2022-07

Formulation, Optimization, and Evaluation of Moringa oleifera leaf polyphenol-loaded phytosome delivery system against breast cancer cell lines

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NM-AIST

https://doi.org/10.58694/20.500.12479/1498

FORMULATION, OPTIMIZATION, AND EVALUATION OF *Moringa oleifera* LEAF POLYPHENOL-LOADED PHYTOSOME DELIVERY SYSTEM AGAINST BREAST CANCER CELL LINES

Jecinta Wanjiru Ndung'u

A Dissertation Submitted in Partial Fulfilment of Requirements for Award of a Degree of Master's in Life Sciences of The Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

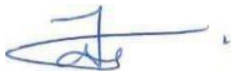
July, 2022

ABSTRACT

Active plant molecules like polyphenols have been repositioned increasingly utilized for managing breast cancer traditionally. However, polyphenols possess poor lipid miscibility, that results in poor bioavailability and decreased efficacy. 'Phytosome' is a plant based nanocarrier that improves the bioavailability of the polyphenols. The purpose of this study was to improve the bioavailability of *Moringa oleifera* Lam leaf polyphenols (Mopp) through encapsulation with phytosomes to enhance their activity on 4T1 cancer cell lines. The Mopp phytosomes were extracted by microwave-assisted extraction. *Moringa oleifera* polyphenols-loaded phytosomes (MoP) were prepared by the nanoprecipitation method and characterized by a dynamic light scattering technique. The ultraviolet-visible spectroscopy determined the percentage entrapment efficiency (EE%). Fourier-transform infrared (FTIR) spectroscopy assessed MoP formation. The dialysis membrane technique investigated *in vitro* drug release while storage stability test analysis identified MoP stability. The *in vitro* cytotoxic activity of MoP on E6 Vero cell lines and anti-proliferative activity on 4T1 cancer cell lines were investigated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, while female Swiss albino mice were used to assess acute toxicity. Results showed that MoP average particle size was 296±0.29 nm, a zeta-potential of -40.1±1.19 mV, and polydispersity index of 0.106±0.002. The Mopp total phenolic content was 50.81±0.02 mg GAE/g, EE% was 90.32±0.11%, while FTIR spectra demonstrated successful formation of phytosomes. The drug release profiles demonstrated biphasic behavior of immediate burst release and subsequent prolonged sustained release. The *in vitro* cytotoxicity assays indicated that the MoP had low cytotoxic effect of 98.84±0.53 µg/mL on E6 Vero cells; while doxorubicin had 68.35±3.51while Mopp was 212.9±1.30 µg/mL. Moreover, MoP exhibited the highest antiproliferative effect on 4T1 cancer cells with an inhibitory concentration of 7.73±2.87 µg/mL and selectivity index >3. The results indicated a significant difference ($p \leq 0.001$) in MoP when compared to Mopp and doxorubicin. *In vivo* investigation confirmed the safety of MoP on mice at a dose below 2000 mg/kg. The present findings suggest that the MoP can serve as an effective and promising formulation for breast cancer drug delivery in cancer therapy.

DECLARATION

I, Jecinta Wanjiru Ndung'u, do hereby declare to the Senate of The Nelson Mandela African Institution of Science and Technology that this dissertation is my original work and that it has neither been submitted nor being concurrently submitted for a degree award in any other institution.

Jecinta Wanjiru Ndung'u		28 July 2022
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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by The Nelson Mandela African Institution of Science and Technology, a dissertation titled **"Formulation, Optimization, and Evaluation of *Moringa oleifera* Leaf Polyphenols-Loaded Phytosome Delivery System against Breast Cancer Cell Lines"** in partial fulfillment of the requirements for the degree of Master's in Life Sciences of The Nelson Mandela African Institution of Science and Technology.

Prof. Hulda Shaidi Swai		28 July 2022
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ACKNOWLEDGMENTS

First and foremost, my sincere gratitude goes to my supervisors, Prof. Hulda Swai and Dr. Elingarami Nkya of the School of Life Sciences and Bioengineering (LiSBE) of the Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania, for their technical, and professional support throughout this study. I am also grateful to Dr. Jeremiah Gathirwa; Principal Research Scientist at the Kenya Medical Research Scientist (KEMRI) in Kenya for his immense mentorship and guidance toward my research work. They consistently supported me and made this work a success.

I acknowledge my sponsor, the Inter-University of East Africa/ World Bank through the Centre for Research, Agricultural Advancement, Teaching Excellence (CREATES) of The Nelson Mandela African Institution of Science and Technology (NM-AIST).

I would also like to thank the Director-General of the Kenya Medical Research Institute in Nairobi Kenya - for the provision of study materials and a conducive environment that allowed conducting my study successfully.

I also appreciate the Center for Traditional Medicine and Drug Research, and KEMRI's staff for the technical, guidance, and emotional support.

Lastly, I would like to appreciate my parents; James Ndung'u, and Esther Wangui for their unfailing support and encouragement during my study. I owe this accomplishment to them.

DEDICATION

This dissertation is dedicated to the Almighty God for good health and breakthrough in all aspects of my studies. I also dedicate it to my family members for their endless support, kindness, devotion, and for believing in my potential. Your generous heart will always be remembered.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
<	Less
>	Greater
≤	Less than or equal
°	Degree
µg	Microgram
℃	Degree Celsius
C=C	Carbon-Carbon double bond
CC_{50}	Cytotoxic Concentration (concentration required to cause alterations in 50% of intact cells)
Cm	Centimeter
C-N	Carbon-nitrogen bond
C-O-C	Carbon oxygen-carbon bond
cP	Specific heat capacity
CREATES	Center for Research, Education, Agriculture Training
CTMDR	Center for Traditional Medicine and Drug Research
Da	Dalton
DLS	Dynamic Light Scattering
EE	Entrapment Efficiency
FTIR	Fourier Transform Infra-Red
G	Grams
GAE	Gallic Acid Equivalent
GAE/g	Gallic acid equivalent per gram
GHz	Gigahertz
H	Hours
IC_{50}	Inhibitory concentration

KEMRI	Kenya Medical Research Institute
mg	Milligram
min	Minutes
mL	Millilitre
Mo	*Moringa oleifera*
MoP	*Moringa oleifera* polyphenol loaded-phytosomes
Mopp	*Moringa oleifera* polyphenols
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
mV	Millivolt
nm	Nanometres
NM-AIST	The Nelson Mandela African Institute of Science and Technology
PBS	Phosphate Buffered Saline
PDI	Polydispersity Index
pH	Potential of hydrogen
rpm	Revolutions per minutes
SEM	Standard error of the mean
THPs	Traditional Health Practitioners
TPC	Total Phenolic Content
W	Watts
WHO	World Health Organization
Wnt/ β	Wingless-related integration site/ Beta
µL	Microlitre

CHAPTER ONE

INTRODUCTION

1.1 Background of the Problem

Breast cancer is a leading global health issue and a common cause of cancer deaths in women (Feng *et al*., 2018). According to Lei *et al*. (2021), approximately 2.3 million breast cancer cases and 685 000 deaths were reported globally in 2020. The incidences of breast cancer and mortalities are rapidly increasing in developing countries, with an estimated 74 072 deaths and 168 690 reported cases in 2018 (Sharma, 2021). It is one of the most common types of women's cancer in Sub-Saharan Africa, with a projection of 416 000 deaths expected between 2020 to 2029 (McCormack *et al*., 2020). As reported by the World Health Organization (WHO), Kenya reported 3107 breast cancer deaths in 2020 (Lei *et al.*, 2021). In Tanzania, case data demonstrated an upward growth trajectory from 2732 in 2012 to 3037 new cases in 2018 and are further anticipated to upsurge by 82% by 2030 (Sood *et al.*, 2021). Notably, without interventions, the burden of breast cancer is bound to rise, exerting tremendous financial and emotional strains on individuals.

Breast cancer is a heterogeneous malignant disease that commonly affects women in the world (Feng *et al.*, 2018). The malignant cells in the breast glands can invade other body parts through lymph nodes or blood vessels. Factors such as tobacco, poor diet, obesity, lack of physical exercise, prolonged and cumulative exposure to progesterone or estrogen, and excessive alcohol intake are the risk factors for breast cancer (Momenimovahed & Salehiniya, 2019). Exposure to ionizing radiation, genetic disorders, and certain bacterial infections such as *Helicobacter pylori*, can also activate breast cancer cells.

Adjuvant therapies for breast cancer include immunotherapy, chemotherapy, and radiation therapy. These not only confer adverse side effects, but some cancer cells can gain resistance and they are relatively costly, especially in developing countries (Kim *et al.*, 2020). These drawbacks have necessitated the search for alternative efficacious and affordable cancer drugs. In this context, medicinal plants are an important alternative approach since they pose fewer side effects in appropriate doses (Gaonkar & Hullatti, 2020; McGrowder *et al.*, 2020). Cognizant of this, plant-derived agents like Docetaxel from *Taxus baccata* are used in some cancer treatments (Da-Rocha *et al.*, 2020).

Moringa oleifera Lam. (Mo*)* leaf is a commonly used herbal medicine for cancer treatment in many African communities. Several *in vitro* studies have demonstrated its efficacy against breast cancer (Gaffar *et al.*, 2019*;* Mumtaz *et al.*, 2021*;* Wanjiru *et al.*, 2018; Wisitpongpun *et al.*, 2020). The efficacy of this plant against breast cancer cells is attributable to polyphenols such as tannins, flavonoids, and terpenoids. Research by Xu *et al*. (2020) revealed improved inhibitory effects of polyphenols on 4T1 cancer cells. Studies by Liu *et al*. (2019), Komath *et al*. (2018), and Zhang *et al*. (2019), have also reported increased bioactive compound bioavailability, thus improving their potential in inhibiting breast cancer cells. Although polyphenols have antineoplastic effects, they are characterized by poor plasma membrane permeability and lipid immiscibility because of their hydrophilic nature, which limits their absorption into the target cells and also lowers their bioavailability and therapeutic efficacy (Dobrzynska *et al*., 2020; Wanjiru *et al*., 2018). *Moringa oleifera* polyphenols (Mopp*)* are also degraded by digestive enzymes and gastric fluids when administered orally (Dobrzynska *et al.*, 2020). An example of a novel drug delivery system using different nanocarriers of the nanomedicine field includes silver that has been conjugated with Mopp to solve the bioavailability challenges, but silver can impose toxicity (Tiloke *et al*., 2018; Tortella *et al.*, 2020; Velidandi *et al*., 2020). Consequently, it is necessary to search for a drug delivery system that is safer and more bioavailable, hence the introduction of nanomedicine in the present study.

Nanomedicine involves comprehensive, monitoring, repair, control, defense, construction, and improvement of biological systems at the molecular level using engineered nanostructures and nanodevices to achieve health benefits. Cancer nanomedicine development has focused on improving the efficacy of cytotoxic agents and improving their delivery into tumor cells. Nanomedicine has been tailored to initiate tumor cell death using hybrid nanostructures, such as phytosomes, which act as a drug delivery vehicle for bioactive compounds (Lombardo *et al*., 2019).

Phytosomes are new drug delivery systems with an enhanced controlled drug release profile. They are made of phospholipids, which are a lipid and cell membrane's major component. Phospholipids are amphipathic molecules with two neutrally charged tails and a positively charged head that renders them miscible in lipid and water conditions. Part of the phosphate group is the oxygen atom, which tends to lose or gain electrons (Chi *et al.*, 2020), which facilitates the modification of polyphenols, generating an amphiphilic complex that expediates their movement across the cell membrane obstruction. Therefore, phytosomes may be a

desirable delivery vehicle to enhance the absorption of polyphenols through biological lipid barriers, thus increasing their efficacy and improving their therapeutic effects. The soy phospholipid is readily available clinically, owing to biodegradability, biocompatibility, and low toxicity compared to its synthetic alternatives. Phytosomes, therefore being the nanocarrier, attract great interest owing to the minimization of the side effects of polyphenols and improvement of their bioavailability.

