Cape Peninsula
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THE ROLE OF GOLD NANOPARTICLES SYNTHESIZED USING EXTRACTS OF *CYCLOPIA INTERMEDIA* INTHE ANTICANCER EFFICACY OF DOXORUBICIN

By

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DECLARATION

I, Jumoke Adebisi Aboyewa, declare that the contents of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Jumoke Adebisi Aboyewa _____ **19/03/2022**

Signed **Date**

ABSTRACT

Colorectal cancer (CRC) is the most common type of gastrointestinal cancer affecting both developed and developing countries. It is a multifactorial disease process with etiology encompassing genetic factors, environmental exposure (including diet), and inflammatory conditions of the digestive tract. Doxorubicin (DOX) is a potent chemotherapeutic drug used for the treatment of different types of cancer but CRCs are nearly unaffected by treatment with DOX. Its use as an adjuvant for the treatment of CRC has been developed; however, adverse effects including cardiotoxicity and nephrotoxicity persist. Given the adverse health risks associated with chemotherapy and other conventional treatment modalities, there is a great need for new, affordable, and effective drugs for CRC treatment. Current research has widely revealed the fundamental role of medicinal plants in CRC therapy. C*yclopia intermedia*, commonly known as Honeybush (HB) is an indigenous South African shrub used to make the popular HB herbal teas which have many health benefits. HB has been reported to possess antioxidant, anti-obesity, antidiabetic, anticancer, and antimicrobial properties. Traditionally, the infusion made from HB leaves is used for the treatment of infections, coughs, sore throat, colds, osteoporosis, prevention of cancer, and asthma. HB has been shown to contain high concentration of monomeric polyphenols and flavonol compounds with mangiferin (MGF) being the most abundant, contributing majorly to the plants' antioxidant and anticancer effects. Moreover, the overall acceptance of medicinal plants and the complexity of phytochemicals present in plants have led to their utilization in the synthesis of biogenic nanoparticles (NPs). Biogenic NPs synthesized from medicinal plant materials often exhibit new or improved properties compared to their bulk plant extracts. The improved bioactivity displayed by biogenic NPs is probably due to the enhanced stability of the bioactive compounds present within the NPs, and increased surface area of NPs. Due to the size (1-100 nm) of the NPs, therapeutic drugs could be encapsulated within the NPs, giving rise to improved delivery and therapeutic effect of drugs at the target cells. This study, therefore, was performed to investigate whether gold NPs synthesized using water extract of HB and MGF would enhance the anticancer effect of DOX in human colon (Caco-2) cancer cell lines.

The first part of the study investigated and compared the phytochemical, total polyphenolic contents (TPC), and antioxidant capacity of fermented HB (FHB) and green/unfermented HB (GHB) plant to determine if these extracts have the reducing capacity to produce biogenic AuNPs. The result revealed that both the GHB and FHB plants exhibited significant phytochemical and antioxidant contents. Further, the phytochemical and antioxidant contents of

GHB and FHB plants were leveraged for the reduction gold salt for the synthesis of gold nanoparticles (AuNPs). While the synthesis of AuNPs from MGF was done following published protocol, the successful synthesis of AuNPs from the GHB and FHB plant extracts begins with the optimization of synthetic parameters such as temperature, reaction time, and concentration of plant extract was achieved. However, the AuNPs synthesized from the GHB plant were smaller in size and more stable compared to those made from the FHB. Therefore, the AuNPs prepared from the GHB plant were used for further studies.

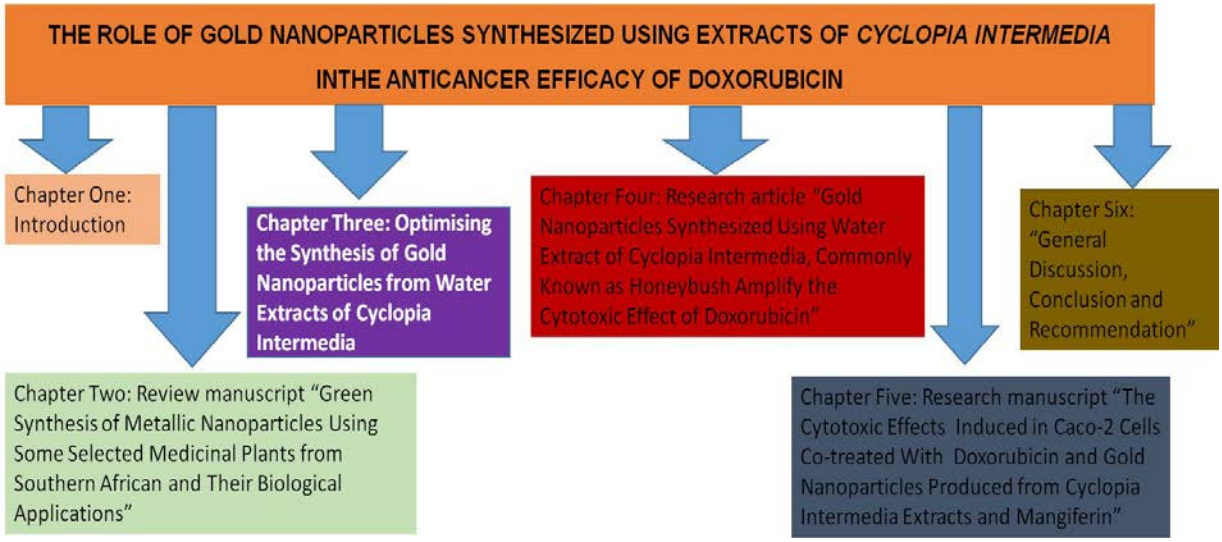
The synthesis of AuNPs using water extract of HB and MGF was investigated and the features of the produced biogenic AuNPs were compared. Although several studies have reported the successful synthesis of AuNPs using MGF, however, HB plant has not been previously used for the synthesis of AuNPs. The synthesis of AuNPs from HBE was done by mixing 2 mg/ml of the plant extract with 1 mM $NaAuCl_4.2H_2O$ at 70°C in a heating block shaking at 600 rpm. These conditions were obtained following the variation of conditions such as temperature, plant extract concentration, pH of plant extract, and reaction time. Besides, the synthesis of AuNPs using MGF was simply done following a previously described method. The biogenic AuNPs were characterized using UV-vis spectrophotometry, Dynamic Light Scattering (DLS), X-ray diffraction spectrophotometry (XRD), High-Resolution Transmission Electron Microscopy (HR-TEM), and Fourier Transform Infrared (FT-IR) spectroscopy. The comparative analysis of the biogenic AuNPs revealed that they exhibit quite similar features and thus, it is believed that MGF being abundantly present in HB plays a significant role in the synthesis of AuNPs from HB.

Further, the cytotoxicity of the biogenic AuNPs was assessed in human colon (Caco-2), glioblastoma (U87), and prostate (PC-3) cancer cells as well as in normal epithelial breast (MCF-12A) cells using cytotoxic MTT assay. Both biogenic AuNPs exhibited very low cytotoxicity against these cells. Besides, the cytotoxic effect of DOX on colon (Caco-2) cancer cells was investigated and the result showed that the cells were inherently resistant to low concentration of DOX (1.56-12.5 µg/ml). The cells only responded to higher doses (25-100 µg/ml) which could cause cardiotoxicity and nephrotoxicity. Although the effect of the biogenic AuNPs and DOX used individually at a concentration of 1000 µg/ml and 1.56 µg/ml, respectively showed a very low cytotoxic effect. However, when the AuNPs and DOX were combined, cytotoxicity was enhanced. This shows that in combination with DOX, the biogenic AuNPs significantly augmented the anticancer effects of DOX in Caco-2 cells leading to enhanced cell death.

Furthermore, the study investigated the combinatorial effect of both MGF-AuNPs and DOX (MGF-AuNPs-DOX) or HB-AuNPs and DOX (HB-AuNPs-DOX) and their possible underlying mechanisms in Caco-2 cells. Assays such as colony formation, adenosine triphosphate (ATP), reactive oxygen generation (ROS)**,** mitochondrial depolarization, and DNA fragmentation assays were used to determine the mechanistic and synergistic effects of the co-treatments in Caco-2 cells. The results showed that the intracellular ATP depletion was aggravated in Caco-2 cells treated with a combination of the biogenic AuNPs and DOX compared to individual treatment. However, treatment with the combination of the biogenic AuNPs and DOX resulted in increased mitochondrial depolarization compared to treatment with biogenic AuNPs and DOX alone. Also, the percentage ROS generation in cells treated with a combination of either MGF-AuNPs-DOX or HB-AuNPs-DOX was minimal but significantly higher compared to untreated Caco-2 cells. Also, the colony formation analysis shows that the combination of either MGF-AuNPs-DOX or HB-AuNPs-DOX inhibits the long-term survival of Caco-2 cells compared to untreated cells. Finally, the DNA fragmentation analysis of Caco-2 cells exposed to the combination of the biogenic AuNPs and DOX shows that the co-treatment enhanced the apoptotic effect of DOX, leading to increased cell death. Together, the findings from this study strongly suggest that the combination of biogenic AuNPs and DOX may be effective in the treatment of CRC, however, additional mechanistic and molecular studies are recommended to unravel the full potential of combination therapy.

PREFACE

This thesis consists of five chapters, and it is presented in an article-based format, the chapters are written according to the guidelines of the journals where they are published or submitted for review. The graphical representation of the thesis is given below;



- Chapter one gives a brief introduction of the study.
- Chapter two (literature review) is written in the form of a review article and has been published in "Plants". The review provides an overview of the biological applications of metallic nanoparticles synthesized using Southern African medicinal plants.
- Chapter 3 describes the optimization experiment that led to achieving the successful synthesis of AuNPs from both GHB and FHB plants. It encompasses the effect of synthestic parameters including temperature, pH, concentration of plant extracts and reaction time.

- Chapters four and five are written as research articles which present the results of the experimental investigations performed in this study as shown in the chart above. Chapter four has been published in “Nanomaterials”. Chapter five is intended to be submitted for publication in “Molecules” and have been formatted accordingly.
- Chapter six is a general discussion, conclusion and recommendation of the study as a whole.

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DEDICATION

This thesis is dedicated firstly to the Almighty God, for it is by Him this work was made possible, and secondly to my father Rev Afolabi Aboyewa, who didn't think that "educating a female child is a waste of money".

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

ABBREVIATION

AgNPs	Silver nanoparticles
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
Au	Gold
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
CIMP	Cytosine preceding guanine island methylator phenotype
CIN	Chromosomal instability
CpG	Cytosine preceding guanine
CRC	Colorectal cancer
DLS	Dynamic light scattering
DMEM	Dulbesco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DPBS	Dulbesco’s Phosphate Buffered Saline
DPPH	Di-phenyl-picrylhydrazyl
EDX	Energy dispersive X-ray
FAP	Familial adenomatous polyposis
FDA	Food and drug agency
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
FT-IR	Fourier transform infra-red

GA	Gum Arabic
HB	Honeybush
HB-AuNPs	Honeybush gold nanoparticles
HB-AuNPs-DOX	Honeybush gold nanoparticles mixed with doxorubicin
HBE	Honeybush extract
HNPCC	Hereditary non-polyposis colorectal cancer
HPLC	High performance liquid chromatography
HPS	Hemartomatous polyposis syndrome
HR-TEM	High resolution-transmission electron microscopy
IARC	International Agency for Research in Cancer
IBD	Inflammatory bowel disorders
KCl	Potassium chloride
MGF	Mangiferin
MGF-AuNPs	Mangiferin gold nanoparticles
MGF-AuNPs-DOX	Mangiferin gold nanoparticles mixed with doxorubicin
MMR	Mismatch repair
MNPs	Metallic nanoparticles
MSI	Microsatellite instability
nm	nanometer
NPs	Nanoparticles
ORAC	Oxygen radical antioxidant capacity
PDI	Polydispersity index
ROS	Reactive oxygen Specie
RPMI	Roswell Park Memorial Institute

SAED	Selected area diffraction
TMRE	Tetramethyl rhodamine ethyl ester
TPC	Total antioxidant contents
UV-Vis	Ultraviolet visible
WHO	World Health Organization
XRD	X-ray diffraction
ZnO	Zinc oxide

DEFINITION OF TERMS

Antioxidants: They are synthetic or natural substances that can scavenge and neutralise free radicals.

Apoptosis: Is a form of programmed cell death that occurs in multicellular organisms. It is used to rid the body of cells that have been damaged beyond repair.

Cancer: It is a broad term. It describes the disease that results when cellular changes cause the uncontrolled growth and division of cells. Some types of cancer cause rapid cell growth, while others cause cells to grow and divide at a slower rate.

Carcinogens: They are substances or factors that can cause cancer.

Cytotoxicity: The toxic effect or quality that a substance presents to the cells.

Green nanotechnology: It is the branch of nanotechnology that utilizes the concepts of green chemistry and green engineering.

Nanoparticles: They are small particles that range between 1 to 100 nm in size.

Nanotechnology: It refers to the manipulation and manufacture of materials and devices at dimensions between 1 and 100 nm.

Phytochemicals: They group of chemicals derived from biological resources especially plant origin. They generally have biological activity in the plant host and play a protective role against diseases.

Reactive oxygen species: They are products of normal cellular metabolism. They are highly reactive chemicals that contain oxygen.

CHAPTER ONE

INTRODUCTION

1.1. Statement of the research problem

Cancer is a multifactorial disease affecting people in both developed and developing countries worldwide. After heart disease, cancer ranks as the second most common cause of death globally (Wolf *et al*., 2018; Bray *et al*., 2018). Despite the substantial advances made in early detection, prevention, and treatment of cancer, new cases and death rates remain high. This challenge in addition to the suffering associated with cancer, rigorous efforts have been prompted to combat this dreaded disease. Conventional treatment incorporating surgery, chemotherapy, and radiotherapy are labelled ineffective due to lack of specificity and associated adverse effects (Torres *et al*., 2018). Thus, the quest for optimal safety and effectiveness of conventional treatment, particularly chemotherapy has recently opened new avenues in nanomedicine. The nanomedicine approach seeks to implement an innovative carrier system to deliver therapeutic agents at diseased targeted sites. In this course, therapeuticis delivered in an orderly manner to target diseased cells without having contact with normal dividing/surrounding cells. The use of nanomaterials, including metallic NPs (MNPs), as carriers/delivery of therapeutics has drawn enormous attention in the last few decades. This nano-delivery approach has been a flourishing strategy for cancer treatment, serving as a critical platform to implement a combined therapy system in a more efficient, more effective, and safer manner. Therefore, this study details the combined anticancer effects of *Cyclopia intermedia* and doxorubicin (DOX) using green nanotechnology and biochemical methodologies. Consequently, the combined technology is perceived to be a safe, affordable, and effective therapy with the promising potential perspective that may reduce the prevalence and menace of cancer.

1.2. Background

Cancer is a multifactorial disorder characterized by complex modification in the genome that arises due to the interactions between the host and the environment (Prager *et al*., 2018; Plummer *et al*., 2016). Cancer cells are known for exhibiting dysfunctional characters such as unresponsiveness to growth signals, uncontrolled cell replication, apoptosis bypass, prolonged angiogenesis, and metastatic (Fouad & Aanei, 2017; Weir *et al*., 2016). The exact mechanism

by which any type of cancer develops is not understood, and the quest for the causes of cancer remains a puzzle. An international symposium organized by the World Health Organization (WHO) in 1950 revealed differences in cancer types around the world. In that symposium, it was discovered that most cancer types are caused by continuous exposure to environmental contaminants, while few develop due to inherited genetic factors (Parsa, 2012). This finding and many more led to establishing the International Agency for Research in Cancer (IARC) in 1965. IARC is a body instructed to conduct multidisciplinary studies on human cancer causes by evaluating epidemiological and experimental evidence.

Evidence has shown that the development and progression of cancer depend on many intrinsic factors within the host's cell (mutations, immune conditions, and hormones) and external factors (chemicals, smoking, drugs, viruses, radiation, and sedentary lifestyles) from the environment. Tobacco smoke and chemicals, including carbon tetrachloride, benzene, vinyl chloride, asbestos, and polychlorinated compounds, are well-known carcinogens. Also, radiation and infectious agents such as viruses and bacteria are cancer inducers, exhibiting different induction mechanisms. Additionally, tobacco smoke, aflatoxin, and radiation are initiating agents. They act by damaging DNA which induces mutations in essential target genes, leading to the development of cancer (Barnes *et al*., 2018). Other carcinogens like phorbol esters and some hormones, particularly estrogen are tumour promoters, which act by stimulating cell proliferation. Estrogen stimulates the development of certain human cancers, including endometrial cancer (Rodriguez *et al*., 2019; Parsa, 2012). These factors combined causes normal cells to lose control mechanisms by proliferating in an uncontrolled manner, producing abnormal cells. The unusually formed cells typically referred to as tumours, grow and affect normal tissue in their locations (benign) and travel through the lymphatic vessels to invade other localities in the body (malignant). This abnormality can appear at any part of the body contributing to the over a hundred types of cancer identified with substantial variation in their morphology and response to treatment.

Colorectal cancer (CRC) is a term that combines colon and rectal cancer. It is the second most deadly and third most commonly diagnosed malignancy globally (Rawla *et al*., 2019). CRC accounted for approximately 1.8 million new cases in 2018, out of which colon cancer accounted for 1,096,000 and rectal cancers, 704,000 (Rawla *et al*., 2019). Like other solid

tumours, CRC is a sporadic or hereditary disease with multiple subtypes classified on their basis of clinical and molecular presentations (Nguyen & Duong, 2018). Approximately 85% of sporadic CRC are characterized by chromosomal instability (CIN) that results from variations in chromosome number and structure (Martínez-A & Van Wely, 2010). These alterations affect the expression of oncogenes and tumour-suppressor genes, which in turn, may activate pathways leading to CRC initiation and progression. The remaining cases of sporadic CRC (15%) exhibit microsatellite instability (MSI) phenotypes that arise due to a defective DNA mismatch repair (Boland & Goel, 2010). The hereditary CRC is distinguished into individuals having either an inherited mutated copy of Adenomatous polyposis coli (APC) (<1%) or hereditary non-polyposis colorectal cancer (HNPCC) (1-3%) or the rare hamartomatous polyposis syndrome (HPS) (<1%) and or those with less penetrant inherited mutations (32%) (Nguyen & Duong, 2018). Although the exact causes of CRC are still unknown, certain factors such as ageing, personal/family history, behaviours, and sedentary lifestyles, as well as tobacco smoke, and specific diets, play prominent roles in CRC development (Haggar *et al*., 2009). CRC is formed when normal glandular epithelial cells are transformed through a series of genetic and epigenetic mutations into a benign adenoma, which may eventually evolve into invasive and metastatic carcinoma (Nguyen & Duong, 2018). According to this sequence of events, at least seven specific mutations are required to develop CRC.

Since the discovery of cancer, the study and development of approaches for effective treatment have become most researchers' hallmarks. Presently, over 60% of global treatment trials with high medical quality concentrate on cancer (Abbas & Rehman, 2018). Although several significant attempts for cancer treatment have been tried, the differential exhibition and metastatic nature of the disease have strained their success (de Sousa Monteiro *et al*., 2014). The use of surgery, chemotherapy, immunotherapy, radiotherapy, and stem cell transplant are the most popular for CRC treatment (Tariq & Ghias, 2016). Moreover, chemotherapeutic drugs, including cisplatin, oxaliplatin, vinblastine, methotrexate, and DOX, have been used to treat various types of cancer. DOX is routinely used for treating several types of cancer, including breast, lung, bladder, stomach, and ovarian cancer (Patra *et al*., 2018; Qi *et al*., 2017). The exact mechanism of action of DOX is complex and not fully understood. However, DOX acts by either intercalating into DNA, inhibiting DNA repair systems or through the generation of free radicals, and disrupting macromolecular biosynthesis (Thorn *et al*., 2011). Despite its wide-spectrum and possible antineoplastic activity, adverse effects, including cardiotoxicity and

nephrotoxicity, persist, thus, limiting the use of DOX in clinical investigation. In general, chemotherapeutic drugs are incorporated with limitations owing to their cost, inability to distinguish cancer cells from non-cancer cells, structural and functional aberrations in healthy cells, increased risk of infection, and allergy (Qi *et al*., 2017). Other side effects include cancer resurgence, fever, pain, swelling, soreness, itchiness, and rash (Qi *et al*., 2017).

Consequently, these limitations ushered in natural therapies as an alternative intervention for cancer treatment (Aiello *et al*., 2019). Herbal extracts have antioxidant compounds such as alkaloids, terpenoids, flavonoids, pigments, and tannins which have higher efficacy and lesser side effects (Aiello *et al*., 2019). These bioactive compounds have been shown to elicit desirable anticancer activity against several types of cancer (Aiello *et al*., 2019; Akay & Yılmaz, 2018). Although the specific mechanism by which they carry out this role is not currently understood. Several tools such as induction of apoptosis, inhibition of cell proliferation, suppression of cancer stimulating enzymes, repair of deoxyribonucleic acids (DNA), stimulating the production of antitumor enzymes, increase in the body defence system and induction of antioxidant effects have been identified (Ajuwon *et al*., 2018; De Sousa Monteiro *et al*., 2014; Mahomoodally, 2013). Some of the plants reported for anticancer activities include *Achillea wilhelmsii*, *Allium sativum L*., *Ammi majus*, *Ammi visnaga*, *Artemis absinthium L., Astragalus cytisus, Astrodaucus orientalis, Avicennia marina, Boswellia serrata, and Cyclopia intermedia*.

Cyclopia intermedia, commonly known as Honeybush (HB), is a South African herbal plant used to make the popular HB herbal tea known for numerous health benefits. HB plant is characterized by woody stems, light yellow flowers, long needle-like or short leaves with a honey-like smell (Joubert *et al*., 2011). HB is known to improve the immune system, protect against inflammatory diseases and offer menopausal relief with phytoestrogenic effects (Magcwebeba *et al*., 2016; Marnewick *et al*., 2009). Moreover, HB has antimicrobial activity, therefore inhibits microbial growth (Magcwebeba *et al*., 2016; Marnewick *et al*., 2009). The anticancer effect of HB through modulation of oxidative stress, inhibition of cell proliferation, and adenosine triphosphate (ATP) production has also been reported (Magcwebeba *et al*., 2016; Marnewick *et al*., 2009). HB contains high concentrations of monomeric polyphenols and flavonol compounds with mangiferin (MGF) and hesperidin identified as the most abundant (Magcwebeba *et al*., 2016). MGF ($C_{19}H_{18}O_{11}$) is an important bioactive compound found

abundantly in different parts of *Mangifera indica* and HB, thus influencing their therapeutic properties. MGF has been extensively studied and reported for its anti-carcinogenic, anti-inflammatory, and anti-oxidative properties (Joubert &de Beer, 2012; Joubert *et al*., 2011; Steenkamp *et al*., 2004). Hence, the chemotherapeutic and chemo-preventative properties of MGF, which is attributed to it antioxidant properties offer its continuous inclusion in natural medicines.

Figure 1.1. *Cyclopia intermedia*

The historical appraisal of natural therapies as remedies for treating most diseases is demonstrated as over 60% of currently used anticancer drugs are derived from natural sources (Cragg & Pezzuto, 2016; Newman & Cragg, 2012). Although the advent of this alternative therapy has undeniably improved cancer patients' care, however, advanced metastases remain untreatable (Wang *et al*., 2012). Moreover, the confusion of normal dividing cells for cancer cells, resistance of cancer cells due to constant mutation, and metastasis also contribute to the reduced efficacy of natural therapies. Hence, the continued quest for cheap yet safe and more effective treatment is the hallmark of the research community. Therefore, cancer research focuses on developing a new treatment regimen for the early and advanced stages of the

disease while working on how best to use existing treatments including chemotherapy for optimal effect.

In the last few decades, combination therapy in clinical practice which harnesses the synergistic, additive, or antagonistic potentials of anticancer agents has been underway. This approach recently ushered in nanomedicine with the advantage of applying nanotechnology techniques to improve the efficacies of anticancer drugs (Jeevanandam *et al*., 2018). Nanotechnology is an essential aspect of science that relies on the synthesis, modification, modulation, and use of materials at the nanometer scale for advanced biotechnology. NPs (NPs) have attracted much interest due to their distinct physicochemical, optical, magnetic, and biological properties (Milaneze *et al*., 2016). They have been successfully employed to deliver bulky size material to solve challenges such as poor bioavailability, low solubility, lack of targeted delivery, and generalized side effects. The application of nanotechnology for drug delivery has opened avenues for possible advances in various diseases, including cancer**.** Recent work has reported an improved efficacy in treatment accomplished by the combinatorial effect of NPs having anticancer properties with chemotherapeutic drugs (Aboyewa *et al*., 2020). Among the well-studied NPs, inorganic NPs, particularly gold NPs (AuNPs), have been reportedly used in combination with anticancer drugs limited by severe side effects.

1.3. The rationale for this study

CRC is a common malignancy in all populations, accounting for approximately 10% of cancer-related death in western countries. Although there has been an increased access to adequate healthcare and screening programs, increasing incidence and high mortality persist in developing countries. The overwhelming impact of CRC management on the economy, symptoms of patients and the effect on the quality of life necessitate this research. Although DOX is a potent and valuable clinical anticancer agent, associated side effects are the major problems limiting its use. CRC is inherently resistant to DOX, and higher doses are required to inhibit their growth effectively. This may result in adverse side effects, including cardiotoxicity and nephrotoxicity. Hence, it is rational to develop alternative treatment modality that will improve the pharmacokinetics and reduce the severe toxicities associated with DOX. NPs, especially those of biological origin in combination with chemotherapeutic drugs can enhance the activity of the later, offering promising improvement in cancer treatment. In this regard,

multiple pathways are targeted for effective treatment of diseased cells. With this approach, the synergic anticancer values of biogenic NPs and anticancer drugs would be explored to full potential. The study, therefore, investigated the efficacy of biogenic AuNPs synthesized using water extract of *Cyclopia intermedia* and MGF combined with DOX as enhanced anticancer agents in CRC.

1.4. Aim

The research study was conducted to investigate the probable synergistic anticancer effect of AuNPs synthesized using water extract of HB and DOX in an i*n vitro* model of CRC treatment. The research study also investigated the mechanism of action of the combination therapy in human colorectal Caco-2 cells through the following;

- Intracellular adenosine triphosphate (ATP)
- Generation of reactive oxygen species (ROS)
- Mitochondrial depolarization
- Cellular uptake and internalization
- Clonogenicity
- DNA fragmentation

1.5. Objective

In an attempt to achieve the stipulated aim mentioned above, the following specific objectives were met:

- To quantify the phytochemical and antioxidant potential of aqueous leaf extracts of HB.
- To synthesis, characterize and evaluate the stability of biogenic AuNPs from water extract of HB.
- To prepare biogenic HB-AuNPs and DOX mixture and evaluate their stability in different biological media.
- To investigate the viability and cytotoxicity of prepared HB-AuNPs-DOX in Caco-2 cells and the levels of ATP and ROS generated.
- To quantify the amount of biogenic AuNPs taken up by Caco-2 cells.
- To investigate the effect of HB-AuNPs-DOX on the mitochondrial membrane status, apoptosis, and long-term survival of Caco-2 cells.

1.6. Research questions

- Will the water extract of HB successfully reduce Au^{3+} in the synthesis of HB-AuNPs?
- What are the characteristic features/morphologies of synthesized HB-AuNPs?
- Will the synthesized biogenic HB-AuNPs be stable in biological media?
- Will the biogenic HB-AuNPs be cytotoxic to Caco-2 cells?
- Will the biogenic HB-AuNPs enhance the cytotoxic effect of DOX in Caco-2 cells?
- Will the biogenic HB-AuNPs be effectively taken up and internalized in Caco-2 cells?
- Will the combination of the biogenic HB-AuNPs and DOX affect the levels of ATP in Caco-2 cells?
- Will the combination of the biogenic HB-AuNPs and DOX generate ROS in Caco-2 cells?
- Will the combination of the biogenic HB-AuNPs and DOX affect the mitochondrial membrane status of Caco-2 cells?
- Will the combination of the biogenic HB-AuNPs and DOX affect the long survival rate of Caco-2 cells?
- Will the biogenic HB-AuNPs enhance the apoptotic effect of DOX in Caco-2 cells?

1.7. Hypothesis

H_0: Biogenic HB-AuNPs will not enhance the therapeutic effect of DOX and will not generate a synergetic reductive effect on the proliferation of Caco-2 cells.

H_1: Biogenic HB-AuNPs will enhance the therapeutic effect of DOX and generate a synergetic reductive effect on the proliferation of Caco-2 cells.

1.8. Significance of the study

This study provides insight into a system that harnessed the probable synergistic effects of biogenic AuNPs and DOX in inhibiting the growth of Caco-2 cells. It also provides information on the mechanism of action of the co-treatment in Caco-2 cells. The research findings will proffer knowledge on how medicinal plants can be used to fabricate MNPs for the delivery of traditional chemotherapeutics with enhanced effects on cancer with little or no side effects. The

information generated from this research will be of relevance to different sectors, including the pharmaceutical industry, medicinal plant research, traditional healers, and independent researchers. The biological activities of the combined therapy might lead to the formation of ground-breaking alternative/ novel drug formulations for cancer treatment that could contribute to effective and enhanced treatment, thus improving the quality of life. This study's research outputs include published manuscripts in various reputable, peer-reviewed journals of high impact factors. Outcomes from this research were also presented at workshops and seminars, which contributed immensely to the pool of scientific knowledge in cancer research.

1.9. Ethical consideration

Ethical approval for this study was obtained from the Research Ethics Committee of the Faculty of Health and Wellness Sciences, the Cape Peninsula University of Technology, South Africa (CPUT/HW-REC 2019/H12).

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CHAPTER TWO: MANUSCRIPT

GREEN SYNTHESIS OF METALLIC NANOPARTICLES USING SOME SELECTED MEDICINAL PLANTS FROM SOUTHERN AFRICA AND THEIR BIOLOGICAL APPLICATIONS

Chapter two is the literature review written in the form of a review article. This chapter provides an overview of the biological applications of metallic nanoparticles synthesized from Southern African medicinal plants. The review article has been published by “Plants”.

plants

MDPI

Review

Green Synthesis of Metallic Nanoparticles Using Some Selected Medicinal Plants from Southern Africa and Their Biological Applications

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Abstract: The application of metallic nanoparticles (MNPs), especially that of silver, gold, cobalt, and zinc as antimicrobial, anticancer, drug delivery, contrast, and bioimaging agents has transformed the field of medicine. Their functions, which are attributed to their physicochemical properties, have gained prominence in various technological fields. Although MNPs can be produced via rigorous physical and chemical techniques, in recent years, a biological approach utilizing natural materials has been developed. With the increasing enthusiasm for safe and efficient nanomaterials, the biological method incorporating microorganisms and plants is preferred over physical and chemical methods of nanoparticle synthesis. Of these bio-entities, plants have received great attention owing to their capability to reduce and stabilize MNPs in a single one-pot protocol. South Africa is home to ~10% of the world's plant species, making it a major contributor to the world's ecological scenery. Despite the documented contribution of South African plants, particularly in herbal medicine, very few of these plants have been explored for the synthesis of the noble MNPs. This paper provides a review of some important South African medicinal plants that have been utilized for the synthesis of MNPs. The enhanced biological properties of the biogenic MNPs attest to their relevance in medicine. In this endeavour, more of the African plant biodiversity must be explored for the synthesis of MNPs and be validated for their potential to be translated into future nanomedicine.

Keywords: green nanotechnology; metallic nanoparticles; medicinal plants; antimicrobial; cytotoxicity; green synthesis

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1. Introduction

Over the years, MNPs (at a size range between 1 and 100 nm) have fascinated scientists and are currently utilized in various fields not limited to medicine, agriculture, and engineering [1–3]. The widespread practical applications of nanomaterials are attributable to their unique optical, catalytic, electronic, and physical properties [4,5]. The synthesis of MNPs

with desired characteristics can be achieved via a variety of physical and chemical processes [6–8]. However, these methods are elaborate, expensive, time-consuming, and potentially hazardous to the environment and living organisms. The urgency to minimize the potential negative impacts of the nanomaterials synthesized via physical and chemical routes has led to the exploration of biological entities. The capability of microorganisms and plants to transform metal ions into MNPs revealed a simple, rapid, cost-effective, and eco-friendly approach for nanoparticle synthesis [9–11]. Microorganisms, including bacteria, fungi, and yeasts, can reduce metallic salts into nanoparticles (NPs) [12–14]. These microbes produce proteins, enzymes, reducing cofactors, peptides, and organic materials that play significant roles in the reduction of metallic salts into MNPs. These molecules can serve as either reducing and/or capping agents. In this regard, numerous microorganisms including *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* have been used for the synthesis of MNPs [13,15,16]. Plants are by far the most important biological components for MNP synthesis, as their universal abundance and lack of pathogenicity offer an advantage over other biological sources. Plant-mediated synthesis is relatively mild, eco-friendly, cost and time effective; with their phytochemicals and bioactive contents acting as reducing, capping, and stabilizing agents [17,18]. Consequently, natural resources are conserved, and, at the same time, opportunities are created for sustainable development. Numerous studies have reported the successful synthesis of biogenic NPs using different plant species [6,8,19]. At their nanoscale size, the NPs exhibit significantly different characteristics, as well as improved bioactivity, compared to their bulk counterpart [6,20]. Despite the increasing awareness and promising benefits reported for plant-mediated NPs, this area of research remains underexplored. Of a fact, more than 80% of the world's population rely on herbs/medicinal plant for treating diverse kinds of diseases, including diabetes, high blood pressure, cancer, and tuberculosis [21]. Moreover, nearly 60% of all synthetic drugs used for clinical purposes are derived from plants which further justify the significance of medicinal plants [22].

South Africa houses about 10% of the world's plant species, with over 3000 plants reported to have significant medicinal benefits [23]. Despite this biodiversity and the promising benefits of plant-mediated MNPs, the utilization of South African medicinal plants for the synthesis of MNPs is still largely underexplored [24]. Recently, indigenous South African plants including *Salvia africana-lutea* [6], *Sutherlandia frutescens* [6], *Galenia africana* [25], *Catharanthus roseus* [26], *Hypoxis hemerocallidea* [27], *Cotyledon orbiculata* [28], and *Aspalathus linearis* [29] were utilized for the fabrication of MNPs. These MNPs with sizes ranging from 5–50 nm were reported to exhibit high antibacterial activities compared to their respective plant extracts. Therefore, considering the vast potential of plants as alternative sources of reducing agents with enhanced bioactivities, continuous research towards the exploration of South African medicinal plant reserves will be of paramount importance.

