+	-	-	13[c], 41[c]
Geranyl acetate	-	-	-	-	+	13[e], 39[e],
(E)-Geranyl acetate	-	-	+	-	+	13[c,e], 39[e], 41[c]
9-Geranyl-p-cymene	-	-	+	-	-	13[e], 39[e],
Germacrene A	-	-	+	-	-	16[c], 40[c]
Germacrene D-4-ol	-	+	-	-	+	17[b], 34[e]
Germacrene B	+	-	-	-	-	37[a]
Germacrene D	+	+	+	-	-	16[c], 17[b], 37[a], 40[c]
Gleenol	-	-	+	-	+	16[c],34[e]
Globulol	+	+	-	+	+	13[a,e], 14[a], 17[b,d], 37[a], 39[e],

α-Guaiene	+	+	+	-	+	13[c], 16[,c], 17[b], 34[e], 37[a], 40[c]
δ-Guaiene	+	-	+	-	-	13[a,c], 14[a], 41[c]
3,7-Guaiadiene	-	-	-	-	+	13[e], 34[e], 39[e]
Guaiol	+	-	-	-	+	16[a,e], 34[e]
α-Gurjunene	+	+	+	-	+	13[a,e], 14[a], 16[c,e], 17[b], 34[e], 37[a], 39[e], 41[c]
γ-Gurjunene	-	-	+	-	+	13[e], 39[e], 40[c]
1-Heptanol	+	-	-	+	+	13[a.e], 14[a], 17[b,d], 34[e]
(E)-2-hexenal	-	-	-	+	-	17[d]
1-Hexanol	+	+	-	+	+	13[a.e], 14[a], 17[b,d], 34[e]
Hexadecanoic acid methyl ester	-	-	+	-	-	38[c]
(Z)-3-hexanol acetate	-	+	-	-	-	17[b]
Heneicosane	-	-	+	-	-	38[c]
Heptenyl acetate	-	-	+	-	-	13[c]
Heptadecyloxirane	-	-	+	-	-	38[c]
Z)-3-Hexen-1-ol	+	-	+	-	-	13[a,c], 14[a], 37[a] , 41[c]
(3E)-3-Hexen-1-yl acetate	+	-	-	-	-	16[a]
(Z)-3-Hexen-1-yl 3-methylbutyrate	+	-	-	-	-	16[a], 37[a]
Hexyl valerate	-	-	-	-	+	13[e], 34[e]
α-Himachalene	-	-	+	-	-	16[c]
β-Himachalene	-	-	+	-	-	16[c]
γ-Himachalene	-	-	+	-	-	16[c]
Himachalol	-	-	+	-	-	43[c]
Humulene	-	-	+	-	-	43[c]
Humulene epoxide	-	-	+	-	-	44[c]
Humulene epoxide I	-	-	+	-	+	13[c,e], 39[e], 41[c]
Humulene epoxide II	+	-	+	-	+	13[a,c,e], 14[a], 16[e], 34[e], 37[a], 39[e], 41[c]

Humulene epoxide III	-	-	+	-	-	13[c], 41[c]
Humulene oxide	+	-	-	-	-	37[a]

Table 2.3 continued

Phytochemistry	***H. cym.*** ***(a)***	***H. foe.*** ***(b)***	***H. odora.*** ***(C)***	***H. pat.*** ***(d)***	***H. pet.*** ***(e)***	**References** ***(a) (b) (c) (d) (e)***
Humulene epoxide II	+	-	-	-	-	14[a], 37[a]
10-Hydroxy calamenene	-	-	+	-	-	13[c]
α-Humulene	+	+	+	+	+	13[a,c,e], 14[a], 16 [c, e], 17[b,d] 34[e], 37[a] , 39[e], 41[c]
β-Hydroagarofuran	-	-	-	-	+	16[e]
neo-Intermedeol	-	-	+	-	-	16[e]
Intermedeol	-	-	+	-	+	16[c,e], 34[e]
1-isopropyl-3-methylbenxene	-	-	+	-	-	38[c]
Isoaromadendrene epoxide	-	-	+	-	-	38[c]
Isocaryophyllene oxide	+	-	+	-	+	13[a,c,e], 14[a], 34[e], 39[e], 41[c]
1-(2-Isopropyl-5-methylcyclopentyl)ethanone	-	-	+	-	-	38[c]
Isoborneol	+	-	+	-	-	16[a,c], 37[a]
iso-ascaridole	-	+	-	-	-	17[b]
Isobornyl acetate	+	-	+	-	-	16[a,c], 37[a]
Italicene	-	+	+	-	+	13[c,e], 17[b], 39[e], 41
endo-Isocamphane	+	-	-	-	-	37[a]
Isoitalicene	+	-	-	-	+	13[e], 16[a], 34[e], 37[a], 39[e]
Cis-Isopulegone	-	-	+	-	-	40[c]
Cis-Jasmone	-	-	+	-	-	40[c]
Juniper camphor	-	-	+	-	-	16[c]
Kaur-15-ene	-	-	-	-	+	13[e], 39[e]

Kaur-16-ene	-	-	-	-	+	13[e], 34[e]
Lavandulol	-	-	-	-	+	16[e], 34[e]
Lavandulyl acetate	-	-	-	-	+	16[e], 34[e]
Lavandulyl isobutyrate	-	-	-	-	+	16[e],34[e]
Lavandulyl isovalerate	-	-	-	-	+	16[e], 34[e]
Levomenol	-	-	+	-	-	43[c]
Ledol	-	-	-	-	+	13[e], 39[e]
Limonen-4-ol	+	-	-	-	-	13[a], 14[a]
trans-1,2-Limonene epoxide	-	-	+	-	-	13[c], 41[c]
Limonene	+	+	+	+	+	13[a,c,e], 14[a], 16 [c,e], 17[b,d] 34[e], 37[a], 39[e], 41[c]
Linalool	+	-	+	-	+	13[a], 14[a], 16[a,e], 34[e], 37[a], 45[c]
trans-Linalool oxide	+	-	-	-	-	37[a]
Longiborneol (=juniperol)	+	-	-	-	+	16[e], 34[e]
Longifolene	-	-	+	-	-	16[c]
Ethyl linolenate	-	-	+	-	-	43[c]
(E)-β-Ionone	-	-	-	-	+	16[e], 34[e]
neo-Menthol	-	-	+	-	-	16[c]

Table 2.3 continued

Phytochemistry	***H. cym.* *(a)***	***H. foe.* *(b)***	***H. odora.* *(C)***	***H. pat.* *(d)***	***H. pet.* *(e)***	**References** ***(a) (b) (c) (d) (e)***
p-Menthone	-	-	+	-	-	40[c]
6-Methyl-5-hepten-2-one	-	-	+	-	+	13[c,e], 34[e], 39[e], 41[c]
cis-p-Mentha-1 (7),8-dien-2-ol	+	-	-	-	-	13[a], 14[a], 42[a]
cis-p-Mentha-2,8-diene-1-ol	-	-	+	-	-	13[c], 47[c]
(+)-p-Mentha-2,8-diene	-	-	+	-	-	38[c]
cis-p-Menth-3-en-1,2-diol	+	-	+	-	-	13[a,c], 14[a], 39[e], 41[c]
Methyl hexyl bourgene	+	-	-	-	-	37[a]

Methyl 6.6-dimethylbicyclo[3.1.1]hept-2-ene-2-carboxylate	-	+	-	-	-	17[b]
4 methylene-2,8,8 trimethyl-2-vinyl-bicyclo nonane	-	-	+	-	-	38[c]
para-Methylanisole	-	-	+	-	-	16[c]
1-Methyl-4-acetyl-cyclohex-1-ene	-	-	+	-	-	13[c], 41[c]
3-Methyl-N-naphthalen-1-yl-benzamide	-	-	+	-	-	38[c]
α-Muurolene	+	-	-	-	+	16[a], 34[e], 37[a], 39[e]
α-Muurolol	-	-	+	-	+	13[c], 16[e], 34[e] , 41[c]
γ-Muurolene	+	-	+	-	+	13[e], 16[a,c,e], 39[e]
t-Muurolol	+	-	-	-	-	14[a]
(E)-Myroxide	+	-	-	-	-	16[a], 37[a]
(Z)-Myroxide	+	-	-	-	-	16[a], 37[a]
Myrcene	+	+	+	+	+	13[a,c,e], 14[a], 16[a,c,e], 17[b,d], 34[e], 37[a], 39[e], 41[c]
Myrtenol	+	+	-	-	-	14[a],15[a], 15[b] ,42[a]
trans-Myrtanol acetate		-	-	-	+	16[e], 34[e]
Myrtenal	-	-	+	-	+	13[c], 16[e] , 41[c]
Myristic acid	-	-	+	-	-	43[c]
Myrtenyl acetate	-	+	+	-	+	13[e], 16[c], 17[b], 34[e], 39[e]
Nerol	+	-	-	-	-	37[a]
(E)-Nerolidol	-	-	+	-	+	13[e], 39[e], 45[c]
2-Nerolidol	-	-	+	-	-	38[c]
Neryl acetate	+	-	-	-	-	37[a]
Neryl valerate	-	-	-	-	+	13[e], 39[e]
2-Nonanol	+	-	-	-	-	13[a], 14[a], 39[a]

(E)-2-Nonenal	-	-	-	-	+	13[e], 34[e], 39[e]
Nonanal	+	-	+	-	+	16[a,c,e]
Nonadecanoic acid, ethyl ester	-	-	+	-	-	38[c]
2-Nonanone	+	-	-	-	-	13[a], 14[a], 42[a]
cis-β-Ocimene	+	-	-	-	-	37[a]
(Z)-β-Ocimene	+	-	+	+	+	13[a,c,e], 14[a], 16[a,c,e], 17[b], 34[e], 37[a], 39[e], 41[c]

Table 2.3 continued

Phytochemistry	***H. cym.* *(a)***	***H. foe.* *(b)***	***H. odora.* *(C)***	***H. pat.* *(d)***	***H. pet.* *(e)***	**References** ***(a) (b) (c) (d) (e)***
(E)-β-Ocimene	+	+	+	+	+	13[a,e], 14[a], 16[a,c,e], 17[b,d], 34[e], 37[a],41[c]
trans-β-Ocimene	+	-	+	-	-	37[a], 40[c]
allo-Ocimene	+	-	-	-	-	16[a]
neo-allo- Ocimene	+	-	-	+	-	16, 17[d]
Methyl octadec-9-en-12-ynoate	-	-	+	-	-	43[c]
9,15-Octadecadienoic acid methyl ester	-	-	+	-	-	38[c]
12-Octadecenoic acid methyl ester	-	-	+	-	-	38[c]
2-Octanol	+	-	-	-	-	13[a], 14[a], 37[a], 42[a]
1-Octen-3-yl acetate	-	-	+	-	-	16[c]
3-Octanol	-	-	+	-	-	13[c], 41[c]
1-Octen-3-ol	+	-	+	-	+	13[a,c,e], 14[a], 39[e], 42[a], 43[c]
1-Octenyl acetate	-	-	+	-	-	13[c],41[c]
Octyl acetate	+	-	-	-	-	37[a]
2-(2-octenyl)-cyclopentanone	-	-	+	-	-	38[c]
9,12,15-Octadecatrienoic acid ethyl ester	-	-	+	-	-	38[c]

Z,E-3,13-Octadecadien-1-ol	-	-	+	-	-	38[c]
Palustrol	-	-	-	-	+	13[e], 39[e]
Palmitic acid	-	-	+	-	-	43[c]
Patchouli alcohol	-	-	-	-	+	16[e],34[e]
β-Patcholene	-	-	+	-	-	40[c]
Phellandral	+	-	-	-	-	37[a]
α-Phellandrene epoxide	+	-	-	-	-	37[a]
β-phellandrene	-	+	-	-	-	17[b]
Perilla aldehyde	+	-	-	-	-	16[a]
Pelugone	-	-	+	-	-	40[c]
Perillen	-	-	+	-	-	13[c], 41[c]
2-Phenylethyl acetate	+	-	+	-	-	13[c], 14[a], 37[a], 41[c]
Phytol	-	-	+	-	-	43[c]
α-Pinene	+	-	+	+	+	13[a,c,e], 14[a], 16[c], 17[d], 34[e] , 41[c], 45[c]
α-Pinene oxide	+	-	-	-	+	13[e], 16[a,e], 34[e], 39[e]
β-Pinene	+	-	+	+	+	13[c,e], 14[a], 16[c,e], 17[d], 34[e], 37[a,], 39[e], 45[c]
cis-Pinocamphone	-	+	-	-	+	16[e], 17[b], 34[e]
trans-Pinocarvyl acetate	-	-	+	-	+	13[e], 16[c,e], 39[e]
trans-Pinocarveol	+	+	-	-	+	14[a], 16[e], 17[b], 34[e], 37[a], 42[a]
Pinocarvone	+	-	-	-	+	13[a], 14[a], 16[c], 34[c] 37[a], 42[a]
cis-Piperitol	-	-	+	-	-	13[c], 41[c]
Piperitone	-	-	+	-	-	40[c]

Table 2. 3 continued

Phytochemistry	***H. cym.*** ***(a)***	***H. foe.*** ***(b)***	***H. odora.*** ***(C)***	***H. pat.*** ***(d)***	***H. pet.*** ***(e)***	**References** ***(a) (b) (c) (d) (e)***

3-phenyl-3-methylbutanoic acid methyl ester	-	-	+	-	-	38[c]
Phthalic acid mono-2-ethylhexyl ester	-	-	+	-	-	38[c]
Phthalic acid, butyl pent-2-en-4-yn-1-yl ester	-	-	+	-	-	38[c]
Piperitenone	-	-	+	-	-	40[c]
Porosadienol	-	-	+	-	-	13[c], 41[c]
Rosifoliol	+	-	-	-	-	14[a], 37[a], 42[a]
Rosefuran	-	-	+	+	-	13[c], 41[c]
Sabinene	+	+	+	+	-	13[a], 14[a], 16[a,c], 17[b,d] , 34[e] , 37[a], 42[a], 44[e]
cis-Sabinene hydrate	+	+	+	+	+	16[a,c,e], 17[b,d], 40[c]
Santalene	-	-	+	-	-	44[c]
trans-sabinene hydrate	-	+	+	-	-	16[c], 17[b]
Safranal	+	-	-	-	+	16[e], 37[a]
Selina-3,7-(11)-diene	-	-	+	-	-	13[c],16[c], 41[c]
Selina-5,11-diene	+	-	+	-	-	13[a,c], 14[a], 37[a], 42[a]
α-Selinene	+	+	+	-	-	13[c] , 17[b], 37[a]
7-epi-α-selinene	-	+	-	-	-	17[b]
γ-Selinene	+	-	+	-	-	37[a], 40[c]
β-Selinene	+	+	+	-	+	16[a,c,e], 17[b], 34[e], 37[a], 40[c] , 41[c]
Spathulenol	+	-	+	-	+	13[c,e], 16[e], 34[e], 37[a], 39[e], 41[c]
α-Terpinene	+	+	+	+	+	13[a,e], 14[a], 16[a,c,e], 17[b,d], 34[e], 37[a], 39[e] ,42[a], 40[c]
α-Terpinenyl acetate	-	-	+	-	-	40[c]

γ-Terpinene	+	+	+	+	+	13[a,c,e],14[a],16[c,e],17[b,d],34[e],37[a], 39[e], 41[c], 42[a]
1-Terpineol	+	-	-	-	-	37[a]
Terpinen-4-ol	+	-	+	-	-	37[a], 40[c], 44[c]
4-Terpineol	+	+	+	-	+	16[c,e], 17[b],34[e], 37[a]
4-Terpineol acetate	-	-	+	-	-	16[c]
α-Terpineol	+	-	+	-	+	13[a,,e], 14[a], 16[a,c,e], 34, 37[a], 39[e], 42[a]
δ-Terpineol	+	-	+	-	+	13[a,e], 14[a], 16[a], 34[e], 37[a], 39[e] , 42[a]
Terpinolene	+	+	+	+	+	13[a,e], 14[a], 16[a,e], 17[b,d], 34[e], 37[a], 39[e], 40[c], 42[a], 45[c],
α- Terpinolene	+	-	-	-	-	37[a]
7-Tetradecyne	-	-	+	-	-	38[c]
6,10,14-trimethyl-2-pentadecanone	-	-	+	-	-	38[c]
3,7,11,16-tetramethyl-hexadeca-2,6,10,14-tetraen-1-ol	-	-	+	-	-	38[c]
6-(p-Tolyl)-2-methyl-2-heptenol	-	-	+	-	-	38[c]
trans-Guai-11-en-10-ol	-	+	-	-	-	17[b]
3-Thujanol	-	-	-	-	+	16[e], 34[e]
α-Thujene	+	+	-	+	-	17[b,d], 37[a]
Thymol methyl ether	-	-	-	-	+	13[e], 34[e], 39[e]
Tricyclene	-	+	+	-	+	,16[c,e], 17[b], 34[e]
Undecanal	-	-	-	-	+	16[e], 34[e]
Valencene	+	-	+	-	+	16[a,c,e], 34[e] , 37[a]
Valeranone	+	+	-	-	+	16[e], 17[b] ,34[e], 37[a]

Viridiflorol	+	-	+	-	+	13[a,c,e], 14[a], 16[e], 34[e], 42[a], 41[c]
Viridiflorene	-	+	+	-	-	16[c], 17[b]
Vulgarol-β	+	-	-	-	-	37[a]
α-Ylangene	+	-	+	-	-	13[a,c,], 14[a], 16[a,c], 41[c], 42[a],

[c]Abbreviation for table 2.3

***H. cym.* (a)** = *Helichrysum cymosum*

H. foe. (b) = Helichrysum foetidum

H. odora (c) = *Helichrysum Odoratissimum*

H. pat. (d) = *Helichrysum Patulum*

H. Pet (e) = *Helichrysum Petiolare*

2.5.1. Reproductive health potentials of Helichrysum Species

Reproductive healthcare comprises of multiple processes involved in the sexual health functions and systems in all life stages in humans (48) and has been identified to be among the prevalent health challenges in Africa. However, the use of pharmaceutical drugs for the treatment and management of reproductive health challenges is known to be very costly or to have adverse side effects (49). As such, indigenous patients in most African countries opt for alternative forms of treatment mainly with plant-based products due to the fact that they are cheap, accessible and culturally acceptable alternatives (49, 50). Consequently, a variety of herbal remedies and their isolated bioactive compounds are used to treat infertility conditions and have demonstrated little or no side effects (51). Despite their effectiveness in traditional medicine, there is a lack of adequate pharmacological data relevant to the discovery of new therapeutic agents (50). Only a few studies have investigated the benefits of the genus *Helichrysum* for ameliorating reproductive health challenges. Some *Helichrysum* species of Southern African origin have been reported to be used traditionally to treat male and female infertility issues, e.g. *Helichrysum caespititium* (DC.), *Helichrysum latifolium*, *Helichrysum nudifolium var. pilosellum*, *Helichrysum odoratissimum (L.)*

Sweet, *Helichrysum platypterum DC* as well as *Helichrysum psilolepis Harv*. (38). There is limited information in literature, especially on the anti-infertility effects of *H. cymosum, H. foetidum, H. odoratissimum, H. patulum and H. petiolare*.

Watcho et al. (52), investigated the effects of the aqueous extract (AE) and the methanol extract (ME) of *H. odoratissimum* in reducing cyclophosphamide (CP)-induced reproductive toxicity in male rats. Different doses of the extracts and CP were orally administered to seven animal groups, viz: 5 mL/kg of CP, 10 mL/kg of CP + distilled water (DW), 10 mL/kg of CP + 5% Tween 80, 0.25mg/kg of CP + clomiphene citrate, 50 and 100 mg/kg of CP and AE while 50 and 100mg of CP and ME were administered to each of the groups (52). The results of the study indicated that there was a significant ($P < 0.001$) decrease in the weight of the seminal vesicles, testosterone levels, sperm count, motility and viability compared to the control. All extracts and doses of *H. odoratissimum* treatment reversed the effects of CP and significantly ($P < 0.001$) increased sperm count, sperm viability and sperm motility ($P < 0.05$). Thus, *H. odoratissimum* could be considered as an alternative therapy in mitigating and preventing reproductive damage caused by cancer treatment (chemotherapy) with CP.

2.5.2. Anti-inflammatory activities

Potent bioactive compounds derived from the genus *Helichrysum* have indicated anti-inflammatory activities, often associated with the occurrence of different flavonoids. For instance, the compound kaempferol has been reported to be involved in the inflammatory process of experimental diabetes (53), while arzanol is known to be involved in the release of such pro-inflammatory mediators as IL-1β, IL-6, IL-8 (11, 54, 55). Therefore, the potential of freshly isolated bioactive compounds from the *Helichrysum* genus in the management of inflammatory responses has been reported and in one of such studies (56), where the aqueous and methanol extracts of *H. cymosum* (whole plant) were investigated through the use of the cyclooxygenase (COX-1) inhibition technique and results showed a value of 52.0% and 100% inhibition respectively. In another study, the 5-lipoxygenase inhibitory activities of the aqueous, methanol and essential oil extracts of *H. odoratissimum* were assessed using three-fold stepwise dilution, and the positive and negative controls were nordihydroguaiaretic acid and Tween®20, respectively. The results showed that the essential oil produced a 5-lipoxygenase inhibitory activity of 50% (IC_{50} values of 22.5 ppm -35.9 ppm) (41).

2.5.3. Antibacterial activities

Most of the available information in literature is focused on the antimicrobial effects of the genus *Helichrysum,* which is so far the predominantly studied activity (11). The five selected *Helichrysum* species are reported to exhibit toxic effects against transformed human kidney epithelial (Graham) cells, (*H. cymosum* and *H. odoratissimum*) as well as growth inhibitory activities (*H. petiolare* and *H. nudifolium*) (26). Despite numerous documented antimicrobial activities, most of the compounds isolated from the *Helichrysum* spp. still need further investigations on their activities (26). The antimicrobial activities of the five *Helichrysum* spp. are presented in Table 2. 4.

Table 2.4. Antibacterial activities of five Helichrysum species

Species	Pathogens	Extract/positive control and part used	Method	Mic values and Activity	Reference
H. cymosum	A	Acetone extract(AE), Essential oil (EO) Helihumulone (H) (Aerial part) Ciprofloxacin (+ control)	disc diffusion and microdilution	MICs: 1 – 8 mg/mL (EO), 0.078 – 0.313mg/mL (AE), 0.016 – 0.125 mg/mL (H). ZI: 3.7 to 8.0 mm (extracts) and 3.7 to 8.0 mm (control) Result; Activities exhibited against	(14, 42)

				E. faecalis, B. cereus, *B. subtilis*, and *S. aureus*.	
	B	Ethanol extracts (Whole plant)	micro plate	MIC: 0.8 to 1.6 mg/ml	(56)
	C	Acetone and methanol extracts, Essential oil (Aerial parts) Ciprofloxacin (+ control 0.01 mg/ml)	disc diffusion	MIC: <0.25 mg/mL and ZI: 7 and 5 mm (extracts against *S. aureus* and *B. cereus*) MIC: 0.0003 mg/mL and ZI: 6mm (control)	(13)
H. Foetidum	D	Chloroform and methanol extract (ratio1:1), (leaf and stem) ciprofloxacin (+ control)	Microdilution	MIC: 0.01 mg/mL and 0.5 mg/mL (Extracts) MIC: 0.0003 mg/mL (control) Activities against *Bacillus cereus* and *Staphylococcus aureus*	(26)

	E	Essential oils	agar diffusion and microdilution	MIC and MBC (3.8 mg/mL to > 7.5 mg/mL)	(57)
	F	Methanol and 7, 4′-dihydroxy-5-methoxyflavanone, 6′-methoxy-2′, 4, 4′-trihydroxychalcone, 6′-methoxy2′, 4-dihydroxychalcone-4′-O-β-D-glucoside, apigenin, apigenin-7-O-β-D-glucoside, and Kaur-16-en-18-oic acid. (Flower and leaf) erythromycin (+ control)	fluorescence-based antibacterial growth inhibition	Concentrations: 85.4% of 1 mg/mL and 21.8% of 0.1 mg/mL (Extract) Inhibition: 75.0%–85.0% against *Bacillus subtilis* at a concentration of 1 mg/mL (all compound)	(31)
H. odoratissimum	G	Acetone and methanol extract neomycin and ciprofloxacin (+controls)	disc diffuse on and broth microdilution	MIC: 0.5 mg/mL to >16.0 mg/mL ZI: from 4.1 mm to 9.4 mm (extracts showed activities *against S. aureus,*	(58)

			E. faecalis, and *B. cereus*)	
H	Acetone extracts (aerial parts)	Agar dilution	0.01 mg/ml to 1.0 mg/ml extract exhibited activities against *B. cereus, B. pumilus, B. subtilis, M. kristinae, S. aureus*, and *E. cloacae*	(59)
I	Acetone and methanol extracts, essential oils (aerial parts), Ciprofloxacin (0.01 mg/mL, + control).	disc diffusion	MIC: <0.25 mg/mL extract exhibited activities *against S. aureus* and *B. cereus*	(13)
J	Chloroform: methanol (1:1) (leaf and stem), ciprofloxacin (+ control)	96-well microplate	4.0 mg/mL and 2.0 mg/mL, Activities exhibited against *S. aureus* and *B. cereus* (leaf and stem).	(26)
K	Essential oils chloramphenicol (25 µg + control) tetracycline (25 µg + control)	Disc diffusion and broth microdilution	MIC: 1.3 mg/mL to 10.0 mg/mL ZI: 6.7 mm to 17.0	(45)

				MIC (control): 1.3 mg/mL to 10.0 mg/mL ZI (control): 6.0 mm to 23.7 mm Volatile oil showed activities against tested organisms.	
H. Patulum	L	Chloroform and methanol extracts (ratio 1:1), (leaf and stem) Ciprofloxacin and amphotericin (+ control)	96-well microplate	MIC: 4.0 mg/ml Antibacterial activities of the extracts was observed on *S. aureus, B. cereus*	(26)
H. Petiolare	M	Aqueous extracts (leaf), ciprofloxacin (50 mg/ml + control)	disc diffusion	ZI: 9 mm to 15 mm ZI (control): 27 mm to 55 mm Extracts exhibited activities against pathogens.	(32)
	N	Chloroform and methanol extracts (ratio 1:1), (leaf and stem),	96-well microplate	MIC: 4.0 mg/mL and 2mg/mL Antibacterial activities were exhibited against	(26)

	ciprofloxacin (+ control)		*S. aureus* and *B. cereus* by the extracts.	
O	Acetone and methanol extracts, essential oils (EO) (leaf), neomycin 30 µg/disc (+ control)	disc diffusion and microtiter plate dilution	MIC: 312.5 µg/mL to 625 µg/mL ZI: 2.5 mm to 9.0 mm extracts MIC: 8000 µg/mL ZI: <1.0 mm which was lower than 6.0 mm MIC: 0.08–0.31 µg/mL control Extracts showed activities against *S. aureus* and *B. cereus* and EO against S. *aureus*.	(39)
P	Acetone and methanol extracts, essential oils (aerial parts), ciprofloxacin (0.01 mg/mL) + control	disc diffusion	MIC: <0.25 mg/mL to 8.0 mg/mL [70]. The extract showed activities against *S. aureus*	(13)

[a] **Pathogens used in table 4**

A = (*Enterococcus faecalis, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Yersinia enterocolitica, Klebsiella pneumoniae, Cryptococcus neoformans and Candida albicans*),

B = (*Bacillus subtilis, S. aureus, E. coli, and K. pneumonia*),

C = (*E. coli, Yersinia enterocolitica, Klebsiella pneumoniae, S. aureus, and B. cereus*),

D = (*Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, and Pseudomonas aeruginosa*),

E = (*Acinetobacter calcoaceticus, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Micrococcus kristinae, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella spp., Salmonella typhi, Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis, and Streptococcus faecalis*),

F = (*Bacillus subtilis*),

G = (*S. aureus, Enterococcus faecalis, B. cereus, P. aeruginosa, K. pneumoniae, Serratia odorifera, and Moraxella catarrhalis*),

H = (*Bacillus cereus, Bacillus pumilus, B. subtilis, Micrococcus kristinae, S. aureus, Enterobacter cloacae, E. coli, K. pneumoniae, P. aeruginosa, and S. marcescens*),

I = (E. *coli, Yersinia enterocolitica, Klebsiella pneumoniae, S. aureus, and B. cereus*),

J = (*S. aureus, Staphylococcus epidermidis, B. cereus, K. pneumonia, and P. aeruginosa*),

K = *B. cereus, B. pumilus, S. aureus, S. aureus, Streptococcus faecalis, E. cloacae, E. coli, K. pneumoniae, P. vulgaris, P. vulgaris, P. aeruginosa, and Serratia marcescens*),

L = (*Bacillus cereus, Staphylococcus aureus, and Staphylococcus epidermidis, Klebsiella pneumoniae, and Pseudomonas aeruginosa, Cryptococcus neoformans*),

M = (*S. aureus, Pseudomonas aeruginosa, and Mycobacterium smegmatis*),

N = (*S. aureus, Staphylococcus epidermidis, B. cereus, K. pneumoniae, and P. aeruginosa*),

O = (*Staphylococcus aureus, Escherichia coli, Bacillus cereus, Bacillus subtilis, Yersinia enterocolitica, and Klebsiella pneumoniae*), **P** = (*E. coli, Y. enterocolitica, Klebsiella pneumoniae, S. aureus, and B. cereus*).

2.5.4. Antioxidant properties

Aladejana et al. (60), evaluated the antioxidant properties of whole plant extracts (acetone, ethanol, boiled and cold aqueous) of *Helichrysum petiolare* using 2, 2'- azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), diphenyl-1-picrylhydrazyl (DPPH), nitric oxide radical (NO) scavenging activities and total antioxidant capacity (TAC) assays. The boiled aqueous extracts exhibited the highest phenolic content of 212.963 mg/g, which suggest that heating might have facilitated a higher extraction of this compound, while, the ethanol extract was high in flavonoids (172.393 mg/g) and proanthocyanidines (65.855 mg/g) contents, suggesting a higher extraction yield for these solvent and a possible stronger antioxidant properties. The acetone extracts showed higher amounts of alkaloids, flavonols and saponins, while the cold aqueous extract had a relatively lower overall phytochemical content. The DPPH and ABTS radical scavenging activities were highest with the boiled aqueous extract with IC50 inhibition values of 0.02 mg/mL and of IC50 0.07 mg/mL respectively while the highest NO inhibition and TAC effects were observed with the ethanol extract (IC50 0.41 and IC50 0.19) respectively. The findings from these studies proposed the use of ethanol and boiled aqueous extracts of *H. petiolare* for medicinal treatments which correlates with the mode of administration in traditional medicine. Lourens et al. (39) also investigated the acetone and methanol extracts and essential oils from the leaves of *H. petiolare* using the DPPH assay and found that the acetone and methanol extracts showed activities with IC50 values of 44.28 μg/mL and 28.70 μg/mL respectively. The findings of this study reveals that the extracts and essential oil exhibited antioxidant activity. However, the activities of extracts were enhanced compared to essential oil.

Franccedil et al. (61) studied the antioxidant activity of the essential oil from the leaves of *H. cymosum* using the DPPH assay and butylated hydroxyl toluene as the positive control. The radical scavenging potential demonstrated by *H. cymosum* was attained at a value of 6.3 g/L compared with butylated hydroxy toluene (BHT) at 7.0 mg/L.

Twilley et al. (62), assessed the antioxidant activity of the ethanol extracts from the leaves of *H. odoratissimum* using the DPPH assay. The plant extract showed a higher scavenging activity with IC50 value of 5.13 ± 0.07 pg/mL. Frum and Viljeo (41), also documented antioxidant activities of the essential oil and methanol extract from the leaves of *H. odoratissimum*, using the DPPH assay. The results showed a decrease in activities for the essential oil with IC50 >100 parts per million (ppm).

2.5.6. Neuroprotective properties

Only a few studies have investigated the neurodegenerative properties of most *Helichrysum* species. Species such as *H. odoratissimum* have been listed among medicinal plants of Southern Africa used for memory and central nervous-related ailments (63), but their neuroprotective properties have not been elucidated. Neuroprotection is an approach used by scientists in managing neurodegenerative diseases, since treatments are mostly palliative (64). Additionally, antidepressant drugs have exhibited numerous side effects, thus hampering the usage of such medication for longer periods of time. Recently, herbal medicines have been acknowledged as alternative approaches to manage neurodegenerative diseases (NDD), seemingly because they are considered to be safer and can potentially be used over longer durations with minimal side effects (65). Studies have shown that plant dietary supplements and foods rich in antioxidant scavenging free radicals, could be used to modulate apoptotic effects and have been reported to exhibit neuroprotective properties (66). Pharmacological activities displayed by some compounds such as arzanol, pholoroglucinol α-pyrone isolated from some species within the genus *Helichrysum*, are known to act as eicosanoid inhibitors on PGE2 synthesis, the NF-κB pathway, while other compounds such as caffeic and quercetin derivatives are known for their antioxidant and anti-inflammatory effects for managing NDD such as mood disorders. Therefore, exploration of these bioactive compounds could be useful in drug discovery and treatment of NDD (65).

2.5.7. Antidiabetic activities

Diabetes is a disease of global concern due to its high prevalence across the world (67), most especially in the developing countries where the management and treatment are still problematic (68). Based on the challenges associated with diabetic treatments, researchers and pharmaceutical industries are now focusing attention on alternative herbal remedies to treat and manage diabetes

(68). However, only a few medicinal plants with hypoglycemic effects have been investigated extensively in both *in vitro* and *in vivo* studies. Some *Helichrysum* species like *H. gymnocomum, H. nudifolium, H. odoratissimum* and *H. petiolare* are well-known antidiabetic plants used by traditional healers in the Eastern Cape region of South Africa (67, 69).

Aladejana et al. (60), evaluated the antidiabetic potentials of ethanol, cold and boiled aqueous extracts of *H. petiolare* on L6 myocytes and HepG2 (C3A) hepatocytes using the following methods α-amylase, α-glucosidase and lipase inhibition assays. The ethanol extracts exhibited cytotoxic effects in the treated cells while the cold and boiled aqueous extracts enhanced glucose uptake in L6 and C3A cell lines. However, the L6 indicated a higher glucose uptake compared to the C3A hepatocyte cell-line following treatment with cold aqueous extract. Also, the best inhibition of α-amylase and α-glucosidase was exhibited by the boiled aqueous extract compared with cold aqueous extract, while the boiled aqueous extract exhibited a lower lipase inhibition than acarbose, an approved dietary treatment for type 2 diabetes mellitus used as the control, with no significant difference observed ($p<0.05$). Another study investigated the hypoglycemic activities of 50, 100 and 150 mg/kg body weight doses of *H. odoratissimum* aqueous leaf extract in alloxan-induced diabetic Swiss albino mice and the diabetic mice showed a non-dose dependent response by decreasing the glucose levels (70).