Previous studies have reported improved anticancer efficacy of nano-phytosome compared to free polyphenols on breast cancer cell lines (Barani *et al.*, 2021; Moeini *et al.*, 2021). Therefore, this research focused on exploring an effective phytosome delivery that overcomes the bioavailability challenge, limits toxicity, and enhances the antitumor effect essential for clinical applications. Although Mo crude extracts have been applied in the management of cancer, there is currently no reported literature on the antiproliferative action of Mo polyphenols loaded phytosomes (MoP) complex on 4T1 cancer cell lines. Thus, this study explored the anti-proliferative efficacy of the formulated MoP complex on breast cancer cell lines and *in vivo* cytotoxic activity using female Swiss albino mice.

1.2 Statement of the Problem

Breast cancer is a prevalent malignancy among women worldwide, causing deaths and accounting for 25% of all cancer (Momenimovahed & Salehiniya, 2019). It is a common type of women's cancer in sub-Saharan Africa with a projection of 416 000 deaths between 2020 and 2029 (McCormack *et al*., 2020). According to WHO, there were 3107 breast cancer deaths were reported in Kenya in 2020 (Lei *et al*., 2021). Mortality and morbidity from breast cancer are projected to increase to 82% from 3037 by 2030 in Tanzania if there will be no curable treatment. Coupled with this, the available treatment approaches, such as chemotherapy and radiotherapy, are weighed down by their toxicity, high costs, and increasing resistance (Kaboli *et al.*, 2020).

Medicinal plants are being used as anticancer alternatives in breast cancer management. However, their effectiveness is largely limited by their phytoconstituents being poorly absorbed, with limited availability to the target cancer cells. This is attributed to their multiple ring structure that limits their absorption by simple diffusion through enterocyte cells to target cells. Also, their hydrophilic phytoconstituents have poor lipid miscibility, thus limiting their bioavailability due to the lipid nature of the cell wall of the target cells (Yang *et al.*, 2020).

Similarly, crude leaf extracts from Mo have proven to display antiproliferative effects on cancer cells (Gaffar *et al*., 2019; Mumtaz *et al*., 2021; Wanjiru *et al.*, 2018; Wisitpongpun *et al*., 2020). However, they are limited by poor absorption and availability of their hydrophilic phytoconstituents to the target cells. Additionally, using the extracts with other nanoparticles like silver nanoparticles to solve this challenge, still imposes toxicity on normal cells (Tiloke *et al*., 2018; Tortella *et al.,* 2020; Velidandi *et al*., 2020). This study, therefore, aimed at exploring the anticancer efficacy of synthesized MoP complex on breast cancer cell lines.

1.3 Rationale for the Study

Assessment of the anti-proliferative effect of Mo phytosomes complex is herein suggested due to safety, target therapy, and the lipophilic nature of the phytosome. Phytosomes resolve challenges of poor absorption and bioavailability of hydrophobic compounds by allowing direct delivery into targeted tumor cells. With improved bioavailability and absorption nature of phytoconstituents, there is improved efficacy of the herbal drug such as polyphenols in the target cells (Komath *et al.*, 2018; Liu *et al.*, 2019; Zhang *et al*., 2019).

Furthermore, phytosome protect the phytoconstituents from destruction by digestive enzymes and offer good stability to phytoconstituents (Mane *et al*., 2020). Phytosomes have been used to design evodiamine therapeutic drugs from anticancer plants and have shown improved stability, increased bio availability, and absorption of the water-soluble phytoconstituents (Kumar *et al*., 2017; Saroha *et al.*, 2020). Owing to the above benefits of phytosome drug delivery systems, the present study incorporated the hydrophilic *Moringa oleifera* phytoconstituents in phytosomes and delivered them to breast cancer cell lines to improve their efficacy. This study, therefore, explored the use of synthesized Mo phytosomes complex and free polyphenols extracts of Mo leaf against breast cancer cell lines. The knowledge created from this study will be important in initiating anticancer drug development process with enhanced absorption and bioavailability, in improving breast cancer management.

1.4 Objectives of the Study

1.4.1 General Objective

To formulate and characterize *Moringa oleifera* polyphenol loaded-phytosomes complex targeting breast cancer cell lines.

1.4.2 Specific Objectives

(i) To isolate *Moringa oleifera* polyphenols (terpenoids, tannins, and flavanoids) present in Mo leaves.

(ii) To formulate and characterize Mo polyphenols loaded-phytosomes system of drug delivery.

(iii) To evaluate the cytotoxicity and antiproliferative effects of Mo polyphenols loadedphytosome complex against breast cancer and Vero cell lines.

(iv) To evaluate *in vivo* acute toxicity assay for Mo polyphenols loaded-phytosomes on female Swiss albino mice.

1.5 Research Questions

(i) What is the amount of total phenolic content in *Moringa oleifera* extracted polyphenols?

(ii) Is Mo nano-phytosome complex capable of delivering the polyphenols on 4T1 breast cancer cell lines?

(iii) Do Mo polyphenols loaded- phytosomes complex induce cytotoxicity on Vero cell lines and enhance *in vitro* antiproliferative effect on 4T1 lines of breast cancer cell lines?

(iv) Is MoP formulation safe on mice?

1.6 Significance of the study

The knowledge created may be crucial to Traditional Health Practitioners (THPs) and local communities in breast cancer management. This information is also important to researchers since it is a reliable selection tool for breast cancer drug development using the locally available FDA-approved drug delivery system which enhances the bioavailability of bioactive compounds with medicinal values.

The study can also stimulate researchers' attention to the underutilized nanomedicine on medicinal drugs as an affordable and sustainable source of potential anticancer drugs. The findings may significantly contribute to policy creation for the use of safe and effective herbal medicine in primary care, particularly in breast cancer therapy.

1.7 Delineation of the Study

This study focused on the formulation, optimization, and evaluation of Mo leaf polyphenols loaded phytosome delivery system against breast cancer cell lines. The Mo leaves were collected at Machame, Moshi, Tanzania. This study failed to cover other *Moringaceae* family members apart from the ones mentioned above. In this sense, the information presented in this article should not be interpreted as referring to the whole *Moringaceae* family or all cancer cell lines.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of Breast Cancer

Breast cancer is a condition that causes abnormal cells to proliferate and divide excessively in the breast gland. Gene mutation in the cell controlling growth in breast cancer can initiate malignancy tumor. This is because they are capable of ignoring growth signals thus dividing uncontrollably (Song *et al.*, 2020). The main carcinogens that can trigger breast cancer include physical carcinogens; ionizing radiation and ultraviolet, biological carcinogens; bacterial infections e.g., *Helicobacter pylori* and chemical carcinogens; asbestos, aflatoxins, and arsenic.

2.2 Breast Cancer Burden

Breast cancer is the most common disease in women and the 5th greatest cancer mortality cause worldwide accounting for an estimated 2.3 million new cases, accounting for 11.7% of all cancer cases and 685 000 deaths (Sung *et al*., 2021). It accounts for 25% of the new cancers and 15% of all cancer death in women (Bray *et al.*, 2018). Even though it is considered a disease in high-income countries, an estimated 58% of breast cancer deaths and 50% of cases occur in developing nations (Joko *et al*., 2020). It is the second highest cause of cancer mortality in sub-Saharan Africa, which constitutes a higher threat if no action is taken. In Kenya, an estimated 3107 breast cancer deaths were reported in 2020 (Lei *et al.*, 2021). Additionally, in Tanzania, it is the second leading cancer and is projected to increase to 82% from 3037 by 2030 (Sood *et al.*, 2021).

2.3 Molecular Basis of Cancer

Signaling pathways such as Human Epidermal Growth Factor Receptor (HER2) Wnt/ β-catenin and estrogen receptors regulate the normal mammary stem cells and development. These signaling pathways also control cell differentiation, cell growth, cell motility, and stem cell proliferation. Malignant breast cancer is a molecular disease in which the cellular pathways that involve alteration of cells (Feng *et al*., 2018). The altered pathways involve genes such as oncogenes HER2, Cellular Myelocytomatosis (c-MYC), and Rat Sarcoma virus (RAS), Epidermal Receptor (ER) genes, tumor suppressor genes Retinoblastoma (RB), Tumor Protein 53 (TP53), Breast Cancer 1 (BRCA1), and Breast Cancer 2 (BRCA2) genes. When these

pathways are altered, this may lead to abnormal cellular proliferation causing cancer (Feng *et al.*, 2018).

2.4 Conventional Drugs

Breast cancer incidence and mortality rates are still rising, thus there is a requirement for urgent investigation on possible management/ treatment methods (Malvezzi *et al.*, 2019). The current approaches used for breast cancer treatment include radiation therapy, surgery, immunotherapy, and chemotherapy (WHO, 2006). The major limitations to these approaches include high costs, lack of specificity, adverse side effects, and resistance to chemotherapeutic drugs, which altogether increase the risks of high mortality rates (Kim *et al.*, 2020). Consequently, these therapeutic gaps in the developing countries have led to the exploration of new lead compounds from medicinal plants that have improved bioavailability, lower-cost alternatives, and high safety (Gaonkar & Hullatti, 2020; McGrowder *et al.*, 2020). The search has led to advances in the field of traditional medicine to develop effective and less toxic treatments for breast cancer.

2.5 Traditional Medicine: Phytomedicine

Traditional medicine is a potential health care primary mode of disease treatment, especially in developing countries, despite the remarkable advancements in modern drugs (Gaonkar & Hullatti, 2020; McGrowder *et al.*, 2020). Phytomedicines and plant mixtures have been used in ailment treatments since ancient times. Medicinal plants possess an opportunity for drug discovery. Some of the current drugs have their origin in traditional medicine in different cultures. According to WHO, medicinal plants play a significant role in sustainable human health. They are also considered cheap, easily available, and have fewer side effects. Despite them being used in ailment management, they are limited in their effectiveness due to their poor absorbability when taken orally.

2.5.1 Overview of *Moringa oleifera* Leaves

Moringa oleifera referred is a deciduous, drought- resistant, and fast-growing tree species in the *Moringaceae* family and native to India. Its common names are drumstick, horseradish, and ben oil tree. It is also referred to as the "wonder tree" or "miracle tree" due to its diverse roles as traditional herbal medicine (Kamran *et al*., 2020). The tree is referred to as "*Mlonge*" in Swahili. It grows up to 10–12 m in height and 45 cm trunk diameter. It is native to India but

has become naturalized in African countries. The leaves contain a great profile of essential element traces and are a great source of vitamins, amino acids, proteins, beta-carotene, and several phenolics (Kamran *et al.*, 2020).

It is also a significant source of fats, iron, potassium, and other nutrients; and thus, it is a highly nutritious plant (Kamran *et al*., 2020). The leaves are most nutritious and a significant source for vitamins C, Vitamin B, beta-carotene, protein, manganese, and vitamin K. Due to these benefits, the leaves have drawn much attention from most researchers. They not only contain various polyphenols which are under research to determine their influence on human health but also have antioxidant, anti-atherosclerotic, tumor-suppressive effects, anti-inflammatory properties, and immune-boosting properties (Dhakad *et al.*, 2019).

2.5.2 Medicinal uses of *Moringa oleifera* Anticancer Therapeutic Effects

Moringa oleifera leaf has shown anticancer, hepatoprotection, antibacterial, antioxidant, and anti-inflammatory properties in *in vitro* and *in vivo* studies. The antiproliferative effect of Mo leaf crude extract on Human Colorectal Carcinoma (HCT116) cell line; human colon cancer and breast cancer have been reported (Tragulpakseerojn *et al.*, 2017). Mo plant being a medicinal plant is considered cheap and readily available, and an alternative source of treatment and management of cancers with minimal side effects (Gaonkar & Hullatti, 2020; McGrowder *et al*., 2020). Research done by Xu *et al.* (2020) showed progress of polyphenols in enhancing ant proliferative effects on 4T1 cancer cells as compared to crude extract. Studies by Liu *et al.* (2019), Konath *et al.* (2018), and Zhnag *et al*. (2019) have also reported improvement in the bioavailability of bioactive compounds, thus improving their efficacy in the management of breast cancer cells.