The present review provides a brief overview of some potent South African medicinal plants utilized for nanoparticle synthesis. Characterizations, as well as the biological applications of the biogenic MNPs, were highlighted. With this update, rigorous research could be directed towards African medicinal plants to produce natural and effective nanoproducts that will revolutionize many technologies and industries, including pharmaceuticals, food, cosmetics, construction, medicine, engineering, and many others.

2. MNPs and Their Application

Nanotechnology is an important aspect of science that relies on the synthesis, modification, modulation, and application of materials within the nanometer scale (1–100 nm) [30–32]. NPs have attracted much interest in the last decades due to their distinct physicochemical, optical, magnetic, and biological properties [30]. Their properties, predominantly convened by their large surface area to volume ratio and size, are absent in bulk materials. Consequently, these unique properties are explored for application in environmental, water, food, biomedical, and space industries [4,33]. MNPs particularly

those of noble metals such as silver and gold NPs have been widely explored in various biomedical fields [34] not limited to tissue engineering, health care, drug delivery, and gene delivery [34–36]. This section will give a brief overview of some of the most widely used MNPs and their application.

2.1. Silver NPs (AgNPs)

AgNPs have drawn considerable research interest due to their superior physical, chemical, and biological characteristics [26,37,38]. Rigorous efforts have been made to explore their integral properties for practical and clinical applications, particularly, as therapeutic and diagnostic agents [27,28]. Previous and recent discoveries have shown that AgNPs exhibit interesting antimicrobial effects against a wide range of microorganisms [6,28]. AgNPs are thus used in water purification and wound dressing as antimicrobial agents [35]. Their use for the production of paints, disinfectants, and some kitchen appliances has also been reported [37,39]. The therapeutic applications of AgNPs in terms of their antiviral, antifungal, anticancer, and antibacterial properties have also been demonstrated [6,27,28]. Currently, AgNPs are commercialized as antimicrobial agents in the pharmaceutical and cosmetic industries and are also utilized to protect against infections in various medical implants or bone cement [40,41].

2.2. Gold NPs (AuNPs)

AuNPs, on the other, hand have attracted significant interest over the last decades owing to their optical and chemical properties. Their potential as diagnostic and therapeutic agents in a variety of medical fields has been documented [42–44]. AuNPs are used as drug carrier/delivery, bioimaging, contrast, photothermal, and anti-angiogenic agents [45,46]. AuNPs were investigated for antibacterial activity and found to display an effective antibacterial effect against gram-positive and gram-negative bacterial strains [47–50]. AuNPs can ferry and deliver hydrophobic and hydrophilic drugs, peptides, antibodies, and small molecule drugs to the targeted tumour site with no toxic effects on normal or surrounding tissues [51]. Moreover, AuNPs provide a platform to attach multiple moieties on their surface; therefore, they are suitable as drug delivery agents [4,52]. AuNPs can be functionalized with therapeutic moieties to exhibit enhanced anticancer effects [51,53]. Additionally, strategies that involve attaching targeted peptides that would possibly target receptors that are exclusively expressed by diseased cells have been reported [53]. AuNPs-conjugates were reported to significantly enhance the selectivity as well as the sensitivity of therapeutic peptides on pancreatic (Panc-1) and colon (Caco-2) cancer cells [53,54]. According to previous and recent studies, biosynthesized AuNPs have achieved significant targeting and selectivity against a variety of cancer cells without any additional molecules [19]. A study by Majoumouo *et al*. [19] demonstrated that the AuNPs synthesized using *Terminalia mantaly* exhibited enhanced cytotoxicity and selectivity on Caco-2, epithelial breast (MCF-7), and liver (HepG2) cancer cells. Another study by Anadozie and co-workers [55] revealed that AuNPs synthesized from water extract of *Xylopia aethiopica* showed excellent anticancer activity on MCF-7 and Caco-2 cells. These reports and many more have opened a limitless opportunity towards the incorporation of AuNPs in molecular diagnostics and therapy.

2.3. Metal Oxide NPs

Copper and copper (II) oxide NPs have found diverse applications in physics and material science engineering. They are strong antimicrobial agents exhibiting excellent disinfecting properties against several infectious organisms [56]. The application of iron oxide NPs in many biomedical applications like gene therapy, stem cells, cancer, and atherosclerosis has been reported [57]. These NPs have been developed as anticancer, antifungal, antimicrobial, and targeted drug delivery agents. Zinc oxide (ZnO) NPs are popularly known for their antimicrobial and anticancer activities. They have been found useful

in food packaging, wastewater treatments, and some personal care products [13]. ZnO NPs were used as preservatives in food packaging to prevent and inhibit the growth of microbes on food materials. Moreover, ZnO NPs proffer toxicity against microorganisms compared to all other metal oxides NPs.

3. Synthesis of MNPs

Consequent to the continuous utility of MNPs in several modern-day applications, the method for their preparation needs to be safe to achieve better control of desirable physicochemical and bio-functional properties. Several methods and modifications have been explored for their synthesis [14]. Over the years, NPs have continuously been used and modified to enable their application in various fields ranging from agriculture and biomedicine. The techniques developed for the synthesis of NPs are classified as top-down and bottom-up. The top-down approach reduces bulk material of interest to NPs, while the bottom-up approach build-up smaller material into the required nanostructure [32]. Further, these techniques are classified into physical, chemical, and biological methods of NP synthesis as depicted in Figure 1. Physical methods mainly operate on a top-down strategy where bulk materials are systematically broken down bit by bit to produce finely divided NPs [4]. The physical methods rely on mechanical pressure, electrical and radiation energy, melting, evaporation, or condensation techniques to produce NPs. Examples include vapor condensation, aerosol, laser ablation, pyrolytic, high-energy ball milling, laser pyrolysis, inert gas condensation, and mechanical crushing processes [4]. The chemical methods involve the use of organic and inorganic substances, including sodium citrate, elemental hydrogen, sodium borohydride, hydrazine, dimethylformamide, and ascorbate as reducing agents for the synthesis of the NPs. Sol-gel, hydrothermal, chemical vapour deposition, microemulsion, and polyol techniques are the commonly used chemical methods [4].

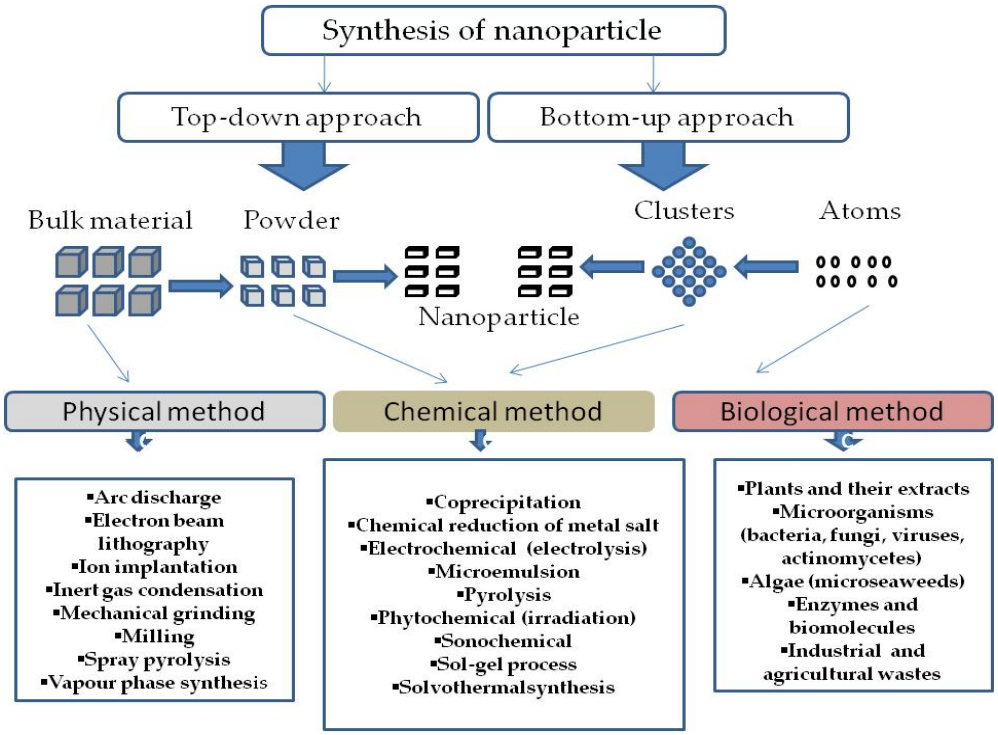

Figure 1. Methods used for the synthesis of MNPs. The different methods are classified as top-down and bottom-up. Adapted from [4].

Although the physical and chemical approaches are the most popular, they are costly, time-consuming, and require sophisticated working conditions. Moreover, their application, particularly in biomedicine, is threatened with toxicological effects on humans, animals, and the environment [58,59]. The search for non-toxic and environmentally friendly protocols which do not use hazardous chemicals and complicated physical techniques in NP synthesis has led to the exploration of biological entities. Thus, microorganisms and plants have emerged as green agents, providing eco-friendly routes for the synthesis of NPs.

3.1. The Advent of Green Nanotechnology

Green nanotechnology is an exciting and emerging area of science and technology that embraces the principles of green chemistry with potential benefits towards sustainability, protection, and overall safety of the human race [60]. The green chemistry methodology introduces a desirable approach to the synthesis, processing, and application of less hazardous chemicals to reduce threats to human health and the environment [61]. The approach requires an in-depth understanding of the raw materials, especially in terms of their fabrication into nanomaterials and the resulting bioactivities that pose little or no hazardous effects on humans and the environment. On this note, nanomaterials can be engineered from natural sources at our disposal with the assurance of minimized potential risks. The use of biological entities as reducing, capping, and stabilizing agents in the synthesis of MNPs is constantly gaining wide attention [25]. The method utilizes microorganisms, and plant products as reducing agents [11], presenting benign and eco-friendly conditions that can improve safety in humans and animals [31].

The exploration of microorganisms such as bacteria, fungi, algae, and viruses as reducing agents for MNP synthesis is becoming more popular among natural product researchers. Microorganisms reduce metallic ions into their corresponding NPs with the help of enzymes produced by their cellular metabolism in two ways. (1) Intracellular pathway, where the metal ions are trapped and reduced inside the microbes (2) extracellular pathway, where the ions are reduced on the microbial cell surface or in the medium [5,12]. Fungi, which are sometimes referred to as "bio-nano factories," have reportedly been used to reduce gold ions to AuNPs because of their capability to secrete large amounts of enzymes and their ability to withstand metal toxicity to a more considerable extent [13].

Yeast, such as *C. albicans* and algae such as *Sargassum wighti* have been successfully used to synthesize stable AuNPs making them good candidates for NP production [30]. Bacteria are also potential bio-factories for NP production, as they can withstand stress exerted by heavy metal toxicity. Some bacterial cultures such as *Pseudomonas stutzeri*, *P. aeruginosa*, *Thiobacillus ferrooxidants*, and *E. coli* were used to synthesize monodispersed MNPs with excellent biomedical applications [2,62]. In another study, the culture supernatants of *K. pneumonia*, *E. coli*, and *Enterobacter cloacae* were used as reducing agents for the rapid biosynthesis of AgNPs [47,62]. Microorganisms have undeniably provided MNPs with significant antimicrobial and anticancer properties [12]. However, the elaborate experimental procedures in terms of isolation, culture preparation, and maintenance have strained this approach. This limitation and others have upsurged the synthesis of MNPs using plant sources. Thus, plant-mediated synthesis is becoming most prominent, offering a quicker and more manageable approach than microbial and other green sources [2,10,34].

The potential of plants to bioaccumulate heavy metals suggests their use in the transformation of metal ions into MNPs. Alfalfa sprouts were the first plant reportedly used for the synthesis of AgNPs [63]. Subsequently, different plant species along with a variety of bioactive compounds have been explored for the production of MNPs including gold, silver, zinc, iron, copper, and platinum. Studies have shown that the reduction and stabilization of MNPs are achieved through the action of diverse compounds like proteins, amino acids, polysaccharides, and phytochemicals like flavonoids, alkaloids,

tannin, and polyphenols present in the plants [64,65]. With the plant-derived approach, synthesis and purification are quicker and easier compared to the microbial-mediated approach [2,63].

3.2.The Synthesis of Plant-Mediated MNPs

The notion that plants can bio-accumulate and reduce metal ions has opened options for considering their use as an alternative route for the synthesis of MNPs. The plant-mediated synthesis of NPs offers advantages over the microbes-mediated approach in simplicity, cost-effectiveness, rapidity, and non-pathogenicity [65]. Several plants including *Zingiber officinale*, *Punica granatum*, *Acalypha indica*, *Ficus benghalensis*, *Galenia africana*, *Terminalia mantaly*, and *Catharanthus roseus* have been reportedly used for the synthesis of MNPs. The synthetic process is initiated by the addition of extracts obtained from plant parts such as leaves, flowers, roots, stems, bark, and fruits into the aqueous solution of metal ions [8]. The phytochemicals present in the plant extracts which include sugar, flavonoids, protein, enzyme, polymer, and organic acid, act as reducing and stabilizing agents. The alkaloids, polyphenols, terpenoids, polysaccharides, amino acids, organic acids, vitamins, and heterocyclic compounds are implicated in the bioreduction process [65], and equally, play significant roles in the capping and stabilization of the bio-synthesized NPs. The exact mechanism and the plant components responsible for plant-mediated synthetic NPs remains complicated [8,19,20]. Several potential mechanisms have been proposed [66] as shown in Figure 2. During NP synthesis, a bioreduction phase occurs where bioactive compounds present in the plant extracts reduce metal ions/salts from their mono or divalent oxidation states to zero-valent states [67]. Subsequently, the reduced metal atoms' nucleation indicated by physical observation of a colour change in the reaction medium takes place. As bioreduction and nucleation continue, a growth phase is reached where smaller particles mechanically interact to form larger particles that are thermodynamically more stable. The final stage of the synthesis is the termination stage, where the bioactive compounds exert their stability potentials and finally define the shape and morphology of the NPs.

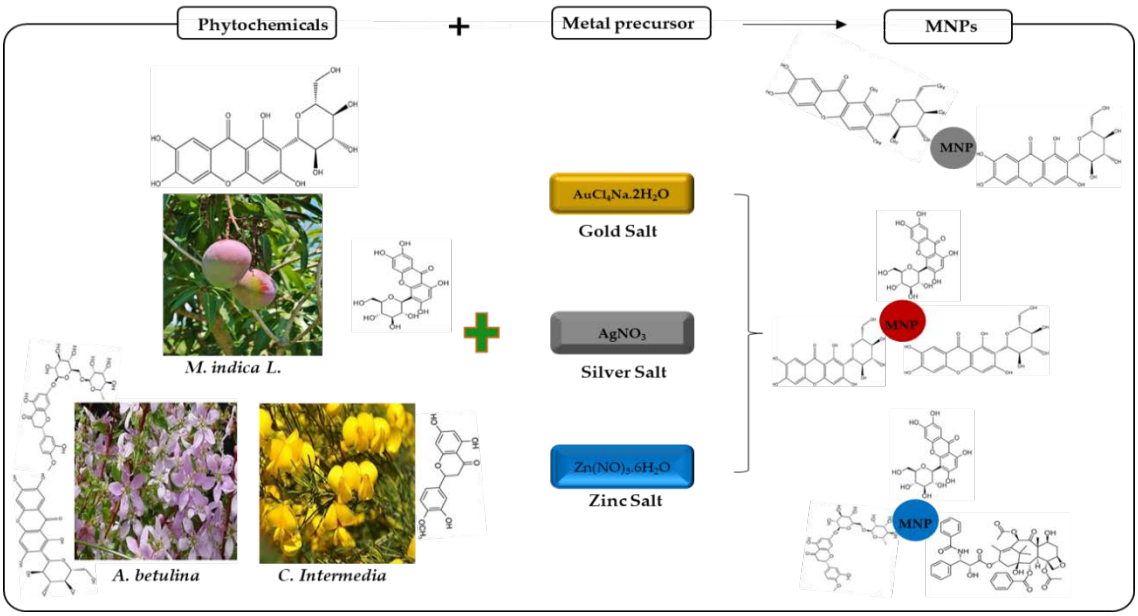

Figure 2. Synthesis of MNPs using plant extracts. Several plant extracts with high reductive capacity actively reduce metallic ions including silver, zinc and gold ions to their corresponding MNPs. Adapted from [20].

Reports suggest that the mechanisms employed in the process of preparing NPs from medicinal plants differ from plant to plant due to the variation in their bioactive molecules, their composition and concentration [6]. The variation between plants and their subsequent interaction with aqueous metal ions as well as factors such as pH, temperature, and reaction time results in NPs exhibiting different physical, chemical, and biological properties [8]. Moreover, the properties of the phytochemicals present in the plants play the most vital role in the bioactivities of the NPs [28]. It is believed that the NPs derived from a particular plant extract may take on the bioactivities displayed by the plant extract [20]. In most cases, the NPs have a superior or higher bioactivity than the plant extract [19]. Such NPs can be engineered as nanoplatforms for molecular diagnosis, effective and targeted delivery of drugs, providing treatments of diseases that can resolve some of the drawbacks associated with traditional medicine [20]. These NPs have the potential to be translated into conventional medicine much faster, hence overcoming the many challenges associated with present-day standard modes of treatment.

4. Biological Application of MNPs Synthesized from Some Selected South African Medicinal Plants

4.1. South African Medicinal Plant Biodiversity

Africa is a continent endowed with remarkable natural biodiversity with over 45,000 different plant species documented [21]. In a comprehensive list containing African medicinal plants, only about 5400 plants have been reported to have medicinal properties. This represents approximately 12% of the total African plants and therefore supports the claim that African plants are under-utilized for medicinal purposes. The interest in Africa's underexplored medicinal plants has recently upsurged; over 60% of recent publications focused on the African medicinal plants and their bioactivities [21]. Despite the increasing research interest, the commercialization of African plants still lags. A global review of commercialized medicinal plants revealed that only 83 African medicinal plants are considered partially or fully commercialized [23], whereas, in Europe and Asia, commercialization is at its maximum peak. Plants have been a major source of medicines in most African countries for thousands of years [68,69] and have played an integral role in basic traditional medicine and healthcare in many developing countries, including South Africa.

South Africa is the third most biodiverse country, and houses one of the world's six floral kingdoms (the Fynbos Biomes) in addition to other biodiversity hotspots, the Succulent Karoo and the Albany-Maputaland corridor [23,70]. Figure 3 shows the floristic region of South Africa. According to the "African Plant Checklist and Database Project", Sub-Saharan Africa houses 50,136 angiosperm taxa, out of which Southern Africa accounts for 22,755 taxa [23]. Moreover, South Africa hosts around 30,000 flowering plant species with over 3000 plants reported to have medicinal uses [21,69]. The global review of commercialized medicinal plants shows that South Africa accounts for 14 out of the 83 commercialized African medicinal plants [71]. The most valuable of all indigenousSouth African plants is the *Aloe vera* L. while those that have received universal recognition include *Agathosmabetulina*, *Aloe ferox*, *Aspalathuslinearis*, *Harpagophytum procumbens*, *Hypoxis hemerocallidea*, *Merwilla natalensis*, *pelargonium sidoides*, *Sclerocarya birrea*, and *Cyclopia intermedia*.

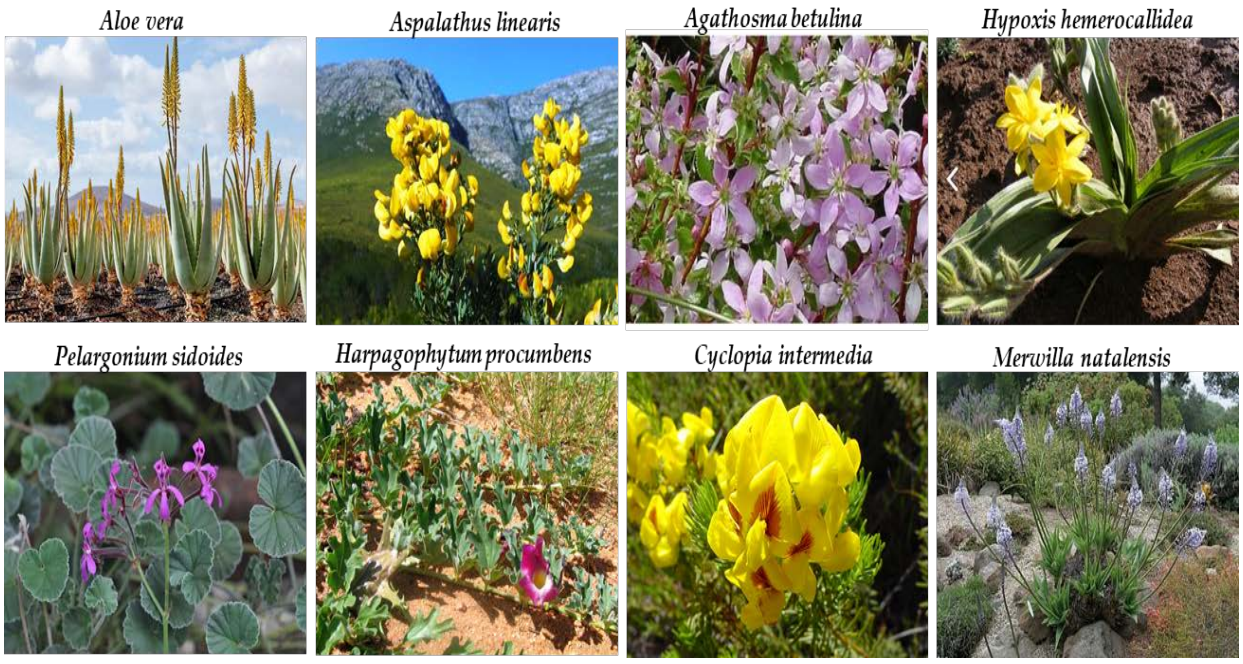

Figure 3. Catalogue of some of the indigenous plants found in the Cape Floristic Region of South Africa.

4.2. MNPs Synthesized from Indigenous SA Plants and Their Application

Medicinal plants have a long history in traditional medicine; over 80% of the South African population relies on the plants to meet their primary health care needs [69]. Medicinal plants offer differential therapeutic properties based on their integral supply of secondary metabolites, viz, flavonoids, alkaloids, phenolics, terpenoids, tannins, glycosides, quinones, steroids, and saponins [72]. However, most of these compounds have low absorption, resulting in a loss of bioavailability and efficacy due to their inability to cross the lipid bilayer of cells [73]. Thus, several phytochemical and pharmacological investigations of these plants and their derivatives revealed impressive in vitro activity and less in vivo efficacy. To facilitate effectiveness, one approach that is increasingly suggested in the literature is the combination of traditional medicinal plants with nanotechnology [74,75]. Nanotechnology is one of the newest approaches with the potential to revolutionize the medical and pharmaceutical fields [19,20,75]. The incorporation of nanosystems in herbal medicine could serve as an effective tool in eradicating the limitations associated with medicinal plants [76]. With the emerging new technologies, these plants can be formulated into newer strategies that can enhance the delivery and efficacy of their phytochemicals [77]. Green nanotechnology has shown great promise in this regard and various medicinal plants have been reportedly used to synthesize MNPs with enhanced biocompatibility and biological activities [18,49]. Hembram *et al*. [78] reported the synthesis of AgNPs using aqueous leaf extracts of *Mentha pulegium*, *Coriandrum sativum*, and *Prosopis cineraria*. This particular study reported that the biogenic AgNPs showed an enhanced anticancer effect in in vitro breast cancer cells [78]. Due to the excellent pharmacological activities displayed by plant mediated MNPs, focus on how traditional plants would benefit the pharmaceutical industry especially in the drug discovery platform is now paramount.

South Africa is rich with a plethora of plants with a long history in traditional medicine for the treatment of infectious and chronic diseases, necessitating their inclusion in nanotechnology. The exploration of South African medicinal plants including *Camellia sinensis*, *Azadirachtaindica*, *Aloe vera*, and

Jatropha curcas plant extracts for the synthesis of a variety of MNPs has been documented. Moreover, a largescale screening of plant species obtained from the Cape floristic region of South Africa including *Aspalathus hispida*, *Indigofera brachystachya*, *Nidorella foetida*, and *Podocarpus falcatus* was successfully used for the synthesis of AuNPs [8]. Interestingly, the investigations regarding the activities of South African plant-mediated MNPs revealed enhanced antimicrobial and anticancer activities compared to the crude plant extract [6]. Despite the number of valuable indigenous plants utilized for MNP production, only very few of them have been explored for biological purposes. Therefore, the present study reviews 12 potent plants commonly used in South Africa traditional medicine in terms of their potential for MNPs synthesis and their biological applications. Some of these plants are indigenous, while others are also found in other parts of the world. A list of some selected South African plants incorporating their traditional use for treating various ailments and their bioactivity through green nanotechnology is given in Table 1.

Table 1. List of selected South African medicinal plants utilized for MNP synthesis and their biological activity.

Plant Species	Indigenous Application	Major Phytochemicals	MNPs	MNP Size (nm)	MNP Bio-Activity
Cyclopia intermedia	Treat constipation, nervousness, cough, eczema, epilepsy and regulate blood pressure [21]	Mangiferin (MGF), isomangiferin, hesperidin and isosakuranetin[79,80]	AuNPs	20	Anticancer [20]
Sutherlandia fructecens	Treat wounds, cancer, diabetes, skin diseases, rheumatism, urinary tract infection, fever, gonorrhoea, kidney and liver problems [6,81]	Saponins, pinitols, flavonoids, triterpenoids, Cannavanine, cycloartane glycosides, flavonol glycosides, and aminobutyric acid [81]	ZnONPs AgNPs	5–25 15–20	Antimicrobial [82] Antibacterial and anticancer [6]
Hypoxis hemerocallidea	Immune booster, purgative, and laxative tonic Treat tuberculosis, urinary tract infection, infertility, cancer, diabetes, and wounds [83,84]	Sterols, norlignane, daucosterols, stanols, hypoxide, sterolins, and β-sitosterol [83]	AuNPs	9–27	Antibacterialand anti-inflammatory[25,27]
Eucomis autumnalis	Reduce fever, urinary diseases, stomach, lower backaches, syphilis and sometimes used to induce labour [85,86]	Homoisoflavanones, terpenoids, and diben-α-pyrones [86]	AgNPs	56	Antimicrobial [87]
Plumbago auriculata	Treat headaches, warts, skin infection, wounds, and fracture [88,89]	Tannins, phenols, alkaloids, saponins, flavonoids, plumbagin, α-amyrin, capensisone, and diomuscinone[88]	AgNPs	15.22	Antimicrobial [7]
Catharanthus roseus	Treat rheumatism, venereal diseases, skin infections, high blood pressure, and	Vinblastine,deoxyvinblastin, vincoline, cathanranthamine, rosicine, leurosine,	AgNPs AgNPs	49 35.55	Antimicrobial and wound healing [41] Larvicidal [26]

	diabetes [90,91]	vindoline, vincristine [92]	AgNPs	6–25	Antimicrobial [76]
Aspalathus linearis	Treat insomnia, stomach cramps, allergies, digestive problems as well as improve appetite [22]	Spalathin, orientin, isoquercitrin, luteolin hyperoside[93,94]	AuNPs RhNPs	44 1.2	Antimicrobial [95]
Indigofera tinctoria	Epilepsy, asthma, stomach ache, bronchitis, and some skin diseases [96]	Saponins, alkaloids, flavonoids, phenolic compounds [97]	AuNPs	6–29	Antibacterial, antifungal, and anticancer [98]
Artemisia herba-alba	Treat anorexia, indigestion, and gastrointestinal problems [99,100]	1,8-cineole, alpha, and beta-thujone, davanone, chrysanthenone, cis-chrysanthenol[99,101,102]	AgNPs	6–29	Antibacterial and mosquito repellant[103]
Centella asiatica	Treat fever, leprosy, syphilis, tuberculosis, leprosy, asthma, epilepsy, mental disorder, minor wounds Consumed as a vegetable and used as a spice [104]	Triterpenoids, centellose, medacassoside, triaponosides, flavonoid quercetin, rutin, kaemferol, patuletin, apigenin, polyacetylenes, phenolic acids, sterols [104]	AgNPs	30–50	Antimicrobial[105]
Galenia africana	Treat venereal sores, eye infections, asthma, tuberculosis, cough, wounds, skin infections and relieve toothache [106]	Trihydroxyflavanone, trihydroxychalcone, dihydroxychalcone, trihydroxy-3-methoxychalcone [107]	AuNPs	9–27	Antibacterial [25]
Sclerocarya birrea	Treat dysentery, rheumatism, malaria, and diarrhea[108,109]	Glucosides, steroids, glycosides, flavonoids, fatty oils, alkaloids, phenols, resins, calcium, phosphorus [108,109]	AgNPs	112	Antimicrobial [87]

4.2.1. *Cyclopia intermedia*

C. intermedia, commonly known as Honeybush (HB), is a popular South African shrub belonging to the family Fabaceae. The plant is an erect, much-branched shrub found in winter rainfall coastal and mountainous areas with a moderate Mediterranean climate [110]. It is a perennial plant with a woody stem that grows up to 1.5 to 3 m tall. HB is characterized by yellow flowers, long needle-like leaves with a honey-like smell [93,94]. The leaves and stems are used to brew the famous South African herbal HB tea known for several health benefits. Traditional healers from South Africa use the infusion prepared from the leaves and stem to relieve constipation, nervousness, cough, eczema, epilepsy, and to regulate blood pressure [21]. HB tea is caffeine-free and contains low tannin, making it a suitable tea that can be drunk at bedtime. The health benefit of HB includes prevention of skin cancer and lowering of blood glucose levels [111]. Additionally, HB offers phytoestrogenic properties against breast cancer by binding to oestrogen receptor subtypes, thus, prevents the growth of breast cancer cells [112]. Its anticancer effects are through its modulation of oxidative stress, inhibition of cell proliferation, and adenosine triphosphate (ATP) production [112]. Studies showed that HB extracts contain polyphenolic compounds which are correlated

with the plant's biological activities. The significant compounds, characterized and isolated from HB, are the xanthones (MGF and isomangiferin) and flavanones (hesperidin, and isosakuranetin) [79,80]. These polyphenols offer the HB plant the ability to improve the immune system, protect against inflammatory diseases, and inhibit antimicrobial and tumour growth [111,112].

The synthesis of AuNPs using HB extracts (HBE) has been previously documented [20]. Using an eco-friendly approach, water extract of HB leaves was used to synthesize HB-AuNPs. The UV-Vis spectroscopic analysis showed that the biogenic HB-AuNPs displayed distinct peaks at 540 nm with a hydrodynamic diameter of 66.74 nm. The XRD analysis confirmed that the biogenic HB-AuNPs were crystalline. The average core size of the HB-AuNPs as determined by transmission electron microscope (TEM) was found to be 20 nm, exhibiting predominantly spherical with some triangular-shaped AuNPs. The FTIR analysis clearly showed the formation of AuNPs and indicated that HBE contains various phytochemicals, particularly polyphenols such as MGF, which could have been one of the phytochemicals that acted as reducing and stabilizing agents for the HB-AuNPs. A further study showed that the HB-AuNPs exhibited selective toxicity against brain (U87), prostate (PC-3), and colon (Caco-2) cancer cells [20]. These effects were compared and found similar to the effects of AuNPs synthesized from MGF (MGF-AuNPs). Thus, the study strongly suggests that MGF is possibly one of the reducing agents for the synthesis of HB-AuNPs [20]. Interestingly, the anticancer effects of both HB-AuNPs and MGF-AuNPs were further augmented when used in combination with doxorubicin [20]. Importantly, the study further investigated the effect of the HB-AuNPs and MGF-AuNPs on normal breast epithelial (MCF-12A) cells and showed that the biogenic MNPs had no toxicity on the cells even at the highest concentration of 1000 μg/mL [20]. This observation was consistent with earlier study that showed that 100 μM AuNPs synthesized using MGF (MGF-AuNPs) isolated from leaf extracts of *M. indica* L had no toxic effect on normal epithelial breast (MCF-10A) cells [51]. Similarly, Majoumouo *et al*. [19] showed that AuNPs synthesized using water extract of *T. mantaly* showed no significant reduction in cell viability of non-tumourigenic skin fibroblast (KMST-6) cells after 24 h treatment. Consequently, these findings present useful information in understanding the therapeutic application of biogenic MNPs, particularly in MNP formulations and dosage regimen.

4.2.2. *Sutherlandia fructecens*

S. frutescens is an indigenous South African medicinal plant found predominantly in the Eastern, Northern, and Western Cape provinces, and some areas of KwaZulu-Natal. It is an attractive leguminous shrub that can grow from 0.5 m up to 1.2 m in height. Also, it has greyish-green leaves finely arranged in a feather-like feature. *S. frutescens* is characterized by transparent bladder-like fruits, large balloon-like seed pods, and orange-red flowers. Traditional healers in South Africa use decoction prepared from *S. frutescens* (leaves, stems, flowers, roots, and pods) to treat wounds, cancer, diabetes, rheumatism, influenza, gonorrhoea, and to reduce body temperature [6,81]. The plant is also used to alleviate diverse kinds of symptoms and conditions like depression and stress. Further, the plant is used to treat urinary tract infections, stomach infections, skin diseases, gonorrhea, kidney and liver problems [82]. The plant has been formulated into capsules, tablets, gels, ointments, and creams, and available in some pharmacies and herbal shops [113].

The phytochemical profile of the plant revealed the presence of saponins, pinitols, flavonoids, and triterpenoids, which are believed to be responsible for its enormous medicinal activities. Additionally, cannavanine, cycloartane glycosides, and flavonol glycosides are also abundantly present in the plant, contributing significantly to its bioactivity [81]. Pharmacological studies of *S. frutescens* extracts revealed evidence of its antiviral, anti-inflammatory, antibacterial, antiproliferative, antimutagenic, antioxidant, and antidiabetic properties [113,114]. An improved quality of life reported in HIV patients following treatment with commercial Sutherlandia tablets further expounded the popularity of the plant as an

effective herbal product [114]. An earlier study reported a dose-dependent decrease in cell number and changes in the morphology of human breast cancer (MCF-7) cells following treatment with an aqueous extract of *S. fructescens* [114]. Moreover, the leaf extracts of *S. frutescens* reduced insulin levels and enhanced glucose uptake in streptotozin-induced diabetes in Wistar rats [115]. Owing to the acclaimed medicinal benefits, it is imperative to investigate the fabrication of *S. frutescens* extracts into MNPs for enhanced bioactivity.