2.5.8. Cytotoxicity

The cytotoxicity of the chloroform and methanol extracts (ratio 1:1), from the leaves and stems of *H. foetidum, H. odoratissimum, H. patulum and H. petiolare* were amongst the 35 South African *Helichrysum* species investigated by Louren et al. (26), using the sulforhodamine B (SRB) assay against transformed human kidney epithelial (Graham's) cells, MCF-7 breast adenocarcinoma and SF-268 glioblastoma cells, at a concentration of 0.1 mg/ml. The cytotoxicity results were as follows*: H. foetidum* (% Graham cells = 32.7 ± 2.1, % SF-268 cells = 57.8 ± 2.1 and % MCF-7 cell = 24.9 ± 0.4), *H. odoratissimum* (% Graham cells = 17.5 ± 0.4, % SF-268 cells = 48.2 ± 1.4 and % MCF-7 cell = 7.4 ± 0.7), *H. patulum* (% Graham cells = 63.8 ± 1.3, % SF-268 cells = 75.2 ± 2.0 and % MCF-7 cell = 37.8), *H. petiolare* (% Graham cells = 59.3 ± 3.4, % SF-268 cells = 76.6 ± 3.0 and % MCF-7 cell = 33.4). These results tend to suggest that the plant species investigated might be toxic against Graham cells at the concentration of 0.1 mg/ml. In another study the cytotoxicity of the chloroform and water-methanol extracts of the aerial part of *H.*

cymosum was investigated against the Vero African green monkey kidney cells using the XTT assay and zearalenone as positive control. The IC50 values shown by the extracts were 36.5 µg/mL (chloroform) and 59.7 µg/mL (methanol-water) and 1.3 µg/mL (control). These result suggest that the extract and the chloroform of the tested specie had more toxic effect toward Vero African green monkey kidney cells compared to the control. (7). Also, the cytotoxicity of essential oil *of H. odoratissimum* was investigated using the brine shrimp assay, with a median lethal concentration of 31.6 µg/mL against the brine shrimp (45) indicating that this specie might be toxic against brine shrimp.

2.5.9. Conclusion

This review reveals important bioactive compounds, medicinal properties and some pharmacological activities of *H. cymosum, H. foetidum, H, odoratissimum, H. petiolare* and *H. patulum*. The diverse medicinal uses reported have shown that these plant have a potential as herbal remedy and necessitate further pharmacological evaluation of all bioactive compound isolated from these species. Therefore, exploring the phytochemical properties and pharmacological activities in correlation with the traditional uses of these plants in further studies will add more knowledge about their medicinal value and also enhance scientific validation of these medicinal plants in drug discovery.

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Authors' contributions (provide if number of authors is more than ONE)

Conceptualization: A.Q Nkemzi and O.O. Oguntibeju; Writing of the original draft: A.Q. Nkemzi; Writing review and editing: A.Q. Nkemzi, O.E. Ekpo and O.O. Oguntibeju; Supervision: O.E. Ekpo and O.O. Oguntibeju. All authors have read and agreed to the published version of the manuscript.

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CHAPTER THREE

In vitro hypoglycemic, antioxidant, anti-inflammatory activities and phytochemical profiling, of aqueous and ethanol extracts of *Helichrysum cymosum*

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Abstract

Background

Diabetes mellitus (DM) is a complicated and multifaceted metabolic disorder that poses significant health challenges for individuals and healthcare systems worldwide. Efforts to understand its pathophysiology and develop novel treatment options for the disease through preclinical research and drug discovery have been the focus of several researchers globally. One of the approaches to tackle this is the use of plant-based products, which are therapeutically effective and affordable.

Purpose

The current study investigated the phytochemical constituents of *Helichrysum. cymosum* aqueous (AQ) and 70% ethanol (ET) extracts, their cytotoxicity, as well as their *in vitro* antidiabetic effects via antioxidant, anti-inflammatory, and selected enzyme inhibition assays.

Materials and methods

Bioactive compounds were identified using High-Performance Liquid Chromatography and UHPLC-ESI-MS. Cytotoxicity was assessed on C3A hepatocyte cells using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Antioxidant activity was determined using the average cellular CellROX® Orange fluorescent intensity, ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The mouse macrophage cell line, RAW 264.7 was used to assess anti-inflammatory activity by measuring the levels of nitrite produced. Enzymatic inhibitory assays such as alpha glucosidase, alpha amylase and pancreatic lipase inhibition were performed to determine the antidiabetic effect of the plant extracts. Also glucose uptake and glucose utilization on C3A hepatocytes and L6myocytes were evaluated.

Results

The results show significantly higher polyphenol and flavonol contents in ET (303 ± 4 mg GAE/g, 183 ± 6 mg QE/g, respectively) compared to AQ (83.7 ± 2 GAE/g, 36.0 ± 1 respectively). ET also showed significantly higher antioxidant activity (DPPH assay: 1893 ± 34 µmol TE/g; FRAP assay: 158 ± 2 µmol AEE/g; and ABTS assay: 1402 ± 26 µmol TE/g) compared with AQ (DPPH assay: 606 ± 8.4 µmol TE/g; FRAP assay: 530 ± 2 µmol AEE/g; and ABTS assay: 450 ± 11 µmol TE/g). HPLC reveals the presence of caffeic acid (AQ), chlorogenic acid (AQ and ET), kaempferol (AQ), and rutin (ET), while UHPLC-ESI-MS analysis identified 43 compounds in both AQ and ET. Cytotoxicity was observed only for ET at a concentration of 250 µg/mL, while both AQ and ET

exhibited cellular antioxidant activity, alpha-glucosidase activity, and minor alpha-amylase activity. There was also significant glucose uptake and its utilization by C3A hepatocytes and L6 myocyte cells. However, the extracts did not exert substantial effect on lipase inhibition in the current study.

Conclusion

The finding reveals that the bioactive compounds present in *H. cymosum* extracts have potential antioxidant, anti-inflammatory, and hypoglycemic effects; therefore, these compounds can be further explored as future candidates for drug discovery.

Keywords

Diabetes mellitus; glucose utilization; metabolic disorder; phytochemical compounds; α-amylase, α-glucosidase

Abbreviation

ANOVA, Analysis of variance; AME, Accurate mass match error; AQ, Aqueous extract; AAE, Ascorbic acid equivalent; DM, Diabetes mellitus; DMSO, Dimethylsulfoxide; DMEM, Dulbecco's Modified Eagle Medium; DNS, 3,5-dinitrosalicylic acid, ET, Ethanol extracts; HPLC, High-Pressure Liquid Chromatography; GAE; Gallic acid equivalent; LPS, Lipopolysaccharide; LC-MS, Liquid Chromatography Mass Spectroscopy; MEM, Minimal Essential Medium; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NED, N-(1-Naphtyl)-ethylenediamine dihydrochloride; PBS, Phosphate-buffered saline; ROS, reactive oxygen species;. T1D, Type 1 Diabetes; T2D, Type 2 Diabetes; TE, Trolox equivalent; UHPLC-MS, Ultra-High Performance liquid chromatography- Mass spectroscopy; QE, Quercetin equivalent

3.1. Introduction

Diabetes mellitus is a complicated chronic metabolic disease represented by hyperglycemia, emanating from carbohydrates, fats, and protein metabolism (Oguntibeju, 2019, Nasab et al., 2020, Dilworth et al., 2021). Hyperglycemia is caused by disrupted insulin production or secretion (Akinyede et al., 2021a). DM is a major public health concern in underdeveloped and developed nations, with a reported incidence of about 451 million individuals conceivably existing with DM, and 693 million people have been projected to be affected by DM by 2045 (Jadalla et al., 2022). The etiology of hyperglycemia in DM is connected to oxidative stress (OS) and inflammation

(Papachristoforou et al., 2020). Oxidative stress is created by an imbalance between excess production and elimination of reactive oxygen species (ROS) beyond the body's natural antioxidant capacity (Omodanisi et al., 2017). ROS production is generated from increased inflammatory mediators and protein glycosylation, the autoxidation of glucose, the formation of superoxide radicals, and advanced glycation byproducts resulting in the alteration of cellular hemeostasis (Omodanisi et al., 2017, González et al., 2023, Sadiq, 2023). Persistent hyperglycemia has a profound impact on the body organs, leading to a wide range of complications like cardiovascular disease, kidney failure, heart attack and stroke, arterial disease, and damage to peripheral nerves (Akinfenwa et al., 2022, Maniruzzaman et al., 2020). The long-term effects of diabetic complications are associated with uninterrupted OS in diabetic cells and hyperpolarization (Dilworth et al., 2021).

Primary intervention in treating DM necessitates regulation of hyperglycemia and decreasing OS and inflammation outcomes (Wang et al., 2021). Therefore, inhibition of key digestive enzymes such as α-glucosidase, α-amylase, and pancreatic lipase plays a beneficial role in the treatment of DM and obesity (Dirir et al., 2022, Lui et al., 2020). Current treatments for DM have been achieved using oral hypoglycemic medications, insulin therapy, management of food intake, and physical exercises, among others (Dilworth et al., 2021, Prasathkumar et al., 2022, Dumbre et al., 2023). However, most DM therapies are known to have undesirable outcomes and are relatively expensive, thereby hampering their use by many people with diabetes (Akinfenwa et al., 2022). It is important to search for an alternative treatment option with minimal side effects and that is more cost effective. Natural remedies have been identified as treatment options to manage DM in most developing countries and several bioactive constituents have been documented and reported to be essential to the treatment realization of DM (Tran et al., 2020, Ansari et al., 2022, Sagbo and Hussein, 2022). Compounds like alkaloids, flavonoids, polyphenols, and tannins, are known to be instrumental in glucose regulation and alleviating hyperglycemic effects (Akinfenwa et al., 2022). Flavonoids obtained from plant extracts have been observed to exhibit antidiabetic properties by inhibiting the action of carbohydrate digestive enzymes (α-glucosidase and α-amylase enzymes) associated with hyperglycemia (Tran et al., 2020, Amoo et al., 2022, Jadalla et al., 2022).

"The genus *Helichrysum* Mill belong to the family Asteraceae, with a global estimate of 500–600 species, 250 of which are of South African origin" (Lourens et al., 2008). Species within this genus

are scented, woolly shrubs with a golden yellow flower (Matanzima, 2014, Leonardi et al., 2018). The species *Helichrysum cymosum*, which is use within the rural communities of South Africa as an herbal remedy to treat diseases like coughs, colds, pains, infected wounds, and headaches, has also been reported to induce trances and attract goodwill from ancestors (Heyman, 2013, Maroyi, 2019). Other therapeutic applications include cardiovascular complications, kidney problems, urinary infections, eye infections, vomiting, insomnia, diarrhea, laxatives, blocked noses, as an immune system booster, for controlling flatulence, for weak bones, as an insect repellant, for skin infections, and for treating varicose veins and influenza (Jadalla et al., 2022). Ealier studies have reported antioxidant, anti-inflammatory, antifungal, antiviral, antimicrobial, anti-diabetic, and cytotoxic effects (Jadalla et al., 2022). Bioactive compounds such as sesquiterpenes and chalcones have been isolated from this species (Matanzima, 2014, Maroyi, 2019, Jadalla et al., 2022). Despite their promising therapeutic potential, a complete spectrum of phytochemical compounds and their biological activities, such as antidiabetic, antioxidant, and anti-inflammatory have not been fully investigated and comprehended. It is based on this background that our research seeks to investigate the effect of aqueous and ethanol extracts on the major digestive enzymes α-amylase, α-glucosidase, and pancreatic lipase, as well as their antidiabetic, antioxidant and anti-inflammatory potential of the isolated bioactive compounds from the extracts.

3.2. Materials and methods

3.2.1. Reagents

RAW 264.7 mouse macrophages were purchased from Cellonex (South Africa). Sulfanilamide Solution and N-(1-Naphtyl)-ethylenediamine dihydrochloride (NED) Solution were made following the manufacturer's instructions and products purchased from Promega. Lipopolysaccharide (LPS) and aminoguanidine were purchased from Sigma-Aldrich (St. Louise, MO, USA). RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from GE Healthcare Life Sciences (Logan, UT, USA). Minimal Essential Medium (MEM) and phosphate-buffered saline (PBS) with and without Ca^{2+} and Mg^{2+} were purchased from Cytiva (Marlborough, MA, USA). Bis-benzamide H33342 trihydrochloride (Hoechst), non-essential amino acids, and penicillin/streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). CellROX® Orange reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). A-amylase type VI-B from Porcine pancreas, acarbose, starch, 3,5-dinitro salicyclic acid, sodium monobasic

and diabasic, α -glucosidase from *Sacchromyces cerevisiae, and* pNPG were all purchased from Sigma-Aldrich (St. Louis, MO, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and Dulbecco's Modified Eagle Medium (DMEM) were procured from GE Healthcare Life Sciences (Logan, UT, USA). All solutions were prepared freshly when needed for the respective assays, unless stated otherwise.

3.2.2. Plant collection

Helichrysum cymosum shoots were harvested in the garden of the Cape Peninsula University of Technology, Bellville campus, Cape Town, South Africa. The plant was authenticated by a botanist (P. Dryfhout) with voucher number 3708 and stored in the herbarium at the Department of Horticultural Sciences, Cape Peninsula University of Technology, Western Cape, South Africa. All experimental protocols and procedures of the study were approved by the Research Ethics Committee (REC) of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, South Africa (ethics approval number *CPUT/HWS-REC2021/H1*).

3.2.3. Extraction preparation

The shoots of *H. cymosum* were washed thoroughly, cleaned, and dried in an oven at 40 °C. The extraction protocol was according to Aladejana et al. (2020), with slight changes. The plants were then crushed to powder using an electric grinder. Subsequently, 200 g of crushed samples were soaked in 2.5 L of 70% ethanol and distilled water, respectively, and then stirred for 48 hours. The extracting solvent, 70% ethanol, was used because it has been reported to give a high yield of phytochemicals (Tourabi et al., 2023). Both ethanol and aqueous extracts were then filtered using a funnel and Whatman No. 1 filter paper. The ethanol extract was concentrated at 70 °C using a rotary vacuum evaporator, while the aqueous extract was freeze-dried. The concentrated extracts were stored at 4 °C in the refrigerator until required.

3.3. Cell culture maintenance

Human hepatoma-derived C3A hepatocytes were purchased from the American Type Culture Collection and maintained in 10 cm culture dishes in complete medium (MEM with 1% non-essential amino acids, 10% FBS, penicillin/streptomycin). The cells were incubated at 37 °C with 5% CO_2 in a humidified environment and sub-cultured after 90% confluence. Test samples of the

plant extracts were prepared by reconstituting in dimethyl sulfoxide (DMSO) 100 mg/mL, then sonicated and stored at 4 °C until used.

3.3.1. MTT Cytotoxicity assay

Cytotoxicity analysis was performed following the procedure in Okaiyeto et al. (2022a) with slight modifications. This analysis was performed to establish a safer concentration of the extracts for subsequent assays. C3A hepatocyte cells were seeded in 96 well plates at 5000 cells/well (100 µL aliquots) and left overnight to attach (24 hours). Five hundred (500) µg/mL dilutions of each extract were prepared independently in a complete medium and a 6-point serial dilution was made with the following concentrations: 15.6 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, and 500 µg/mL. From each dilution, 100 µL aliquots were added to 100 µL of attached cells in the 96 well plate, thus yielding final concentrations of 7.8 µg/mL, 15.6 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, and 250 µg/mL. The cells were treated for 48 hours at 37°C, 5% CO_2, and 30 µM Melphalan (100 mM stock) was used as a positive control due to its reported cytotoxic effect on cells (Alrifaie 2023). The Treatments were aspirated from the wells and 100 µL MTT (0.5 mg/mL) in the complete medium was added to each well and cells were incubated for 3 hours. After that, 100 µL DMSO was added to each well, and absorbance was measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). The graphs of the percentage cytotoxicity against the concentrations of the extracts were plotted using GraphPad Prism 5.

3.3.2. Cellular Antioxidant Assay

The cellular antioxidant activity (CAA) was assessed according to the procedure described by Wolfe and Liu. (2007). The assay was used to quantify the antioxidant activity of phytochemicals in food extracts and dietary supplements in live cells using an oxidant in conjunction with a fluorescent probe specific for the detection of reactive oxygen species (ROS). The experimental design discussed below is such that the observed activity reflects both the direct ROS scavenging activity of the sample as well as potential changes in the inherent capacity of the cells to resist oxidative stress, as cells were pre-treated with the sample for 24 hours before exposure to TBHP.

The C3A hepatocytes were seeded in 96-well plates at a density of 2 x 10^4 cells/well in 100 µL aliquots and left overnight to attach. Afterward, treatments were prepared in a complete medium

and added to the cells, and 100 μM catechin was added as a positive control and incubated for 24 hours. Oxidative stress was induced by adding tert-butyl hydroperoxide (TBHP) to the culture/treatment medium at a final concentration of 30 μM and incubating for 2 hours. The culture/treatment medium was gently aspirated, and 100 μL of staining solution [CellROX® Orange (5 μM) and Hoechst 33342 (5 μg/mL) in PBS with Ca^{2+} and Mg^{2+}] was added to each well. Plates were incubated for 30 minutes (protected from light), and fluorescent micrographs were captured immediately using an ImageXpress Micro XLS Widefield Microscope (Molecular Devices) with a 10x Plan Fluor objective using DAPI and TRITC (tetramethylrhodamine isothiocyanate) filter cubes. Acquired images were analyzed using the MetaXpress software and the Multi-Wavelength Cell Scoring Application Module. Antioxidant activity was determined using the average cellular CellROX® orange fluorescent intensity. All values obtained were calculated using GraphPad Prism 5.

3.3.3. Anti-inflammatory activity

Anti-inflammatory analysis was conducted according to the procedure of Shauli et al. (2023) with minor changes. The RAW 264.7 cells were seeded in RPMI1640 culture medium supplemented with 10% FBS (RPMI complete medium) into 96-well plates at a density of 1 x 10^5 cells per well and allowed to attach overnight.

The following day, the spent culture medium was removed and 50 μL sample aliquots (diluted in RPMI complete medium) were added to give final concentrations of 50, 100, and 200 μg/mL. To assess the anti-inflammatory activity, 50 μL of LPS (final concentration of 500 μg/mL)-containing medium was added to the corresponding wells. Aminoguanidine (AG) was used as the positive control at 100 μM, and cells were incubated for a further 24 hours. To quantify NO production, 50 μL of the spent culture medium was transferred to a new 96-well plate. Sulfanilamide solution and NED solution were prepared as per the manufacturer's instructions. A 50 μL Sulfanilamide solution was added to the spent culture medium and incubated for 10 minutes in the dark at room temperature. Fifty (50) μL NED solution was then added to each well and further incubated for 5-10 minutes in the dark at room temperature. The absorbance was measured at 540 nm (BioTek® PowerWave XS spectrophotometer), and a nitrite standard curve (using sodium nitrite dissolved in a culture medium) was used to determine the concentration of NO in each sample.

3.4. Determination of hypoglycaemic effect.

3.4.1. Alpha amylase Inhibitory activity

Alpha amylase activity was determined as described in Aladejana et al. (2020), using the 3,5-dinitrosalicylic acid (DNS) method with slight modifications. Both aqueous and ethanol extracts were dissolved in their various solvents. Different concentrations (10, 50, 100, 250, 500, and 1000 µg/mL) of the plant extracts were placed in test tubes (40 µl) each. Into the extract solutions, 160 µl of distilled water were added. Afterward, 400 µl of starch solution (0.5 g starch in 50 ml phosphate buffer) was added into the test tubes to initiate the reaction. Subsequently, 200 µl of α-amylase (4 units/ml) was incubated at 35 °C for 10 minutes to start the reaction. From the incubated solution, 200 µl was placed in a different test tube, and 100 µl of DNS (20 mL of 30 g of sodium potassium tartrate tetrahydrate mixed with 50 mL of 1 g of 3,5-dinitrosalicylic acid solution and 20 ml of 2 M NaOH at 90–95 °C) was added to end the reaction and boiled for 15 minutes. The mixed solution was then cooled down to a normal temperature, and 900 µl of distilled water was used to dilute the solution. The sample blank was prepared using a similar concentration of the plant extracts without the enzymes, and another blank with 100% enzyme activity was prepared with buffer in place of the plant extract. Acarbose was used as a positive control and was prepared similarly to the test samples, as described above. From the test sample, blanks, and positive control, 200 µl of each mixture was placed in a 96-well plate, and the absorbance was measured at 540nm using a UV spectrophotometer. The percentage inhibition of the enzyme α-amylase was determined using the equation (As-Ac/Ac) x 100.

3.4.2. Alpha-glucosidase activity

Alpha-glucosidase activities of aqueous and ethanol extracts of *H. cymosum* were evaluated following the procedure described by Erukainure, (2018) with minor modifications. Briefly, 50µl of both plant extracts at different concentrations (10, 50, 100, 250, 500, and 1000 µg/mL) and α-glucosidase from *Sacchromyces cerevisiae* (1.0 Unit/mL) were prepared in phosphate buffer 100mM at pH 6.8, placed in a 96-well plate, and incubated for 15 minutes at 37°C. Afterward, 100 µL of 5 mM p-Nitrophenyl-α-D-glucopyranoside (pNPG) solution prepared in phosphate buffer (100 mM, pH 6.8) was added to the reaction mixture and incubated for an additional 20 minutes

at 37°C. Acarbose was used as a positive control, and the absorbance was measured at 405 nm. The percentage inhibition was calculated with the formula below:

$$\%\ alpha\ glucosidase\ inhibition = \frac{(\text{absorbance of control} - \text{absorbance of test sample})}{\text{absorbance of control}} \text{ X } 100$$

3.4.3. Glucose uptake and utilization

Glucose uptake and its utilization were evaluated using C3A hepatocytes and L6 myocytes following the procedure in van de Venter et al. (2008), with some modifications. Both cells (C3A hepatocytes and L6 myocytes) were seeded, respectively, in 96-well plates (2×10^4 cells/well, 100 µL aliquots) and left overnight to attach. Various concentrations of treatments were prepared in a complete medium and added to the cells, followed by incubation for 24 hours (two plates) and 48 hours (two plates) for each cell type. After 24 and 48 hours of incubation, cell culture/treatment medium (5 µL for C3A; 10 µL for L6), was removed from the respective plates and transferred into new 96 well plates (A), which were sealed and stored at -20°C until required. The rest of the medium was aspirated, and cells were washed with 100 µL PBS and 25 µL of incubation buffer (RPMI-1640 diluted with PBS containing 0.1% bovine serum albumin (BSA) to a final glucose concentration of 8 mM) was added to cells (C3A and L6). Insulin (1 µg/mL) was used as a positive control, and the cells were then incubated for another 4 hours. Some of the culture medium (5 µL) was transferred to a new 96-well (plate B). Afterward, 200 µL of glucose oxidase reagent (3 mM phenol, 0.4 mM 4-aminoantipyrine, 0.25 mM EDTA, and 2.5 U/mL horseradish peroxidase in 0.5 M PBS (pH 7.0) with 1 mU/mL glucose oxidase from *Aspergillus niger*) was added to the plates (A and B), respectively, and incubated for 15 minutes at room temperature. Cell-free wells containing incubation buffer and complete culture medium were used as glucose standards. The absorbance was then measured at 510 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). Glucose uptake and consumption were determined as a function of the concentration of glucose (mM) remaining and expressed as the difference between the mean of the standard and test samples. The MTT assay was further used to determine cell viability during the analysis.

3.4.4. Pancreatic lipase inhibition

Pancreatic lipase inhibition for the aqueous and ethanol extracts was performed following the procedure described by Pringle et al. (2021). Different concentrations of sample extracts were established at 500, 250, 125, 62.5, and 31.25 µg/mL. A volume of 10 µL of the extracts and 5 µL of porcine pancreatic lipase enzyme (100 mg mL^{-1} prepared in 100 mM Tris-HCl, pH 8.0) were pre-incubated at 37 °C for 15 minutes. Subsequently, 170 µL of the substrate (*p*-nitrophenyl palmitate (pNPP) [1 mg/mL in isopropanol), with reaction buffer (gum arabic (1 mg mL^{-1}), sodium deoxycholate (2 mg mL^{-1}) and Triton X-100 (5 µL per mL) prepared in 100 mM Tris-HCl (pH 8.0)] was added to the mixture containing extracts and enzymes and incubated at 37°C for 25 minutes. Pancreatic lipase activity was then determined by measuring the absorbance at 405 nm using a BioTek® PowerWave XS spectrophotometer, and 100 µM Orlistat was prepared and used as a positive control. The percentage of pancreatic lipase inhibition was calculated according to the equation:

$$\% \textit{ Lipase inhibition} = \frac{\text{A405nm of blank} - \text{A405nm of test sample)}}{\text{A405nm of blank}} \text{ X } 100$$

3.5. Phytochemical Screening and antioxidant properties of aqueous and ethanol extracts of *Helichrysum cymosum* (HC)

3.5.1. Determination of Total polyphenols content (TPC) of *H. cymosum*

The total polyphenol content of the aqueous and ethanol extracts of the plant was determined using the Folin-Ciocalteu method described by Singleton et al. (1999). Briefly, 25 µL of the extract solution was mixed with 25 µL of Folin-Ciocalteu reagent (0.1M) and 100 µL of 7.5% Na_2CO_3 in a 96-well plate and then incubated for 30 min at room temperature. The absorbance was measured at 765 nm. TPC was estimated using gallic acid as standard and results were expressed as milligram of gallic acid equivalents per 1 gram of dry weight (mg GAE/g dry weight).

3.5.2. Determination of Total Flavonol Content (TFC)

Total flavonols for both aqueous and ethanol extracts were determined according to the procedure by Mazza et al. (1999). Into each well plate, 12.5 µL of the extracts, 12.5 µl of 0.1% HCl in 95% EtOH, and 225 µl of 2% HCl were added, while quercetin was used as the standard. The plate was

left for 30 minutes at room temperature, and absorbance was measured at 360 nm. All results were expressed as milligrams of quercetin equivalents per gram (mg QE/g).

3.5.3. Determination of Flavanol content.

The flavanol content of the AQ and EtOH extracts was determined as described by McMurrough and McDowell, (1978) using a calorimetric procedure, whereby the flavanols in the extracts react with 4-(Dimethylamino)-cinnamaldehyde giving a light blue coloration. Catechin was used as standard, and the plate was kept for 30 minutes, after which absorbance was measured at 640 nm. Results were expressed as milligrams of catechin equivalents per gram (mg CE/g).

3.5.4. Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed to determine the total antioxidant capacity of *H. cymosum* aqueous and ethanol extracts. The experimental procedure for this assay was adopted from Benzie and Strain (1996). The plant extract (0.05 g) was weighed and dissolved in 60% ethanol and centrifuged for 1 minute at 4000 rpm after which 10 µL of extracts at different concentrations were placed in triplicate in each well, and 300 µL of FRAP reagent (10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ), 20 mM $FeCl_3$, and acetate buffer (pH 3.6)) was added into the same wells. Ascorbic acid was used as a standard, and the plates containing the samples and standard were incubated for 30 minutes at 37 ^{o}C and the absorbance reading was later measured at 593 nm. All the results were expressed as µM ascorbic acid equivalent per gram dry weight (µmol AAE/g).

3.5.5. Trolox equivalent antioxidant capacity (TEAC)

TEAC analysis measures the capacity of antioxidants to scavenge ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation, according to the method in Re et al. (1999). An ABTS stock solution containing (8 mM ABTS and 3 mM potassium persulfate) was prepared 24 hours before the experiment. A volume of 275 µL ABTS reagent was mixed with 25 µL of extract samples and were placed into the 96 well plates, and Trolox was used as a standard. This was incubated for 30 minutes, and the absorbance was read at 734 nm using a plate reader. TEAC results were expressed as µmol TE/g.

3.5.6. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay of *H. cymosum* was determined according to the procedure in Sharma and Bhat, (2009) with some modifications. Different concentrations of the extracts were diluted, and 25 µL of each dilution was placed into a 96-well plate in triplicate. Afterward, 275 µL of freshly prepared DPPH solution (24 mg DPPH with 100 mL methanol) was added to each well. The mixture was incubated in the dark for 30 min, and the absorbance was measured at 517 nm using a microplate reader. Trolox was used as a standard, and measurements were recorded as (µmol TE/g).

3.5.7. High-Performance Liquid Chromatography (HPLC)

The phenolic acid and flavonoid content of the aqueous and ethanoic extracts of *H. cymosum* were determined by high-performance liquid chromatography (HPLC) (Agilent Technology 1200 series, Bellefonte, USA) using a diode array detector and a C18 column (5µm (4.6 mm x 150 mm i.d.). Briefly, into the HPLC column 20 µl of samples were automatically injected, and the mobile phase composition consisted of 0.1% acetic acid in water (A) and 0.1% Acetic acid in methanol (B) at a flow rate of 1 mL/min. A gradient elution was performed, running from 80:20 (A: B) to 20:80 (A: B) over 30 minutes. Compound detection was done at 320 nm and 360 nm, with the peaks of specific phenolic acid and flavonoid standards being documented based on retention time (Vongsak et al., 2012). The calculation for the concentration was obtained using the equation below and the results were expressed in mg/mL.

$$\frac{\text{Area of standard}}{\text{Area of the sample}} \times 20\ \mu g/ml$$

3.5.8. Qualitative analysis of phytochemicals from the extract of Helichrysum cymosum (Aqueous Q1 and Ethanol Q2 extracts)

The phenolic contents of aqueous and ethanoic extracts of *H. cymosum* were determined by LC-SM analysis, which comprises a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (MS) attached to the ultra-performance liquid chromatography (UPLC) (Waters, Milford, MA, USA) following the procedure reported by Idris et al. (2024) with slight modifications. ‘’Briefly, the ultra-performance liquid chromatography (UPLC) attached to the MS with 88 waters acquity and 89 was used for high-resolution UPLC-MS analysis. Electrospray ionization was used in

negative 90 mode with a cone voltage of 15 V, desolvation temperature of 275 °C, a desolvation gas at 650 91 L/hr, and the rest of the MS settings optimized for best resolution and sensitivity''. Two (2 µL) samples were injected into the UPLC column, and the mobile phase composition consisted of solvent A (0.1% formic acid) and B (acetonitrile containing 0.1% formic acid) (Idris et al., 2024). The gradient for solvents alternated linearly from A at 100% for 1 minute to 28% B in 22 minutes. Subsequently, it moved to 40% B in 50 seconds and a wash step for 1.5 minutes for 100% B, and then 40% B in 50 seconds and a wash step for 1.5 minutes for 100% B. It was equilibrated again to the initial conditions for 4 minutes. The following conditions were maintained for the column: flow rate: 102 0.3 mL/min, temperature: 55 °C. Ion mobility was set at IMS 104. The wave velocity was set at 332 m/s and the wave height at 20.2 V. Data were processed by scanning (from *m/z* 150 to 1500 in resolution mode as well as in MS^E mode). In the MS^E mode, the initial MS data was taken at a low collision energy (4 V), and the next one at a collision energy ramp (40–100 V). The reference mass (Leucine enkaphalin) was calibrated with sodium formate to obtain the appropriate mass measurements. Polyalanine was used for the 105 calibration and calculations.

Identification of compounds and designated names given was achieved through accurate mass matching using XCMS, which was connected to the following databases (MassBanks, Metlin, NIST, and ReSpect). All identified compounds were considered unidentified if the accurate mass match error (AME) was greater than 5 ppm (Zubarev and Makarov, 2013). The mass fragmentation patterns of compounds that were accessible were searched from these databases (Idris et al., 2023). The few phenolic compound standards that were spiked under similar LC-MS conditions and fragmentation patterns were identified based on retention time, mass fragmentation, and ionization mode (Okaiyeto et al., 2022a, Idris et al., 2023). Since UPLC-ESI-QTOF-MS could identify many compounds, therefore, all standards could not be obtained. Hence, information on MS and MS^2 fragmentation ions from the literature and databases was used to annotate these compounds. To minimize false annotations, the number of carbon atoms in the supposed identified compounds was estimated. Standard mixtures and chemical markers ranging from (3.9, 7.8, 15.6, 31.3, 62.5, 125.0, and 250.0 mg/L) were used for quantification. The calibration and linear curve were tested at the range of standard solution concentrations (mg/L) by plotting the peak areas against the standards and a correlation coefficient measured using linear regression, and an R-value greater than 0.99 was considered linear.

3.6. Statistical Analysis

All results were evaluated by one-way analysis of variance (ANOVA), followed by a Bonferroni post-test using GraphPad Prism 5. Results were shown as a mean ± standard error of the mean, and statistical significance $P < 0.05$ was measured as substantial variation in the mean.

3.7. Results and Discussion

Phytochemical contents present in plants are known for diverse medicinal healing potentials when applied (Al-Mustafa et al., 2021, Moriasi et al., 2021)**.** Bioactive constituents of the genus *Helichrysum* have been well established as having anti-bacterial, antioxidant, anti-diabetic, anti-cancer, anti-inflammatory, and hepatoprotective potentials (Lourens et al., 2008, Lourens et al., 2011, Jadalla et al., 2022). The activities of this genus are linked to the occurrence of vital biomolecules such as flavonoids, phenolics, phloroglucinol derivatives, and others (Lourens et al., 2008). Furthermore, the species *H. cymosum* has been recently reported to have antidiabetic potential (Jadalla et al., 2022), antimalarial, antibacterial, and toxicity effects (Van Vuuren et al., 2006). In this research, the phytochemical constituents of *H. cymosum* were confirmed to contain secondary metabolites such as polyphenols and flavonols, which might be linked to the pharmacological potentials demonstrated by the plant. Thus, the current findings agree with other findings on this species (Akinyede et al., 2021b, Jadalla et al., 2022). Additionally, the findings revealed that the ethanol extracts had the greatest polyphenols and flavonols (303 ± 4 GAE/g, 183 ± 6 mg QE/g, respectively) compared to the aqueous extract (83.7 ± 2 GAE/g, 36.0 ± 1 mg QE/g, respectively), possibly because ethanol is a relatively much better extracting solvent for phenolic compounds (Al-Mustafa et al., 2021).

Antioxidant components in plants play a major role in modulating oxidative stress (OS)-induced complications (Benoite and Vigasini, 2021, Akinyede et al., 2021a, Akinyede et al., 2021b). Diabetic complications have been associated with OS as one of its fundamental causes, and this occurs when there is a buildup of free radicals, which are detrimental to human health, causing overproduction of mitochondrial superoxides, β-cell dysfunction, and diabetes vascular damage (Giacco and Brownlee, 2010, Jadalla et al., 2022). Cell damage and mutation are known to be caused by free radicals, and natural antioxidant products are recognized to contribute significantly to clearing free radicals. Literature reports have shown that the inhibition of OS through antioxidants is essential in diabetes therapies, especially by protecting β-cell dysfunction in

diabetes. Hence, the application of antioxidant-rich substances as therapeutic agents is plausible (Kifle and Enyew, 2020).