This is contributed due to the presence of chemical components like flavones, flavonoids, tannins, anthocyanins, quinones, isoflavones, lignins, coumarins, isocatechins, catechins, which are classes of polyphenols that possess antioxidant properties that contribute to their anticancer potential (Xu *et al.,* 2020). However, polyphenols and flavonoids, which are responsible for the antiproliferative activity of these bioactive constituents, have low oral and topical absorption either due to their considerable molecular weight or poor lipophilic nature. This limits their effectiveness on the target cells (Dobrzynska *et al.*, 2020; Gaffar *et al.*, 2019; Ndung'u *et al.,* 2018). To solve this problem, research has previously been conducted using Mo silver nanoparticles as a system for drug delivery. The studies have reported improved

potential anti-proliferative agents against breast cancer. However, the methods still impose toxicity due to toxic metals being used toxicity (Tiloke *et al*., 2018; Tortella *et al.*, 2020; Velidandi *et al.*, 2020). Therefore, there is a need to develop a safer delivery method for poorly absorbed and low-oil immiscible anticancer compounds to the target cell without imposing toxicity. There is therefore need to search for a drug delivery system that is safer and more bioavailable, hence the introduction of nanomedicine in the present study.

2.6 Nanotechnology and Nanomedicine

Nanotechnology alongside other concepts such as nanostructures, nanoparticles, and nanomaterials, has become a significant scientific research topic and technological development. Nanotechnology involves manipulation and utilization of nanometer-scale devices with multiple applications (Lombardo *et al*., 2019). The greatest application of nanotechnology is in biotechnology and health. The emergence of nanotechnology and nanomedicine brings medicine together with aim of improving existing treatments. Nanomedicines, therefore, involves the utilization of nanotechnology in the diagnosis, treatment, and regulation of biological systems. In nanomedicine, molecules and atoms are manipulated to generate nanostructures indistinguishable from biomolecules in size to enhance their interaction with human cells. Nanomedicine has ushered in drug delivery vehicle platforms. Currently, nano delivery strategies such as phytosomes are being generated. The constructs are modified with moiety to enhance target delivery. Traditional convectional drugs suffer from non-specific distribution which results in the accumulation of the drugs in healthy organs, thus increasing toxicity. Phytosomes are the main mainstay within the field of nanomedicine that aims at utilizing nanoscale of 1 to 100 range of constructs to enhance delivery and efficacy of bioactive compounds with medicinal benefits.

2.7 Phytosomes

‘Phyto’ implies plant, whilst ‘some’ means cell-like. Therefore, phytosomes are advanced herbal formulations that contain herbal extract bioactive phytoconstituents of phytomedicine bounded by a lipid (Kumar *et al*., 2017). Most phytomedine bioactive compounds are water soluble and include compounds such as terpenoids, glycosides, flavonoids, tannins that possesses wider therapeutic effects. Phytosomes being a vesicular drug delivery system have the potential of encapsulating hydrophilic phytoconstituents and modifying them to have lipophilic nature. This encapsulation prevents the phytoconstituents from destruction by the

digestive secretions (Chi *et al.,* 2020). Thus, their absorption and bioavailability are enhanced and the efficacy of phytoconstituents is improved compared to conventional herbal extracts. Additionally, they assure the availability of phytoconstituents to the target cells and have shown improved efficacy compared to the crude conventional water phytoconstituents (Chi *et al*., 2020).

2.7.1 Principle of Phytosome Technology

The phosphotidylcholine consists of bifunctional compounds. The phosphatidylcholine has hydrophilic choline (serine) moiety and lipophilic phosphatidyl moiety. The dual property of phytosomes makes them good and effective emulsifiers (Chi *et al.,* 2020). The choline head binds to the phytochemical constituents including flavonoids, phenols and terpenoids provide direct complexation with phosphatidylcholine, while the phosphatidyl portion envelops the choline-phytoconstituents complex forming a molecular phytosomes complex.

2.7.2 Advantages of Phytosomes

While phytosomes consist of a few phospholipids' molecules unit bonded together, liposomes consist of aggregates of many units of phospholipids but without bonding to them specifically. Liposomes have been reported to be a touted delivery system but their dietary supplements are not yet met. Studies have reported phytosomes as being markedly improved efficacy with better absorptions and better stability due to the chemical bond formed between phospholipid and phytoconstituents (Chi *et al.,* 2020). Their breakthrough model includes assured delivery to tissue, significantly greater benefits, enhance bioavailability and they do not compromise nutrients' safety.

Phytosomes form a chemical bond thus better stability between phytoconstituents and phospholipids. They improve phytoconstituents dose because of the higher bioavailability of phytoconstituents in phytosomes' structure. They also increase the duration of action of the phytoconstituents (Kumar *et al.,* 2017). They also resist the action of digestive enzymes and gut bacteria and are more stable when in gastric secretions. The phosphotidylcholine used in forming the phytosomes possesses therapeutic properties due to its hepatoprotective property, giving an enhanced synergistic effect. They enhance lipid insoluble phytoconstituents absorption via both topical and oral routes which increases their bioavailability and enhances therapeutic effects.

2.7.3 Phytosome as a Carrier of Phytoconstituents

Phytosome is an advancement of herbal drug delivery that intend to efficiently manage health (Kumar *et al.*, 2017). They have been shown to improve the therapeutic benefits of medicinal plants. This enhanced bioavailability nature of phytosomes is a result of increased solubility, hydrophilicity and enriched drug absorption in the systemic circulation, and decreased hepatic metabolism. Previous studies by Chi *et al*. (2020) showed improved efficacy of phytosomes, thus suggesting the improved antiproliferative efficacy on targeted cancer cells.

Despite them having numerous advantages, phytosomes are not prevalent in the market. The present study thus hypothesized that the phytosome technology could curb this challenge because they enhance the phytoconstituents' bioavailability and absorption in the target cells (Chi *et al.*, 2020). This study, therefore, aims at studying Mo nanophytosome complex therapeutic effects on breast cancer cell lines.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and Reagents

Dichloromethane (99% purity), ethanol, and double-distilled water were procured from Sigma-Aldrich Inc. Soy phosphatidylcholine (Lipoid) was obtained from Biotec lab ltd. The 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT) and Trizol ™ reagent was obtained from Sigma. All reagents were of analytical grade. The 4T1 mammary carcinoma cells and Vero cell lines were ordered from the American Type Culture Collection (Manassas, VA, USA) and Dulbecco's modified Eagle's medium from Gibco, Life Technologies, Inc., USA). The Fetal Bovine Serum (FBS) 10%, L-glutamine 1%, and 1% antibiotics (streptomycin) were purchased from Gibco.

3.2 Ethical Considerations

Before commencing the study, clearance was sought from the Kenya Medical Research Institute; Scientific and Ethics Review Unit (SERU) and the Animal Care and Use Committee (ACUC). Approval certificates number KEMRI/SERU/CTMDR/CSCP099/4204 and KEMRI/ACUC/24.05.2021 were respectively issued. All the laboratory procedures and protocols were followed and no human sample was used in the study.

3.3 Sample Collection and Preparation

Moringa oleifera leaves were collected from May 20th to 26th, 2021 at Machame, Moshi, Tanzania, where they were in use by the THPs and local communities for breast cancer management. The collection was done with the assistance of a botanist, the area herbalist, and an authentication voucher number JWN/MO/05/2021 awarded by a professional taxonomist. The samples were taken to The Nelson Mandela African Institution of Science and Technology (NM-AIST), sorted and air-dried for seven days, and then pulverized into fine powder. Whatman's filter paper (No. 1) was used to filter the materials, after which they were subjected to a Microwave-assisted extraction technique as described in Section 3.4.

3.4 Microwave-Assisted Extraction

A domestic microwave oven (Akai 24001), with 800 W total capacity operating at 2.54 GHz, was employed for Mopp extraction. Approximately 5.0 g of plant samples were sonicated in distilled water (100 mL). The mixtures were irradiated at (750 W, 90s) as outlined by Sachez-Camargo *et al.* (2021) with slight modifications. The prepared mixture was then filtered using Whatmans' No. 1 filter paper, concentrated by a freeze drier, and the extracted yield determined gravimetrically. The yield of extracts was calculated according to Gonfa *et al*. (2020) as shown in Equation 1.

$$\text{Percentage Yield} = \left(\frac{W2\text{-}W1}{W0}\right) \times 0\text{-}W \qquad (1)$$

Where W2= Weight of blank container, W1= Weight of extract with container, W0= Initial dried sample weight

3.5 Estimation of Total Phenolic Content

The quantification of Total Phenolic Content (TPC) was assessed following the FolinCiocalteu method (Cao *et al.*, 2020) with slight modifications. Triplicate tests were conducted in the experiment. The results were expressed as equivalents of mg of gallic acid (GAE) per 100 g of dry Mo sample (mg GAE/ 100 g). This was computed as natural compound (gallic acid) equivalent (GAE) using Equation 2.

$$T = \frac{CXV}{M} \qquad (2)$$

Where;

T- is the total phenolic content in mg g^{-1} of the extracts as GAE,

C- is the concentration of gallic acid established from the calibration curve in mg mL^{-1}

V- is the volume of the extract solution in mL

M- is the weight of the extract in gm

The prepared sample was then put in an air-tight bottle and transferred to the Centre for Traditional Medicine and Drug Research (CTMDR) at the Kenya Medical Research Institute (KEMRI) laboratories in Kenya for further analysis.

3.6 Phytosome Synthesis

Moringa oleifera phytosome formulation was prepared using a thin-layer hydration method (Wijiani *et al.*, 2020). Briefly, the lipoid was dissolved in dichloromethane, while the Mopp was diluted with 90% ethanol. The mixture was then poured into a round-bottom flask, sonication for 10 min, and subjected to a BUCHI Mini spray dryer B-290 (Inlet and outlet temperature; 100 ℃, pump rate 25; aspirator; 100) for nitrogen gas to flow and the solvent to evaporate. An even and a thin film layer MoP complex was formed.

3.7 Characterization of Phytosomes

3.7.1 Particle Size Distribution, Zeta Potential, and Polydispersity Index

The particle size distribution, zeta potential, and Polydispersity Index (PDI) for the MoP complex were assessed using the Dynamic Light Scattering (DLS) technique particle size analyzer comprising of a Malvern, Zetasizer Nano computerized system using the protocol by El-Far *et al*. (2021). The following parameters were applied; temperature of 25 °C, wavelength of 633 nm, 173° light scattering angle, a 1.33 refractive index of the medium, and 0.8872 cP medium viscosity.

3.7.2 The Percentage Entrapment Efficiency

The percentage entrapment efficiency (percentage EE) of Mopp in phytosome complex was obtained by the ultracentrifugation method as previously outlined by Abd El-Fattah *et al*. (2017). The MoP formulations were briefly centrifuged for 90 minutes at 4°C and 15 000 rpm. The supernatant was separated and analyzed for TPC as previously described using a UV spectrophotometer at 290 nm. The percentage content of polyphenol entrapped was then calculated using the formula in Equation 3.

$$\text{Entrapment efficiency (\%)} = \left(\frac{\text{Total drug added-unentrapped drug}}{\text{Total drug}}\right) \times 100 \qquad (3)$$

3.7.3 Fourier Transform Infrared Spectroscopy

The Fourier-transform Infra-red (FTIR) (JASCO 4700 ATR-FT/IR) spectral analysis of MoP complex was used to determine the chemical stability and structure of the compound using the protocol previously described by Thiruvengadam and Bansod (2021). Potassium bromide pellets were freshly prepared to avoid any moisture effect, mixed with 0.5 mL of the sample,

then placed beneath a fixed probe of FTIR and scanned over a spectrum of 4400 to 400 cm^{-1} wavenumber region.