S. fructescens was first reported for the synthesis of Zinc Oxide (ZnO) NPs [82]. In this particular study, the water extracts of the plant were used to reduce zinc salt for the synthesis of ZnO NPs. The exact mechanism by which the ZnO NPs were formed is not yet fully understood. It was proposed that phytochemicals present in the plant donated hydrogen atoms, which resulted in the release of Zn^{2+} ion. Afterward, the Zn^{2+} reacted with the polyphenols of the plant, resulting in the formation of ZnO NPs. The FTIR measurement revealed a vibrational peak at region 1400 cm^{-1} which corresponds to biomolecules that are involved in the formation of the ZnO NPs. The TEM images revealed spherical-shaped NPs with the core size ranging between 5 and 25 nm. The antibacterial investigation against *E. coli*, *S. aureus*, *P. aeruginosa*, and *E. faecalis* using agar well diffusion assay revealed that *S. frutescens*ZnO NPs inhibited the growth of all test strains. Although this particular study reported an impressive antibacterial activity for *S. frutescens*ZnO NPs, however, the mechanism of the antibacterial effect was not fully captured. Evidence shows that NPs can pass through the membrane to interact with cellular components of the bacterial cell wall thus induce oxidative stress that subsequently leads to cell death (Figure 4). The *S. frutescens*ZnO NPs also exhibited a dose-dependent effect in lung (A549) cancer cells with approximately 93.4% cell death following a 24-h treatment [82]

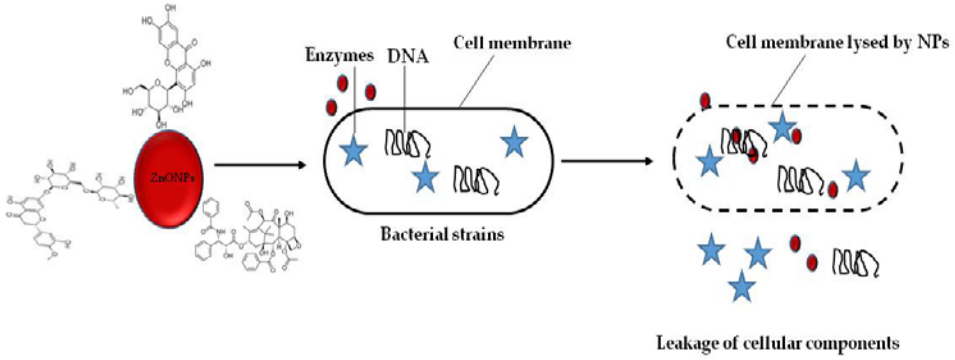

Figure 4. Mechanism of ZnO NPs against bacterial strains. ZnO NPs exhibited remarkable inhibition of cell growth and cell death in bacterial strains following the direct interaction between the ZnO NPs and the bacterial cell surface. Adapted from [82].

S. frutescens leaf extracts were also used in the synthesis of AgNPs following incubation of water extract of S.frutescens with $AgNO_3$ in a simple and easy method [6]. The *S. frutescens*AgNPs were predominantly spherical and polygon-shaped and had an average core size between 15 and 20 nm. The various shapes and sizes indicated that more than one phytochemical was involved in the reduction and possibly capping of the AgNPs. This claim was validated by the overlapping peaks in the FTIR spectra of the extracts and the AgNPs. While the water extract *S. frutescens* showed antibacterial activity at concentrations up to 50 mg/mL, the biogenic AgNPs displayed a significant bacterial growth inhibition against *S. epidermidis* and *P. aeruginosa*. The minimum inhibitory concentration (MIC, lowest

concentration that inhibits visible growth) of the AgNPs was ~66 fold lower than the concentration of the plant extract (50 mg/mL) that failed to inhibit growth [6].

4.2.3. *Hypoxis hemerocallidea*

H. hemerocallidea of the hypoxidaceae family is an indigenous Southern African plant, found abundantly in South Africa, Swaziland, Lesotho, and Botswana. The plant, commonly known as 'African potato', 'miracle plant', 'molic', and 'star flower' is enlisted in Southern Africa as an indigenous medicinal plant with potential health benefits [84]. The plant is characterized by strap-like hairy leaves, yellow star-shaped flowers, and thick green hairy stems. Currently, *H. hemerocallidea* is commercialized as a natural product with potent applicability in drug development [23]. The traditional use of *H. hemerocallidea* as a strengthening tonic, purgative, and laxative, or to treat tuberculosis, urinary tract infection, infertility, cancer, diabetes, and wounds has been documented [83]. The promising anticancer properties reported for *H. hemerocallidea* prompted its inclusion as one of the South African plants used for the treatment of cancer in the Eastern Cape Province of South Africa. The popularity of the plant became more prominent following its recommendation for human immunodeficiency virus (HIV) patients as an immune booster [23].

The plant is composed of hypoxoside, which is believed to be the main component responsible for its numerous medicinal activities. Sterols, norlignane, daucosterols, stanols, hypoxide, sterolins, and β-sitosterol are also reported to play significant roles in the plants' therapeutic activities. Also, *H. hemerocallidea* is laced with trace elements including, copper, zinc, and manganese, thus used as pro-fertility supplements. Evidence-based laboratory investigation indicated that extracts obtained from African potato possess numerous pharmacological properties including antidiabetic, anti-inflammatory, antioxidant, antihyperglycemic, analgesic, and anticancer [84].

The interesting biological properties entrusted in this plant led to its fabrication into MNPs with enhanced bioactivity. The aqueous leaf extract of *H. hemerocallidea* was explored in the synthesis of AuNPs (Hy-AuNPs). The reduction of the gold salt was achieved by the phytochemicals that contain the O-H, CH_3, and C-O functional groups commonly found in flavonoids, terpenoids, carbohydrates, and phenolic compounds. The resulting AuNPs with hydrodynamic size range from 10–45 nm displayed selective effect against *P. aeruginosa*, *S. epidermidis*, *E. coli*, and *S. aereus* [27]. In another study, Elbagory *et al*. [27] compared the physicochemical properties of the Hy-AuNPs with AuNPs synthesized using water extract of hypoxoside (a compound isolated from *H. hemerocallidea*) and found that they were similar. This suggests that hypoxoside is involved in the synthesis of Hy-AuNPs. Furthermore, both AuNPs had immunomodulatary effects and reduced the pro-inflammatory cytokines in macrophages (THP 1) and natural killer (NK-92) cells [27].

A recent study by Aremu *et al*. [116] reported the successful synthesis of AgNPs using ethanolic extracts of *H. hemerocallidea*. The study showed that the resulting AgNPs with an average diameter of 6–20 nm exhibited significant antibacterial activity against *Bacillus cereus*, *Streptococcus pneumonia, P. aeruginosa, Moraxella catarrharis*, and *E. coli*. Interestingly, a combined effect of the AuNPs with broad-spectrum antibiotic, streptomycin showed that the AuNPs synergistically enhance the antibacterial effect of streptomycin up to 30–52% [116].

4.2.4. *Eucomis autumnalis*

E. autumnalis of the Asparagaceae family is a deciduous, summer-growing bulb characterized by its extraordinary floral arrangement with wavy, soft, and fleshy flowers [87]. They are widely distributed in open grasslands and marshes across all the provinces of South Africa, as well as some neighbouring countries like Botswana, Zimbabwe, and Malawi [85]. Although the bulb is believed to be toxic if consumed on its own, a decoction prepared in water or milk is safe for treating fevers, urinary diseases,

lower backaches, syphilis, and at times used to induce labour [85]. Many constituents, including homoisoflavanones, terpenoids, and diben-α-pyrones, have been reportedly isolated from this plant [86]. The traditional use of *E. autumnalis* as anti-inflammatory agents is popularly accepted, hence, it is recommended for the manufacture of non-steroidal anti-inflammatory drugs [85]. It is traditionally used to treat wounds, fractures and to relieve pain often associated with surgical procedures [85].

The phytochemicals present in water extract of *E. autumnalis* plant were used for the reduction of $AgNO_3$ to AgNPs [87]. The resulting brownish-black colour connotes the formation of AgNPs [40]. The UV-Vis spectrophotometric analysis of the formed MNPs gives SPR bands at 423 nm, confirming the presence of AgNPs. FTIR analysis of biosynthesized AgNPs signals the contribution of polyphenols and aromatic compounds in the bioreduction process. The biosynthesized *E. autumnalis*AgNPs revealed the hydrodynamic size of 56 nm and predominantly spherical shape AgNPs. These AgNPs were further evaluated for their antimicrobial activities against *Listeria monocytogenes*, *Enterococcusfaecalis*, *K. pneumonia*, and *Acinebacter baumannii*. The result indicates that the AgNPs displayed enhance antimicrobial effects over its bulk plant extracts in all test stains [87]. Consequently, this finding revealed that *E. autumnalis*AgNPs have promising antibacterial properties against bacteria strains.

4.2.5. *Plumbago auriculata*

P. auriculata is a bushy evergreen shrub belonging to the family Plumbaginaeae. It is a native South African plant but is also distributed in other tropical and subtropical regions of the world, including Sri Lanka and India [89]. In South Africa, *P. auriculata* is predominantly found in the Southern and Western Cape to the balmy subtropical province of Kwazulu-Natal. It is commonly known as Cape leadwort in English or blousyselbos in Afrikaans [88]. Traditionally, *P. auriculata* is used to treat headaches, warts, skin infections, wounds, and fractures [89]. The plant is reported to contain a wide range of phytochemicals, including tannins, phenols, alkaloids, saponins, flavonoids and, proteins. Bioactive compounds including plumbagin, α-amyrin, capensisone, and diomuscinone have been isolated from the plant [89]. A study shows that most of the pharmacological activity, including antimicrobial, anticancer, and anti-inflammatory effects of the plants, is conferred by its biomarker compound plumbagin [89]. Plumbagin is an effective inhibitor of cell growth, displaying a broad-spectrum anticancer effect against a wide range of cancers, including breast, liver, prostate, pancreatic, and ovarian cancers [89].

Earlier, the successful synthesis of AgNPs from *P. auriculata* leaf and calyx extracts had been reported [7]. In the study, the bioreduction of Ag^+ ions by the aqueous extracts was demonstrated in an eco-friendly manner. In the process of synthesis, a colour change from yellowish-brown to dark brown and light yellow to dark brown was observed for the leaf and calyx extracts, respectively. The colour change confirmed the bioreduction of Ag^+ to Ag^0 [35]. The biogenic AgNPs so produced denoted by the appearance of absorption peaks between 420 and 460 nm. The AgNPs were relatively spherical to oblong in shape with a core size diameter of 15.22 and 26.5 nm for leaf and calyx extracts, respectively. FTIR spectra revealed that the phytochemicals containing -OH and C=O groups in both extracts were involved in the bioreduction and stabilization of AgNPs [35]. The antibacterial activities of both biogenic AgNPs revealed their desirable antibacterial effects against *E. coli*, *K. pneumoniae*, *S. typhimurium*, and *S. aureus* as compared to their respective bulk plant extracts. The study was the first to report the antibacterial activities of AuNPs using water extracts of *P. auriculata* against gram-positive and gram-negative multidrug resistance bacteria [7]. Consequently, the effective antibacterial activity of the biogenic AuNPs opens a new path in antibacterial drug discovery.

4.2.6. *Catharanthus roseus*

C. roseus, also known as Madagascar periwinkle or Vincarosea, is a well-studied medicinal plant. It is a perennial herb characterized by a woody base with glossy bright green leaves and pink to white flowers

[117,118]. *C. roseus* originated from Madagascar and was imported to South Africa as an ornament, but has now become widely distributed in major parts of South Africa including KwaZulu-Natal, Limpopo, and Gauteng Provinces [119]. *C. roseus* has been extensively studied and shown to contain over 130 phytochemicals with vindoline implicated as its principal marker compound [120]. Other major constituents isolated from *C. roseus* include vincristine and vinblastine which are well-known anticancer drugs used for treating Hodgkin's lymphoma and leukemia [121]. Most of the alkaloids isolated from this plant include deoxyvinblastine, vincoline, cathanranthamine, rosicine, and leurosine have diverse pharmacological activities [118].

Traditionally, a decoction made from leaf extracts is used to treat rheumatism, venereal diseases, skin infections, high blood pressure, diabetes mellitus, and many others [26,91]. In Madagascar, the extract from the plant is used as purgative, vomitive, and for relieving toothache [119,122]. Antioxidant and pharmacological activities of *C. rosues* such as antidiabetics, antimicrobial and anti-inflammatory activities, have been reported. The wound healing potential of ethanolic leaf extracts of *C. roseus* showed an enhanced wound contraction in Wistar rats [123]. Verma and Singh [122] reported the antimicrobial influence of leaf extract of *C. roseus* against *S. citreus*, *S. aureus*, *E. coli*, and *P. aeruginosa*, indicating *C. roseus* as an excellent antibacterial agent.

Different parts of *C. roseus*, including stem, root, leaf, and flower, have been explored for the synthesis of AgNPs. For example, an eco-friendly synthesis of AgNPs using aqueous leaf extract of *C. roseus* was reported [41]. Here, the bioactive constituents of *C. roseus* leaf extracts were used to reduce silver salt for the synthesis of *C. roseus*AgNPs. The observed colour change from light yellow to brown following incubation of *C. roseus* leaf extract and $AgNO_3$ solution indicated the reduction of Ag^+ to AgNPs. This was confirmed by the appearance of an absorption peak at 425 nm. Additional characterization revealed biosynthesized AgNPs of an average size of 49 nm having crystalline nature. FTIR techniques adopted to investigate the plausible mechanism behind the formation of these AgNPs revealed the presence of amide group possibly contributed by enzymes/proteins present in the plant extract. These compounds were proposed to be responsible for the reduction of $AgNO_3$ to AgNPs. The antioxidant activity of the biogenic crystalline AgNPs investigated using 2,2-Diphenyl-1-picrylhydrazyl (DPPH)-free radical scavenging assay showed a dose-dependent increase in the DPPH inhibition percentage up to 82% at 300 μg/mL. Also, these AgNPs showed good antimicrobial effects against *E. coli*, *K. pneumoniae*, *P. aeriginosa*, *S. aureus*, *C. koseri*, and *C. albicans* when compared to the plant extract or $AgNO_3$ alone [76]. Further study revealed the wound-healing activity of the biosynthesized NPs as it had enhanced wound closure and reduced wound size in male albino mice compared to untreated animals with no evidence of microbial contamination [41].

Another study reported an eco-friendly approach for the synthesis of AgNPs from the aqueous root extract of *C. roseus* in the presence of silver nitrate [26]. A colour change from light yellow to dark brown indicated the presence of AgNPs, which was confirmed by SPR at 423 nm. The resulting spherical shaped AgNPs with sizes ranging between 35 and 55 nm. FTIR analysis indicates the presence of alkanes and aliphatic amines, which were believed to be responsible for the bioreduction and stabilization of the AgNPs. These AgNPs were evaluated for their larvicidal activity against fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus* and it was observed that the biogenic AgNPs had enhanced larval mortality effect when compared to *C. roseus* root extracts [26]. Thus, the study revealed the potential of AgNPs synthesized using *C. roseus* as strong anti-larvalcidal agents.

Similarly, AgNPs synthesized using an aqueous extract of *C. roseus* flower were reported. FTIR spectra indicate the presence of aldehydes, carbonyls, alcohols, phenols, alkanes, and aliphatic amines. The spherical-shaped biosynthesized AgNPs having an average size ranging between 6 and 25 nm showed remarkable antibacterial activity against *E. coli*, *S. aureus*, *K. pneumonia*, and *B. subtilus* [76]. Other parts of *C. roseus* including stem have also been explored for the synthesis of AgNPs and their antimicrobial activity has been documented. Given the high efficacy of AgNPs of *C. roseus* as strong

antibacterial, antioxidant, antilaraicidal, and wound healing agents, it is recommended that other biological applications including anticancer property and the synthesis of other metallic NPs should be investigated.

4.2.7. *Aspalathus linearis*

A. linearis, belonging to the family Fabaceae, is an endemic South African plant widely distributed in the Western Cape Province of the country. *A. linearis* is cultivated to brew the popular herbal Rooibos tea. It is frequently used traditionally to treat insomnia, stomach cramps, allergies, digestive problems as well as to improve appetite [21]. Previous and recent in vitro and in vivo studies have implicated *A. linearis* as a rich source of phenolic compounds. Bioactive compounds viz, aspalathin, orientin, isoquercitrin, luteolin, and hyperoside have been reportedly isolated from *A. linearis* [79,94,112]. These are believed to be responsible for their antioxidant, immunomodulatory, anti-inflammatory, antidiabetic, and chemoprevention effects [94].

The synthesis of AuNPs using *A. linearis* tea leaves to optimize the antifungal activity of some commercial antifungal discs has been documented [88]. In this study, a green route approach was attempted for the synthesis of AuNPs from the *A. linearis* plant where the aqueous plant extract was mixed with $NaAuCl_4$ and stirred for 30 min at room temperature. A colour change from yellow to ruby-red in the reaction mixture indicating the formation of AuNPs showed a strong resonance peak at 529 nm. The biogenic AuNPs with an average size of 44 nm demonstrated excellent in vitro stability in different biological media at varying pH levels [95]. The resulting biogenic AuNPs were investigated for their enhanced antifungal activities against Aspergillus spp using disc diffusion assay. In this particular study, the AuNPs were coated around eight commercial antifungal discs (clotimazole, nystatin, flucytosine, fluconazole, econazole, ketoconazole, miconazole, and amphotericin) and their zones of inhibition were compared to those coated with pristine. The AuNPs attached better onto the antifungal disc compared to the pristine coated antifungal disc resulting in an enhanced antifungal activity against Aspergillus spp. This study revealed AuNPs as good antifungal agents that could be of importance in medical and veterinary applications. The later part of their study investigated the toxicity of the biosynthesized AuNPs against human Hep-G2 liver cancer cells, and the result indicated that the AuNPs showed no significant effect on the cells [95].

The aqueous leaf extract of *A. linearis* was also used for the synthesis of rhodium (Rh) NPs at room temperature [29]. The disappearance of the deep orange colour typical of Rh precursor to a lit orange, following the addition of the Rh precursor to the aqueous extract of *A. linearis*, indicated the formation of RhNPs [29]. The *A. linearis* extracts showed near-infrared (NIR) absorbance in the optical range of 225–600 nm, however, the peak almost completely disappeared in the presence of the RhNPs which is as a result of the reduction of RH^{3+} to Rh^0 [29]. The RhNPs were non-agglomerated and had an average size of approximately 1.2 nm. Further characterization revealed that the RhNPs were amorphous [29]. Apart from RhNPs, other metallic NPs, including zinc oxide and cobalt, have been reportedly synthesized from *A. linearis* and fully characterized [124], but nothing is reported regarding their biological applications.

4.2.8. *Indigofera tinctoria*

I. tinctoria, belonging to the Fabaceae family, is an erect, branched perennial plant found predominantly in widespread tropical Africa. The plant is characterized by its greyish-brown stem and dark-green leaves [125]. *I. tinctoria* species is reported to be a major source of the colour indigo, and the leaves have widely been cultivated, extracted, and processed for the production of indigo dye [96]. Leaf infusion of *I. tinctoria* is traditionally used to treat epilepsy, asthma, stomach ache, bronchitis, and some skin diseases. An investigation of its phytochemical contents revealed that the plant contains reducing sugars, saponins, carbohydrates, alkaloids, flavonoids, and phenolic compounds [97].

Recently, Vijayan *et al*. [98] reported the first-time synthesis of AuNPs from aqueous leaf extract of *I. tinctoria*. A change in colour from light yellow to violet after 30 seconds of exposure to microwave irradiation was observed for the AuNPs, which was later confirmed by an SPR peak at 545 nm. Further characterization regarding the morphology of the AuNPs revealed that the NPs were crystalline in nature, triangular, spherical, and hexagonal, and having a particle core size ranging between 6 and 29 nm [98]. An investigation of the antibacterial and antifungal activities of the AuNPs revealed some degree of antimicrobial effects against *B. pumilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *Aspergillus fumigatus*, and *Aspergillus niger* with noticeable zones of inhibition. In addition, the study further evaluated the anticancer effect of *I. tinctoria* AuNPs on human lung (A549) cells using the 3-[4,5-dimethythiazol-2yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The result revealed that the biogenic *I. tinctoria* AuNPs showed enhance toxicity against lung cancer (A549) cells with evident changes (shrinking) in cellular morphology compared to the bulk plant extract [98]. Therefore, the findings from this study clearly showed that AuNPs synthesized from water extracts of *I. tinctoria* can be effectively used as powerful weapons against cancer cells, particularly lung cancer as well as against bacteria cells.

4.2.9. *Artemisia herba-alba*

A. herba-alba, also known as the white wormwood, is a perennial shrub that grows commonly on the dry steppes of the Mediterranean regions in Northern Africa, Western Asia, and South-Western Europe [21]. Its leaves are strongly aromatic and covered with fine glandular hairs. It is used as an antiseptic and antispasmodic, as well as a treatment for anorexia, indigestion, and gastrointestinal problems [100]. Major constituents of *A. herba-alba* include 1,8-cineole and appreciable amounts of alpha and beta-thujone as well as other oxygenated monoterpenes in addition to davanone, chrysanthenone, and cis-chrysanthenol [126]. Phytochemical investigation of the aerial part of *A.herba-alba* revealed two new natural sesquiterpene lactones [103] in addition to artemisin, an endoperoxide sesquiterpene lactone and the quaianolide structural type. These compounds, particularly those isolated from its essential oil, tend to provide Artemisia with a wide range of bioactivity, including antibacterial, antiseptic, antifungal, and choleretic activities [127]. *A. herba-alba* is commonly used as a remedy for enteritis, menstrual pain, nervous problems, and various intestinal disturbances among the Bedouins in the Negev desert of Israel [21,102]. Essential oil from this plant showed antibacterial and antispasmodic activities in rabbits [121]. Also, an aqueous extract of aerial parts of the plant with a hypoglycemic effect in alloxan-induced diabetic rabbits and mice has been reported [99].

The first record of *A. herba-alba* mediated synthesis of AgNPs was reported [102]. The appearance of a yellowish-brown suspension following the mixing of $AgNO_3$ solution with the water extracts of *A. herba-alba*, in addition to the peak at 425 nm, indicated the formation of AgNPs. Further characterization of the fabricated *A. herba-alba*AgNPs revealed spherical shaped NPs with a size range of 43 and 74 nm. FTIR analysis of the fabricated AgNPs indicated relevant absorption peaks corresponding to N-H stretching from peptide linkages and C-C stretching vibrations of aromatic amines. The fabricated AgNPs were tested on Indian and Saudi Arabian strains of Anopheles, Aedes, and Culex mosquitoes and reported to be a source of green nanoinsecticides against mosquito vectors [124]. Also, the bacteria growth inhibition effect of the biogenic *A.herba-alba*AgNPs was demonstrated against *B. subtilis*, *K. pneumonia*, and *S. typhi*. This study proposed that the fabricated *A. herba-alba*AgNPs have relevant insecticidal and bactericidal activities against species of high public health importance [102].

4.2.10. *Cantella asiatica*

C. asiatica is a herbaceous, perennial plant native to South Africa, Asia, and the South Pacific. In South Africa, the decoction of *C. asiatica* is used to treat fever, leprosy, syphilis, tuberculosis, asthma, epilepsy, mental disorder, minor wounds, and it is also consumed as a vegetable and used as a spice [21].

C. asiatica has been extensively studied to contain pentacyclic triterpenoids, centellose, centelloside, and medacassoside, which are believed to proffer its healing potential [104]. The major chemical constituents found in this plant are triterpene saponosides, flavonoid derivatives viz quercetin, rutin, kaemferol, patuletin, apigenin in addition to polysaccharides, polyacetylenes, phenolic acids, and sterols [104]. An investigation of the neuroprotective activity of *C. asiatica* demonstrated that the plant can inhibit acetylcholinesterase, a key enzyme implicated in the pathogenesis of Alzheimers disease [128]. Moreover, the wound healing property of ointment, cream, and gel formulated from aqueous extract of this plant showed enhanced wound healing properties in Wistar rats by increasing collagen content and tensile strength at the wound site [129]. Other studies also documented the cognitive-enhancing, antioxidant, antidepressant, antiepileptic, sedative, and anxiolytic properties of *C. asiatica*.

The synthesis of AgNPs using aqueous leaf extracts of *C. asiatica* L. was developed by Rout and colleagues [105]. Upon incubation of plant extracts with silver salt at room temperature, stable AgNPs were produced without the involvement of toxic chemicals as capping agents. Biosynthesized AgNPs were spherical with a size range between 30 and 50 nm. Agar well diffusion assay revealed effective antimicrobial activity of biosynthesized *C. asiatica*AgNPs against *S. aureus* [105].

In a similar study, Thakkar *et al*. [63] synthesized AgNPs using aqueous leaf extracts of *C. asiatica*. In this particular study, the antimicrobial activity of both the aqueous extracts of *C. asiatica* and its resulting AgNPs were compared against *S. aureus*, *P. aeruginosa*, and *E. coli*. Results showed that leaf extracts of *C. asiatica* had low activity compared to those exhibited by its biogenic AgNPs. This finding suggests that *C. asiatica*AgNPs interact with the bacteria cell wall more than the extracts and thus describe the AuNPs as stronger antimicrobial agents compared to its bilk plant extract [63].

4.2.11. *Galenia africana*

G. africana is a major group of flowering plants belonging to the family Aizoaceae. *G. africana* commonly referred to as kraalbos, geelbos, or perdebos, is found abundantly in Namaqualand, Northern Cape, some parts of Karoo, and Eastern Cape Province of South Africa. The plant is characterized by leaves growing in the opposite direction, soft woody shrublet (0.5–1.5 m high), and acute apex flowers. The plant is used to treat venereal sores, eye infections, asthma, tuberculosis, cough, wounds, and skin infections. Locals masticate the leaves of *G. africana* to relieve toothache [106]. The earlier phytochemical investigation reported by Mativandlela *et al*. [107] showed three known flavonoids viz, trihydroxyflavanone, trihydroxychalcone, dihydroxychalcone, and a new trihydroxy-3-methoxychalconeare present in the *G. africana* [107].

The first-time synthesis of AuNPs from *G. africana* was demonstrated by [25], where the phytochemicals present in aerial parts of *G. africana* were utilized for the reduction of gold salts to form Galenia-AuNPs. The change in colour from light yellow to red colour following 1 h incubation at 70°C indicated the appearance of Galenia-AuNPs [25]. The AuNPs had absorption spectra at 534 nm with varying shaped crystalline AuNPs with core sizes ranging between 9 and 27 nm. The different shapes were an indication that different bioactive compounds present in the plant extract actively participated in the reduction process as equally revealed by FTIR. The biogenic AuNPs were stable in various biological media and exhibited antibacterial effects against *S. aureus*, *E.coli*, *S. epidermidis*, and *P. aeruginosa*. These microbes are very common in wound infections, consequently Galenia-AuNPs could be recommended as potent treatment regimen for wound infections. To address the toxic effects of biogenic AuNPs in non-cancer cells, this particular study further reported that the Galenia-AuNPs have excellent biocompatibility in KMST-6 cells [25]. The Galenia-AuNPs at a concentration of 32 μM following a 24 h treatment showed no significant reduction in the cell viability [25]. On the other hand, Hossain *et al*. [130] investigated the toxic effect of biogenic AgNPs synthesized from water extract of Andrographis

paniculata stem in healthy male Wistar rats. The result showed that there was no significant toxic effect on the liver and kidneys following an intravenous treatment of the rats with either 2 mg/kg or 5 mg/kg body weight. More specifically, there was no significant difference in serum biomarkers and creatine levels between the treated and control animals [130]. Thus, biogenic MNPs are believed to offer excellent biocompatibility and can be recommended for future therapeutic applications [25].

4.2.12. *Sclerocarya birrea*

S. birrea is a medium-sized to a large deciduous tree with an erect trunk, having its leaves mostly crowded at the end of the branches. The plant is commonly found in riverine, grasslands, and bushlands as well as in well-drained, loamy, and sandy soil [68]. *S. birrea* is predominantly found in the Phalaborwa area in Limpopo [108]. Traditionally, the decoction of *S. birrea* is used to treat dysentery, rheumatism, malaria, and diarrhea [109]. The leaves are eaten as vegetables, while the fruits and plums, including alcoholic drinks, jams, and juice produced from the plant, are consumed in most countries of West Africa [109]. Medicinal value credited to this plant is traced to their secondary metabolite contents, including, steroids, glycosides, flavonoids, fatty oils, alkaloids, phenols, and resins, as well their calcium, and phosphorus contents. Moreover, traditional healers in West Africa use the infusion prepared from the stem bark of the plant to treat diabetes [109].

In an earlier study, Lediga and colleagues explored the phytochemicals present in *S. birrea* plant extracts for the synthesis of AgNPs [87]. The resulting brownish-black colour in the reaction medium confirmed the formation of AgNPs which was confirmed by the SPR band at 440 nm. The FTIR analysis of biosynthesized AgNPs indicated the presence of polyphenols and aromatic compounds, which were believed to play major roles in the bioreduction process. The hydrodynamic size of *S. birrea*AgNPs was 112 nm and the NPs were predominantly spherical. The AgNPs NPs were evaluated for their antimicrobial activities against *L. monocytogenes*, *E. faecalis*, *K. pneumonia*, and *A. baumannii*. The result displayed the enhanced antimicrobial effect of *S. birrea*AgNPs over its bulk plant extracts exhibiting the highest inhibition against *A. baumannii* [87].

4.3. Preclinical and Clinical Application of MNPs

Despite the highlighted potential of biosynthesized MNPs, very few of them have been critically evaluated for in vivo purposes [131] and only one was evaluated in human trials [74]. This suggests that many of the MNP formulations have not sufficiently accomplished the pharmaceutical regulation to warrant their clinical application [132]. Nonetheless, pre-clinical studies so far have provided compelling evidence of the potential clinical application of the biogenic MNPs. Some of their biomedical applications are highlighted in Section 2, where they are used as antimicrobial, wound healing [41], drug delivery [20,74], and therapeutic agents [19,28]. The outcomes of these nanomaterials, supported by the longstanding use of the medicinal plants further encourage their translation into clinical practice. In fact, the biogenic AgNPs have potential to replace the chemically synthesized MNPs due to their biocompatibility. Additionally, the biosynthesized MNPs are capable of targeting and killing diseased cells without attaching targeting and therapeutic moieties [19].

The wound healing property of biogenic *C. roseus*AgNPs was reported in mice. Further, the study observed sign of microbial contamination, pus formation, inflammation and bleeding in untreated mice, whereas, mice treated with the *C. roseus* AuNPs showed none of these symptoms [41]. Independent studies demonstrated the anti-tumor effect of AuNPs loaded with resveratrol (RES-AuNPs) in breast (MDAMB-231), pancreatic (PANC-1), prostate (PC-3) cancer cells [77], as well as in mice bearing liver tumours [133]. In both studies the RES-AuNPs exhibited stronger anti-tumour effect when compared to free resveratrol [77,133]. MGF-AuNPs were the extensively studied, and demonstrated to have antitumor effects on various cancer cells alone [75] or in combination with chemotherapeutic drugs [20]. MGF-

AuNPs was also shown to significantly reduce tumour volumes in mice implanted with PC-3 tumour xenografts within three weeks of administration. This particular study further revealed that 80% of the intratumoural administered radiolabelled MGF-AuNPs were localized in the tumour site within 30 min of treatment and this was maintained for 24 h [75]. These encouraging pre-clinical results prompted the work of Khoobchandani *et al*. [74] that investigated the clinical efficacy of biogenic MNP formulation in metastatic breast cancer patients. The study established Nano Swarna Bhasma (NSB), a nanodrug prepared through the proprietary combinations of phytochemicals from *Emblica officinalis*, *Mangifera indica*, *Curcumin longa*, *Acacia nilotica*, and *Glycyrrhiza glabra* with the AuNPs synthesized using *Mangifera indica* peel extract [74]. The NSB exhibited selective toxicity against human breast (MDA-MB-23) cancer and non-cancer aortic endothelial (HAEC) cells [74], and reduced tumour size in Severe Combined ImmunoDeficient mice. More importantly, the study also reported significant therapeutic benefits in breast cancer patients treated with NSB and these benefits were more pronounced when usedin combination with antitumour drugs (doxorubicin and cyclophosphamide). Although, NSB is the first biogenic MNP to be used in human trials [74], there are a number of other MNPs that have been approved by the Food and Drug Administration (FDA) for clinical trials. These include AuNPs approved for cancer (NCT00356980), diabetes (NCT02837094), dental (NCT03669224), and skin (NCT02219074) therapy; AgNPs for skin infections (NCT03752424); and iron oxide NPs for cancer therapy (NCT02033447) and imaging (NCT04261777).

5. Conclusions and Future Perspectives

It is evident from literature that biogenic MNP formulations undeniably have broad-spectrum propensity against a wide array of diseases, however, their translation into conventional medicine suffers from a number of drawbacks, particularly the lack of information regarding their fate in vivo. According to literature, various biological models incorporating plants, mammalian cells, microorganisms, fish and mammals have been used to evaluate the toxicity of plant mediated MNPs, yet none has been able to sufficiently establish the exact mechanism involved in their toxicity. On the other hand, quite a number of pre-clinical/clinical investigations have evidently reported interesting antimicrobial, wound healing, anticancer, antioxidative, anti-inflammatory, immunomodulatory, and cytotoxic activities of these biogenic MNPS. It is therefore recommended that considerable efforts need to be devoted towards assessing the efficacy and safety of these natural MNP formulations in clinical research. More importantly, detailed description of the methods for the synthesis, purification, exact composition of the MNP formulations as well as the dosage should strictly be provided to allow for its reproductivity. In this regard, MNP formulations composed mainly of biological material could serve as better options over chemically synthesized MNPs, thus their translation into conventional medicine could be much faster, with the benefit to overcome the many challenges associated with the present-day standard modes of treatment.

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CHAPTER THREE

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Abstract

The field of nanotechnology is gaining an increased interest in plant science, particularly for the production and application of nanomaterials. The aim of the study was to investigate the ability of *Cyclopia intermedia* (*C. intermedia*), commonly known as Honeybush (HB) to reduce gold ions resulting in the synthesis of gold nanoparticles (AuNPs). In this study, water extracts of green HB (GHB) and fermented HB (FHB) were studied for the production of AuNPs. This is the first time this plant was investigated in this way. The synthetic process which simply involves mixing the plant extract with gold salt was optimized by varying conditions such as reaction time, plant extract concentration, pH, and temperature. A reaction time of 1 hr using 2 mg/ml of the green HB plant extract, at a pH of 4.5 and at a reaction temperature of 70°C produced the best conditions for the synthesis of green HB-AuNPs (GHB-AuNPs). These conditions are similar to the conditions used for the synthesis of fermented HB-AuNPs (FHB-AuNPs), except that 4 mg/ml of the extract produced the best results. The observation of a colour change from light yellow to ruby red suggested the successful synthesis of AuNPs and this was confirmed using the UV-Vis spectrophotometric analysis. The AuNPs were purified and analysed using the Dynamic Light Scattering (DLS) to determine their hydrodynamic size, polydispersive index (PDI) and zeta potential. The study reports that both the green and fermented HB plant extracts have excellent reducing potentials for the production of AuNPs.