The current study investigated the *in vitro* antioxidant activities of *H. cymosum* AQ and ET extracts by assessing FRAP, TEAC (ABTS), and DPPH as shown in table 3.1. The result demonstrated stronger antioxidant activity for the ethanol extract, and the highest effects were seen with DPPH (1893 ± 34 µmol TE/g), followed by FRAP (158 ± 2 µmol AEE/g), and then ABTS (1402 ± 26 µmol TE/g). Similarly, the antioxidant activities of the AQ extract were highest in DPPH (606 ± 8.4 µmol TE/g), followed by FRAP (530 ± 2 µmol AEE/g) and ABTS (450 ± 11 µmol TE/g). Thus, the DPPH, FRAP, and TEAC assays showed the strongest activities for the ET compared to the AQ.

The high antioxidant activity of the ET is surely linked to the presence of phytochemical constituents such as polyphenols and flavonols. Flavonoids, which are polyphenol and phenolic compounds, are known to possess potent antioxidant effects in mitigating the development of oxidative stress-related diseases. The phenolic antioxidant is known for interfering with the accumulation of ROS and other free radicals through the transfer of hydrogen atoms from its hydroxyl group, whereas the flavonoids are well established for their oxidative stability potentials by scavenging ROS and free radicals via oxidation, converting them to more stable and less reactive free radical forms (Akinyede et al., 2021a, Jadalla et al., 2022). Flavonoids are capable of reducing free radicals through quenching, chelating radical intermediate compounds, and upregulating or protecting antioxidant defense systems (Otang et al., 2012), and natural antioxidants can avert oxidative stress-related diseases such as diabetes (Benoite and Vigasini, 2021).

In general, the antioxidant activities exhibited by both AQ and ET extracts of *H. cymosum* could be linked to the reported phytochemicals they possess, displaying good scavenging activities. *H. cymosum* essential oils are known to have radical scavenging activities with a free radical scavenging concentration of SC_{50} = 6.3 g/l), in line with other studies (François et al., 2010). Additionally, an investigation by Adewinogo et al. (2022) presented the antioxidant potential of the essential oils of *H. cymosum* as having moderate antioxidant activities, in comparison to the current study, in which only the extracts exhibited strong antioxidant activities.

Table 3.1: Phytochemicals compound and antioxidant capacity of H. cymosum extracts.

Compounds/ antioxidant capacity	Aqueous extract	Ethanol extract
Polyphenols (mg GAE/g)	83.7 ± 2	303 ± 4
Flavonols (mg QE/g)	36.0 ± 1	183 ± 6
Flavanols (mg CE/g)	ND	ND
ABTS (µmol TE/g)	450 ± 11	1402 ± 26
FRAP (µmol AEE/g)	530 ± 2	158 ± 2
DPPH (µmol TE/g)	606 ± 8.4	1893 ± 34

All values are presented as mean ± SD (n=3). GAE (gallic acid equivalent), AAE (ascorbic acid equivalent), TE (trolox equivalent), CE = Catechin equivalents, QE (quercetin equivalent), ND none detected.

3.7.1. High-Pressure Liquid Chromatography (HPLC) analysis

HPLC was used at 320 and 350 nm, respectively, for both the aqueous and 70% ethanol extracts, as shown in Figures 3.1 (A and B) and 3. 2 (A and B). Specific flavonoids were examined from both extracts, as shown in Table 3.2 below. The findings disclose compounds such as: caffeic acid (AQ), chlorogenic (AQ and ET), kaempferol (AQ), and rutin (ET). Further screening was then performed using UHPLC-ESI-MS.

Table 3.2: Phytochemicals compound of Aqueous and 70% ethanol extract of H. cymosum identified using high-pressure liquid chromatography (HPLC).

Compounds	Unit	Aqueous extract	Ethanol extract
Caffeic acid	mg/g	0.28	0.00
Chlorogenic acid	mg/g	4.48	21.64

Kaempferol	mg/g	0.49	0.00
Rutin	mg/g	0.00	6.74

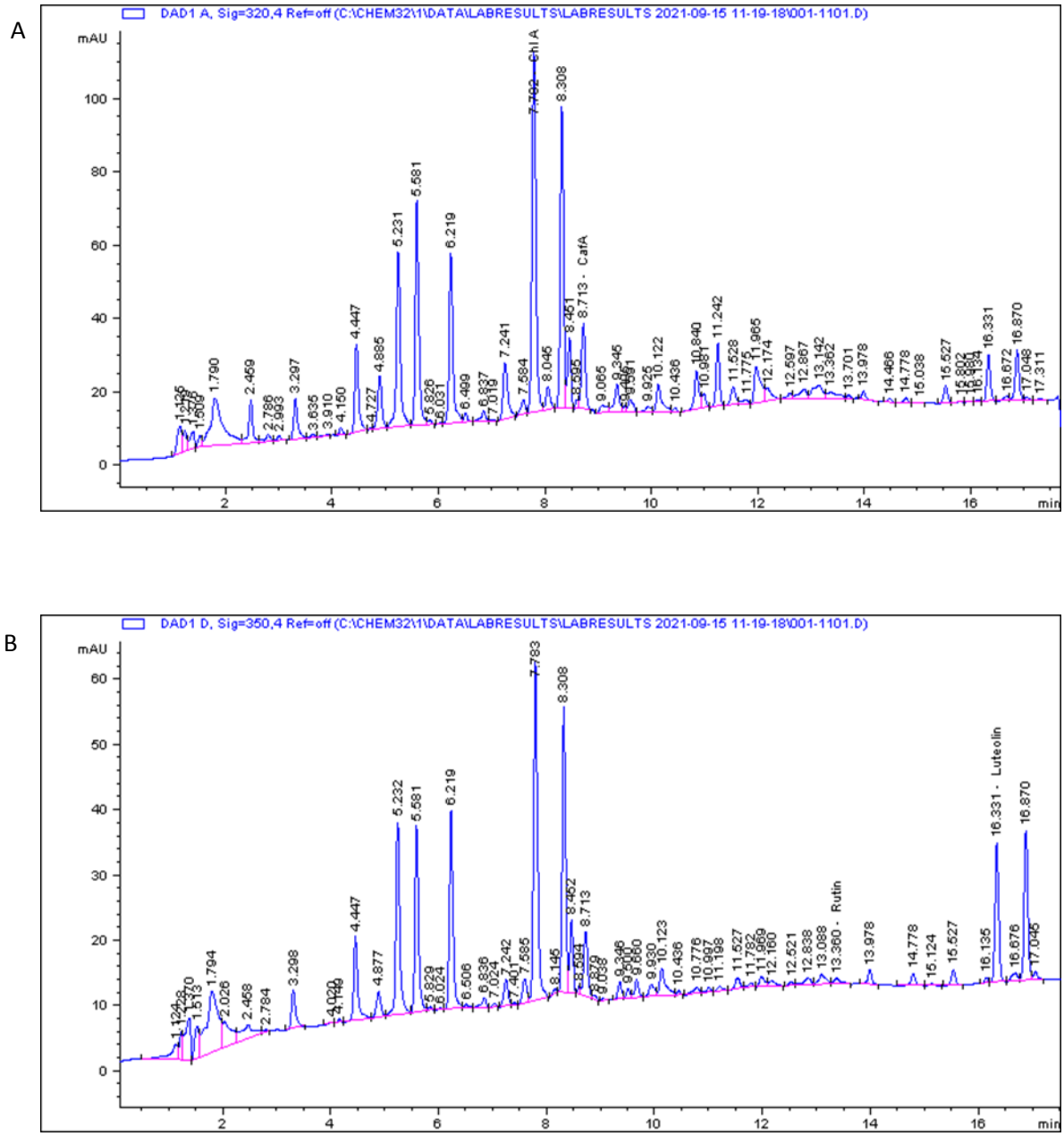


Figure 3.1: HPLC chromatogram at 320nm (A) and 350nm (B) of aqueous extract of *H. cymosum*.

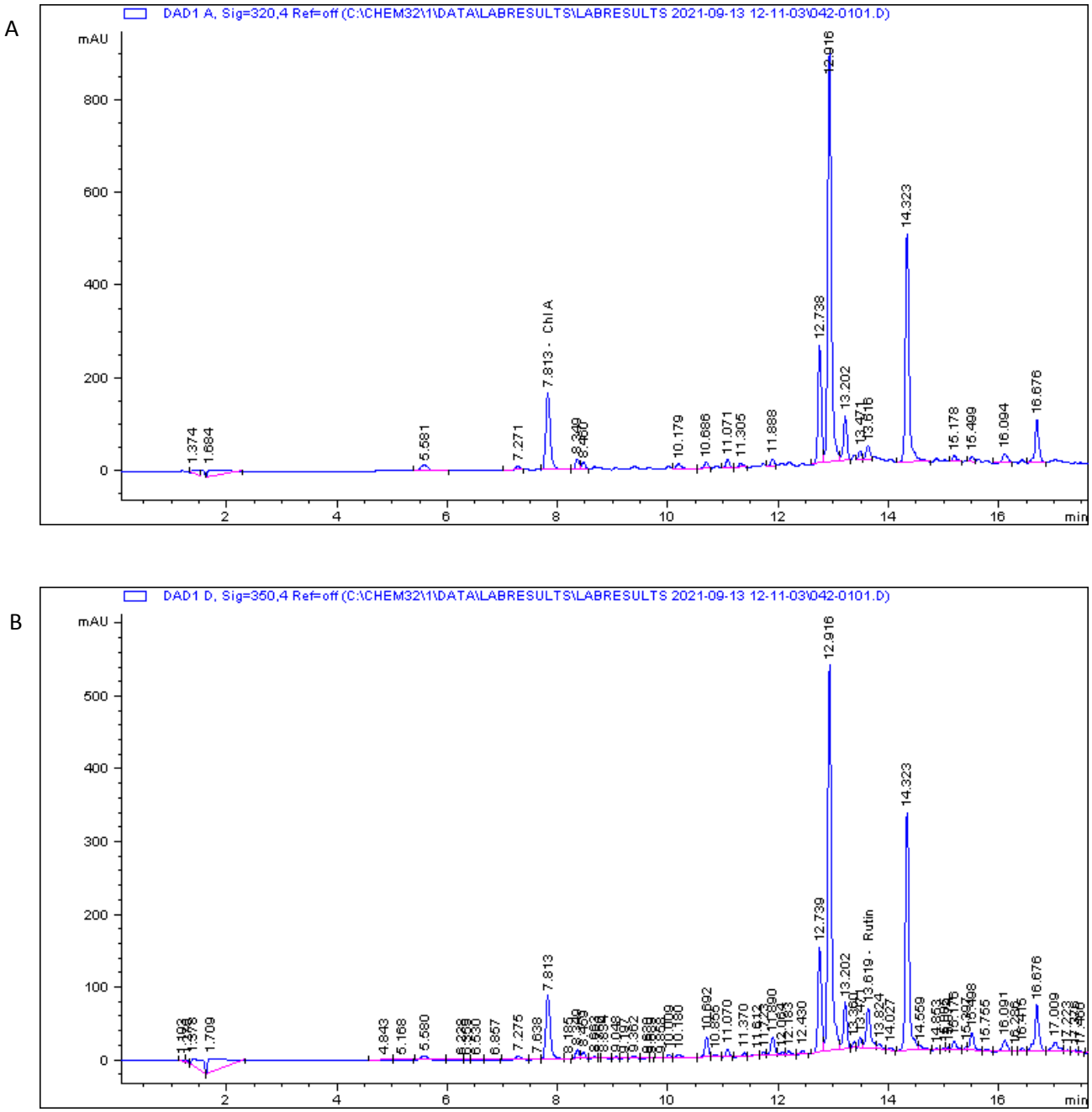


Figure 3.2: HPLC chromatogram at 320nm (A) and 350nm (B) of 70% ethanol extract of *H. cymosum*.

3.8. Characterization of identified compounds isolated (UPLC) from *H. cymosum*

The ultra-performance liquid chromatograph (UPLC) was used to screen the phytochemicals present in the *H. cymosum* aqueous and 70% ethanol extracts, of which a total of 43 phytochemical

derivatives were identified. These phytochemicals include hydoxycinnamic acids, flavan-3-ols, flavonol, helihumulone, and other compounds, as shown in Table 3.3.

3.8.1. Identification of hydroxycinnamic acids

Mono, di, and tri acyl chlorogenic acid were identified at peaks, 10-14, 22-26 and 27 respectively, presenting maximum absorption wavelengths at 300 and 326 nm of UV maxima (Idris et al., 2023). These spectra exhibited a deprotonated molecular ion at *m/z* 353 of mono caffeoyl quinic acids (mono-CQAs) and *m/z* 515 of di caffeoyl quinic acids (di-CQA). Fragmentation in the MS/MS produced *m/z* 191 (QA), which gave a dehydrated quinic acid moiety (*m/z* 173) and caffeic acid (*m/z* 179) as prominent fragments. The isomers were assigned using the hierarchical keys and order of elution sequence from the reversed-phase column previously developed as 3-O-caffeoylquinic acid (3-O-CQA) < 1-O-caffeoylquinic acid (1-O-CQA) < 5-O-caffeoylquinic acid (5-O-CQA) < 4-O-caffeoylquinic acid (4-O-CQA). Also, 4-O-CQA and 3-O-CQA were differentiated by the intensity of the characteristic ions of chlorogenic acids (Idris et al., 2023, Idris et al., 2024).

In the former, *m/z* 173 is the base peak, and in the latter, *m/z* 191 is the base peak (Crozier et al., 2009). Generally, CGAs with more hydroxyl groups in quinic tend to be more hydrophilic compared to those with more free axial hydroxyl groups. Meanwhile, the caffeoyl residue is easily removed in the course of fragmentation: 1 > 5 > 3 > 4 (Clifford et al., 2005). Given that hydroxyl groups are situated at the axial in positions 1 and 3, and the equatorial in positions 4 and 5 (Clifford et al., 2005, Idris et al., 2024), for di-O-CQAs, the more the additional caffeoyl groups are attached to free equatorial hydroxyl groups (owing to steric interactions), the stronger the retention (Clifford et al., 2005, Idris et al., 2024). Meaning, the loss of the caffeoyl group (C) is likely to be in the order of 1-C > 5-C > 4-C > 3-C (Clifford et al., 2005). This enabled peaks 22-26 to be classified as "1,3 di-O-CQA, 3,4 di-O-CQA, 1,5 di-O-CQA, 3,5 di-O-CQA, and 4,5 di-O-CQA" respectively, since the elution order is "1,3-diCQA <<< 1,4-diCQA << 3,4-diCQA < 1,5-diCQA < 3,5-diCQA << 4,5-diCQA" (Clifford et al., 2005). The prominent ions for di-CQAs are 4,5-di-CQA, which has the base peaks *m/z* 353, 191, 179, and *m/z* 173, while 3,5-di-CQA has *m/z* 353 and 179 as base peaks (Schram et al., 2004). Di CQAs gave a maximum absorption wavelength of UV at 326 nm.

Earlier investigations on the chlorogenic profiling of *H. cymosum* revealed the presence of dicaffeoyl quinic acids from the acetonic extract (Matanzima, 2014). Peak 27 was identified as a tricaffeoylquinic acid using an accurate mass match, while Peak 5 was ambivalently recognized as p-Coumaric acid ethyl ester based on the presence of *m/z* 119 that would symbolize the decarboxylated coumaroyl. Additionally, a free dimethoxy cinnamic acid was allocated at peak 2.

3.8.2. Identification of flavan-3-ols

Many flavan-3-ols were in oligomeric/ or polymerized forms (peaks 18–21) and identified from the stereoisomers catechin and epicatechin. The monomers catechin, epicatechin, gallocatechin, and gallo(epi)catechin gave various lengths of polymers called proanthocyanidins (PAs). The two types of *B*-type PAs are linked by C4-C8 or C4-C6 interflavan linkages and *A*-type PAs have additional C2-C5 or C2-C7 interflavan ether-linkages, between the oligomers (Singh et al., 2018; Jimoh et al., 2024). The order of hydrophobicity for flavan-3-ol monomers in reversed-phase liquid chromatography, i.e. "(-/+)-epicatechin > (-/+)-catechin > (-/+)-epigallocatechin > (-/+)-gallocatechin," also enabled identification of the monomers and the oligoners (Jimoh et al., 2024). The order of elution from the column is in the opposite direction. Thus, (-/+)-gallocatechin elutes earlier, and epicatechin comes out last. Diastereoisomers; "(–)-epicatechin (2R,3R) and (+)-catechin (2R,3S) resolve easily on reverse phase column packings, but (–)-epicatechin (2R,3R) and (+)-epicatechin (2S,3S), and (–)-catechin (2S,3R) and (+)-catechin (2R,3S)", do not (Clifford and Kuhnert, 2022).

Also, flavan-3-ols elute according to their degree of polymerization (DP), firstly eluting the monomers and then the different oligomers (López-Cobo et al., 2016). As reported by other authors, A-type procyanidins eluted before B-type procyanidins (Wallace and Giusti, 2010, López-Cobo et al., 2016, Gao et al., 2018). Catechin and epicatechin exhibit fragments *m/z* 151, 135 (due to retro Diels Alder (RDA) fragmentation) upon collision-induced dissociation (CID) of their precursor ions $[M-H]^-$; *m/z* 289 (Idris et al., 2023, Jimoh et al., 2024). In addition, "fragment ions *m/z* 245 ($[catechin-H-44]^-$, loss of CO_2], *m/z* 205 $[catechin-H-84]^-$, loss of flavonoid A ring) and *m/z* 179 ($[M-H-110]^-$, loss of flavonoid B ring)" were consistent with that of literature (Escobar-Avello et al., 2019, Jimoh et al., 2024). Based on the above information, the assignment is indicated and represented in Table 3.3.

Table 3.3: Phytochemicals screened from extract of Helichrysum cymosum (Aqueous Q1 and Ethanol Q2 extracts) using LCMS.

Peak	t_R (min)	UV λmax (nm)	*m/z* $[M-H]^-$	MS/MS	Tentative name	Sample	Identification
1	1.39		248.9	181, 1	Piperic acid	Q1, Q2	Jimoh et al. (2024)
2	1.49		225.0	179, 215	Dimethoxycinnamic acid monohydrate	Q1, Q2	Idris et al. (2024)
3	1.62		317.0	215, 165, 195, 174	Benzoyl galactonic acid Derivative	Q2	New
4	1.70		239.1	229, 207, 193	Flavonol	Q1, Q2	Idris et al. (2024)
5	1.80		191.0	149, 119	p-Coumaric acid ethyl ester	Q1, Q2	Akinyede et al. (2022)
6	1.88		133.0	133, 7	Malic acid	Q2	Abd Ghafar et al. (2020)
7	11.38		272.9	175, 192	Esculetin derivative	Q2	Idris et al. (2024)
8	12.84		175.1	145	Esculetin	Q1, Q2	Idris et al. (2024)

9	13.47		203.1	185, 2	L-Tryptophane	Q2	Soto Mayer, (2019)
10	13.67	323	353.1	179, 135, 191	3-caffeoylquinic acid	Q1, Q2	Yang et al. (2022)
11	16.09	326 sh	353.1	191, 173	1-caffeoylquinic acid	Q2	Yang et al. (2022)
12	16.17	326	353.1	191, 179	5-caffeoylquinic acid	Q1, Q2	Akinyede et al., (2022)
13	16.24	325	353.1	179	4-caffeoylquinic acid	Q2	Idris et al., (2023)
14	16.71	322	179.0	135	Caffeic acid	Q1	Akinyede et al. (2022), Idris et al. (2023)
15	18.39	373	565.1	519, 169	Galloyl derivative	Q2, Q1	Idris et al. (2024)
16	19.02		423.1	Unfragmented	Helihumulone	Q2	Matanzima, (2014)
17	20.13	266	455.1	173, 225	O-Methylated (+)-Catechin gallate	Q1	Nešović et al. (2020)
18	20.56	280	611.1	457, 367, 225	(+)-gallocatechin-3-Ogallate - derivative	Q1	Alperth et al. (2019)
19	20.61	280	611.1	575, 197, 357	(-/+)-gallocatechin-derivative	Q2	Abu-Reidah et al. (2014)

							Alperth et al. (2019)
20	21.32	282	609.1	197	(-/+)-(Epi)gallocatechin-(epi)gallocatechin	Q1, Q2	Alperth et al. (2019)
21	21.39	341	611.1	575, 4	(-/-)-(Epi)gallocatechin-(epi)gallocatechin	Q2	Alperth et al. (2019)
22	22.62	325	515.1	353, 173, 179, 191	1,3-Dicaffeoylquinic acid	Q2	Yang et al. (2022)
23	23.13	327	515.1	191, 353, 179, 135	3,4-Dicaffeoylquinic acid	Q2	Yang et al. (2022)
24	23.18	327 sh	515.1	353, 179, 191	1,5-Dicaffeoylquinic acid	Q2	Yang et al., (2022), Idris et al. (2023)
25	24.09	327 sh	515.1	353, 173, 179, 191, 135	3,5-Dicaffeoylquinic acid	Q2	Akinyede et al. (2022), Yang et al. (2022), Idris et al. (2023)
26	24.11		515.1	353	4,5-Dicaffeoylquinic acid	Q2	Yang et al. (2022)

27	24.37	293, 327	677.1	467, 5	3,4,5-Ttricaffeoylquinic acid		Yang et al., (2022)
28	24.47	340	681.2	331, 3	6-methoxyquercetin derivative	Q2	Idris et al., (2024)
29	24.53	341	315.0	300, 271, 255	O-Methyl quercetin		Mohamed et al. (2024)
30	24.59	289	327.2	215, 265, 293	oxo-dihydroxy-octadecenoic acid	Q1	Idris et al. (2023), Idris et al. (2024), Jimoh et al. (2024)
31	24.67	295	329.2	265, 293, 227, 129	Trihydroxy-octadecadienoic acid	Q1	Idris et al. (2024), Jimoh et al. (2024)
32	24.83		299.1	255	Quercetin	Q2	Matanzima, (2014)
33	24.89	340	285.1	255, 265	Kaempferol	Q2	Matanzima, (2014)
34	24.92	289, 335	287.1	255, 265, 255, 269	Dihydrokaempferol	Q2	Idris et al. (2024)
35	25.01	280, 330	265.1	149	Magnolol	Q2	Li et al. (2023, Jimoh et al. (2024)

36	25.02	287	265.1/ 293.2	150	2'-Deoxyguanosine monohydrate	Q1	New
37	25.17	283	265.1	150	Magnolol	Q1, Q2	Li et al. (2023, Jimoh et al. (2024)
38	25.87	280, 323	423.2	279			
39	26.34		341.1	150, 265, 293, 279	Magnolol derivative	Q1, Q2	Li et al. (2023), Jimoh et al. (2024)
40	26.36		461.2	379, 341, 265, 150, 249	Pentoside derivative	Q2	New
41	26.43		341.1	150	Not identified	Q2	New
42	26.56		341.1	157	Not identified	Q2	New
43	27.20		238.8	239	Not identified	Q1, Q2	New

3.8.3. Identification of flavonols

Our study showed the presence of kaempferol and quercetin aglycones in confirmation with earlier reports (Matanzima, 2014), which was confirmed in this study at peaks 32–33 (Figure 3B). However, some methylated and 6-methoxy quercetin derivatives of quercetin are being reported here for the first time. In addition, dihydrokaempferol has also been noticed at peak 34 (Figure 3B), as previously reported in the literature for their MS/MS fragment ions (Dias et al., 2013).

3.8.4. Identification of other compounds

Key among these compounds is helihumulone which has previously been isolated from the genus *Helichrysum* (Bohlmann et al., 1979, Matanzima, 2014) was reported with MS1 ion at *m/z* 423.0919 and its high presence is indicated in this study (Figure 3B). Some other compounds include carboxylic acids, piperic acid, malic acid, polyunsaturated fatty acids; oxo-dihydroxy-octadecenoic acid, and trihydroxy-octadecadienoic acid. Fragmentation of the carboxylic acids occurs through the release of one or two water (18 Da) and carbon dioxide (44 Da) molecules or both (62 Da), and is characterized by the ion fragments $[M - H\text{-}18]^-$ and CO_2, $[M - H\text{-}44]^-$, or $[M - H\text{-}62]^-$ (Okaiyeto et al., 2022b, Idris et al., 2023). Amino acid at peak 9 was identified in negative ion modes as tryptophan, based on a deprotonated molecular ion peak $[M\text{-}H]^-$ at *m/z* 203.0670 (Gouveia and Castilho, 2009). Additionally, its MS/MS spectrum displayed a fragment ion $[M\text{-}H\text{-}NH_3]^-$ at *m/z* 186. It is generally a fragmentation, generating the diagnostic 2-carboxy spiro [cyclopropane-indolium] fragment ion when ionized in positive mode.

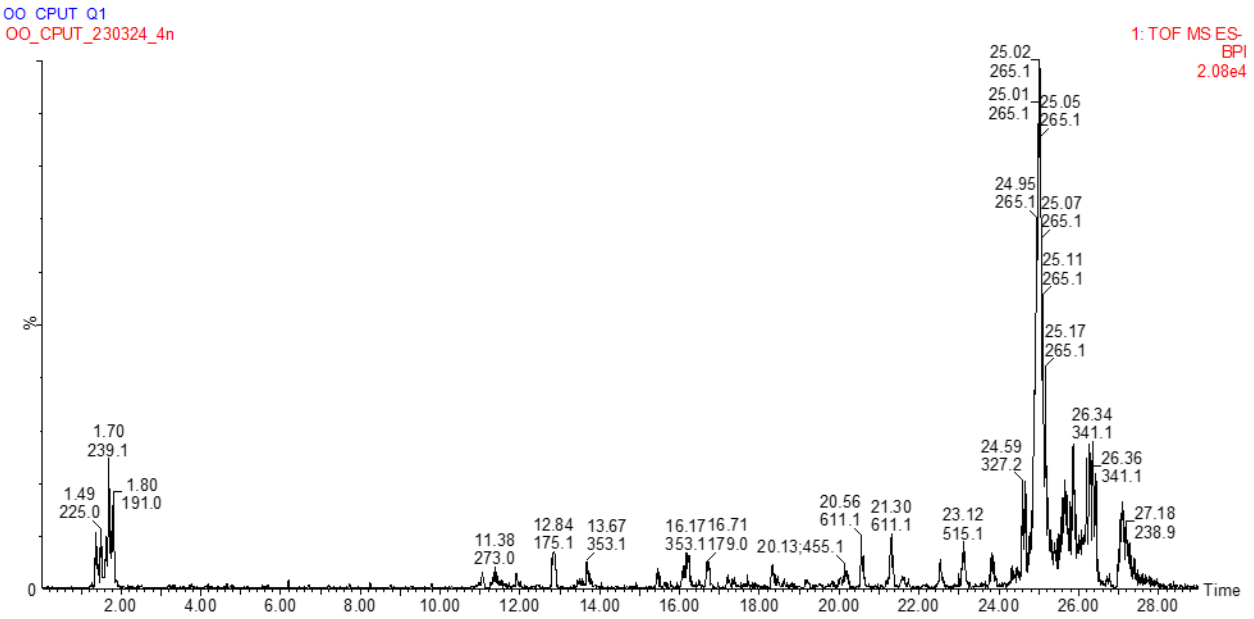


Figure 3.3A: Shows UHPLC-ESI-MS base peak chromatogram for aqueous extract of *H. cymosum* analyzed in the negative ion mode.

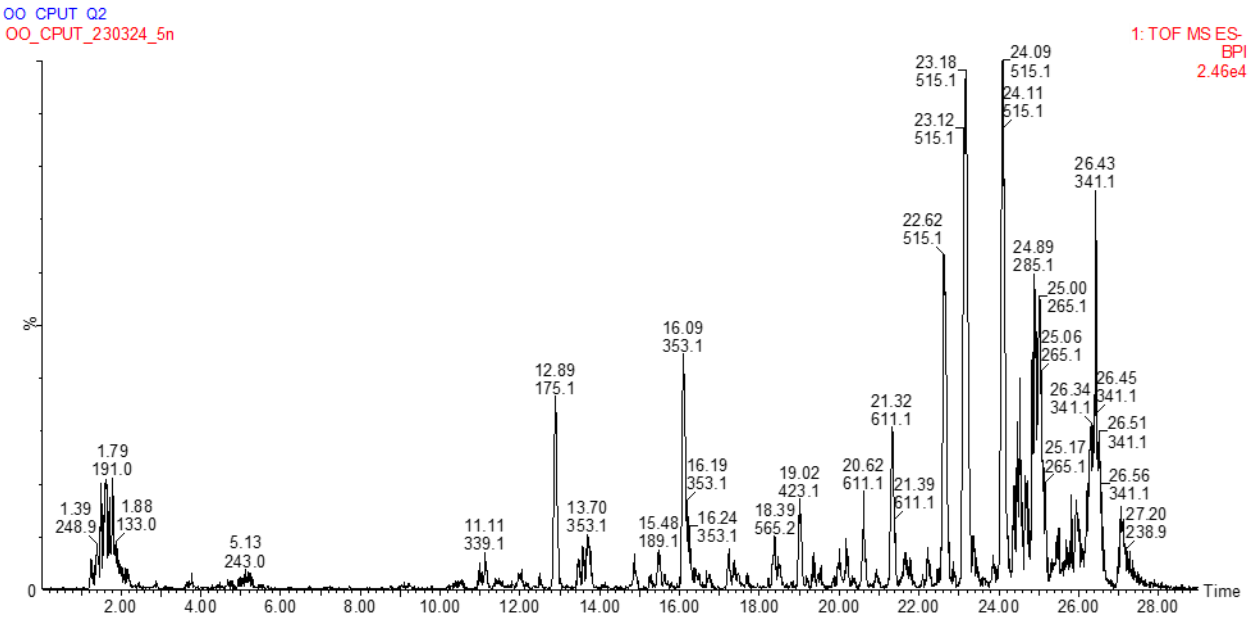


Figure 3.3B: Shows UHPLC-ESI-MS base peak chromatogram for 70% ethanol extract of H. cymosum analyzed in the negative ion mode.

3.9. Evaluation of antidiabetic activities of aqueous and ethanol extracts.

3.9.1. Alpha glucosidase inhibitory activity

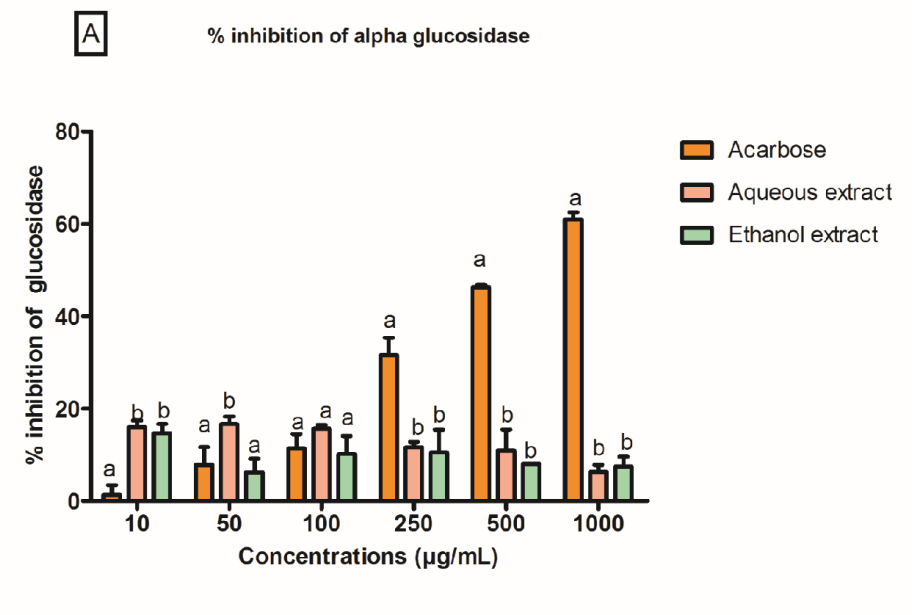
Alpha glucosidase is an enzyme located in small intestines at the brush border membrane of the enterocytes, where it breaks down dietary carbohydrates into monosaccharides for absorption. The enzymatic inhibition of alpha-glucosidase has been the main approach used in antidiabetic drug development since this enzyme is involved in carbohydrate metabolism to glucose (Kawamura-Konishi et al., 2012, Ghani, 2015, Visvanathan et al., 2021). Antidiabetic activities of most drugs and medicinal plants have been associated with the ability to inhibit enzyme activities (Oboh et al., 2014, Erukainure, 2018). In the present study, the alpha-glucosidase inhibitory activities by the aqueous (AQ) and 70% ethanol (ET) extracts of *H. cymosum* was assessed and compared with acarbose (Figure 3.4A). The results reveal significant inhibition by the aqueous extracts at lower concentrations (10 µg/mL and 50 µg/mL) while at 100 µg/mL AQ and the control, the same rate of inhibition with no significant ($p > 0.05$) difference was displayed. Equally, the ethanol extract showed maximum inhibition at (10 µg/mL) and at (50 µg/mL and100 µg/mL) with no significant ($p > 0.05$) difference compared to the standard drug acarbose. The current study revealed that the trend of alpha-glucosidase inhibitory activity is at its highest potential at lower concentrations and

declines steadily with increased concentrations. The lowest inhibition was observed at 250 µg/mL, 500 µg/mL, and 1000 µg/mL, respectively compared to acarbose**.** The finding suggests that *H. cymosum* contains phytochemical such as flavonoid and polyphenols which have a strong affinity to the enzyme binding site better at lower concentration that higher concentration (Ramkumar et al., 2010, Proença et al., 2022). The lower inhibitory effect at higher concentration could be attributed to saturation of the enzyme active site by the compounds or binding to other areas of the enzyme rather than the active side by lowering it inhibitory effects. Another potential factor might be due to competitive inhibition, where both compound and substrates contend for occupancy of the enzyme active site, thereby affecting their enzyme binding affinity (Proença et al., 2022). Nevertheless, the alpha-glucosidase inhibition presented in this study can be linked to the presence of the following compounds; quercetin, chlorogenic acid, catechin and its derivatives, caffeic acid, kaempferol and which have also been reported in other studies to be an effective alpha-glucosidase inhibitors (Lin et al., 2016, Tian et al., 2016, Erukainure, 2018, Pimpley et al., 2020). The observed, hypoglycemic effect displayed by *H. cymosum* extracts, accords to earlier investigation by Jadalla et al. (2022) in which *H. cymosum* methanoic extract exhibited strong alpha-glucosidase inhibitory activity with IC_{50} value of 12.94 ± 0.2 µM 18.16 ± 1.2 and 44.44 ± 0.2 µM respectively. Enzymatic inhibition of alpha-glucosidase by *H.cymosum* portrays a potent hypoglycemic agent that can be used to regulate the amount of blood glucose by limiting glucose absorption into the blood stream (Erukainure, 2018).