3.7.4 *In vitro* Drug Release Study of Polyphenol from *Moringa oleifera* Phytosomes

In vitro drug release of MoP was assessed using a dynamic dialysis method with slight modifications (Yu *et al*., 2019). A dialysis bag at 4000 Da with phosphate-buffered saline (PBS) at 0.02 M concentration and 7.4 pH was used in this study. The dialysis bags allowed the released polyphenols to permeate to the release medium. Briefly, the equivalent of 10 mg of Mo phytosomes was dispersed in 2.0 mL of PBS, added into a dialysis bag, and then changed into an Erlenmeyer flask with 100 mL PBS media. The system was then kept on a magnetic stirrer at 37 °C at 100 rpm under controlled conditions. The release media were wholly withdrawn and swapped with fresh PBS solution at designated time intervals from 0 to 8 h, then at 12, 24, and 72 h. The release media (media with the samples) were filtered using 0.45 Millipore filter paper and measured against fresh PBS media as blank at λmax of 425 nm by spectrophotometry. Mathematical models were employed to analyze the drug release kinetics used in this study, which includes Zero order, First order, Hixson-Crowell, Higuchi, and Korsemeyer Peppas models. The correlation coefficient (R^2) and release exponent (n) were used to decide the best-fit kinetic model and the method of the drug release out of the different release kinetics are presented in Table 1.

Table 1: Mathematics model equations for different release kinetics

Kinetic model	Equation
First order model	$\log C = \log C_0 - K t/2.303$
Zero order model	$Ct = C_0 + K_0 t$
Higuchi model	$Q = KH \times t^{1/2}$
Korsemeyer Peppas Model	$\log(Mt/M) = \log K_{kp} + n \log t$,

The percentages of polyphenols released were plotted as a function of time according to various kinetics models (Gouda *et al.,* 2017) using Equation 4.

$$\text{Percentage Polyphenols release} = \left(\frac{Ct}{Ci}\right) \times 100 \quad (4)$$

Where Ct is the sample concentration for each time point and Ci is the initial concentration.

3.7.5 *In vitro* Bioaccessibility Determination of MoP and Mopp

The MoP and Mopp were evaluated for their bioaccessibility in a simulated gastrointestinal tract (GIT) model consisting of the mouth, stomach, and intestine according to the method of Grgić *et al*. (2020). The prepared samples were then exposed to the simulated gastric and small intestine phases.

(i) Simulated Salivary Fluid in Mouth Phase

Simulated salivary fluid phase (SSF) was prepared using 0.328 g/L ammonium nitrate, 1.594 g/L sodium chloride, 0.202 g/L potassium chloride, 0.636 g/L potassium phosphate, 0.198 g/L urea, 0.308 g/L potassium citrate, 0.146 g/L lactic acid sodium salt and 5 g/L porcine gastric mucin type II. An aliquot of 4 mL of each extract was mixed with 4 mL of simulated saliva and the pH of the mixture adjusted to 6.8. The mixture was shaken continuously for 10 minutes at 100 revs while maintaining temperature at 37 °C.

(ii) Simulated Gastric Fluid (SGF)

The SGF was prepared with a slight modification of the methods of Shah *et al.* (2016) and Grgić *et al*. (2020). Two grams of sodium chloride, 7.0 mL of hydrochloric acid (420 g/L) and pepsin (3.2 g) was dissolved in 1 L of double distilled water and the pH adjusted to 1.2 using 1 M HCl. The sample from the mouth phase mimicking the bolus was mixed with SGF phase at a ratio of 50:50. The pH of the two-phase mixture was adjusted to 2.0 using 1 M NaOH and incubated at 37 °C for 2 h with continuous shaking at a speed of 100 rev/min.

(iii) Small intestinal phase

About 15 mL of the digested sample from the gastric phase was mixed with 8.25 mL simulated intestinal buffer solution. An aliquot of 1.87 mL fresh bile extract, 30 µL of 0.3 M calcium chloride, and 3.75 mL of pancreatin solution were also added and the volume topped up to 30 mL using deionized water. The temperature was maintained at 37°C and pH adjusted to 7.0 with 1 M NaOH. Approximately 1.5 mL lipase suspension (at a concentration of 60 mg/mL) was dissolved in phosphate-buffered saline (PBS) and added to the mixture. The mixture was then allowed to shake for 2 h while monitoring the pH (pH was maintained at 7.0) to mimic intestinal digestion process. NaOH (0.25 M) was used to neutralize the fatty acids released

from the lipid digestion while maintaining the pH of 7.0. The mixture was then shaken at 100 rpm for 6 h at 37 °C.

(iv) Measurement of Bioaccessibility

At the end of in vitro digestion, digested sample was used to measure the percentage bioaccessibility. Triton X-100 (1%) was added to the MoP sample to rupture the lipid membrane, and the mixture was vortexed and later centrifuged at 20 000 rpm for 30 min at 4 ˚C. The supernatant was collected and filtered. The filtrate was then fractioned and phenolic compounds solubilized. The total phenolic content was quantified and bioaccessibility calculated as follows:

$$Bioaccessibility\ (\%) = CDigesta/\ Ci * 100$$

3.7.6 *In vitro* Storage Stability Tests

A short-term stability test of the MoP complex was performed immediately upon preparation and then at regular time intervals, according to Lang *et al.* (2021). Briefly, 10 mL of MoP complex were stored at 25 °C room temperature, respectively for 25 days. As an essential indicator to assess the short-term stability of MoP, the drug-loading residual content was evaluated at predetermined time intervals (0 to 8 and 25 days), respectively. An aqueous of free polyphenols were also prepared in the same way and used for comparison.

3.8 Cell Viability

The cell cytotoxicity of the MoP complex against 4T1 cancer and Vero (E6) cell lines was assessed by MTT assay basing on the procedure by Alhakamy *et al.* (2020). Briefly, a monolayer in the exponential growth phase was trypsinized, after which trypan blue was used to count the viable cells. Cells (1×10^6 mL^{-1}) were seeded in a 96-well microtitre plate in minimum essential media with bovine serum (100 µL) and incubated in 5% CO_2 at 37 °C. After 24 h, the cells were exposed to 20 µL of MoP added in triplicate at initial concentrations of 1000 uL followed by three-fold serial dilutions and incubated for 72 h. Thereafter, 50 µL MTT dye was added and set at 37 °C for 2 h. After that, approximately 100 µL Dimethyl Sulfoxide was added to the solubilized formazan crystals and absorbance was read at 570 nm by a 96-well microplate reader (Thermo Fisher, Multiscan Go Spectrophotometer model). Doxorubicin was used as a standard drug (positive control). The percentage cytotoxicity was determined

using untreated cells as the negative control and expressed in CC_{50} values to infer a concentration that altered 50% of intact cells. In addition, the half-maximal inhibitory concentration (IC_{50}) of 4TI by MoP was assessed and calculated using the GraphPad Prism 6 Software, USA. The experiment was carried out three times. The cytotoxicity percentage was computed using the background-corrected absorbance as shown in Equation 5.

$$\% \text{ Cytotoxicity} = \left(\frac{1-\text{absorboance of the experiemental well}}{\text{Absorbance of negative control well}}\right) \times 100 \quad (5)$$

The proliferative effects were calculated using the formula shown in Equations 6 – 8.

$$\text{Proliferation rate} = \frac{\text{At-Ab}}{\text{Ac-Ab}} \quad (6)$$

$$\% \text{ Viability} = \frac{\text{At-Ab}}{\text{Ac-Ab}} \text{ x } 100 \quad (7)$$

$$\% \text{ inhibition} = 100 - \frac{\text{At-Ab}}{\text{Ac-Ab}} \text{ x } 100 \quad (8)$$

Where;

At = Absorbance value of test compound

Ab = Absorbance of blank

Ac = Absorbance of negative control

3.9 *In vivo* Experiment for Acute Toxicity Studies using Swiss Albino Mice

Female Swiss albino mice (18-22 g) aged six to eight weeks were obtained from the KEMRI animal home. They were then housed under standardized conditions before the experiment. A total of 27 mice were weighed, picked at random, and split into nine groups. The mice were marked on their tails for easy identification. Additionally, they were fed water and mice food pellets *ad libitum*. The cages were kept at 25°C while ensuring lighting regulation. The acute toxicity experiment was conducted according to the Organization for Economic Cooperation and Development and slight changes to the protocol by Dongmo *et al*. (2019).

Briefly, the mice were fasted for 3 h before dosing but given water *ad libitum* only. An estimated 0.2 mL of the drugs were prepared in PBS at 50, 300, and 2000 mg/mL concentrations based on 1 mL/kg of the mice's body weights. The mice were observed for

general behavior, body weight changes, and mortality for the first 5 hours and subsequently every day for 14 days after treatment.

The following parameters were considered; mortality, signs of acute toxicity, and behavioural changes (aggression, paralysis, unusual vocalization, agitation, sedation, tremors, convulsions, ataxia, diarrhoea, piloerection, catatonia, unusual locomotion, grooming, fasciculation, sleep, coma, prostration and asphyxia, hypo and hyperactivity and tremors) (Dongmo *et al*., 2019). All mice were weighed, immediately after treatment on the 1^{st} day, then on the 7^{th} and 14^{th} days, respectively. All mice were sacrificed at the end of the experiment, and the spleen, heart, kidneys, liver, lungs, intestines, ovaries and brains of each mouse were dissected and examined for abnormalities.

3.10 Data Management and Statistical Analysis

The raw data was transferred into the Microsoft excel sheet and used in calculating mean absorbance values. The Origin 2019b software was used in to obtain the TPC. Graph pad software was used to calculate concentrations needed to inhibit 50% cell growth. The quantitative values obtained per treatment were converted to percentage inhibition. All the data were shown as mean ± SEM; the standard error of the mean. Variance analysis was performed to compare the models and the insignificant lack of fit for each output element was determined on the p-value. The very small probability values of the selected model for each output element indicated that the model was significant for the data set ($p<0.05$). Differences in p –value < 0.05 were deemed statistically significant. Figures, graphs, and tables were used to give a clear presentation of the data obtained from the study.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Determination of Total Phenolic Content

Folin Ciocalteu's method using gallic acid as the standard was used to estimate the TPC for MoP. The plot had a slope (m) = 1.5305 and an intercept of 0.0181. The standard curve equation was $y = 1.5305_x + 0.0181$, R^2=0.998 as seen in Fig. 1.

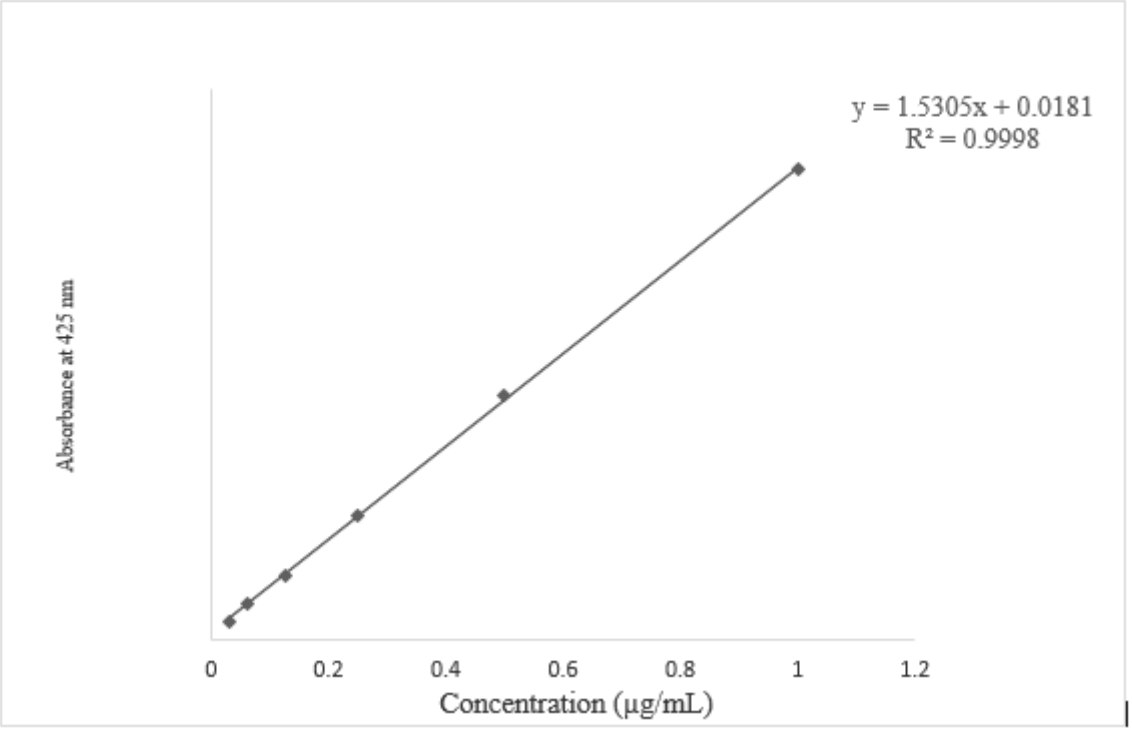


Figure 1: Total phenolic content of standard gallic acid (R^2 values are a representation of the mean data set of n=3)

The concentration used ranges were 1 (100 %), 0.5 (50%), 0.25 (25%), 0.125 (12.5%), 0.0625 (6.25%), and 0.03125 (3.125%) µg/mL as shown in Fig. 1. The TPC were calculated according to the above linear equation of the calibration curve for gallic acid; $y = 1.5305_x + 0.0181$, R^2 = 0.9998, Where X represents the amount of gallic acid in µg and y the absorbance.