Keywords: Biogenic synthesis, *Cyclopia intermedia*, Fermented honeybush, Green/Unfermented honeybush, Nanotechnology

3.1. Introduction

The inclusion of natural (biological) entities in nanotechnology has gained wide attention, particularly in their application in medical fields (Patra *et al*., 2018; Shah *et al*., 2015). This approach known for its simplicity, rapidity, cost-effectiveness and eco-friendliness is more superior to both the physical and chemical methods of nanoparticles' (NPs) production. This approach recruits living organisms which includes extracts of microorganisms, fungi and plants as reducing and stabilizing agents towards the synthesis of NPs (Dube *et al*., 2020; Bhagyanathan &Thoppil, 2018; Elbagory *et al*., 2017), that are less harmful to humans and animals (Mittal *et al*., 2013). Interestingly, plant and plant derivatives are becoming the preferred reducing agents to synthesise these NPs as they are quicker and easier to produce compared to other sources (Madkour, 2017; Rai *et al*., 2013; Thakkar *et al*., 2010). Plants contain secondary metabolites, including flavonoids, polyphenols, alkaloids, polysaccharides as well as biomolecules such as proteins, amino acids and vitamins that have been implicated in the bioreduction of metal precursors (Mohamad *et al*., 2014). These metabolites can also play significant roles as capping and stabilizing agents during the synthesis of NPs (Paosen *et al*., 2017). Numerous plants including *Salvia africana-lutea, Sutherlandia frutescens*, *Azadirachta indica*, and *Aspalathus hispida* (Dube *et al*., 2020; Elbagory *et al*., 2016)have been used successfully for the production of AuNPs. The synthesis which involves a reaction between the plant extracts and the metal precursor is often affected by different factors not limited to reaction time, temperature, pH and concentrations of plant and metal precursor. Therefore, optimization of these parameters is necessary to achieve the ideal conditions (which vary from plant to plant) for NP synthesis.

C. intermedia, belonging to the family of Fabaceae is a leguminous shrub that grows in the fynbos botanical zone of South Africa. The plant material, particularly, the stem and leaves are used to brew the herbal HB tea known for its many health benefit (Dube *et al*., 2017; Marnewick *et al*., 2009). Both the GHB and FHB plants are rich in polyphenols including mangiferin, isomangiferin, hesperitin and hesperidin, which provide the potential antioxidant, immune-boosting and cancer preventative benefits (Ajuwon *et al*., 2018; Magcwebeba *et al*., 2016; Joubert & de Beer, 2012; Marnewick *et al*., 2009). Traditionally, the fermented HB tea is used to relieve symptoms of infections, which include cough, sore throat, runny nose, fever, mild

tiredness, nausea, vomiting, diarrhea, head or body aches and sneezing (Ajuwon *et al*., 2018; Dube *et al*., 2017).

In this study, water extracts of GHB and FHB were investigated for the synthesis of AuNPs. In the process of synthesis, conditions such as temperature, reaction time, plant extract concentration, and pH were varied to achieve the ideal conditions for the synthesis of AuNPs. The reactions which were performed at the different conditions were analysed using UV-vis spectroscopy to ascertain the presence of AuNPs. Both the green and fermented plant extract produced AuNPs at 2 mg/ml and 4 mg/ml of the plant extract, respectively. The optimal reaction conditions for both extracts were 70°C temperature, 1 hr reaction time, and pH of 4.5. Successfully synthesized AuNPs were purified and analysed using DLS to determine their hydrodynamic size, PDI and zeta potential.

3.2. Materials and Methods

3.2.1. Sample preparation

Dried green and fermented HB plant materials denoted GHB and FHB, respectively were donated to CPUT Oxidative Stress Research Centre by the Rooibos Ltd (Clanwilliam, South Africa). The plant materials were stored in sealed plastic containers and kept at 25°C in a dark room. The water extracts of both GHB and FHB were done according to the method described by Dube *et al*. (2017). Briefly, 100 g of the HB leaves were dissolved in 1000 ml of boiled double-distilled water and kept on a magnetic stirrer without heat for 24 hr. After, the infusion was filtered through Whatman's No 1 and 0.45 µm filters. The resulting supernatant was freeze-dried using FreeZone 25L Freeze Dry (Labconco, Kansas City, MO, USA). The dried extracts were stored at 4 °C in sterile sealed containers for further use.

3.2.2. Phytochemical analysis

Quantitative measurement of polyphenols, flavonols, and flavanols was performed on the water extract of GHB and FHB using spectrophotometry and colorimetry techniques.

3.2.2.1. Determination of total polyphenolic contents (TPC)

Total polyphenols were determined using the method described previously (Genwali *et al*., 2013). This involved the use of Folin-Ciocalteu reagent and sodium carbonate. Briefly, 25 µl of

samples followed by 125 µl of 200 mM Folin reagent was dispensed into each well of a greiner plate and allowed to rest for 5 min. Thereafter, 100 µl of 7.5% aqueous sodium carbonate was added to the wells. A blue colour complex was formed from the reaction between phenolic compounds and Folin's reagent. The plates were incubated at room temperature for 2 hr. The absorbance was measured at 765 nm. The intensity of this colour-complex measured is directly proportional to the concentration of phenolic compounds present in the sample. Gallic acid (0, 20, 50, 100, 250, and 500 mg/L) in 10% ethanol was used to prepare the standard curve. Results were expressed as microgram gallic acid equivalents per gram dry mass of the plant (mgGAE/g DM).

3.2.2.2. Determination of flavonol content

Flavonol content was determined using quercetin (0, 5, 10, 20, 40, and 80 mg/L) in distilled water as a standard (Shahidi & Ambigaipalan, 2015). 10 mg of the extracts were weighed and dissolved in water. After, 12.5 µl of samples were pipetted into the 96-well plates. 12.5 µl of 0.1% HCl was added to each well, followed by 225 µl of 2% HCl. This was left to incubate for 30 min at room temperature. Readings were taken at 360 nm, and results expressed as microgram quercetin equivalent per gram dry mass of the plant (mgQE/g DM).

3.2.2.3. Determination of flavanol content

Spectrophotometric determination of flavanols was done using DMACA reagent (Shahidi & Ambigaipalan, 2015). DMACA reacts with flavanols to form a characteristic light blue colour. 1 mM Catechin hydrate was used to prepare the calibration curve at the following concentrations; 0, 5, 10, 25, 50, 100 µM. Briefly, 25 µl of the sample was dispensed into each well of a greiner 96-well plate and 275µl of DMACA reagent was added to each well. Plates were incubated for 30 min at room temperature. Readings were taken at 640 nm. The intensity of the colouration is directly proportional to the concentration of flavonols in the sample. Results were expressed as mg catechin equivalent per gram dry mass of the plant (mg Catechin/g DM).

3.2.3. Antioxidant capacity

The water extract of GHB and FHB were investigated for antioxidant capacities, viz; ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and 2,2-

diphenyl-1-picrylhydrazyl (DPPH) using standard spectrophotometric and colourimetric techniques.

3.2.3.1. ORAC assay

The ORAC assay kinetically measured the peroxyl-radical absorbing potential of the antioxidants present in the extracts according to the method previously described by Chanda & Dave (2009). Trolox; a water-soluble analog of vitamin E, was used as the standard antioxidant reference. Working solutions were 1.2 mM fluorescein solution, 75 mM phosphate buffer (pH 7.4), and AAPH solution. Samples were diluted (x10) using phosphate buffer. Briefly, 12 µl of samples were dispensed into a black 96-microwell plate, followed by 138 µl of fluorescein solution. After that, 50 µl of AAPH was added, and readings were taken. The change in fluorescence of the reaction mixture was monitored over 2 hours and recorded every minute (excitation = 485 nm and emission = 535 nm). Results were determined with a regression equation that relates Trolox concentrations with the net area under the kinetic fluorescein decay curve ($y = ax^2 + bx + c$). The ORAC values were expressed in micromoles of Trolox equivalents per gram of sample (µmolTE/g sample).

3.2.3.2. FRAP assay

The ferric reducing power of the GHB and FHB was determined using spectrophotometry according to the method of Seng *et al*. (2013). The stock solution (FRAP reagent) was prepared with acetate buffer (300 mM, pH 3.6), TPTZ solution, $FeCl_3.6H_2O$ solution, and distilled water. The solution mix was in a ratio of 10:1:1:2, respectively. 10 µl of the sample followed by 300 µl of FRAP reagent was dispensed into the microplate. After 30 min of incubation at room temperature, the absorbance was determined at a wavelength of 593 nm. Ascorbic acid was used as the reference standard and results expressed as micromoles of ascorbic acid per gram of extract (µmolAAE/g sample).

3.2.3.3. DPPH assay

The DPPH radical scavenging ability of the GHB and FHB was determined using spectrophotometry according to the method of Seng *et al*. (2013). Briefly, 10 µl of the sample followed by 300 µl of the DPPH reagent was dispensed into the microplate. The reaction mixture was incubated for 30 min at room temperature. Afterward, the absorbance was determined at a

wavelength of 734 nm. Trolox was used as the reference standard and results expressed as micromoles of Trolox equivalent per gram of extract (µmolTE/g sample).

3.2.4. High-performance liquid chromatography (HPLC) analysis

The phenolic compounds present in water extract of GHB and FHB plants were determined using the HPLC techniques described by Dube *et al*. (2017) with slight modification. On the HPLC system (Agilent Technology 1200 series, Bellefonte, USA), a G1315C diode array detector and a C18 column of 5 µm (4.6 mm x 150 mm i.d) were used to separate the compounds. The chromatographic conditions were as follows; 20 µl sample injection volume, column set at 23°C, flow rate set at 1 mL/min for 15 min. Detection was performed at a wavelength of 280 and 300 nm. Peaks were identified on the retention time of MGF and hesperidin standards. The analytical signals were monitored at 2-20 mV potential applied. To determine the concentrations of the compound present in the extract, the following equation was used:

$$X\ mg/L = \left({}^{Area\ of\ sample}/_{Area\ of\ standard} \right) \times 20\ mg/L$$

Results were expressed in mg/g:

$$X\ mg/L \times extractionvolume\ (L)/weight\ (g)$$

3.2.5. Synthesis of biogenic AuNPs

This study reports for the first time, the synthesis of AuNPs using water extract of GHB and FHB. Since it was a first-time study, optimization for the ideal synthesis of AuNPs from GHB and FHB plant extracts were investigated.

3.2.5.1. Optimization of conditions for the synthesis of AuNPs

The optimization for the synthesis of AuNPs using water extract of GHB and FHB was done because this study is the first to report the synthesis of AuNPs using GHB and FHB extracts.

Synthetic conditions such as temperature (25, 50, 70 and 100°C), pH (4.33, 5.09, 6.13, 7.08, 8.05, 9.11.10, and 11.05), and concentration of plant extract (0.5, 1, 2, 4, 8, 16, 32 and 64 mg/ml) and time reaction (0, 1, 3, 6, 12, 18 and 24 hr) were considered for the synthesis of HB-AuNPs. The mixture of the HB extracts (20 µl) and 1 mM sodium tetrachloroaurate (III) dehydrate ($NaAuCl_4 \cdot 2H_2O$) (100 µl) at different conditions listed above was done in a griener flat-bottom 96 well plate as previously described by Elbagory *et al*. (2016).

3.2.5.2. Biogenic synthesis of GHB-AuNPs and FHB-AuNPs

Sequel to the optimization experiment for the synthesis of the AuNPs, the following ideal conditions were selected; 2 mg/ml of plant extract, the temperature at 70°C, plant extract pH of 4.5, and 1 hr reaction time for the synthesis of GHB-AuNPs. While, 4 mg/ml of plant extract, the temperature at 70°C, plant extract pH of 4.5, and 1 hr reaction time for the synthesis of FHB-AuNPs. The synthesis was upscaled by adding 10 ml of the plant extracts at pH 4.5 (2 mg/ml for GHB and 4 mg/ml for FHB) dissolved in distilled water to 50 ml of 1 mM $NaAuCl_4 \cdot 2H_2O$ in a glass beaker (Elbagory *et al*., 2016). The mixture was kept in a shaking incubator (40 rpm) for 24 hr at 70°C.

3.2.6. Confirmation of AuNPs using UV-Vis spectroscopic technique

The principle of UV-vis spectroscopy is based on the absorption of ultraviolet light (UV) or visible light by chemical compounds, which results in the production of distinct spectra. Spectroscopy is based on the interaction between light and matter. When matter absorbs light, it undergoes excitation and de-excitation, resulting in the production of a spectrum. Figure 3.1 explains the typical principle of the UV-visible spectrophotometric technique. Metal NPs are related with precise absorbance bands in characteristic peaks at absorptions which is due to the surface plasmon resonance (SPR) of the NPs (Hanan *et al*., 2018).

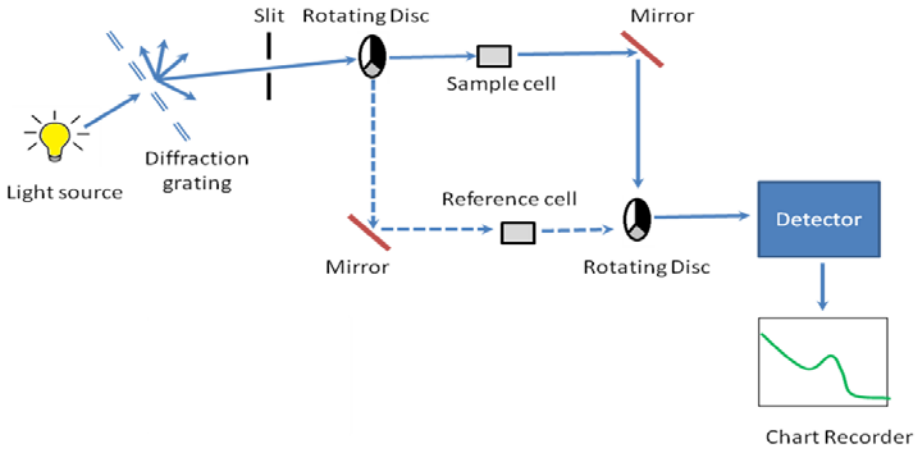

Figure 3.1: Typical principle of the UV-visible spectrophotometric technique (Ms et al., 2019)

In this study, the UV-vis spectroscopic technique was adopted to confirm the presence or absence of AuNPs in the reaction mixtures. Here, 100 µl of the reaction mixture was dispensed into a greiner 96 flat bottom plate and the spectrum of the AuNPs was determined using a POLARstar Omega microtitre plate reader (BMG Labtech, Ortenberg, Germany) at a wavelength range of 400–800 nm.

3.2.7. Dynamic light scattering (DLS) analysis of purified AuNPs

DLS technique is a standard method used to determine the average diameter of materials in the nanometer scale dispersed in a liquid medium. The technique is based on the Brownian motion of dispersed particles in suspension. As the incident light hits the suspension, the intensity and direction of the light beam are both transformed and scattered into a detector. DLS measures the scattered light from a laser that passes through a colloid and mostly depends on Rayleigh scattering from the suspended NPs (Zheng *et al*., 2019). Figure 3.2 depicts the basic setup of a DLS measurement system.

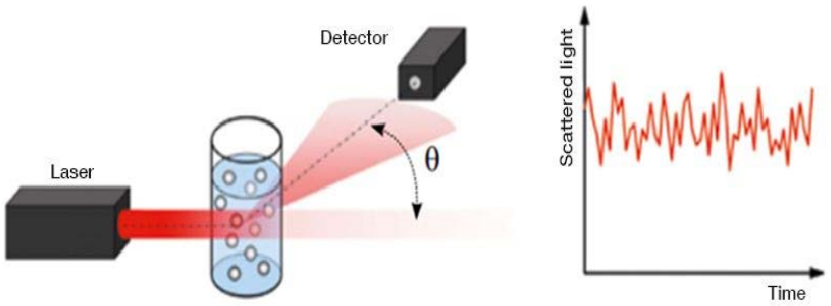

*Figure 3.2: The basic setup of a DLS measurement system. The sample is contained in a cuvette. The scattered light of the incident laser can be detected at different angles (*Zheng et al., 2019)

The effect of purifying the AuNPs in terms of identifying whether the process of washing would affect the hydrodynamic size, polydispersity index (PDI), and zeta potential of the biogenic AuNPs were determined using DLS (Zetasizer Nano ZS90, Malvern Instruments Ltd, UK). 1 ml of either purified or unpurified biogenic AuNPs was placed into a polystyrene cuvette for hydrodynamic size and PDI measurement. While for zeta potential measurement, 1 ml of either purified or unpurified biogenic AuNPs was placed in a disposable capillary cell (DTS1070) cuvette for the zeta potential analysis. The cuvettes were placed in the cuvette compartment of the Zetasizer machine and measured. The results of the purified AuNPs were compared with those of the unpurified AuNPs.

3.3. Results and Discussion

3.3.1. Phytochemical analysis of GHB and FHB

The water extract of GHB and FHB were successfully prepared in boiled distilled water without further modification. The extracts obtained from both plant materials were screened for the presence of flavanols, flavonols, and polyphenols, as depicted in Table 3.1. The comparative flavanol analysis of GHB and FHB revealed that GHB contained higher amounts of flavanols (approximately 8.38 mg/g) than FHB (approximately 4.67 mg/g). Flavanols are a highly diversified and multi-substituted subgroup of flavonoids. They have the hydroxyl group bound on the C3 position of the flavanoid skeleton. They are structurally different from other flavanoids in that there is no double bond between C2 and C3 and no carbonyl in the C4 position. Flavanols

are found abundantly in plant foods and fruits, including tea leaves, cocoa beans, grape seeds, bananas, apples, apricot, peaches, and various berries.

The levels of flavonols were very low in GHB (0.3 mg/g) as well as in FHB (0.11 mg/g). Flavonols are one of the essential sub-groups of flavanoids with a ketone group. They are structurally identified with a hydroxyl group on the C3 position in the flavonoid skeleton. They are widely distributed in various parts of the plant, including leaves, fruits, and vegetables. Flavonols are also present in glycosylated form in higher plants. Numerous *in vitro* and *in vivo* studies have shown that flavonols demonstrate excellent antioxidant, antiviral, anti-inflammatory, and anti-thrombogenic activities.

Moreover, the TPC of GHB and FHB depicted in Table 3.1 showed that the TPC of GHB (0.1827 mgGAE/g) was significantly higher than that of the FHB (0.696 mgGAE/g) plant. Although the reduced flavonols, flavanols and TPC observed for FHB compared to GHB was possess diverse biological properties such as antioxidant, anti-apoptosis, and antiaging, expected since the process of fermentation often results in the alteration of the plants' chemical composition. Therefore, this suggests that GHB may have more reducing capacity compared to FHB. Polyphenols represent a wide variety of secondary metabolites in plants and plant products. They are primarily derivatives and isomers of flavones, isoflavones, flavonols, catechins, and phenolic acids. Polyphenols anticarcinogen, anti-inflammatory, anti-atherosclerosis, cardiovascular protection, and improvement of endothelial function. Other functions of polyphenols include neuroprotective effect on anti-ageing and neurodegenerative diseases, antimutagenic, maintenance of gastrointestinal health, and effects on digestive enzymes. Moreover, polyphenols are also known for an antiallergic, antidiabetic, and protective effects on immune cell functions. Thus, polyphenols are often described as the main ingredients responsible for the pharmacological activities of medicinal plants.

Table 3.1: Phytochemical analysis of GHB and FHB

Phytochemical constituents	GHB	FHB
Flavanols (mg/g)	8.3753 ± 0.0018	4.6624 ± 0.0062
Flavonols (mg/g)	0.3000 ± 0.0004	0.1142 ± 0.0002
TPC (mgGAE/g)	0.1827 ± 0.0006	0.0696 ± 0.0010

Result is expressed as mean value ± standard error of mean (SEM)

3.3.2. Antioxidant capacity of GHB and FHB

The estimation of antioxidant potential and reducing capacity of GHB and FHB using DPPH, ORAC, and FRAP assays was carried out, and the results are shown in Table 3.2. DPPH free radical assay is based on an electron-transfer that produces a violet solution in ethanol (Seng *et al*., 2013). This free radical is reduced to give a colourless solution in the presence of an antioxidant molecule. The comparative DPPH radical scavenging potential of GHB and FHB revealed that GHB (10.26 µmolTE/g) has a higher antioxidant capacity than HBE (2.13 µmolTE/g). The same trend was observed for ORAC with GHB exhibiting higher activity (50.85 µmolTE/g) than FHB (19.15 µmolTE/g). Moreover, the FRAP analysis of GHB was higher (11.58 µmolAAE/g) than for FHB (2.58 µmolAAE/g). Both GHB and FHB have remarkable antioxidant capacity, but GHB exhibits a higher capacity. It is not surprising that antioxidants can alter the oxidation level of metal ions by supplying electrons. Redox reaction also occurs during MNPs formation from corresponding aqueous salts. Plant extracts contain reducing substances that transfer electrons to the metal ions producing MNPs. Since antioxidant potential and total reducing capacity vary among different plant species, it is expected that plants with higher reducing capacity will be more potent in reducing metallic ions to MNPs.

Table 3.2: Antioxidant capacity of GHB and FHB

Antioxidant capacity	GHB	FHB
DPPH (µmolTE/g)	10.26 ± 0.02	2.13 ± 0.06
ORAC (µmolTE/g)	50.85 ± 0.15	19.15 ± 0.08
FRAP (µmolAAE/g)	11.58 ± 0.04	2.58 ± 0.07

Result is expressed as mean value ± SEM

3.3.3. HPLC analysis of HB plant extract

The HPLC analysis of the water extract of GHB and FHB plant confirmed that the two most abundant polyphenols present in HB are mangiferin and hesperidin (Ajuwon *et al*., 2018; Dube *et al*., 2017; Joubert & de Beer, 2012). Figure 3.3 shows the chromatogram for aqueous extract of GHB and FHB at a wavelength of 300 nm. The additional peaks revealed in the chromatogram represent other compounds that have been previously identified (De Beer *et al*., 2012). These compounds include isomangiferin, hesperetin, eriodictyol, naringenin, luteolin,

diosmetin, tyrosol, narirutin, and many others not identified in this study due to the unavailability of their standards.

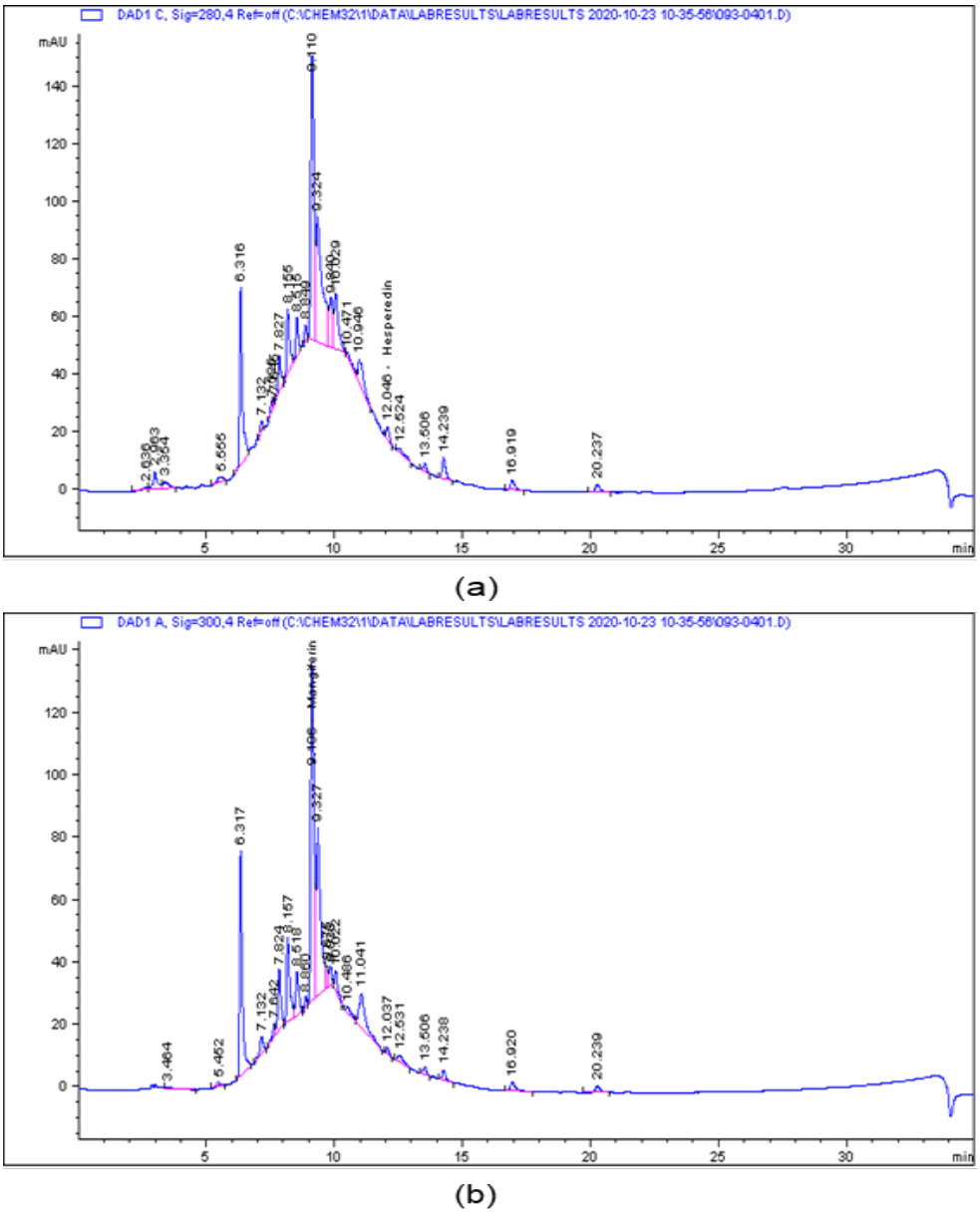

Figure 3.3: Chromatogram of the separation of the water extract of GHB (A) and FHB (B) at 300 nm wavelength

3.3.4. The optimum condition for the synthesis of biogenic AuNPs

Chemical method is among the most important approaches in AuNPs synthesis. This method utilizes chemicals such as sodium citrate sodium borohydride, hydrazine, elemental hydrogen, dimethylformamide and ascorbate for the reduction of Au ions into AuNPs. However, these

chemicalsare toxic and hazardous to human health and environs, hence limiting their use, particularly, in biomedical application (Majoumouo *et al*., 2020). Interestingly, plants contain phytochemicals (flavonoids, phenolic acids, carotenoids, and isoflavones) that can also reduce Au ions to produce AuNPs. GHB and FHB also contain these phytochemicals and the results of antioxidant capacity discussed in section 3.4.2 indicate that both GHB and GHB should be able to reduce Au ions for AuNPs production.

Additionally, the process of reducing metal ions, coupled with the formation of the NPs, is affected by various factors. Apart from variations in the phytochemical content of the different plant extracts, other factors such as the concentration of metallic ions/salts, concentration of plant extracts, the pH of the reaction mixture, reaction temperature, and reaction time have been shown to affect the synthesis of NPs as well as their shapes and sizes (Patra *et al*., 2018; Mukherjee *et al*., 2016). In this regard, a process of optimization is needed to identify the most suitable parameters for NP synthesis (Khandel*et al*., 2018). Although numerous studies have reported the synthesis of AuNPs from various plant extracts, differences in plants' compositions necessitate optimization for each plant extract. Since this study is the first to report the synthesis of AuNPs using water extracts of GHB and FHB, it is therefore necessary to optimize the above-mentioned synthetic factors to obtain the ideal conditions for the synthesis of AuNPs. It should be noted that the successful synthesis of biogenic AuNPs following incubation of reacting species is often characterized by a colour change from light yellow (that comes from the mixture of the plant extract and the Au salt) to ruby red. The appearance of the ruby red colour, which is due to the excitation of surface plasmon vibrations, represents spectroscopic signature of AuNPs. The presence of AuNPs can be confirmed by subjecting the sample to UV-vis ananlysis. The appearance of absorbance peak often at wavelength between 500 and 600 nm further confirms the excitation of AuNPs' SPR (Elbagory *et al*., 2016). In this study, factors which include the concentration of plant extract, pH of the reaction mixture, reaction time, and temperature of the reaction mixture were optimized to obtain the ideal conditions for the synthesis of biogenic AuNPs. The UV-Vis spectra of all reactions at different synthetic conditions are depicted in Figure 3.4.

3.3.4.1. The optimum reaction temperature for the synthesis of AuNPs

Temperature is a critical factor that affects the synthesis of NPs. It has been demonstrated that the rate of synthesising NPs from plant sources can be controlled by changing the temperature of the reaction medium (Dube *et al*., 2020; Elbagory *et al*., 2016). In this particular study, biogenic AuNPs were synthesised over a 24-hr period at 25°C and 70°C as described by Elbagory *et al*. (2016). It was observed that for all reactions at 25°C, no visible colour change was seen immediately after the reactive substances containing GHB extracts were incubated. However, visible colour changeswereonly observed for the reactions containing 4 mg/ml and higher concentrations of GHB after 1 hr reaction time (and this was maintained for 3, 6, 12, 18 and 24 hr reaction time) at 25°C. However, for FHB, no visible colour changes were seen for all reactions at all reaction time at 25°C. On the contrary, immediate colour changes were observed for all reactions for both GHB and FHB at 70°C. All reaction mixtures were further investigated for the presence of an absorbance peak using the UV-vis spectrophotometer. The λ_{max} as determined by the UV-vis for all reactions for the synthesis of GHB is shown in Table 3.3.

Table 3.3: The λmax of all reactions for the synthesis of GHB-AuNPs at 25°C and 70°C

Plant extract concentration (mg/ml)	λmax (nm)								λmax (nm)							
	25°C								70°C							
	0 hr	0.5 hr	1 hr	3 hr	6 hr	12 hr	18 hr	24 hr	0 hr	0.5 hr	1 hr	3 hr	6 hr	12 hr	18 hr	24 hr
0.5	*	*	*	*	*	*	*	*	546	540	548	548	548	540	540	542
1	*	*	*	*	*	*	*	*	542	540	542	548	550	548	540	548
2	*	*	548	540	542	538	534	532	540	538	532	538	536	536	534	532
4	*	560	546	568	548	568	548	540	542	540	540	542	540	538	540	538
8	545	542	540	542	558	548	560	568	540	540	542	538	538	540	538	540
16	*	*	568	568	560	548	564	560	544	540	542	538	536	540	540	548
32	*	*	566	560	558	548	560	560	548	540	546	544	540	540	548	540
64	*	*	564	562	558	560	560	560	542	540	538	540	548	538	540	548

*No nanoparticle was synthesized at this condition.

Studies have shown that NPs synthesized at 70ºC give a sharper and narrow SPR bands compared to those formed at a lower temperatures (Dube *et al*., 2020). Elbagory *et al*. (2016) showed that AuNPs synthesized from *Eriocephalus africanus* at 70°C exhibited a distinct and

narrow SPR band compared to the AuNPs synthesized at a lower temperature. In the present study, the synthesis of AuNPs was reported for GHB at both 25°C and 70°C with those synthesized at 70°C having distinct and narrow absorbance peaks. However, the synthesis of AuNPs from GHB was observed only at 70°C. Therefore, this study agrees with the previous research in that AuNPs synthesized at 70°C for all reactions produced narrow SPR bands compared to those synthesized at 25°C. This indicates that higher temperature causes high reaction kinetics, thus reducing the rate of synthesis. As a result, 70°C was selected as the optimum temperature for both GHB-AuNPs and FHB-AuNPs synthesis due to the immediate colour change observed in the reaction mixtures as well as the presence of distinct and narrow absorbance peaks that correspond to the presence of AuNP. The λ_{max} as determined by the UV-vis for all reactions for the synthesis of GHB is shown in Table 3.4.

Table 3.4: The λmax of all reactions for the synthesis of FHB-AuNPs at 25°C and 70°C

Plant extract concentration (mg/ml)	λmax (nm)								λmax (nm)							
	25°C								70°C							
	0 hr	0.5 hr	1 hr	3 hr	6 hr	12 hr	18 hr	24 hr	0 hr	0.5 hr	1 hr	3 hr	6 hr	12 hr	18 hr	24 hr
0.5	*	*	*	*	*	*	*	*	556	550	548	548	540	548	548	540
1	*	*	*	*	*	*	*	*	546	540	542	540	538	540	548	542
2	*	*	*	*	*	*	*	*	540	548	546	540	548	538	540	540
4	*	*	*	*	*	*	*	*	540	540	540	540	538	538	540	536
8	*	*	*	*	*	*	*	*	548	548	550	548	542	540	538	538
16	*	*	*	*	*	*	*	*	540	548	540	548	540	538	548	540
32	*	*	*	*	*	*	*	*	548	546	542	540	548	540	548	542
64	*	*	*	*	*	*	*	*	550	542	540	542	540	548	540	548

*No nanoparticle was synthesized at this condition.

3.3.4.2. The optimum concentration of plant extracts for AuNPs synthesis

The amount of plant extract in terms of the volume and concentration affects the time required for NP formation, hence influencing the physical and chemical properties of the formed NPs. Here, the concentrations of the plant extracts used were 64, 32, 16, 8, 4, 2, 1 and 0.5 mg/ml. These plant extracts were mixed with 1 mM $NaAuCl_4 \cdot 2H_2O$ as described by Elbagory *et al*. (2016). The reactions were monitored for colour change at different time intervals for 24 hr, and the UV vis spectrum was recorded at time intervals, as shown in Figure 3.4. The appearance of a colour change from light yellow to ruby red was observed for reaction containing 8 mg/ml of

GHB immediately after incubation at 25°C. Further, reactions containing 4, and 8 mg/ml of GHB extracts at 25°C changed from light yellow to ruby red after 0.5 hr of incubation. Additionally, reactions containing 16 mg/ml of GHB and higher changed from light yellow to deep purple, which could mean the formation of aggregated NPs. It was also observed that the mixture containing 1, 2, 4, and 8 mg/ml yielded immediate colour change from light yellow to ruby red at 70°C for GHB. While mixtures containing 4 and 8 mg/ml of FHB yielded immediate colour change from light yellow to ruby red at 70°C. The reactions containing 0.5, 16, 32, and 64 for GHB produced a purple colour; the same was noted in those containing 0.5, 1, 2, 16, 32, and 64 for FHB at 70°C. This observation indicated these reactions produced aggregated AuNPs. Although, it was observed that the intensity of the absorbance increased with an increase in the concentration of plant extracts, only the reaction containing 2 mg/ml of GHB produced distinct and narrow SPR bands at both 25°C and 70°C. Similar observations were detected for FHB and only the mixture with 4 mg/ml of extract showed distinct and narrow SPR band at 70°C. Therefore, 2 mg/ml of GHB was selected as the ideal concentration of plant extract for the synthesis of GHB-AuNPs while 4 mg/ml of FHB was selected as the ideal concentration of plant extract for the synthesis of FHB-AuNPs.