3.9.2. Alpha amylase Inhibitory activity

Alpha amylase is a digestive enzyme that enables the breakdown of polysaccharides into glucose and maltose (Kaur et al., 2021). The alpha-amylase inhibitory activities of aqueous and ethanol extracts of *H. cymosum* at various concentrations is shown in Figure 3.4 B. The results show that there was substantial inhibition of alpha-amylase at the lowest concentration (10 µg/mL) for both aqueous and ethanol extracts, approximate to that of the control (acarbose), while the other concentrations show moderate activities, but the efficacy was lesser than that of acarbose. Earlier study by Jadalla et al. (2022) reported no measurable alpha-amylase inhibitory activity of *H. cymosum* methanoic extracts**.** Therefore, the lower activity exhibited by both extracts in this study could be ascribed to the minimal interaction and binding affinity of the phytochemical constituents with alpha-amylase (Lu et al., 2017, Kaur et al., 2021). Perhaps, the different compound present

in *H. cymosum* were engaged in a competitive mode with the substrate for same binding site of the enzymes, thus preventing the action of the compound in inhibiting alpha amylase enzyme (Zhang et al., 2015). Inhibition of carbohydrate- hydrolyzing enzyme activities by compound such phenolic is based on their ability to bind with proteins. Phenolic compounds such as flavonoids and phenolic acid have been associated with antidiabetic activities via enzyme inhibition (Agarwal and Gupta, 2016). Other bioactive compounds such as anthocyanins, carotenoids, flavonoids, phenolic, and vitamins have equally been reported to inhibit α-amylase in diabetic patients, by preventing the mechanisms that are involved in the development of diabetes (Agarwal and Gupta, 2016).


A
% inhibition of alpha glucosidase
% inhibition of glucosidase
80
60
40
20
0
10
50
100
250
500
1000
Concentrations (µg/mL)
Acarbose
Aqueous extract
Ethanol extract

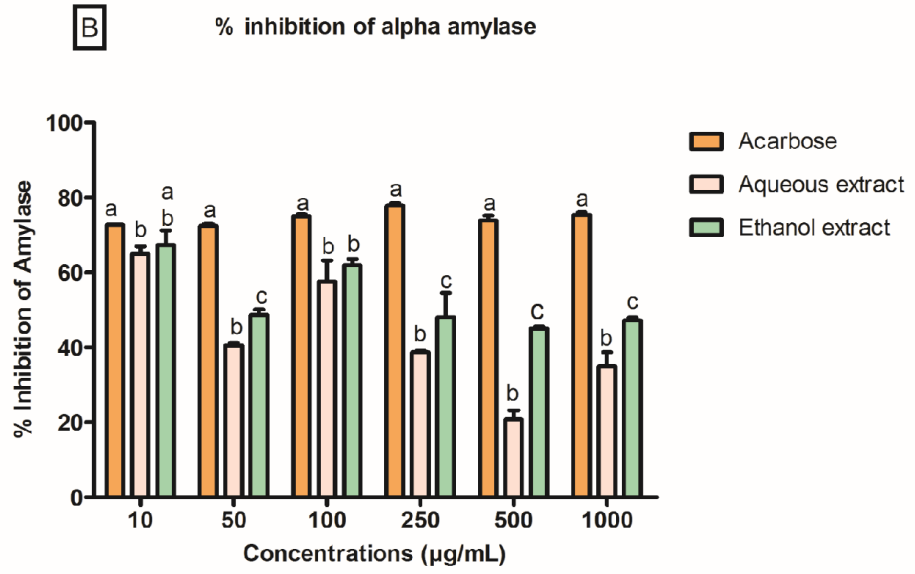

B
% inhibition of alpha amylase
% Inhibition of Amylase
100
80
60
40
20
0
10
50
100
250
500
1000
Concentrations (µg/mL)
Acarbose
Aqueous extract
Ethanol extract

Figure 3.4: α-glucosidase (A) and α-amylase (B) inhibitory activities of aqueous and 70% ethanol extracts of Helichrysum cymosum. Data were express as mean ± SD; n = 3. The bars with different letters shows significantly different ($p < 0.05$). The % inhibition was compared to the control acarbose.

3.9.3. Pancreatic lipase inhibition

The pancreatic lipase inhibitory activity of *H. cymosum* aqueous and ethanol extracts is shown in (Figure 3.5). The findings show a lesser and significant ($p < 0.05$) inhibitory effect on lipase enzyme at all concentration for both extracts compared to the standard drug orlistat (control), suggesting that the biomolecule present in *H. cymosum* might have very low affinity for pancreatic lipase enzyme binding site, thus hindering it effectiveness (Li et al., 2021). Similarly, a study by Pringle et al. (2021) showed that *A. linearis* has no inhibitory effects on pancreatic lipase. Despite the diminished effect of *H. cymosum* extracts toward pancreatic lipase enzyme, the bioactive compound could still exert its therapeutic effect in combating obesity and hypertriglyceridemia in type 2 diabetes via other mechanistic pathways such as AMPk-activated protein kinase, PI3K, phosphatidylinositol 3-kinases; AKT, protein kinase (Lv et al., 2019, Savova et al., 2023, Nethengwe et al., 2024). Therefore, the importance of extracts' action through these pathways PI3K/AKT/ AMP, cannot be omitted, they could have a downstream effect on insulin action and activation of different organs like adipose tissue, liver and muscle. Studies have shown that activation of these signaling pathways PI3K/AKT/ AMPk /GSK3 have antidiabetic and lipid modulatory effect by the extract of *Hypericum attenuatum* Choisy in mice (Lv et al., 2019). The findings suggest that antidiabetic modulatory effect H. *cymosum* extracts is more dominant toward carbohydrate metabolizing enzymes with a less impact on triglycerides. However, the adipogenic approach could be the potential mechanism via which the extracts could potentially modulate obesity (Savova et al., 2023).

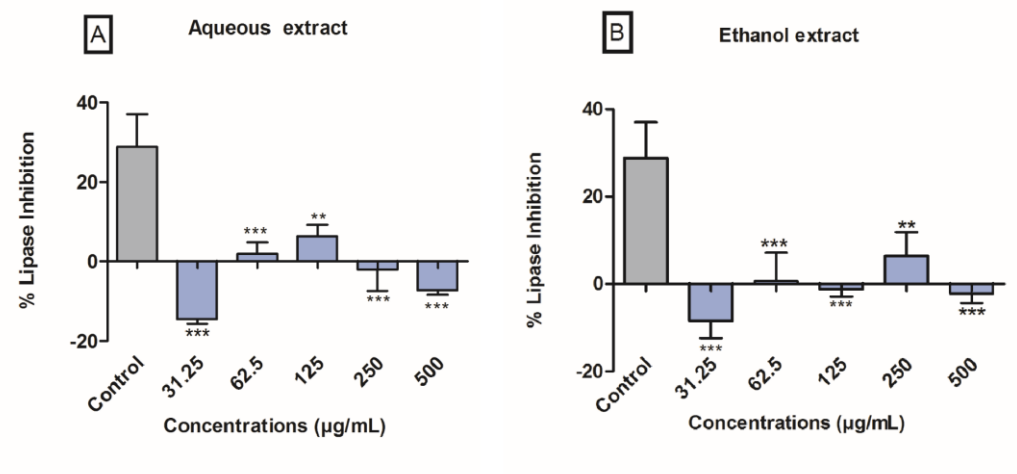


Figure 3.5: Pancreatic lipase inhibition of aqueous (A) and 70 % ethanol (B) extracts of H. cymosum. Error bars indicate the standard deviation of the mean of four replicates. The * on the bars indicate significant difference ($p < 0.05$) compare to the control orlistat (100 µM).

3.9.4. Cytotoxicity assays (MTT)

The cytotoxicity effect of aqueous (AQ) and 70% ethanol (ET) extracts were examined on C3A hepatocyte using melphalan as positive control. Cells were treated with AQ and ET extracts at various concentrations (7.8 µg/mL; 15.6 µg/mL; 31.25 µg/mL; 62.5 µg/mL; 125 µg/mL and 250 µg/mL) for 48 hours. The AQ extracts (Figure 3.6A) did not show any significant difference ($p > 0.05$) in cell viability at all concentrations compared to the untreated control, however, there was a significant increase ($p < 0.05$) by *H. cymosum* AQ extracts at all concentration compared to the melphalan treated control. From this finding, it was observed that the AQ extracts gradually increases cell viability at lower concentrations up till (7.8 µg/mL; 15.6 µg/mL; 31.25 µg/mL) and then slowly decreases at higher concentrations (62.5 µg/mL; 125 µg/mL and 250 µg/mL), suggesting that *H. cymosum* AQ extracts gradually enhanced cell viability to a certain ranges of concentration and then start to induce cellular stress on cells, hence decrease in cell proliferation, although there was no significant difference ($p > 0.05$) compared to the untreated control. The results demonstrated the safe use of AQ extracts at all concentrations. The ET extract (Figure 3.6B) exhibited a significant ($p < 0.05$) increase in cell proliferation from (7.8 µg/mL; 15.6 µg/mL; 31.25

µg/mL; 62.5 µg/mL; 125 µg/mL) compared to melphalan treated control and no significant change ($p > 0.05$) with 250 µg/mL concentration. When compared to the untreated controls, the ET extracts of *H. cymosum* showed no significant difference from the lowest concentration up to 125 µg/mL but at 250 µg/mL, there was a significant decreased cell viability, which was indicative of toxicity ($p < 0.05$). The findings indicates that the ET extracts is safe for use at lower concentrations, whereas higher concentration pose a toxicity threat and requires careful evaluation.

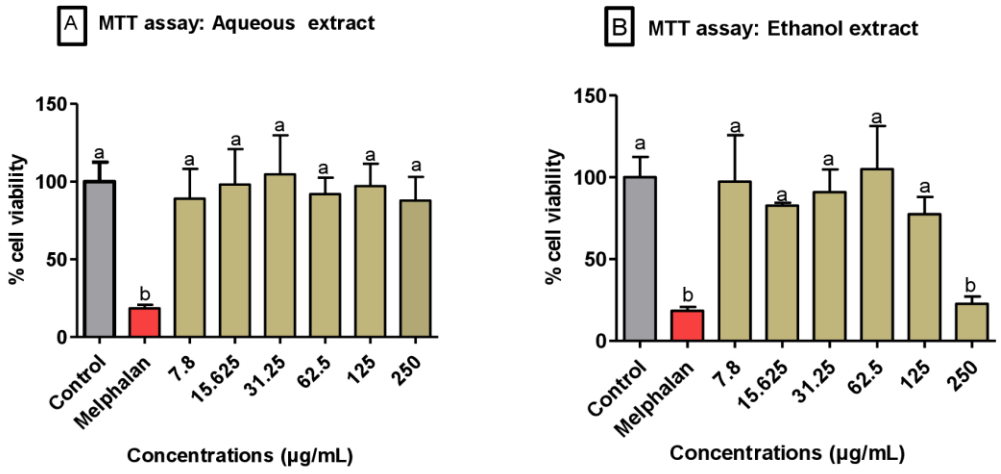


Figure 3. 6: Cytotoxicity evaluation of aqueous (A) and 70% ethanol (B) extracts of H. cymosum against C3A cells after 48 hours. Melphalan was used as positive control. Error bars indicate standard deviation of mean four replicate. Bars with different letters present significant different ($p < 0.05$) compared to the untreated control.

3.9.5. Anti-inflammatory activities and cellular antioxidant activities.

The mouse macrophage cell line, RAW 264.7, is a well-characterized and popular model to investigate the anti-inflammatory potential of test samples. Cells were cultured in multi-well plates and activated by exposure to LPS which induces the expression of iNOS with concomitant

nitric oxide formation. Changes in NO production were determined by measuring the levels of nitrite in the culture medium. Simultaneous evaluation of cell viability (MTT assay) was used to confirm the absence of cytotoxicity in the test sample. The current study adopted this model to evaluate the anti-inflammatory potentials of *H. cymosum* aqueous and 70% ethanol extracts. The observed anti-inflammatory activity as shown in Figure 3.7 (A, B, C, and D) indicated a decrease in nitrite concentration in response to LPS activation of RAW macrophages with no effect on cell viability ($p > 0.05$), as seen with the AG-treated cells. The AQ treated cell shows a higher NO production at all concentrations with no effect on cell viability ($p > 0.05$) (Figure E and F). The AQ treated cells showed no significant ($p > 0.05$) inhibition of NO compared to LPS treated cells and its cell viability, whereas control and the AG treated showed a significant ($p < 0.05$) reduction in NO with no effect on cell viability (Figure 3.7 A, B, C, and D).

The ethanol extract (Figure3.7 B) exhibited a slight decrease in NO production at 50 µg/mL ($p < 0.05$) compared to LPS treated cells and could possess anti-inflammatory potential, however, the ethanol extract showed cytotoxicity at concentrations of 100 and 200 µg/mL with significant ($p < 0.05$) inhibition of NO, but the results of these concentration cannot be considered (Figure 3.7 B and D). Similarly, macrophage activation was indicated by a significant ($p < 0.05$) reduction in NO production with significant decline in cell viability for the ethanol treated cells (Figure F and H). It could be said that the ethanol extracts possess anti-inflammatory effect at minimal concentrations but toxicity concerns for higher concentrations. For the aqueous extracts, the ineffectiveness on NO inhibition, despite maintaining cell viability does not roll out its potency toward inflammatory responses, it action could be of importance via other anti-inflammatory inhibitory channel or routes such as cytokines production (Nemudzivhadi and Masoko, 2014).

Oxidative stress and inflammatory response have been associated with the accumulation or buildup of free radicals (Diaz et al., 2012, Nemudzivhadi and Masoko, 2014). Reactive oxygen species (ROS) have been implicated in mediating cytokine productions through the activation of transcription factors like NF-κB, indicating that there is a connection between ROS and cytokines to initiate an inflammatory response (Nemudzivhadi and Masoko, 2014). However, the inflammatory response is well known to be a protective mechanism against ailments, but on the flip side, unending inflammatory actions could result in tissue dysfunction (Ravipati et al., 2012, Nemudzivhadi and Masoko, 2014, Okaiyeto et al., 2022a). Additionally, excess accumulation of

free radicals can interact with pro-inflammatory molecules resulting in the production of superoxides and peroxynitrites which can cause permanent cell membrane damage (Ravipati et al., 2012). Previous studies have uncovered natural anti-inflammatory compounds such as phenolic and flavonoids to play an important role as anti-inflammatory agents (Diaz et al., 2012, Ravipati et al., 2012, Wen et al., 2015). The anti-inflammatory effects shown by the ethanol extract is suggestive of the presence of phenolic compounds. Therefore, interfering with the production of ROS by natural antioxidants is an effective strategy to overcome and prevent OS and its subsequent damage to organs (Palomino et al., 2022).

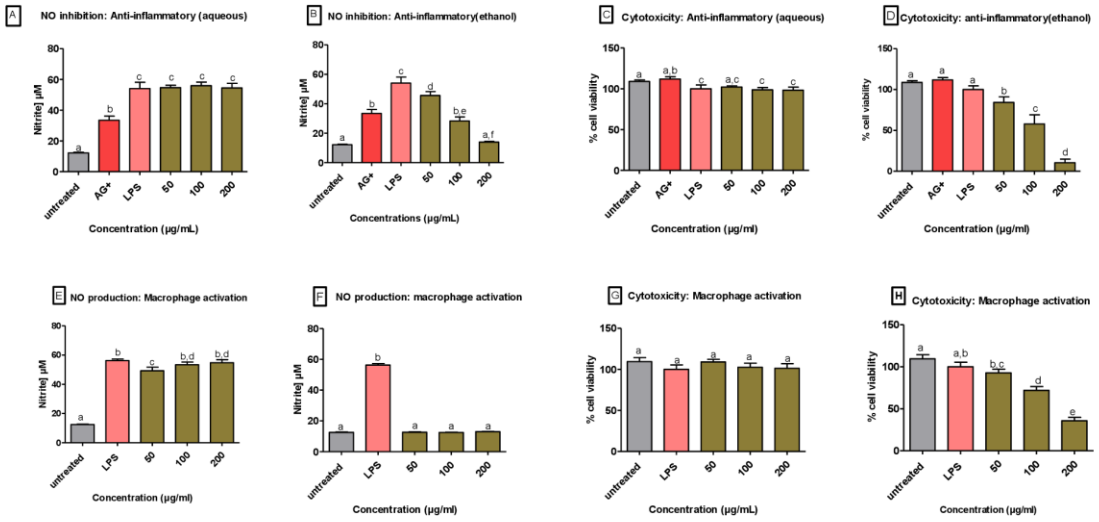


Figure 3.7: Nitric oxide production and inhibition of LPS-activated macrophages (A, B, E, and F), and their corresponding cell viability (%) (C, D, G, and H) after 24 hours treatments with aqueous and 70 % ethanol. Error bars indicate standard deviation of mean four replicate. Bars with different letters present significant different ($p < 0.05$) compared to the AG- control and untreated control.

3.9.6. Cellular antioxidant activities

Cellular antioxidant activity (CAA) is used to quantify the antioxidant capacity of bioactive compounds or phytochemicals in cells, using an oxidant in conjunction with a fluorescent probe specific for the detection of reactive oxygen species (ROS) (Wolfe and Liu, 2007, Wen et al., 2015). This approach provides knowledge by considering the antioxidant behaviors, uptake distribution, and metabolism in physiological conditions (Wen et al., 2015). In the current study, CAA analysis was used to determine the antioxidant activities of *H. cymosum* aqueous and ethanol extracts in C3A hepatocytes using (TBHP) and the CellROX® Orange reagent (Molecular Probes®, Life Technologies, USA). TBHP, a short-chain organic hydroperoxide, is commonly used to induce cellular oxidative stress as its metabolism results in the production of peroxyl and alkoxyl radicals (Yang et al., 2020). CellROX® Orange is a cell-permeable, reduced, non-fluorescent dye that becomes brightly fluorescent upon oxidation by ROS, with excitation/emission maxima of 640/665 nm, respectively. The observed activity reflects both the direct ROS scavenging activity of the samples, as well as any potential changes in the inherent capacity of the cells to resist oxidative stress as cells are pre-treated with aqueous and ethanol extracts for 24 hours before exposure to TBHP (Martín et al., 2001, Singh et al., 2018, Norfaizah, 2020).

The cellular antioxidant activity of aqueous and ethanol extracts was determined in C3A cells using TBHP as an oxidant and CellROX® Orange as a quantitative indicator of ROS. Catechin (100 µM) was used as a positive control to indicate antioxidant activity as shown in Figures 3.8(A and B), indicating that the aqueous extract was not cytotoxic at all concentrations, whereas significant cytotoxicity was observed in C3A hepatocytes after treatment with the ethanol extract (62.5 and 125 µg/mL), hence, their antioxidant capacity at those concentrations cannot be reliably interpreted. On the other hand, the aqueous extract exhibited antioxidant activity at a treatment concentration of 31.25 and 125 µg/mL. Although the reduction at 62.4 µg/mL was not statistically significant, the ethanol extract showed significant inhibition of TBHP-induced ROS production at 31.25 and 62.5 µg/ml.

One previous study has shown that dietary antioxidants could as well act as pro-oxidants in cell culture systems and might result to cellular damage (Martin et al., 2008), thus the variation of antioxidant activities of the different doses in the current study suggest that higher concentrations

of test sample can enhance oxidative stress damages. However, ethanol extracts were cytotoxic at these concentrations (62.5 and 125 μg/mL) after 24 hours treatment. The cytotoxic effect observed by the ethanol extract at these concentrations could be attributed to the identified phytochemical compounds such as flavonoids (Olabiyi et al., 2020, Okaiyeto et al., 2022b). These compounds may disrupt of the trans-membrane protein flux, hence inhibiting cell proliferation (Sohn et al., 2013).

Previous studies showed that assessing the ROS produced directly in living cells is a pointer to oxidative damage. The pro-oxidant TBHP, a short-chain organic hydroperoxide, is commonly used to induce cellular oxidative stress as its metabolism results in the production of peroxyl and alkoxyl radicals (Martín et al., 2001, Martin et al., 2008). It was observed that the ROS produced in cultured cells during this study was reduced significantly at all ranges of concentrations with the aqueous extracts and at lower concentrations for ethanol extract. This reveals that the natural antioxidants present in *H. cymosum* could reduce the generation of ROS in the C3A cell lines, thereby preventing oxidative stress damage to cells. Thus, the quenching of ROS by the *H. cymosum* extracts could improve oxidative stress damage. The aqueous extract showed promising activities at all tested concentrations, while the effects of the ethanol extract were seen at lower concentrations. Thus, lower concentrations of ethanol should be considered for use in future studies.

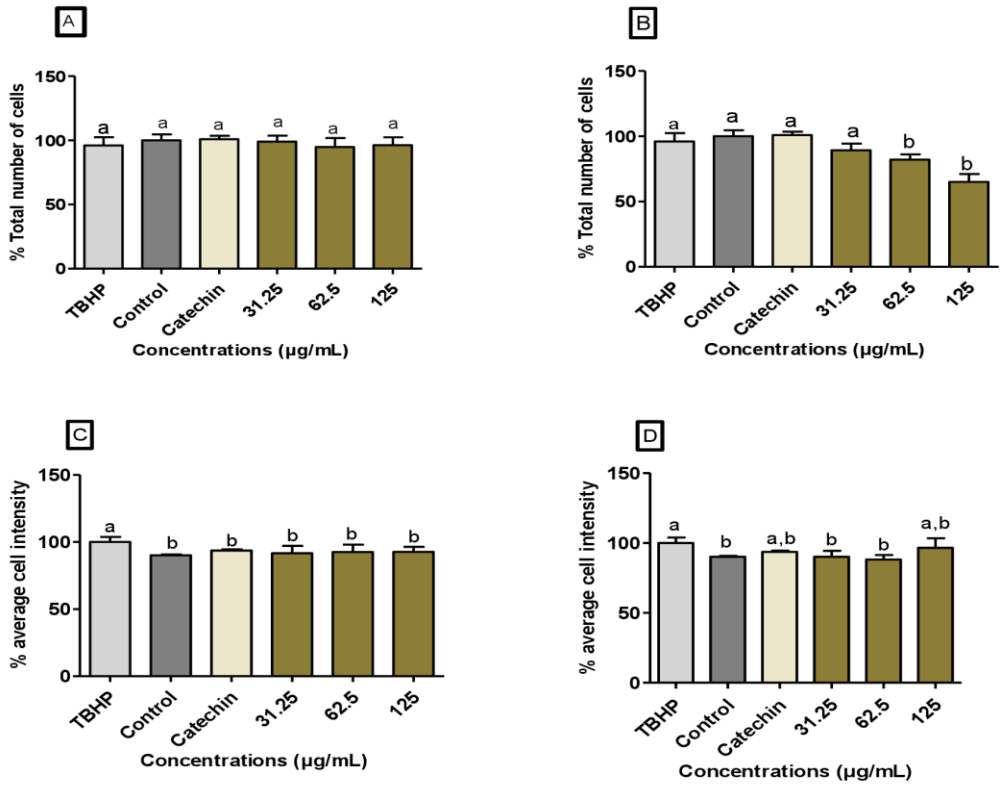


Figure 3.8: Cellular antioxidant activity of C3A cells: Total number of cells (%) (A and B) and Average Intensity (C and D), after 24 hours of treatment with aqueous (A and C) and 70 % ethanol (B and D). Catechin (100 µM) was used as a positive control and significant toxicity ($p<0.05$ compared to TBHP). Error bars indicate standard deviation of quadruplicate values obtained from a single experiment. Different letters above the bar for each given concentration compared to the control are significantly different ($p < 0.05$).

3.9.7. Glucose Uptake and utilization in C3A hepatocytes and L6 myocyte cell

Glucose uptake and its utilisation are important regulatory pathways used in the clearance of glucose in the bloodstream in normalising blood glucose levels (Chadt and Al-Hasani, 2020). Figure 3.9.1 (A, B, C, and D) shows glucose utilisation by C3A hepatocytes after 24- and 48-hour treatment with aqueous and ethanol extracts. The AQ 24 hours treatment demonstrated progressive increased in glucose utilization from lowest to the highest in a concentration-dependent order

compared to the untreated control, a significant increase ($p < 0.05$) was noted at higher concentrations (30, 60, and 120 µg/mL) (Figure 3. 9.1 A). Meanwhile the 48 hours AQ treatments showed a minor and insignificant increase across all concentrations compared to the control ($p > 0.05$) (Figure 3.9.1 C), Also cell viability was >70% across all concentrations, therefore, no toxicity was recorded.

ET (24 h) shows continuous increase in glucose utilization across all concentrations, a higher and significant ($p < 0.05$) glucose utilization was attained at 15 and 30 µg/mL, with no toxicity, whereas toxicity was observed at 60 µg/mL compared to the control (Figure 3.9.1 B), whereas, for the ET 48 h treatment, the highest glucose utilization was attained at lower 3.75 µg/mL and 15 µg/mL ($p < 0.05$) and toxicity was exhibited at the highest concentration (30 and 60 µg/mL) (Figure 9.1 D). The glucose utilization for L6 myocyte cells after 48 hours for both AQ and ET shows that the cells utilized all the available glucose (Figure 3.9.2 C and D). For the 24 hours glucose utilization in a L6 myocyte cells, a very small substantial increase was observed after normalizing with MTT cell viability although statistically insignificant ($p < 0.05$) at 15 µg/mL for AQ and .7, 7.5 and 15 µg/mL for ET (Figure 3.9.2 A and B, respectively)

Glucose uptake in L6 Myocyte cells shown in Figure 3.9.3 (A, B, C, and D) demonstrated a significant increase in glucose uptake at 15, 30, and 60 µg/mL (Figure 3.9.3 A) and 7.5, 15 and 30 µg/mL for (Figure 3.9.3 B) after 24 hours compared to control ($p < 0.05$) and no significant difference ($p > 0.05$) compared to Insulin. After 48 hours treatment, samples show no significant increase compared to the control (Figure C and D), no toxicity was recorded, and cell viability was >70% at all concentrations. Glucose uptake by C3A hepatocyte cells within 48 hours of treatment as shown in Figure3.9.3 (F and G), demonstrated a comparable glucose uptake to that of the control within all concentrations for A (Figure3.9.3 F). Likewise, ET (3.9.3 G) exhibited similar rate in glucose uptake across the various concentrations except at 7.5 µg/mL which had a slight decline compared to the untreated control, while insulin had a higher glucose uptake compared to all concentrations in both AQ and ET extracts. The observed glucose uptake and its utilization by C3A and L6 myocytes could have be enhanced by bioactive phytochemical constituent of *H. cymosum*. The findings reveal that the phenolic compound present in H. *cymosum*, could potentially modulate glucose metabolism by influencing cellular uptake in glucose responsive cells

(muscle and fat), by enhancing its glucose uptake and utilization (Abifarin et al., 2021, Olaokun et al., 2017).

Studies have established the important role played by glucose transporter in the distribution of glucose via cell surface membrane to storage organs (muscles and adipose tissues) and in maintaining normal blood glucose levels (Okaiyeto et al., 2022b). However, GLUT4 remains the most projected among other GLUTs for insulin regulation and glucose transport in adipose and skeletal muscles (Pereira et al., 2017, Chadt and Al-Hasani, 2020). GLUT4 facilitates glucose uptake via insulin action which stimulates the transportation of GLUT4 proteins across the plasma membrane (Satoh, 2014, Sayem et al., 2018, Joost et al., 2002, Leto and Saltiel, 2012). Insulin promotes glucose uptake by increasing the concentration of Glut4 protein at the plasma membrane more than the basic activities of the transporters (Simpson et al., 2001, Watson et al., 2004, Wang et al., 2020).

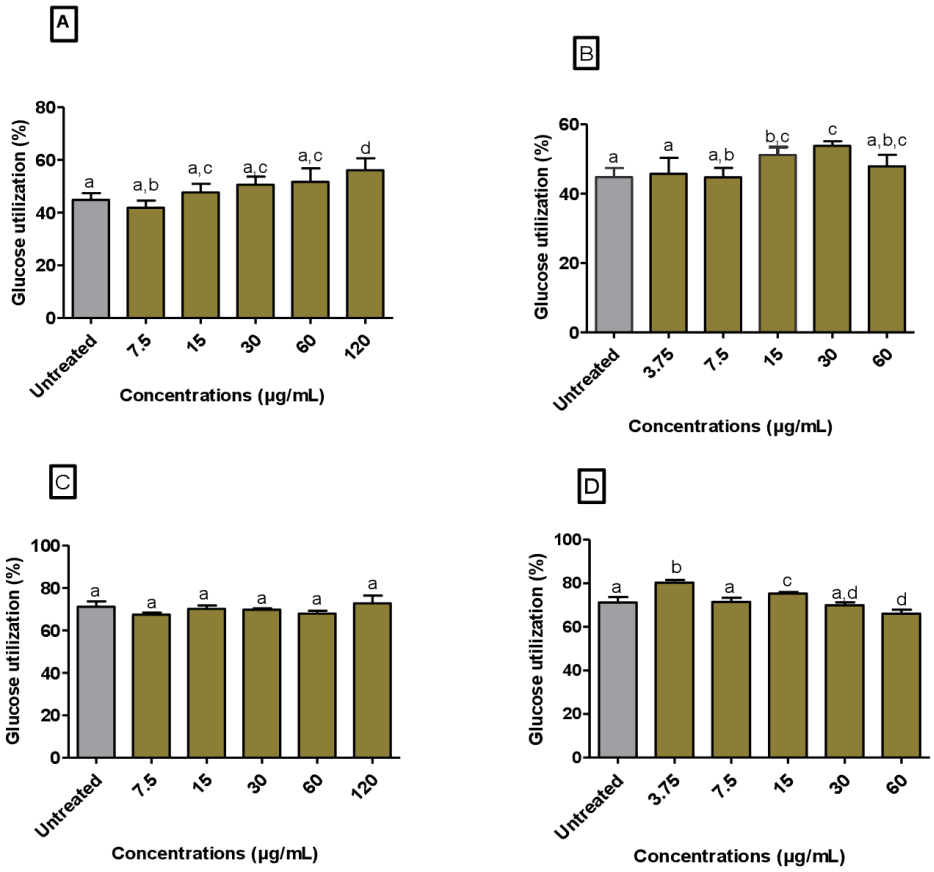


Figure 3.9.1: Glucose utilization (%) after 24 hours (A and B) and 48 hours (C and D) of treatment by *H. cymosum* in C3A hepatocytes. Results were normalized to cell viability as determined using the MTT assay. Error bars indicate the standard deviation of the mean of four replicates. Letter above the bars indicates a significant difference ($p < 0.05$) compared to the control.

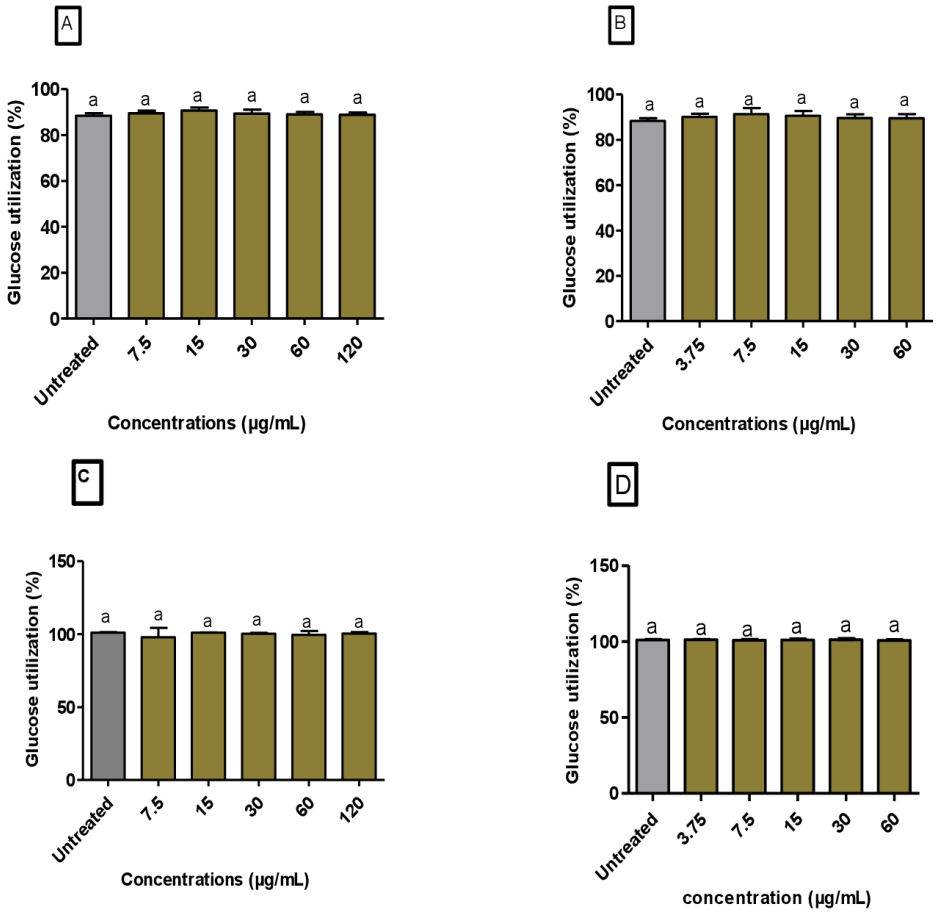
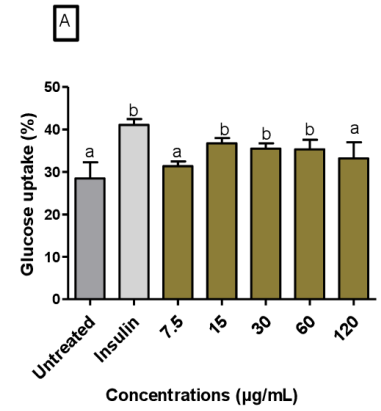


Figure 3.9.2: Glucose utilization (%) after 24 hours (A and B) and 48 hours (C and D) of treatment by *H. cymosum* in L6 myocytes cells. Results were normalized to cell viability as determined using the MTT assay. Error bars indicate the standard deviation of the mean of four replicates. Letter above the bars indicates a significant difference ($p < 0.05$) compared to the control.