4.1.2 Evaluation and Characterization of *Moringa oleifera* Phytosomes Formulation

(i) Particle size, Polydispersity Index, and Zeta Potential

Mean polydispersity index (PDI) was analyzed using the DLS technique. The average MoP formulated size was found to be in the range of 137.6±1.47 to 296±0.29 nm, and the PDI ranged from 0.106±0.002 to 0.204±0.011 as seen in Fig. 2.

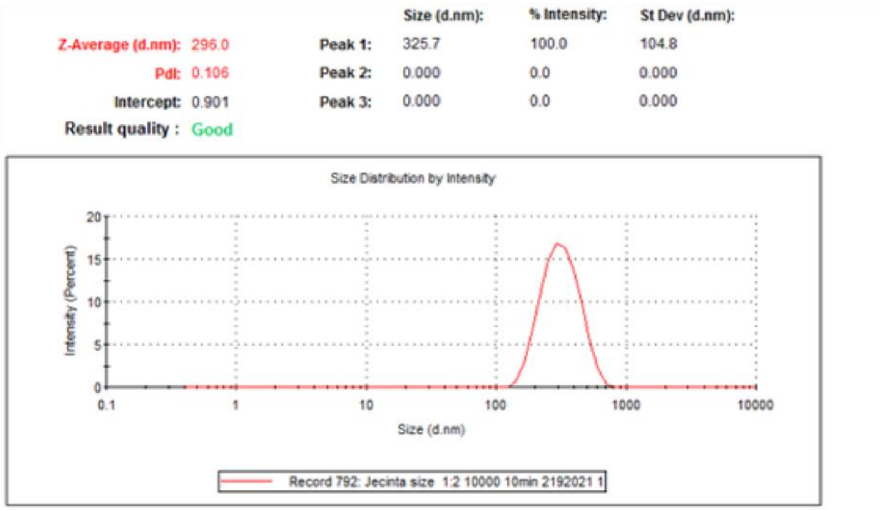


Figure 2: The average particle size and PDI of the optimized phytosomes formulation

The zeta potential is a measure of the colloidal dispersion stability. The zeta potential value (32.3±2.55 to -40.1±1.19 mV) obtained for MoP was greater than −30 mV, indicating excellent stability as shown in Fig. 3.

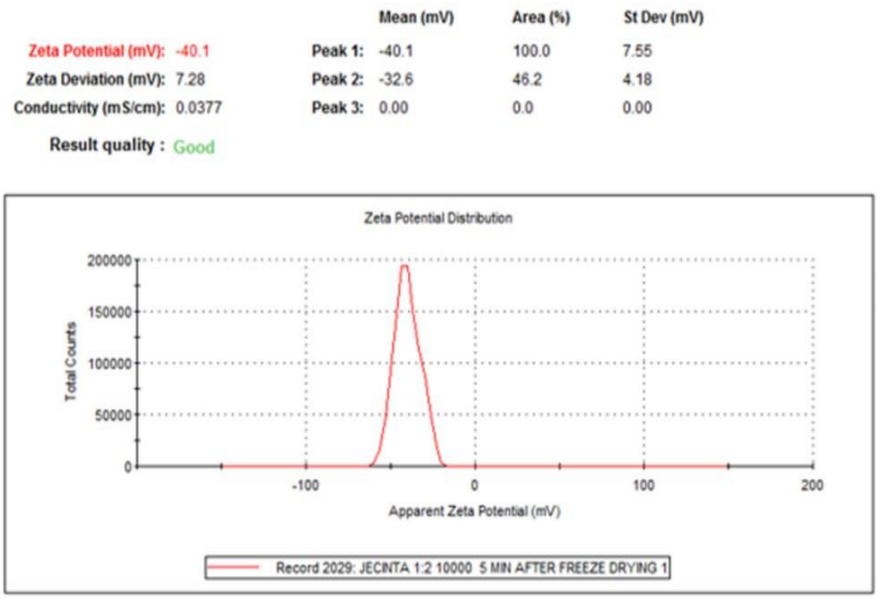


Figure 3: Zeta potential of optimized Mo phytosomes formulation

(ii) Total Phenolic Content Comparisons and Percentage Entrapment Efficiency

The TPC of Mopp at 1 mg/mL concentration was 50.81±0.02 mg GAE/g while MoP was 45.89±0.27 mg GAE/g of Mo dry sample as shown in Fig. 4 and Table 2. This is an indication of the high TPC in the extracted Mopp. The high percentage EE simply indicated the flawless loading of Mo polyphenols on the phytosome nanoparticles with negligible loss during the process, as shown in Table 2.

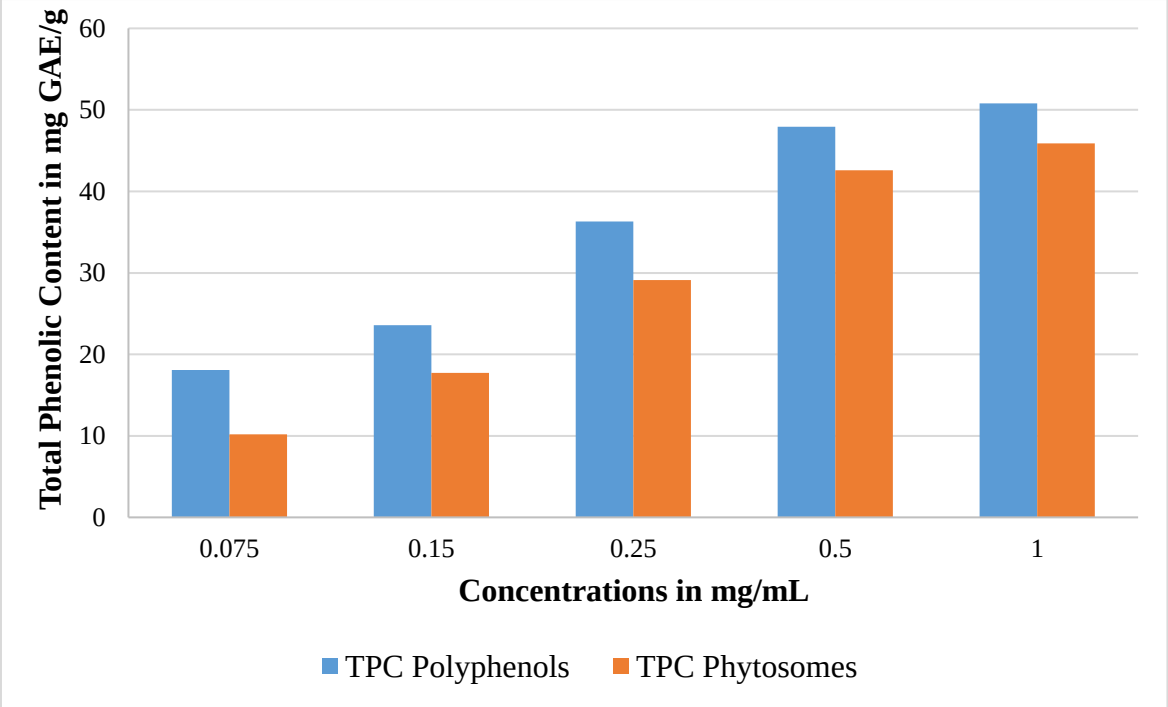


Figure 4: The total phenolic content of Mopp before and after MoP complex formulation

Table 2: Total phenolic content and the encapsulated efficiency

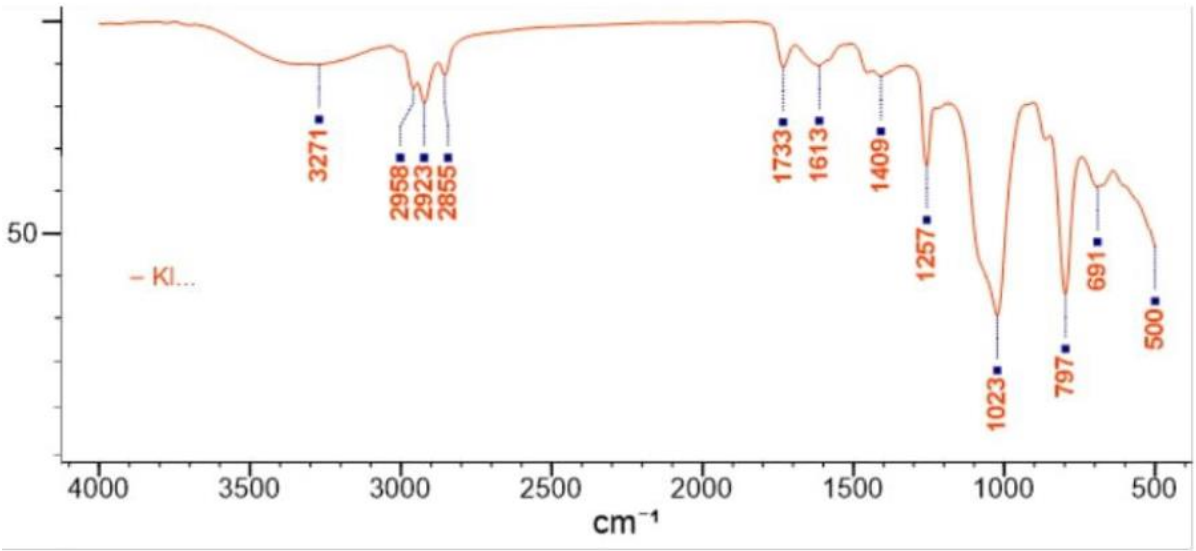
Concentration mg/mL	TPC Polyphenols in GAE/g	TPC Phytosme GAE/g	%Encapsulation Efficiency
1	50.81±0.02	45.89±0.27	90.32±0.11
0.5	47.93±0.13	42.60±0.33	88.88±0.41
0.25	36.29±0.05	29.11±1.13	80.21±2.26
0.15	23.59±0.17	17.71±0.71	75.07±2.5
0.075	18.07±1.12	10.19±0.13	56.39±1.35

(iii) Fourier Transform Infrared Spectroscopy

The FTIR spectra asserted the formation of Mo phytosomes as in Table 3 and Fig. 5 (a, b, and c).