3.3.4.3. Optimum reaction time for the synthesis of biogenic AuNPs

The reaction time required for the synthesis of NPs is also a key factor to be considered during the synthesis of biogenic NPs. The reaction mixtures were subjected to UV-vis analysis at 0, 0.5, 1, 3, 6, 12, 18 and 24 hr (Figures 3.4 and 3.5). It was observed that as the reaction time increased, the absorbance peak as well as the colour intensity of the reaction also increased.In this study, the highest colour intensity were observed for reactions incubated for 24 hr, however, the most distinct and narrow absorbance peaks were exhibited for reactions incubated for 1 hr. Consequently, present study considered that the optimum reaction time for the synthesis of both GHB-AuNPs and FHB-AuNPs is1 hr.

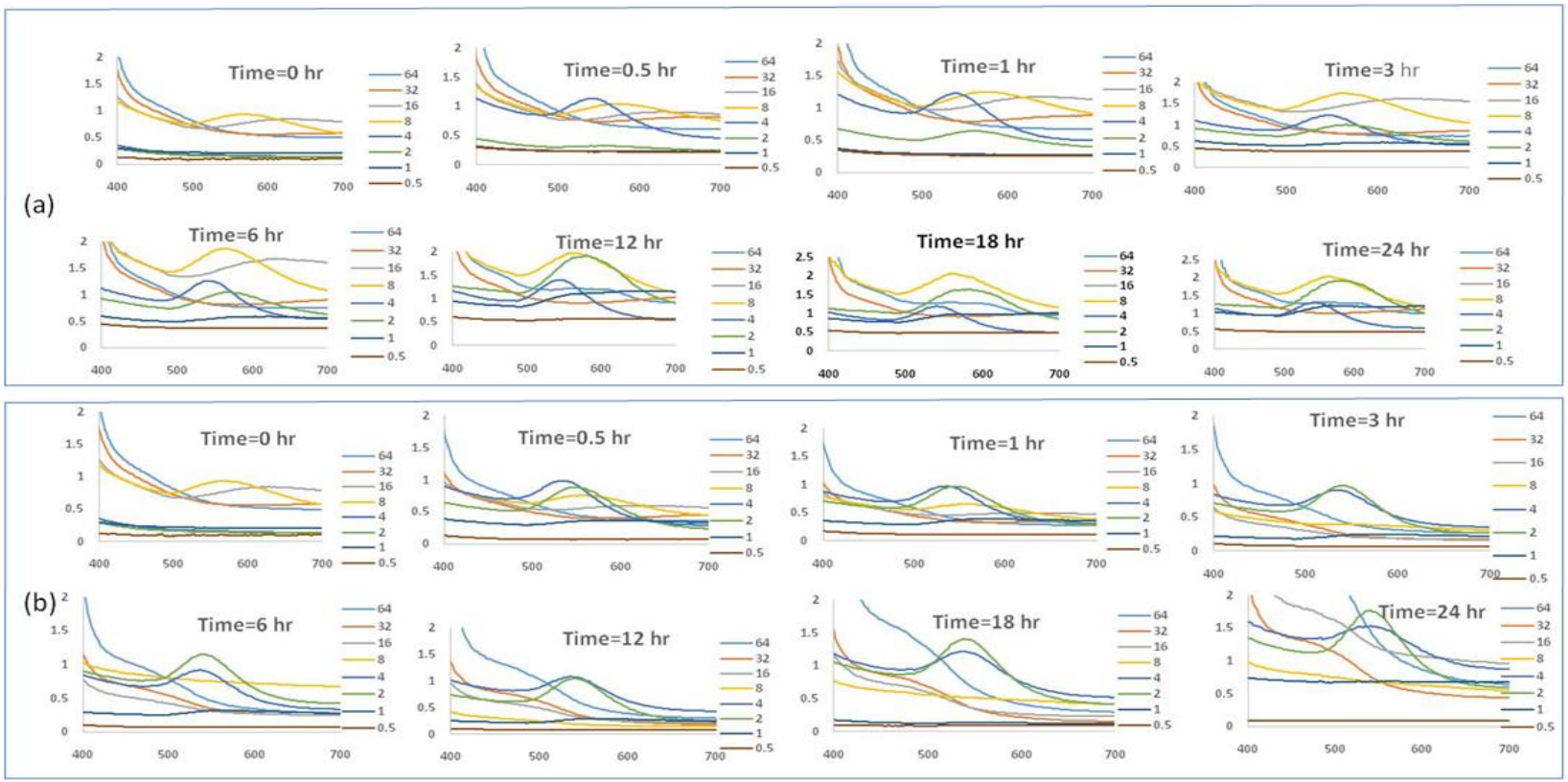

Figure 3.4: The UV-Vis spectra of all reactions for the synthesis of GHB-AuNPsat 25°C (A) and 70°C (B). Plant concentrations (0.5, 1, 2, 4, 8, 16, 32 and 64 mg/ml) and reaction time (0, 0.5, 1, 3, 6, 12, 18 and 24 hr) were considered as well for the optimization of GHB-AuNPs synthesis.

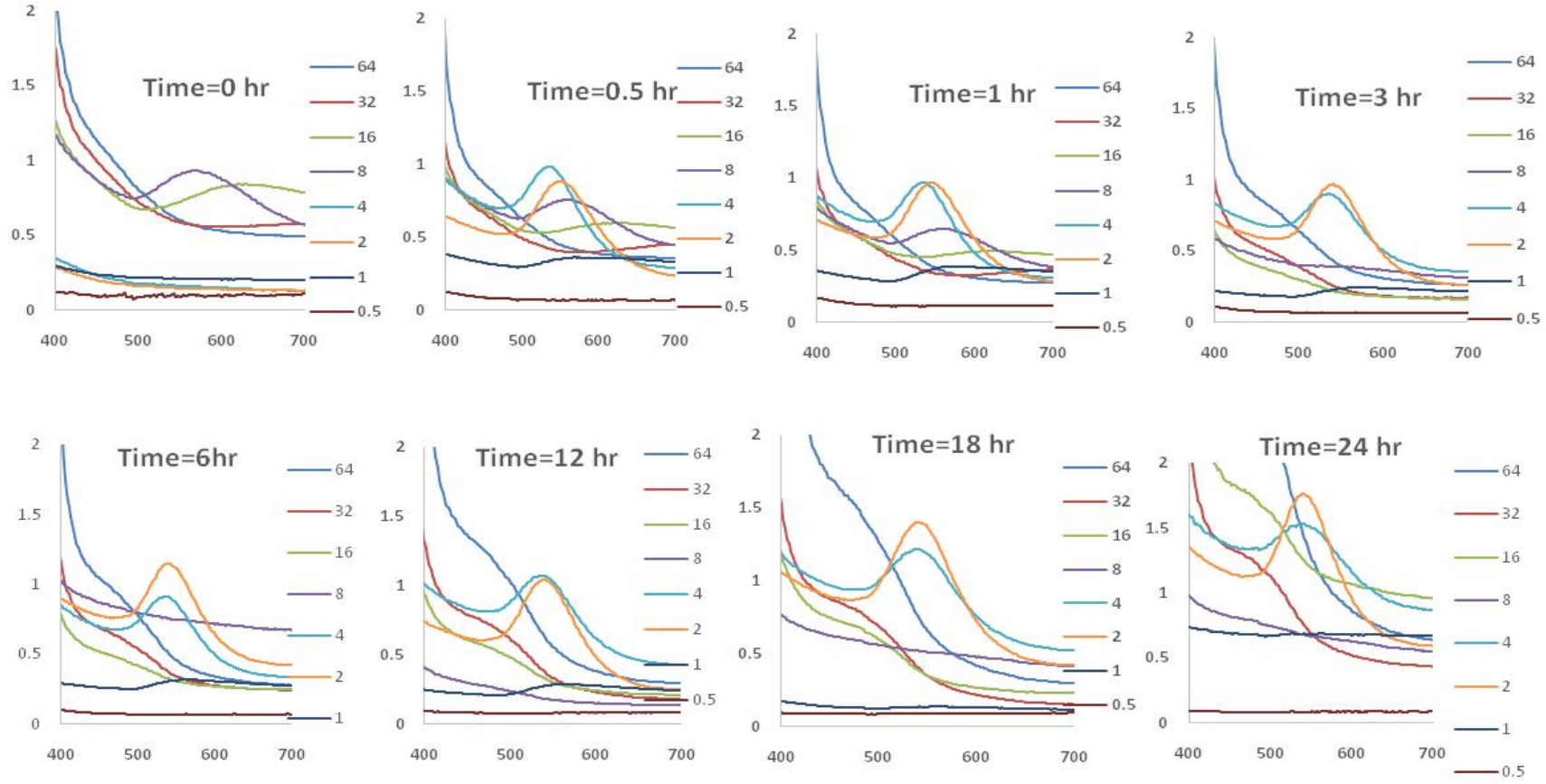

*Figure 3.5: The UV-Vis spectra of all reactions for the synthesis of FHB-AuNPs*at 70°C. *Plant concentrations (0.5, 1, 2, 4, 8, 16, 32 and 64 mg/ml) and reaction time (0, 0.5, 1, 3, 6, 12, 18 and 24 hr) were considered as well for the optimization of FHB-AuNPs synthesis*

3.3.4.4. Optimum pH of plant extracts for the synthesis of GHB-AuNPs and FHB-AuNPs

pH is an essential factor that affects the formation of NPs synthesized via plant-mediated approach. The variation in the size and shape of the resulting NP is greatly influenced by the reaction medium's pH (Dube *et al*., 2020). In this study, 2 mg/ml of GHB and 4 mg/ml of FHB were selected as the ideal concentration for HB-AuNPs synthesis. To determine the optimal pH and reaction temperature, reactions were performed at different pH (between 4.33 and 11.05) and different temperatures (25, 50, 70, and 100°C). It was observed that a colour change from light yellow to ruby red was observed for all reactions, which is indicative of the successful synthesis of AuNPs. Figure 3.6 shows the UV-vis spectra of the reaction mixtures with their corresponding absorbance peaks. In this study, it was observed that pH 4.33, which is the plant extract's pH produced the most distinct and narrow absorbance peaks. Therefore, pH 4.33 was selected as the ideal pH of GHB and FHB for AuNP synthesis.

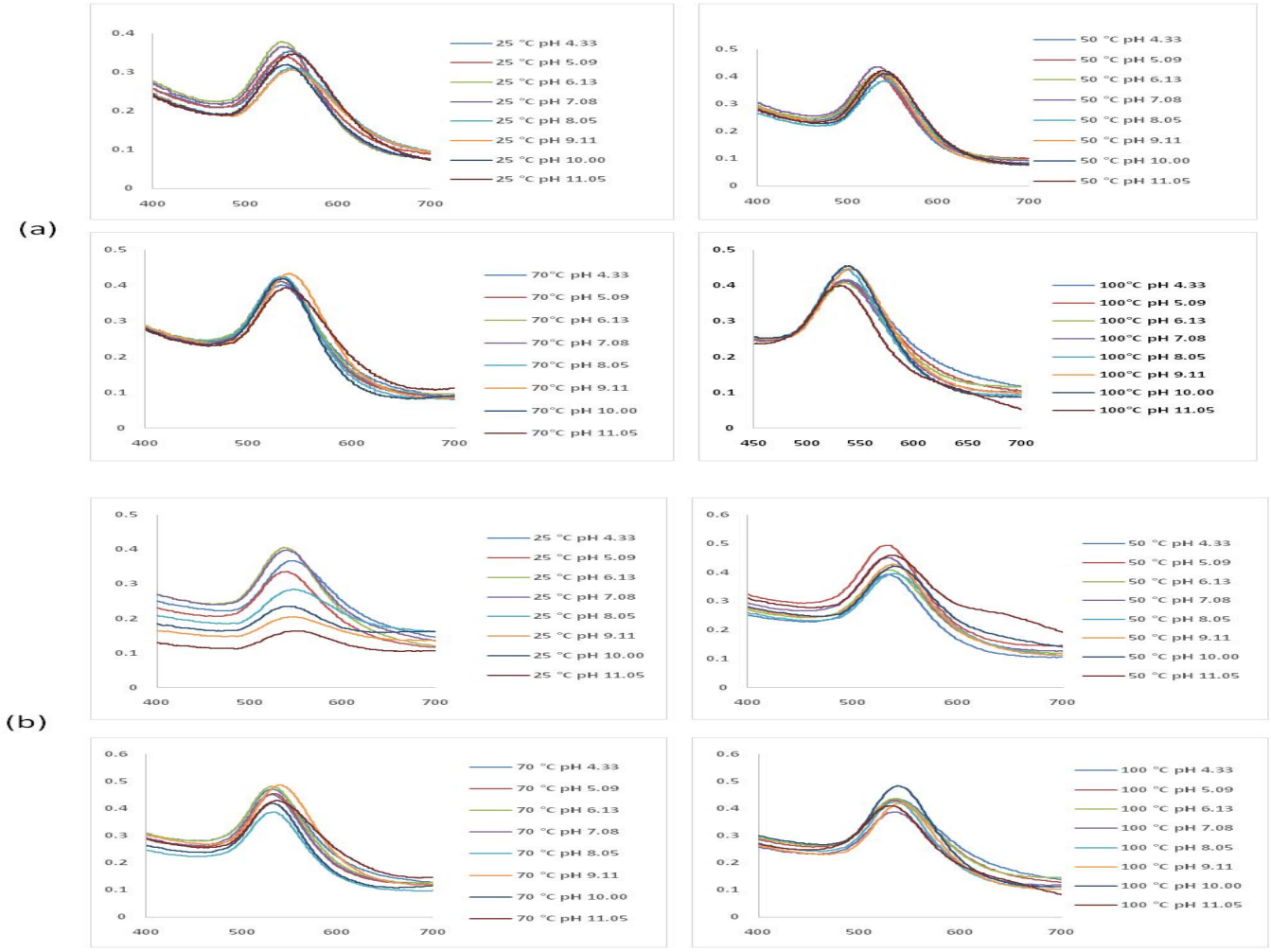

Figure 3. 6: The effect of plant pH on the synthesis of GHB (A) and FHB (B). 2 mg/ml of GHB and 4 mg/ml of FHB were varied at different pH (4.33, 5.09, 6.13, 7.08, 8.05, 9.11.10, and 11.05) for the synthesis of GHB-AuNPs and FHB-AuNPs, respectively at differen temperatures (25, 50, 70 and 100°C).

3.3.5. Purification and DLS analysis of biogenic AuNPs

The excess $NaAuCl_4 \cdot 2H_2O$ and plant extract that did not partake in the formation of the AuNPs must be removed by washing the NPs with water. However, this can affect the size and charge of the AuNPs resulting in aggregation. For this reason, the AuNPs were washed in water and analysed by DLS. Table 3.5 summarises the changes in size, charge and PDI of the AuNPs after 3 rounds of washing with water. The result showed that the hydrodynamic size of both GHB-AuNPs and FHB-AuNPs was increasing following each wash. Additionally, aggregation of AuNPs was physically observed at the third wash particularly for the FHB-AuNPs. Similar increment following each wash was observed for the zeta potential as well as the PDI of both AuNPs. Therefore, for this study, synthesized AuNPs were washed only two times to avoid the aggregation of the AuNPs.

Table 3.5: The effect of purification (by washing) on the size, zeta and PDI of biogenic AuNPs

DLS analysis	Activity	GHB-AuNPs	FHB-AuNPs
Size	Before wash	52.89 ± 0.01	96.5 ± 0.06
	First wash	68.35 ± 0.12	98.8 ± 0.02
	Second wash	85.62 ± 0.06	108.8 ± 0.02
	Third wash	86.05 ± 0.01	144.0 ± 0.07
Zeta	Before wash	-11.1 ± 0.00	-11.1 ± 0.01
	First wash	-22.5 ± 0.04	-14.2 ± 0.00
	Second wash	-29.5 ± 0.01	-14.9 ± 0.00
	Third wash	-28.8 ± 0.09	-25.8 ± 0.06
PDI	Before wash	0.394 ± 0.04	0.551 ± 0.07
	First wash	0.302 ± 0.01	0.582 ± 0.05
	Second wash	0.298 ± 0.06	0.485 ± 0.00
	Third wash	0.559 ± 0.08	0.540 ± 0.05

Result is expressed as mean value ± SEM

3.4. Conclusion

For the first time, water extracts of both GHB and FHB plants were used to reduce gold ions to produce AuNPs. The successful synthesis of the NPs was achieved following the optimization of parameters such as temperature, pH, reaction time and concentration of plant extracts that often affect the synthetic process. Overall, the ideal conditions for the synthesis of AuNPs from the GHB were 2 mg/ml of plant extract, a reaction temperature of 70°C, pH of 4.33 and reaction time at 1 hr. Additionally, the ideal conditions for the synthesis of AuNPs from FHB were 4 mg/ml of plant extract, temperature at 70°C, pH at 4.33 and reaction time at 1 hr. Additionally, this study report the stability of the synthesized biogenic AuNPs in water after wash. Thus,

present study strongly suggests that both the GHB and FHB plants are good candidates for the effective reduction of gold precursor to produce biogenic AuNPs.

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CHAPTER FOUR: MANUSCRIPT

GOLD NANOPARTICLES SYNTHESIZED USING EXTRACTS OF *CYCLOPIA INTERMEDIA*, COMMONLY KNOWN AS HONEYBUSH AMPLIFYTHE CYTOTOXIC EFFECTS OF DOXORUBICIN

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Article

Gold Nanoparticles Synthesized Using Extracts of *Cyclopia intermedia*, Commonly Known as Honeybush, Amplify the Cytotoxic Effects of Doxorubicin

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Abstract: *Cyclopia intermedia* (*C. intermedia*) is an indigenous South African shrub used to prepare the popular medicinal honeybush (HB) tea. This plant contains high levels of mangiferin (MGF), a xanthonoid that was reported to have numerous biological activities, including anti-tumor activity. MGF and extracts that contain high concentrations of MGF, such as extracts from *Mangifera indica* L. or mango have been used to synthesize gold nanoparticles (AuNPs) using green nanotechnology. It has previously been shown that when AuNPs synthesized from *M. indica* L. extracts are used in combination with doxorubicin (DOX) and Ayurvedic medicine, the anti-tumor effects appear to be augmented. It has also been demonstrated that MGF used in combination with DOX resulted in enhanced anti-tumor effects. In this study, *C. intermedia* (HB) and MGF were used to synthesize HB-AuNPs and MGF-AuNPs, respectively. The physicochemical properties of the AuNPs were characterized by the UV-Visible Spectroscopy (UV-Vis), dynamic light scattering (DLS), Fourier transform infra-red spectroscopy (FTIR), X-ray diffraction spectroscopy (XRD) and high-resolution transmission electron microscopy (HR-TEM). The cytotoxicity of HB-AuNPs and MGF-AuNPs were assessed on human colon (Caco-2), prostate (PC-3) and glioblastoma (U87) cancer cells; as well as normal breast epithelial (MCF-12A) cells using the MTT assay. Both HB-AuNPs and MGF-AuNPs demonstrated relatively low cytotoxicity in these cells. However, when these nanoparticles were used in combination with DOX, the cytotoxicity of DOX was significantly augmented.

Keywords: *Cyclopia intermedia*; honeybush; green synthesis; gold nanoparticles; mangiferin; nanotechnology

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1. Introduction

Several studies have reflected on the bio-activity of mangiferin (MGF), the xanthonoid

Article

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Keywords: *Cyclopia intermedia;* Honeybush; Green synthesis; Gold nanoparticles; Mangiferin; Nanotechnology

1. Introduction

Several studies have reflected on the bio-activity of MGF, the xanthonoid is known for the treatment of several health problems not limited to diabetes [1], inflammation, obesity, cancer [1,2] and osteoarthritis [2,3]. The health-benefits of MGF have prompted a rigorous research to find other sources of this valuable compound [4,5]. While the extracts from mango (*Mangifera indica L.*) fruit peel [6], seeds, leaves [2,7,8], bark and roots [2,7] have been shown to contain this compound, MGF is also found abundantly in other plant species such as *Salacia chinensis*, *Swertia chirata*, *Hypericum aucheri* and *C.*

intermedia, [6,9,10]. The xanthones (MGF and isomangiferin) and flavanones (hesperitin and hesperidin) are the major components of *C. intermedia* extracts, and are thought to be responsible for most of its pharmacological effects including anti-diabetic, anti-cancer, anti-obesity, antioxidant and antimicrobial activities [11,12].

Independent studies have reported that MGF has excellent anti-cancer effect; however its exact molecular mechanism has not been fully elucidated. Previous evidence clearly indicates that MGF could inhibit the proliferation of cancer cells via the induction of apoptosis, and its ability to regulate apoptotic pathways via multiple targets has been documented [13]. A study by Zou *et al*. showed that MGF induced apoptosis of A459 human lung cancer cells by activating NF-κB and caspase-dependent pathways [14]. Pan *et al*. reported the suppression of Bcl-xL and XIAP expression and the blocking of nuclear entry of NF-κB in HL-60 human acute myeloid leukemia cells treated with MGF [15].

It has also been reported that the co-administration of MGF and the chemotherapeutic drug, DOX can promote the anti-cancer effects of DOX in U-937 human myeloid leukemia cells [16]. Another study showed that MGF sensitized the response of MCF-7 breast cancer cells to DOX by suppressing the expression of p-glycoprotein [17]. It is also believed that MGF might be a promising agent to reverse DOX-resistance in cancer cells and is thus considered as a potential chemosensitizer for DOX therapy [17].

Numerous studies reported on the use of green nanotechnology to synthesize AuNPs from *M. indica L*. [18-20] and MGF [6,9]. While MGF is a major phytochemical constituent of the peel of *M. indica L*., it is not known if MGF is involved in the synthesis of the AuNPs when synthesis is done using a plant extract. Al-Yasiri*et al*. reported the synthesis of radiolabelled AuNPs (MGF-^{198}AuNPs) using MGF. The study also showed that MGF-^{198}AuNPs was retained in prostate tumors resulting in a significant reduction of tumor size [6]. It was also recently demonstrated that Nano Swarna Bhasma, AuNPs synthesized using a mixture of plant phytochemicals from *M. indica L*. peel and proprietary combinations of *Emblica officinalis*, *M. indica L*., *Curcumin longa*, *Acacia nilotica* and *Glycyrrhiza glabra*; was highly toxic to the MDA-MB-231 breast cancer cells [9]. Experiments using Severe Combined ImmunoDeficient (SCID) mice showed that this treatment (Nano Swarna Bhasma) reduced breast tumor sizes in these animals. Importantly, the study also showed in a pilot human clinical investigation that breast cancer patients who received the Nano Swarna Bhasma treatment in addition to conventional anti-tumor drugs (DOX and cyclophosphamide) experienced significant therapeutic benefits [9].

In the present study, we investigated if AuNPs can be synthesized using water extracts produced from the *C. intermedia*, which also has a high MGF content. *C. intermedia*, commonly known as honeybush (HB), is indigenous to the South-Western and South-Eastern parts of South Africa [21]. The leaves of HB are commonly used to make herbal tea which has many health benefits. HB is rich in antioxidants and the health benefits include the treatment of infections, coughs, sore throat, colds, osteoporosis, prevention of cancer and asthma [11]. The genus *Cyclopia* consist of some 20 species of flowering plants in the legume family, Fabaceae [4]. Several species which include *C. longifolia*, *C. subternata*, *C. sessiliflora*, *C. maculata*, *C. genistoides* and *C. intermedia* grow naturally in the wild in various regions of South Africa. To our knowledge, this plant has not previously been used for the synthesis of AuNPs. The study demonstrated the synthesis of AuNPs using leaf extract of *C. intermedia*. We showed that these nanoparticles had low cytotoxicity on the selected cells but appeared to significantly augment the cytotoxic effects of DOX in Caco-2 cells when used in combination with DOX.

2. Results

2.1. Biosynthesis of HB and MGF AuNPs

HB is rich in polyphenols, of which MGF (62.721 mg/g) and hesperidin (40.742 mg/g) are the most abundant [22]. Although the synthesis of AuNPs has been reported for both MGF and hesperidin [6,10,23], MGF with its four hydroxyl groups and a glucose unit exhibits stronger bioreduction of metal precursors to form nanoparticles. It is therefore likely that MGF found abundantly in *M. indica* L. and HB extracts (HBE) could act as both reducing and stabilizing agent during the synthesis of AuNPs. However, due to the complex and diverse nature of the phytochemicals present in plant extracts it is almost impossible to pinpoint a particular phytochemical responsible for the bioreduction and stabilization of the nanoparticles. Of interest, phenolic compounds (caffeic acid, gallic acid) and flavonoids (anthocyanidins, isoflavones, flavones and flavols) were reported to play a major role in the synthesis of metallic nanoparticles [24,25]. This is due to the high nucleophilic character of hydroxyl and carbonyl groups as well as their excellent binding affinity to metal ions [26,27].

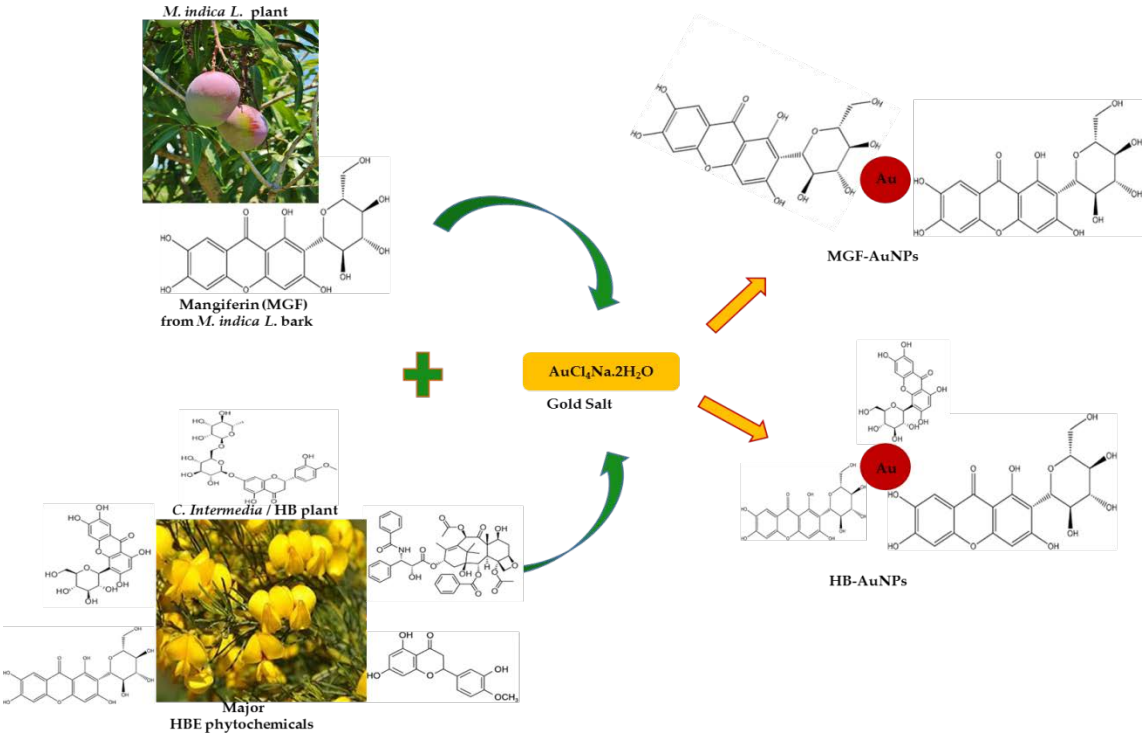
Polyphenolic compounds such as flavonoid and phenols are the major constituents of antioxidants in most plant species and their antioxidant activity is mainly accredited to their redox properties. As a result, they can act as reducing agents by aiding in the reduction of metallic ions into nanoparticles [28]. It is known that plants with a higher Total Phenolic Content (TPC) show high antioxidant activity and consequently possess excellent reductive capacity necessary for the synthesis of nanoparticles [29]. The phytochemical constituents, TPC and antioxidant capacity of HBE and MGF were compared and displayed in Table 1. MGF was commercially obtained; it was isolated from M. indica L. bark and had the purity of ≥98% (TLC). The comparative phytochemical analysis of HBE and MGF revealed that 10 mg/ml HBE contained high amounts of flavanols (8.38 mg/g) and it was not detected in the MGF. Flavonols were present in HBE (0.3 mg/g), but at a much lower concentration than in the MGF (8.7 mg/g). The TPC was also significantly higher in MGF (1.30 mgGAE/g) than in HBE (0.18 mgGAE/g). A previous study has shown that plants with high TPC show higher reducing capacities for nanoparticle synthesis. Goodarzi *et al*. reported the successful synthesis of nanoparticles from various plant extracts and reported that *Zataria multiflora* has the highest TPC and thus more potent in nanoparticle synthesis [30]. The antioxidant activity of HBE as measured by DPPH radical scavenging, ORAC and FRAP activities were also lower for HBE compared to MGF. Several plant extracts with high reductive capacity have been reported to actively reduce metallic ions including silver and gold ions to their corresponding metallic nanoparticles [31,33]. This reductive capacity can be easily traced to the secondary metabolite content of the plants which includes polyphenols, flavonoids and steroids. Numerous studies have also demonstrated that these metabolites in the aqueous medium act as both reducing and stabilizing agents for the synthesis of metallic nanoparticles [34,35]. This study revealed that HBE exhibited high reductive capacity and based on this was likely to reduce gold ions to form AuNPs.

Table 1. Phytochemical analysis and antioxidant capacity of HBE and MGF.

Phytochemical constituents	HBE	MGF
Flavanols (mg/g)	8.3753	-
Flavonols (mg/g)	0.3000	8.6742
TPC (mgGAE/g)	0.1827	1.2992
DPPH (µmolTE/g)	10.2601	75.3811
ORAC (µmolTE/g)	50.8520	376.2916
FRAP (µmolAAE/g)	11.5828	94.8750

-, Not detected.

AuNPs were successfully synthesized using both HBE and MGF and the possible mechanism for AuNP synthesis is depicted in Scheme 1. During synthesis, a distinctive ruby red color change was observed within 10 mins after sodium tetrachloroaurate (III) dehydrate and HBE was combined at 70°C to produce HB-AuNPs. In comparison, the reaction with MGF was almost immediate (<2 mins). For the synthesis of MGF-AuNPs, Gum Arabic (GA) was added to stabilize the nanoparticles. GA is a non-toxic glycoprotein polymer commonly used as a stabilizer in the food and pharmaceutical industries. It has been reportedly used to functionalize and stabilize AuNPs owing to the excellent binding affinity of its abundant carboxyl groups to other biomolecules. The introduction of GA resulted in a color change from brown (for MGF without GA) to a ruby red color that appeared immediately, indicating the formation of stable MGF-AuNPs.



Scheme 1: Possible mechanism for green synthesis of biogenic MGF-AuNPs and HB-AuNPs.

2.1.1. Characterization of MGF-AuNPs and HB-AuNPs

HB-AuNPs and MGF-AuNPs were characterized by UV-Visible spectrophotometry, DLS and HRTEM analysis. HB-AuNPs and MGF-AuNPs produced maximum UV-Vis absorption peaks at 540 and 538 nm, respectively (Figure 1), which corresponds to the surface plasmon resonance (SPR) for the AuNPs.

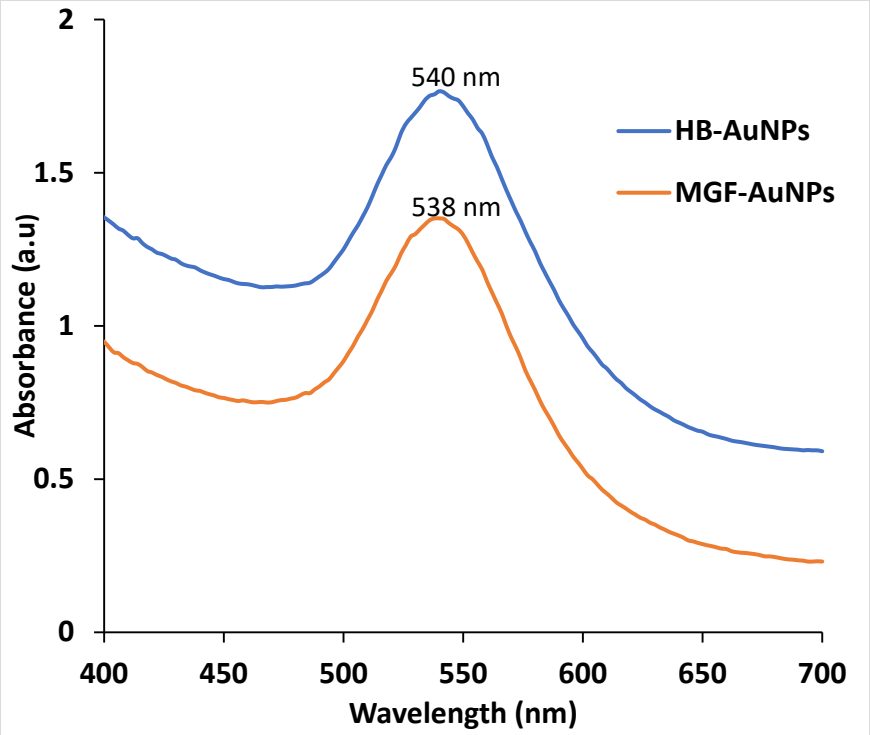


Figure 1. UV-Visible absorption of HB-AuNPs and MGF-AuNPs.

The hydrodynamic diameter, polydispersity index (PDI) and zeta potential of the biogenic AuNPs were measured by DLS. As shown in Table 2, the Z-average size of HB-AuNPs and MGF-AuNPs were similar. The diameter of HB-AuNPs and MGF-AuNPs were 66.74±9.7 nm and 65.50±15.15 nm, respectively. The Zeta potential and PDI of HB-AuNPs was -23.45±1.4 mV and 0.57±0.01, respectively. MGF-AuNPs had a zeta potential of -27.87±2.54 mV and a PDI of 0.43±0.07. The characteristics of HB-AuNPs and MGF-AuNPs as measured by DLS were very similar. Nanoparticles with a zeta potential between -30 and +30 mV are stable and will not aggregate in solution [36]. This suggests that HB-AuNPs and MGF-AuNPs are stable in solution.

Table 2. DLS analysis of HB-AuNPs and MGF-AuNPs.