A
Glucose uptake (%)
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10
20
30
40
50
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b
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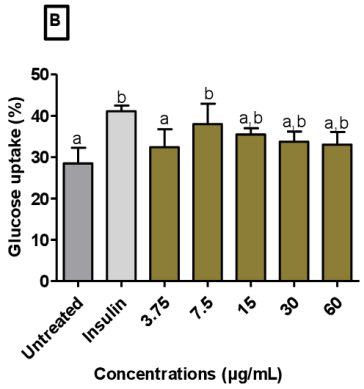
b
b
b
a
Untreated
Insulin
7.5
15
30
60
120
Concentrations (µg/mL)


B
Glucose uptake (%)
0
10
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30
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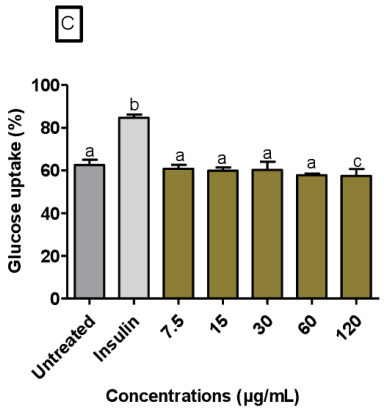
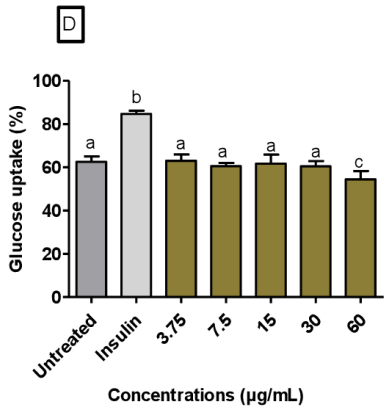
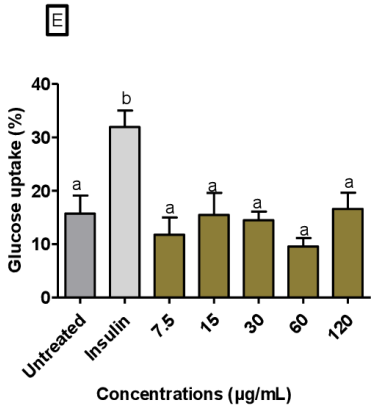
a b
Untreated
Insulin
3.75
7.5
15
30
60
Concentrations (µg/mL)


C
Glucose uptake (%)
0
20
40
60
80
100
a
b
a
a
a
a
c
Untreated
Insulin
7.5
15
30
60
120
Concentrations (µg/mL)


D
Glucose uptake (%)
0
20
40
60
80
100
a
b
a
a
a
a
c
Untreated
Insulin
3.75
7.5
15
30
60
Concentrations (µg/mL)


E
Glucose uptake (%)
0
10
20
30
40
a
b
a
a
a
a
a
Untreated
Insulin
7.5
15
30
60
120
Concentrations (µg/mL)

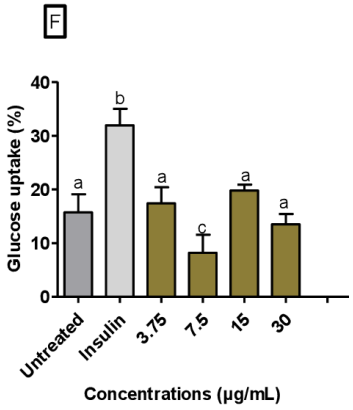

F
Glucose uptake (%)
0
10
20
30
40
a
b
a
c
a
a
Untreated
Insulin
3.75
7.5
15
30
Concentrations (µg/mL)

Figure 9.3: Glucose uptake (%) by L6 myocytes and in C3A hepatocytes, following 48-hour pre-treatment with H. cymosum aqueous and ethanol extracts. Results were normalized to cell viability as determined using the MTT assay. Error bars indicate the standard deviation of the mean of 4 replicates from a single experiment. Different letters on the bars signify statistics significance ($p < 0.05$) compared to the untreated control.

3.10. Conclusion

Phytochemicals are known to play a major role in glucose regulation and in ameliorating hyperglycemia. The findings from the current study showed the presence of important flavonoids, flavonols, and phenolic compounds in the aqueous and ethanol extracts of *H. cymosum*. Both extracts demonstrated potent hypoglycemic, antioxidant, antidiabetic, and anti-inflammatory activities, which play significant roles in the complications of diabetes mellitus. To our understanding, this study was the first to report potential *in vitro* antidiabetic effects of the aqueous and 70% ethanol extracts of *H. cymosum*. The findings from the plant extracts make them reliable sources of potential plant-based lead compounds for the development of antidiabetic drugs. However, further investigations, especially for the mechanism of actions of these identified phytochemical compounds as well as *in vivo* studies are recommended.

Credit authorship statement

Achasih Quinta Nkemzi: Conceptualization, Data curation, methodology, formal analysis, Investigation, Validation, Writing original draft, Writing review & editing**. Fanie Rautenbach**: methodology Formal analysis, review & editing. **Kunle Okaiyeto**: Data curation, methodology, review & editing. **Nasifu Kerebba**: methodology Formal analysis, writing original draft, review & editing. **Omolola Oyenihi**: Data curation, methodology, review & editing. **Okobi Eko Ekpo**: Supervision, Conceptualization, methodology, Investigation, Validation, administration, Writing review & editing. **Oluwafemi Omoniyi Oguntibeju**: Funding acquisition, resources, supervision, Conceptualization, investigation, administration, validation, and review & editing.

Declaration of competing Interest

The authors declare that they have no competing interests.

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CHAPTER FOUR

Antidiabetic, anti-inflammatory, antioxidant and cytotoxicity potentials of green-synthesized zinc oxide nanoparticles using the aqueous extract of *Helichrysum cymosum*

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Abstract

The current research involved the synthesis of zinc oxide nanoparticles (ZnO-NPs) using an aqueous extract of *Helichrysum cymosum* shoots, and subsequent characterization via different analytical methods such as UV-vis spectroscopy, Scanning electron microscope (SEM), Energy

dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD), Transmission electron microscope (TEM), and zeta potential. The biological effects of the ZnO-NPs were then tested against C3A hepatocyte cells and L6 myocyte cell lines via series of analysis, including, cytotoxicity, antioxidant, anti-inflammatory, antidiabetic effect via enzymatic inhibition. The UV-Vis analysis showed a maximum absorption spectrum at 360, and the TEM analysis reveals a spherical and hexagonal structures, with an average dimension of 28.05 - 58.3 nm, and the XRD reveals a crystalline hexagonal structure. The zeta potential evaluation indicated that the ZnO-NPs are relatively stable at -20 mV, and the FTIR analysis identified some important functional group associated to phenolics, carboxylic acid and amides that are responsible for reducing and stabilizing the ZnO-NPs. The synthesized ZnO-NPs demonstrated cytotoxic effects on the cell lines at higher concentrations (125 µg/mL and 250 µg/mL), complicating the interpretation of the results of the inflammatory and antioxidant assays. However, there was a significant ($p < 0.05$) increase in the inhibitions of pancreatic lipase, alpha-glucosidase, and alpha-amylase, indicating beneficial antidiabetic effects.

Keywords: Alpha-glucosidase, alpha-amylase, hexagonal structure, C3A hepatocyte, L6 myocyte, characterization.

4.1. Introduction

Nanotechnology applications span a wide range of scientific fields, including agriculture, cosmetics, the food industry, material science, engineering, medical science, etc. (Akintelu and Folorunso 2020; Paul et al. 2020; Singh et al. 2021, Okaiyeto et al. 2021). The unique dimension (1 - 100 nm) and easy permeability of nanoparticles across biological barriers have attracted much attention (Abel et al. 2021; Falih et al. 2021; Singh et al. 2021)**.** Several approaches have been used to synthesize nanoparticles, including chemical, physical, and biological methods (Varadharaj et al. 2020; Brayami et al. 2020; Donga and Chanda 2022). However, the use of chemical and physical methods has become unattractive because it is time-consuming, complicated, costly and often associated with toxicity and limited biocompatibility (Donga and Chanda 2022). Therefore, alternative biosynthesis routes using plant extracts have recently gained attention as they pose little or no environmental hazard.

This “green” method is preferable because its protocols are simple, affordable, clean, and involve the use of eco-friendly solvents for extraction; thus, this method can be considered appropriate for large-scale production of nanoparticles (Ochieng et al. 2015; Brayami et al. 2020; Aldeen et al. 2022; Govindan et al. 2020). Important biological molecules present in the different parts of a plant, e.g. alkaloids, amino acids, flavonoids, and proteins, are known to play a vital role during green synthesis by reducing metal ions to metal nanoparticles and stabilising the synthesized nanoparticles (Ochieng et al. 2015; Aldeen et al. 2022).

Nanoparticles synthesized from metal and metal oxides such as gold (Au), silver (Ag), selenium, copper (Cu), copper (II) oxide (CuO), zinc oxide (ZnO), platinum (Pt) and palladium (Pd) have presented great potential when applied in different domains (Donga and Chanda 2022; Gebre 2023). Zinc oxide nanoparticles (ZnO-NPs) have gained significant attention among other metal nanoparticles due to their specific physical and chemical properties (Jiang et al. 2018; Issam et al. 2021) such as semiconducting properties with a band gap of 3.37 eV and a high excitation energy of 60 meV. Other notable properties include biological, chemical, electrical, physical, environmental, biocompatibility, low cost, and non-toxic properties (Thema et al. 2015; Kumar et al. 2018; Abel et al. 2021**;** Donga and Chanda 2022). In addition, ZnO nanoparticles synthesized from different parts of plants have been reported with diverse biological activities such as antimicrobial, anti-inflammatory, antioxidant, cytotoxicity, antidiabetic, anticancer, and photocatalytic properties (Saratale et al. 2018; Govindan et al. 2020; Ifeanyichukwu et al. 2020; Ahmed 2022**;** Donga and Chanda 2022). ZnO is predominately used in metal nanoparticle surveys (Ifeanyichukwu et al. 2020), and the green biosynthesis approach (ZnO) has been reported to be a promising option for producing ZnO-NPs. Metal oxide NPs have been explored as potential therapeutic agents for ailments such as diabetes (Thema et al. 2015; Mishra et al. 2017).

Diabetes mellitus (DM) is a complex and chronic metabolic disorder emanating from increased blood sugar levels (hyperglycemia) (Ashrafizadeh et al. 2020). It results from impaired glucose metabolism due to abnormal insulin secretion, action, or both (Dhas et al. 2016; Brayami et al. 2020). Prolonged DM has been associated with severe health complications such as nephropathy, neuropathy, retinopathy, and liver damage which, if left unattended to, might result in severe morbidity and mortality (Virk 2017). An increase in DM worldwide has been associated with ageing, lifestyle, and urbanization. Global epidemiological data indicates a significant rise in the

number of individuals with DM over the past three decades, increasing from 160 million to 410 million. The prevalence rate is estimated at 420 million individuals in 2019, and is projected to reach 690 million by 2040 (Jayarambabu et al. 2020). The management of DM has been a serious public health concern and requires strategic interventions (Badeggi et al. 2020). Several efforts in managing DM have been reported, such as lifestyle and nutritional changes and pharmacological intervention. An alternative way of managing DM is the use of herbal medicine, which has shown promising results and possesses hypoglycemic potential (Virk, 2018).

Zn is a trace element that plays a critical enzymatic and cellular role in the human body, such as immune functions, apoptosis, metabolic regulations, oxidative equilibrium, and metabolic and signal transduction. Zinc metal has been reported to ameliorate diabetic complications such as nephropathy and cardiomyopathy by enhancing the mechanisms of insulin signalling pathways (SanTang 2019). Owing to the importance of zinc to the human body and the quest for alternative medicine to treat DM, ZnO-NPs have been the preferred option used to deliver zinc in many disease therapies because of the beneficial role played in numerous enzymatic and cellular activities of the body (Ahmed 2022). The role played by ZnO-NPs in reducing mRNA inflammatory cytokines through the inhibition of activation of NF-kB, has also been documented (Paul et al. 2020).

Several ethnopharmacological studies have revealed the beneficial use of plant-mediated medicines in treating different ailments, since plant-based medicinal options are known to be affordable, reliable, and less toxic (Ansaril et al. 2022, Sagbo and Hussein 2022). Furthermore, the emergence of plant-based mediated nanoparticle research has increased in recent years due to beneficial biomolecules such as alkaloids, flavonoids, glucosides phenolic, and protein they possess, which are responsible for the reduction of metal ions to nanoparticles (Okaiyeto et al. 2021). The important role played by these plant phytochemical constituents has prompted the search for novel antidiabetic and anti-inflammatory drugs with minimal toxicity**.** *H. cymosum* belongs to the Asteraceae family and has been reported to possess notable biomolecules such as flavone, 5-hydroxy-8 methoxy-7- prenyloxyflavonone helihumolone, helichromachalcone and phloroglucinol derivatives, sesquiterpenes and chalcones (Van Vuuren et al. 2006; Lourens et al. 2011; Heyman 2013; Jadalla et al. 2022). These biomolecules have been tested in different biomedical applications such as antioxidant, anti-inflammatory, antifungal, antiviral,

antimicrobial, anti-diabetic, and cytotoxicity activities from these species (Matanzima 2014; Jadalla et al. 2022; Maroyi 2019). Jalladia et al. (2022) have documented the alpha-amylase and alpha-glucosidase activities of *H. cymosum*. To the best of our knowledge, our study is the first study reporting the antidiabetic, anti-inflammatory, and cytotoxicity potentials of ZnO-NPs synthesized from the aqueous extract of *H. cymosum*. The investigation entails using aqueous extracts of South African *H. cymosum* species to synthesize ZnO-NPs from zinc nitrate hydrate. After that, detailed characterization was carried out on the synthesized ZnO-NPs, and their biological activities were investigated.

4.2. Materials and Methods

4.2.1. Plant collection and extract preparation

Helichrysum cymosum shoots were harvested in the garden of the Cape Peninsula University of Technology, Bellville campus, Cape Town, South Africa. The plant was authenticated by a botanist (P. Dryfhout) with voucher number 3708 and stored in the herbarium at the Department of Horticultural Sciences, Cape Peninsula University of Technology, Western Cape, South Africa. Afterwards, the plant was thoroughly washed with distilled water and dried in an oven at 40 ^{o}C, then crushing using an electric grinder. The aqueous extract was obtained by boiling 100 g/L at 100 ^{o}C for 30 min and then allowed to cool at room temperature. The plant extract was then filtered using a Whatman No. 1 filter paper (Sadiq et al. 2021). The filtrate extract of *H. cymosum* was then stored at 4 °C for subsequent use.

4.2.2. Biosynthesis of ZnO-NPs of aqueous extract of *H. cymosum*.

The biosynthesis of ZnO-NPs was carried out as described by Ifeanyichuku et al. (2020) as illustrated in Fig. 1. Briefly, 9.47 g of zinc nitrate hydrate salts 0.1 M was dissolved in 500 ml of double distilled water and stirred till the complete dissolution of the salt. A volume of 500 ml of *H. cymosum* extract was added to the Zn salt mixture and allowed to stir until all the salt was dissolved, followed by 2 M NaOH to adjust the pH to 12 (Issam et al. 2021). The entire mixture was stirred for 3 h at 60 ^{o}C. A pale-yellow precipitate was formed, and the mixture was then allowed overnight at room temperature for complete synthesis. The precipitate formed was then collected, centrifuged at 6000 rpm, and washed 3 times using distilled water to remove impurities. The ZnO-NPs were left overnight to dry in an oven at 80 ^{o}C, and the resulting powder was further

calcined at 400 ºC for 3 h in a furnace. A white powder was obtained and crushed using a mortar and pestle to fine powder, and later used for characterization. The ZnO-NPs samples for cell culture analysis were prepared by reconstituting in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL, and the mixture was then sonicated for proper dissolution and stored at 4 °C until used.

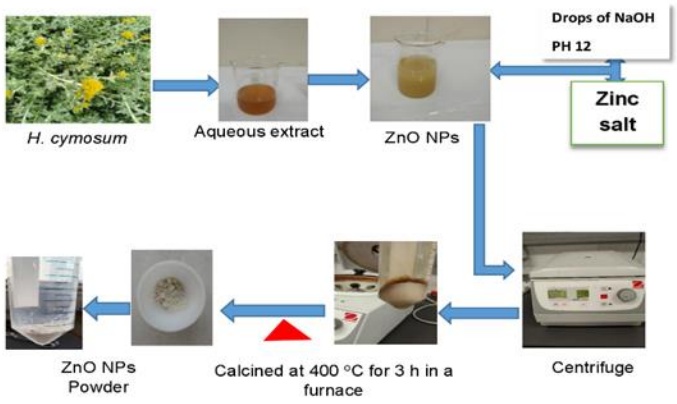

Fig. 4. 1 Pictorial illustration of the synthesis process of ZnO-NPs from aqueous extract H. cymosum shoots

4.2.3. Characterization of synthesized ZnO-NPs

Ultraviolet-visible (UV-vis) spectroscopy (BMG LABTECH-SPECTROstar-Nano, Germany) at spectra range from 280 to 500 nm was used to confirm the formation of ZnO-NPs after synthesis. Origin Pro 8 software was used to plot the data obtained. Information on the stability and dispersion of the ZnO-NPs were determined Zeta potential {(Nano-zs90, country) equipped with both the Zetasizer and zeta potential}. The morphology and crystalline nature of the ZnO-NPs were evaluated using an X-ray diffractometer (XRD), which provides information about symmetry, size, and shape. The Perkin Elmer Spectrum (Version 10.4.2) ATR-FTIR spectrometer was used to determine the phytochemical compounds involved in the reduction and stabilization of the nanoparticles. FTIR of the ZnO-NPs was performed at a wavelength range of 4000 - 500 cm^{-1}. Scanning electron microscopy (SEM) (JEOL JSM-6360LV, Tokyo, Japan) equipped with energy dispersive X-ray analysis (EDX) (Noran SIX 200 Energy Dispersive X-ray (JOEL, Ltd,

Tokyo, Japan) was used to examine the morphology and microstructure (SEM) and purity and elemental compositions (EDX) of the ZnO-NPs. The shape and size distribution of the synthesized ZnO-NPs were analyzed using Transmission electron microscopy (TEM) (Tecnai T20 TEM, LaB_6 filament) at 200.0 Kev BF mode.

4.3. Cell culture

Human hepatoma-derived C3A hepatocytes were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and maintained in 10 cm culture dishes in complete medium (minimal essential medium (MEM) with 1% non-essential amino acids, 10% foetal bovine serum (FBS), 1% penicillin/streptomycin). The cells were incubated at 37 °C, with 5% CO_2 in a humidified environment, and subculture post 90% confluence.

4.3.1. Cellular Antioxidant Assay (CAA)

The cellular antioxidant activity of ZnO-NPs was examined on C3A hepatocyte cells. One hundred microliters (100 µL) of aliquots of cells were seeded at a density of 2×10^4 cells/well and left to attach overnight. The ZnO-NPs treatment (15 µg/mL. 63 µg/mL, 31.25 µg/mL and 62.5 µg/mL) was reconstituted in a complete medium, and 100 µM catechin was used as a positive control and incubated for 24 hours. Oxidative stress was induced by adding tert-butyl hydroperoxide (TBHP) to the culture/treatment medium at a final concentration of 30 µM and incubated for 2 h. The culture/treatment medium was gently aspirated, and 100 µL staining solution [CellROX® Orange (5 µM) and Hoechst 33342 (5 µg/mL) in PBS with Ca^{2+} and Mg^{2+}] was added to each well. Plates were incubated for 30 min (protected from light), and fluorescent micrographs were captured immediately using an ImageXpress Micro XLS Widefield Microscope (Molecular Devices) with a 10× Plan Fluor objective using DAPI and TRITC (tetramethylrhodamine isothiocyanate) filter cubes. Acquired images were analyzed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Antioxidant activity was determined using the average cellular CellROX® Orange fluorescent intensity.

4.3.2. Cytotoxicity analysis of ZnO-NPs of *H. cymosum*

The cytotoxic effect of the synthesized ZnO-NPs of *H. cymosum* was assessed on C3A cells. In 96 well plates, 100 µL (5000 cells/well) were seeded and left for 24 h to attach. Five hundred (500) µg/mL of ZnO-NPs dilution was prepared in a complete medium and a 6-point dilution of different

concentration was made (15.6 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, and 500 µg/mL). Afterwards, 100 µL aliquots from each dilution was added to 100 µL of attached cells in the 96 well plate, thus yielding final concentrations of 7.8 µg/mL, 15.6 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, and 250 µg/mL. The treated cells were allowed for 48 h at 37 °C, 5% CO_2, and 30 µM Melphalan (100 mM stock) was used as a positive control. The treatments were aspirated from the wells, and 100 µL MTT (0.5 mg/mL) in the complete medium was added to each well and cells were incubated for 3 h. Thereafter, 100 µL DMSO was added to each well, and absorbance was measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

4.3.3. Anti-inflammatory activity using RAW 264.7 mouse macrophages

The anti-inflammatory activity of ZnO-NPs was evaluated following the procedure of Shauli et al. (2023) with slight modification. The RAW 264.7 cells were seeded at a density of 1×10^5 cells per well in a 96-well plate and left overnight to attach. The culture medium was removed, and 50 µL of sample aliquots (diluted in RPMI complete medium) were added to give final concentrations of 50, 100, and 200 µg/mL. Thereafter, 50 µL of LPS (final concentration of 500 µg/mL)-containing medium was added to the corresponding wells, and Aminoguanidine (AG) was used as the positive control at 100 µM, followed by incubation for 24 h. The spent culture medium (50 µL) was transferred to a new 96-well plate to quantify nitric oxide (NO) production. Sulfanilamide solution and NED solution were prepared as per the manufacturer's instructions. An aliquot volume of 50 µL sulfanilamide solution was added to the spent culture medium and incubated for 10 min in the dark, at room temperature. Fifty (50) µL NED [N-(1-Naphtyl)-ethylenediamine dihydrochloride] solution was added to each well and further incubated for 5-10 min in the dark at room temperature. Absorbance was measured at 540 nm (BioTek® PowerWave XS spectrophotometer), and a nitrite standard curve (using sodium nitrite dissolved in culture medium) was used to determine the concentration of NO in each sample.

4.3.4. Alpha amylase Inhibitory activity

Alpha-amylase inhibitory activity was conducted as described by Aladejana et al. (2020), using the 3, 5-dinitrosalicylic acid (DNS) method with slight modifications. Forty microliters (40 µl) of different concentration ranges of ZnO-NPs test samples were placed in test tubes. In the same test

tubes, 400 µl of starch solution (0.5 g starch in 50 ml phosphate buffer) and 200 µl α-amylase (4 units/ml) were added and incubated at 35 °C for 10 min to start the reaction. After incubation, 200 µl of the test samples were transferred into new test tubes, and 100 µl of DNS (20 mL of 30 g of sodium potassium tartrate tetrahydrate mixed with 50 mL of 1 g 3, 5-dinitro salicylic acid solution and 20 ml of 2 M NaOH at 90-95 ^{o}C) was added to stop the reaction. The solution was then boiled for 15 min and allowed to cool at room temperature. Subsequently, distilled water (900 µl) was added to dilute the solution. Two hundred microliters (200 µl) of sample, blanks, and positive control were prepared and placed in a 96-well plate, and the absorbance was measured at 540 nm using a UV spectrophotometer. The percentage inhibition of the enzyme α-amylase was determined using the equation.

$$\%\ alpha\ amylase\ inhibition = \frac{(\text{absorbance of control} - \text{absorbance of test sample})}{\text{absorbance of control}} \times 100$$

4.3.6. Alpha-glucosidase activity

The alpha-glucosidase activity of ZnO-NPs was assessed according to the procedure described by Erukainure (2018). Briefly, 50 µl of both plant extracts at different concentrations (1000, 500, 250, 100, 50, 10 µg/mL) and α-glucosidase (1.0 Unit/mL) prepared in phosphate buffer 100 mM at pH 6.8 were placed in a 96-well plate and incubated for 15 min at 37 °C. Afterwards, 100 µL of 5 mM p-Nitrophenyl-α-D-glucopyranoside (pNPG) solution prepared in phosphate buffer (100 mM, pH 6.8) was added to the reaction mixture and incubated for an additional 20 min at 37 °C. Acarbose was used as a positive control, and the absorbance was measured at 405 nm. The percentage inhibition was calculated with the formula below:

$$\%\ \text{alpha glucosidase inhibition} = \frac{(\text{absorbance of control} - \text{absorbance of test sample})}{\text{absorbance of control}} \times 100$$

4.3.7. Glucose uptake and utilization

With modifications, glucose uptake and utilization were evaluated using C3A hepatocytes and L6 myocytes following the procedure in van de Venter et al. (2008). Both cells (C3A and L6) were seeded in 96 well plates (2×10^4 cells/well, 100 µL aliquots) and left overnight to attach. Various concentrations of treatments were prepared in a complete medium and added to the cells, followed by incubation for 24 h and 48 h for each cell type. After incubation, cell culture/treatment medium

(5 µL for C3A; 10 µL for L6) was removed from the respective plates and transferred into new 96 well plates (A), which were sealed and stored at -20 °C until required. The rest of the medium was aspirated, and cells washed with 100 µL PBS and 25 µL incubation buffer (RPMI-1640 diluted with PBS containing 0.1% bovine serum albumin (BSA) to a final glucose concentration of 8 mM) were added to cells (C3A and L6). Insulin (1 µg/mL) was used as a positive control, and the cells were then incubated for another 4 h. Some culture medium (5 µL) was transferred to a new 96 well (plate B). Afterwards, 200 µL of glucose oxidase reagent (3 mM phenol, 0.4 mM 4-amino antipyrine, 0.25 mM EDTA and 2.5 U/mL horseradish peroxidase in 0.5 M PBS (pH 7.0) with 1 mU/mL glucose oxidase from *Aspergillus niger*) was added to the plates (A and B), respectively and incubated for 15 min at room temperature. Cell-free wells containing incubation buffer and complete culture medium were used as glucose standards. The absorbance was then measured at 510 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA. Glucose uptake and consumption were determined as a function of the concentration of glucose (mM) remaining and expressed as the difference between the mean of the standard and test samples. The MTT assay was further used to determine cell viability.

4.3.8. Pancreatic lipase inhibition

Pancreatic lipase inhibitory activity of ZnO-NPs was performed following the procedure described by Pringle et al. (2021). A volume of 10 µL of sample at different concentrations and 5 µL of porcine pancreatic lipase enzyme (100 mg mL^{-1} prepared in 100 mM Tris-HCl, pH 8.0) was pre-incubated at 37 °C for 15 min. Afterwards, 170 µL of the substrate (*p*-nitrophenyl palmitate (pNPP) [1 mg/mL in isopropanol) with reaction buffer (gum arabic (1 $mgmL^{-1}$), sodium deoxycholate (2 mg mL^{-1}) and Triton X-100 (5 µL per mL) prepared in 100 mM Tris-HCl (pH 8.0)] was added to the mixture containing extracts and enzyme and incubated at 37 °C for 25 min. Pancreatic lipase activity was then determined by measuring the absorbance at 405 nm using a BioTek® PowerWave XS spectrophotometer, and 100 µM Orlistat was prepared and used as a positive control. The percentage of pancreatic lipase inhibition was calculated according to the equation:

$$\%\ Lipase\ inhibition = \frac{\text{A405nm of blank} - \text{A405nm of test sample)}}{\text{A405nm of blank}} \times 100$$

4.4. Statistical Analysis

Results are expressed as a mean ± standard error of the mean. Differences between the means were determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-test. All analyses were performed with GraphPad Prism 5. The difference between the mean values of $P < 0.05$ was considered statistically significant.

4.5. Results

The UV-Vis spectrometry analysis was the preliminary step to confirm the synthesis of zinc metal oxide nanoparticles by the surface plasmon resonance band (SPR) (Issam et al. 2021). The UV-Vis spectrum was scaled on a wavelength range from 280 – 500 nm for *H. cymosum*-mediated ZnO-NPs, as shown in Fig. 4.2. The results show a broad peak range of 354 to 360 nm, confirming a successful formation of ZnO-NPs with a maximum absorption peak observed at 360 nm.

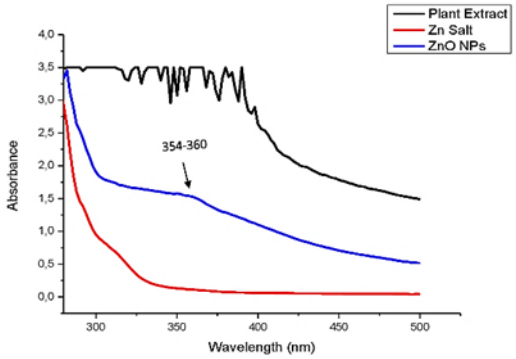

Fig. 4.2 UV–vis spectroscopy analysis of ZnO-NPs synthesized form aqueous extract of H. cymosum

4.5.1. Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) of H. cymosum ZnO-NPs

The SEM analysis was used to study the surface morphology of synthesized ZnO-NPs from *H. cymosum* aqueous extract, and the results are represented in Fig. 4.3.1a. The images showed an aggregate of closely packed irregular hexagonal shapes of the ZnO-NPs. The EDX results depicted in Fig. 4.3.1b reveal two fundamental elements, Zn and O, on the spectra of *H. cymosum*-mediated ZnO-NPs. An additional peak of C was identified and could be associated with the bioactive compound capping during ZnO-NPs formation.

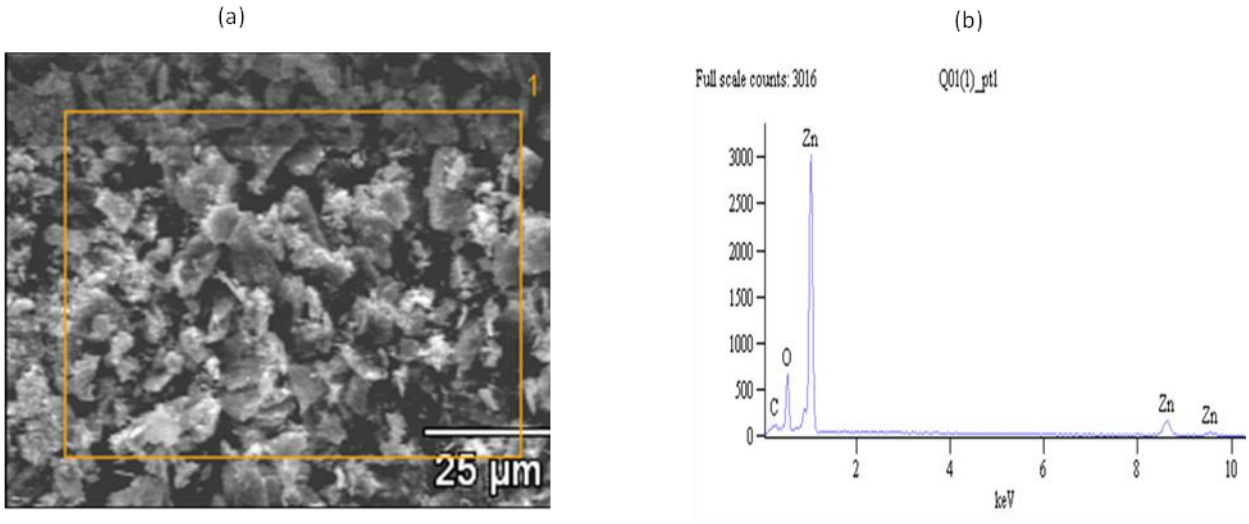

Fig. 4.3.1 Scanning electron microscopy (a) and EDX spectrum (b) analysis of aqueous extract of H. cymosum synthesized ZnO-NPs

4.5.2. TEM analysis

The surface morphology of the ZnO-NPs was interpreted using TEM at different magnifications, as shown by the micrographs in Fig. 4.3.2. The resulting images showed an agglomeration of nanoparticles with dark spots. The nanoparticles were spherical and hexagonal, with an average dimension of 28.05 - 58.3 nm. The dark areas observed might have resulted from the agglomeration of the NP during synthesis, which corroborates with observations from other studies in the literature (Mbenga et al. 2022; Mkhize et al. 2022).

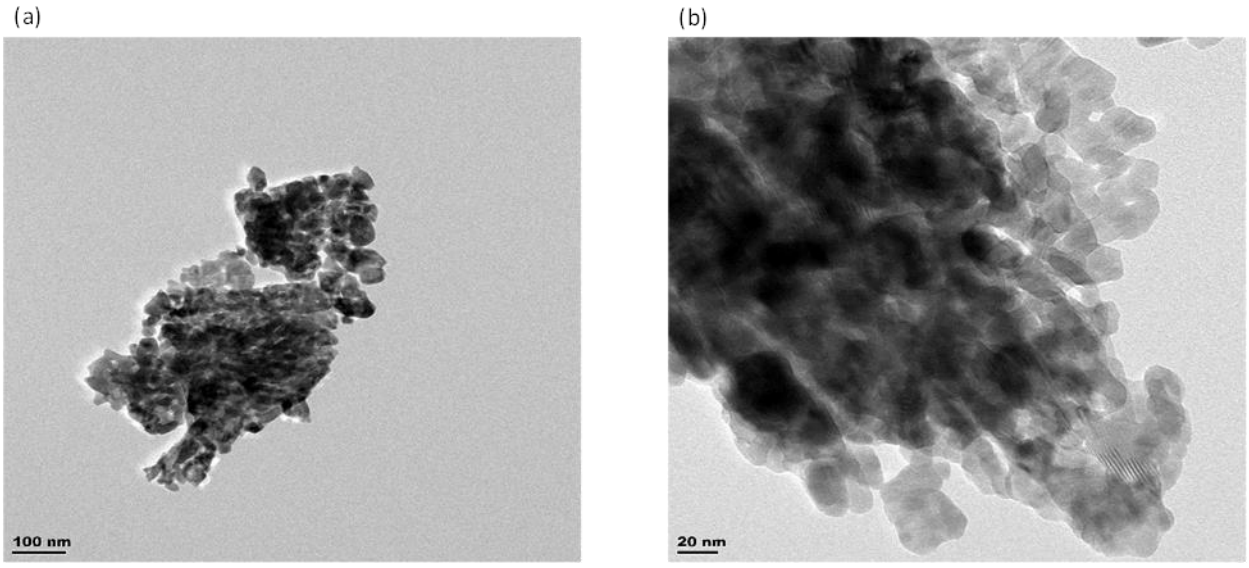

Fig. 4.3.2 TEM analysis (a and b) of aqueous extracts of H. cymosum-mediated zinc oxide nanoparticles

4.5.3. Zeta potential

The dispersion stability of nanofluid was measured by the absolute value of zeta potential (Fig. 4.3.3). In the present study, the synthesized ZnO-NPs of *H. cymosum* showed a negative charge with good stability of -20.8 mV.