Table 3: Fourier Transform Infra-Red and the functional groups present in Mo phytosomes

S/No	Frequency Range (cm^{-1})	Functional Group Identified
1	3271	Hydroxyl compound
2	2958, 2923 and 2855	CH and CH2 stretching aliphatic group
3	1733	Carbonyl group
4	1613	C=C Unsaturated Compounds
5	1409	Stretching -C=O inorganic carbonate
6	1257	C-N amide 111 band
7	1023	C-O-C Group
8	797	C-H
9	691	C-S Linkage



(a)

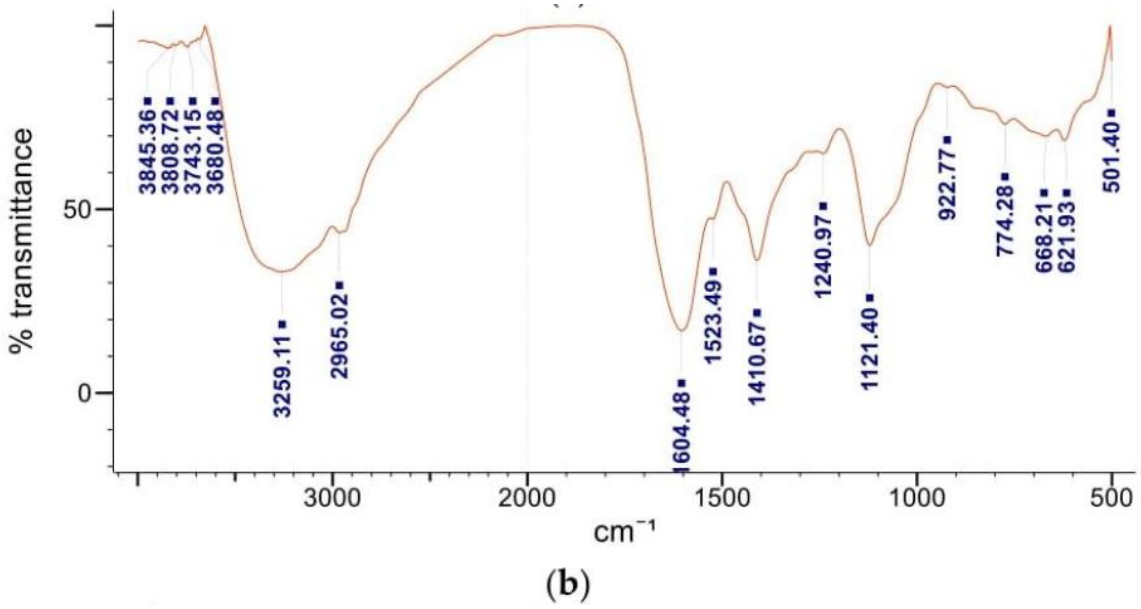


Figure 5: **Fourier Transform Infra-Red spectra of Mo phytosomes (a), Mo polyphenols FTIR spectra (b), and Phospholipid (c)**

(iv) *In vitro* Drug Release

The *in vitro* drug release of optimized MoP complex and Mopp is shown in Fig. 6. while the drug release for different kinetic models with their correlation coefficient is shown in Tables 4 and 5.

Table 4*:* ***In vitro*** **drug release data for different kinetic models**

Time (Hrs)	Square Root of Time	Log Time	Cumulative* Percentage Drug Release ±SD	Log Cumulative Percentage Drug Release	Cumulative Percent Drug Remaining	Log cumulative Percent Drug Remaining
1	1	0	18.2306	1.2608	81.7694	1.9126
2	1.4142	0.3010	22.7721	1.3574	77.2279	1.8877
3	1.7321	0.4771	29.8258	1.4746	70.1742	1.8461
4	2.0	0.6021	32.9598	1.5180	67.0401	1.8263
5	2.2361	0.6990	40.0134	1.6022	59.9866	1.7781
6	2.4494	0.7782	41.6890	1.6200	58.3110	1.7658
7	2.6458	0.8451	42.6274	1.6297	57.3726	1.7587
8	2.8284	0.9031	43.4316	1.6378	56.5683	1.7525
24	4.8990	1.3802	50.6032	1.7042	49.3967	1.6936
48	6.9282	1.6812	52.2118	1.7178	47.7882	1.6793
72	8.4853	1.8573	53.4852	1.7282	46.5147	1.6676

Table 5: The correlation coefficient for different kinetic models

Formulations	Zero Order (R^2)	First Order (R^2)	Higuchi (R^2)	Korsmeyer Peppas (R^2)
Phytosome	0.5203	0.59	0.6877	0.9306

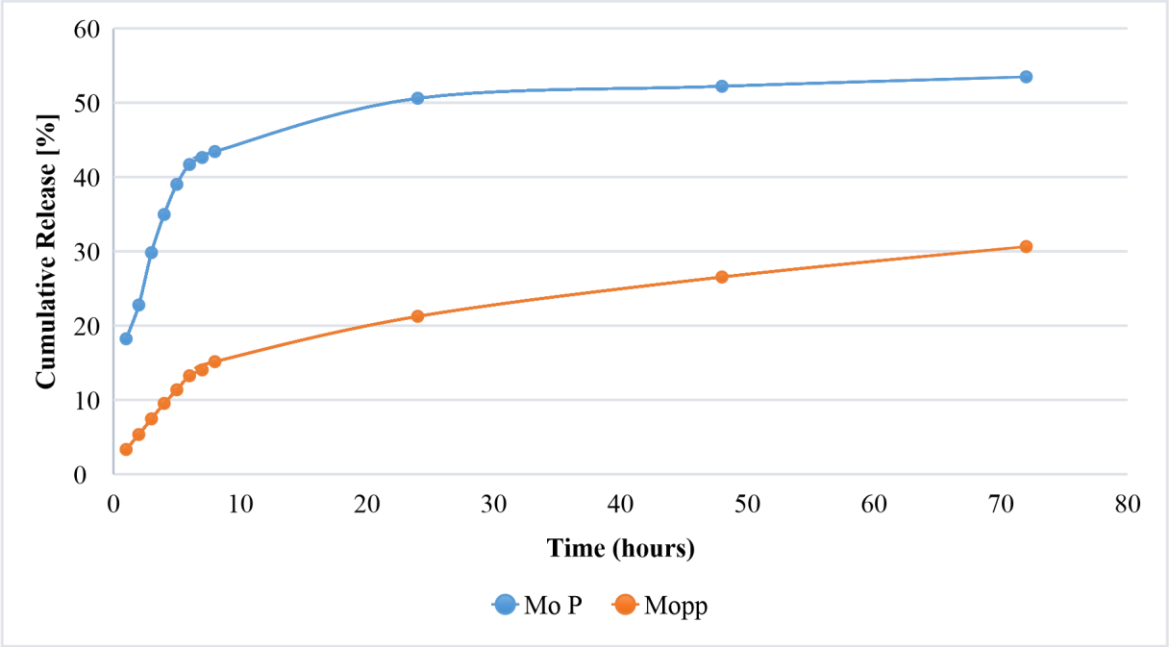


Figure 6: ***In vitro*** **release profile of optimized Mo phytosomes compared to free Mo polyphenols in phosphate buffer saline pH 7.4 at 37±0.5 ℃ (mean ± sd, n=3)**

(v) ***In vitro*** **Bioaccessibility**

As shown in Fig.7, there was a significant difference in percentage bioaccessibility of polyphenols in the GIT when TPC of Mopp was compared to MoP (p-value 0.001) after in vitro digestion. Therefore, these results proved the successful effect of phytosomes as a carrier Fig.7.

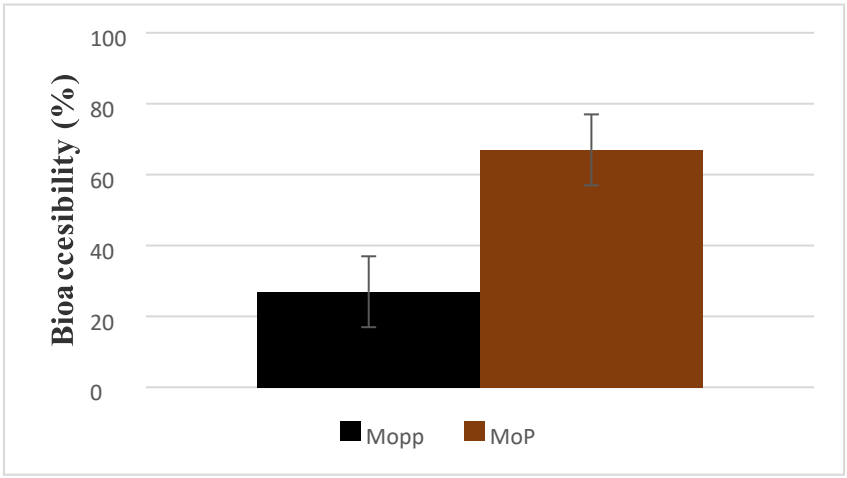


Figure 7: Bioaccessibility comparison of Mopp and MoP after exposure to simulated gastrointestinal conditions

(vi) Physical Storage Stability Test

Table 6 shows the *in vitro* stability test for MoP carried out at room temperature for 25 days. The MoP average size, zeta potential, particle distribution, and PDI was used in the analysis of the physical change of the structure of phytosomes.

Table 6: The *in vitro* storage stability for MoP at 25 ℃, room temperature the data expressed as mean ±SD

Number In Days	Average Particle Size	Zeta Potential	Polydispersity Index
1	220.3±0.12	-38.3±1.14	0.11±0.02
5	227.9±1.11	-40.9±3.56	0.13±0.11
10	229.6±0.20	-41.1±2.48	0.14±0.04
15	231.7±1.34	41.4±1.52	0.17±0.16
20	236.5±2.53	-42.7±31	0.19±0.03
25	239.6±2.46	-42.8±2.53	0.19±0.07

The Mo phytosomal formulations characterization parameters by DLS technique after day 1 to 25 days of storage at 25 ℃ showed an average zeta size range of 220.3±0.12 to 239.6±2.46 nm. The Zeta potential ranged between -38.3±1.14 to -42.8±2.53 mV while the PDI ranged between 0.11±0.022 to 0.19±0.065 as shown in Table 6. No significant change was observed in MoP particle average size, zeta potential, and polydispersity index for the 14 days (p value> 0.05).

4.1.3 Phytosomes, Polyphenols, and Doxorubicin Effects on Vero E6 (Normal) Cell Lines

Moringa oleifera polyphenols and MoP had CC_{50} > 90 µg/mL in the cytotoxicity studies except for doxorubicin. The CC_{50} value of the Vero cell ranged between 68.35±3.51 and 212.9±1.30 µg/mL. The inhibitory concentration for Doxorubicin, free Mopp, and the MoP complex were 3.04±0.27, 39.94±0.10 and 7.73±2.87, while the selectivity index that measures the drug's ability to control cancer growth was 2.07±1.30, 5.34±0.13 and 12.79±0.50 µg/mL respectively (Fig.8).

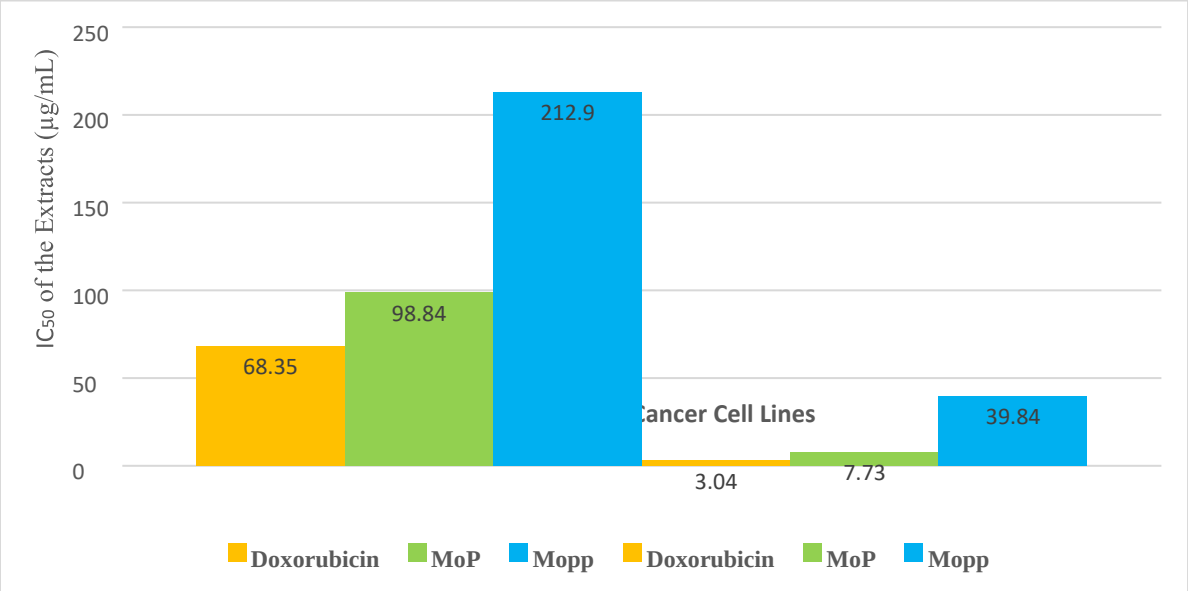


Figure 8: Concentration of the drug that reduces cell viability by 50% (CC50) and Concentration that inhibits 50% of breast cancer cells (IC50) of Doxorubicin (standard drug) and Extract (free polyphenols and encapsulated polyphenols) comparisons toward Vero cell lines and 4T1 cell lines