AuNPs	Z-average size (nm)	PDI	Zeta potential (mV)
HB-AuNPs	66.74 ± 9.7 nm	0.571 ± 0.01	-23.45 ± 1.4
MGF-AuNPs	65.50 ± 15.15 nm	0.432 ± 0.07	-27.87 ± 2.54

- Results are expressed as mean ± SEM.

HRTEM image analysis showed similar characteristics between HB-AuNPs and MGF-AuNPs. Both HB-AuNPs and MGF-AuNPs are polydisperse with most nanoparticles having predominantly spherical shapes and some triangular shapes. The core diameter of the nanoparticles varied from 5 to 45 nm (Figures 2a and 2b). The average core size for HB-AuNPs was 20 nm (Figure 2c) and 26 nm for MGF-AuNPs (Figure 2d).

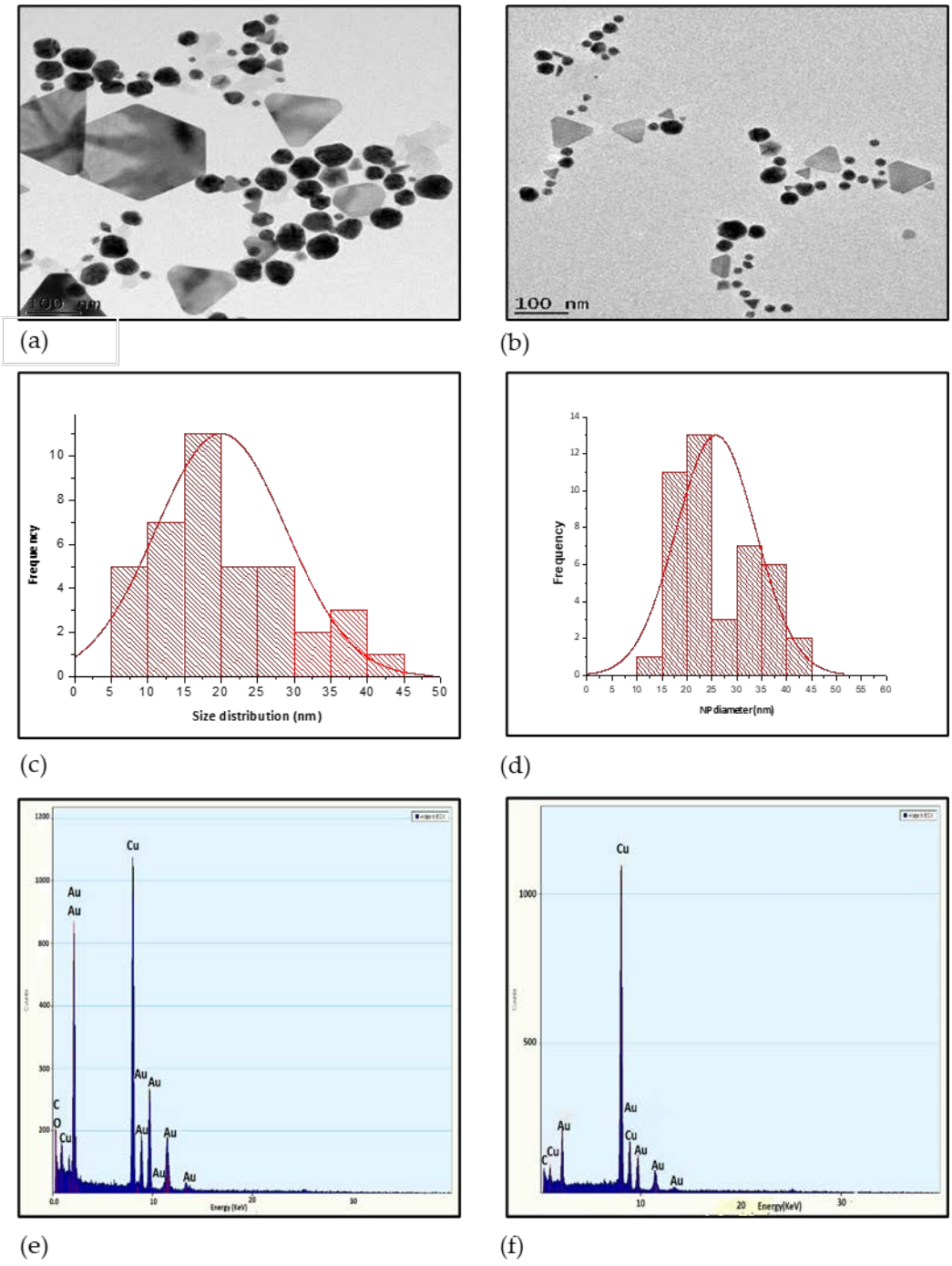


Figure 2. Morphology and EDX profiling of the biogenic-AuNPs. HRTEM micrographs of HB-AuNPs (a) and MGF-AuNPs (b); particle size distribution of HB-AuNPs (c) and MGF-AuNPs (d); EDX profiles of HB-AuNPs (e) and MGF-AuNPs (f) for their chemical composition.

The EDX was employed to analyze the composition of the AuNPs. The result clearly confirmed the presence of Au in both HB-AuNPs (Figure 2e) and MGF-AuNPs (Figure 2f). The peaks that correspond to Cu and C in the EDX spectra of the two AuNPs could be due to the carbon-coated copper grid while the

O in the HB-AuNPs is from one of the phytochemicals present in the HBE as previously argued by Wang *et al* [37].

The XRD and SAED patterns in Figure 3 demonstrated the crystalline nature of the HB-AuNPs and MGF-AuNPs. The XRD pattern for the two AuNPs revealed five peaks corresponding to standard Bragg's reflection (111), (200), (220), (311) and (222) of face center cubic (fcc) lattice for AuNPs [38]. The more intense peaks correspond to (111) and (200) planes for HB-AuNPs (Figure 3a) and MGF-AuNPs (Figure 3b), which indicates the preferential growth of AuNPs at that orientation [32,39].

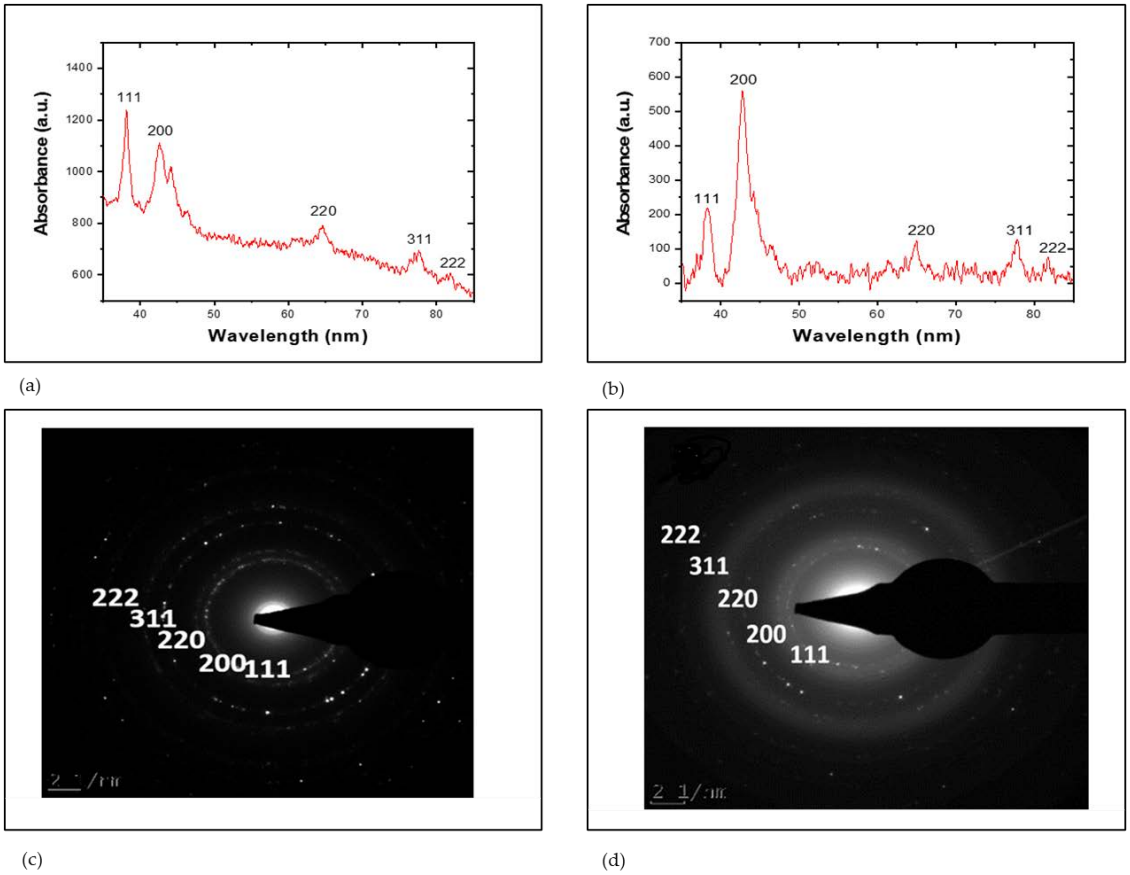


Figure 3. XRD and SAED patterns of the biogenic-AuNPs as determined by XRD Spectroscopy and HR-TEM. XRD patterns of HB-AuNPs (a) and MGF-AuNPs (b); SAED patterns of HB-AuNPs (c) and MGF-AuNPs (d).

The SAED patterns of HB-AuNPs and MGF-AuNPs in Figures 3c and 3d, respectively, also confirmed the appearance of ring-like peaks similar to the XRD fcc corresponding to (111), (200), (220), (311) and (222). The fcc nature of gold observed for HB-AuNPs and MGF-AuNPs further gives a clear indication that HB-AuNPs and MGF-AuNPs are composed of pure crystalline gold.

The FTIR analysis was used to investigate the functional groups of the phytochemicals in HBE, MGF, HB-AuNPs and MGF-AuNPs. The FTIR spectra were compared to identify the functional groups of the phytochemicals present in HBE and MGF that are likely to be responsible for the reduction of gold ions to their zero-valence form. Figure 4a showed peaks at 3747 and 3299 cm^{-1} that are indicative of the presence of hydroxyl and amine groups, respectively. The O-H stretching is for alcohols which could be

polyphenols and polysaccharides. The reduction in the intensity of peak at 3299 cm^{-1} in AuNPs is evidence of the participation of the hydroxyl functional groups. This takes place when alkoxide ions are generated which will then serve as a reducing agent [40]. According to Ovais *et al.*, polyphenols are the chief reducing and/or capping agents in the plant-mediated green synthesis of metallic nanoparticles [41]. The bands at 2921 cm^{-1} for both the HBE and AuNPs, and an additional 2671 cm^{-1} for the latter are due to the C-H stretching of aromatic compounds. This provides further support for the presence of polyhydroxy aromatic compounds [31]. In both the HBE and the HB-AuNPs, the peak at 1411 cm^{-1} was due to bending vibration as a result of SP^2 hybridization which is common to alkenes and aromatic carbons [42]. The bands at 1064 cm^{-1} were observed for both the extract and the AuNPs corresponding to the C-O stretching of alcohols and carboxylic acids. This band is also assigned to the C-N stretching of aliphatic amines [43] mentioned earlier at 3747 cm^{-1} peak, thereby confirming the involvement of aliphatic amines in the formation of the HB-AuNPs. The weak bands at 2103 and 2140 cm^{-1} correspond to C=N stretching, an indication that amides may also be involved in the synthesis of the HB-AuNPs [44].

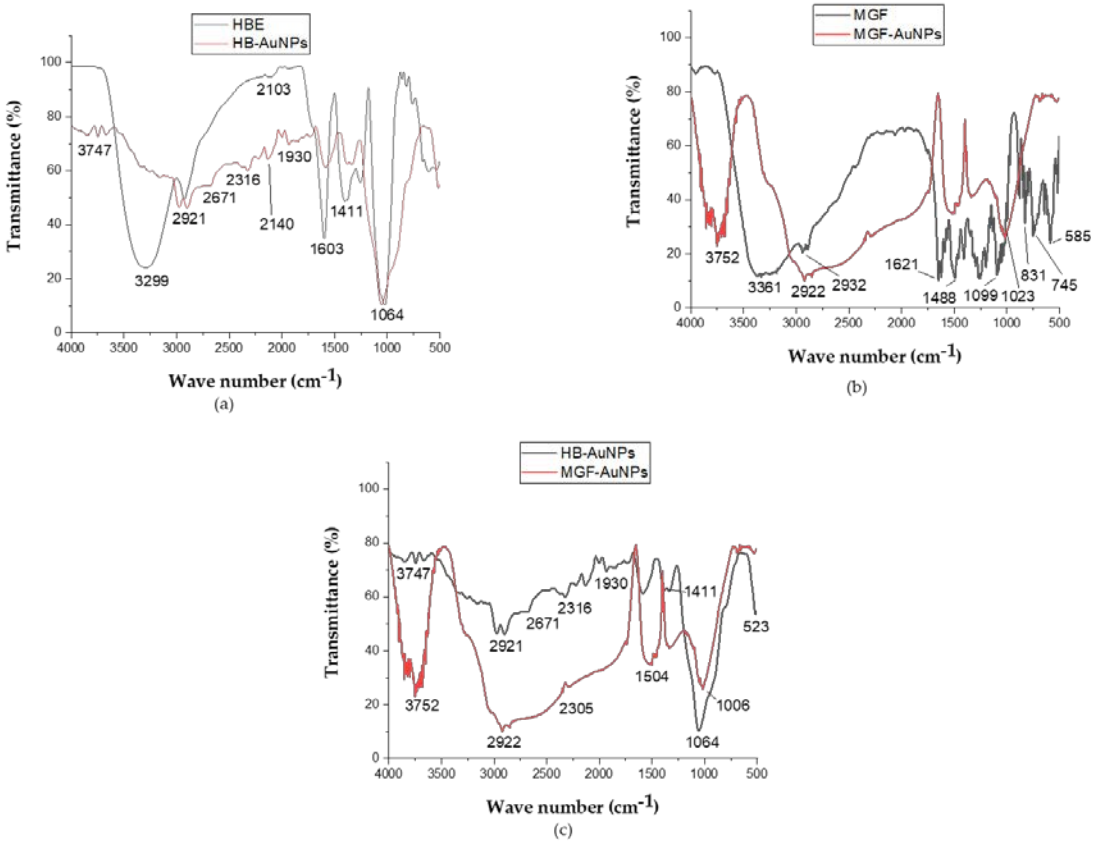


Figure 4. FTIR spectra of the HB and MGF extracts and their respective biogenic-AuNPs. (a) HB-Extract and HB-AuNPs; (b) MGF-Extract and MGF-AuNPs; (c) HB-AuNPs and MGF-AuNPs.

The FTIR spectra of MGF and MGF-AuNPs are shown in Figure 4b. The 3362 cm^{-1}peak in the MGF is indicative of OH-alcohol stretching, its absence in the MGF-AuNPs suggests the involvement of the hydroxyl functional groups in the synthesis of MGF-AuNPs. In addition, the sharp peak at 2069 cm^{-1} corresponding to C-H stretching vibration in the FTIR spectra of MGF was significantly reduced in FTIR spectra of MGF-AuNPs. This further shows that C-H functional groups could also be involved in the reduction of gold ions [45]. Furthermore, the disappearance of peaks at 1488 and 1099 cm^{-1} corresponding

to C-O-C and RCH_2OH in MGF-AuNPs indicates the removal of the glucose unit from MGF [10]. Interestingly, these peaks are retained in HB-AuNPs indicating that the glucose units found in MGF might not be involved in the reduction and stabilization of HB-AuNPs. Overall, polyphenols, amines, polysaccharide, and amides are responsible for the formation of HB-AuNPs; while the hydroxyl and glucose units are involved in the formation of MGF-AuNPs. The FTIR spectra of HB-AuNPs and MGF-AuNPs showed some features that are quite similar as shown in Figure 4c. The FTIR suggests that the hydroxyl moieties which are also present in MGF were actively involved in the biosynthesis of HB-AuNPs.

2.2. In vitro stability of HB-AuNPs and MGF-AuNPs

The stability of HB-AuNPs and MGF-AuNPs was evaluated at 0 and 24 hrs incubation in various biological media and buffers; these include Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI 1640), Bovine serum albumin (BSA), fetal bovine serum (FBS) and Dulbecco's Phosphate Buffered Saline (DPBS). The spectra indicating the presence of the AuNPs still remained following a 24 hr incubation in various solutions as depicted in Figure 5. However, a change in the SPR and their corresponding absorbances was observed especially for AuNPs in the two media (Figure 5 (a, f, b and g)) and FBS (Figure 5 (d and i)). HB-AuNPs showed an increase in absorbance (0.716 to 1.316) after 24 hr incubation in DMEM (Figure 5a) while the MGF-AuNPs showed a moderate and negligible change in absorbance (0.642 to 0.63) and a redshift in the SPR (Figure 5f). These changes suggest that the AuNPs (especially HB-AuNPs) might be reacting with the components within the media and buffers resulting in increased dispersion quality of the AuNPs leading to the observed changes [46]. In addition, the two AuNPs were found to be highly stable in water at room temperature.

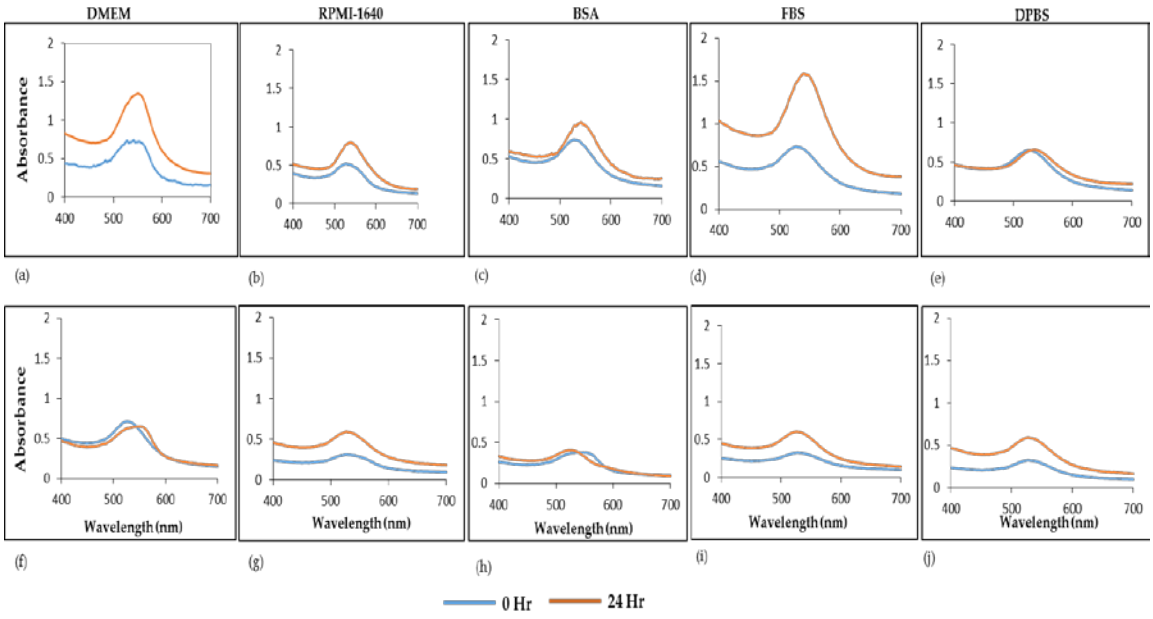


Figure 5. Stability of AuNPs in biological media at 0 and 24 hrs incubation at 37°C. UV-Vis spectra of HB-AuNPs (a-e) and MGF-AuNPs (f-j) in DMEM (a,f), RPMI (b,g), BSA (c,h), FBS (d,i) and DPBS (e,j).

2.3. Cytotoxicity of HB-AuNPs and MGF-AuNPs

The effects of HB-AuNPs and MGF-AuNPs were tested on cancer (Caco-2, U87 and PC-3) and non-cancer (MCF-12A) cell lines. The cells were treated with the nanoparticles (doses from15.62 to 1000 μg/ml) and their viability was assessed by the colorimetric MTT assay after 24 hrs. The extracts did not show cytotoxicity on the four cell lines after 24 hrs treatment at the same concentrations (data not shown).

Of note, both the HB and MGF have been reported to have antioxidant, antimutagenic and anti-cancer activities [47,48]. HB is known to improve the immune system, protects against inflammatory diseases, offers menopausal relief with phytoestrogenic effects, and has antimicrobial effects [48,49]. HB primarily exerts its anticancer effects through modulation of oxidative stress, inhibition of cell proliferation and inhibition of adenosine triphosphate production, all of which are closely associated with its high concentrations of monomeric polyphenols and flavonol compounds [49]. .MGF on the other hand has demonstrated using both *in vitro and in vivo* to have a broad-spectrum effect on several types of cancers. Evidence suggests that MGF exerts its anticancer potential by down-regulating inflammation, inhibiting cell cycle, offering protection against oxidative stress and DNA damage, enhancing apoptosis and inhibiting cell growth/invasion in malignant cells [13]. Studies have credited the pharmacological benefits of MGF-containing plants to MGF [12]. Intensive research has been done on *M. indica L* as the main source of MGF to substantiate these claims. Interestingly, AuNPs synthesized from whole extracts and MGF from *M. indica L* showed enhanced bio-activity when compared to the extracts alone.

The present study reports on the effect of AuNPs synthesized from HBE and MGF in cancer (Caco-2, U87 and PC-3) and non-cancer (MCF-12A) cells.The HB-AuNPs and the MGF-AuNPs showed similar toxicity towards the four cell lines as shown in Figure 6. At concentration of either HB-AuNPs (15.62-250 μg/ml) or MGF-AuNPs (15.62-125 μg/ml), the viability of the MCF-12A cells was over 100% (Figure 6a). This suggests that the biogenic AuNPs at these concentrations seem to protect the cells and thus lead to increment in their cell proliferation. The viability of MCF-12A cells was not significantly affected by either HB-AuNPs or MGF-AuNPs even at high concentrations. However, both HB-AuNPs and MGF-AuNPs behaved differently on the cancer cells. Significant reduction in cell viability was observed in U87 (Figure 6b) and PC-3 (Figure 6d) cells at concentrations ranging from 31.25 to 1000 μg/ml, while the viability of Caco-2 was only affected at concentrations ranging from 500 to 1000 μg/ml (Figure 6c). These results suggest that the toxicity of both HB-AuNPs and MGF-AuNPs are fairly low in the Caco-2 cell lines compared to the other cell lines. The U87 cells were the most susceptible to the effects of MGF-AuNPs and HB-AuNPs. As summarized in Table 3, MGF-AuNPs and HB-AuNPs inhibited 50% (IC_{50} values) of U87 cell growth at 85.9 and 121.4 μg/ml, respectively. While a concentration higher than 1000 μg/ml was required to get IC_{50} values in the other three cell lines. Based on their cytotoxic profile, HB-AuNPs and MGF-AuNPs have very similar biological activity. Both HB-AuNPs and MGF-AuNPs exhibited negligible cytotoxic effects on the MCF 12A cells even at the highest (1000 μg/ml) concentration. These indicate that the two AuNPs might be biocompatible when used *in vivo*. Independent studies have reported the biocompatibility and non-toxicity effects of biogenic AuNPs, MGF-AuNPs (up to 100 μM) was shown to be non-toxic on non-cancerous breast (MCF-10A) cells following a 24 hr treatment [10]. Another study also reported that AuNPs synthesized from *Hibiscus sabdariffa* extracts had very little toxic effect in normal 293 cells [50]. The negligible toxicity of AuNPs in non-cancerous cells reported in the present study, in addition to earlier reports further supports the preference of biogenic AuNPs for various biological applications. However, it should be noted that the four cell lines used in this study are derived from four different tissue origins. While the non-cancerous MCF-12A is derived from breast tissue, Caco-2, U87 and PC-3 were derived from colon, brain, and prostate, respectively.

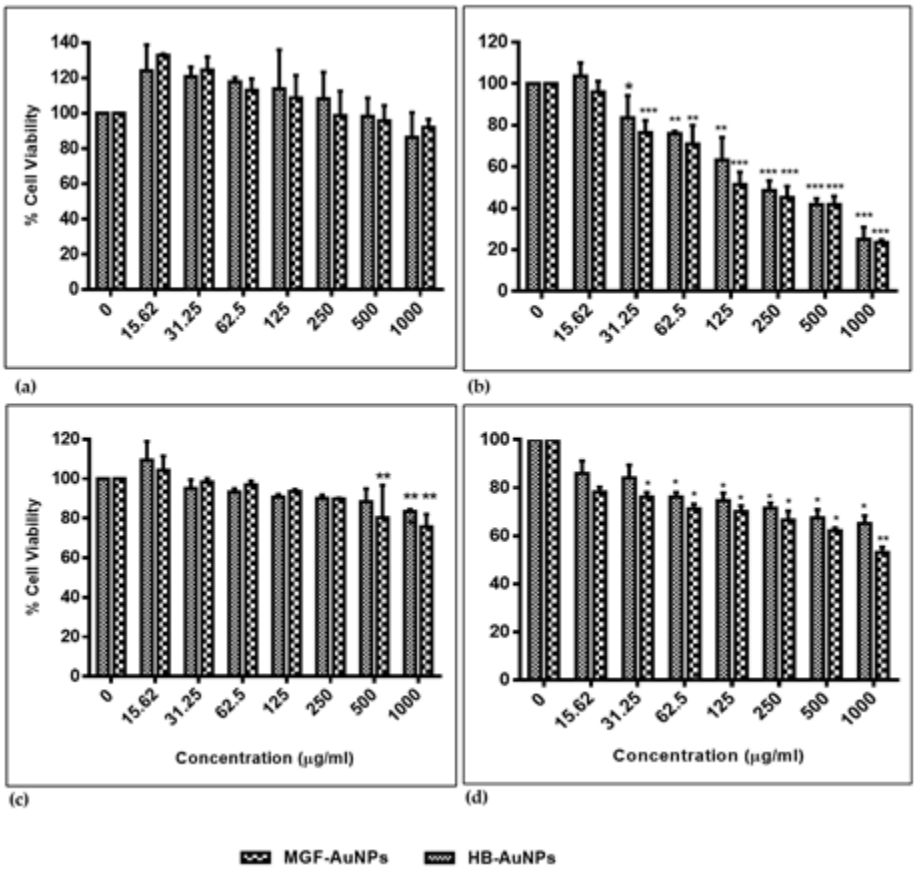


Figure 6. Investigation of the cytotoxic effects of HB-AuNPs and MGF-AuNPs in human cell lines. The *in vitro* effects of HB-AuNPs and MGF-AuNPs were tested in MCF-12A (a) U87 (b), Caco-2 (c) and PC-3 (d) cells. Data represented as the mean from three independent experiments. Error bars represent the standard error mean (± SEM). Data was considered to be statistically significant if $p < 0.05$, *** $p < 0.001$, **$p < 0.01$, * $p < 0.05$.

Table 3. IC_{50} values of the AuNPs.

AuNPs	IC_{50} (µg/ml)			
	MCF-12A	**U87**	**Caco-2**	**PC-3**
HB-AuNPs	>1000	121.4	>1000	>1000
MGF-AuNPs	>1000	85.9	>1000	>1000

AuNPs have attracted significant interest over the last decades owing to their ease of synthesis, surface functionalization, chemical stability, biocompatibility,low-toxicity and enhanced permeability and retention [51,52]. As a result, AuNPs are now widely researched for their potential in drug carrier/delivery, bioimaging, diagnosis and therapeutics. An earlier report demonstrated the significant anticancer effect of MGF-AuNPs against human PC-3 prostate cancer cells [6]. Khoobchandani *et al*. reported a 100% clinical benefit in breast cancer patients treated with biogenic AuNPs compared to standard anticancer drug [9]. Resveratrol-AuNPs reduced cell viability of MDAMB-231, PANC-1

(pancreatic cancer cells) and PC-3 cancer cells in a dose-dependent (20-220 μg/ml) manner after treating for 24 and 48 hrs[53]. Consequently, the work presented here and previous studies describe promising results towards the integration of AuNPs, particularly of biological origin in cancer therapy regimen.

2.3.1 *Co-treatment of Caco-2 cells with DOX and the biogenic AuNPs*

Due to their unique optical properties, AuNPs have many applications in medicine and can be used as drug delivery, photothermal, drug-sensitizing agents, among others. The drug-sensitizing effect of both HB-AuNPs and MGF-AuNPs was tested on the Caco-2 cells which had showed resistance towards their treatment. The Caco-2 cells were treated with a combination of DOX and the AuNPs at least-toxic concentrations. As shown in Figure 7a, DOX showed a dose-response induced cell death after 24 hrs of treatment with 1.56 – 1000 μg/ml of the drug. DOX showed a significant cytotoxic effect on Caco-2 cells at higher concentrations (6.25-100 μg/ml), and negligible effects were observed at 1.56 μg/ml which was selected for the co-treatment with the AuNPs.

After 24 hrs, the viability of cells treated with free DOX (1.56 μg/ml), HBE (1000 μg/ml), MGF (1000 μg/ml), MGF-AuNPs (1000μg/ml) and HB-AuNPs (1000μg/ml); reduced by ≤ 15% (Figure 7b). However, co-treatments with either DOX (1.56 μg/ml) and MGF-AuNPs (1000μg/ml) or DOX 1.56 μg/ml) and HB-AuNPs (1000μg/ml) further augmented the cytotoxic effect of DOX. While the viability of cells treated with DOX only or the AuNPs only reduced by about 10%, the viability of cells treated with DOX and MGF-AuNPs, or DOX and HB-AuNPs, reduced by 40% and 70%, respectively. This data suggests that both MGF-AuNPs and HB-AuNPs appear to work in synergy with DOX to enhance the cytotoxic effect of DOX, and this effect was more pronounced on co-treatment with the HB-AuNPs.

Of a fact, DOX is a potent and valuable clinical anticancer agent; associated side effects and the development of resistance to DOX are the major problems limiting its use [54]. Cancer cells, especially colorectal cancer, are inherently resistant to DOX and require higher doses (as shown in this study) to effectively inhibit their growth. This consequently may result in adverse side effects including cardiotoxicity and nephrotoxicity [55]. Strategies that can sustain its efficacy while minimizing its off-target toxicity has been one of the major areas of research focus. And through co-treatment with metallic nanoparticles, this may be possible. An independent study reported the enhanced effect of DOX on human breast cancer (MCF-7) cells when used in combination with iron oxide nanoparticles [56]. In another study, the sensitivity of ovarian A2780 cancer cells to cisplatin was enhanced when used in combination with silver nanoparticles synthesized from curcumin [57]. The present study proposed that biogenic MGF-AuNPs and HB-AuNPs increased the sensitivity of Caco-2 colon cancer cells to low concentration of DOX by offering synergistic effect in inhibiting their growth. The findings from this study indicated that AuNPs synthesized from pure MGF and HBE that contains substantial amount of MGF enhanced the efficacy of DOX.

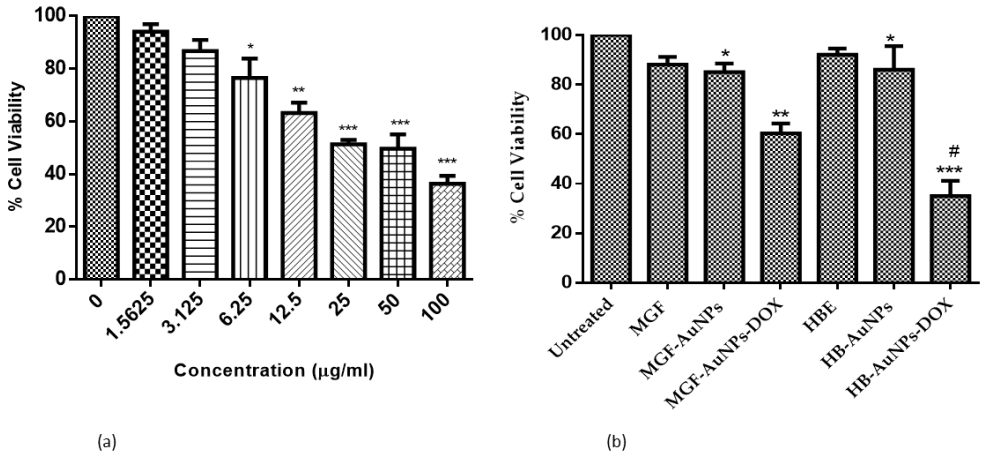


Figure 7. Co-treatment of Caco-2 cells with AuNPs and DOX enhanced cell death compared to individual treatments. (a) shows the viability of Caco-2 cells following treatment with increasing concentrations (1.56-100 μg/ml) of DOX for 24 hrs. (b) shows the viability of Caco-2 cells following treatment with MGF (1000 μg/ml, HBE (1000 μg/ml), MGF-AuNPs (1000 μg/ml), HB-AuNPs (1000 μg/ml) and DOX (1.56 μg/ml) in combination with either MGF-AuNPs (MGF-AuNPs-DOX) or HB-AuNPs (HB-AuNPs-DOX). Data represented as the mean from three independent experiments. Error bars represent the standard error mean (± SEM). Data was considered to be statistically significant if $p < 0.05$. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # <0.05 HB-AuNPs vs MGF-AuNPs.

3. Materials and Methods

3.1. Sample Preparation

MGF and GA powder were purchased from Sigma (St Louis, MO, USA). DOX was bought from Wuhan Sunrise Technology Development Company Limited (China). All these products were used as they were without any modification. Dried HB plant materials were donated to the Oxidative Stress Research Centre (Cape Peninsula University of Technology, South Africa) by the Rooibos Ltd. (Clanwilliam, South Africa). The plant materials were stored in sealed plastic containers and kept at 25°C in a dark room.

MGF, GA and DOX stock solutions were prepared in double distilled water. MGF and GA were prepared fresh. The HBE were prepared by boiling 100 g of the HB leaves in 1000 ml of double distilled water and kept on a magnetic stirrer without heat for 24 hrs. Afterwards, the infusion was filtered through Whatman's No 1 followed by 0.45 μm filters. The resulting supernatant was freeze dried using FreeZone 25L Freeze Dry (Labconco, Kansas City, MO, USA). The dried extracts were stored at 4 °C in sterile sealed containers for further use.

3.1.1. Phytochemical analysis and antioxidant capacity

The aqueous HBE, MGF and GA (1 mg/ml) were investigated for the presence of flavanols, flavonols, total polyphenolic contents (TPC) and antioxidant capacity viz, ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) using standard biochemical methods [58].

3.2. Synthesis of HB and MGF AuNPs

HB-AuNPs were synthesized according to the previously described method [33] with few modifications. Briefly, 1 ml of the 2 mg/ml HBE dissolved in distilled water was mixed with 5 ml of 1 mM sodium tetrachloroaurate (III) dehydrate (Sigma Aldrich, St. Louis, MO, USA). The mixture was kept in a shaking incubator (40 rpm) for 1 hr at 70°C.