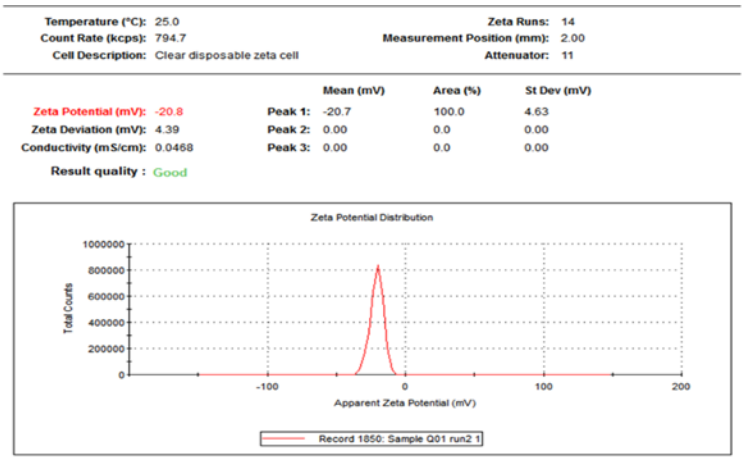

Fig. 4.3.3 Zeta potential (mV) of ZnO-NPs synthesized from aqueous extract of H. cymosum shoot

4.5.4. FTIR analysis of ZnO-NPs

The surface functional groups of the synthesized ZnO-NPs of *H. cymosum* were investigated using FTIR analysis at wavelengths of 4000 - 500 cm^{-1}. Different peaks characterizing biomolecules were identified from the spectra (Fig. 4.3.4). The results reveal a broad peak at 3388 and 3261 cm^{-1}corresponding to the O-H stretching vibration and to the alcohol and phenols functional group, or water molecules present in the extract (Bayrami et al. 2018; Mhkize et al. 2022; Unni et al. 2022). The absorption peak of 2895 and 2894 cm^{-1} signifies the stretching for C-H bonds (alkanes), peak 1650 cm^{-1}, 1641 cm^{-1} C=O, primary amide, 1399 cm^{-1} (NO_2). From 1083 cm^{-1},1066 cm^{-1}, and 1015 cm^{-1} are allocated to the presence of -N-H, -C-O, and =C-H and are linked to aliphatic amine, phenol, and carboxylic acid). The peak ranges from 788 cm^{-1}, 725 cm^{-1}, 690 cm^{-1}, 637 cm^{-1}, 603 cm^{-1}, and 560-500 cm^{-1}) are indicative of the N-H characteristic of amines; these spectrum peaks

corroborate the formation of ZnO-NPs (Donga and Chanda 2022). The above peaks belong to the vibration stretch of the plant extract, and ZnO-NPs oven-dried at 80 ° C are indicative of the biomolecule present at the surface of ZnO-NPs responsible for capping and stabilizing the bio-fabricated nanoparticles. The calcined FTIR spectra at 400 °C also retain some peaks which could be allotted to the following functional group (Bayrami et al. 2018, Unni et al. 2022). According to Akintelu et al. (2022), biomolecule functional groups such as –O-H, C=O, C=C, C-N, C-H, and N-H are good reducing agents for ZnO-NPs synthesis.

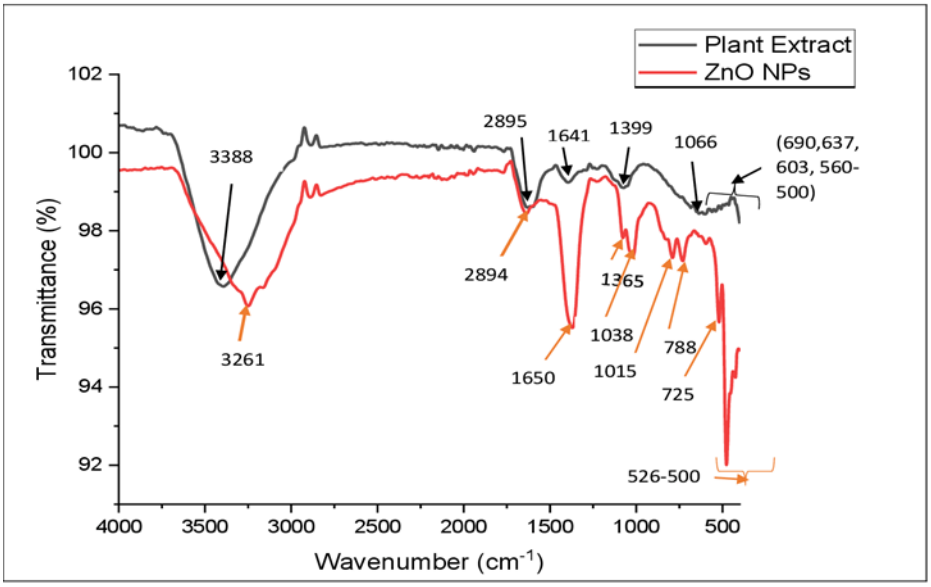

Fig. 4.3.4 FTIR spectrum of aqueous extract of H. cymosum-mediated ZnO-NPs. The different peaks represent the identified functional groups

4.5.5. X-ray diffraction (XRD)

Fig. 4.3.5 depicts the XRD patterns of ZnO-NPs of *H. cymosum*. The results reveal prominent diffraction peaks at 2θ = 31.64°, 34.31°, 36.11°, 47.46°, 56.48°, 62.71°, 66.17°, 67.84°, 68.99°, 77.17°, 89.57°, which correspond to the crystal reflection planes 100, 002, 101,102, 110,103, 200, 112, 202 of the hexagonal structure of ZnO phase. The matching of the peaks was according to the reported standard values in the joint committee on powder diffraction standards (JCPDS) card No: 036-1451 (Obeizi et al. 2020; Darezereshki et al. 2021).

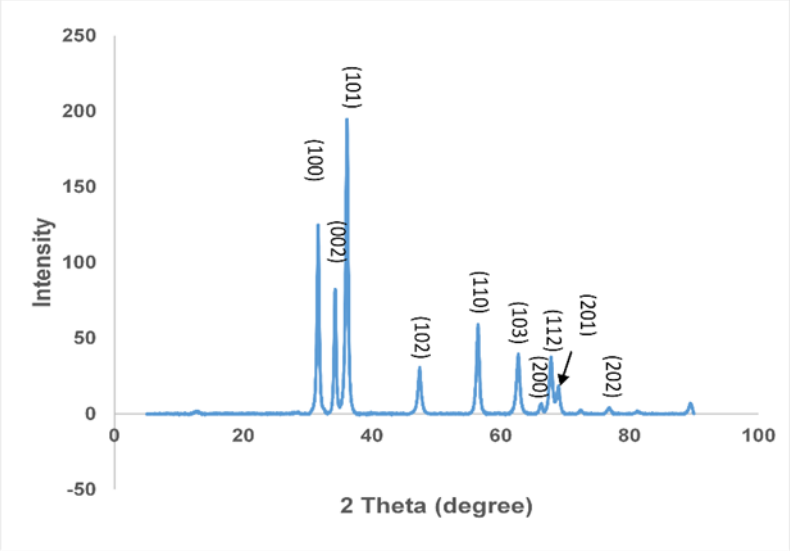

Fig. 4.3.5 XRD spectra of ZnO-NPs of aqueous extract of H. cymosum

4.6. Cytotoxicity assay (MTT)

H. cymosum synthesized ZnO-NPs were evaluated for toxicity on C3A cells; the results are represented in Fig. 4.4. The cells were treated for 48 h with different concentrations (7.8 µg/mL, 15.6 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, and 250 µg/mL). The synthesized ZnO-NPs at lower concentrations (7.8 - 62.5 µg/mL) did not cause any substantial change in cell viability compared to the control, but a significant change ($p < 0.05$) was noted compared to the positive control. Meanwhile, at higher concentrations (125 µg/mL and 250 µg/mL), there was a sharp decline in cell viability which was significant ($p < 0.05$) compared to the control. In summary, the ZnO-NPs were safe up to 62.5 µg/mL, beyond this limit, a cytotoxic effect was observed.

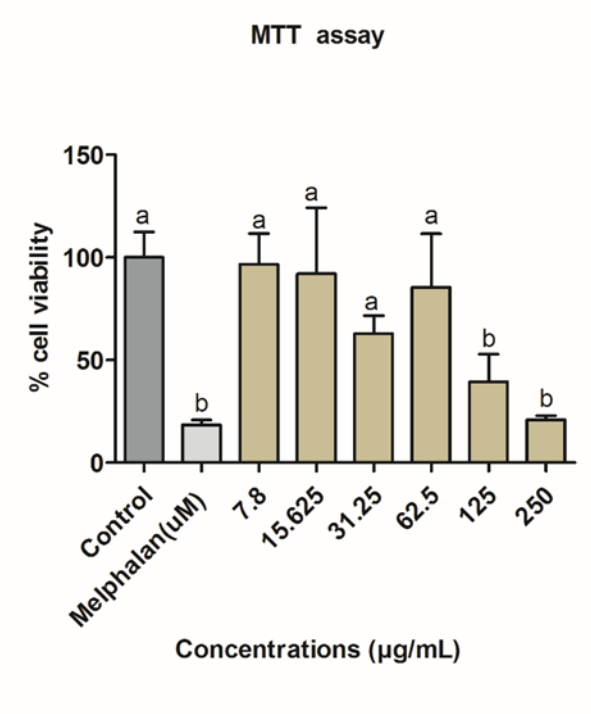

Fig. 4.4 Represent the % cell viability of C3A hepatocytes cells treated with *H.cymosum* ZnO-NPs for 48 h. Each bar represent mean ± SD of four replicate wells, melphalan was used as a positive control. Bars with different letters present significant different ($p < 0.05$) compared to the untreated control

4.7. Cellular antioxidant activities (CAA)

After treatment, the cellular antioxidant activity of the synthesized ZnO-NPs showed significant toxicity at 62.5 µg/mL (Fig. 4.5a). Therefore, the antioxidant capacity at this concentration cannot be reliably interpreted. In addition, the ROS level also increased at a similar concentration (62.5 µg/mL) as indicated by the average cell intensities.The remaining concentrations (15.63 and 31.25 µg/mL) did not induce CAA activity (Fig. 4.5b).

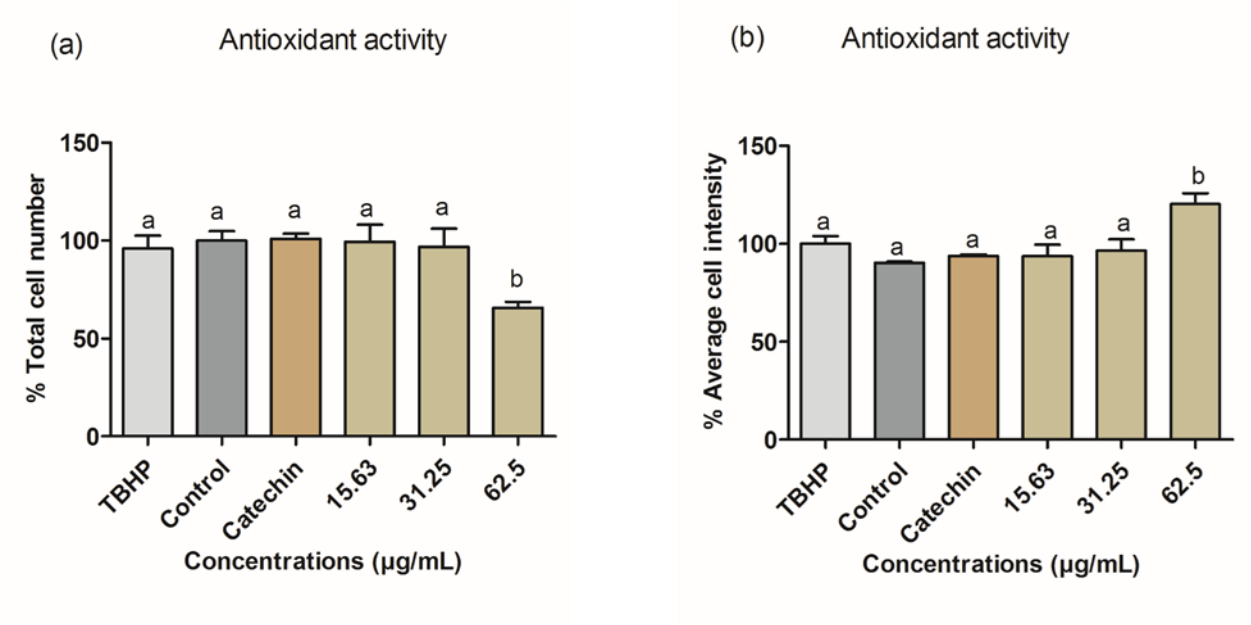

Fig. 4.5 Represent % total number of cells (a) and % average cell intensity (b) after 24 h of treatment of C3A cells with ZnO-NPs. Error bars represent the mean ± SD of four replicate wells, Catechin (100 µM) was used as a positive control and significant toxicity ($p<0.05$ compared to TBHP). Different letters above the bar for each given concentration compared to the control are significantly different ($p < 0.05$)

4.8. Anti-inflammatory activities

In Fig. 4.6, the anti-inflammatory activity is indicated by the decrease in nitrite concentration in response to LPS activation of RAW macrophages with no effect on cell viability, as seen with the AG-treated cells which significantly ($p < 0.05$) decreased the nitrites less than 40% (Fig. 4.6a) with increase cell viability above 100% (Fig. 4.6b). This shows a better anti-inflammatory activity compared to the ZnONPs treatments. The findings showed that the 50, 100 and 200 µg/mL concentrations of ZnO-NPs could substantially decrease NO production (Fig. 4.6a and 4.6c) but are cytotoxic at all tested concentrations (Fig. 4.6b and 4.6d) with a significant reduction ($p < 0.05$) in cell viability. Therefore, the NO results were not considered.

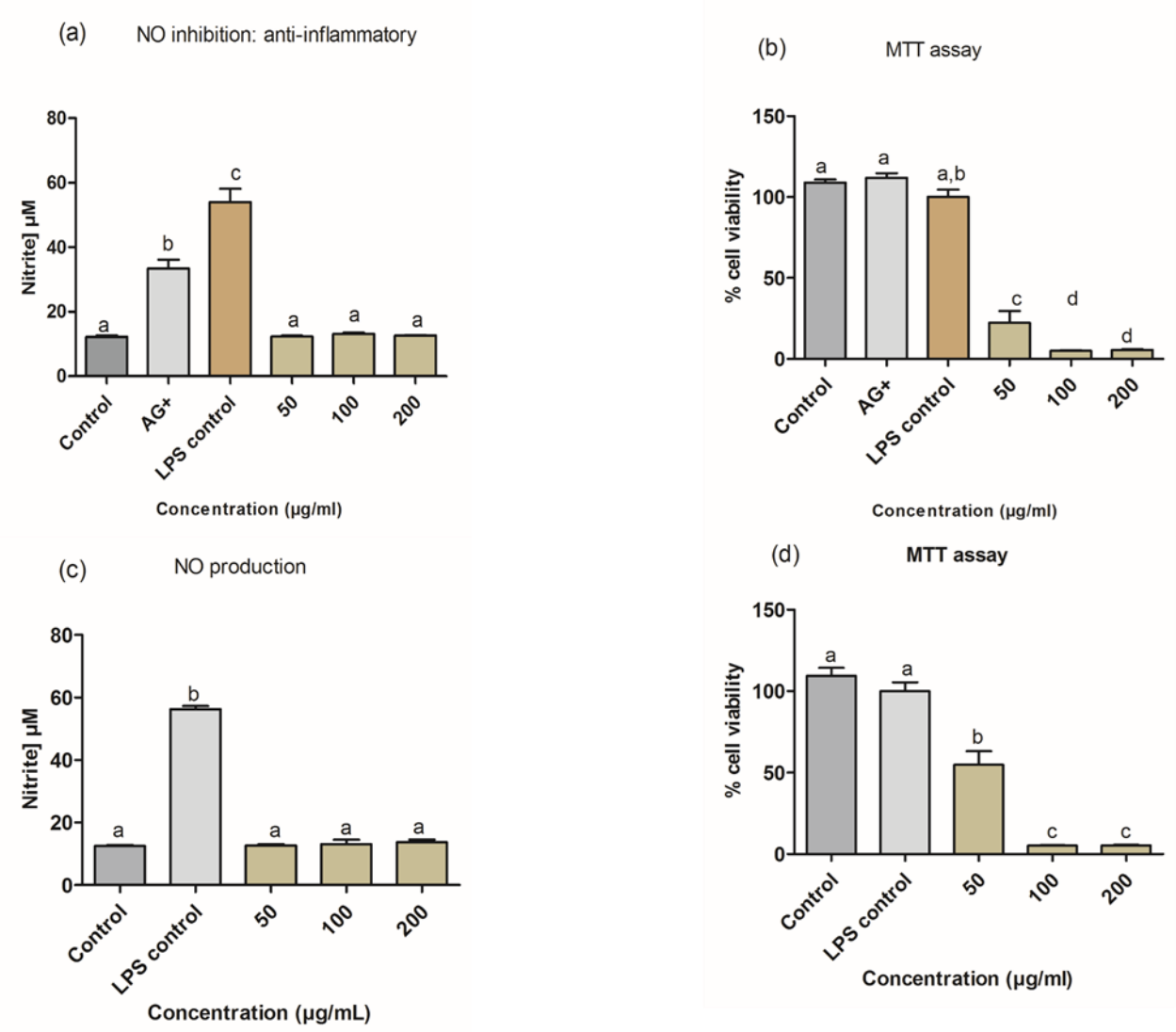

Fig.4.6 Nitric oxide production (a) and inhibition of LPS-activated macrophages (c), and their corresponding cell viability (%) (b and d respectively) after 24 h treatments with ZnO-NPs. Error bars indicate the mean ± SD of four replicate wells. Bars with different letters present significant different ($p < 0.05$) compared to the AG- control and untreated control.

4.9. Glucose uptake and glucose utilization

The glucose uptake and utilization by C3A hepatocyte and L6 myocyte cells were used to estimate the antidiabetic activity of ZnO-NPs of *H. cymosum*. The C3A cells exposed to ZnO-NPs showed a significant increase in glucose utilization at 15, 30, and 60 µg/mL within 24 h of treatment Fig. 4.7 (a). After 48 h of treatment, ZnO-NPs exhibited an equal rate of glucose utilization from 7.5 - 60 µg/mL compared to the control Fig. 4.7(b), and cytotoxicity was observed at 120 µg/mL for

both 24 h and 48 h (Fig. 4.7 a and b). The MTT was used to normalize glucose uptake and utilization data to compensate for any differences in cell numbers due to exposure to treatment. The L6-treated cells within 24 h showed a small but significant increase in glucose utilization at 30 µg/mL Fig. 4.7 (c). After 48 h, the cells utilized all the available glucose (Fig. 4.7d). As a result, no differences were observed between the treatments. Cytotoxicity was seen at the highest concentrations (60 and 120 µg/mL) for 24 and 48 h (Fig. 4.7 c and d). Fig. 4.8 (a, b, and c) shows that exposure to ZnO-NPs increased glucose uptake at concentrations of 7.5, 15, and 30 µg/mL within 24 hours and 15 and 30 µg/mL after 48 h. cytotoxicity was observed at (60 and 120 µg/mL) in (Fig. 4.8 a and b) and 120 µg/mL in (Fig 4.8. c).

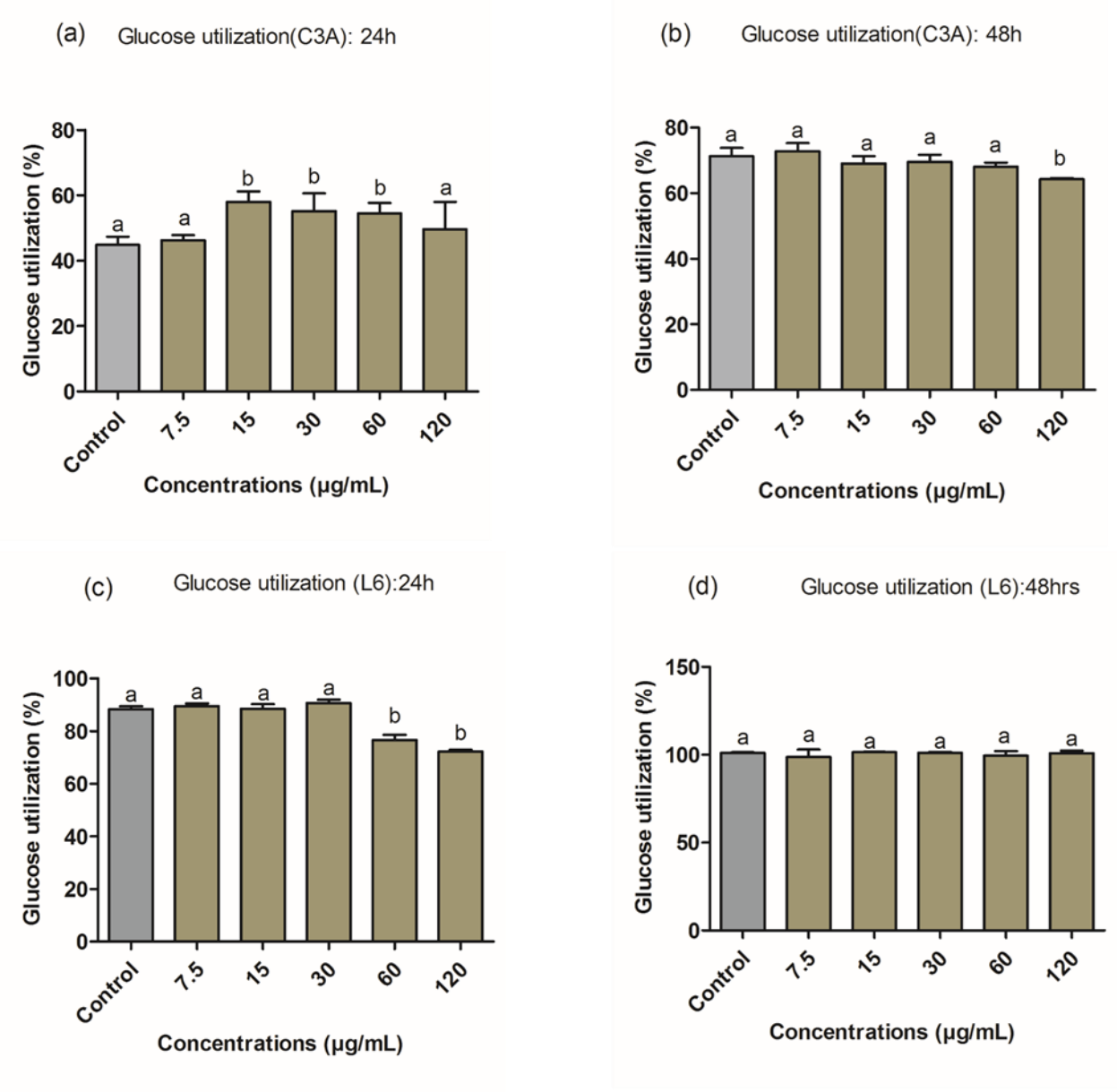

Fig. 4.7 Glucose utilization (%) after 24 h (a and c) and 48 h (b and d) of treatment by H. cymosum ZnO-NPs in C3A hepatocytes and L6 myocytes. Results were normalized to cell viability as determined using the MTT assay. Error bars indicate the standard deviation of the mean of four replicates wells. Letter above the bars indicates a significant difference ($p < 0.05$) compared to the control

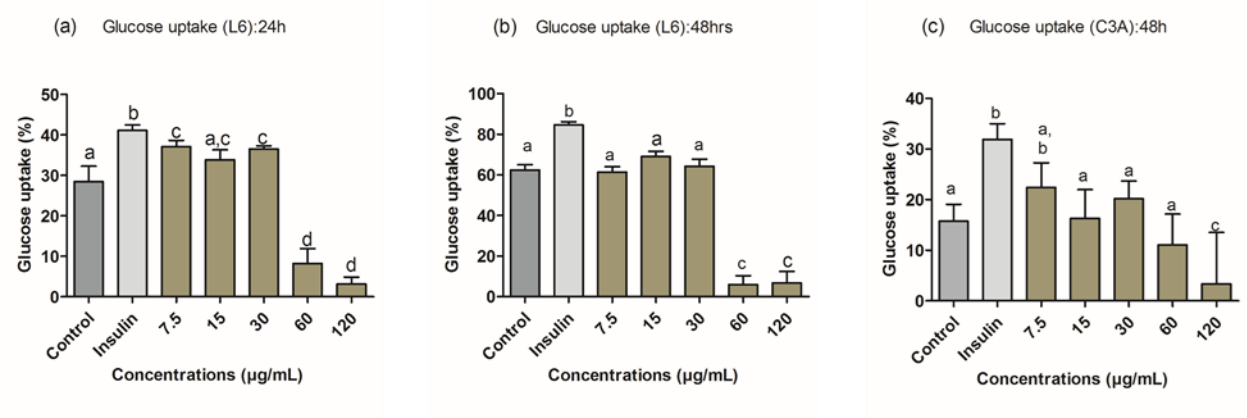

Fig. 4.8 Glucose uptake (%) by L6 myocytes and in C3A hepatocytes, following 24 h (a) and 48 h (b and c) pre-treatment with H. cymosum ZnO-NPs. Results were normalized to cell viability as determined using the MTT assay. Error bars indicate the standard deviation of the mean of 4 replicates wells. Different letters on the bars signify statistics significance ($p < 0.05$) compared to the untreated control

4.10. Pancreatic lipase inhibition

Pancreatic lipase inhibition is a promising mechanism to combat obesity and hypertriglyceridemia (Lunagariya et al. 2014). Inhibition was minimal at 31.25 and 62.5 µg/mL but gradually increased from 125-500 µg/mL compared to the control (Fig. 4.9.1). However, statistical significance was not achieved at 125-500 µg/mL compared to the control (Orlistat:100µM).

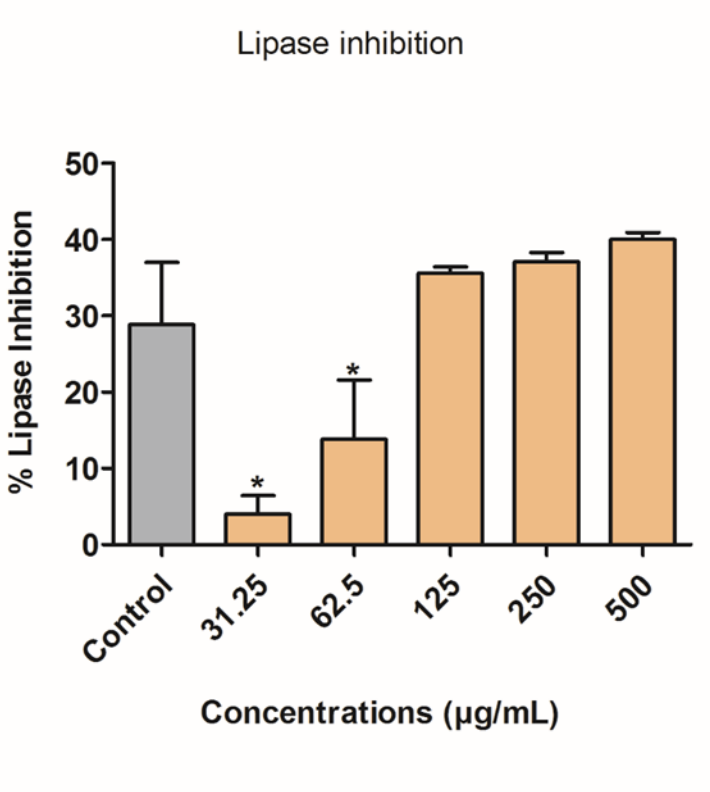

Fig 4.9.1 Pancreatic lipase inhibition of H. cymosum ZnO-NPs. Error bars indicate the standard deviation of the mean of four replicates. The * on the bars indicate significant difference ($p < 0.05$) compare to the control orlistat (100 µM)

4.11. Alpha-glucosidase and alpha-amylase

The antidiabetic effect of the synthesized ZnO-NPs of *H. cymosum* was evaluated using alpha-glucosidase and alpha-amylase inhibitory assay. A standard drug-acarbose was used as a positive control. The ZnO-NPs inhibited alpha-glucosidase significantly ($p < 0.05$) from 10 to 100 µg/mL. Subsequently, the control (acarbose) showed higher inhibition than the tested ZnO-NPs from 250 to 1000 µg/mL (Fig. 4.9.2a). Fig. 4.9.2b indicates that ZnO-NPs exhibited a comparable level of inhibition of alpha-amylase at 10-50 µg/mL to that of the control with no significant difference. The higher concentrations (100-1000 µg/mL) showed a slight decline compared to the control with a significant difference. The present findings reveal that the lower concentrations of ZnO-NPs of *H. cymosum* have a higher anti-hyperglycemic effect and could be used in mitigating blood glucose levels.

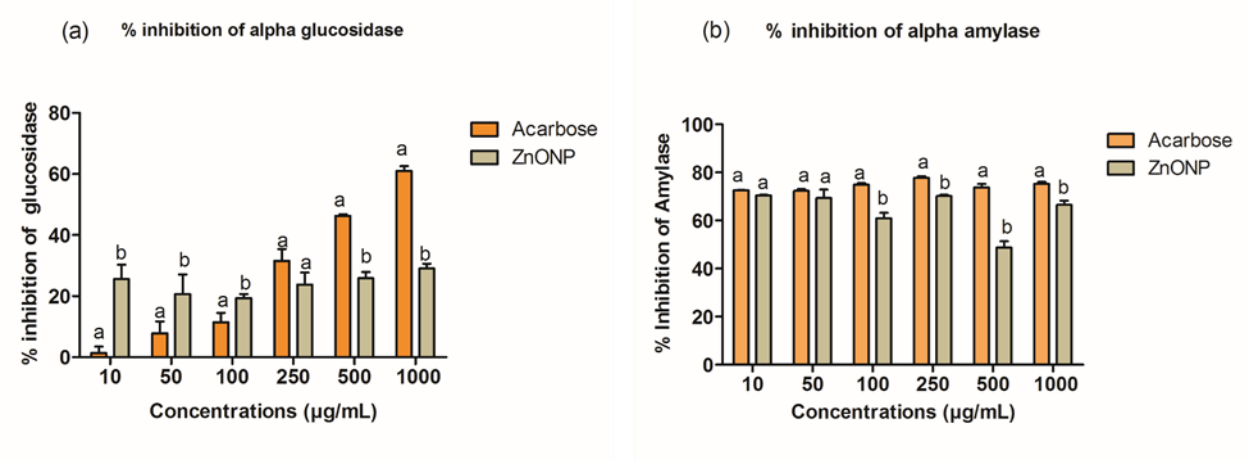

Fig.4.9.2 α-glucosidase (a) and α-amylase (b) inhibitory activities of *H. cymosum* ZnO-NPs. Data were express as mean ± SD; n = 4. The bars with different letters shows significantly different ($p < 0.05$). The % inhibition was compared to the control acarbose

4.12. Discussion

Nanoparticles have received substantial attention due to their unique size range, making them suitable for medical and biological applications (Tang et al. 2019; Siddiqui et al. 2020, Ifeanyichukwu et al. 2020). In the current study, *H. cymosum*-mediated zinc oxide nanoparticles were characterized using UV-vis spectroscopy, Scanning electron microscope (SEM), Energy dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD), Transmission electron microscope (TEM), and zeta potential. The UV-vis (Fig. 4.2) showed a successful formation of ZnO-NPs ranging from 354 to 360 nm, attaining its maximum at 360, which aligns with similar peaks reported in the literature (Ifeanyichukwu et al. 2020). The SEM analysis (Fig. 4.3.1a) showed a cluster of irregular hexagonal shapes of the ZnO-NPs, suggesting that the high surface area of the NPs might have caused them to stick together (Sundrarajan et al. 2015; Mkhize et al. 2022; Unni et al. 2022). The compounds Zn, O and C were identified from EDX analysis (Fig. 4.3.1b). The presence of the C peak could be associated with the bioactive compound capping ZnO-NPs during formation. Similarly, Bayrami et al. (2020) reported Zn, O, and C on ZnO-NPs of *Urtica dioica* extract. Other studies have reported that Zn and O are elemental composition of ZnO-NPs, signifying the purity of the elemental compositions present in ZnO-NPs (Bayrami et al. 2020; Mbenga et al. 2022).

The TEM analysis also reveals a cluster of spherical and hexagonal-shaped nanoparticles with an average dimension of 28.05 - 58.3 nm (Fig. 4.3.2 a and b). The dark areas observed might have resulted from the agglomeration of the NP during synthesis, corroborating other studies (Mbenga et al. 2022; Mkhize et al. 2022)**.** The zeta potential showed an excellent dispersion stability of nanofluids at -20.8 mV (Fig. 3.3)**.** The results revealed that the negatively charged molecules found on the ZnO-NPs are involved in capping and stabilizing the nanoparticles (Alarmdari et al. 2020; Mkhize et al. 2022). In a study by Mohana and Sumathi (2020), the Zeta potential of *Agaricus bisporus*-mediated ZnO-NPs was -20.5 mV with good stability and aligns with our present finding. Mkhize et al. (2022) also reported -23 mV and good stability. Previous studies have established that nanoparticles with −25 mV and +25 mV are likely to be more stable (Mahobia et al. 2016; Mkhize et al. 2022). However, the colloidal dispersion stability with absolute zeta potential has been reported to be 30 mV for ZnO-NPs. Thus, the lower colloidal stability of the ZnO-NPs dispersion could have influenced the possible aggregation of the nanoparticles (Hidayat 2018). In addition, the nanoparticles' composition and medium of dispersion are known to influence the surface charge of the particles (Mahobia et al. 2016).

The FTIR peaks reveal the presence of important functional groups capping the ZnO-NPs, such as alkanes, amines, carboxylic acids and phenols (Fig. 4.3.4) (Bayrami et al. 2018; Mhkize et al. 2022; Unni et al. 2022; Donga and Chanda 2022). These functional groups played a positive role in the reduction of zinc (Zn^{2+}) and stabilizing the ZnO-NPs during synthesis (Ochieng et al. 2015; Ifeanyichukwu et al. 2020; Aldeen et al. 2022) The XRD analysis reveals prominent peaks which correspond to the crystal reflection plane of the hexagonal structure of ZnO-NPs phase (Fig 4.3.5). The high-intensity diffraction peaks signify the formation of the superior crystalline quality of the nanoparticles (Obeizi et al. 2020; Alrajhi et al. 2023). The crystalline quality and purity of the ZnO-NPs might be influenced by the presence of a stabilizing agent capping the surface of the ZnO-NPs of *H. cymosum* (Alrajhi et al., 2021). Our finding aligns with other reported XRD peaks in previous studies, confirming the formation of a crystalline monoclinic structure (Bhattacharya et al. 2020; Obeizi et al. 2020; Alrajhi et al. 2023; Sadiq et al. 2021; Rashwan et al. 2022). Zinc oxide nanoparticles, amongst other metal oxides, play an important role in many enzymatic and cellular activities and have been well investigated and applied in diseases such as cancer, diabetes, etc. (Tang 2019; Ahmed 2022).

Diabetes mellitus (DM) is a metabolic disorder arising from limited insulin secretion or action during carbohydrate, fat, and protein metabolism, resulting in a rise in blood sugar levels (hyperglycemia) (Rehana et al. 2017; Kifle and Enyew 2020; Gadoa et al. 2022). There are several antidiabetic drugs; however, they are associated with side-effects (Haase et al. 2008; Gadoa et al. 2022). One of the important ways to manage DM is by targeting and decelerating the hydrolyzing enzyme (α-glucosidase and α-amylase) activity. Enzymes such as α-glucosidase and α-amylase play a major role in carbohydrate metabolism and absorption. Therefore, the inhibition of the activities of these enzymes could help lessen the burden of post-prandial hyperglycemia (Rehana et al. 2017). Several hyperglycemic drugs, including acarbose, miglitol, and voglibose, are suitable inhibitors of α-glucosidase and α-amylase enzyme activities, but these drugs are known to be associated with adverse effects (Rehana et al. 2017). Owing to these drugs' limitations, immense interest in plant-derived sources of inhibitors with minimal side effects is currently being explored (Rehana et al. 2017). Bioactive compounds isolated from plants have proven to be potent hypoglycemic agents that can be used in drug development and treatment options for ailments (Kifle and Enyew 2020). Additionally, green synthesized nanoparticles from metals such as gold, iron, silver, zinc, and their oxides, have been reported for their medical and biological applications (Alkaladi et al. 2014; Gadoa et al. 2022).