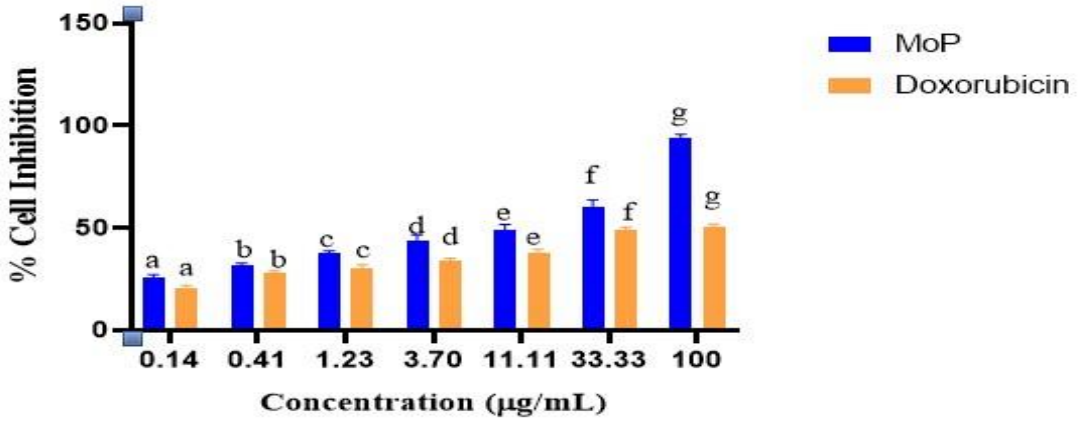


Figure 9: Percentage cell inhibition comparison for MoP and doxorubicin at different dosages. Bars with different letters per concentration are significantly different ($p < 0.05$) according to Student's t-test analysis

4.1.4 *In vivo* Toxicity Studies

The oral administration of Mopp and the MoP at doses of 50, 300, 300, and 2000 mg/kg doses resulted in no signs of acute toxicity. The weight range was as shown in Table 7.

Table 7: The weight variation of Swiss albino female mice during the 14 days of free Mopp and MoP complex and control oral administration. The data are expressed as mean ± SD

Concentration of Polyphenols and Phytosomes of *Moringa oleifera*	Weight (g) Day 1	Weight (g) Day 7	Weight (g) Day 14
50mg/kg Polyphenols	21.60±1.21	25.50±0.32	27.0±2.71
300mg/kg Polyphenols Group 1	24.12±0.10	28.15±0.37	29.20±1.33
300mg/kg Polyphenols Group 2	23.65±0.22	26.60 ±0.49	27.61±1.42
2000mg/kg Polyphenols	23.39±0.28	26.1±1.68	27.67±2.79
50mg/kg Phytosomes Complex	24.67±1.36	28.20±1.32	28.50±2.45
300mg/kg Phytosomes Complex	25.0±1.31	27.67±1.53	28.28±0.27
300mg/kg Phytosomes Complex	22.0±0.62	25.14±1.02	25.67±1.38
2000mg/kg Phytosomes Complex	26.65±2.29	26.51±1.92	27.06±0.03
Control	23.00±3.02	25.15±2.58	27.11±1.26

4.2 Discussions

The ultimate goal of the present study was to develop natural lipid nanoparticles with anti-cancer properties. Despite having potent anti-oxidant, liver protective, anti-inflammatory, and anticancer properties, polyphenols are limited in their efficacy due to their poor bioavailability and these results in a poor clinical trial outcome. This study aimed at preparing MoP to overcome this problem while ensuring safety. The phytosome technology relies on forming intermolecular bonding between phosphatidylcholine and polyphenols. A significant amount of polyphenols was successfully extracted and then tested for their TPC.

The Folin-ciocalteu method using gallic acid was used to draw a standard curve that was used to calculate the TPC for MoP and free Mopp. The method was based on electron transfer in an alkaline medium from phenolic compounds to phosphomolybdic phosphotungstic acid complexes in folin to form blue colored complexes (PmoW11O40)-4 as determined by spectrophotometry at 760 nm. The calibration curve was used to measure the linearity of the method and a correlation coefficient of R^2 =0.9998 was an indication of a good linearity concentration as shown in Fig. 1. The gallic acid conformed to Beer's Law with a regression coefficient (R^2) = 0.9998 since it met the acceptance linearity criteria for a value not less than 0.990 (Duan *et al*., 2020).

The phenolic content in the Mo phytosomes was found to be lower than in the Mopp, indicating that not all the polyphenols were encapsulated in the phytosomes complexes during optimization and preparation but a significant amount was incorporated in the phytosomes complex. Typical phenolics have been shown to possess antioxidant, antiproliferation, and anticancer activities alongside phenolic acids and flavonoids. In this study, the MoP drug delivery complex system was successfully developed. Phytosomes were used to deliver polyphenols to the target to improve the absorption, bioavailability, control of drug release, and drug distribution, thereby enhancing the passive targeting to cancerous cells, retention of polyphenols, permeability effect, and efficacy, producing better results than conventional herbal extracts.

The particle size distribution and zeta potential values for the formulated MoP were also analyzed. The results indicated that the MoP complex microsphere size was in the range of < 300 nm as shown in Fig. 2. The average particle size distribution showed that the MoP particles were homogeneously distributed as supported by the PDI value < 0.3 (Fig. 2). This agrees with Piazzini *et al*. (2019) that the best homogeneity is PDI value < 0.5. The zeta potential is an essential parameter that measures colloidal dispersion such as phytosome stability. A higher electrostatic repulsion between the particles indicates more excellent stability. According to the present findings as shown in Fig. 3, zeta potential was >-40.1 mV, implying that the prepared MoP had good physical stability.

The percentage entrapment efficiency of Mopp was verified by the Folin-Ciocalteau spectrophotometric method and validated as shown in Table 2. Previous studies have reported that the formulations percentage EE depends on drug solubility and bonding interaction leading to matrix formation. Therefore, the present data support results from other studies like those by Pal *et al.* (2021) in which polyphenols demonstrated higher affinity towards Phosphatidylcholine and their capability to establish an intermolecular bonding to help retain its stability and specificity.

The FTIR was used to study the possible interactions between polyphenols from *Moringa oleifera* and phospholipids. The Infra-Red spectra were recorded in the range of 4000 – 400 cm^{-1}. The obtained Infra-Red spectra were interpreted for their functional groups from polyphenols, phosphatidylcholine, and phytosomes at their respective wave number (cm^{-1}) as indicated in Fig. 5 and Table 3. The spectra showed a broad peak at 3271 cm^{-1}, representing the aliphatic alcoholic (−OH) group usually present in the polyphenolic groups. Phospholipids

shielded the peaks at 1613cm^{-1} (C=C), which indicates the presence of unsaturated compounds usually found in fatty acids in the phytosomes. The spectra also showed fatty acids' long-chain bands of the phospholipid molecule at 2958, 2923, and 2855 cm, $^{-1}$ indicating phytosomes formation as shown in Figure 5. The band at 1257 cm^{-1} indicates the association of stretching vibration of C-N usually found in the choline moiety in phytosomes. The spectral band at 1023 cm^{-1} is attributed to symmetric stretching of the C-O-C vibration mode present in phospholipids and polyphenols, and this indicates the successful formation of the Mo phytosomes complex. The absorption at 1613 cm^{-1} in the complex spectrum indicates the formation of hydrogen bonds and existence of electrostatic interactions between polyphenols and phospholipids as shown in Fig. 5 (a), all of which confirm the successful formation of the phytosomes.

To analyze the mechanism of MoP and how the polyphenols are released from the drug delivery system, the polyphenols from the MoP complex were compared at different time points. The polyphenols drug release profiles *in vitro* studies indicated a sustained and controlled release. A fast polyphenol release of 43.43% was observed at the end of the 8th hour, followed by a sustained drug release of 53.49% over the remaining 24, 48, and 72 h for MoP as observed in Fig. 6. Although a burst release profile was observed in the MoP and Mopp profiles, the release was higher in the Mo phytosomes formulation as shown in Fig. 6. The initial drug release burst was due to the drug being entrapped near the surface, while the sustained release was attributed to its release diffusion from the phytosomes, this also agreeing with the study by El-Lakany *et al.* (2018). The *in vitro* release study data were fitted into different kinetic models, and the best fit was achieved by the highest correlation coefficient. Korsmeyer Peppas model release kinetics with linear regression $R^2 = 0.9306$ was the best fit for this study as indicated in Tables 4 and 5. The drug release from Korsmeyer equation showed that diffusion and erosion could be the drug release mechanism, agreeing with the results by Parashar *et al*. (2019). In our study, 'in vitro release testing" was used as a measure of drug dissolution as documented previously by Shen and Burgess (2013) . This is considered an important tool for quality control purposes as well as for prediction of the in vivo performance of drug delivery involving nanocarrier systems and has become an essential quality control test of drug development since it was officially adopted in the United State Pharmacopeia (USP) in 1970 (Dokoumetzidis *et al*., 2006). There is no standard pharmacopeial/regulatory in vitro dissolution/release test currently available for nanoparticulate systems. However, extensive efforts have been made to develop suitable *in vitro* dissolution/release testing methods for nanoparticulate delivery systems. Current methods are broadly divided into three categories, namely membrane diffusion

methods (such as dialysis methods), sample and separation methods, and continuous flow methods.

Membrane diffusion methods (dialysis methods) are the most widely investigated for the in vitro dissolution release testing of nanoparticulate systems. In these methods, the nanoparticulate systems are separated from the release medium through dialysis membranes that are permeable to the free drug but impermeable to the nanoparticles. Dialysis methods have been widely used to investigate in vitro drug dissolution/release profiles of liposomes, emulsions, polymeric nanoparticles (Wang *et al*., 2019), as well as lipid nanocarriers.

The free Mopp bioaccessibility mean percentage was 26.95 ± 0.02, showing extensive degradation, whereas the MoP bioaccessibility mean percent was 66.98 ± 0.01%; indicating a better stability than the Mopp as shown in Fig. 7. The low bioaccessibility of Mopp was due to compromised conditions (digestive enzymes and the acidic environment) of the gastrointestinal tract. These findings are in line with previous reports where low bioaccessibility of free phenolic compounds was seen after in vitro digestion as compared to encapsulated polyphenols (Shah *et al.*, 2016; Grgić *et al*., 2020). This might be attributed to low absorption due to larger molecular weight and thus limited bioavailability.

The storage stability for the Mo phytosomes results was presented at 25 ℃. There was a small variation in average size, PDI, and Zeta potential over the 25 days ($p > 0.05$), which shows that the sample remained stable when exposed at room temperature as shown in Table 6. This shows that the formulated phytosomes complex retained its charge, and the tendency for aggregation at 25 ℃.

The criteria usually used for *in vitro* cytotoxicity after 72 h of exposure time for the U.S. National Cancer Institute indicate that an $IC_{50} < 4$ µg/mL for pure compounds and $IC_{50} < 20$ µg/mL for crude extracts is considered highly antiproliferative. Additionally, an IC_{50} value less than 30 µg /mL is considered to be antiproliferative, while an IC_{50} value of 30 - 100 µg /mL is considered moderately antiproliferative and above 100 µg/mL is indicated as inactive (Livingstone, 2001).

The *in vitro* cytotoxicity results showed that MoP was non-toxic to normal cells and highly selective on breast cancer cells compared to free polyphenols. Doxorubicin was toxic to 4T1 cell lines as the selectivity index was ≤ 3. Generally, the Mo phytosomes inhibited the proliferation of the 4T1 cancer cells. The findings thus indicated that a low concentration of

MoP at 0.14 μg/mL did not affect cell growth much, whereas the treatment with 0.41, 1.23, 3.70, 11.11, 33.33, 100 μg/mL of MoP inhibited the growth of 4T1 cells in a dose-dependent manner as shown in Fig. 9. There was a significant difference between the cytotoxicity of the Mopp and MoP ($p \leq 0.05$), and their activity was not comparable to that of the positive control.

The *in vitro* antiproliferative activity of Mo leaf crude extract has been considered in previous studies (Gaffar *et al.*, 2019; Mumtaz *et al*., 2021; Ndung'u *et al.*, 2018; Wisitpongpun *et al.*, 2020) and showed an inhibitory concentration above 100 μg/mL. In this study, the inhibitory concentration for Mopp was 39.94±0.10 μg/mL while that of MoP complex was 7.73±2.87 μg/mL as shown in Fig. 8, which is an indication that Mopp in phytosomes complex had an improved efficacy compared to the earlier reported Mo crude extract. A study by Xu *et al.* (2020) reported improved inhibitory effects of polyphenols on 4T1 cancer cells. Studies by Liu *et al*. (2019) and Zhang *et al*. (2019) have reported improved bioavailability of bioactive compounds, thus improving their potential inhibitory effect on breast cancer cells. The present studies agree with the above-mentioned reported studies and other researchers such as Moeini (2021), where phytosomes and other lipid phospholipids nanocarrier improved the bioavailability of bioactive compounds and consequently their efficacy in inhibiting the growth of breast cancer cells. In this study, *Moringa oleifera* phytosomes formulation; and antiproliferative effects on 4T1is being reported for the first time.

The oral administration of the free Mopp and Mo phytosomes at a dose from 50 up to 2000 mg/kg resulted in no clinical signs of acute toxicity nor did they influence the body weight of the Swiss albino mice during a short period of 5 h and in a prolonged period of 14-day of observation (Table 7). There was no mortality in both control and treated groups across the different doses of the drugs. Additionally, there were no abnormalities in the autopsy organs. Moreover, there were no significant body weight changes during the 14 days of study. Despite that *in vivo* safety of the MoP complex and Mopp on Swiss albino mice is being reported for the first time, the study agrees with the *in vitro* safety studies on Vero E6 cell lines (normal cells). This suggests that the *in vivo* novel the MoP complex can be safely administered orally up to up to 2000 mg/kg.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In the present study, the MoP complex was successfully formulated, characterized and shown to improve bioavailability of Mopp, especially in inhibiting 4T1 cancer growth. That is an indication that the MoP complex can be a reliable candidate for improved drug dosage in breast cancer therapy. This study concludes that the novel MoP has better physical characteristics and reduced cytotoxicity to free Mo polyphenols, and they possess antiproliferation potential on breast cancer cell lines than the free Mo polyphenols. The strong antiproliferation activity could be attributed to the high content of polyphenol compounds encapsulated by the present phytosomes that enhanced apoptosis. The MoP were also safe in the *in vivo* study up to 2000 mg/kg dosage. This dose can therefore be used for further studies in determining the *in vivo* studies in determining antiproliferative activity of MoP complex.

5.2 Recommendations

Despite the revealed background on antiproliferative efficacy of MoP on 4T1 cancer cell lines, further studies are still needed in the following areas:

(i) Surface and size characterizations of the novel *Moringa oleifera* polyphenol-loaded phytosomes using scanning electron microscope and transmission electron microscope respectively.

(ii) The molecular mechanism of action of the MoP in the *in vitro* studies on 4T1, to identify the genes triggered by MoP in inhibiting breast cancer cell growth.

(iii) Comprehensive *in vivo* sub-acute assay to evaluate further the efficacy of the novel MoP in mice

(iv) *In vivo* studies on antiproliferative effects of MoP complex on breast cancer which can be done through Otambo chair initiative

These aforementioned activities were beyond the scope of this study due to budgetary and time limitations.

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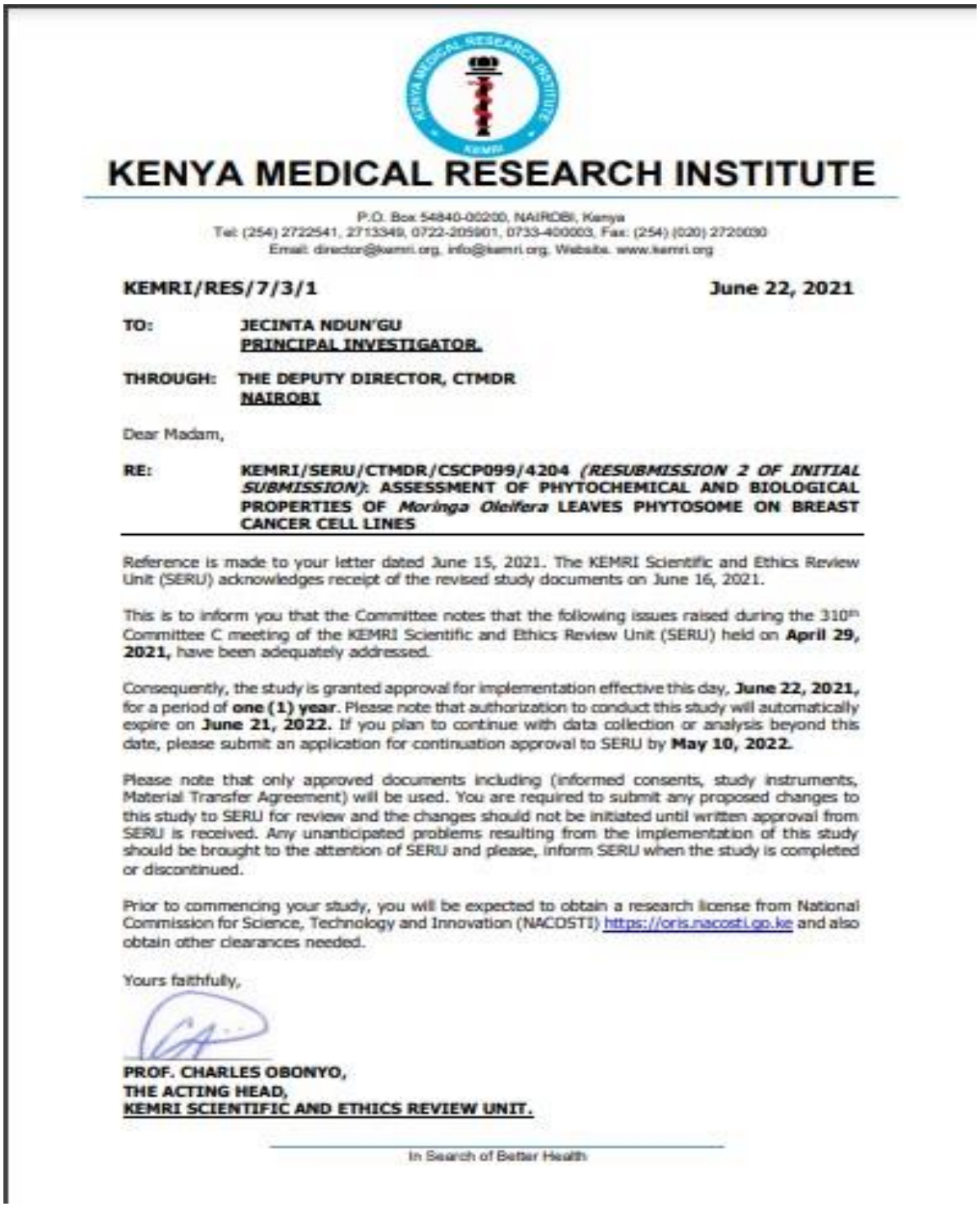
APPENDICES

Appendix 1: Request for permission from KEMRI to do the research



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KEMRI/RES/7/3/1 **June 22, 2021**

TO: **JECINTA NDUN'GU**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DEPUTY DIRECTOR, CTMDR**
NAIROBI

Dear Madam,

RE: **KEMRI/SERU/CTMDR/CSCP099/4204 *(RESUBMISSION 2 OF INITIAL SUBMISSION)*: ASSESSMENT OF PHYTOCHEMICAL AND BIOLOGICAL PROPERTIES OF *Moringa Oleifera* LEAVES PHYTOSOME ON BREAST CANCER CELL LINES**

Reference is made to your letter dated June 15, 2021. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on June 16, 2021.

This is to inform you that the Committee notes that the following issues raised during the 310th Committee C meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **April 29, 2021,** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **June 22, 2021,** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **June 21, 2022.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **May 10, 2022.**

Please note that only approved documents including (informed consents, study instruments, Material Transfer Agreement) will be used. You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and please, inform SERU when the study is completed or discontinued.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours faithfully,

PROF. CHARLES OBONYO,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

In Search of Better Health

Appendix 2: Output 1: Oral Presentation

BACKGROUND

The active plant molecules have been relocated increasingly for managing breast cancer traditionally. However, phytoconstituents such as polyphenols have high polarity and possess a large molecular size thus cannot be absorbed by simple diffusion to target cells by passive diffusion. The polyphenols also possess a poor lipid miscibility and this results to poor bioavailability and decreased efficacy. 'Phytosome' plays a very important role of improving bioavailability, prolong the retention time and also facilitate absorption of the polyphenols.

Methods

Phytosome being a novel formulation system loaded with polyphenols and phosphatidylcholine complex was prepared by a nanoprecipitation technique combined with solvent evaporation method with an aim of developing a drug delivery system. The gallic acid was used as a standard and the total phenolic content expressed as mg/g gallic acid equivalents (GAE). The zeta-potential, average particle size, encapsulation efficiency, polydispersity index, solubility and residual drug-loading content was used to evaluate the *Moringa oleifera* polyphenols-loaded phytosomes. Additionally, Fourier transform infrared spectroscopy was used to evaluate the integrity of the polyphenols-loaded phytosomes. Cytotoxicity and the anti-proliferative activity of *Moringa oleifera* nanophytosome complex against normal Vero cell lines and 4T1 (Breast) cancer cell lines were assessed by the MTT cell viability assay.

Results

Gallic Acid Equivalent standard curve equation was $Y = 0.0061x + 0.0396$ with R^2 value = 0.9991. Electrophoretic light scattering described a dispersion with an average particle size of 250.1000± 0.2300nm, polydispersity index of 0.2160± 0.0550 to 0.8700± 0.1170, and a zeta-potential value of -45.4000± 1.4000 mV. The stability tests for *in vitro*, demonstrated that the average particle size had no evident change at different storage conditions of +4^{o}C, -20 ^{o}C, -80 ^{o}C and at room temperature of 25 ^{o}C. The 50% cytotoxic concentration value of normal Vero cell lines for nanophytosome was 98.4400±1.4350 µg/ml and 212.9000±1.3000 µg/ml for *Moringa oleifera* polyphenols. The half maximal inhibitory concentration (IC_{50}) for co-encapsulated nano-phytosome was 7.7300 µg/ml and for the free polyphenols of *Moringa oleifera* was 39.8400 µg/ml on 4T1 cancer cell lines.

Conclusion

In vitro cytotoxicity assays using VERO cell lines, indicated that the polyphenol-loaded phytosomes had remarkable cytotoxicity. The *In vitro* anti-ploriferative effect revealed inhibitory effect on 4T1 cancer cell lines growth. These findings indicates that the *Moringa oleifera* polyphenol-loaded phytosomes had better effects of prolonging retention time and promoting absorption than the free polyphenols. This shows that it can be used as a sustained delivery system for bioactive compounds with poor lipid immiscibility. Additionally, it can offer an effective and promising formulation for drug delivery for breast cancer therapy.

Appendix 3: Output 2: Certificate of Oral Conference Presentation

Appendix 4: Certificate of Publications

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CERTIFICATE OF PUBLICATION

Certificate of publication for the article titled:

Formulation, Optimization, and Evaluation of *Moringa oleifera* Leaf Polyphenol-Loaded Phytosome Delivery System against Breast Cancer Cell Lines

Authored by:

Jecinta Wanjiru; Jeremiah Gathirwa; Elingarami Sauli; Hulda Shaid Swai

Published in:

Molecules **2022**, Volume 27, Issue 14, 4430

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Basel, July 2022