MGF-AuNPs were synthesized in a similar method and only differ in sample preparation and temperature used. Briefly, 4.2 mg of MGF (Sigma Aldrich) was dissolved in boiled 6 ml of double distilled water. To this mixture, 12 mg of GA powder was added as a stabilizer and the mixture was kept on a magnetic stirrer with constant stirring at 80°C until MGF was completely dissolved. Then 0.2 ml of the MGF-GA mixture was added to 1 ml of warm 1 mM sodium tetrachloroaurate (III) dehydrate. Heat was switched off after the color changed to ruby red, and the mixture was continuously stirred on a heating block (200 rpm) for further 60 mins at room temperature.

3.2.1. Characterization of the biogenic-AuNPs

The biogenic AuNPs were washed thrice in double-distilled water and centrifuged at 14,000 rpm for 15 min, the AuNPs were resuspended in equal volume of double distilled water. The SPR of the AuNPs was determined using a POLARstar Omega microtitre plate reader (BMG Labtech, Ortenberg, Germany) at a wavelength range of 400–800 nm. The hydrodynamic diameter, PDI and zeta potential of the AuNPs were determined using Malvern Zetasizer instrument (Malvern Ltd., UK). Perkin Elmer Spectrum two Fourier-Transform infrared (FTIR) Spectrophotometer (Waltham, MA, USA) was used to determine the functional groups in the HBE, MGF, and their respective AuNPs. High-Resolution Transmission Electron Microscope (HRTEM) linked with Energy Dispersive X-ray Spectroscopy (EDX) was used to determine the morphology and crystalline nature of the AuNPs.

3.2.2. Stability of the AuNPs in biological buffers

The *in vitro* stability of HB-AuNPs and MGF-AuNPs was determined by observing changes in the SPR band (UV-vis spectra) following incubation in various biological media. Stability study was done by adding equal volumes of AuNPs with BSA, FBS, DPBS, DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (pen-strep; Sigma) and RPMI 1640 supplemented with 10% FBS and 1% pen-strep following a previous method [33]. The absorbance of the mixture was read at 0 and 24 hrs using a POLARstar Omega microtitre plate reader.

3.3. Effects of biogenic AuNPs on non-cancerous and cancerous cells

3.3.1. Cell culture

The human colon (Caco-2), prostate (PC-3), glioblastoma (U87) cancer and non-cancer breast (MCF-12A) cell lines were all purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, United States). Caco-2 and U87 cells were maintained in DMEM supplemented with 10% FBS and 1% pen-strep. PC-3 cells were maintained in PRMI 1640 supplemented with 10% FBS and 1% pen-strep. MCF-12A cells were maintained in DMEM/F12 supplemented with 10% FBS, 1% pen-strep, 10 µg/ml insulin (Roche, Germany), 10 ng/ml epidermal growth factor (Sigma) and 0.5 µg/ml hydrocortisone (Sigma). The cells were cultured in a humidified 5% CO_2 incubator at 37°C.

3.3.2. Cell viability assay using MTT assay

The effect of the biogenic AuNPs and DOX on Caco-2, PC-3, U87, and MCF-12A cells was determined using MTT reagent following a previous protocol with slight modifications. The cells were

individually seeded into sterile 96-well microtiter plates at a density of $1X10^5$ cells/ml and incubated at 37°C for 24 hrs. Afterwards, the growth medium was replaced with 100 µl of either the AuNPs or DOX prepared in growth medium and further incubated for 24 hrs. Then, 10 µl of MTT reagent (5 mg/ml MTT) was added in each well and further incubated for 3 hrs at 37°C. The MTT-medium mixture was replaced with 100 µl of dimethylsulfoxide (Kimix, Cape Town, South Africa) and the absorbance was measured on a POLARstar Omega microtitre plate reader at 570 nm and a reference wavelength of 700 nm. Cell viability was calculated in reference to the untreated (negative) control. The concentration of the AuNPs that inhibited 50% cell viability (IC_{50}) in cancer cells was calculated using Graphad Prism software [59,60].

The cell viability was also assessed for the Caco-2 cellstreated with DOX and those co-treated with DOX and AuNPs. Here, the least toxic concentration of DOX on Caco-2 cells was evaluated by exposing the cells to increasing concentrations of DOX (1.56-100µg/ml). This concentration was used for the co-treatment with AuNPs. Caco-2 cells were co-treated for 24 hrs with either DOX (1.56 µg/ml) and HB-AuNPs or DOX (1.56 µg/ml) and MGF--AuNPs (15.62 µg/ml) and the viability of the cells were assessed using the MTT assay as previously described.

4. Conclusion

This study shows for the first time the synthesis of AuNPs from an extract of *C. intermedia*. The study also shows a possible synergistic effect between DOX and AuNPs (HB-AuNP) produced from *C. intermedia*, as well as between DOX and AuNPs (MGF-AuNP) produced from MGF. The physicochemical characteristics and bioactivities of MGF-AuNPs and HB-AuNPs appear to be similar. Considering that HB-AuNPs was produced using an extract of *C. intermedia*, which contains a high concentration of MGF, it is possible that MGF is involved in the synthesis of HB-AuNPs. It is also likely that the synergistic effect between DOX and MGF-AuNPs, as well as DOX and HB-AuNPs can be ascribed to MGF, since several studies have suggested that MGF can enhance the anti-tumor effects of DOX. However, the mechanism of this synergistic effect needs to be investigated further and the involvement of MGF in the synthesis of HB-AuNPs from *C. intermedia* extract must be elucidated. The cytotoxic effects of this co-treatment must be investigated on a larger panel of cancer and normal cell lines to establish the selectivity of the treatment to specific cancers.

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CHAPTER FIVE: MANUSCRIPT

THE CYTOTOXIC EFFECTS INDUCED IN CACO-2 CELLS CO-TREATED WITH DOXORUBICIN AND GOLD NANOPARTICLES PRODUCED FROM *CYCLOPIA INTERMEDIA* EXTRACTS AND MANGIFERIN

This chapter is intended to be submitted for publication in “Molecules” and has been formatted accordingly

molecules

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Article

The cytotoxic effects induced in Caco-2 cells co-treated with doxorubicin and gold nanoparticles produced from *Cyclopia intermedia* extracts and mangiferin

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Abstract: Mangiferin (MGF) is a natural and valuable polyphenol found in significant levels in many plant species including *Cyclopia intermedia* (*C. intermedia*). In our previous study, we reported the successfully synthesis of gold nanoparticles (AuNPs) using MGF and water extract of *C. intermedia* and showed that these nanoparticles have very low cytotoxicity on human colon (Caco-2) cancer cells. Although further study showed that the biogenic AuNPs appear to significantly augment the cytotoxic effects of doxorubicin (DOX) in Caco-2 cells when used in combination with DOX, however, the mechanism of the enhanced effect is not fully understood. In this present study, we examined the uptake of the biogenic AuNPs for possible cellular internalization. Additionally, we investigated the synergistic and mechanistic effects of the combination of biogenic AuNPs and DOX on Caco-2using assays such as adenosine triphosphate (ATP), reactive oxygen species (ROS), mitochondrial depolarization, colony formation, APOPercentage and DNA fragmentation assays. The study showed that the biogenic AuNPs were effectively taken up by the cancer cells, which in turn, enhanced the sensitivity of Caco-2 to DOX. Moreover, the combination of the biogenic AuNPs and DOX caused rapid depletion of ATP levels, reduced ROS production, mitochondrial depolarization, inhibition of the long-term survival and induction of apoptosis in Caco-2 cells. Although the study provided new insight in understanding the mechanism of the combined effect of the biogenic AuNPs and DOX, further mechanistic and molecular studies are recommended to fully understand their synergistic anticancer efficacy.

Keywords: Gold nanoparticles; *Cyclopia intermedia*; Mangiferin; Colony formation; Cellular uptake; DNA fragmentation; Mitochondrial depolarization.

1. Introduction

The combination of anticancer drugs with natural compounds has emerged as a new and interesting strategy to overcome drug resistance. Several plants and phytochemicals including polyphenols,

alkaloids and carotenoids are potent chemosensitizers having ability to inhibit or even reverse drug resistance [1,2].

MGF, a natural flavonoid found abundantly in many plant species has been studied extensively for its therapeutic properties. Several *in vitro* and *in vivo* studies show evidence for the use of MGF for cancer chemoprevention or in combination with chemotherapeutic drugs as an anticancer treatment [3–6]. It is speculated that the anticancer effects of MGF is attributed to its anti-inflammatory, anti-apoptotic and anti-proliferative properties. Evidently, MGF possesses a unique anticancer property in that it has the ability to reverse drug resistance in DOX-resistant cancer cells and is thus recommended as potential chemosensitizer for DOX therapy. In the work of Louisa *et al*, MGF was able to sensitize DOX-resistant MCF-7 breast cancer cells to DOX [7]. Takeda *et al*. also claimed that the combination of MGF with adriamycin (DOX), vincristine and melphalan resulted in enhanced cytotoxic effect of these anticancer drugs in multiple resistant myeloma cells [8]. Considering that MGF has drug-resistance-reversal potential, an opportunity to exploit MGF-rich plants such as; *Mangifera indica*, *Salacia chinensis*, *Swertia chirata*, *Hypericum aucheri* and *Cyclopia intermedia* (commonly known as honeybush, HB) for possible chemoresistance activity is currently underway.

It was recently shown that the cytotoxic effects of DOX can be enhanced in Caco-2 cancer cells when used in combination with gold nanoparticles synthesized using MGF (MGF-AuNPs) [9]. This study also showed that biogenic AuNPs synthesized using water extracts of *C. intermedia* (HB-AuNPs) have a similar synergistic effect. *C. intermedia* is endemic to the fynbos biome of South Africa and it is widely known for its numerous biological activities, viz; antioxidant, antimutagenic, antimicrobial and antidiabetic activities [10–12]. Infusions prepared from the leaves of HB is used for the treatment of infections, coughs, sore throat and colds [13]. Studies done earlier revealed that HB show protective effects against skin infection, inflammatory diseases and cancer including breast, prostate and uterus cancers [11,13,14]. The evaluation of the bioactive constituents of HB revealed the presence of several phenolic compounds including MGF. MGF-AuNPs and HB-AuNPs displayed similar physico-chemical characteristics, which strongly suggest that MGF present in HB extracts play a significant role in the reduction of metal ions to metallic nanoparticles (MNPs) [9].

The use of plant-mediated biogenic nanoparticles in combination with existing anticancer agents presents effective treatment regimen in cancer therapy [15]. Mukherjee *et al*. reported that biogenic AuNPs synthesized using *Peltophorum pterocarpum* and conjugated with DOX (b-Au-PP-Dox) significantly inhibited the proliferation of human lung (A549) and skin melanoma (B16-F10) cancer cells. The study also showed that b-Au-PP-Dox was more effectively taken up and released compared to DOX alone, thus, resulted in a significant reduction of tumor growth in C57BL6/J female mice [16].

DOX remains one of the most potent wide-spectrum drugs in cancer therapy. A study by Wang et al. revealed that DOX induced apoptosis in cardiomyocytes by activating p53 protein [17]. Additionally, several studies reported the role of ROS generation in DOX-induced tumor killing (Asensio-Lo´pez *et al*., 2017; Zhou *et al*., 2001). DOX has also been shown to damage mitochondrial DNA leading to dysfunction of the mitochondria and therefore respiration. This causes the depletion of ATP that subsequently leads to apoptosis [20]. Despite the highly therapeutic effect of DOX, its use is limited because it is associated with cardiotoxicity and nephrotoxicity [21,22]. Several strategies aimed at reducing the toxicity of this drug has been explored including the development of the nanodrug Doxil. Doxil is the first drug delivery system based on PEGylated (polyethylene glycol coated) liposome technology. Doxil and other liposomal formulations of DOX including ThermoDox, LipoDox, Myocet and Caelix were designed to be more tolerable and more effective than free DOX. In cancer patients who have a high risk of cardiac disease, liposomal-DOX formulations offered increased survival compared to free DOX [23].

While the study by Aboyewa *et al* demonstrated the potential of MGF-AuNPs and HB-AuNPs to enhance the toxicity of DOX, this study investigated the underlying mechanisms of toxicity induced in

Caco-2 cells. Several bioassays, which include the colony formation, ATP, ROS, mitochondrial depolarization and DNA fragmentation assays, were employed to determine the mechanistic and synergistic effects of the co-treatments in Caco-2 cells. The cellular uptake of MGF-AuNPs and HB-AuNPs for possible delivery and internalization in Caco-2 cells was also investigated. The study reported that the biogenic AuNPs were effectively taken up by the cancer cells, which in turn, enhanced the sensitivity of Caco-2 to DOX. Moreover, the combination of the biogenic AuNPs and DOX caused rapid depletion of ATP levels, reduced ROS production, increased mitochondrial depolarization, and inhibited the long-term survival by induction of apoptosis in Caco-2. Overall, the study reported that the combination of either MGF-AuNPs and DOX or HB-AuNPs and DOX exhibited similar cytotoxicity on Caco-2 cells. Additionally, the findings from the study strongly suggest that the biogenic AuNPs facilitated the sensitization of Caco-2 cells to DOX and potentiated the anticancer efficacy of DOX [9]. Although the study provided new insight in understanding the mechanism of the combined effect of the biogenic AuNPs and DOX, further mechanistic and molecular studies are recommended to fully understand their synergistic anticancer efficacy.

2. Results and Discusion

2.1 Co-treatment of cells with HB-AuNPs and DOX

Previously, the cytotoxicity of HB-AuNPs was investigated against MCF-12A (non-cancerous breast cell line) and three cancer cell lines i.e. Caco-2 (colon), U87 (brain) and PC-3 (prostate) cells. The AuNPs were non-toxic to the MCF-12A cells at concentrations ≤1000 μg/ml, and demonstrated differential anticancer effects on the three cancer cell lines. The U87 cells were the most susceptible, followed by PC-3 cells at concentrations starting from 31.25 μg/ml. The Caco-2 cells were resistant to the HB-AuNPs treatments, significant effects were observed at ≥ 500μg/ml. Interestingly, when the Caco-2 cells were exposed to 1000 μg/ml of HB-AuNPs combined with less-toxic concentration of DOX (1.56 μg/ml), the cell viability was significantly reduced to 40 % viability [9]. The HB-AuNPs-DOX co-treatment was also evaluated in other cell lines, as shown in Figure 1. Caski and HeLa cells (cervical cancer cells) responded slightly better to the treatment when compared to the Caco-2 cells. The HT-29 cells, also a colon cancer, showed a resistance towards the co-treatment with a viability of 76%. The effects of HB-AuNPs-DOX co-treatment were negligible in non-cancer skin fibroblasts (KMST-6) and triple negative breast cancer (MDA-231) cells. The different responses between the two colon cancer cell lines (Caco-2 vs HT-29 cells), suggested that the co-treatment might be targeted at specific cellular mutations which might be reduced on the HT-29 cells.

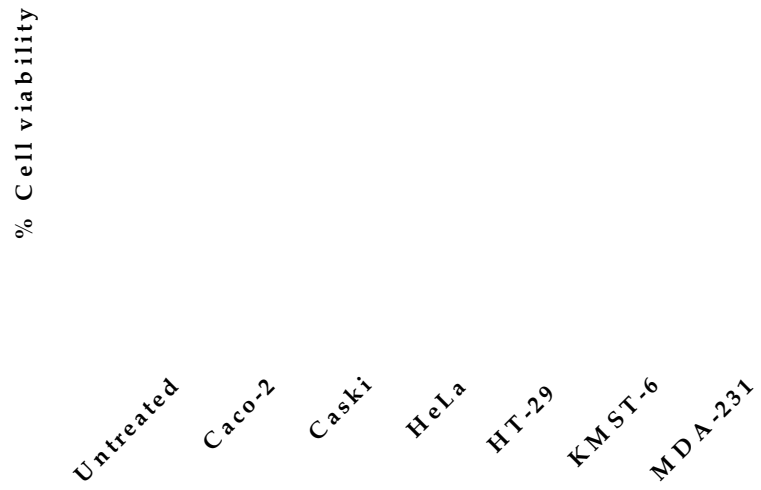
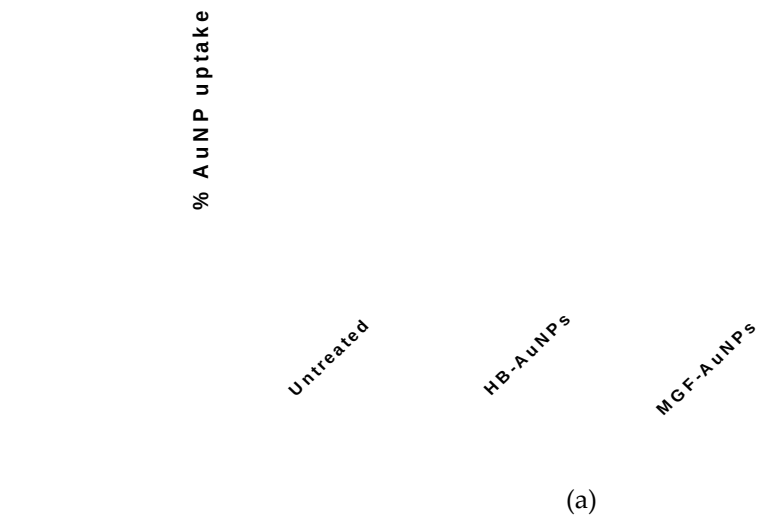


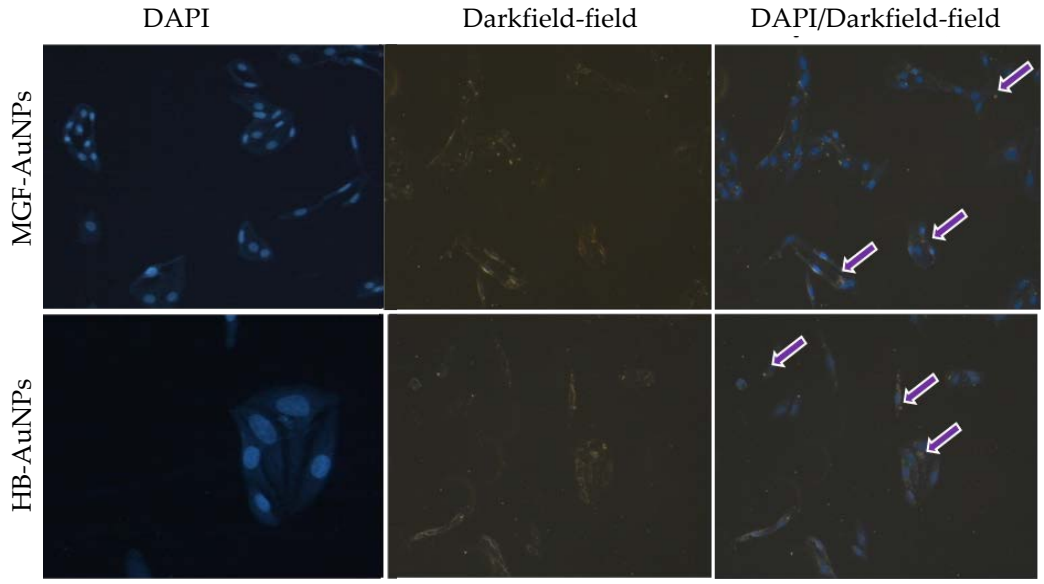
Figure 1: Co-treatment of cells with HB-AuNPs with DOX selectively enhanced cell death in colon and cervical cancer cells. Error bars represent the standard error mean (± SEM). Data was considered to be statistically significant if $p < 0.05$; *** $p < 0.001$, **$p < 0.01$.

2.2 Cellular uptake and internalization of biogenic AuNPs in Caco-2 cells

The potential of NPs for application in medicine is credited to their ability to pass through the biological barrier and enter the cell to exert their function. NPs enter the cell mainly through endocytic pathways, which involve the engulfment of NPs into membrane invaginations. Once inside, the NPs form endocytic vesicles, which are then transported to specialized intracellular sorting/trafficking compartments. In nanomedicine, the safe entry of NPs into the cells is a crucial step to obtain high therapeutic efficacy. In this study, the uptake of MGF-AuNPs and HB-AuNPs in Caco-2 cells were studied by incubating the cells with the AuNPs for 24 hr as described previously [24]. Figure 2a shows the percentage of gold recovered from Caco-2 cells in relation to the total amount of AuNPs the cells were exposed to. A similar percentage of gold was recovered from Caco-2 cells exposed to either MGF-AuNPs (24%) or HB-AuNPs (29%). Additionally, dark field microscopy showed the presence of the biogenic AuNPs inside the cells (Figure 2b). In this study, it was observed that the biogenic AuNPs were efficiently taken up by Caco-2 cells, but on their own exhibited very low cytotoxicity as demonstrated previously [9].



(a)



(b)

Figure 2: Cellular uptake and internalization of AuNPs in Caco-2 cells. Analysis of AuNP uptake in Caco-2 cells by ICP-OES (a) and dark-field microscopy (b) Dark field microscopic analysis-arrows are pointing at the AuNPs inside the cells.

2.3 Mechanisms of co-treatment with biogenic AuNPs and DOX

2.3.1 The effect of MGF-AuNPs-DOX and HB-AuNPs-DOX on ATP levels

The mitochondria represent a dynamic organelles that play crucial and essential roles in overall cellular and survival of an organism [25]. They serve as important gate-keepers of cells and provide a

promising approach for monitoring the fate of cells. Mitochondria constitute the most prominent source of ATP and are implicated in the management of several cellular function including physiological metabolism, stress responses and cell death [26]. Normally, cells maintain stable levels of intracellular ATP and this stability is believed to offer a requisite for normal cell functioning. It also suggests that although transient changes in intracellular ATP which could be due to physiological activity often occur, prolonged changes may compromise the viability of the cells, leading to pathological consequences [27]. Accordingly, the mitochondria which originally serve as a supporter of life can rapidly switch to being a promoter of cell death. Therefore, it is not surprising that mitochondrial dysfunction is associated with cell death and subsequent development of diseases [28]. Consequently, direct evaluation of in vitro activity and function of the mitochondria might be useful in evaluation protocol as an adequate, more objective and desirable parameter of cell survival and death.

In the present study we investigated the intracellular ATP levels in Caco-2 cells exposed to HBE, MGF, DOX, biogenic NPs, and a combination of DOX and the biogenic NPs. While all these treatments resulted in a significant reduction in ATP levels in Caco-2 cells, the ATP levels was especially lower in cells treated with MGF-AuNP-DOX or HB-AuNP-DOX (Figure 3). The ATP levels were reduced in the co-treatments compared to the individual treatments.to the ATP levels were 50% and 47% in cells treated with DOX and MGF-AuNPs, respectively, and reduced to 35% in cells treated with MGF-AuNPs-DOX. Similarly, treatment with HB-AuNPs-DOX reduced intracellular ATP levels to 28% compared to DOX alone (50%) and HB-AuNPs alone (36%). It is well known that the main function of the mitochondrial is to provide energy in form of ATP for the cell survival, and any pathophysiological alterations in mitochondrial often lead to impaired mitochondrial functions and subsequently result in decreased ATP production and cell death. Therefore, the present study proposes that the cytotoxic effect of exhibited by either MGF-AuNPs-DOX or HB-AuNPs-DOX in Caco-2 cells is related to the reduced ATP levels. Further, this finding suggests that the reduced ATP levels could be as a result an alteration in the mitochondrial structure.

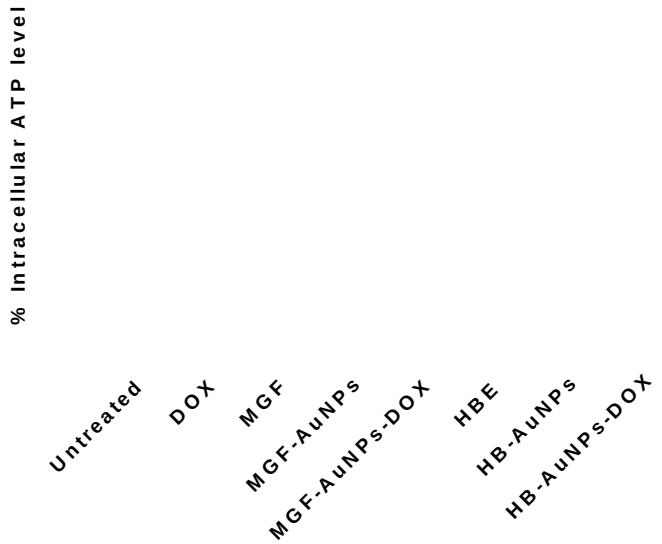


Figure 3: Intracellular ATP levels in Caco-2 cells co-treated with the biogenic AuNPs and DOX. Notes: Results are expressed as mean ± standard error of the mean (SEM). Data was considered to be statistically significant if $p < 0.05$, *** $p < 0.001$, **$p < 0.01$.

2.3.3 The effect of MGF-AuNPs-DOX and HB-AuNPs-DOX on mitochondrial function

Mitochondria play crucial roles in numerous biological activities, and cellular damage may lead to the depolarization of the mitochondrial and cause a decrease of the membrane potential [29]. Depolarization of the mitochondrial has been shown to be associated with impaired mitochondrial functions including reduced oxidative capacity, enhanced ROS production and reduced ATP production [30]. The changes in mitochondrial membrane potential exhibited by either MGF-AuNPs-DOX or HB-AuNPs in Caco-2 cells investigated, Figure 4 shows that all the treatments increased the number of cells with depolarized mitochondria. While mitochondrial depolarization was increased to 25% and 37% in cells treated with DOX and HB-AuNPs, respectively; it was considerably increased to 52% in cells treated with HB-AuNPs-DOX. Similarly, while mitochondrial depolarization increased to 25% and 34% in cells treated with DOX and MGF-AuNPs, respectively, mitochondrial depolarization increased to 46% in cells treated with MGF-AuNPs-DOX. Interestingly, it was observed that the effect of DOX was enhanced by almost 2-fold when used in combination with the biogenic AuNPs compared to DOX alone. This data suggests that biogenic AuNPs seem to work in synergy with DOX to enhance the effects of DOX on mitochondrial function. This effect further explains the observed increase in cell death exhibited by Caco-2 cells treated with either MGF-AuNPs-DOX or HB-AuNPs-DOX. Several studies have established that anticancer agents proffer toxicity on cancer cells via the induction of ROS which subsequently affect mitochondrial processes, leading to cell death. In this study, Caco-2 cells exposed to single treatment of either the biogenic AuNPs or DOX induced higher levels of ROS compared to cells treated with either MGF-AUNPs-DOX or HB-AuNPs. However, impairment of mitochondrial function in Caco-2 cells following exposure to the co-treatment (MGF-AuNPs-DOX or HB-AuNPs-DOX) was more severe compared to the single treatment of either the biogenic AuNPs or DOX. Therefore, this study infers that the increased cell death observed in Caco-2 following treatment with either MGF-AuNPs-DOX or HB-AuNPs-DOX could be as a result of the damage contributed by alteration in mitochondrial processes [31].

Figure 4: The effect of MGF-AuNPs-DOX and HB-AuNPs-DOX on mitochondrial function. The percentage relative mitochondrial potential in Caco-2 cells exposed to various treatments. Data represented as the mean from three independent experiments. Error bars represent the standard error mean (± SEM). Data was considered to be statistically significant if $p < 0.05$, *** $p < 0.001$, **$p < 0.01$, * $p < 0.05$.

2.3.3 The apoptotic effect of biogenic AuNPs and DOX combination

The cytotoxic assays used in this study indicate that treatment with combination of biogenic AuNPs and DOX, induced cell death in Caco-2 cells. Therefore, we assayed for some of the hallmarks of apoptosis i.e the DNA fragmentation (Apodirect BD Pharmingen™) and externalization of the phosphatidyl serine (PS, APOPercentage). DNA fragmentation results from the activation of endonucleases during apoptosis [26]. In the later steps of cell death, chromosomal DNA is cleaved into smaller fragments through the action of endonucleases. The APO-Direct assay can be used to label these DNA fragments with FITC-dUTP using the TdT enzyme which incorporates FITC-dUTP into the 3′-hydroxyl-DNA ends of the DNA fragments found in apoptotic cells. The DNA fragmentation results depicted in Figure 5A indicate that treatment with DOX, MGF, HBE, MGF-AuNPs, HB-AuNPs, MGF-AuNPs-DOX and HB-AuNPs-DOX induced fragmentation of DNA; however, the effect was more pronounced with the co-treatments. The number of cells that underwent apoptosis was also evaluated using the APOPercentage assay (Figure 5B). The results showed a significant high degree of apoptosis in Caco-2 cells treated with HB-AuNPs and HB-AuNPs-DOX where approximately 20% and 65% of cells were apoptotic, respectively.

Figure 5: Co-treatment with biogenic AuNPs and DOX induces cell death on Caco-2 cells through apoptosis by causing DNA Fragmentation (A) and externalization of PS (B). The cells were treated with DOX (1.56 µg/ml), High Dox used as a positive control(7.8 µg/ml), (MGF (1000 µg/ml), MGF-AuNPs (1000 µg/ml), HB-AuNPs (1000 µg/ml), MGF-AuNPs-DOX (1000 µg/ml MGF-AuNPs; 1.56 µg/ml DOX) and HB-AuNPs-DOX (1000 µg/ml HB-AuNPs; 1.56 µg/ml DOX). Data represented as the mean from three independent experiments. Error bars represent the standard error mean (± SEM). Data was considered to be statistically significant if $p < 0.05$, *** $p < 0.001$, **$p < 0.01$.

Apoptosis is a natural mechanism adopted by living organisms for the removal of damaged or unwanted cells. It is a highly regulated process that plays vital roles in the pathogenesis of a number of pathological and physiological processes including cancer. It has been documented that DOX induce apoptosis by first inhibiting topoisomerase II. This action leads to the generation of free radicals and DNA damage which subsequently results in cell death [32]. An earlier study reported that DOX induced apoptosis in breast (MCF-7) cancer cells following treatment with 4 µM of DOX [33]. A study by Wang et al. (2004) showed that 0.5 µM DOX induced apoptosis in PA-1 (human ovarian teratocarcinoma) and human breast adenocarcinoma (MCF) via caspase-3 activation and DNA fragmentation [17]. These reports in addition to several others show that DOX remains one of the most potent wide-spectrum drugs in cancer therapy. However, CRCs are inherently resistant to DOX which at higher doses causes cardiotoxicity and nephrotoxicity [21,22]. Unfortunately, the mechanism by which these cells develop the resistance is not fully known. Xiong and Xiao suggested the activation of steroid receptor co-activator, while Kubiliūtė et al. reported the involvement of epithelial mesenchymal transition, cell adhesion and motility [34,35]. Thus, strategy to overcome drug-resistance as well as restoring the sensitivity of cancer cells to DOX becomes necessary. A study by Khaleel et al. (2016) showed that the combination of resveratrol and didox restored the resistance of HT-29 cells by decreasing the IC_{50} of DOX from 0.88 µM to 0.47 µM [36]. The present study strongly suggest that the co-treatment with the biogenic AuNPs and DOX targets multiple apoptotic pathways leading to increased cell death in Caco-2 cells compared to either biogenic AuNPs or DOX alone.

2.3.4 The effect of the biogenic AuNPs and DOX combination in ROS levels

Although the elevation of ROS levels appears to be one of the paradigms for NP-mediated toxicity [37], the direct toxicity of NPs without the influence of excessive ROS levels has been reported previously [38]. Consequently, high ROS production may not necessarily be required for NP-induced toxicity [39]. On the other hand, DOX induces death in cancer cells by increasing cellular ROS levels. Several studies have shown that DOX-induced cardiotoxicity is mainly caused by ROS production (Wang *et al*., 2010). Accordingly, the present study also investigated what effect of co-treatment with the biogenic NPs and DOX had on the ROS levels of Caco-2 cells using the CM-H_2DCFDA fluorescent probe.

The result showed a significant increase in the percentage of ROS positive cells in DOX or MGF-AuNP-DOXtreated Caco-2 cells when compared to untreated control (Figure 6), which suggest increased production of ROS. Interestingly, the HB-AuNPs and MGF-AuNPs exhibited non significant difference in ROS levels when compared to the untreated control. It is well known that HB possesses high concentrations of polyphenols such as hesperedin, MGF, caffeic acid, and gallic acid (Aboyewa et al., 2021). Polyphenols are powerful antioxidants which scavenge oxygen derived compounds or prevent the formation of ROS [10]. It is possible that the high nucleophilic properties of the hydroxyl and carbonyl groups in these polyphenols are involved in the capping of metal ions during the formation of AuNPs and thus prevent the formation of oxygen radicals. Although, this finding contradicts the report of other studies that suggest biogenic AuNP-induced toxicity is related to elevated ROS generation. However, the study is consistent with earlier findings that reported the induction of (lung cancer) cell death without significant increase in ROS levels [38,39]. In this study, the increase in ROS production observed in Caco-2 cells treated with DOX was expected and in agreement with previous studies [18,19]. It is possible that the reduced ROS level in Caco-2 cells co-treated with the biogenic AuNPs and DOX was mediated by the combine effects of both treatment, leading to reduced proliferation [40]. Additionally, it could be that the AuNPs may have offered protection against DOX-induced ROS in Caco-2 cells. Thereby, leading to the activation of a number of pathways, some may reduce oxidative stress while others may lead to increased cell death. Thus, the study clearly shows that the anticancer efficacy of DOX in Caco-2 could be dependent on ROS induction while the anticancer efficacy of MGF-AuNPs, HB-AuNPs and HB-AuNPs-DOX are not. Hence, present study suggests that the combination of biogenic AuNPs and DOX mediated cytotoxicity is independent of the ROS.

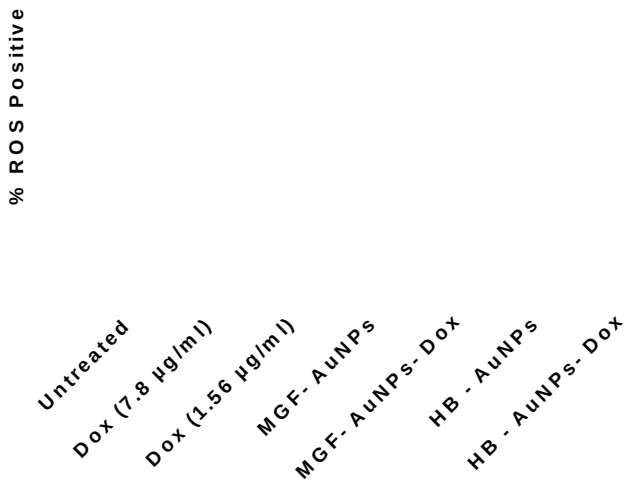


Figure 6: Co-treatment with biogenic AuNPs reduces ROS in Caco-2 cells. ROS positive Caco-2 cells treated with DOX (1.56 µg/ml), High Dox used as a positive control (7.8 µg/ml), MGF-AuNPs (1000 µg/ml), HB-AuNPs (1000 µg/ml), MGF-AuNPs-DOX (1000 µg/ml MGF-AuNPs; 1.56 µg/ml DOX) and HB-AuNPs-DOX (1000 µg/ml HB-AuNPs; 1.56 µg/ml DOX). Error bars represent the standard error mean (± SEM). Data was considered to be statistically significant if $p < 0.05$, *** when compared to the untreated group.