The findings from the present study showed that ZnO-NPs effectively inhibited higher α-glucosidase at lower concentrations. Meanwhile, at higher concentrations, the rate of inhibitory activity of ZnO-NPs did not progress enough compared to acarbose (Fig. 4.9.2a). The ZnO-NPs showed a strong inhibitory effect against α-amylase; the highest impact with a substantial difference was at the lower concentration (70%) (Fig. 4.9.2b). The inhibitory activity of ZnO-NPs against α-glucosidase and α-amylase could be due to phytochemical compounds capping the nanoparticles (Kifle and Enyew 2020). Compounds such as polyphenols and flavonoids isolated from plants are well-known for inhibiting alpha-glucosidase and alpha-amylase (Kifle and Enyew 2020). In addition, the ZnO-NPs showed potent lipase inhibition at higher concentrations. Pancreatic lipase is an enzyme responsible for converting triglyceride into fatty acid and glycerol (Ashraf et al. 2021). Several complications, such as DM, cardiovascular diseases, and hypertension, are associated with obesity caused by high levels of fat in the body (Ashraf et al. 2021). To avoid these complications, it is necessary to slow down the rate of enzyme activity involved in breaking down triglyceride into fatty acids and glycerol. Drugs such as Orlistat are

commonly used to inhibit pancreatic lipase activity in obese cases. Recently, other sources of inhibitors have been reported to come from plants. Compounds such as flavonoids, phenolics, tannins and saponins suppress enzymatic activity (Ashraf et al. 2021). The current finding shows that ZnO-NPs have a strong inhibitory effect on lipase activity (Fig. 4.9.1). It was observed that ZnO-NPs at higher concentrations demonstrated good inhibition compared to the standard drug, orlistat. Similarly, Meer et al. (2022) and Ashraf et al. (2021) reported better lipase inhibition of ZnO-NPs derived from *L. sativum* and *Boerhavia difusa* linn seeds, respectively.

In the current study, ZnO-NPs showed a remarkable glucose uptake and glucose utilization in the treated cells when compared to the control (Fig. 7a) and (Fig. 4.8 a, b and c), suggesting an excellent anti-diabetic agent for potential drug development (Asani et al. 2017; Virgen-Ortiz et al. 2020). Other *in vitro* studies showed that ZnO-NPs could potentially improve glucose transporter (GLUT-4) and increase β-cell proliferation (Asani et al. 2017; Virgen-Ortiz et al. 2020), by maintaining stable glucose metabolism. Glucose transporters (GLUTs) play a major role by regulating tissue-specific glucose uptake in organs such as adipose tissue, liver, and skeletal muscles, ensuring the blood glucose level is well regulated (Chadt and Al-Hasani 2020). A combined action of GLUT-2 and GLUT-4 transporters facilitates glucose clearance in the bloodstream. GLUT-2 increases insulin secretion and binding to its receptors, helping to increase the level of GLUT-4 in the plasma membrane and inhibition of hepatic gluconeogenesis, thus promoting glucose transportation to storage organs (Wang et al. 2012, Chadt and Al-Hasani 2020).

The ability of ZnO-NPs to reduce nitrite (NO) concentration in response to LPS activation of RAW macrophages was used to determine the anti-inflammatory activities. A substantial decrease in NO levels was observed at all concentrations, with cytotoxicity also noted at these concentrations (Fig. 4.6 a, b, c and d), therefore the results need to be interpreted with caution. Other previous studies have reported that ZnO-NPs could reduce allergic inflammatory responses (Kim and Jeong 2015). Generally, macrophages are known to be involved in the initiation, maintenance, and resolution of inflammatory reactions within the immune systems (Watanabe et al. 2019). Activation of these macrophages through lipopolysaccharide (LPS) binding to TLR4 activates the release of inflammatory mediators such as interleukin (IL)-1 β and NO. Nitrites are known to play a fundamental role as mediators in cellular communications. However, when produced in excess, it

can result in complications like neurological disorders, septic shock, rheumatoid arthritis, and autoimmune disease (Kim and Jeong 2015).

The cellular antioxidant activity of green-synthesized ZnO-NPs was determined in C3A cells using TBHP as an oxidant and CellROX® Orange as a quantitative indicator of ROS. Catechin (100 μM) was used as a positive control to indicate antioxidant activity, as shown in Fig. 4.5 (A and B). It was noted that ZnO-NPs did not induce CAA activity at lower concentrations, but there was a significant increase at the highest concentration, which was also cytotoxic. The CAA activity at this concentration could not be inferred because of toxicity. The toxic effect and increased ROS production shown by the ZnO-NPs could be linked to insufficient bioactive reducing metabolites in suppressing the production of ROS (Nagajyothi et al. 2015). Other studies have shown that phytochemical capping of ZnO-NPs could suppress ROS production and exhibit minimal toxicity, contrary to observations from our current study (Liu et al. 2010; Wu et al. 2014; Nagajyothi et al. 2015). Also, the elevated ROS generation might be due to damage to the mitochondrial electron transport chain, which then alters protein activity and subsequent cell death or apoptosis (Bhattacharya et al. 2020; Zhang et al. 2023). Based on our findings, it could be assumed that the lower concentration of ZnO-NP (Fig. 5) exhibited good CAA activity, whereas, at the highest concentration, there was toxicity and cell death due to ROS generation due to mitochondrial damage and oxidative stress (Choudhury et al. 2017; Wang et al. 2018; Zhang et al. 2023).

Similarly, previous studies have reported increased ROS generation following treatment with increased concentrations of ZnO-NPs (Choudhury et al. 2017). The generation of ROS has been attributed to the release of excessive intracellular zinc ions; thus, treatment with ZnO-NPs could trigger a singlet oxygen state ($^1O_2$) (Choudhury et al. 2017; Lekki-Porębski et al. 2023). Based on these reports, it is plausible to suggest that ZnO-NPs are toxic with increasing concentration (Bandeira et al. 2020. This finding corroborates other findings that have reported the cytotoxic effects of ZnO-NPs on many different cells (Anitha et al. 2018; Mkhize et al. 2022).

4.13. Conclusion

The current study synthesized and characterized ZnO-NPs of *H. cymosum* aqueous extract. The ZnO-NPs were further assessed for antidiabetic, anti-inflammatory, cellular antioxidant, and cytotoxicity potentials. Notably, the biological activities of ZnO-NPs were enhanced by the

presence of the phytochemical compounds acting as the capping agents. The excellent inhibitory activity of α-glucosidase, α-amylase, lipase inhibition glucose uptake, and utilization demonstrated by ZnO-NPs make it a promising candidate that could be optimized further and used as a lead in the production of antidiabetic drugs. The anti-inflammatory results could not be adequately interpreted because of the observation of toxicity. Further *in vitro* and *in vivo* antidiabetic studies of ZnO-NP are recommended. This study is the first to explore the antidiabetic potential of ZnO-NPs synthesized using aqueous extract of *H. cymosum*.

Authors Contribution

Achasih Quinta Nkemzi: Conceptualization, Data curation, methodology, formal analysis, Investigation, Validation, Writing original draft, Writing review & editing. **Kunle Okaiyeto**: Data curation, methodology, review & editing. **Omolola Oyenihi**: methodology, review & editing. **Chinyerum Sylvia Opuwari**: methodology, review & editing. **Okobi Eko Ekpo**: Supervision, Conceptualization, methodology, Investigation, Validation, administration, Writing review & editing. **Oluwafemi Omoniyi Oguntibeju**: Funding acquisition, resources, supervision, Conceptualization, investigation, administration, validation, and review & editing.

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Conflict of Interest

The authors declare that they have no competing interests.

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CHAPTER FIVE

The *in vitro* protective effects of *Helichrysum cymosum* on TM4 Sertoli cell

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Abstract

Phytocompounds continue to play an integral role for the treatment of many disease conditions, possibly due to their many reported bioactive properties. *Helichrysum cymosum* (*H. cymosum*) is an aromatic shrub known to possess important bioactive constituents, demonstrating numerous biological activities. The study aimed to evaluate the protective effects of two extracts of *H. cymosum* (aqueous AQ and 70% ethanol ETOH) on cell viability, morphological changes, production of reactive oxygen species (ROS), total antioxidant capacity (TAC), superoxide dismutase (SOD) and glutathione (GSH) activities in TM4 Sertoli cells. The results obtained showed no significant effect of *H.cymosum* extracts on TM4 Sertoli cell viability after 24h ($p>0.05$), and a significant decrease after 48h ($p<0.05$). Also, cell structure integrity was maintained at all concentrations compared to the positive control (10% DMSO) in which the cells appeared shrunken and round shaped. Antioxidant enzyme activity assessments showed that SOD,

GSH and TAC had good scavenging properties compared to the controls for both extracts after 24 h and 48 h treatment, although no statistically difference was recorded. ROS production was also maintained by both extracts after 24 h and 48 h treatment compared to the positive and negative controls, albeit with no statistically significant differences. In conclusion, *H. cymosum* extracts may contain potent bioactive components such as flavonoids that possibly work synergistically to mitigate OS-induced infertility in males. Further investigations on these extracts are recommended, including *in vivo* experiments to validate their use for the formulation of herbal supplements and in the drug discovery process.

Keywords: Antioxidant capacity, phytocompounds, glutathione, Superoxide dismutase, cytotoxicity, *Helichrysum cymosum*.

5.1. Introduction

Infertility is a global health challenge which affects 40–50% of couples in the world (Kaltsas, 2023). A couple is considered infertile if they are unable to conceive over one year after having regular and unprotected sex (Nantia et al., 2009, Takalani et al., 2023). The problem of infertility has historically been associated with the females, however, there is increasing evidence of male infertility in literature (Abdillahi and Staden, 2012, Sylvest et al., 2018; Agarwal et al., 2021). Sexual dysfunctions have been reported in 25%-63% of women and 10%-52% men (Dutta and Sengupta, 2018). The majority of male infertility cases are linked to idiopathic sperm disorders, sexual dysfunction and anatomical sperm abnormalities (Abdillahi and Staden, 2012; Semenya and Potgieter, 2012; Dutta and Sengupta, 2018); however, environmental, physiological and genetic factors have also been implicated (Bansal and Bilaspuri, 2010; Brehm, 2019).

The pathogenesis of male reproductive dysfunction involves complex endogenous pathways (Petricca et al., 2023), and the involvement of oxidative stress (OS) has been linked to the disproportionate production of reactive oxygen species (ROS) and the antioxidant defense mechanism (ADM) of the body (Chang et al., 2008; Kaltsas, 2023; Petricca et al., 2023). OS can lead to cellular damage if the levels of ROS (free radicals, peroxides, etc.) production are not brought under control by the body's ADM (Bansal and Bilaspuri, 2010). Such damage could affect the sperm protein, lipid and DNA (Kumbhare et al., 2023) resulting in infertility complications. Studies have shown that OS can be modulated by the use of oral antioxidants to enhance the body's

defense mechanism (Kumbhare et al., 2023; Liew et al., 2024; Siddique et al., 2024). Antioxidants have the capacity to lessen OS-induced damages through a number of mechanisms (Kaltsas. 2023; Bajaj et al., 2024).

The current treatment and management of male infertility involves the use of injectable and oral medications. The injectable medications include follicle-stimulating hormone (FSH), human chorionic gonadotropin (hCG), human menopausal gonadotropin (hMG), gonadotropin-releasing hormone (GnRH) (Alexander et al., 2023; Mansour. 2023; Fink et al., 2024), while oral medications like clomiphene citrate, anastrazole, clomiphene-tamoxifen combination, etc. have also been reported (Chua et al., 2013; Panner Selvam et al., 2023). These known medications are known to be generally effective for treating male infertility, they are generally costly, have many side effects and require prolonged use. These factors necessitate the search for alternative treatment options such as herbal therapies (Ho and Tan 2011; Dutta and Sengupta, 2018), or combination therapies (Nantia et al., 2009).

Herbal therapies are used in different parts of the world to treat different disease conditions and enhance general health (Malviya et al., 2016), and most contain abundant compounds with potent antioxidant properties which play a major role in alleviating the OS induced in different disease conditions (Olabiyi et al., 2020; Liew et al., 2024). *H. cymosum*, commonly known as gold carpet or yellow-tipped strawflower in English, goute tapyt in Afrikaans, and impepho in isiXhosa or isiZulu, belongs to the Asteraceae family of branched, aromatic, perennial shrubs with yellow flowers. It is distributed along the eastern coastline of South Africa, from KwaZulu-Natal through to the Western Cape and known to possess great medicinal value (Van Vuuren et al., 2006; Matazima et al., 2014; Philander, 2011; Giovanelli et al., 2018; Mayori, 2019).

The known major traditional uses of *H. cymosum* include treatment of cough and cold, pains, infected wounds, headache and "goodwill to ancestors". The minor medicinal applications include blocked nose, immunity boost, cardiovascular problems, diarrhea, dizziness, eye problems, flatulence, improvement of appetite, influenza, as insect repellent, for insomnia, kidney problems, laxative, menstrual pain, pertussis, pulmonary problems, skin infections, urinary problems, varicose veins, vomiting, and weak bones (Louren et al., 2008; Giovanelli et al., 2018). Some of the bioactive compounds identified in *H. cymosum* include helihumulone, helichromanochalcone, 5-hydroxy-8-methoxy7-prenyloxyflavanone, α-pinene, Δ-3-carene β-caryophyllene, 1, 8-cineole,

trans-caryophyllene, and (Z)-β-ocimene (VanVuuren et al., 2006; Mayori, 2019). These compounds have demonstrated diverse biological activities like antioxidant, antifungal, anti-inflammatory, antiviral, antimalarial, and cytotoxicity (Louren et al., 2008; Matanzima 2014; Jadalla et al., 2022).

It is expected that phytoconstituents that cause significant reduction in testosterone, LH, and FSH levels as well as those that impair testicular steroidogenesis, could be useful sources of male contraceptives (Abdillahi and Staden, 2012; Dike et al., 2023; Ibitoye et al. 2023). The anti-infertility potentials of some *Helichrysum* species like *H. odoratisssimum* have been reported (Watcho et al., 2019), but there are no studies reported in the literature on the reproductive benefits of the *Helichrysum* species of South Africa. Thus, the current study seeks to explore the protective effects of *H. cymosum* on sertoli cell functions as evaluated by cytotoxicity, ROS generation, total antioxidant capacity, SOD and GSH activity experiments. Sertoli cells are known to play a key role in spermatogenesis by regulating and supporting germ cell development (Monsees et al., 2000; Opuwari, 2009; Chang et al., 2017; Olabiyi et al., 2020).

5.2. Methodology

5.2.1. Plant collection

H. cymosum shoots were harvested in the garden of the Cape Peninsula University of Technology, Bellville campus, Cape Town, South Africa. The plant was authenticated by a botanist (P. Dryfhout) with voucher number 3708 and stored in the herbarium at the Department of Horticultural Sciences, Cape Peninsula University of Technology, Bellville, Cape Town, South Africa.

5.2.2. Extract preparation

The shoots of *H. cymosum* were washed thoroughly, cleaned, and dried in an oven at 40°C. The extraction protocol was adapted from Aladejana et al. (2020a) with some modifications. The plants were then crushed to powder using an electric grinder. Subsequently, 200 g of the crushed samples were soaked in 2.5 L of 70 % ethanol and distilled water, respectively, and stirred for 48 hours. Both the ethanol and aqueous extracts were then filtered using a funnel and Whatman No.1 filtered

paper. The ethanol extract was concentrated at 70°C using a Rotary vacuum evaporator while the aqueous extract was freeze-dried. The concentrated extracts were stored at 4°C in the refrigerator until required.

5.3. Cell culture maintenance

TM4 mouse Sertoli cell lines were purchased from the American Type Culture Collection (ATCC) and maintained in 25 cm culture dishes in complete culture medium (Dulbecco's Modified Eagle's medium (DMEM) / F12 Ham nutrient mixture supplemented with 2.5% fetal bovine serum (FBS), 5% horse serum, 1% penicillin/streptomycin).The cells were incubated at 37°C, 5% CO_2 in a humidified environment, and sub-cultured after confluence. Test samples of the plant extracts were prepared by reconstituting in 0.1% dimethyl sulfoxide (DMSO) at a concentration of 1mg/mL, then sonicated and stored at 4°C until used. Different concentrations of the extracts (120 µg/mL, 60 µg/mL, 30 µg/mL, 15 µg/mL, 7.5 µg/mL and 3.75 µg/mL) were prepared in complete medium DMEM. The negative control was prepared in (0.1% DMSO in DMEM) and positive control in (10% DMSO in DMEM).

5.3.1. Cell viability (MTT assays)

The viability of TM4 cells was evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) according to the procedure in Olabiyi et al. (2020). TM4 cells were seeded in 96 well plates at 4000 cells/well (200 µL aliquots) and left overnight to attach (24 hours) and the media removed. The cells were then exposed to the different concentrations (120 µg/mL, 60 µg/mL, 30 µg/mL, 15 µg/mL, 7.5 µg/mL and 3.75 µg/mL) of the extracts reconstituted in complete DMEM medium, the negative control containing (0.1% DMSO in DMEM) and the positive control containing (10% DMSO in DMEM) and incubated for 24 or 48 hours. Subsequently, all treatments were aspirated from the wells and 100 µL MTT (1mg/mL) in a complete medium was added to each well and incubated for 3 hours. After incubation, the supernatant was removed, and 100 µL DMSO added to each well to dissolve the formazan crystals. The absorbance was measured at 560 nm and the background at 750 nm using a plate reader (FLUO Star® Omega, BMG Labtech). The graphs of the percentage cytotoxicity against the concentrations of the extracts were plotted using GraphPad Prism 5. The results were expressed as percentage of control, according to the equation below.

$$\% \textit{ cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

5.3.2. Cell morphology

The effects of the aqueous and 70 % ethanol extracts of *H. cymosum* on TM4 cell morphology was performed using 96 well plates. Into each well, 200 µL/well (4000 cells) were seeded and allowed for 24 hours to attach. Later cells were treated with different extract concentrations (120 µg/mL, 60 µg/mL, 30 µg/mL, 15 µg/mL, 7.5 µg/mL and 3.75 µg/mL) for 24 and 48 hours respectively. The morphological changes after treatment were then observed using the Invitrogen™ EVOS™ XL Core imaging system, and micrographs captured at 10 X magnification. More than one image was captured for each concentration.

5.4. Cellular antioxidant enzyme assays

5.4.1. Cell lysate preparation

TM4 cells (1 million cells/ mL) were seeded into a six well plate and allowed overnight (24 h) to attach. The cells were then treated with different concentrations (120 µg/mL, 60 µg/mL, 30 µg/mL, 15 µg/mL, 7.5 µg/mL and 3.75 µg/mL) of the aqueous and 70% ethanol extracts, respectively. The control wells comprised of negative control (0.1% DMSO in DMEM) and positive control (10% DMSO in DMEM). All treated plates were incubated for 24 h and 48 h respectively. After that the supernatant was removed, rinsed with PBS followed by scrapping of cells and addition of 1mL of 1% SDS to all the wells and transferred to Eppendorf tubes and sonicated in cool ice for 10 seconds. After sonication, samples were centrifuged for 10 mins at 2500 r m p to obtain the lysates and stored at -20^{o}C for subsequent use (Lowry et al., 1951; Bara and Kaul, 2018).

5.4.2. Glutathione (GSH) levels

The level of reduced GSH was determined based on the oxidation of GSH by 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB) according to the procedure by Ellman, 1959, modified by Polycarp et al. (2015), with minor modifications. Briefly, GSH standard concentrations (20, 40, 60, 80 and 100 µM) were prepared from reduced L-glutamine (GSH) stock (1 mM) and DBTN reagent 1mM was prepared separately.

Test samples and standards (13.3 µl) were each placed into 96 well plates in triplicates. Thereafter, 15.3 µl of PBS and 33.3 µl DBTN reagent were added to initiate the reaction and then shaken for 30 seconds. The reaction mixture was allowed to incubate at room temperature for 5 minutes, and absorbance measured at 412 nm.

5.4.3. Superoxide dismutase activity (SOD)

The SOD activity was measured on cell lysates, which is based on the ability to inhibit auto-oxidation of pyrogallol (Marklund and Marklund, 1974). The analysis was performed according to a modified procedure adapted from Bara and Kaul (2018). A volume of 8.3 µl of samples (cell lysates) were placed in triplicates in a 96 well plate, and 238.3 µl DTPA-Tris-HCL buffer {1mM DTPA and 5mM TRIS-HCL (pH 8.2)} was added into the sample wells and 246.6 µl to the blank wells. A 3.4 µl of pyrogallol (15mM) was added to the sample and blank mixtures, excluding the sample blanks. The change in absorbance per minute for 3 minutes was recorded on a plate reader at 420 nm. The result is express as percentage of control according to the equation below.

$$\%\ cell\ viability = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

5.4.4. Total antioxidant capacity (TAC)

The total antioxidant capacity of the *H. cymosum* extracts was measured using the colorimetric assay 2, 2´-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) following the procedure described by Greifová et al. (2022). This method is based on oxidation of colorless reduced ABTS to a blue-green color cation $ABTS^{+}$ using hydrogen peroxide in an acidic reaction medium (pH 3.6). Briefly into a 96 well plate 20 µl of samples and standards were placed and 200 ul of 0.4M acetate buffer (0.4 M glacial acetic acid + 0.4 M anhydrous sodium acetate, PH 5.8) was added and first read at 660 nm. Subsequently, 20ul of 10mM ABTS prepared by dissolving in {2mM hydrogen peroxide (30% H2O2) in 30 mM acetate buffer (pH 3.6)} was added. The mixture was incubated in the dark for 5 minute and absorbance read at 660nm**.** The results were expressed as percentage of control.

5.4.5 Intracellular ROS production

Intracellular ROS production in TM4 was determined using the method by Banerjee et al. (2019), involving 2, 7- dichlorofluorescein diacetate (DCF-DA), with slight modifications. Briefly, twenty microliters (20 µl) of the sample (cell lysates) were placed in to a 96 well plate in triplicates with 152 µl Hank's buffer and 30 µl of DCF-DA (5uM). The mixture was incubated at 37 ^{0}C for 15 minutes and the wavelength recorded at 485 nm excitation and 520 nm emission, incubated and read for a second time using Spectro star® Omega (supplier) absorbance microplate reader. Results were expressed as percentage ROS production according to the calculation below.

5.5 Statistical analysis

Values were expressed as a means ± standard errors of the means of three repeated experiments. Differences between the means were determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-test. All analyses were performed with GraphPad Prism 5. A difference in mean values was considered statistically significant if $p < 0.05$.

5.6. RESULTS

5.6.1. Effects of *H. cymosum* extracts on cell viability

The *H. cymosum* AQ and ETOH extracts were assessed on TM4 Sertoli for cell viability after 24 h and 48 h treatments, to establish a safer concentration as shown in Fig. 5.1. Cells were exposed to the following concentrations (120 µg/mL, 60 µg/mL, 30 µg/mL, 15 µg/mL, 7.5 µg/mL and 3.75 µg/mL), and the results showed no significant changes in cell viability after 24 h in all tested concentrations for both AQ and ETOH extracts ($p>0.05$), compared to the negative control. Meanwhile, the positive control declined significantly ($p < 0.05$) compared to all other treatments (Fig. 5.1 A and C).

Following 48 h treatment, the AQ extracts exhibited a slightly significant ($p < 0.05$) decrease in cell viability was recorded in almost all the tested concentrations compared to the control except the lowest concentration (3.75 µg/mL) (Fig 5.1. B). However, the percentage cell viability was significantly ($p < 0.05$) higher than the positive control. On other hand, the cells exposed to the ETOH extracts for 48 h showed a significant decrease ($p < 0.05$) in cell viability for all concentrations compared to the negative control (Fig. 5.1 C) and a significant increase ($p < 0.05$) compared to the positive control (Fig. 5.1 D).

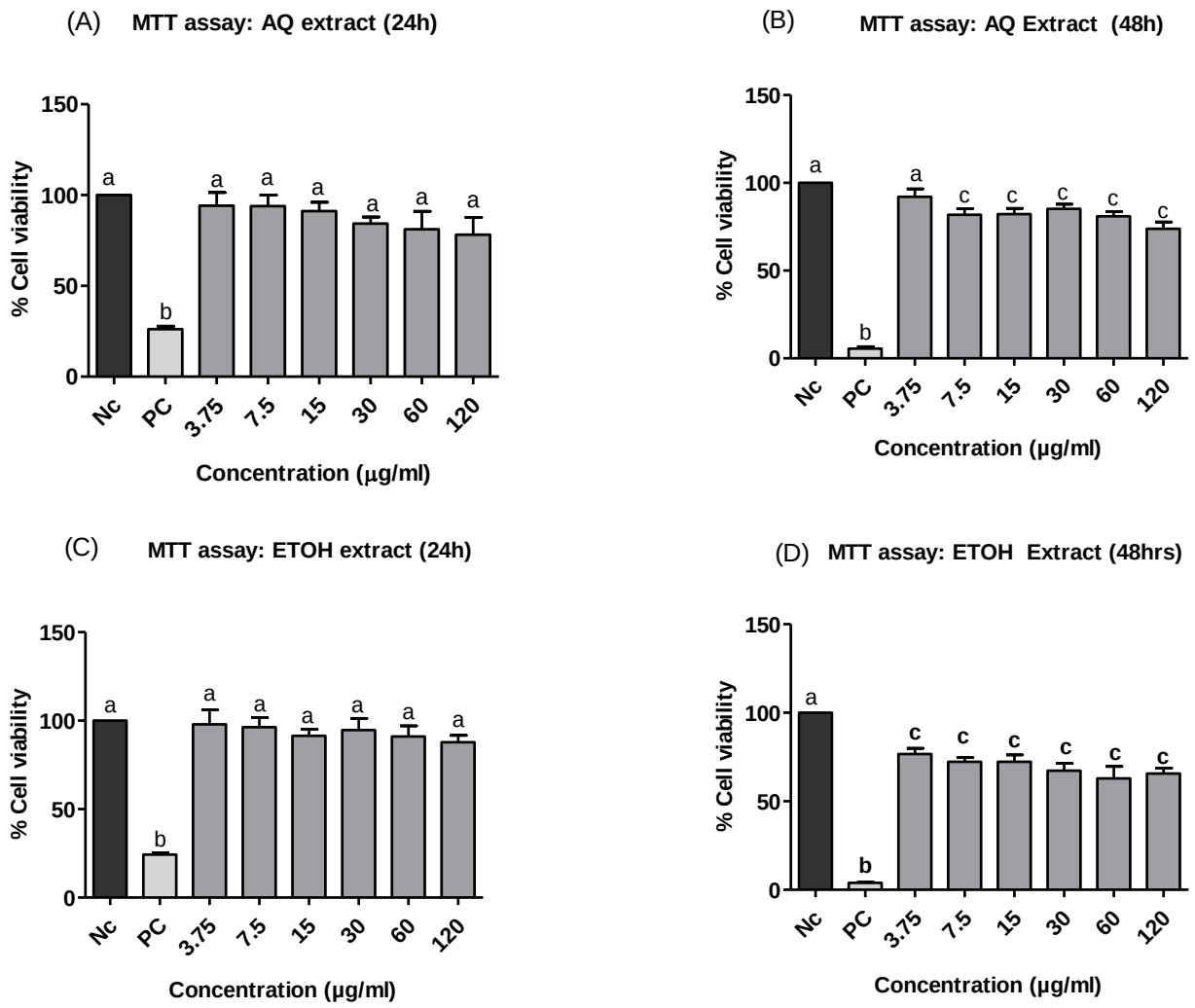


Figure 5. 1. The effect of *H. cymosum* (A and B) aqueous (AQ) and (C and D) 70% ethanol (ETOH) extracts on TM4 Sertoli cells viability treated for 24 h and 48 h period. Results are represented as means ± SEM of three repeated experiments (n = 3), 10% DMSO in DMEM was used as positive control and 0.1% DMSO in DMEM as negative control. Bars with different letters represent significant difference ($p < 0.05$), while bars with similar letters represent no significant difference ($p>0.05$) compared to the negative control.

5.6.2. Superoxide Dismutase activity (SOD)

The SOD activity was measured on TM4 Sertoli cells treated with AQ and ETOH extracts for a period of 24 h and 48 h. Both extracts (AQ and ETOH) showed no significant difference at all concentrations compared to the NC and PC after 24 h exposure (Figs. 5.2 A and C). Likewise, the

48 h AQ extracts did not show any significant change at all concentration compared to both controls NC and PC, but the positive control decline by 9% difference in SOD activity compared to the other treatments without any significant difference Fig. 5.2B. The ETOH extract after 48 h treatment showed no significant difference across all concentration comparatively to the controls (NC). But a significant difference was observed at (120 µg/mL, 60 µg/mL, 30 µg/mL, 7.5 µg/mL and 3.75 µg/mL) compared to the PC Fig. 5.2D.

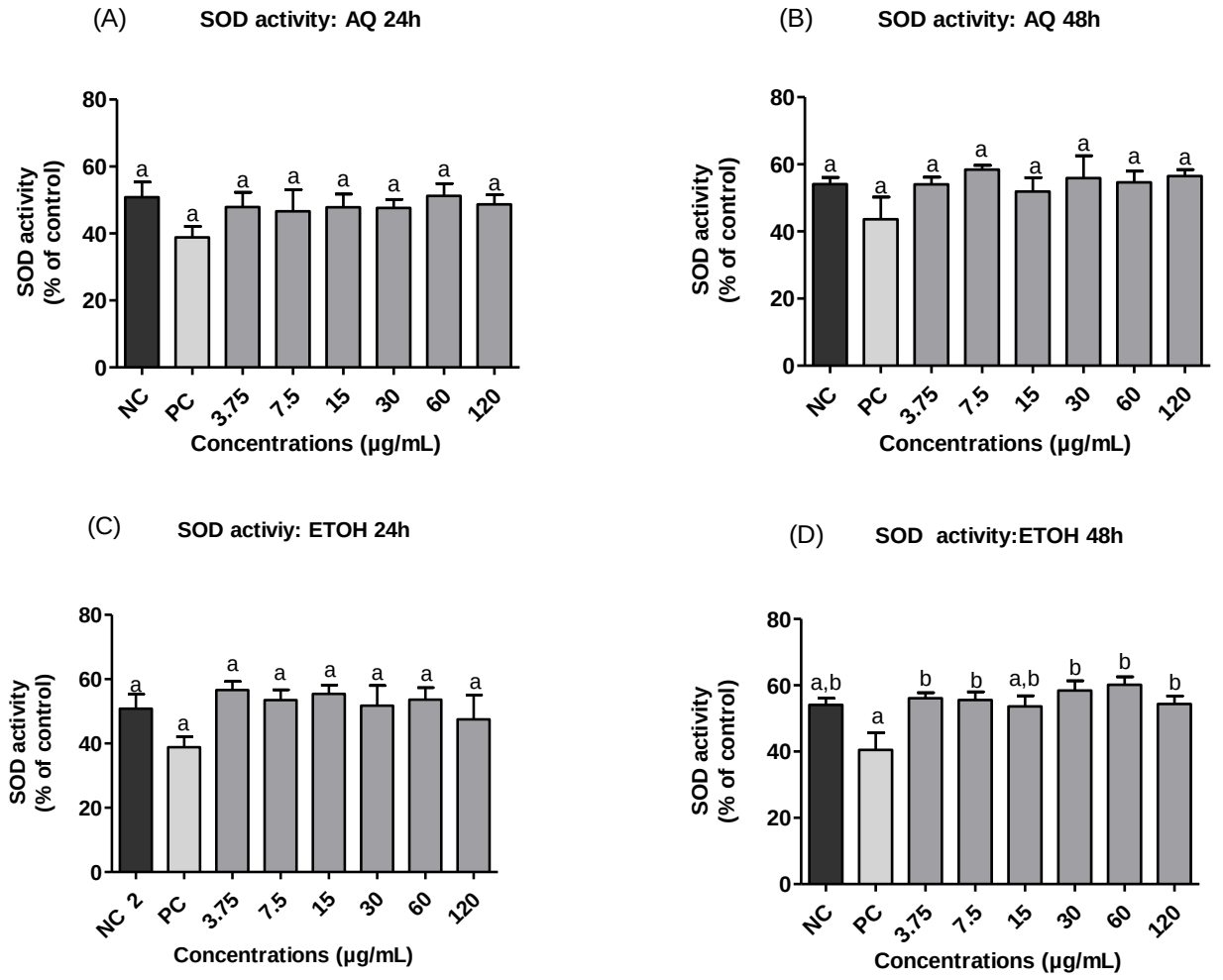


Figure 5.2. Superoxide dismutase (SOD) enzyme activity after exposure to *H. cymosum* (A and B) aqueous (AQ) and (C and D) 70% ethanol (ETOH) on TM4 Sertoli cells for 24 h and 48 h period respectively. Values represent mean ± SEM of three repeated experiments (n=3), 10% DMSO in DMEM was used as positive control and 0.1% DMSO in DMEM as negative control.

Bars with different letters represent significant difference ($p < 0.05$), while bars with similar letters represent no significant difference ($p>0.05$) compared to the negative control.

5.6.3. Glutathione (GSH)

The levels of reduced GSH were measured after exposure to *H. cymosum* AQ and ETOH for 24 h and 48 h duration (Fig 5.3). The AQ extracts showed slight increases at most concentrations for both 24 h and 48 h treatments, with no statistically significant differences ($p>0.05$) seen compared to the negative control. However, the positive control exhibited significant ($p < 0.05$) reduction in glutathione levels compared to all tested concentrations (Figs. 5.3A and B).

For the ETOH extracts treated for 24 h duration, exhibited no significant difference ($p>0.05$) in GSH level compared to the negative control, with a significant ($p < 0.05$) increase compared to the positive control (Fig 5.3C). However, Fig 5.3D shows statistically significant decreases ($p < 0.05$) only at the highest two concentrations (60 and 120 µg/mL) compared to the negative control and no substantial difference at lower concentrations ($p>0.05$) after 48 h period. The positive control showed statistically significant ($p < 0.05$) reduction in glutathione levels compared to all tested concentrations (Figs. 5.3C and D).

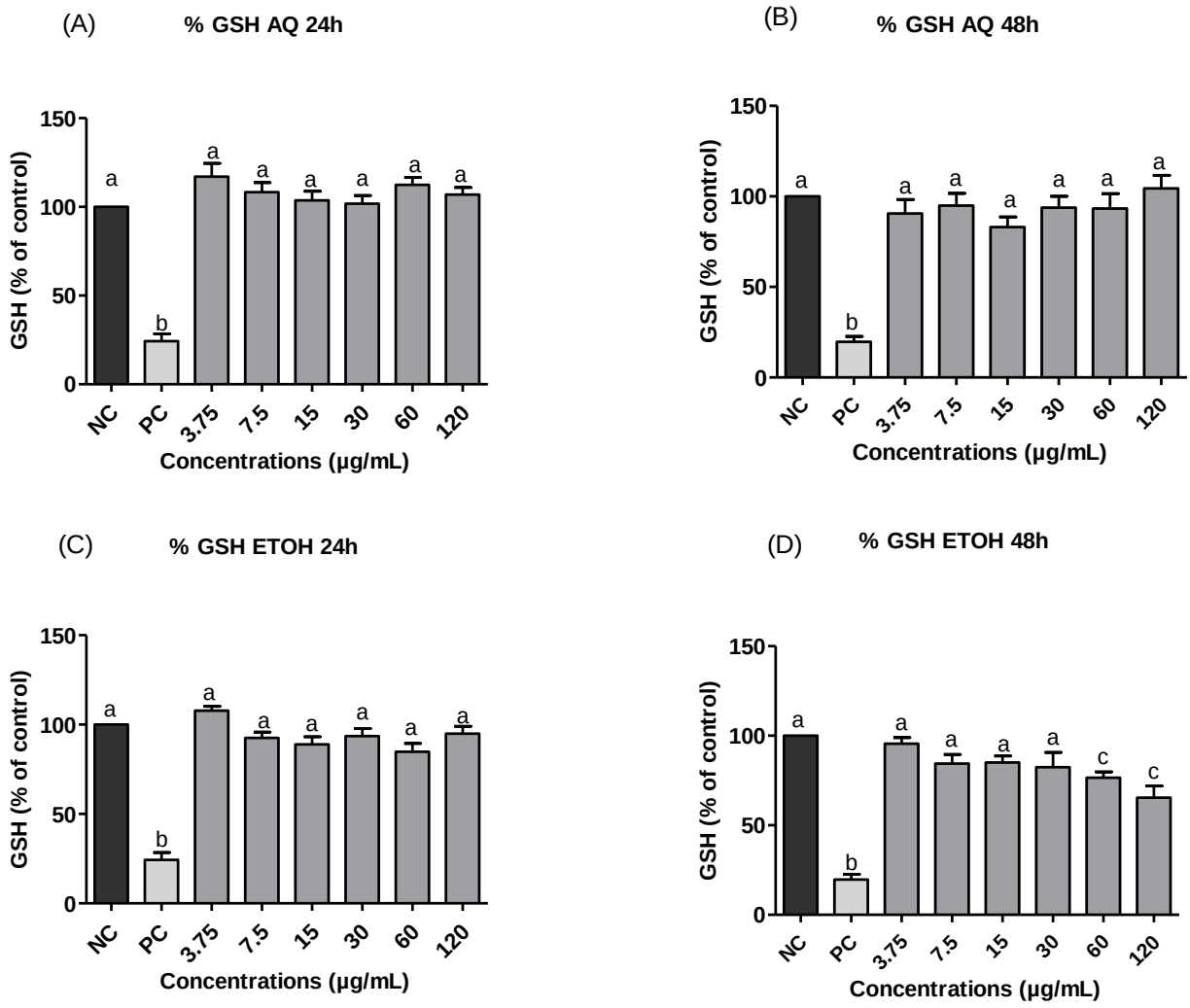
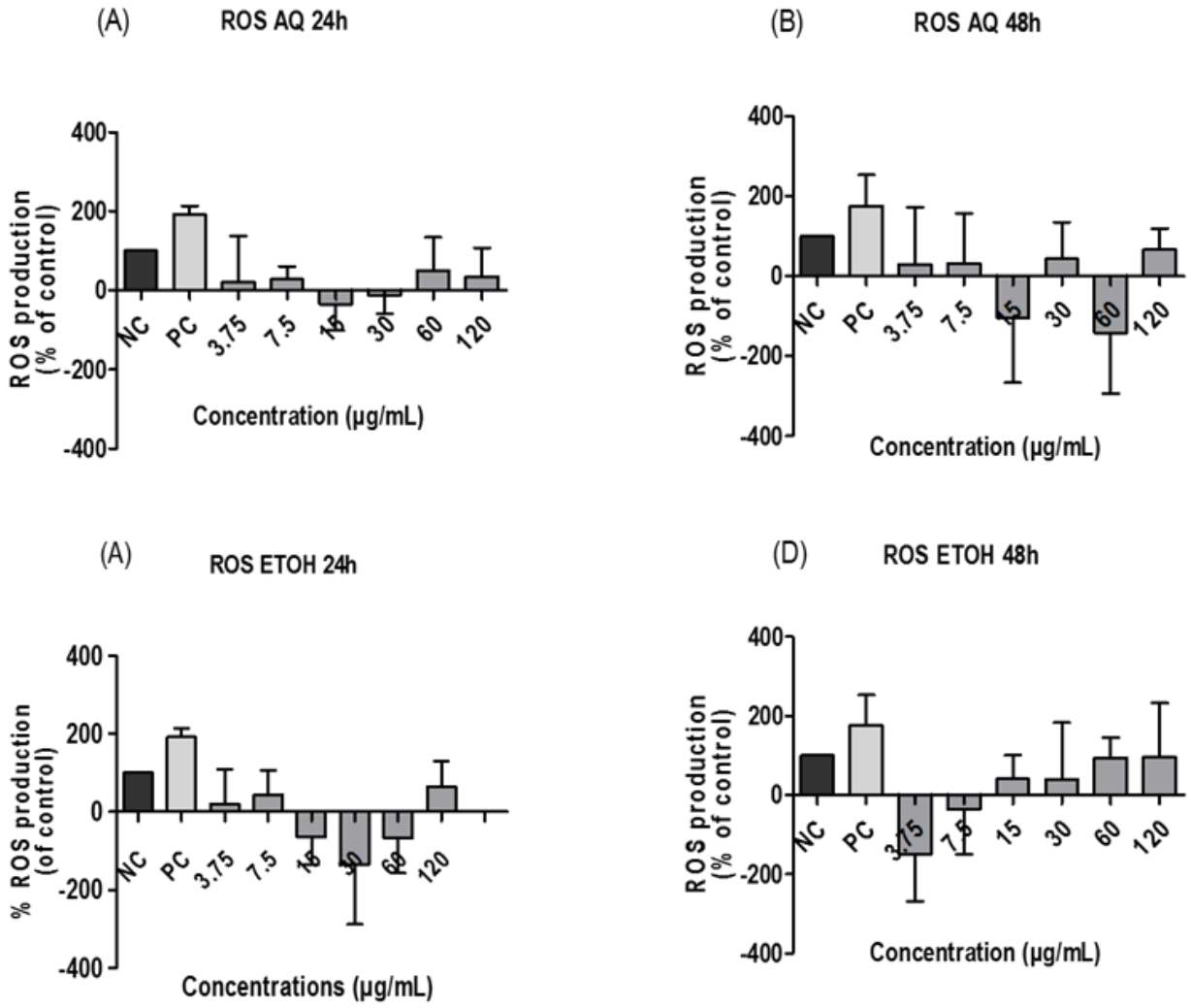


Figure 5.3. Effect of H. cymosum aqueous (A and B) and ethanol (C and D) extracts on Glutathione level after 24 and 48 hours exposure to TM4 Sertoli cells. Values are represented as mean ± SEM of three repeated experiments (n=3), 10% DMSO in DMEM was used as positive control and 0.1% DMSO in DMEM as negative control. Bars with different letters represent significant difference ($p < 0.05$), while bars with similar letters represent no significant difference ($p>0.05$) compared to the negative control.

5.6.4. Evaluation of intracellular ROS production

In the current study, both the AQ and ETOH extracts of *H. cymosum* mitigated the generation of ROS at all tested concentrations for both AQ and ETOH treated for 24 h and 48 h durations

respectively, maintaining ROS levels below both the positive control (10% DMSO in DMEM) and negative control (0.1% DMSO in DMEM) (Figs. 5.4 A-D).



5.6.5. Total antioxidant capacity (TAC)

No significant change was noted for the total antioxidant capacity after treatment, for both extracts and treatment times when compared to the control ($p > 0.05$). Although the TAC decreased in the PC group compared to the NC, this was insignificant ($p > 0.05$); Fig. 5.5A-D).

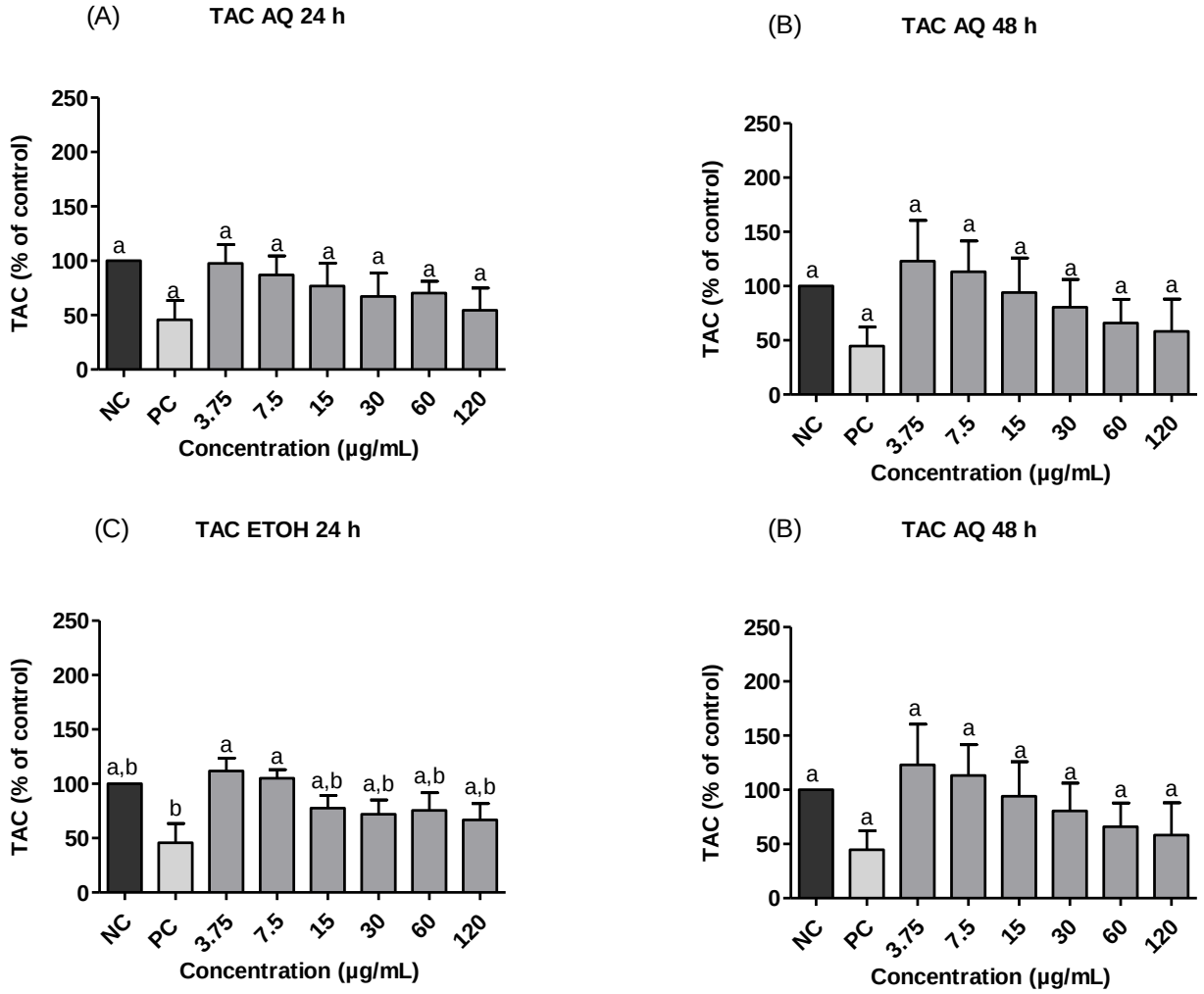


Figure 5.5. Total antioxidant activities of *H. cymosum* aqueous (A and B) and ethanol (C and D) extracts after 24 and 48 hours exposure to TM4 Sertoli cells. Values are represented as mean ± SEM of three repeated experiments (n=3), 10% DMSO in DMEM was used as positive control and 0.1% DMSO in DMEM as negative control. Bars with different letters represent significant difference ($p < 0.05$), while bars with similar letters represent no significant difference ($p>0.05$) compared to the negative control.

5.6.6. Cell morphology changes after treatment

TM4 cell morphology was observed after treatment with the AQ and ETOH extracts of *H. cymosum* for 24 h and 48 h, and the micrographs below taken. The images show normal dividing cells without any effect on cell structure integrity for both extracts at all concentrations and

negative control, suggesting that the treatment where not toxic to the cells (Fig 5.5A and B). The positive control displayed significant changes in structural integrity of the cells for both 24 h and 48 h, which was noted by cell shrinkage and round shape caused by the toxic effect of DMSO 10% (Fig 5.5A and B).

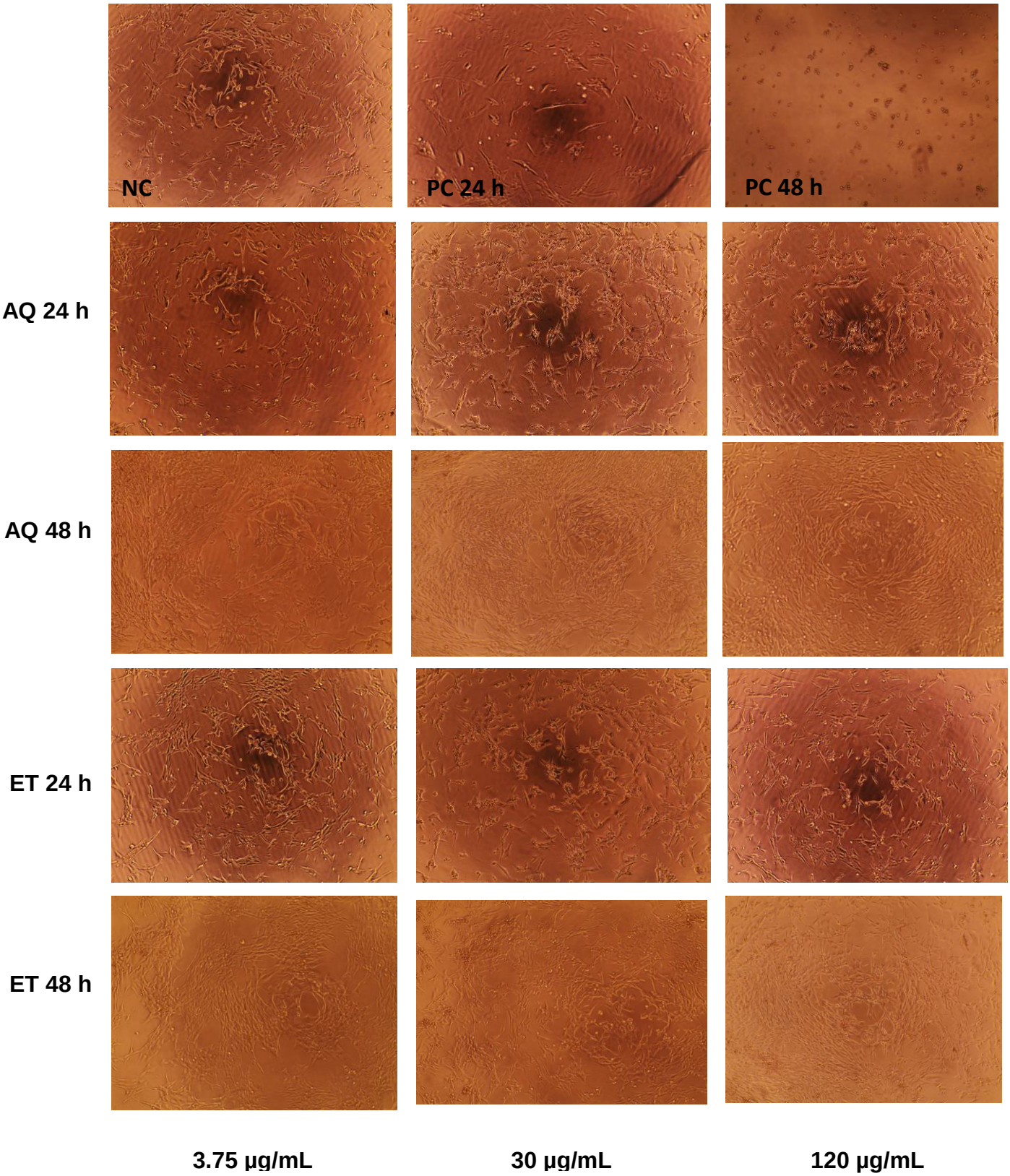

NC
PC 24 h
PC 48 h
AQ 24 h
AQ 48 h
ET 24 h
ET 48 h
3.75 µg/mL
30 µg/mL
120 µg/mL

Figure 5. 5 B. Micrograph images of TM4 sertoli cells captured at (10x) magnification after treatment to AQ and 70% ethanol extracts for 48 hours.

5.7. Discussion

Infertility has become a serious global health issue affecting many couples in their reproductive age and posing profound social, psychological and economic challenges (Agarwal et al., 2020; Kaltsas, 2023). The contributing factors to male subfertility or infertility are multifaceted (Agarwal et al., 2020), with oxidative stress (OS) being one key factors known to play a significant role (Petricca et al., 2023). A major risk factors in male infertility is the mismatch often seen between ROS production and its neutralization by the body's natural antioxidant defense systems, resulting in elevated of ROS levels in the semen (Chang et al., 2008; Adewoyin et al., 2017; Koshevoy et al., 2021). Herbal therapies are well known for their abundant antioxidant constituents which are known to alleviate OS in many disease conditions (Nantia et al., 2009; Olayibi et al., 2020). Thus, in this study the antioxidant effects of *H. cymosum* extracts (AQ and ETOH) were evaluated.

Firstly, the MTT assay was used to evaluate the cytotoxicity of both extracts to determine a safer concentration. The findings showed that overall, both extracts did not significantly reduce the viability of the Sertoli cells at all concentrations and treatment durations (Fig. 5.1A and 5.1C). Previous studies on TM4 Sertoli cells have reported toxicity at a higher concentrations of 100 or 1000 μg/mL after treatment with *Phyllanthus amarus* (Olayibi et al., 2020). The lack of cytotoxicity observed in the current study may due to the concentrations used being lesser than 1000 μg/mL. The positive control displayed significant reduction in cell viability, demonstrating toxicity effects on the TM4 Sertoli cells interestingly, the changes in the cell morphology aligns to the results of cell viability performed in this study.

Further investigation was done to determine the antioxidant potential of *H. cymosum* extracts by analyzing the following assays: SOD, GSH, ROS, and TAC. Superoxide dismutase (SOD) is a vital antioxidant detoxifying enzyme found in cells and acts as a preliminary defense system against reactive oxygen species (ROS) by eliminating the superoxide anion and converting it to hydrogen peroxide hence preventing the toxic effects of these free radicals (Ighodaro and

Akinloye, 2017; Islam et al., 2022). Following treatment with the ETOH and AQ extracts of *H. cymosum* (24 h), SOD activity showed no significant changes at all concentrations compared to the controls (Fig 5.2). Similarly, the SOD activities for the AQ and ETOH extracts (48 h) did change significantly between the respective concentrations and the controls. Study by Oyedemi et al. (2010) reported an increase SOD inhibition when exposed to plant extract (*S. hennigsii*). More and Makola, (2020) also reported an elevated SOD activity with 90–50% activity after treatment with *Solanum sisymbriifolium* extracts. Hence, findings suggest that for H.cymosum to fully activate its catalytic effects requires cofactors such as copper, iron and manganese to exert its full catalytic potential when metabolizing free radical (Zheng et al., 2023)

Another significant antioxidant is glutathione (GSH) which regulates and balanced the oxidation and reduction processes in cells, thereby maintaining proper cell functions. (Aoyama, 2021; Averill-Bates, 2023). The multifunctional intracellular antioxidant, glutathione (GSH) plays a major role in counteracting oxidative stress, and also participates in several metabolic functions like regulating the cell cycle, calcium homeostasis, enzyme activity as a cofactor, DNA repair, activation of transcription factors, etc. (Forman et al., 2009; Georgiou-Siafis and Tsiftsoglou, 2023; Haaften et al., 2023). The efficacy of glutathione is controlled by a balance between its active (GSH) and inactive (GSSG) forms. The active form facilitates glutathione to carry out its cellular functions whereas the inactive form is produced from neutralized free radicals (Di Giacomo et al., 2023). Therefore, a balance between these two forms of glutathione is important in maintaining cellular health, whereas an imbalance could result in accumulation of free radicals and inflammatory cytokines, cellular dysfunction, rapid aging, risk of chronic degenerative diseases, etc. (Novelli et al., 2022; Al-Temimi et al., 2023).

The findings from the present study showed only minor increases in GSH levels for AQ extract 24 h and 48 h (Fig. 5.3 A, B, and C) and the ETOH extract (24 h), as well as a dose-dependent decrease at 48 h in ETOH extract -treated cells (Fig. 5.3D), with no significant differences noted. Similarly, in another study reported ($p<0.05$) significant increased GSH levels in treatment group of smoker given *Allium sativum* extract compared to smokers and control groups (Savira et al., 2023). Likewise, Falang and Pierre (2022) reported increased in GSH content in male rats treated with *Zizuphus mauritiana* compound p-coumaroyl alphitolic acid (ACA) compared to the control. Despite no significant difference observed in this study the slight increase in GSH content suggest

that the *H.cymosum* extracts is capable of enhancing GSH biosynthesis, however, to conclusively confirm assertion, further investigation needs to be conducted to determine if the extracts(AQ and ET) could promote GSH recycling or have an effects on its biosynthesis. (Al-Temimi et al., 2023; Haaften et al., 2023)

The measurement of reactive oxygen species (ROS) is also a marker used to determine oxidative stress damage in biological samples. In this study, a substantial decrease in ROS levels was observed (Fig. 5.4) compared to the control for both extracts following treatment times. Extracts for 24 and 48 h, indicating that exposure to the *H. cymosum* extracts did not induced any oxidative stress damage even with the highest concentrations. Instead the AQ and ET extracts showed significant reduction in ROS production, which suggest the potential antioxidant action of metabolites such as flavonoids present in the plant (Jallada et al., 2022). our findings aligns with earlier reported that demonstrated increased ROS levels in rats exposed to the drug, Valproic acid (VPA), causing valproate-induced oxidative stress which was reversed by the antioxidant action of *Moringa oleifera* (Ertik et al., 2022). Accordingly, Jambor et al., (2022), also demonstrated significant reduction ROS production after treatment Lepidus at 5, 10, and 25 µg/mL, these results match the current observation shown by *H. cymosum* treatments on ROS production, although our study showed significant reduction at all concentrations. This indicates that the phytochemicals present in plants could have strong antioxidant effects to reverse oxidative stress. Phytocompounds like flavonoids have been extensively reported to possess antioxidant properties, possibly through scavenging reactive oxygen species (ROS) and activating the cellular antioxidant systems (Ramli et al., 2023).

Antioxidants are known to either scavenge the free radicals formed or intercept the radical generating process (Olabiyi et al., 2020). In this study, cells treated with the AQ and ETOH extracts for 24 h and 48 h, had increased TAC at lower concentrations (3.75 µg/mL and 7.5 µg/mL), with no significant difference compared to the controls (Fig 5). Overall, the observed increase in antioxidant activity (SOD, GSH and TAC) is directly proportional to a decrease in ROS production Fig. 5.(2, 3, 4 and 5).

5.8. Conclusion

The present study is the first to explore the antioxidant activities, cytotoxicity and morphological changes of AQ and ETOH extracts obtained from *H. cymosum* shoots on TM4 Sertoli cell lines.

The outcome showed that *H. cymosum* extracts possess antioxidant properties that could neutralize the effects of oxidative stress damage. Four antioxidant parameters were analyzed in this study. The SOD, GSH and TEAC (ABTS) analysis showed better antioxidant scavenging activities especially at the lower concentrations for both 24h and 48h.

Extracts exhibited decrease ROS production at all concentrations compared to the control. Additionally, no cytotoxic effect was observed except for the ETOH that decreased at the highest concentration with no statistical significance, suggesting the plant can promote cell proliferation, which is supported by no effect on the morphology of the cells. Therefore *H. cymosum* is promising plant candidate that contain bioactive molecules that can further be explored to develop novel antioxidant drugs to manage male subfertility. However, further *in vitro*, *in vivo* and clinical research on specific compounds and mechanisms responsible for the antioxidant activities is recommended.

Authors Contribution

Achasih Quinta Nkemzi: Conceptualization, Data curation, methodology, formal analysis, Investigation, Validation, Writing original draft, Writing review & editing **Chinyerum Sylvia Opuwari:** Conceptualization, methodology, review & editing. **Okobi Eko Ekpo**: Supervision, Conceptualization, methodology, Investigation, Validation, administration, Writing review & editing. **Oluwafemi Omoniyi Oguntibeju**: Funding acquisition, resources, supervision, Conceptualization, investigation, administration, validation, and review & editing.

Declaration of competing Interest

The authors declare that they have no competing interests.

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CHAPTER SIX

General Discussion and Conclusion

6.1. General discussion

DM and its complications have been shown to increase morbidity and mortality rates worldwide (Wang et al., 2022). It has become a public health emergency due to continuous escalation over the past three decades (Antini et al., 2024). This disease involves a complex network of factors, propelled by higher levels of obesity, marked by a variety of concomitant health conditions and increase risk of untimely death, leading to economic and social burdens (Chan et al., 2020, Antini et al., 2024). The economic burden imposed by DM on national economy is very large, with an estimated 966 billion USD spent in 2021 on global health care (Wang et al., 2022). DM is also known to affect several physiological systems and tissues including male reproductive organs. DM is amongst the leading cause of male infertility especially type 2 diabetes mellitus. The action of chronic inflammatory response and oxidative stress in DM can lead to compromised sperm quality and increased DNA fragmentation (Fan et al., 2024). As of present, there are numerous synthetic drug options used to manage this disease, however, diabetic patients are often discouraged from these medications due to high cost and unpleasant side effects (Wang et al., 2024). As a result, there is a demanding need to discover new, safe and effective drug. Therefore, alternative and complementary therapies have become a common and convenient treatment option for DM (Peng et al., 2023).

The use of medicinal plants as a source of medicine has been a cultural practice for ages (Mukhtar et al., 2020). Herbal remedies were fabricated from different plant parts and used for treating ailments such as cancer, diabetes and malaria (Mohammed et al., 2014, Raimi et al., 2021). Ethno-pharmacological studies have reported over 1200 medicinal plants with hypoglycemic effect (Mukhtar et al., 2020). Diverse medicinal plants have been reported from Africa, these plants are used by indigenous population for their general health and wellbeing (Okaiyeto et al., 2021). Despite the beneficial role of these plants to health, most of these plant species are still under

explored and information on efficacy and mechanism of action of many plant species is still not uncovered. Also, safety of these herbal mixtures need to be considered especially toxicity issues. Earlier studies have highlighted toxicity concern when using high doses of plant, hence, knowledge of recommended doses before use is necessary (Okaiyeto et al., 2021). Some species of the genus *Helichrysum* have been used for antidiabetic treatments, however the species *H. cymosum* is consumed to treat different illnesses but the antidiabetic potential and mechanism of actions have not been fully explored. This study was performed to identify the phytochemical compounds responsible for the following biological activities; hypoglycemic, antioxidant, anti-inflammatory and reproductive potential, and to establish safer concentrations of aqueous, ethanol and zinc oxide nanoparticles from *H. cymosum* shoots**.**

The principal findings of this study has been discussed in the previous three chapters. The first part was presented in chapter three, this study was performed using the aqueous and ethanol crude extracts of *H. cymosum*. The bioactive compounds present were quantitatively identified, and antioxidant capacity determined, followed by anti-inflammatory and antidiabetic evaluation. The results revealed the presence of important phytochemical compounds such as polyphenol, flavonols, flavan-3-ols, hydroxycinnamic acids, helihumulone and others compounds. These compounds demonstrated strong antioxidant capacity (DPPH, FRAP, and TEAC) for both extracts. The ethanol extracts displayed a better antioxidant activity compared to aqueous extracts. Natural antioxidants are known to prevent diseases associated with OS, the antioxidant activity of *H. cymosum* can be associated to the high polyphenols present (Kifle and Enyew, 2020, Akinyede et al., 2021a). The compounds demonstrated an important role in mitigating OS diseases and are known for stabilizing OS by scavenging free radicals and converting it to more stable and less reactive form (Akinyede et al., 2021a, Jadalla et al., 2022). The extracts were also able to inhibit carbohydrate hydrolyzing enzymes (alpha amylase and alpha glucosidase inhibition), and strong glucose uptake and its utilization. The modulatory effect carbohydrate metabolism is due to the presence of high polyphenols contained in *H. cymosum* extracts (Abifarin et al., 2021, Olaokun et al., 2017). From this study, the lower concentrations of AQ and ET extracts exhibited potent glucose lowering potentials than higher concentrations. The result showed that the high concentration could be linked to the observed toxicity at this concentration. The ethanol extracts exhibited anti-inflammation potential by interfering in the production of nitric oxide.

The second part of this study was carried out using zinc oxide nanoparticles synthesized from aqueous extracts of *H. cymosum*, the findings was reported in chapter 4. The synthesized ZnONPs were characterized by analyzing the following: UV-vis spectroscopy, Scanning electron microscope (SEM), Energy dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD), Transmission electron microscope (TEM), and zeta potential. The current study reveal a successful formation of ZnONP at a maximum wavelength of 360nm, similar peak have been reported by (Ifeanyichukwu et al. 2020). The SEM results showed a cluster of irregular and hexagonal shapes with an average size dimension range of 28.05 - 58.3 nm obtained from TEM analysis. The EDX analysis identify the presence of Zn, O, and C, the additional C peak could have resulted from the phytochemical compounds capping the ZnONPs (Bayrami et al., 2020).The zeta potential showed an excellent dispersion stability of nanofluids at -20.8 mV. The study also uncovered important functional groups such as alkanes, amines, carboxylic acids and phenol from FTIR analysis. These functional groups are crucial role in the formation and stabilization of the ZnONP. (Ochieng et al. 2015; Ifeanyichukwu et al. 2020; Aldeen et al. 2022). The XRD diffraction patterns showed superior crystalline quality of the nanoparticles, which correspond to earlier reported (Bhattacharya et al. 2020; Obeizi et al. 2020; Alrajhi et al. 2023; Rashwan et al. 2022).

After characterization, the antidiabetic potential was assessed using enzymatic assays α-glucosidase and α-amylase and pancreatic lipase inhibition. The absorption of glucose was also determined via glucose uptake and its utilization assays on C3A hepatocytes cells and L6 myocytes cells. These enzymes play a crucial role in carbohydrate metabolism and inhibition of its activities is very necessary in mitigating postprandial hyperglycemia (Rehana et al. 2017). An effective inhibition of α-glucosidase and α-amylase and pancreatic lipase by ZnONPs was demonstrated in this study. However, the inhibitory effects of alpha glucosidase were highly effective at lower concentrations compared to the higher concentrations. The inhibitory effects of the enzymes could be associated to important phytochemicals such polyphenols and flavonoids capping the ZnoNPs (Kifle and Enyew 2020). The high lipase activity shown is in accordance to study conducted by (Meer et al. 2022). A notable glucose uptake and its utilization by the cells was shown by the ZnONP compared to the control, which is indicative of its ability to maintain a stable glucose metabolism (Chadt and Al-Hasani 2020). Hence, our study agrees with other reports on ZnONP

how it could potentially enhance glucose transporter (GLUT-4) and increase β-cell proliferation (Asani et al. 2017; Virgen-Ortiz et al. 2020). The anti-inflammatory activity assed was based on the ability to reduce nitrite concentration in response to LPs activation of RAW macrophage, which shows a substantial reduction, however cytotoxicity was also noted which interfered with the interpretation of the results. Similarly, the CAA activity was elevated at a higher concentration than lower concentration.

The third part of this study investigated the cytotoxic effects and antioxidant activities of *H.cymosum* aqueous and ethanol extracts on TM4 Sertoli cell lines which is presented in chapter 5. The findings showed that both extracts and treatment duration maintain cell viability compared to the negative control, however a significant reduction was observed by the positive control compared to all the treatments for 24 h period. For the 48 hours treatment, a slight but significant reduction was observed for both extracts, except at the lowest concentration 3.75 µg/mL that exhibited no change compared to the control. Besides, the slight reduction of treatment compared to the negative control, treatments still maintain higher cell viability when compared to the positive control. The cell viability is in accordance with morphological observation**.**

The following antioxidant parameters SOD, GSH, ROS, and TAC were evaluated in current research. The SOD activity showed no significant difference in SOD activity for all treatments and its duration compared to the control. Contrary to our study, Moore and Makola, (2020) reported an increase in SOD activity by 90–50% after treatment with *Solanum sisymbriifolium* extracts. For the GSH level, our findings presented a minor increase for AQ extracts within 24 and 48 h period and ETOH 24 h, however the ETOH 48 h showed a concentration-dependent decrease with a significant reduction observed at the higher concentrations (60 and 120 µg/mL). Also extracts showed an increase in TAC analysis at lower concentration, although statistical difference was not shown. The ROS activity measurement showed a significant reduction in ROS by the extracts compared to the positive control and negative control at all treatment times. The findings showed that *H. cymosum* have a positive influence on antioxidant activity by reducing ROS levels and minor elevation of GSH, although no significant elevation was observed in SOD and TAC, suggesting that H. cymosum extracts requires cofactors such as copper, iron and manganese to exert its full catalytic potential when metabolizing free radical (Zheng et al., 2023). Nevertheless,

the bioactive constituents present in the extracts exhibited protective effect on TM4 Sertoli cells against oxidative stress damage (Nantia et al., 2009; Olayibi et al., 2020).

6.2. General conclusion

The present study investigated 3 samples; aqueous extract, 70% ethanol extract and synthesized ZnONPs obtained from the shoots of *Helichrysum cymosum*. The findings identified valuable bioactive compounds posing excellent antioxidant capacity. All samples effectively inhibited major carbohydrate digestive enzymes, suggestive of a good antidiabetic agent that could be employed in herbal remedies to mitigate and treat type 2 diabetes and associated complications. However, toxicity measures should be considered when using the samples at higher concentrations of ethanol and ZnONPs. The findings also revealed that both extracts (AQ and ETOH) are promising herbal agent that can be explored and used to modulate male subfertility.

6.3. Recommendations

This study shed light on *H.cymosum* plants, DM and male reproduction, providing valuable insight to understand their mechanism of actions. Our study suggest further investigation In vivo and clinical surveys in human clinical surveys, in order to fully comprehend and validate its antidiabetic capabilities, and potential treatment option for type 2 diabetes. Additionally, the study suggests further investigation on underlying biological mechanism such as sperm quality, DNA fragmentation, testosterone levels and others antioxidant parameters contributing to male infertility, beyond this findings in order to uncover new insights and potential therapeutic target exerted by *H.cymosum*.

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