2.4 The effect of the biogenic AuNPs and DOX combination on long-term survival of Caco-2 cells

The clonogenic assay was performed to determine the reproductive viability of Caco-2 cells following treatment with either MGF-AuNPs-DOX or HB-AuNPs-DOX. The assay essentially tests every cell in the population for its ability to undergo unrestricted division [41]. It is a standard method for assessing the efficacy of radiation or cytotoxic agents in vitro. The assay is based on the ability of a single cell to survive and grow into a colony of at least 50 cells after a short-time of exposure to therapeutics [42]. In this study, Caco-2 cells were treated for 24 hr with various treatments including MGF-AuNPs-DOX, and HB-AuNPs-DOX, and then allowed to grow for fourteen days with regular change of growth medium. The colonies formed were counted to estimate the differences in cell survival between treated and untreated cells. As shown in Figure 7 the number of colonies formed following treatment with DOX, MGF, MGF-AuNPs, HBE and HB-AuNPs were significantly reduced compared to untreated cells. The capability of cells to form colonies following treatment with either MGF-AuNPs-DOX or HB-AuNPs-DOX was even more severely affected as indicated in Figure 5. The number of colonies formed after treatment with DOX, MGF-AuNPs, and HB-AuNPs alone was 115, 299 and 369, respectively; only 24 and 20 colonies were formed after treatment MGF-AuNPs-DOX and HB-AuNPs-DOX. The reduction in colonies formed with the combination of biogenic AuNPs and DOX is suggestive of the high cytotoxicity and damaged caused by the combination therapy. Thus, the combination of either HB-AuNPs and DOX

or MGF-AuNPs and DOX has a robust anticancer effect in Caco-2 cells by preventing the recovery of the cells from the treatment. Overall, the combination of the AuNPs and DOX has therapeutic potential for CRC treatment because targeting the clonogenic subset of cancer cells is strongly believed to be essential for successful cancer therapy [41].

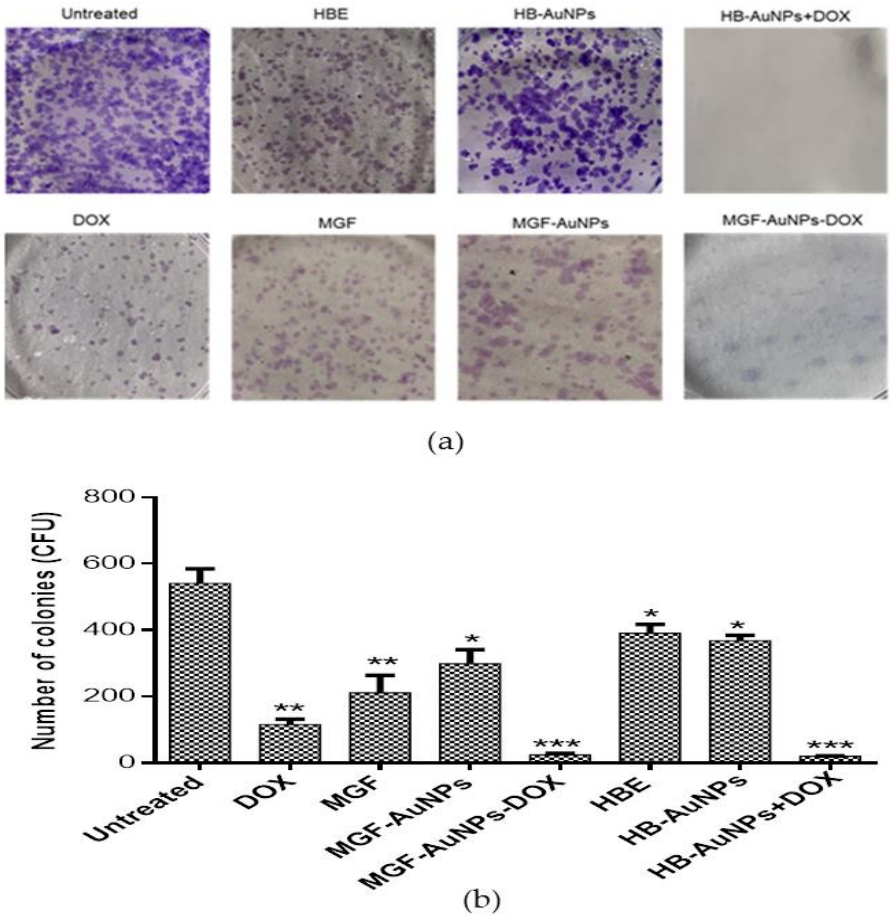


Figure 7: The combination of biogenic AuNPs and DOX inhibits the survival of Caco-2 cells. The pictorial images of the colonies formed (A) and the number of colonies formed (B) following treatment of Caco-2 cells with DOX, MGF, MGF-AuNPs, HBE, HB-AuNPs, and HB-AuNPs-DOX. Data represented as the mean from three independent experiments. Error bars represent the standard error mean (± SEM). Data was considered to be statistically significant if $p < 0.05$, *** $p < 0.001$, **$p < 0.01$, * $p < 0.05$.

3. Materials and Methods

3.1 Synthesis and characterization of biogenic MGF-AuNPs and HB-AuNPs

The synthesis and characterization of the biogenic MGF-AuNPs and HB-AuNPs using MGF and water extracts of HB plant, respectively; was performed as described previously [9]. The biogenic AuNPs

were characterized using UV-Vis spectroscopy, dynamic light scattering (DLS), Fourier-transform infrared spectroscopy (FTIR), X-ray Diffraction (XRD), and High-Resolution Transmission Electron Microscopy (HR-TEM).

3.2 In vitro cytotoxicity using MTT assay

The human colon (Caco-2 and HT-29), cervical (Caski and HeLa), breast (MDA-231) cancer cell lines; and the non-cancer skin fibroblast (KMST-6) cell line were purchased from American Type Culture Collection (Manassas, Virginia, United States). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Roche, Germany) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco). Cells were cultured in a humidified 5% CO_2 incubator at 37°C. The cytotoxicity of the MGF-AuNPs-DOX and HB-AuNPs-DOX was performed as described previously [9]. Briefly, the cells were seeded at 1 x 10^5 cells/ml and treated for 24 hr with the mixture of MGF-AuNPs and DOX (MGF-AuNPs-DOX) and HB-AuNPs and DOX (HB-AuNPs-DOX) as described previously [9]. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and represented as percentage cell viability.

3.3 Biogenic AuNP cellular uptake study

3.3.1 Analysis of AuNP uptake by inductively coupled plasma - optical emission spectrometry (ICP-OES)

To evaluate the internalization of the biogenic AuNPs in Caco-2 cells, cellular uptake was performed according to published protocols [24] with some modifications. Briefly, cells were cultured in a 12-well plate at a density of 1 x 10^5 cells/ml and incubated for 24 hr at 37°C in a 5% CO_2 humidified atmosphere. Afterwards, the cells were treated with single concentration of either HB-AuNPs or MGF AuNPs (1.56 µg/ml), and incubated for 24 hr. The cells were harvested by trypsinization and centrifuged at 3000 rpm for 3 min. The resulting cell pellets were washed twice with PBS to remove unbound AuNPs, and re-suspended in 2 ml aqua regia (HCl: HNO_3 in ratio 3:1) (Kimix, Cape Town, South Africa) and digested at 90°C for 2 hr. The digested samples were diluted to 10 ml with 2% HCl (Sigma) and analyzed for Au content using the Varian 710-ES ICP-OES (CA, USA). TraceCERT®, 1000 mg/L Au in HCl (Sigma) was used as a standard. The amount of AuNPs taken up by cells was calculated based on the concentration of Au detected in each sample.

3.3.2 Dark-field microscopic analysis for cellular internalization of AuNPs

To further investigate the internalization of the biogenic AuNPs, dark-field microscopic technique was employed according to the method described previously [43]. The cells were sparsely plated (≤ 5000 cells/well) on 12 mm coverslips placed in 6-well culture dishes and incubated for 24 hr at 37°C in a 5% CO_2 humidified atmosphere. Afterwards, the growth media was removed from the dishes and attached cells were incubated with 1 ml of either MGF-AuNPs or HB-AuNPs for 12 hr. After, the cells were washed thrice with PBS and fixed in 4% para formaldehyde for 15 min. The fixative was washed off with PBS, the coverslips were inverted on the slide mounted with 4′,6-diamidino-2-phenylindole (DAPI) and viewed under the dark-field microscope using Leica DM2500 LED optical microscope (Leica Microsystems CMS GmbH, Germany).

3.4 Investigation of the mechanism of co-treatment

3.4.1 Cell culture and treatments

Caco-2 cells were seeded either in 96-well (1 x 10^5) or 12-well (2 x 10^5 cell/ml) microtiter plates and incubated for 24 hr at 37°C in a 5% CO_2 humidified atmosphere. After 24hours, the medium was replaced with complete medium containing MGF-AuNPs-DOX (1000 μg/ml MGF-AuNPs and 1.5 μg/ml DOX), HB-AuNPs-DOX (1000 μg/ml HB-AuNPs and 1.5 μg/ml DOX), HBE (1000 μg/ml), MGF (1000 μg/ml), MGF-AuNPs (1000 μg/ml), HB-AuNPs (1000 μg/ml), Low DOX (1.5 μg/ml) and High dox (7.8 μg/ml). Cells treated with growth medium without any treatments served as negative control. The cells were treated cells for 24 hr at 37°C.

3.4.2 Mitochondrial ToxGlo assay

The intracellular adenosine triphosphate (ATP) levels in Caco-2 cells co-treated with either MGF-AuNP-DOX or HB-AuNP-DOX were assessed using the Mitochondrial ToxGlo assay (Promega, Madison, WI, USA) as described previously [26]. Briefly, cells were seeded in a white-walled 96-well microplates and treated for 24 hr. Following the treatments, 100 μl of the Mitochondrial ToxGlo reagent was added to each well and the plate was incubated for 20 min. Thereafter, the ATP levels were quantified by measuring the luminescence on a PolarStar Omega Plate Reader (BMG Labtech, Ortenberg, Germany).

3.4.3 Flow cytometer analysis

The mitochondrial depolarization, DNA fragmentation, APOPercentage and Reactive oxygen species (ROS) assays were analyzed by flow cytometer and processed as follows. After 24 hr treatments, both the adherent and detached cells were harvested and centrifuged at 3000 rpm for 5 min. Following staining, the cells were analyzed by BD Accuri C6 flow cytometer (Erembodegem, Belgium). Acquisition was done in log mode and approximately 10000 events per cell sample were acquired [44]. The data was analyzed using Flowjo 10.6.1, 2018 (Flowjo Software, LLC, USA).

3.4.3.1 Mitochondrial depolarization assay

The effects of the co-treatments on the mitochondrial membrane potential ($\Delta\Psi m$) of cells were investigated using tetramethyl rhodamine ethyl ester (TMRE) labelling as described previously [45]. Caco-2 cells were treated as described above. Afterwards, cells were harvested by gentle trypsinization, washed with PBS and resuspended in media containing 50 nM TMRE (Molecular Probes, USA). Following 30 min incubation, the cells were washed, re-suspended in PBS and the changes in mitochondrial membrane potential in live cells were assessed using a BD Accuri C6 flow cytometer.

3.4.3.2 DNA fragmentation assay

Cells were treated with a MGF-AuNPs-DOX (1000 μg/ml MGF-AuNPs and 1.5 μg/ml DOX) or HB-AuNPs-DOX (1000 μg/ml HB-AuNPs as described previously [9]. The induction of apoptosis was investigated using the APO-DIRECT™ Kit (BD, Pharmigen, CA, USA). According to the manufacturer's instruction, Afterwards, cells were re-suspended in 1% (w/v) paraformaldehyde in PBS and placed on ice for 30 min. Cells were washed twice in 5 ml PBS, centrifuged and placed in 1 ml 70% (v/v) ice cold ethanol. After 24 hr, the cells were transferred into labelled centrifuged tubes, centrifuged for 5 min at 3000 rpm and suspension ethanol was discarded carefully. To each cell pellets, 1 ml of wash buffer was added, and centrifuged as before. This wash step was repeated. Afterwards, the pellets were resuspended in 50 μl of the DNA Labelling Solution and incubated for 2 hr at 37°C. Following incubation, 1 ml of rinse buffer was added to each tube and centrifuged at 300 x g for 5 min. The rinsing process was repeated and the supernatant was discarded. Cell pellets were re-suspended in 0.5 ml PI/RNase staining buffer in the

dark and afterwards, analyzed by flow cytometry. The cell population was differentiated into four distinct quadrants; live cells (BrdU-/PI-) on the lower left quadrant, early apoptotic cells (BrdU-/PI+) on the upper left quadrant, late apoptotic cells (BrdU+/PI+) on the upper right quadrant and necrotic cells (BrdU+/PI-) on the upper right quadrant. Pseudo dot plot analysis was used to represent the fluorescence distribution and the percentage of fluorescent cells in each quadrant was obtained using Flowjo 10.6.1.

3.4.3.3 APO*Percentage* assay

The percentage of Caco-2 cells that underwent apoptosis following treatments were measured using the APO*Percentage* dye (disodium salt of 3,4,5,6,-tetrachloro-2′,4′,5′, 7′-tetraiodofluorescein, TCTF) as previously described [46]. Afterwards, the cells were stained with APO*Percentage*dye at 1:160 ratio prepared in media (Biocolor, County Antrim, UK). The cells were then analyzed with BD Accuri C6 flow cytometer.

3.4.3.4 ROS assay

The effects of the co-treatments on intracellular ROS generation was evaluated using 5-(and -6)-chloromethyl-2^1,7^1 -dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) probe (Molecular Probes, USA) according to the previous method [26]. The cells were treated as described above. After treatment, the cells were gently removed by trypsinization, washed with 1 x PBS and re-suspended in 7.5 μM of CM-H_2DCFDA prepared in 1 x PBS and incubated for 30 min at 37°C as per the manufacturer's instructions. Thereafter, the cells were washed with PBS and analyzed using BD Accuri C6 flow cytometer.

3.4.4 Colony formation assay

To investigate whether MGF-AuNPs-DOX or HB-AuNPs-DOX will inhibit long-term survival of Caco-2 cells, a colony formation assay was performed according to the previously described method [42]. Briefly, Caco-2 cells were seeded in 60 mm petri dishes at a density of 1 x 10^5 cells/ml and incubated in a humidified 5% CO_2 incubator for 24 hr. Afterwards, the cells were treated for 24 hr as previously described. Cells were harvested and re-plated in 35 mm dishes at a density of 500 cells/dish and incubated at 37°C. Their growth was monitored for a period of 14 days with regular changes of growth medium. Cells were fixed and stained with 0.5% crystal violet (Sigma) prepared in 100% methanol (Sigma). A digital camera was used to capture the image of the dishes and the numbers of colonies formed were counted using BioSpectrum Imaging System (UVP, Upland, CA, USA).

4. Conclusions

This study investigated the possible mechanistic approach employed by the co-treatment with the biogenic AuNPs and DOX in the induction of cytotoxicity in Caco-2 cells. The results generated from this study showed that the combination of the biogenic AuNPs and DOX induced death of Caco-2 through rapid depletion of ATP levels, ROS reduction, mitochondrial membrane depolarization and induction of DNA fragmentation. Additionally, the co-treatment also inhibited long-term survival of Caco-2 cells. Together, the findings from this study strongly suggest that the combination of biogenic AuNPs and DOX exhibited significant cytotoxic effectiveness in in vitro colon (Caco-2) cancer cells. However, other colon cancer cells in addition to mechanistic and molecular studies are recommended for further study to unravel the full potential of this combination therapy and the exact mechanisms that induced cell death in colon cancer cells.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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Sample Availability: HB-AUNPs-DOX and MGF-AuNPs-DOX are available from the authors.

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CHAPTER SIX

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

6.1. General Discussion

Nanotechnology is a branch of nanoscience that deals with the manipulation of particles at their nanometer scale to obtain certain desirable properties that enhance their use in the treatment of various metabolic disorders as well as other life-threatening diseases including cancer (Aboyewa *et al*., 2021a; Majoumouo *et al*., 2020; Ahmad *et al*., 2019; Ovais *et al*., 2018). Southern Africa is rich with a plethora of plants having a long history of use in traditional medicine for the treatment of infectious and chronic diseases. With the emerging new technologies, these plants can be useful in developing alternative therapeutics (Majoumouo *et al*., 2020; Patra *et al*., 2018) in the treatment of various disease conditions including cancer. Green nanotechnology has shown great promise in this regard as various medicinal plants have been reportedly used to synthesize MNPs with enhanced biocompatibility and various biological activities (Salem & Fouda, 2020; Marslin *et al*., 2018). The use of biologically synthesized NPs is a blooming field in cancer therapy and it has paved the way for designing drug delivery systems with promising therapeutic activities.

This study was divided into three sections. Section one focused on the synthesis and characterization of AuNPs using HB extracts and commercially acquired pure MGF (a polyphenol found abundantly in HB). Section two focused on the cytotoxicity mediated by the biogenic AuNPs in human colorectal (Caco-2), glioma (U87), and prostate (PC-3) cancer cells as well as in normal epithelial breast (MCF-12A) cells. Section three focused on the combination of the biogenic AuNPs with a potent anticancer drug, DOX in the attempt to enhance the efficacy of DOX in human Caco-2 cells.

The green synthesis of NPs using biological sources is fast becoming a promising alternative to the conventional physical and chemical synthetic methods (Ms *et al*., 2019; Jain & Mehata, 2017). In the last decade, *in vitro* biological approach where plants and plant derivatives are employed for the bioreduction of metal ions to form MNPs has been vigorously pursued. Plants are rich sources of secondary metabolites including polyphenols, flavonoids, and

steroids and numerous studies have demonstrated that these metabolites in an aqueous medium act as both reducing and stabilizing agents for the synthesis of non-aggregated MNPs (Ovais *et al*., 2018). Thus, biogenic MNPs have achieved indispensable significance owing to the diverse roles played by biomolecules in directing their physicochemical characteristics. Although various plant species including *Dendopanax morbifera* (Wang *et al*., 2016), *Aspalathus linearis* (Diallo *et al*., 2015), *Galenia africana* (Elbagory *et al*., 2017)*, Elaeis quineensis* (Ahmad *et al*., 2019), and *Mangifera indica* (Vimalraj *et al*., 2018) have been employed for the synthesis of MNPs, however, the exact mechanism, particularly the key biomolecules responsible for the reduction of metal ions and the subsequent stabilization of the biogenic MNPs was not fully captured. The precise identification of bioactive compounds involved in producing MNPs is vital in controlling their physical and chemical characteristics for a potential application.

In this study, the phytochemical contents, viz; flavanols, flavonols, and total polyphenol present in GHB and FHB extracts were characterized. Phytochemicals are natural bioactive compounds widely distributed in plants and other forms of life, playing significant roles in the medicinal properties ascribed to some plants. Polyphenolic compounds in particularly are known to exhibit a wide range of bioactive properties not limited to antioxidative, antiproliferative, hypolipidemic, and anti-inflammatory effects (Kooti *et al*., 2017; Mansouri *et al*., 2015; Sharopov *et al*., 2015; Rymbai *et al*., 2013). In this study, both GHB and FHB displayed high phytochemical content but higher contents were seen in GHB. Additionally, the antioxidant potential and reducing capacities of both GHB and FHB were determined using ORAC, FRAP, and DPPH activity. Similar to the result obtained from the phytochemical contents, both extracts displayed strong antioxidant capacities with GHB displaying higher activity than FHB. This observation agrees with the study done by Dube *et al*. (2017) that showed that GHB plant material contain higher phytochemical and antioxidant activities compared to their fermented counterpart. This could possibly be due to the loss of some polyphenolic and other bioactive compounds during the process of fermentation (Joubert *et al*., 2008; Dube *et al*., 2017). It is well known that phenolic compounds are the major antioxidants of most plant species and their antioxidant activity is mainly due to their redox properties which is responsible for the reduction of metal ions during MNP synthesis. Therefore, plant extracts with higher total reducing capacity should be able to effectively produce MNPs. In this study, both GHB and FHB were investigated for the preparation of AuNPs in a low-cost, green synthesis approach. Since the study is the first to

investigate the synthesis of AuNPs using GHB and FHB, optimization of synthetic parameters such as reaction time, reaction temperature, pH and concentration of plant extracts were considered. Overall, a reaction time of 1 hr using 2 mg/ml of the GHB plant extract, at a pH of 4.5 and at a reaction temperature of 70°C produced the best conditions for the synthesis of GHB-AuNPs. These conditions are similar to the conditions used for the synthesis of FHB-AuNPs, except that 4 mg/ml of the extract produced the best results. Although the successful synthesis of AuNPs was achieved using GHB and FHB, AuNPs synthesized using GHB were smaller and more dispersed. The synthesis of smaller and more dispersed AuNPs produced using GHB could have been as a result of its high TPC. It is known that plants with a higher TPC show high antioxidant activity and consequently possess excellent reductive capacity necessary for the synthesis of smaller and non-aggregated nanoparticles (Subramanian *et al*., 2013). A previous study has shown that plants with high TPC show higher reducing capacities for NP synthesis. Goodarzi *et al*. (2014) reported the successful synthesis of NPs from various plant extracts and reported that *Zataria multiflora* has the highest TPC and thus more potent in NP synthesis (Goodarzi *et al*., 2014). Consequently, subsequent study focused mainly on the AuNP made from GHB. GHB is rich in polyphenols including MGF, isomangiferin, hesperitin and hesperidin, with MGF being the most abundant (Ajuwon *et al*., 2018; Dube *et al*., 2017). Although previous studies have reported the synthesis of AuNPs using MGF (Patra *et al*., 2018; Al-Yasiri*et al*., 2017), in agreement, this study also successfully synthesized MGF-AuNPs using MGF (Aboyewa *et al.,* 2021b).

Furthermore, the biogenic AuNPs (HB-AuNPs and MGF-AuNPs) were thoroughly characterized for their physicochemical properties using UV-Vis spectroscopy, DLS, XRD, HR-TEM, and FTIR techniques (Aboyewa *et al*., 2021b). The UV-Visible spectrum displayed distinct peaks at 540 and 538 nm for HB-AuNPs and MGF-AuNPs, respectively, which correspond to the appearance of AuNPs. The appearance of absorption peak in the region of 500-600 nm reported in this study is similar to earlier observation (Patra *et al*., 2018; Al-Yasiri *et al*., 2017). A study done by Elbagory *et al*. (2016) reported that AuNPs synthesized from South African plants including *Aspalathus hispida*, *Nidorella foetida* and *Salvia africana-lutea* showed maxima absorbance between 500 and 600 nm (Elbagory *et al*., 2016). Similarly, AuNPs synthesized using leaf extract of *Terminalia mantaly* produced absorbance peak in the region between 500 and 600 nm (Majoumouo *et al*., 2020). DLS analysis of HB-AuNPs and MGF-AuNPs showed a hydrodynamic diameter of 66.74 nm and 65.50 nm, zeta potential value of -

23.45 mV and -27.87 mV, and PDI of 0.571 and 0.432, respectively. The XRD analysis confirmed that both AuNPs were crystalline. The HR-TEM analysis revealed that both biogenic AuNPs were predominantly spherical with few triangular-shaped AuNPs, exhibiting average core sizes of 20 and 26 nm for HB-AuNPs and MGF-AuNPs, respectively. The EDX measurement confirmed the presence of gold in the biogenic AuNPs while FTIR analysis clearly showed the formation of AuNPs from GHB and MGF and indicated that GHB contains various phytochemicals, particularly polyphenols such as MGF, which possibly work as reducing and stabilizing agents for the synthesized AuNPs. Overall, both HB-AuNPs and MGF-AuNPs displayed very similar properties which strongly suggest that MGF present in the HB extract could have actively participated in the synthesis of HB-AuNPs. Similar observation was reported by Elbagory *et al*. (2019), showing that AuNPs synthesized using *Hypoxis hemerocallidea* extracts exhibited similar physical and chemical properties with those synthesized from hypoxoside. This particular study suggests that hypoxoside one of the major phytochemicals found abundantly in the corms of *H. hemerocallidea* may have actively contributed to the synthesis of AuNPs from *H. hemerocallidea* (Elbagory *et al*., 2019). Also, Ahmad *et al*. (2019) demonstrated that the successful synthesis of AuNPs using aqueous leaf extracts of *Elaeis quineensis* were achieved via the involvement of its gallic acid contents (Ahmad *et al*., 2019). These reports further support the claim that phytochemicals present in plant materials act to reduce metal ions to their corresponding MNPs. Moreover, the stability of the biogenic AuNPs were investigated in various biological media; DMEM, FBS, DPBS, RPMI, and BSA. The stability of NPs in biological media is crucial to preserve their utility in biological response. The study showed that both biogenic AuNPs were stable in these media and this further encouraged the practical application of the biogenic AuNPs in biological study.

The interest in AuNPs synthesized using medicinal plants has grown enormously owing to their potential application in various biomedical fields (Gomes *et al*., 2015; Vijayakumar & Ganesan, 2012) including tissue engineering and health care (Balaji *et al*., 2015; Rai *et al*., 2013; Mishra *et al*., 2013). Biogenic AuNPs have been reported to exhibit antimicrobial, antifungal, anti-inflammatory, antioxidant, immunomodulatory, and anticancer activity (Shikha *et al*., 2020; Wongyai*et al*., 2020; Katas *et al*., 2019). Similar bioactivities have been identified for plant extracts and these bioactivities can be ascribed to the presence of bioactive phytochemicals. These phytochemicals can also participate in the synthesis of biogenic NPs. It is therefore likely

that these phytochemicals may also be responsible for the bioactive observed for biogenic NPs. The *in vitro* cytotoxicity of biogenic AuNPs in various cancer cells including human epithelial lung (A549), triple-negative breast (MDA-MB-231), pancreatic (PANC-1), and colon (HT-29) cancer cells have been reported (Wang *et al*., 2019; Mata *et al*., 2016; Parveen & Rao, 2015). AuNPs, due to their size and their unique optical and chemical properties, are used as drug carrier/delivery, bioimaging, contrast, photothermal and antiangiogenic agents (Elbagory *et al*., 2016). MGF has been shown to have various biological activities, which include antiproliferative, anti-inflammatory and anticancer. In this study, the cytotoxic effect of HB-AuNPs and MGF-AuNPs was investigated in human colorectal (Caco-2), prostate (PC-3), and glioma (U87) cancer cells as well as non-cancerous breast (MCF-12A) cells. The results showed that both biogenic AuNPs exhibited selective toxicity in this panel of cell lines with Caco-2 cells exhibiting lower sensitivity than the other cancer cell lines. This observation corroborates with the finding of (Majoumouo *et al*. (2020) that showed that biogenic AuNPs synthesised using *Terminalia mantaly* showed selective toxicity in Caco-2, MCF-7 and HepG2 cells. On the other hand, AuNPs synthesised from MGF were reported to exhibit non-toxic effect in MCF-10A cells. This shows that selectivity may be as a result of the variation in cell types as well as the physicochemical characteristics of the biogenic AuNPs.

DOX is an FDA-approved drug used to treat a wide range of human cancers including ovarian, kidney, breast, lung, thyroid, multiple myeloma, and gastric cancers. DOX and its analogs belong to the class of anthracycline drugs which act by intercalating within DNA base pairs, thereby inhibiting both DNA and RNA synthesis, causing free-radical mediated oxidative damage leading to DNA damage and the induction of cellular apoptosis. Although, DOX is not often used for treating colorectal cancer, it has shown a potential synergistic effect against refractory colorectal cancer when used in combination with racemic verapamil (Xiong& Xiao, 2018). The use of DOX is limited because of its severe dose-limiting side effects that include cardiotoxicity and nephrotoxicity that often persist after many years of its discontinuous use (Wakharde *et al*., 2018). Additionally, multidrug resistance, cancer cell recurrence, non-specific targeting, and poor bioavailability characterized by DOX have rendered this treatment procedure a failure. Evidently, MGF possesses a unique anticancer property in that it has the ability to reverse drug resistance in doxorubicin-resistant cancer cells and is thus recommended as potential chemosensitizer for DOX therapy (Louisa *et al*., 2014). In this regard, there is a possibility that HB-AuNPs and MGF-AuNPs which presumably contains MGF can also

augment the effects of DOX**.** Caco-2 cancer cells have been shown to be inherently resistant to low concentrations of DOX and only responded to higher doses (Sonowal *et al*., 2017). Consequently, in this study a combination therapy system was investigated where Caco-2 cells were treated with the combination of the biogenic AuNPs and DOX (HB-AuNPs-DOX and MGF-AuNPs-DOX; at their least toxic concentrations). The result of the combination therapy shows 40% and 70%, respectively. The same finding has been earlier demonstrated by Mukherjee *et al*. (2016), showing that biosynthesized AuNPs of *Peltophorum pterocarpum* conjugated with DOX (b-Au-PP-Dox) significantly inhibited the proliferation of human lung (A549) and skin melanoma (B16-F10) cancer cells (Mukherjee *et al*., 2016). These data suggest that both biogenic AuNPs appear to work in synergy with DOX to enhance the cytotoxic effect of DOX (Aboyewa *et al*., 2021b). Further, the study investigated the underlying mechanism of the enhanced effect of DOX and the biogenic AuNPs combination using assays including ATP, ROS, TMRE, colony formation, and APO-Direct assays. Results indicated that the combination of the biogenic AuNPs and DOX disrupt cellular activities through rapid depletion of ATP levels, reduced ROS, altered mitochondrial membrane function, induced apoptosis, and inhibited long-term survival of Caco-2 cancer cell lines which ultimately resulted in their death.

Overall, the findings from this study revealed that water extracts of HB successfully reduced gold ions into AuNPs and demonstrated that MGF, present in HB plays a crucial role in the synthetic process. Further, the biogenic AuNPs have selective cytotoxicity against human colorectal (Caco-2), prostate (PC-3), and glioma (U87) cancer cells as well as normal epithelial breast (MCF-12A) cells with Caco-2 cells showing the least sensitivity to the AuNPs. Additionally, the biogenic AuNPs was able to re-sensitize Caco-2 cells to the anticancer effect of DOX, thus leading to increased inhibition of their proliferation.

6.2. Conclusion

The study demonstrated the potential use of combination therapy with biogenic NPs produced from HB extract and its efficacy in *in vitro* model of colorectal cancer. The study was able to establish that the combination of the biogenic gold NPs and doxorubicin offer enhanced anticancer effect through different mechanisms of action against Caco-2 cells compared to the use of either the biogenic AuNPs or doxorubicin alone. Furthermore, the study successfully demonstrated that the biogenic gold NPs re-sensitized Caco-2 cells to DOX, which subsequently caused significant cell death. This discovery suggests that the combined use of

biogenic NPs with chemotherapeutic drugs could be used in the future as an alternative cost-effective treatment strategy for cancer therapy.

6.3. Recommendation

This study has no doubt established that the biogenic gold NPs combined with DOX offered improved anticancer effect in human CRC. However, aAdditionally, research of the effect of the combination of the biogenic AuNPs and DOX on genes and protein expression that will provide further insight into the mechanistic and molecular mechanism of the treatment is warranted. Finally, it is recommended that the co-treatment modality be explored in other cancer and non-cancerous cell lines.

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Aboyewa, J.A., Sibuyi, N.R.S., Meyer, M. & Oguntibeju, O.O. 2021b. Gold nanoparticles synthesized using extracts of cyclopia intermedia, commonly known as honeybush, amplify the cytotoxic effects of doxorubicin. *Nanomaterials*, 11(1): 1–16.

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ADDENDUM

ADDENDUM 1: RESEARCH OUTPUTS

PUBLISHED ARTICLE

- **Aboyewa J.A**., Sibuyi N.R.S., Meyer M. and Oguntibeju O.O. (2021). Gold Nanoparticles Synthesized Using Extracts of*Cyclopia intermedia*, commonly known as Honeybush, Amplify the Cytotoxic Effect of Doxorubicin. Nanomaterials, 11: 132:1-16

- **Aboyewa J.A**., Sibuyi N.R.S., Meyer M. and Oguntibeju O.O. (2021). Biological Applications of Metallic Nanoparticles Synthesized from Some Selected South African Medicinal Plants. Plants, 10: 1-24.

ARTICLES UNDER REVIEW

- **Aboyewa J.A**., Sibuyi N.R.S., Meyer M. and Oguntibeju O.O. (2021). Gold Nanoparticles of *Cyclopia intermedia* Enhanced Anticancer Effect of *D*oxorubicin: A Mechanistic and Synergistic Approach in Human Colorectal Cancer (A research article intended to be submitted to Molecules).

ADDENDUM 2: ETHICS CLEARANCE

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
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Tel: +27 21 959 6917
Email: sethn@cput.ac.za

15 July 2019
REC Approval Reference No:
CPUT/HW-REC 2019/H12

Dear Ms Jumoke Aboyewa

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Ms Aboyewa for ethical clearance on 20 June 2019. This approval is for research activities related to student research in the Department of Biomedical Science at this Institution.

TITLE: **Gold Nanoparticles of Cyclopia Intermedia (Honeybush): An insight into the Molecular Mechanism of its Anticancer Effect in Prostate Cancer**

Supervisor: Prof M Meyer and Prof O Oguntibeju

Comment:

Approval will not extend beyond 16 July 2020. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Dr. Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences

Cape Peninsula
University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HWS-REC)
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23 July 2020
REC Approval Reference No:
CPUT/HW-REC 2019/H12 (renewal)

Faculty of Health and Wellness Sciences

Dear Ms Jumoke Aboyewa,

Re: APPLICATION TO THE HWS-REC FOR ETHICS CLEARANCE - RENEWAL

Approval was granted by the Health and Wellness Sciences-REC to Ms Aboyewa for ethical clearance on 20 June 2019. This approval is for research activities related to student research in the Department of Biomedical Sciences at this Institution.

Title: Gold nanoparticles of *Cyclopia intermedia* (honeybush): An insight into the molecular mechanism of its anticancer effect in prostrate cancer

Supervisors: Prof M Meyer and Prof O Oguntibeju

Comment:
Approval will not extend beyond 24 July 2021. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards,

M. le Roes-Hill

Dr Marilize Le Roes-Hill
Deputy Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences